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Bioremediation of contaminated soil with hydrocarbons discharged from liquid petroleum gas filling refineries by *Burkholdaria cepatia*

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***Abstract*---**Commercial, industrial, and military activity, largely in the 19th and 20th centuries, have led to environmental pollution that can threaten human health and ecosystem function, liquid gas petroleum (LPG) products are the major sources of energy for industry and daily life that cause environmental contamination during various stages of production, transportation, refining and use. Screening of bacterial isolate by using clear zone techniques and biomass and optical density. Results revealed that isolate Burkholdaria cepatia showed a high ability for hydrocarbons biodegradation and this isolate identified depending on morphological cultural, gram stain, microscopic features, biochemical tests, and VITEK2 compact. In this study, Burkholdaria cepatia had been examined to degrade hydrocarbons in soil. Such strain which is isolated from petroleum hydrocarbon contaminated soil has the ability to utilize a variety of hydrocarbons substrates. Optimal conditions that include (time, temperature & pH) were studied for hydrocarbons biodegradation by Burkholdaria cepatia, results showed with high growth and hydrocarbons biodegredation. Biodegradation Experiments were carried out in lab scale and the growth of microorganisms was investigated directly and indirectly. Results showed an extent of biodegradation more than 80% can be achieved within 10 days using.

***Keywords*---**bioremediation, contaminated soil, gas filling refineries, hydrocarbons, LPG.

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1. Introduction

Soils are complex mixtures of air, organic matter, minerals, water, and countless organisms that are the decaying remains of once-living things. It forms at the surface of land; it is the skin of the earth. Soil is capable of supporting plant life and is vital to life on earth [1]. The main reason for contamination of soil is resulting from the presence of anthropogenic activities and emergencies, agricultural and industrial activities lead to release of large amounts of petroleum hydrocarbons into the environment that caused high concerns in recent [2]. Soil contaminated with hydrocarbons, is the major global concern today because form serious hazard to human health, causes organic pollution of ground water which limits its use, economic loss, environmental problems, and decreases the agricultural productivity of the soil [3], release of hydrocarbons into the environment whether accidentally or due to human activities is a main cause of water and soil pollution [4]. After using gas cylinders many times, amount of waste were accumulates, these residues sourced from either production or reactions occur during the marketing, these residues are mainly high carbonic number in liquid compounds and black solid waste (polymer compounds). [5] .The use of bioremediation for hydrocarbons contamination treatment can overcome some of limitations of physical and chemical treatments and provides a means for cost-effective removal. Such method is recently receiving favorable publicity as promising environmentally friendly treatment technologies for the remediation of hydrocarbons [6]. For this reason, bioremediation has been considered a potentially useful tool in the cleaning of oil spill and the treatment of oil residues [7]. Many soil microorganisms transform hydrocarbons into nontoxic compounds or mineralize them to inorganic compounds; hydrocarbons are degraded in soil mainly by bacteria and fungi [8] and [9], this natural microbiological activity is applied in bioremediation to reduce the concentration and/or toxicity of various pollutants, including petroleum products [10]. These processes take place in the natural environment, and their end-products are carbon dioxide and water [11]. The *Burkholderia cepacia* complex consists of nine genomic species first described by Walter Burkholder of Cornell University in 1949 when he determined it to be the cause of bacterial rot of onion bulbs.[12]. *Burkholderia cepacia* rod-shaped, free-living, motile Gram- negative bacteria ranging from 1.6- 3.2 µm. They have been found to possess multitrichous polar flagella and pili used for attachment, Burkholderia cepacia can be found in soil, water, and commonly found on plant roots, animals, and humans [13], they are of significant environmental interest, they can degrade a large variety of toxic compounds They are distinctive in their ability to metabolize a broad range of organic compounds as carbon and energy sources, a practice using biological compounds to remove hazardous compounds and pollutants and this makes them extremely useful in bioremediation of soil and groundwater contaminated with chlorinated hydrocarbons and herbicides [14].

2. Materials and Methods

2.1 Collection of samples of contaminated soils

Samples of contaminated soil and control, liquid, and solid wastes were collected from soil around gas filling refineries, sampling of topsoil (0-15 cm) using a stainless hand trowel, each sample of soils and solid wastes were collected in sterile plastic bags, while liquid samples collected in sterile glasses bottles, then samples transported to the laboratory and stored at 4°C until using in the isolation of hydrocarbon degrading bacteria.

2.2 Isolation of bacteria

One gram of each sample added to 9ml of distal water and shacked to homogenize and serial dilutions were prepared for each sample, 0.1ml of each dilution were spread upon nutrient agar medium and incubated at 37°C for 24 hour, then bacterial colonies different in morphological characteristics were purified by sub culturing on nutrient agar medium until pure culture was obtained and stored in refrigerator at 4°C until use in next experiments [15].

2.3 Screening of bacterial isolates

Twenty four bacterial isolates were obtained from soil samples which screening to select the most efficient bacteria in biodegradation for next tests.

Determination of biomass

Biomass tests were done for bacterial isolates, after reactivation in nutrient broth at 37 °C for 24 hours. Isolates were inoculated with 100 ml of sterilize modified mineral salt medium containing 1% ml of LPG wastes in 500 ml flask and incubated at 30°C in a shaker incubator at 150 rpm for 5 days. Then, the flasks were taken out and bacterial activities were stopped by adding 1N-HCl to elevate the acidity of medium (pH less than 2). Solvent of acetone: hexane (1:3) was added to separate hydrocarbons from the liquid culture. Medium poured in centrifuge tubes and centrifuged by cooling centrifuge (4°C) at 10000 rpm for 30 minutes to allow the biomass to separate from the supernatant [16].

Two layers were observed, top layer containing solvents and liquid petroleum gas wastes, while lower layer containing water and cells. Top layer (supernatant) poured in beaker, and the aqueous layer gently discharged out. The cells then transported to weighted glasses bowls, dried in oven at 70 °C for 24 hours and weighing [17]. The highest biomass recorded was 3.26g/100ml.

Determination of bacterial growth by optical density

Isolates were inoculated with 100 ml of sterilize modified mineral salt medium MMSM containing 1% of LPG wastes in 500ml flasks and incubated at 30 °C in a shaker incubator at 150 rpm for 5 days. Turbidity parameter used to determined bacterial growth by daily measured of optical density of culture medium and control at wavelength 540 nm starting from zero time to 5th day of incubation. After 5days of incubation measured optical density, higher optical density value is 0.1.

2.4 Identification of bacterial isolate

Identification of bacterial isolate by morphological, microscopic observations, biochemical characteristics, and morphological as well as identified by VITEK2 compact.

3. Results and discussion

3.1 Optimum condition for *B. cepacia* growth

Temperature

Optimum temperature was studied by using modified mineral salt medium MMSM inoculated with *B. cepacia* and incubated for 8 days in shaker incubators at different temperatures 30, 35, and 40°C, growth of B. cepacia were detected by measured optical density by a spectrophotometer (UV–9200) at wavelength 540nm, highest values of OD recorded at 30°C as 0.401, from the results and statistical analysis, significant difference ($p<0.05$) was found among means of values of OD at temperature 30°C, while no significant difference ($p<0.05$) was observed among means of values of OD at other temperatures 35 °C, and 40 °C and when compare means of OD values with value of LSD ($p\leq0.05$) the best temperature for growth of bacterial isolate *B. cepacia* was 30°C, as shown in Table (1) and (2), and Fig. (1) [18].

Table (1) Optical density (540 nm) for *B. cepacia* at 30, 35, 40 C°, pH 5, 7, 9, and 8days incubation

Bacterial isolate	Temp.	pH	Absorption							
			1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day
B. cepacia	30	5	0.01	0.016	0.023	0.031	0.037	0.047	0.05	0.056
		7	0.03	0.033	0.029	0.103	0.123	0.313	0.313	0.401
		9	0.018	0.021	0.025	0.033	0.041	0.043	0.047	0.055
	35	5	0.014	0.016	0.015	0.022	0.037	0.047	0.05	0.056
		7	0.013	0.02	0.024	0.037	0.043	0.09	0.102	0.154
		9	0.011	0.019	0.021	0.025	0.037	0.039	0.037	0.04
	40	5	0.002	0.003	0.013	0.015	0.014	0.019	0.021	0.023
		7	0.015	0.013	0.023	0.025	0.028	0.032	0.038	0.04
		9	0.013	0.019	0.02	0.025	0.027	0.03	0.032	0.035

Table (2) Statistical analysis of effects of temperature and pH on growth of *B. cepacia* after 8days incubation

TEMP. / PH	30C°	35C°	40C°	LSD
PH=5	A0.034±0.01 b	A0.032±0.01 a	B0.014±0.008	0.015

			b	
PH=7	A0.168±0.15 a	B0.060±0.05 a	B0.027±0.01 a	0.090
PH=9	A0.035±0.01 b	A0.029±0.01 a	A0.025±0.007 a	0.011
LSD	0.091	0.032	0.009	

Means with the different small letters in the same column differ significantly (P≤ 0.05)
Means with the different capital letters in the same row differ significantly (P≤ 0.05)

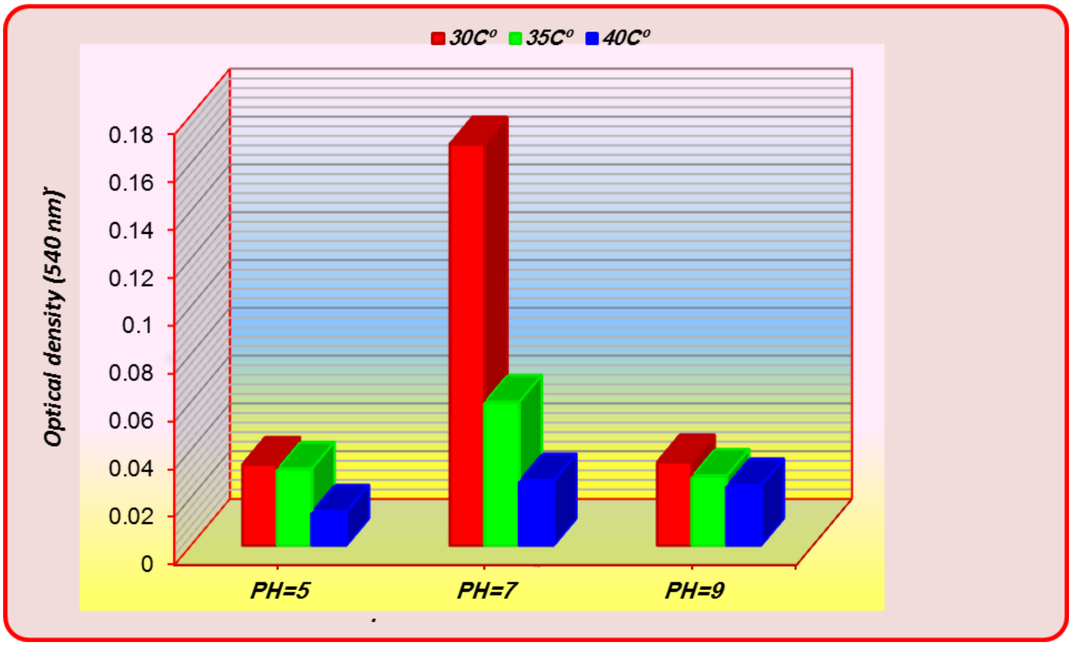

Figure (1) Effects of different pH, temperatures on growth of *B. cepacia* after 8 days incubation

3.1.2 pH

Optimum pH was studied by using MMSM medium inoculated with *B. cepacia* and incubated for 8 days in shaker incubator incubator at 150 rpm and different pH 5, 7, and 9, growth of *B. cepacia* were detected by measured optical density, from results were obtained and statistical analysis for influence, significant difference ($p<0.05$) was found among means of values of OD at pH 7, while no significant difference ($p<0.05$) was observed among means of values of OD at pH 5 and pH 9, and when compare means of OD values with value of LSD ($p \leq 0.05$)

the best pH for growth of bacterial isolate *B. cepacia* was 7, as shown in Table (1) and (2) and Fig. (1).

Incubation period

Optimum incubation period was studied by using sterilize modified mineral salt medium prepared (100ml) in 500ml flask, then added 1ml of hydrocarbons (LPG wastes), inoculated and control flasks (without bacterial culture) incubated in a shaker incubator at 30 °C 150 rpm for 8 days, optical density of the culture and control were measured daily from zero time to 8th day of incubation by a spectrophotometer (UV–9200) at waves length 540 nm, significant difference ($p<0.05$) was found among means of values of OD from 5th to 8th days of incubation period , while no significant difference ($p<0.05$) was observed among means of values of OD at 1st , 2nd , and 3rd days of incubation period and when compare means of OD values with value of LSD ($p\leq0.05$) the best incubation period for growth of bacterial isolates was 5th to 8th days of incubation period and this clearly appears in Tables (3) and (4) and Fig. (3) and (4).

Table (3) Statistical analysis of effects of Incubation period on growth of *B. cepacia* at different temperature

TEMP. / Days	30C°	35C°	40C°
First day	0.019 a ± 0.010	0.013 c ± 0.002	0.010 d ± 0.007
Second day	0.023 a ± 0.009	0.018 bc ± 0.002	0.012 cd ± 0.008
Third day	0.026 a ± 0.003	0.02 bc ± 0.005	0.019 bcd ± 0.005
Fourth day	0.056 a ± 0.041	0.028 bc ± 0.008	0.022 abcd ± 0.006
Fifth day	0.067 a ± 0.049	0.039 abc ± 0.003	0.023 abc ± 0.008
Sixth day	0.134 a ± 0.155	0.059 abc ± 0.027	0.027 ab ± 0.007
Seventh day	0.137 a ± 0.153	0.063 ab ± 0.034	0.030 ab ± 0.009
Eighth day	0.171 a ± 0.199	0.083 a ± 0.062	0.033 a ± 0.009

LSD P ≤ 0.05	0.185	0.047	0.013

Table (4) Statistical analysis of effects of Incubation period on growth of *B. cepacia* at different pH

TEMP. / Days	PH=5	PH=7	PH=9
First day	0.009 d ± 0.006	0.019 b ± 0.009	0.014 d ± 0.004
Second day	0.012 d ± 0.008	0.022 b ± 0.010	0.020 cd ± 0.001
Third day	0.017 cd ± 0.005	0.025 ab ± 0.003	0.022 cd ± 0.003
Fourth day	0.023 bcd ± 0.008	0.055 ab ± 0.042	0.028 bc ± 0.005
Fifth day	0.029 abcd ± 0.013	0.065 ab ± 0.051	0.035 ab ± 0.007
Sixth day	0.038 abc ± 0.016	0.145 ab ± 0.148	0.037 ab ± 0.007
Seventh day	0.040 ab ± 0.017	0.151 ab ± 0.144	0.039 a ± 0.008
Eighth day	0.045 a ± 0.019	0.198 a ± 0.185	0.043 a ± 0.010
LSD P ≤ 0.05	0.022	0.175	0.011

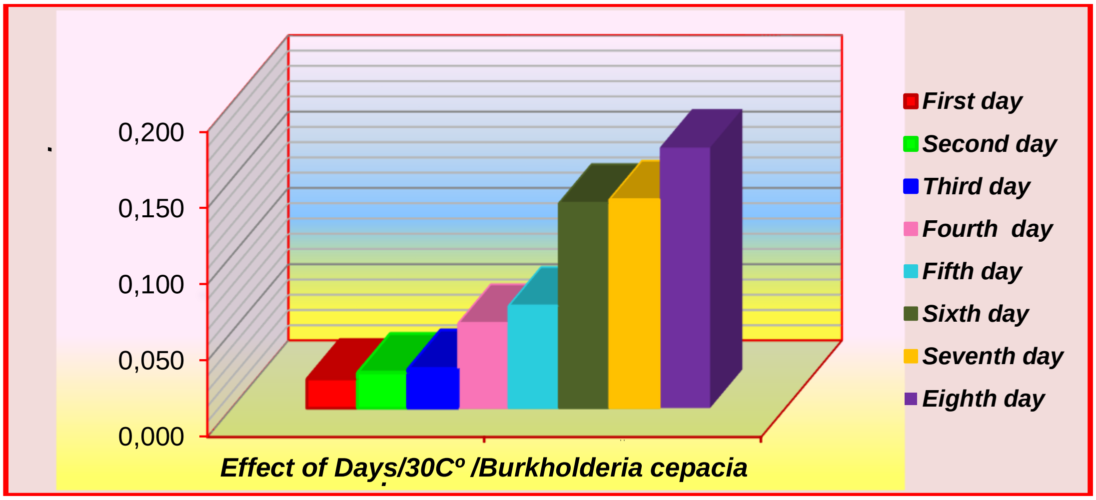

Figure (3) of Effects of incubation periods on growth of *B. cepacia* at optimum temperature

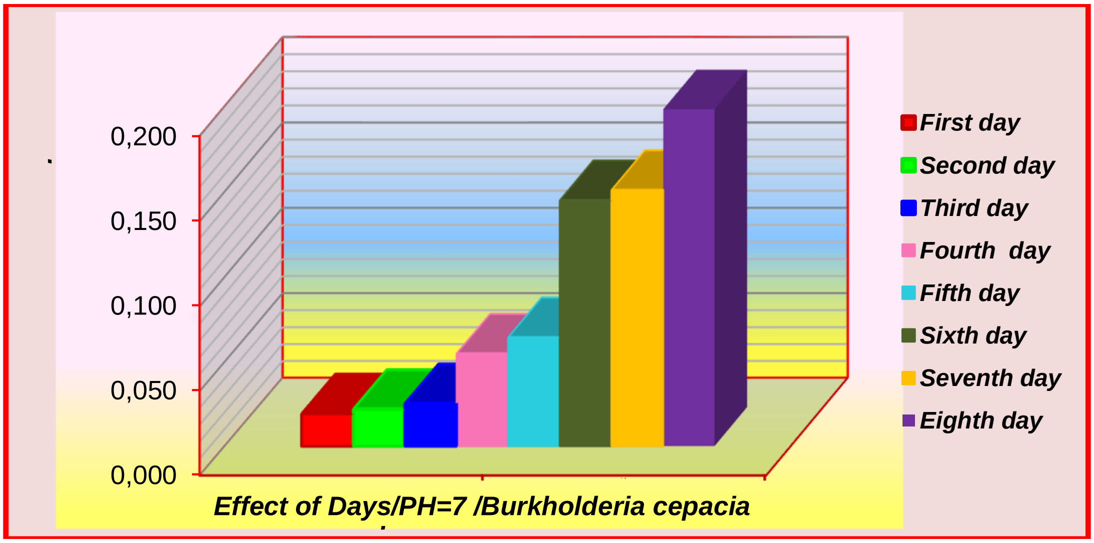

Figure (4) Effects of incubation periods on growth of *B. cepacia* at optimum pH

4. Discussion

Wastes, solid and liquid accumulated in the bottom of liquid petroleum gas cylinders and discharged from gas filling refineries to soil led to high contamination of soil. Therefore in this study trying to applicated bioremediation techniques to reduce pollution from these refineries. *B. cepacia* appeared high efficiency in bioremediation. In this study exampled optimum condition for growth of *B. cepacia* by measured optical density at wavelength 540nm, the result showed that The highest OD values obtained from optimization tests is (0.401) after 8 days at 30oC and pH 7.Therefore the optimum conditions for growth of B. cepacia was 30oC, pH 7, and growth increased with increase incubation period 8 days while biomass reach to 7.944 g/l after 10 days of incubation.

Conclusion and Recommendation

From the results of the present laboratory scale investigations, the following conclusions can be drawn, pollutants discharged from LPG refineries out of the american society of testing and materials ASTM limitations 6.7-3300 ppm., and the results indicate the high ability of the *Burkholderia cepacia*, to degrade hydrocarbons; hence, it can be very useful for environmental protection from wastes of gas filling refineries, and dominant of gram negative bacteria in contaminated soil with hydrocarbons due to have ability in consuming these compounds. The following points are recommended, regenerating bacteria by using cloning and recombinant DNA methods to produce bacteria have high ability to degrade hydrocarbons, more studies in this field is highly required which can be helped to reduce the harmful effects of soil contamination by wastes of gas filling refineries and modern techniques must be used in gas filling refineries to reduce the pollution of soil.

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