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Isolation and Characterization of Naphthalene-degradation Bacteria from Qurugol Lake Located at Azerbaijan

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Bacteria have different decomposing enzymes, a feature which makes them outstanding among all other microbes. One of the major methods to reduce various pollutants is applying biologic method which is known as bioremediation. The aim of the present study was tried to analyze the existence of naphthalene-degradation bacteria phenotypically and genotypically in Qurugöl Lake, located in East Azerbaijan Province in Iran. For this aim at the end of each rainy season samples were taken from the locations of the lake which were the most polluted. These samples were incubated in a specific medium where naphthalene was the only carbonic source of energy for providing the poly aromatic hydrocarbons (PAHs) energy. The single colonies were isolated and the primary phonotypic and biochemical tests were carried out. After that, for genotypic analysis, their 16S rRNA genes were separated and sequenced. Considering the fact that the decomposing bacteria of PAHs compounds, which were taken from the lake, were isolated, it became obvious that part of polluting process is done by these bacteria. The significant bacteria in this case are *bacillus* sp. and closely related type strains include *B. pumilus* (99.9%) Which are capable of decomposing PAHs efficiently in 5% salt concentrations also *Shewanella* sp. and closely related type strains include *Sh. Xiamenensis* (99.1%) which are capable of decomposing PAHs efficiently in 1/5% salt concentration. On the basis of phenotypic and phylogenetic results, it is recommended that isolated RCRI7(=RCRI8) represents a novel strain of the species *Sh. xiamenensis* and The GenBank/EMBL accession number for the 16S rRNA gene sequences of strain RCRI7 is GQ988720. The level of DNA–DNA relatedness between strain RCRI7 and phylogenetically the closest related strain, *Shewanella xiamenensis* JCM 16212^T was 51%.

Key words: Naphthalene, Qurugöl Lake, *bacillus* sp, *shewanella* sp, 16S rRNA gene.

Asphaltenes, heterocyclic, aliphatics and aromatics are four oil components which are regarded as the main chemical pollutants[1]. Among these four groups, cyclic aromatic compounds are regarded as stable compounds due to having benzene rings and since they afflict great harm to mammals, they are of great concern[2]. In fact they are regarded as one of the toxic and mutagen compounds which can cause cancer and can easily get solved and replaced with in fatty tissues. These compounds can be found abundantly in water and land ecosystems. They pollute the environment and as a result natural sources are wasted. The main factors involved in the entrance of these compounds to the environment include: industrial sewage,

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waste water, home sewage, oil material extractions, the factories involved in making medicine and drugs, paint, plastic and insecticides. Naphthalene is the first in cyclic aromatic compounds which is a common pollutant in water and its poisonous feature is proved since it can hinder mitochondrial respiration[2]. One way to reduce pollutants, especially PAHs, is biologic approach and applying microorganisms which is known as bioremediation. Bacteria have different decomposing enzymes[3], a feature which makes them outstanding among all other microbes[4]. Some researches reports about bioremediation available such as: the isolation of Bacillus cereus from phenanthrene bearing environment and their growth capability in the presence of phenanthrene[5], the isolation of strains from mycobacterium sp. bacteria with phenanthrene decomposing feature[6,7] reporting the new space in Polaromonas sp, of naphthalene degrading bacteria[8], molecular characterization of bacteria isolated from waste electrical transformer oil[9], and assigning two naphthalene degrading bacteria belonging to the genera *Paenibacillus* and *Pseudomonas* isolated from a highly polluted[10].

In this study the samples for examining the existence of PAHs-decomposing bacteria were taken from Qurugöl Lake, which is located in the mountainous area of East Azerbaijan Province in Iran. This small lake is located on the intercity transportation road and is exposed to different pollutants. Some PAHs decomposing cultures were analyzed and were phylogenetically identified. Also the capability of theses cultures for efficient decomposing in the presence of different salt concentrations were analyzed.

Objectives

The aim of the present study was tried to analyze the existence of naphthalene-degradation bacteria phenotypically and genotypically in Qurugöl Lake, located in East Azerbaijan Province in Iran.

MATERIALS AND METHODS

Sample collection and isolation

Twelve samples from four different areas of the Qurugöl Lake, located in East Azerbaijan (Fig. 1), at the end of rainy seasons were collected. The samples were taken from 25 cm depth and were transferred in sterile containers. Then they were transported to the RCRI (rice and citrus research institute) laboratory. For isolation and identification of PAHs-degrading bacteria the ONR 7a medium was used[11].

Chemical

Naphthalene was purchased from Merck-Schuchart (Hohenbrunn bei München, Germany).

Each 10 ml of seawater samples was placed into a 500ml baffled flask and supplemented with 100 ml of sterile ONR 7a broth, and 100 mg of naphthalene. They mixed at 30°C on a rotary shaker operating at 150 rpm for several weeks until a yellowish orange. Naphthalene was the sole carbon and energy source for surviving bacteria in this test. 1mg/ml of naphthalene was dissolved in acetone and they were over sprayed onto a solid agar medium, (The solution PAHs was sterilized by filter).

ONR 7a containing

Solution 1

NaCl; 22.79 g, Na_2SO_4; 3.98 g, KCl; 0.72 g, NaBr; 83.00 mg, $NaHCO_3$; 31.00 mg, H_3BO_3; 27.00 mg, NaF; 2.60 mg, NH_4Cl; 0.27 g, Na_2HPO_4 x 7 H_2O; 89.00 mg, TAPSO; 1.30 g.

Solution 2

$MgCl_2$ x 6 H_2O; 11.18 g, $CaCl_2$ x 2 H_2O; 1.46 g, $SrCl_2$ x 6 H_2O; 24.00 mg and **Solution 3:** $FeCl_2$ x 4 H_2O; 2.00 mg.All solutions were mixed after autoclaving and getting coaled at least at 50°C.

100 micro liter from suspension inoculated onto ONR 7a agar plates with naphthalene as sole carbon source. The cultures were incubated at 28±2°C for several weeks. The surfaces of the plates were opaque, and the colonies surrounded by a clear zone were purified further by restreaking on the same type of plates (Kasai, Y., *et al*., 2002). Colonies were taken marine broth medium containing (per liter): Bacto peptone, 5.0 g; Bacto yeast extract, 1.0 g; Fe(III) citrate, 0.1 g; NaCl, 19.45 g; $MgCl_2$ (dried), 5.9 g; Na_2SO_4 , 3.24 g; $CaCl_2$, 1.8 g; KCl, 0.55 g; Na_2CO_3, 0.16 g; KBr, 0.08 g; $SrCl_2$, 34.0 mg; H_3BO_3, 22.0 mg; Na-silicate, 4.0 mg; NaF, 2.4 mg; $(NH_4)NO_3$, 1.6 mg; Na_2HPO_4, 8.0mg; with pH 7.2±0.5. For identification and characterization. The cells were grown in marine broth at 30 °C and stored at -70 °C in broth medium supplemented with 30% (w/v) glycerol.

Standard Gram staining method[12] and

also Gram reaction was tested with KOH lysis test technique[13].

Biochemical tests were carried out for determination of oxidase, catalase, gelatin liquefaction, and ability to hydrolyse starch, Tween 20, Tween 80 and casein, production of indole, H2S and nitrate reduction by Macfaddin[14].

In the next step the capability of the isolated bacteria was analyzed in producing extra-cellular amylase enzyme[15], extra-cellular protease enzyme[16], extra-cellular lipase enzyme[16], extra-cellular nuclease enzyme[17], extra-cellular inulinase enzyme[18], extracellular pectinase enzyme[19], extra-cellular carboxymethyl cellulose enzyme[16], extra-cellular pullulanase enzyme[20] and extra-cellular chitinase enzyme[21]. All tests were carried out at optimum NaCl concentration of each isolate.

Also the ability of using various carbon sources, the production of acid from sugars and the physiological profile of the new strain (RCRI7) was performed using the API 20E, API 20 NE and API 50 CH B/L kits. API ZYM and ID 32E were used to identify the enzyme activities of the strains according to the manufacturer's (bioMerieux) instructions.

Measurement of PAH degradation and NaCl test of colonies

Naphthalene was detected spectrophotometrically (200-800 nm). For integration of the chromatograms and quantification of the PAH amount, the software EXCEL was used.

The optimal concentration of NaCl for Naphthalene-degradation determined in ONR 7a broth medium with different concentration of NaCl: (0, 1.5, 3, 4.5, 6, 7.5, 10, 12.5, 15, 17.5 and 20 percent).

The experiment pursuing, 250ml erlenmeyer flasks contain; 100 ml of ONR 7a medium with different concentration of NaCl including; 1mg/ml of a PAHs used, every concentration was supplied from triplicate flasks. After preparing bacteria Suspension 10^5 cfu, it was added to each flasks, mean while a bacteria free flask also was used as a control. The flasks were incubated at 28±2°C with 150 rpm for 10 days [22].

Following the incubation process, the remaining amount of naphthalene was extracted out of the medium and its concentration and transmittance percentage was determined by spectrophotometer. The percentage of hydrocarbon degradation was calculated based on the results obtained.

Naphthalene-degradation was detected by spectrophotometer absorbance at 480 nm wave lengths daily also the incubation flasks sterilized by filter before measuring for separated bacteria cells and suspense exanimate, the used filters case to increase resolution. This work lasted for 10 days. The control flask was used for Calibration[23].

The 16S rRNA gene sequences

For genotypic characterization, DNA was isolated from the isolates according to the method described by Corbin[24] with some modifications. For phylogenetic analysis based on the 16S rRNA gene sequence, the genes were amplified using PCR technique in the presence of forward 16F27 (5' -AGA GTT TGA TCT GGC TCA G- 3') and reverse 16R1488 (5' -TAC CTT GTT AGG ACT TCA CC- 3') primers[3].

The amplified fragments were purified using Roche kit (Germany) and then sequenced by utilizing forward and reverse and 3 other designed primers; H400 (5'-GGG TTG TAA AGC ACT TTC AG-3'), H550 (5'-CCA GTA ATT CCG ATT AAC GC-3') and H900 (5'-ACT CAA ATG AAT TGA CGG GG-3') used for PCR amplification by *Macrogen* company (Korea).

The 16S rRNA gene reads were assembled using Chromas pro software and aligned using the multiple sequence alignment program CLUSTAL X (version 1.83) (25). Phylogenetic trees were constructed using neighbor-joining, method in MEGA version 4 software package[26].

DNA-DNA hybridization

DNA-DNA hybridization between strain RCRI7 and reference strain, *Shewanella xiamenensis* JCM 16212^T, was performed by BCCM/LMG. DNA-DNA Hybridizations were performed in the presence of 50% formamide at 42 °C between strain RCRI7 and *Shewanella xiamenensis* JCM 16212^T, according to a modification of the method described by Ezaki[27]. The DNA-DNA relatedness percentage reported is the mean of 6-7 hybridizations.

RESULTS

Four isolates were obtained from twelve water samples collected from Qurugöl Lake. The results of phylogenetic tree analysis using NCBI

Table 1. Growth and substrate utilization characteristics of RCRI26 and RCRI7 observed in this. These tests were done in Marine agar medium

RCRI7	RCRI26	Characteristic	RCRI7	RCRI26	Characteristic
-	-	D-Galactos	+	+	Catalase
-	+	D-Fructose	+	+	Oxidase
+	+	D-Xylose			Growth with NaCl
-	-	Adonitol	+	-	0%
-	-	Dulcitol	%3	%12.5	NaCl tolerant
-		Sorbitol			Temperature for growth°C
		Enzyme test	5-45	0-40	Range
+	-	Amylase	28	28	Optimum
+	+(rapid)	Lipase	6-9	6-9	PH
-	-	Chitinase			Acid production form
-	+	Protease	-	-	D-Arabinos
-	-	DNase	+	+	D-Glucose
-	-	Pullulanase	-	+	D-Mannos
-	-	Indol test	-	+	L-Arabinose
+	+	Nitrate reduction	+	-	Citrate

and EZtaxon data bank indicates that the isolated bacteria with naphthalene decomposition capability is similar to bacillus sp and closely related type strains include B. pumilus (99.9%) two strains; RCRI26 and RCRI27 and Shewanella sp. and closely related type strain include Sh. Xiamenensis (99.1%) strain; RCRI7(=RCRI8)(Fig. 2). Also through the analysis of the decomposed PAHs compounds in 480 nanometer wave length, during 10 days of testing it became clear that naphthalene decomposition in salt concentration increases proportionately when slat concentration ranges from zero to 5% and in 5% percent, it is capable of decomposing PAHs efficiently by strains RCRI26 and RCRI27.

In media where the concentration of salt is more than 5%, the decomposing capability decreases proportionately and in %10 salt concentration no decomposition takes place. Strain RCRI7 (=RCRI8) is capable of decomposing PAHs efficiently in 1/5% salt concentrations. In addition to the salt concentration, time has a crucial role in the decomposition process. As the time passes, the level of decomposition increases

Fig.1. Geographical location of Qurugçl Lake located at Azerbaijan by google earth

accordingly. The results gained from measuring the absorption in 480 nanometer shows that in optimum NaCl concentration for each strain, the level of decomposition on the tenth day is more than the preceding days. It is concluded that the microorganisms adapt themselves with the percentage of salt concentration, as a result the necessary enzymes for the decomposition increase and the growth and reproduction of bacteria goes up as the time passes.

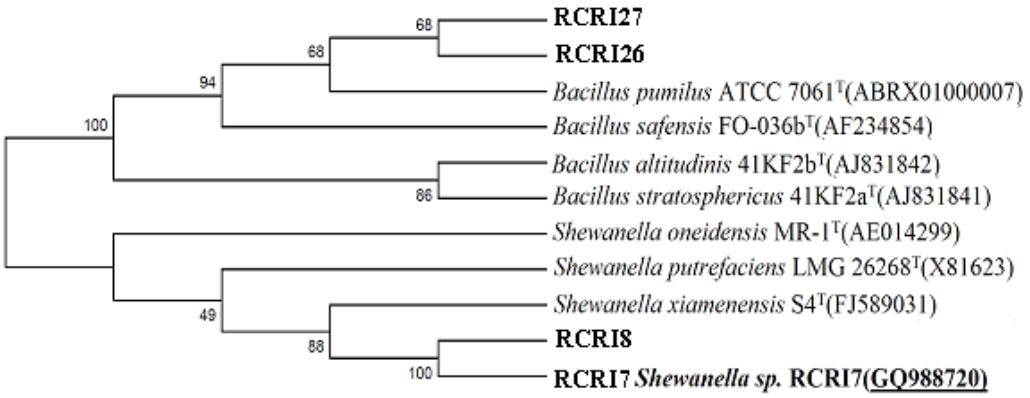

Fig. 2. Phylogenetic relationships of the members of isolated strains, based on a partial sequence of the 16S ribosomal RNA gene. The sequence alignment was performed using the CLUSTAL-X program and the tree was generated using the neighbor-joining method in MEGA

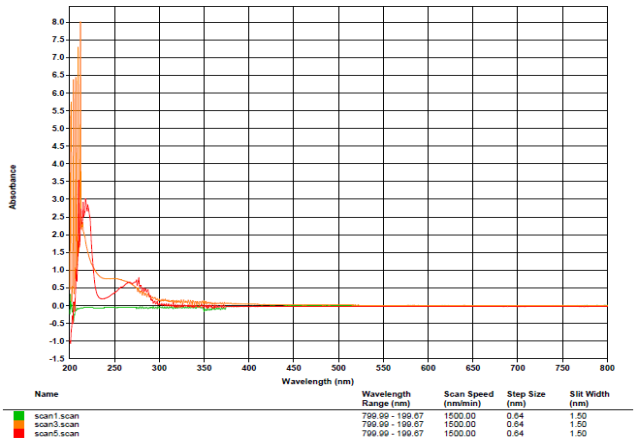

Fig. 3. Effect of RCRI7 strain on the biodegradation of naphthalene, blue line consist of ONR 7a medium, Green line consist of naphthalene and acetone, Red line consist of medium with RCRI7, Orange line consist of supernatant from medium with RCRI7 and violet line is just naphthalene

DISCUSSION

Cyclic aromatic compounds are more stable since they have benzene rings and as the number of benzene rings goes up, their microbic decomposition becomes more difficult[28]. Since naphthalene is a two-ring aromatic compound, in comparison with other aromatic compounds, it is decomposed by bacteria faster and more easily.

Based on the gained results it was clarified that as the concentration of salt goes higher than optimum concentration, the capability of bacteria to use substrate and decompose poly-cyclic aromatic compounds goes down, this is because their metabolic variety goes down. Most of the strong decomposing bacteria of naphthalene are gram negative bacteria especially of the kind *pseudomonas*. But among gram positive bacteria *bacillus sp*. are stronger than others. These bacteria can decompose cyclic aromatic compounds which

have three benzene rings and use them as carbon and energy source. In case there isn't enough access to carbonic sources, bacteria *bacillus sp*. are apt to use aromatic compounds especially naphthalene. Of course in case the concentration of aromatic compounds is more than normal, they act as toxic material and the bacteria won't be able to grow and decompose them, therefore to be able to survive the bacteria will change into spores.

The bacteria isolated for this study (strain; RCRI26 and RCRI27) are garm positive rod shape bacteria which are taken from the 25 cm depth of qurugçl Lake. Since in this part of the lake the maximum oxygen needed for the activation of aerobic and anaerobic bacteria is easily available, later on the anaerobic conditions are not available and thus the decomposition of PAHs compounds is not possible. The decomposing bacteria produce large colonies with pallid color in a marine agar medium. The shape of the colonies is irregular and the angle of the colonies is flat. Their growth in mediums with 1.5, 3, 4.5, 6, 7.5 and 10 percent of NaCl concentration is evident. But in a salt-free medium and mediums up to 12 percent of salt concentration they are not able to grow. Thus it can be concluded that these bacteria are halotolerant bacteria.

The other point is that, even though the studied bacteria are capable of growing at 0-40°C with PH range of 6-9, the efficient growth is at 28 °C with PH grade of around 7± 0/5(Table 1).

The biochemical experiments shown that the strains RCRI26 and RCRI27 are capable to product, oxidaize, catalaze, Lipase and protease enzymes but are not capable of producing gelatinize, chitinase, DNase, Pullulanase and indolase enzymes (Table 1).

The phylogenetic tree showed that strains RCRI7 (=RCRI8) (= JCM 17276^T) fall within the radiation of a cluster composed of *Shewanella* species e"99.1%. 16S rRNA gene sequence similarity with *Shewanella xiamenensis, Shewanella putrefaciens* and *Shewanella oneidensis* (Fig.2).

They could produce lipase and amylas, but they were not able to produce other hydrolytic enzymes. The GenBank/EMBL accession number for the 16S rRNA gene sequences of strain RCRI7 (=RCRI8) is GQ988720.

The isolated bacterium is a Gram-negative, rod shaped and motile. The cells produce large colonies with yellowish color in a marine agar medium. The shape of the colonies is circular and the angle of the colonies is convex and their edges are entire. The cells grow in the absence of NaCl and also in the presence of 0.5, 1, 1.5, 2 and 3 percent NaCl (optimum 0-0.5%).

The cells are able to grow at 5-45 ºC with pH range of 6-9 with the efficient growth at 28 °C and pH of 7±0.5 (Table1).

The strain RCRI7 (=RCRI8) can grow on MacConkey agar, nutrient agar, blood agar, brain heart infusion (*BHI*) and LB Media but not sea water agar. The biochemical experiments indicated that the strain RCRI7 is capable to produce oxidase, catalase, amylase, urease, tyrosinase, ornithine, esculinase and gelatinase but is not capable to produce indolase, arginine and lysine decarboxylase and phenylalanine deaminase, according to lab-made tests. It is positive for reduction of nitrate to nitrite.

According to the API 50CH test, acid is produced from D-glucose, esculin, N- acetyl glucosamine and D-maltose. But not from D-ribose, D-xylose, D- galactose, D-fructose, methyl-α-D-glucopyranoside, D-celobiose, glycerol, erythritol, D-arabinose, L-xylose, adonitol, methyl-β-D-xylopyranoside, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-α-D-mannopyranoside, amygdaline, arbutine, inuline, D-saccharose (sucrose), D-trehalose, genitiobiose, D-turanose, and potassium 5-ketogluconate glycogen, xylitol, D-lyxose, D-tagatose, D-fucose, L- fucose, D-arabitol, L-arabitol, potassium gluconate and potassium 2-ketogluconate.

In API 20NE, D-glucose, N-acetyl-glucosamine, D-maltose and malic acid are assimilated, but L-arabinose, D-mannose, D-mannitol, potassium gluconate, capric acid, adipic acid, trisodium citrate, and phenylacetic acid are not assimilated.

Moreover, in API ZYM, the strain RCRI7 has enzyme activities for lipase, α-glucosidase, α-maltosidase, and L-aspartic acid arylamidase activities, but does not have enzyme activity for β-Glucoronidase, N-Acetyl-β-glucosaminidase, β-galactosidase, α-glucosidase, α-glactosidase, and malonate. Sodium pyruvate acidification is negative.

By taking into consideration all the above mentioned facts it can be concluded that microorganisms of Qurugöl have an efficient role in bioremediation (Fig. 3), that is to say, the polluted water starts getting decomposed by microorganisms in the lake as it enters the lake. It is also possible that part of the pollution entered into the lake may get eradicated physically or chemically, e.g., through oxidation. Having said that, by separating the decomposing bacteria (PHAs) from Qurugöl Lake, it has been clarified that part of the purification process of the lake is done by these bacteria.

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Authors' Contributions

Study concept: tarhriz and Khodaverdi Darian. Analysis and interpretation of data: yazdani, Hejazi, Rahimi. Drafting of the manuscript: Yahaghi,. Critical revision of the manuscript.

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The authors declared that they had no competing interests.

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