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Biological treatment of hydrocarbons contaminated soil by *Serratia ficaria*

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***Abstract*---**Petroleum is one of the most important substances consumed by man at present times, a major energy source in this century, petroleum oils can cause environmental pollution during various stages of production, transportation, refining and use, petroleum hydrocarbons pollutions ranging from soil, ground water to marine environment, become an inevitable problem in the modern life, current study focused on bioremediation process of hydrocarbons contaminants that remaining in the bottom of gas cylinders and discharged to the soil. Twenty-four bacterial isolates were isolated from contaminated soils all of them gram negative bacteria, bacterial isolates screening to investigate the ability of biodegradation of hydrocarbons, these isolates inoculated with modified mineral salt media containing 1% hydrocarbons for five days in shaking incubator 150 rpm at 30°C. Then measured optical density by a spectrophotometer (UV–9200) at waves length 540nm, biomass, where three isolates appeared highest ability to growth than others isolates. These three bacterial isolates were diagnosed by morphological features, gram stain, microscopically examination, biochemical tests, as well as by using VITEK 2 Compact device. One of three isolates was selected and result of identification of this bacterium showed that belonged to Serretia ficaria. Also in this research study of optimal conditions (incubation period, pH and temperature) for the growth of bacterial isolates and consumption of hydrocarbons, where the results indicated that the optimum temperature was 30°C and pH 7 , while optimum incubation period range between 5 to 8 days of incubation, after 10 days of incubation, bioremediation reach to more than 82%

***Keywords*---**biological treatment, bioremediation, contaminated soil, hydrocarbons, Serratia ficaria.

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1 Introduction

Soil is a privileged habitat for microorganisms and is the most biodiverse environment on Earth [1]. Soil microorganisms are a very important part of the environmental ecosystems, which could play an important role in ecological and biodegradable function processes in contaminated soils [2]. Also play a major role in adjust energy flow and cycle of matter by digesting plant, animal, and oil residues, and play a pivotal role in growth and development of agricultural crops, balance of the soil ecosystem 3]. Anthropogenic activities, commercial, industrial, agricultural, and military activity, largely in the 19th and 20th centuries, considers the main reasons for contamination problems of soil [4] and [5], and that lead to release of large amounts of petroleum hydrocarbons into the environment that can threaten human health and ecosystem function and caused high concerns in recent years [6] and [7].

Petroleum oils can cause environmental pollution during various stages of production, transportation, refining and use, petroleum hydrocarbons pollutions ranging from soil, ground water to marine environment, become an inevitable problem in the modern life [8] and [9]. Soil contaminated with hydrocarbons including gas cylinders residues, 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, release of hydrocarbons into the environment whether accidentally or due to human activities is a main cause of water and soil pollution [10].

bioremediation is environment friendly process that utilized a range of communities of microorganisms in combination with series of techniques to decontaminate polluted sites [11]. Due to their high metabolic diversity and high adaptability, microorganisms are able to live in the most varied of natural and artificial habitats created by environmental contamination. Different microbes can use a great variety of refractory pollutants, thus permitting their use in ex and in situ bioremediation. Bioremediation is the act of degradation, removal, reduction, transformation of contaminants or pollutants to less harmful substances through biological means [12].

The ability of microorganisms to bioremediation of pollutants is based on their oxidation and decomposition (biodegradation), assimilation or transformation into non-toxic compounds such as $CO2$ and $H2O$ (biotransformation) [13] and [14]. Serratia ficaria was first described in 1979 by Grimont et al. Straight rods with rounded ends. Serratia are widespread in the environment, are capable of thriving in diverse environments, including water, soil, and the digestive tracts of various animals [15] (Petersen and Tisa, 2013, the bacterium is an opportunistic human pathogen, for hospitalized humans facultatively anaerobic, endosporeformingrod-shaped bacteria of the Enterobacteriaceae family capitalizing on its ability to form tight-knit surface communities called biofilms [16].

2 Materials and Methods

2.1 Soil samples

Soil samples and wastes were collected from soil around gas filling refineries to isolate the hydrocarbons degrading bacteria, sampling of topsoil (0-15cm) 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

2.2.1 One gram of contaminated soil added to 50ml sterilize modified mineral salt medium in 250ml flasks and incubated at 30°C in a shaker incubator at 150 rpm for 5days, after incubation period, prepared series dilution of each flask(10-1 to 10-8), then 1ml of each dilution speared on the surface of nutrient agar plates and incubated at 37 °C for 24-48 hours, then selective morphological different colonies and streaking on nutrient agar and repeated several times until obtained pure culture and stored in refrigerator at 4°C until used in next experiments.

2.2.2 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 [17].

2.3 Screening of bacterial isolates

Twenty four bacterial isolates were obtained from soil samples, in primary screening; only five isolates were selected from theses twenty four bacterial isolates, and then in secondary screening only one bacterial isolated was selected that more efficient in biodegradation process.

2.3.1 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 hydrocarbons 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 [18]. 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 [19]. The highest biomass recorded was 5.047g/100ml.

2.3.2 Determination of bacterial growth by optical density

Isolates were inoculated with 100 ml of sterilize modified mineral salt medium MMSM containing 1% of hydrocarbons 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.

2.4 Optimization of physical parameters
2.4.1 Temperature
Optimum temperature was studied by using modified mineral salt medium MMSM inoculated with *S. ficaria* and incubated for 8 days in shaker incubator at 150 rpm at different temperatures 30, 35, and 40°C, growth of *S. ficaria* were detected by measured optical density by a spectrophotometer (UV–9200) at waveslength 540nm**.**

2.4.2 pH
Optimum pH was studied by using MMSM medium inoculated with *S. ficaria* and incubated for 8 days in shaker incubator incubator at 150 rpm and different pH 5, 7, and 9, growth of *S. ficaria* were detected by measured optical density by a spectrophotometer (UV–9200) at waveslength 540nm

2.4.3 Incubation period
Optimum incubation period was studied by using sterilize modified mineral salt medium prepared (100ml) in 500ml flask, then added 1ml of hydrocarbons, 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.

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

2.5.1 Morphological, cultural characteristics and microscopically examination
Bacterial colonies differ in their appearance, shape, size, margin, and surface features of colonies on nutrient agar plate were studied and a loopfull of the culture was fixed on a slide, and stained by Gram stain to examine Gram reaction to identify the groups of bacteria whether this isolate is a gram positive, gram negative, regularity, color, shape, and arrangement [20].

2.5.2 Biochemical test medium

Biochemical tests were used in the identification of bacteria, included: lactose fermentation test, oxidase test, cetrimide agar, catalase test, nitrate reduction test and indole test, and motility test [21] and [22].

2.5.3 Identification of bacteria using VITEK 2 device

There are 47 biochemical tests and one negative control well. Final identification results are available in approximately 10 hours or less, this device contains 64 biochemical tests. The gram negative card is based on establish biochemical methods and newly developed substrates measurement carbon source utilization, enzymatic activities, and resistance [23]. Vitek 2 gram negative correctly identified 96.8% of the isolates, including 6.4% low discrimination with the correct species listed, misidentifications occurred at 3.0% and no identifications occurred at 0.2%.

3. Results and discussion

3.1 Optimization of physical parameters

3.1.1 Temperature

When tested Optimum temperature by using modified mineral salt medium MMSM inoculated with *S. ficaria* and incubated for 8 days in shaker incubators at different temperatures 30, 35, and 40°C, growth of *S. ficaria* were detected by measured optical density by a spectrophotometer (UV–9200) at waveslength 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 *S. ficaria* was 30°C, as shown in Table (1) and (2), and Fig. (1) [24].

Table (1) Optical density (540 nm) for *S. ficaria* 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
S. ficaria	30	5	0.012	0.017	0.014	0.022	0.025	0.032	0.037	0.041
		7	0.042	0.048	0.094	0.112	0.099	0.163	0.224	0.285
		9	0.021	0.025	0.02	0.026	0.037	0.051	0.102	0.151
	35	5	0.012	0.017	0.014	0.022	0.036	0.038	0.037	0.039
		7	0.033	0.035	0.046	0.06	0.057	0.058	0.099	0.106
		9	0.025	0.023	0.026	0.03	0.028	0.031	0.029	0.037
	40	5	0.002	0.019	0.025	0.028	0.025	0.03	0.028	0.037
		7	0.022	0.021	0.027	0.03	0.033	0.035	0.031	0.047
		9	0.01	0.015	0.022	0.026	0.023	0.027	0.03	0.032

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

PH \ TEMP.	30Cº	35Cº	40Cº
PH=5	0.025 b ± 0.011	0.027 b ± 0.012	0.024 a ± 0.010
PH=7	0.133 a ± 0.085	0.062 a ± 0.027	0.031 a ± 0.008
PH=9	0.054 b ± 0.048	0.029 b ± 0.004	0.023 a ± 0.007
LSD P ≤ 0.05	0.059	0.018	0.009

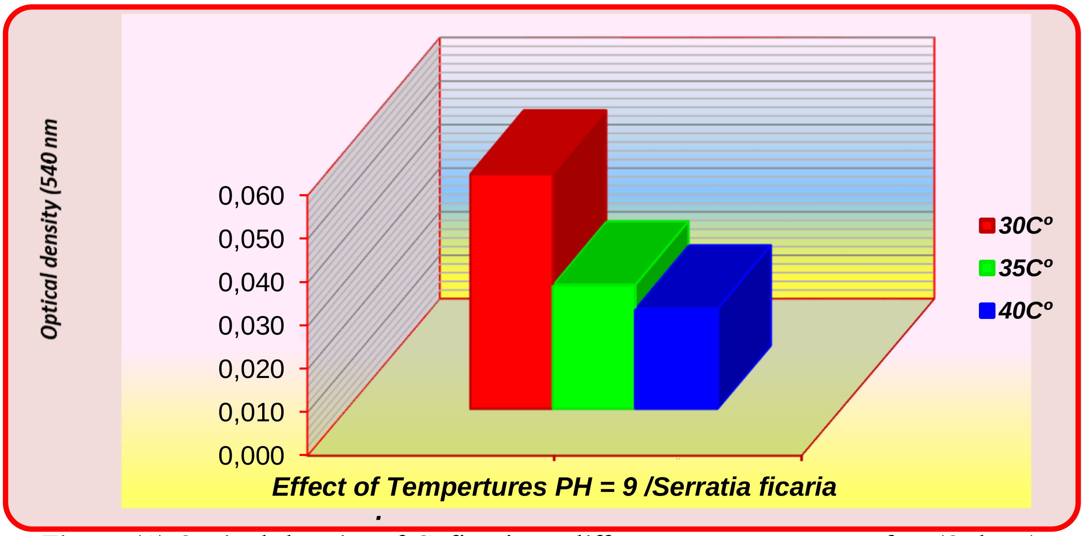

Figure (1) Optical density of *S. ficaria* at different temperatures after (8 days) incubation

3.1.2 pH

After incubated for 8 days in shaker incubator at 150 rpm and different pH 5, 7, and 9, then tested optimum pH for growth of *S. ficaria* and growth of *S. ficaria* 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 ($p\leq0.05$) the best pH for growth of bacterial isolate B. cepacia was 7, as shown in Table (1) and (2) and Fig. (2) and (3).

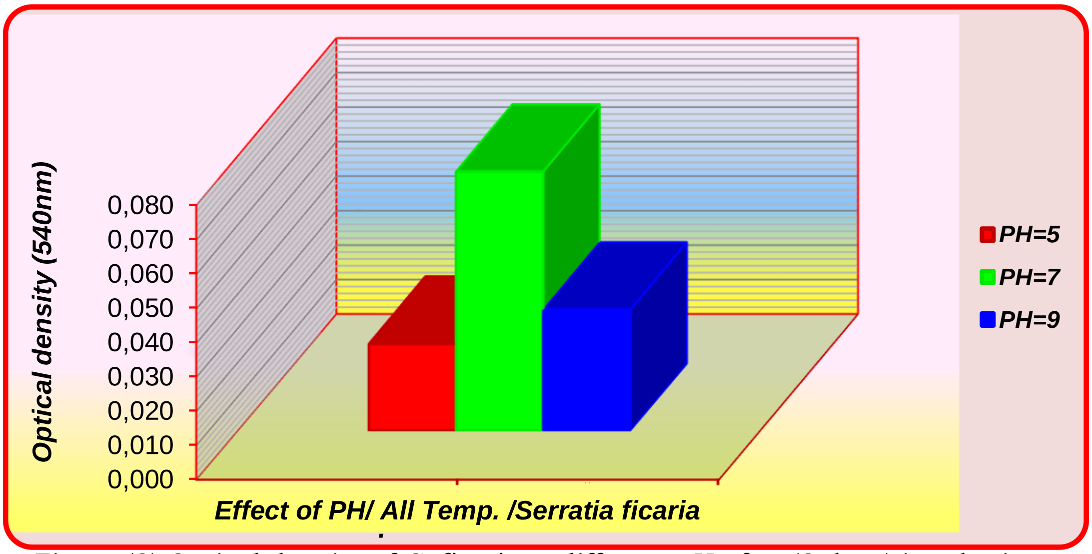

Figure (2) Optical density of *S. ficaria* at different pH after (8 days) incubation

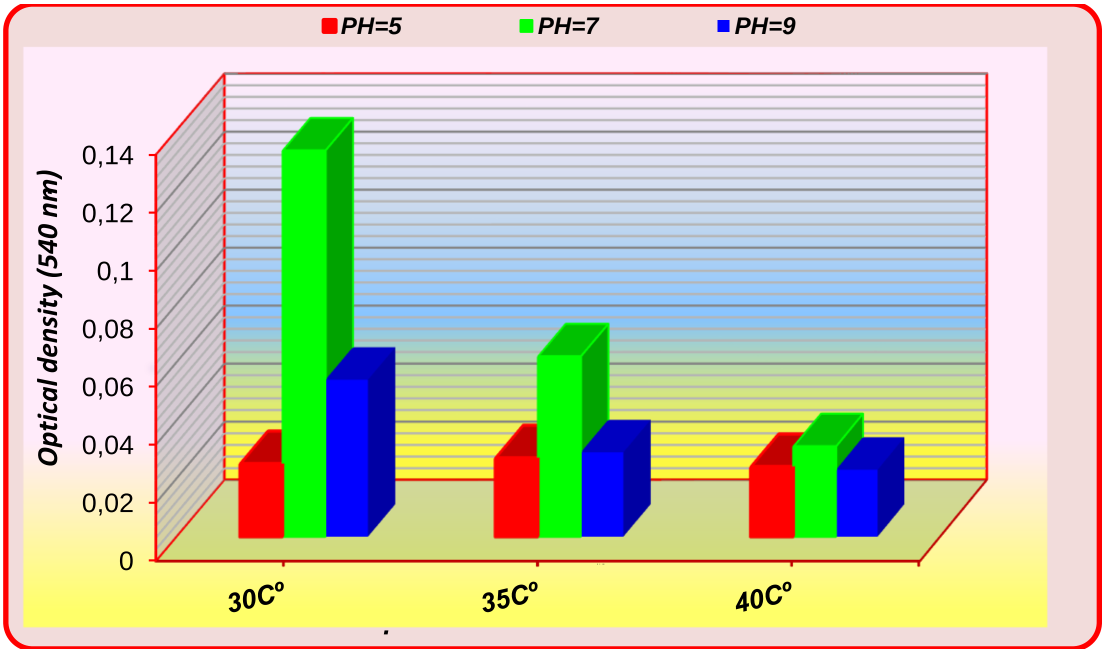

Figure (3) Effects of different pH, temperatures on growth of *S. ficaria* after 8 days incubation

3.1.3 Incubation period

Optimum incubation period for growth of *S. ficaria* 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 *S. ficaria* at different temperature

TEMP. / Days	30C°	35C°	40C°
First day	0.025 b ± 0.015	0.023 a ± 0.011	0.011 d ± 0.010
Second day	0.030 b ± 0.016	0.025 a ± 0.009	0.018 cd ± 0.003
Third day	0.043 b ± 0.045	0.029 a ± 0.016	0.025 bc ± 0.003
Fourth day	0.053 ab ± 0.051	0.037 a ± 0.020	0.028 b ± 0.002
Fifth day	0.054 ab ± 0.040	0.040 a ± 0.015	0.027 bc ± 0.005
Sixth day	0.082 ab ± 0.071	0.042 a ± 0.014	0.031 ab ± 0.004
Seventh day	0.121 ab ± 0.095	0.055 a ± 0.038	0.0430 ab ± 0.002
Eighth day	0.159 a ± 0.122	0.061 a ± 0.039	0.039 a ± 0.008
LSD P ≤ 0.05	0.115	0.040	0.009

Table (4) Statistical analysis of effects of Incubation period on growth of *S. ficaria* at different Ph

TEMP. / Days	PH=5	PH=7	PH=9
First day	0.009 e ± 0.006	0.032 b ± 0.010	0.019 b ± 0.008
Second day	0.018 d ± 0.001	0.035 ab ± 0.014	0.021 b ± 0.005
Third day	0.018 d ± 0.006	0.056 ab ± 0.035	0.023 b ± 0.003
Fourth day	0.024 cd ± 0.003	0.067 ab ± 0.041	0.027 ab ± 0.002
Fifth day	0.029 bc ± 0.006	0.063 ab ± 0.033	0.029 ab ± 0.007
Sixth day	0.033 ab ± 0.004	0.085 ab ± 0.068	0.036 ab ± 0.013
Seventh day	0.034 ab ± 0.005	0.118 ab ± 0.098	0.054 ab ± 0.042
Eighth day	0.039 a ± 0.002	0.146 a ± 0.124	0.073 a ± 0.067
LSD $P \leq 0.05$	0.008	0.113	0.050

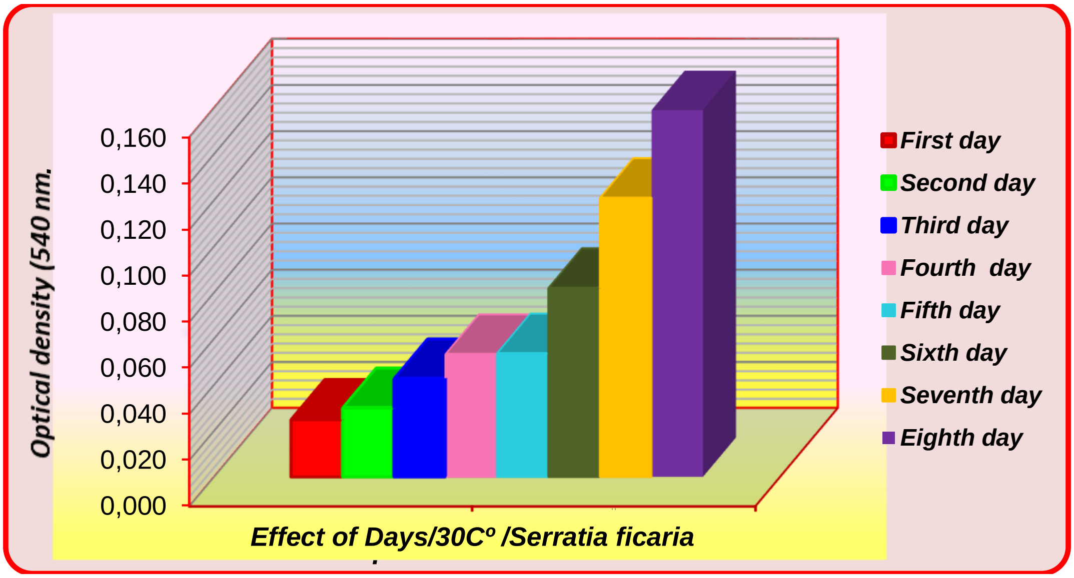

Figure (3) Optical density of *S. ficaria* at different pH and temperatures after (8 days) incubation

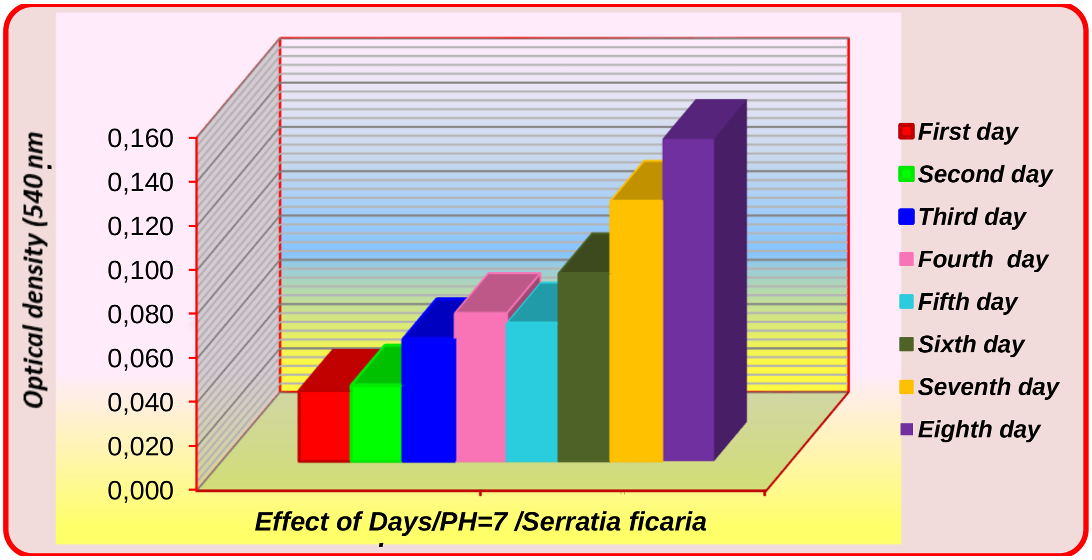

Figure (4) Optical density of *S. ficaria* at different pH and temperatures after (8 days) incubation

4. Discussion

Wastes that 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. *S. ficaria* appeared high efficiency in bioremediation. In this study exampled optimum condition for growth *S. ficaria* by measured optical density at wavelength 540nm, the result showed that The highest OD values obtained from optimization tests is 0.285 after 8 days at 30ºC and pH 7.Therefore the optimum conditions for growth of *S. ficaria* was 30°C, pH 7, and growth increased with increase incubation period while biomass reach to 5.047g/100ml after 10 days of incubation with more than 82% percentage of removal of hydrocarbons.

5 Conclusion and Recommendation

From the results of the present laboratory scale investigations, the following conclusions can be drawn, the results indicate the high ability of the *S. ficaria*, 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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