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Native Bacterial Mixed Culture: A Proportionate Solution for Refinery and Petrochemical Wastewaters

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ABSTRACT

Background: Heat, chemical and organic pollution are three types of environmental pollution, caused by refinery and petrochemical industries. Problems caused by hear and chemical pollutants are currently resolved to some extent but organic pollution such as Poly Aromatic Hydrocarbons (PAHs) are still considered as important problems of industry and environment.

Objectives: A laboratory study was carried out to investigate the effects of native bacterial mixed culture (BMC) isolated from mixtures of refinery and petrochemical wastewaters for treatment of wastewaters of refinery and petrochemical industries.

Materials and Methods: All bacteria were isolated from two refineries and two petrochemical plants of Iran. Several bacterial strains from both kinds of wastewater were mixed and two final stock culture collections (BMC_a and BMC_b), showed the ability to improve the growth among strains. BMC_a was added to the refinery wastewater (activated sludge influent sample) and BMC_b was added to petrochemical wastewater (activated sludge influent sample). The effects of continuous and non-continuous aeration at high and low pressures, along with the effects of nutrient addition in the beginning of experiment versus sequential addition at specific time intervals, were studied.

Results: Native BMC, when continuous high level aeration was used, decreased chemical oxygen demand (COD) in refinery and petrochemical wastewaters for about 81% and 63%, respectively. Gradual addition of nutrients increased COD removal of refinery and petrochemical wastewaters to 85% and 87%, respectively.

Conclusions: Native BMCs from mixture of refineries and petrochemical wastewaters can be an effective method of wastewater treatment of both regional refinery and petrochemical plants. High pressure continuous aeration and gradual nutrient addition to the native BMCs can improve bioremediation of organic wastewater in different industries.

Keywords: Agricultural Inoculants; Environment; Petroleum; Waste Management

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Implication for health policy/practice/research/medical education:
Native Bacterial Mixed Cultures from mixture of refineries and petrochemical wastewaters can be used for wastewater treatment of both regional refinery and petrochemical plants as an effective and inexpensive method to reduce environmental oil industries induced contamination.

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1. Background

Heat, chemical and organic pollution are three types of environmental pollution, caused by refinery and petrochemical industries. Problems related to the first and second agents have been resolved to some extent but organic pollutants such as poly aromatic hydrocarbons (PAHs) are still considered as important problems of industry and environment (1). Pollution caused by petroleum industry strongly affects the environment, fauna (2, 3) and especially humans (4-7).

Effluent of these plants in developing countries are not exactly similar to that of developed countries but also there is a very wide range in developing countries; in other words, chemical oxygen demand (COD) and other specifications such as pH of wastewater differ in a wide range. On the other hand, environmental management of refineries and petrochemical plants is hard in developing countries due to the low allocated budget.

Bioremediation seems worth of further studies as an effective and also less expensive method (8). Generally, bacteria are considered to be the most powerful organisms for bioremediation (9-11). Aerobic bioremediation method is the better for treatment of organic materials which are soluble (12, 13). Adding nutrients (nitrogen and phosphorus) to the treating material has positive effects on treatment of organic wastewaters (14, 15). Various species have different affinities to several substances; therefore, mixed cultures can be more effective in comparison with pure ones (16). In some studies, native species have been acting more effectively than non-native ones (9, 17). We could not find extensive investigations on evaluation of isolated BMCs from mixture of refinery and petrochemical wastewaters for treatment of a refinery or a petrochemical industry pollutants separately.

2. Objectives

The purpose of this study was to evaluate the features of Bacterial Mixed Culture (BMC), isolated from the mixture of effluents of refinery and petrochemical plants in Iran, and studying the results of aeration and adding nutrients at the final treatment of refinery or petrochemical plants' effluents.

3. Materials and Methods

3.1. Wastewater Sampling

Five separate sampling runs were operated for bacterial isolation, pilot study, aeration tests (2 times) and nutrient addition test, respectively. First sampling run was taken from influent and effluent of aeration unit and effluent of clarifiers, from two biggest petroleum refineries of Iran with crude oil influent (Tehran and Khoozestan) and two petrochemical plants of Iran with wide range of petrochemical products (Arak and Abadan). In the next four sampling runs, samples were collected from influent of activated sludge tank of wastewater treatment unit of one of petrochemical plants (Arak Petrochemical) and one of two refineries (Tehran Oil Refinery).

Samples were collected in sterile bottles and transferred at 4° C for bacterial experiments. Sampling runs were performed from April to July of 2009. The mean minimum and maximum temperature were 22° C and 43.5° C, respectively. The mean minimum and maximum atmospheric humidity during sampling were 27% and 59%, respectively.

3.2. Isolation of Bacteria

Samples were neutralized and after adding Tween® 80 (Merck, Germany), they were left on the rotator for 5 minutes at 150 RPM. Dilutions of 10^{-1} to 10^{-4} of the samples were prepared and inoculated on Muller Hinton agar and Peptone Yeast Extract Agar (PYEA) (Merck, Germany) and incubated at 37°C for 72 hrs. Colonies were isolated and cultured on Trypticase Soy agar (Merck, Germany) in order to make pure cultures. Isolated bacteria were identified by colonial morphology, Gram strain and biochemical tests. They were kept frozen at -20° C in Trypticase Soy broth to which 15% (v/v) glycerol was added.

3.3. Bacterial Mixed Culture Preparation

After thawing the frozen bacteria, they were cultured on Brain Heart Infusion (BHI) agar (Merck, Germany) and sub cultured twice. According to our three rules, the bacteria were randomly divided into 24 BMCs (each containing 4 to 6 species). The first rule was that a *Pseudomonas spp*., should be present in each BMC as a Gram-negative bacilli in order to produce a biosurfactant reagent (18). The second rule was that at least one strain of *Bacillus spp*. should be present in each BMC which is known as the main factor for bioremediation of organic wastewater (19). The third rule was that Acinetobacter spp. for removal of organic materials (especially compounds containing phosphorus) (20) and *staphylococcus saprophyticus* for removal of organic materials (especially urea) (21) were added to BMCs if stock culture collections showed the ability to improve the growth as compared to other strains.

Bacterial suspension solutions with turbidity of 0.5 McFarlan standard (1×10^{-8} CFU/mL) were prepared from each species (22). Twenty microliters from each species' suspension were mixed together in BHI broth medium to make 24 BMCs. They were placed on the shaker and were incubated for 24 hours. The serial dilutions of 10-3 to 10-6 were prepared and were cultured on BHI agar medium. After 24 hours, plates were investigated to see if there were errors in the three mentioned conditions.

Eight BMCs were selected and species of these BMCs were put together randomly in various combinations, following the three mentioned rules. Fifteen BMCs were prepared and were evaluated for growth of all inoculated species. Eleven BMCs were chosen according to the three mentioned rules. Two milliliter aliquots of 11 BMCs' BHI were added to refinery and petrochemical wastewater samples separately. The volume of each sample was 2 liter. They were placed on the shaker and were incubated for 24 hours. Serial dilutions of 10-3 to 10-6 were prepared and were cultured on BHI agar medium. After 24 hours, plates were investigated to see if there were errors in the three above mentioned conditions. This step was repeated for several times. Then the chosen BMCs were used for synchronizing the amounts of species' colonies in each BMC. Colony counting was performed by Acolyte colony counter (Synbiosis Company, USA). Based on the results, 100 microliters of Gram-negative bacilli and 50 microliters of other bacterial suspensions were added to final five BMCs.

From 24 BMCs inoculums, finally four BMCs were selected for refinery wastewater and two BMCs chosen for petrochemical wastewater. One of BMCs was common for both refinery and petrochemical wastewaters.

After several sub culturing with different amount of species, one BMC from 4 BMCs of refinery grew in 10^{-3} to 10^{-6} (equal amounts of all species). This BMC was named BMC_a and was selected for refinery wastewater treatment.

From two BMCs of petrochemical, one BMC was common with one of the four BMCs of refinery. The amounts of species weren't equal in several dilutions of this BMC. Consequent to several sub culturing, the second BMC grew in 10^{-3} to 10^{-6} with equal amounts of all species. This BMC was named BMCb and was chosen for petrochemical wastewaters treatment.

3.4. Pilot Studies

A second sampling run was performed. Primarily, 250 cc of wastewater was taken as control sample. COD (The amount of oxygen consumed for oxidation of organic compounds), total nitrogen (including nitrate, nitrite and ammonia, and phosphate levels, representing total phosphorus) were measured by standard methods for the examination of water and wastewater (23). Two and a half liter of refineries' effluent was transferred to beaker a1 and 2.5 liter of petrochemical effluent was transferred to beaker b1.

Simultaneously, 100µL of species present in the BMCa and BMC_b, which contained 12×10^{-8} CFU per mL, were added to 5 mL BHI broth and after incubation for 6 hours, were ready to use at the log phase. Two and a half mL of the BMC_a was poured to beaker a1 and 2.5 cc of the BMC_b was poured to beaker b1. The beakers were placed in a shaker incubator, at 90 rev/min at 37° C. Two hundred and fifty mL of liquid was taken from each beaker every 4 hours and the amount of COD, nitrogen and phosphorus was measured in this sample.

3.5. Aeration

Third sampling run was performed. Air stones were added to the bottom of the beakers of each sample and connected to a separate pump. The beakers ad1 (refineries' effluent containing BMC_a) and bd1 (petrochemical effluent containing BMC_b) were aerated continuously at 6 mbar air pressure. Beakers ad2 (refineries' effluent containing BMC_a) and bd2 (petrochemical effluent containing BMC_b) were aerated non-continuously at 6 mbar air pressure (at 15 minute intervals). Aeration was performed for 5 seconds. The beakers were kept in the incubator. Samples were taken from both beakers every 4 hours, and COD, nitrogen and phosphorus contents were measured.

Fourth sampling run was done in polyethylene beakers and renewal recultured BMC_a and BMC_b broths were added to the beakers. The pump started to perform aeration in ad1H beaker (containing BMC_a and effluent of refineries) and bd1H (containing BMCb and petrochemical effluent) continuously for 24 hours at high level (9 mbar). Aeration was performed continuously for 24 hours but at low level (3 mbar) in ad1L (containing BMC_a and effluent of refineries) and bd1L (containing BMCb and petrochemical effluent). Every 4 hours, a 100 mL sample was taken from each beaker and its COD content was measured.

3.6. Nutrients

Within beaker ad1Hp (containing BMC_a and effluent of refineries with high level continuous aeration) and beaker bd1Hp (containing BMCb and petrochemical effluent with high level continuous aeration), nutrients, namely nitrogen and phosphorus minerals (80 mg KNO3 as a mineral source of nitrogen and 20 mg Na2HPO4 as a source of mineral phosphorus) (Merck Company, Germany) were added at the beginning of the experiment. The same amount of nutrients were added to the beakers ad1Hi (containing BMC_a and effluent of refineries with high level continuous aeration) and bd1Hi (containing BMC_b and petrochemical effluent with high level continuous aeration). Nutrients were then added gradually at 6 hour intervals from the beginning of experiment, at hours 0, 6, 12 and 18. Samples were taken from both beakers every 4 hours. COD and pH of the samples were measured.

4. Results

Ninety one pure colonies were isolated from first sampling from wastewaters of 2 oil refineries and 2 petrochemical plants. Fifty two cases were Gram-positive and 39 were Gram-negative. Fifty eight isolates were bacillus, 31 coccus and 2 coccobacillus. Thirty three species of bacteria were lactose fermenter and 50 species were non-lactose fermenter, where 8 cases did not grow on MacConkey medium.

BMC_a contained one Gram-positive bacillus (*Bacillus* *marcerans*), two Gram-negative bacilli (*Enterobacter amnigenus* and *Pseudomonas aeroginosa*), one Gram-negative coccobacilli (*Acinetobacter spp.*) and one Gram-positive coccus (*Staphylococcus saprophyticus*). BMC_b contained two Gram-negative bacilli, *Serattia marcescense* and *Pseudomonas aeroginosa*, one Gram-positive bacillus (*Bacillus marcerans*) and one Gram-positive coccus (*Staphylococcus saprophyticus*).

Mean COD of last four sampling runs from influent of activated sludge tank was 890 for Tehran oil refinery and 1080 for Arak petrochemical. Mean nitrogen and phosphorus were 65 mg/L and 6 mg/L, respectively for Tehran oil refinery wastewater and were 24 mg/L and 86 mg/L for Arak Petrochemical wastewater, respectively. BMC_a caused a reduction in COD, 60%, in nitrogen, 16%, and 45% in phosphorus. COD, phosphorus and nitrogen was reduced 25%, 32% and 53% respectively in presence of BMC_b.

Continuous aeration was more effective in the treatment of wastewaters comparing with non-continuous one. Effects of continuous and non-continuous aeration on nitrogen, phosphorus and COD is shown in *Table 1*. Effects of high and low levels of continuous aeration on COD is reported in *Table 2*. High level continuous aeration caused more reduction in treatment of both refinery and petrochemical wastewaters. Effects of gradual nutrient addition at 6 hours intervals on COD is reported in *Table 3*.

Table 1. Variation of Chemical Oxygen Demand (COD), Total Nitrogen (N) and Total Phosphorus (P) due to the effects of BMC_a and BMC_b and continuously 1) and Non-continuously 2) Aeration at 6 mbar, in Refinery and Petrochemical Effluents.

Beaker Name	Wastewater Sample	BMC Name	Kinds of Aeration	N (mgl^{-1})	P (mgl^{-1})	COD Removal (mgl^{-1})
ad1	Refinery	a	1	- 17%	-35%	64%
ad2	Refinery	a	2	- 89%	-68%	21%
bd1	Petrochemical	b	1	- 61%	-21%	37%
bd2	Petrochemical	b	2	- 78%	-40%	18%

Table 2. Variation of Chemical Oxygen Demand (COD) Due to the Effects of BMC_a and BMC_b and High (9 mbar) and Low (3 mbar) Level Continuous Aeration in Refinery and Petrochemical Effluents.

Beaker Name	Wastewater sample	BMC Name	Aeration (mbar)	COD Removal (mgl^{-1})
ad1L	Refinery	a	3	65%
ad1H	Refinery	a	9	81%
bd1L	Petrochemical	b	3	24%
bd1H	Petrochemical	b	9	63%

Table 3. Variation of Chemical Oxygen Demand (COD) Due to the Effects of BMC_a and BMC_b and High Level Continuous Aeration Beside Nutrient Addition in Refinery and Petrochemical Effluents.

Beaker Name	Wastewater Sample	BMC Name	Aeration (mbar)	Nutrient Addition	COD Removal (mgl^{-1})
ad1Hp	Refinery	a	9	B[a]	67%
ad1Hi	Refinery	a	9	G[b]	85%
bd1Hp	Petrochemical	b	9	B	37%
bd1Hi	Petrochemical	b	9	G	87%

[a] B, at the beginning of experiment
[b] G, gradually

Notably, pH of samples, taken from activated sludge tank of petrochemical wastewater, was 7.84, whereas it was 7.44 in samples of refineries' effluent. The pH was changed in each sampling. At the last sampling, which was done after nutritional addition at the beginning of experiment, pH of samples taken from activated sludge tank of refinery wastewater changed from 7.33 to 5.50 but it had no change with the gradual nutrient addition. The pH of Petrochemical wastewater changed from 7.56 to 5.61 after addition of nutrients at the beginning of experiment but was not changed by gradual nutrient addition.

5. Discussion

According to worldwide environmental reports, environmental problems from effluents of certain petrochemicals and refineries of developing countries are a trouble (24-26) and improving wastewater treatment processes based on an inexpensive method (i.e. native bacteria) is suggested for these developing countries.

Several effective agents are already reported for treatment of wastewater by bioremediation technologies; such as aeration (12, 13), nutrient addition (14-15), usage of native micro organisms such as bacteria, fungi and algae (9, 17), usage of mixed cultures (16) and etc.

Fungi have some unusual mechanism for bioremediation, in comparison with other microorganisms and provide them with several advantages for pollutant degradation, but the complexity of these mechanisms has also made development of a technology as a viable method of bioremediation slow to develop (27). The ability of algae to absorb metals more than other microbial agents has been recognized but its COD removal effect is lower than others, so algae is utilized in addition with other microorganisms and not only by itself (28).

In this study we prepared native bacteria as a mixed culture, isolated from combination of refinery and petrochemical wastewater plants of a country and were used for waste treatment of refinery as well as petrochemical wastewater of the same country. High level continuous aeration comparing with low level, reduced COD of petrochemical wastewater more than two times but COD removal of refinery wastewater was just 16% more. In this study gradual addition of nutrients versus its addition at the beginning of the experiment, decreased COD of refinery and petrochemical wastewaters for an extra 18% and 50%, respectively. This shows that nutrient addition, especially in a gradual format, affects bioremediation of petrochemical organic wastewaters in a considerable rate. On the other hand, gradual addition of nutrients with intervals in both kinds of wastewaters didn't alter pH and so can be used in the treating process without interaction with pH and bacterial growth rate.

Rasouli Kenari et al. reported non-efficient nitrogen removal during present conventional activated sludge process of Tehran Oil Refinery (29) and they suggested simultaneous nitrification and denitrification processes along with conventional activated sludge process to achieve standard levels of Institute of Standards and Industrial Research of Iran's government for nitrogen level of refinery wastewater effluent (30). We did not add any extra process to activated sludge plants and just we suggest charging activated sludge plants by native BMC made from mixture of both refinery and petrochemical wastewater plants. By our BMC, beside COD removal, nitrogen decreased to 7 mg/L which is lower than standard level (standard allowed concentration of Iran is 10 mg/L).

Shokrollahzadeh et al. have shown that ascendant pollutants of Abadan Petrochemical's wastewater were normal-alkanes (C10 – C21), aromatics and polycyclic hydrocarbons and they showed the microbial diversity of activated sludge contents of Abadan petrochemical and by using several bacteria plus a mold (Trichoderma spp.) could achieve maximum reduction of 89% in COD of petrochemical organic wastewater (31); their BMC contained *Pseudomonas*, *Bacillus* and *Acinetobacter genera* same as our final BMC for petrochemical plants. Our BMC could achieve COD removal, in amount of 87% which is close to their COD removal. However our petrochemical sample was from Arak petrochemical with a wider range of petrochemical products.

A limitation of this method is that, effluent composition of several oil companies are different, and even the products of an oil company change periodically; so BMCs' construction may be affected in an industrial scale.

We suggest that native BMC systems isolated from mixture of refinery and petrochemical wastewaters can be used for the wastewater treatment of refinery and petrochemical industries if studied more in industrial scales.

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Authors' Contribution

Mahmoud Ghazi Khansari: Conception and Design and Critical Revision of the Article

Mohammad Mahdi Zamani: Conception and Design, Obtaining Funding and Writing the Article

Seyedeh Hamideh Mortazavi: Laboratory Analysis (Environmental section)

Marzieh Aligholi: Laboratory Analysis (Microbiology section) and Scientifically Revision of the Article

Hodiseh Mahmoud Janlou: Laboratory Analysis (Microbiology section) and Critical Revision of the Article

Malihe Sadat Poormasjedi Meibod: Literature Search and Conception and Design

Samira Khodi Aghmiuni: Data Collection and Data Analysis

Naghmeh Ghanadian: Laboratory Analysis (Biochemistry section)

Mehrdad Azin:Data Interpretation and Administrative Technical Scientifically Revision of the Article.

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