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ISOLATION AND IDENTIFICATION OF PETROLEUM PRODUCTS MIXTURE DEGRADING BACTERIA IN ACTIVATED SLUDGE FROM MUNICIPAL WASTEWATER TREATMENT IN ASSALUYEH REGION (IRAN) AND THEIR GROWTH KINETICS ASSESSMENT

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ABSTRACT

The oil products such as diesel oil, gasoline and kerosene are the most important environmental pollutants. Extraction, transportation, storage or misusing of these products has caused environmental pollution. Today, bioremediation method with the help of exogenous and indigenous microorganisms could be used and the polluted environment back to desirable primary situation. The goal of this research is isolation and identification of petroleum products degrading bacteria of from activated sludge of Assaluyeh region in Iran and to assess their growth kinetics. The samplings were done of two treatment plants. Isolation of degrader bacteria was accomplished by samples culture on basic mineral medium. By broth and agar basic mineral medium and emulsification test was utilized to screening strongest strains. The kinetics assessment of bacteria in oil products mixture evaluated for 7 days. In this study 5 bacteria including *Pseudomonas* sp, *Bacillus* sp, *Corynebacterium* sp, *Acintobacter lowffi* and *Alcaligenes faecalis* as stronger petroleum products degrading bacteria have been detected. According to emulsification tests and bacteria growth assay in basic mineral medium and growth kinetics *Pseudomonas* sp and *Bacillus* sp were introduced as the most powerful degrader bacteria respectively. Findings showed that activated sludge contains araised potential populace and varieties of bacteria which have high capabilities for hydrocarbons degradation in petroleum products. By more assessments, stronger bacterial strains could be found inactivated sludge

which have more concordance with petroleum products mixture and can be applied for bioremediation of polluted areas.

Keywords: **Bioremediation, Activated Sludge, Petroleum Products, Growth Kinetics, *Pseudomonas***

INTRODUCTION

The importance of environmental pollution controlling has exceeded along with cities expansion, population increase and industrial development. Wastewaters are one of the reasons for environmental pollution which should be refined and returned back to water circulation in the nature **[1]**. Wastewater treatment might be taken place by mechanical or physical, chemical and biological methods **[2]**. Microorganisms are used in biological treatment. These microorganisms are able to absorb the organic materials and nutrients such as nitrogen and phosphorus, for their growth. They need carbon resources for their cellular synthesis and energy resources for their survival. Carbon resources for cellular synthesis are CO_2 or organic compounds available in wastewater and energy resources are including the light or chemical compounds which are accessible in wastewater **[3]**. The activated sludge process is the most common biological process for sanitary and industrial wastewater refining. Activated sludge contains various numbers of microorganisms. The majority of bacteria in activated sludge belong to gram negative genera **[4]**. Various studies have been developed regarding bacteria isolation from activated sludge. **Kampfer, 1997, [5]**, detected some filamentous bacteria in activated sludge by utilizing the microscopic, immunologic methods and oligonucleotide probes marked by 16S rRNA and 23S rRNA. **Sharifi-Yazdi *et al*., 2001, [6],** isolated and identified existing bacteria in activated sludge by industrial treatment process of wastewater. During this research, gram-negative bacteria were dominant in activated sludge and majority of isolated gram negative bacteria belonged to genus *Flavobacterium*, while 22% of isolated bacteria belonged to genus *Pseudomonas.* The only gram-positive bacteria in this report were belonged to genus *Micrococcus* **[6]**. The use of enormous amounts of petroleum products contributes highly to environmental pollutions. Spills of Hydrocarbons occur from several causes, including leakage from tanks, blowouts and dumping of waste petroleum products. The elevated loading of petroleum hydrocarbons in soil causes a significant decline in soil quality, and these soils have become unusable

[7]. Bioremediation is a biological method by microorganisms for reducing different pollutants. Bacteria are most important than the other microbs because of having various degrading enzymes **[8]**. There are two bioremediation techniques which can be used in all the available technologies of treatment in order to try to maximize its efficiencies: the biostimulation, in which there is the increase of the indigenous populations activity by adding nutrients and/or a terminal electron acceptor, and the bio-augmentation, in which there is the increase of the pollutant degradation potential by adding exogenous degrading microbial strains **[9]**. In some soils where bioremediation is not possible by microorganisms, bioaugmentation has introduced as a process for increasing bioremediation rate **[10]**. There are many studies on bioremediation of petroleum products contaminated by exogenous bacteria. **Aislabie *et al*., 2006, [11]**, studied hydrocarbons bioremediation on contaminated polar soils. Climate conditions such as soil temperature fluctuations, low level of nutrient, humidity and improper pH restricting factors for microbial activities and decline hydrocarbons biodegradation. **Tahhan *et al*., 2011, [12]**, increased degradation of total petroleum hydrocarbons by inoculating two selected bacteria from soil. In their study, inoculation was performed in two period of time 62 days and 198 days and TPHs were decreased more than 30%. Through other research, researchers implemented a bioremediation in petroleum contaminated areas by utilizing the activated sludges bacteria of minicipal wastewater system. **Rojas *et al*., 2007, [13]**, isolated and detected bacteria from mixture of activated sludge related to wastewater treatment system. In this study five microorganisms detected: *Psedomonas fluorescens, Pantoea* sp*, Chrysomon luteola, Proteus peenneri* and *Serratia* sp. It revealed that theses isolated bacteria from mixture of activated sludge are able to degrade derived compounds from wastewater systems such as 1,1,2-trichloroethane and ethyl benzene during oil and petrochemical process. Activated sludge is rich in terms of bacterial aggregation. The South Pars Special Economic Energy Zone in Assaluyeh (Bushehr, Iran) has petrochemical and gas and oil refinery so, pollutions have distributed in this region, consequently. The aim of this research is isolation and identification of petroleum products mixture degrading bacteria from activated sludge of Assaluyeh region in Iran and to assess their growth kinetics.

MATERIALS AND METHODS

Sampling

Activated sludge samples were taken from two treatment plants based in 1, 2 and 5 camping sites in Assaluyeh at the South Pars Special Economic Energy Zone for the isolation petroleum productions mixture degrading bacteria. Samples put into flasks containing ice, and carry to the laboratory in less than 24 hours. The experiment was initiated on the sludge activated in the laboratory.

Counting Bacteria

After transferring the samples, the diesel oil, gasolin and kerosen degrading bacteria were counted by the total viable plate count method. In this method the dilution from 10^{-1} and 10^{-9} was prepared from activated sludge samples by physiological serum, then culture in nutrient agar medium, containing 0.5 ml of each petroleum products and nutrient agar medium without petroleum products by surface plate method. The cultured plates, then incubated at 30°c for 48 h. After incubation, the number of colonies was counted in culture with and without petroleum products (cfu/g) **[14, 15]**.

Isolation of Diesel Oil, Gasoline and Kerosene Degrading Bacteria from Activated Sludge

The medium mineral basal salt (MBS) was used to enrichment and doing more tests. Firstly, for enrichment purpose 95 ml of bacterial basal medium poured in the 250 ml flasks, then 5ml sample of activated sludge were added to flasks. These flasks were completed by 1 ml of petroleum products (diesel oil, gasoline and kerosene) as the only source of carbon and energy. Enriched medium in 30°c was incubated in shaker incubator along with aeration for one week. This process was done unit the environment became completely turbid to more bacterial enrichment **[16, 17]**. Next, samples were further cultured on the MBS agar medium contain diesel oil, gasoline and kerosene. The plates were incubated in 30°c for 3-5 days and out wardly different bacterial colonies were purified consecutively on medium mineral basal containing petroleum products and then on the blood agar medium **[18]**.

Growth Assessment of Selected Strains in Mineral Basal Medium Contain Petroleum Products Mixture

After bacterial strains isolation, they were cultured in basal mineral solid and liquid medium with mixtures of substrates as carbon resource and different concentration to screen the best and most protent strains. Those bacteria that had begun to grow in the minimum of time and also had most turbidity were chosen to next step **[19]**.

Emulsification Test

In this process, emulsification test was used based on **Francy *et al.* method in 1991** within a minimal salt solution (MSS). Bacterial suspension was prepared based on 0.5 MacFarland. The suspension were inoculated to tubes contain MSS medium with 0.5 ml diesel oil, gasoline and kerosen mixture and after were mixed by vortex machine. Culture media were incubated under 30°c within the three days. This process repeated for every selected colony. After incubation, tubes were mixed again by vortex, and incubated under 30°c for more 2 h. Emulsification rate was reported from 0 to 4. In this stage, emulsification test was repeated several time to more accurateness. In tubes related to each bacterium was surveyed emulsification average rate were included as strong bacteria strains **[20]**.

Identification of Diesel Oil, Gasoline and Kerosene Degrading Bacteria

After diesel oil, gasoline and kerosene degrading strains selection, to identify these bacteria, the colonies were morphologically evaluated. Tests which were conducted on the colonies including gram staining, morphological characteristics study, oxidase and catalase reaction, sugar test (lactose, sucrose, glucose fermentations), along with-other diagnostic tests (IMVIC) **[21, 17]**.

Growth Kinetics

Optical density (OD) assay in 600 nm was used for evaluation of isolated bacteria growth curve in different concentrations of petroleum products mixture (contain dieasl oil, gasolin and kerosen). In order to determine the bacteria growth 20 ml of petroleum products mixture broth medium (with different concentrations) were poured into separate erlenmeyer flasks. Then, the bacterial suspension was prepared based on the 0.5 MacFarland standard and 5 ml of that was added to each culture medium. Three erlenmeyers were used for each bacteria suspension. In each erlenmeyer flask 1, 3 and 4.5 ml concentration of petroleum products mixture were added. In this study, one erlenmeyer was used as control for each bacterial suspension. In the control medium, there was basal medium and bacterial suspension without petroleum products. Then, all of the erlenmeyers were incubated under 30°c and 120 rpm for 7 days in shaker incubator. Finally, absorption measurement in different concentrations in 600 nm were evaluated by spectrophotometer every 12 h. So bacteria growth curve was illustrated **[22, 17]**.

Statistical Analysis

The statistical of obtained result was performed by SPSS software and ANOVA test.

RESULTS

Isolation and Identification of the Strongest Petroleum Products Mixture Degrading Bacteria

About 40 colonies were isolated in this stage and 25 isolated colonies with more growth power and turbidness were selected. Emulsification average rate for all selected bacteria was higher than 2.5 that represented selection of the potent petroleum products degrading bacterial strains in this study. Totally, 5 bacteria having above emulsification rate had been selected for identification and evaluation the growth kinetics **(Table 1)**. These selected strains were *Acintobacter lowffi, Alcaligenes faecalis, Corynebacterium* sp, *Bacilus* sp and *Pseudomonas* sp.

Counting Bacteria

The results of bacterial counting showed that maximum number of bacteria was 3.625±0.016 in control medium and minimum number in the medium containing petroleum products that was 3.235±0.016. There was a significant difference at the 5% level between these 2 medium **(Figure 1)**.

Average counted bacteria in comparison between two stations showed that, maximum number of bacteria was 3.473±0.010 in station (camp) 1,2 and minimum of that was 3.361±0.010 in station (camp) 5. These two groups had significant differences at 1% level **(Figure 2)**.

Growth Kinetics

The growth curve of the strains on different concentrations of petroleum products mixture (diesel oil, gasolin and kerosene) showed that 4 bacteria including *Alcaligenes lowffi, Alcaligenes faecalis, Bacillus* sp and *Corynebacterium* sp had been prospered the best growth in petroleum products low concentration (1.5 ml). This bacterial growth rate had been decreased with high concentration **(Figure 4, 5, 6, 7)**. But *Pseudomonas* sp had maximum growth rate in high oil products concentration (4.5 ml) and decreasing the oil products concentration led to decreasing its growth rate **(Figure 3)**. *Pseudomonas* sp had maximum OD (0.42) in 600 nm wavelength and 4.5 ml concentration after 96 h among other bacteria. *Bacillus* sp was in the second degree with 0.254 nm (OD) in 1ml after 120 h.

In this study, Based on growth kinetics evaluation, *Pseudomonas* sp and *Bacillus* sp were identified as the most powerful isolated

strains respectively in order to degradation of petroleum products mixture.

Table 1: Evaluation of Identified Strains Based on the Petroleum Products Emulsification in Minimal Salt Solution

Identified bacteria	Emulsification average power
***Pseudomonas* sp**	**4.0**
***Bacilus* sp**	**3.9**
A. lowffi	**3.4**
A. faecalis	**2.8**
***Corynebacterium* sp**	**2.6**

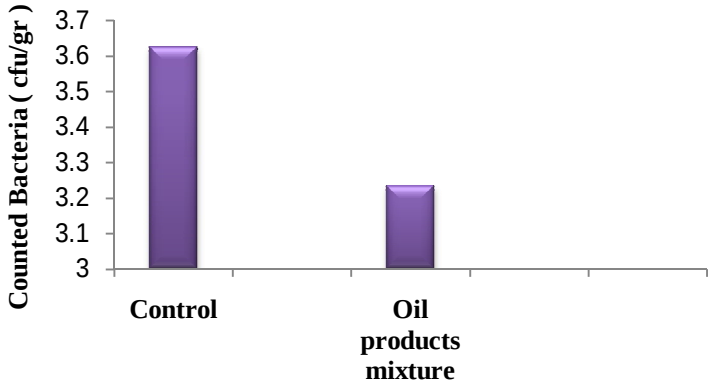

Figure 1: Counted Bacteria in Control Medium and the Medium Containing Petroleum Products

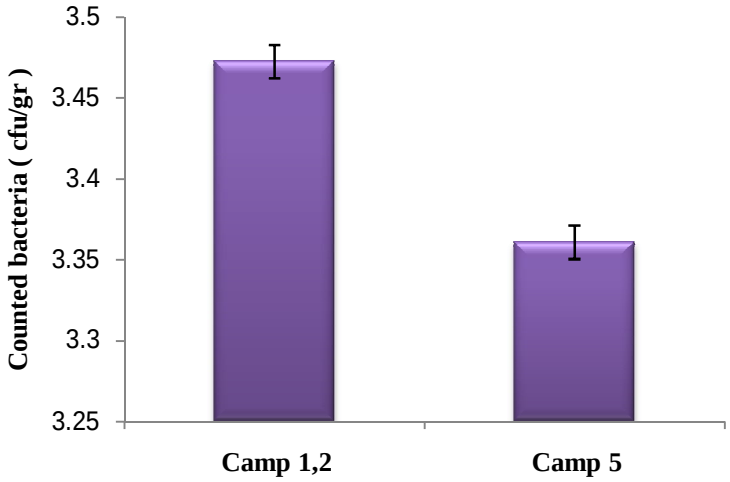

Figure 2: Counted Bacteria in Different Stations

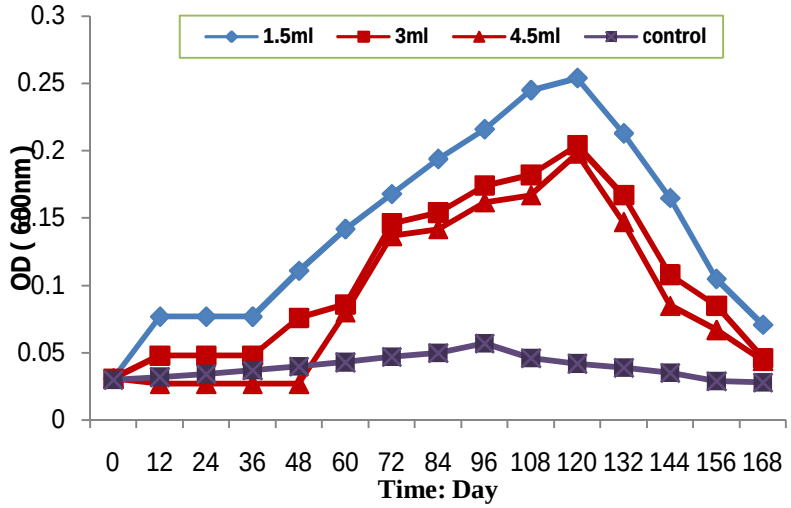

Figure 3: ***Pseudomonas*** **sp Growth Curve in Different Concentrations of Petroleum Products Mixture**

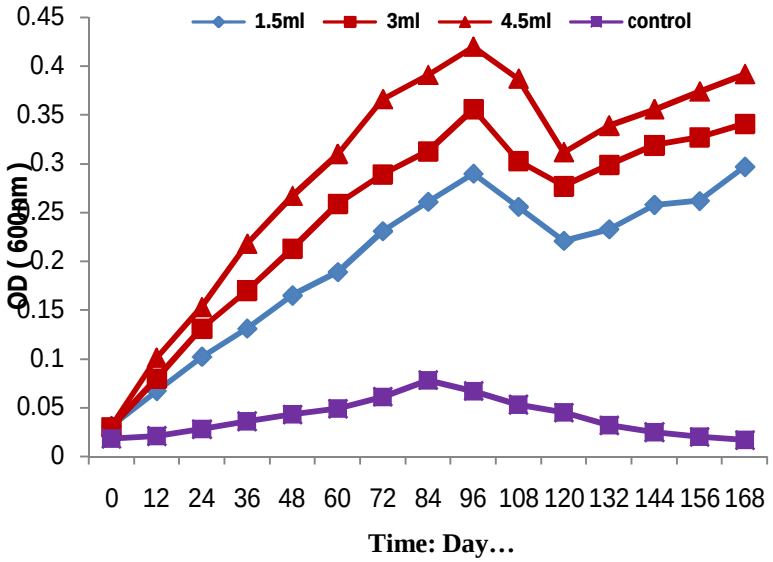

Figure 4: ***Bacillus*** **sp Growth Curve in Different Concentrations of Petroleum Products Mixture**

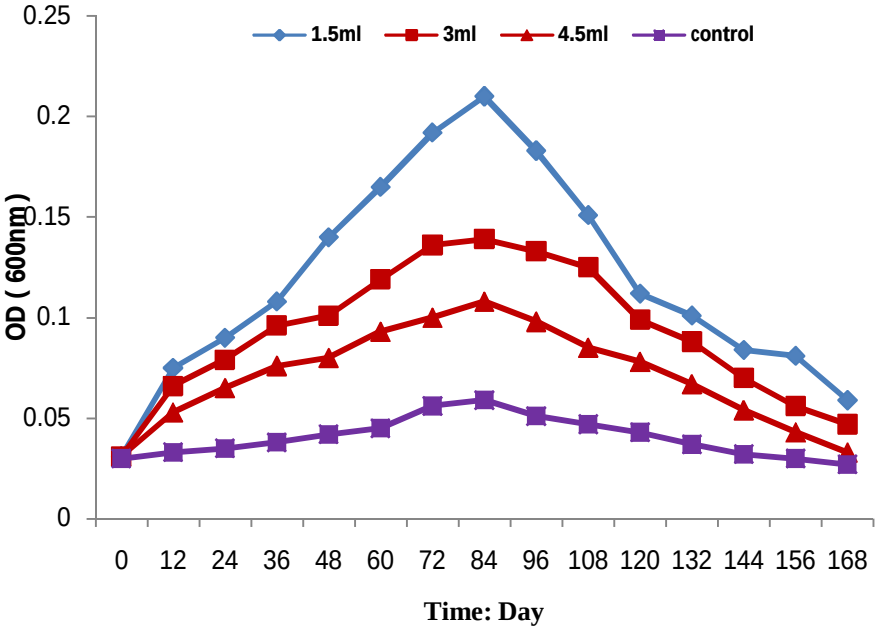

Figure 5: *A. lowffi* Growth Curve in Different Concentrations of Petroleum Products Mixture

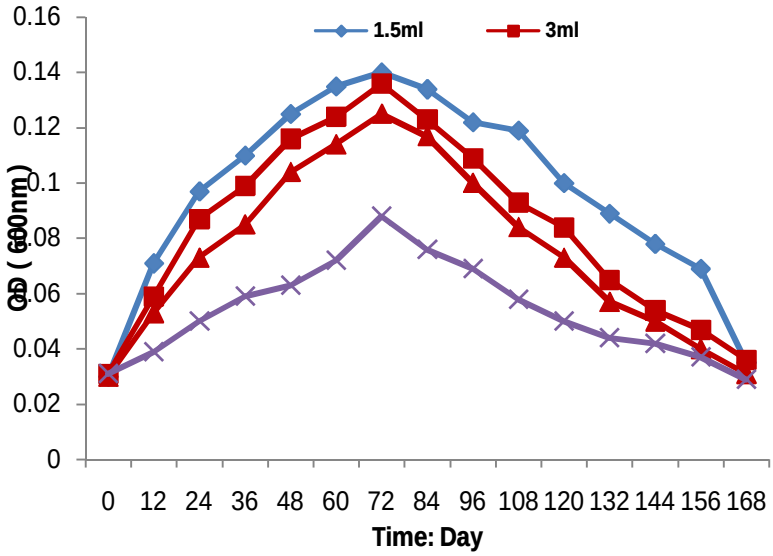

Figure 6: *A. faecalis* Growth Curve in Different Concentrations of Petroleum Product Mixture

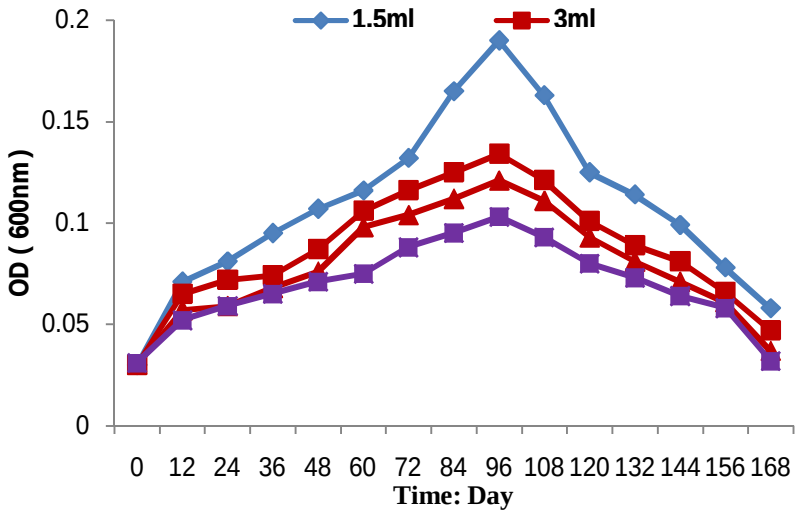

Figure 7: *Corynebacterium* sp Growth Curve in Different Concentrations of Petroleum Product Mixture

DISCUSSION

Petroleum is a crucial source of pollution in countries whit oil reserves. Recently, chemical, physical and biological methods have been used to clean up the petroleum contaminations. These methods have restricting influences with high costs. Bioremediation among the other methods is a provident technology for treating the petroleum hydrocarbons contaminated sites **[23]**. Bioaugmentation might be a worthwhile way to increment of contaminated soils bioremediation **[10]**. Many environments shave thickly populated of microorganisms which could be used for cleansing the polluted areas.

Roberto *et al.*, 2003, [24], studied bioremediation of oil-gas contaminated soil at the South Pole. In this research, cold-tolerant bacterium as exogenous microorganisms (B-2-2) was assessed for bioaugmentation process. Bioaugmentation with B-2-2 strain improved the bioremediation efficiency (75% of the hydrocarbon was removed). In the present study, activated sludge was used for isolation and identification of petroleum products degrading bacteria. *Alcaligenes lowffi, Bacillus* sp, *Corynebacterium* sp, *Pseudomonas* sp and *Alcaligenes faecalis* were identified and isolated as the strongest petroleum products (diesel oil, gasoline and kerosene) degrading bacteria. The emulsification tests were used for selecting the most powerful degrader bacteria. Samples with 2.6 – 4.0 emulsification average rate were selected as index bacteria. Results of emulsification tests showed that *Pseudomonas* sp has highest emulsification

rate (4) which could emphasis its great power for petroleum product degradation in high concentration. *Pseudomonas* sp has different degrader enzymes that help it to grow in high concentration. Also the *Pseudomonas* is capable using different substrates to produce biosurfactants. These biosurfactants increase oil hydrocarbons emulsion and change the surface tension of them **[25]**. Therefore, emulsification properties been demonstrated to enhance the hydrocarbons degradation in the environment **[26]**.

Results of turbidity test showed that culture medium of *Pseudomonas* sp altered to green color and this alteration for *Bacillus* sp and *Corynebacterium* sp was from clear yellow to dark yellow. The culture medium color change showed bacteria produced the secondary metabolites during petroleum products degradation and turning them to more simple compounds. In the present study, evaluation of growth kinetics of 4 bacteria including *Alcaligenes lowffi, Alcaligenes faecalis*, *Bacilus* sp and *Corynebacterium* sp showed that low growth rate of bacteria is due to increasing the petroleum products concentration. Reducing bacterial growth rate might be due to toxicity of petroleum products diesel oil, gasoline and kerosene and their hydrocarbon structures **[27]**. In Iran **Kafilzadeh *et al*., 2011, [17]**, studied growth kinetics of Pyrene degrading bacteria. In this study, they observed bacteria growth decreased in high concentration of aromatic compound. In the present study, growth rate of *Pseudomonas* sp increased in high concentration petroleum products mixture. This result was same as what **Nnamchi *et al*., 2006, [28]**, had achieved. They had proven direct relation between bacterial growth rate and concentration of considered hydrocarbon.

CONCLUSION

In this research, the most powerful bacteria for degradation of petroleum pollutants, such as diesel oil, gasoline, and kerosene were identified by isolating bacteria from activated sludge of Assaluyeh wastewater treatment. Therefore, activated sludge bacteria could be used for bioaugmentation and bioremediation contaminated areas with petroleum products.

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