Quinoline Biodegradation by *Bacillus Licheniformis* Strain *CRC-75*

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ABSTRACT: *A bacterial culture was isolated from oil-contaminated soil based on its ability to metabolize the quinoline as the sole source of nitrogen. In this research Bacillus licheniformis strain was identified based on the result of 16S rRNA analysis. Optimized conditions were obtained with full factorial experimental design method using design expert software at temperature: 32°C, agitation speed: 200rpm, quinoline concentration: 400 ppm.The efficiency of Bacillus licheniformis for biodegradation of quinoline at the optimum conditions was determined to be 35%. Two-phase cultivation media were used with 5, 10, 15 and 20% heavy crude oil concentration in aqueous media. Resting cells of Bacillus licheniformis was shown to be capable of removing about 25% of total nitrogen in 5% heavy crude oil. GC analysis showed a decreasing trend in the activity of this strain against crude oil concentration*

KEY WORDS: *Quinoline, Bacteria, Petroleum, Biodenitrogenation, Bacillus licheniformis.*

INTRODUCTION

Crude oil is a heterogeneous mixture of organic molecules including all-hydrocarbon alkenes and aromatics, as well as sulfur and nitrogen–containing heteroaromatic compounds [1]. Many applications of crude oil are hindered by the presence of sulfur and nitrogen-containing compounds [2,3]. It is known that the chemical and physical petroleum refining processes are currently used to remove most of the nitrogen–containing organic compounds in crude oil [1,4-6]. Quinoline, nitrogen containing heterocyclic compounds, is component of shale oil, crude oil, petroleum products and coal tar, whose combustion leads to the formation of nitrogen oxides (NO_X) [1,7,8].

Quinoline is the most widely studied organonitrogen compound. This is considered to be representative of many organonitrogen compounds typically found in petroleum. Many aerobic and anaerobic microbial cultures that can degrade quinoline have been found [9-17].

Nitrogen heterocyclic compounds can deactivate refining catalysts and can also contribute to chemical instability of refined petroleum products. Therefore, it is necessary to establish a method for removing these nitrogen compounds from crude oil for global environmental protection.

To date, there has been an increasing interest in the use of microorganisms to treat heterocyclic nitrogenous compounds because such a bioprocess enables selective degradation and proceeds under milder conditions than the chemical and physical processes, which need

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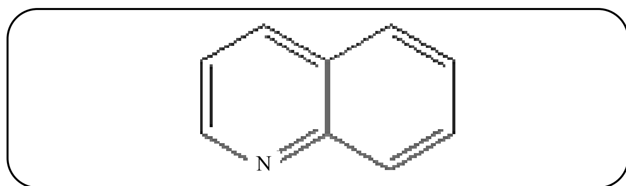

Fig. 1: Quinoline structure [29].

high-temperature and high-pressure conditions [18-27]. However these technologies are not yet available for large-scale applications so future research must investigate proper modifications for industrial applications of these processes [28].

In this research, *Bacillus licheniformis* strain was isolated from petroleum-contaminated soil and quinoline (Fig. 1) was used as the sole source of nitrogen. This compound is soluble in water, alcohol, ether and carbon disulfide with boiling point: 238°C and specific weight: 1.0879 [29]. In addition quinoline degradation was performed in two liquid-phase system consisting water and petroleum oil.

EXPERIMENTAL SECTION

Culture and preculture media

Chemicals: quinoline was obtained from Fluca Company .The other culture compounds were purchased from Merck Company with high purification degree.

LB broth (purchased from Himedia) was used as a preculture medium to increase the microorganism populations. For the screening of quinoline degrading microorganisms, an organic nitrogen free synthetic (ONFS) medium (Table 1) was used [20].

The initial pH of medium was adjusted to 7. 1200 ppm of quinoline was added as the sole source of nitrogen and 20 g/L (6.66 g of each) glucose/glycerol/succinate was used as carbon source. The medium was sterilized at 121°C for 15 min except for the glucose, which was sterilized at 110°C for 20 min.

Screening of quinoline degrading microorganism

Eighty environmental samples of soil, wastewater and petroleum sludge were obtained from petroleum and coal processing, compost and other sites where contamination with petroleum hydrocarbons exist. These samples were collected from Abadan, Bandar Abbas, Shiraz, Kharg, Masjed Soleyman, Ahwaz, Aghajari and Gachsaran oil production sites. First the samples are suspended in preculture medium. A portion of this suspension was added to new preculture medium. After 24 hours of cultivation at 28°C and 160 rpm agitation speed, the cells were harvested and washed twice with mineral medium and suspended in the same mineral medium to show optical density (O.D.) Approximately a population of 2.7×10^5 (cells/ml) was added to mineral medium containing 1200 ppm quinoline and then incubated at 28°C with shaking at 160 rpm for 7 days.

Study of selective consumption of quinoline by Bacillus licheniformis strain

To identify strains selectivity of the consumption quinoline, 6 different growth conditions were performed as shown in (Table 2) and described as follows: [20]

Quinoline as the sole source of nitrogen, alternative carbon source (glucose/ glycerol/ succinate) was available.

No nitrogen compounds of any kind were present, but alternative carbon (glucose/glycerol / succinate) source was available.

Only alternative nitrogen (ammonium sulphate) and carbon (glucose/ glycerol/ succinate) sources were available and quinoline was not present (positive control).

Quinoline was present as well as alternative source of carbon (glucose/ glycerol/ succinate) and nitrogen (ammonium sulphate).

Quinoline as the sole source of carbon and nitrogen.

Quinoline as the sole source of carbon (alternative nitrogen source (ammonium sulphate) was available.

These six growth conditions were the base of bacterial ability to metabolize organonitrogen compounds. Carbon and nitrogen source other than quionoline were supplied as 20g/L and 20 mM, respectively.

Optimization of growth condition

For optimization of growth condition, the results of quinoline reduction were studied with changing 4 factors at 2 levels using full factorial method by Design Expert Software from Stat-Ease Company (U.S.A). These factors were temperature, pH, quinoline concentration, and agitation speed [30-32].

Crude oil treatment with bacteria

Bacillus licheniformis was incubated in medium with

Table1: An aqueous organic Nitrogen free synthetic medium.

Material	value (g/L)
KH_2PO_4	2.60
Na_2HPO_4	4.20
$MgSO_4.7H_2O$	0.20
$MnSO_4$. (4 -6)H_2O	0.02
$FeSO_4.7H_2O$	0.05
$Na_2MoO_4.2H_2O$	0.001
$CaCl_2$	0.02

Table 2: Nitrogen bioavailability assey.

Condition	Carbon source	Nitrogen source
1	glucose/ glycerol/ succinate	Quinoline
2	glucose/ glycerol/ succinate	-
3	glucose/ glycerol/ succinate	ammonium sulphate
4	glucose/ glycerol/ succinate	Quinoline-ammonium sulphate
5	Quinoline	Quinoline
6	Quinoline	ammonium sulphate

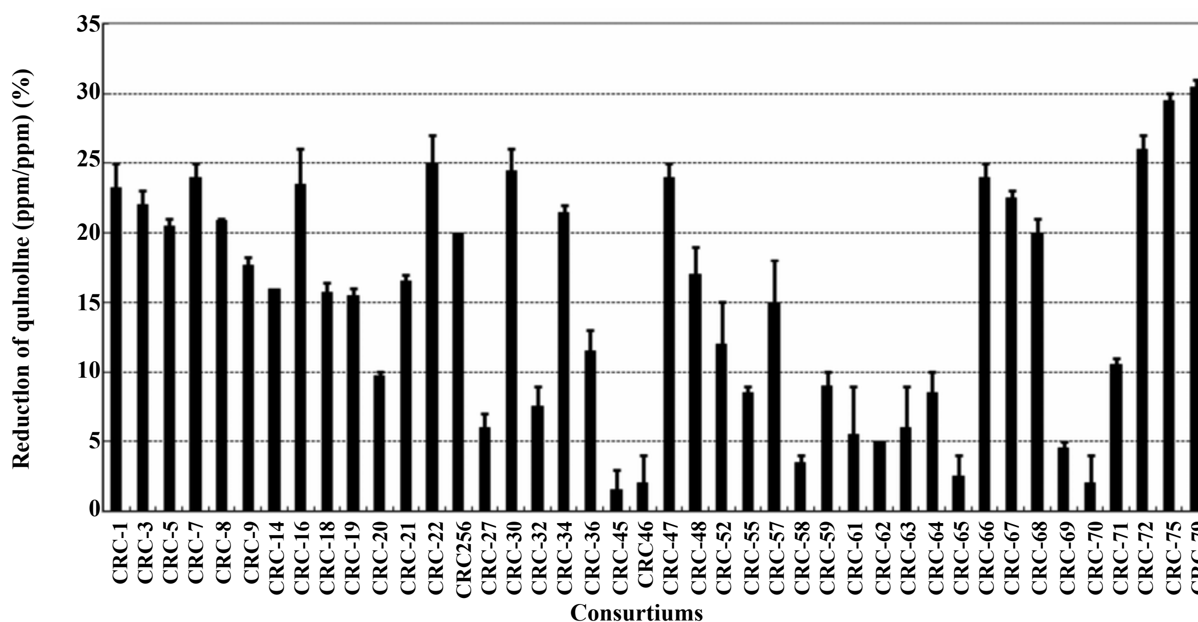

Fig. 2: Reduction of quinoline by all consortiums.

400ppm quinoline as a nitrogen source for 4 days at 28°C. Cells were recovered by centrifugation at 3000 rpm for 20 min at 4°C, then washed twice with phosphate buffer 50mM and pH=7.0, and recovered at 3000 rpm for 20 min at 4°C and then suspended in the ONFS medium.

The cell suspension was cooled at 4°C. The suspension of bacteria (pH=7.0±0.1) with 2×10^8 (cells/mL) was added to ONFS medium containing 5%, 10%, 15% and 20%(v/v) of crude oil (shiraz refinery feed) and then this reaction mixture was shacked at 28ºC for 20 days.

Analyzing Method

For analyzing bacterial growth, the turbidity (OD) of the culture broth was monitored at 600 nm by Beckman UV-Vis DU520 spectrophotometer. Quinoline analysis was carried out by gas chromatography method (438A model) using fused silica capillary column CP-Sil 5/CB (0.23mm i.d. × 50m length) operating at 200ºC with the injection port at 300ºC and detector at 320ºC.

The nitrogen content in crude and treated oil was measured by ASTM D-4629 method. Boiling point distribution was carried out in a GC column (CP3800varian with Column W.cot ultimatetal).

RESULTS AND DISCUSSION

Screening of selective bacteria

In the preliminary screening, about 76 consortiums were isolated From 80 environmental samples. These microbes were screened for their ability to utilize quinoline as the sole source of nitrogen in a screening medium as described in materials and methods (Fig.2).

Out of 76 consortiums, seventeen strains were isolated

Table 3: Screening of Quinoline-degrading microorganisms.

Strains	Degradation (%)
Control	0
CRC1	23.22
CRC3	22
CRC5	20
CRC7	24
CRC8	21
CRC16	23.5
CRC22	25
CRC26	20
CRC30	24.5
CRC34	21.5
CRC47	24
CRC66	24
CRC67	22.5
CRC68	20
CRC72	26
CRC75	29.5
CRC79	30.5

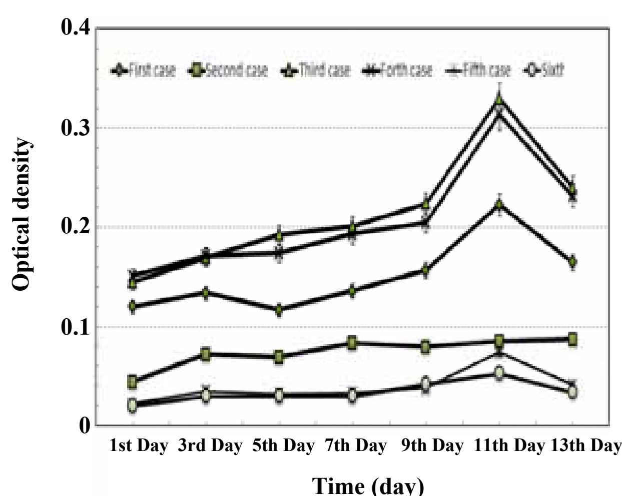

Fig. 3: Growth of strain CRC-75 in 6 different test conditions.

(Table 3) because they showed better ability to degrade quinoline. A complete screening of the seventeen strains showed strain CRC-79 had better quinoline degradation than strain CRC-75 but strain CRC-75 was chosen since it could withstand high-level concentration of quinoline.

Characteristics of CRC-75 strain

Strain CRC-75 is a gram-positive aerobic rod, which is catalase-positive and can utilize glucose, arabinose, xylose, annitole and fructose [33]. Based on the results of its partial sequencing of the 16S rRNA gene, this strain closely resembles B*acillus licheniformis* with 100% homology. The phenotypic characteristics of strain CRC-75 are summarized in Table 4.

Selectivity of Bacillus licheniformis (CRC-75) to consume quinoline

Growth tests with Bacillus *licheniformis* were performed according to the nitrogen bioavailability assay procedures as preciously described in materials and methods. Results of this study were described as follows bearing in mind that O.D. (λ=600 nm) was measured daily in all cases.

First Case: Increasing of OD during growth period of CRC-75 in Fig. 3 and quinoline reduction in Fig. 4, demonstrated the capability of the cleavage of ring and consumption of quinoline as nitrogen source.

Second Case: In Fig. 3, considerable change of OD was not observed so confirming that the culture medium was not a good source of nitrogen (bacteria can not grow without nitrogen source).

Third Case: presence of easy source of nitrogen and carbon in culture medium caused the maximum growth of bacteria (Fig. 3).

Fourth Case: The growth of CRC-75 in medium containing quinoline and ammonium sulphate as nitrogen source was high and despite presence of easy source of nitrogen, quinoline was decreased (Fig. 3). This confirmed the ability of *Bacillus licheniformis* to break C-N bond without any constraint.

Fifth Case: In this case considerable growth of *Bacillus licheniformis* was not observed (Fig. 3), and little reduction of quinoline was obtained (Fig. 4). This confirmed the bacteria didn't use quinoline as carbon source.

Sixth Case: similar to fifth case, *Bacillus licheniformis* couldn't grow well with quinoline as carbon

Table 4: Phenotypic characteristics of B.licheniformis CRC-75.

Characteristics	Strain CRC-75
Shape	Rods
Size	0.6-0.7µm × 2.5-3.5µm
Amino peptidase Test	-
KOH Test	-
Catalase	+
Spores	Ellipsoid
Anaerobic growth	+
VP reaction	+
pH in VP	6.0
Maximum Temperature growth positive up to Growth	55ºC
Medium pH 5.7	+
NaCl 2%	+
10%	+
Acid form	
D-glucose	+
L-arabinose	+
D-xylose	+
D-mantled	+
D-fructose	+
Gas from glucose	-
Hydrolysis of	
Starch	+
Gelatin	+
Casein	+
Tween 80	+
Esculine	+
Use of	
Citrate	+
Propionate	+
Indolreaction	-
Phenyalanine deaminase	-
Arginine dihydrolase	+

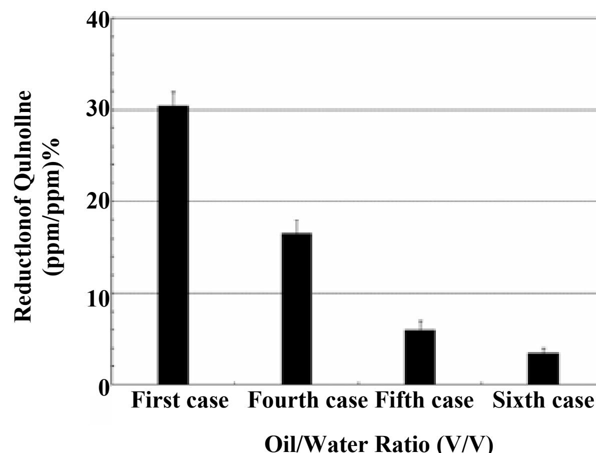

Fig. 4: Reduction of quinoline in different test conditions.

source (Fig. 3). Also Fig. 3 shows the inability of the bacteria to use quinoline as the sole source of carbon.

Eventually, we obtained a pure culture that yielded nitrogen bioavailability assay results indicating that quinoline was used as nitrogen but not as a carbon source.

Optimization of growth condition

All effective parameters on quinoline reductions were considered. These parameters were temperature, pH, agitation speed and concentration of quinoline. For optimization, full factorial method with center point was used. Design summary are expressed in Table 5. By using this method 17 sets of tests were carried out shown in Table 6 [34-35]. The optimum conditions were obtained as shown in Table 7. The analysis proved that quinoline reduction by software and experimental methods showed close correlations.

Crude oil treatment with Bacillus licheniformis

Bacillus licheniformis was grown at optimum condition. After 4 days, the cells were harvested by centrifuge to obtain resting cells. Then resting cells were added to a solution that was prepared with certain amount of oil in (ONFS) (5, 10, 15, 20v/v). After 5 days the amount of nitrogen in oil was measured as shown in Fig. 5.

CONCLUSIONS

Some bacteria isolated from eighty samples of oil-contaminated sites, were capable of breaking carbon-nitrogen bond of quinoline as the sole source of nitrogen. Because of quinoline toxicity in bacterial growth we

Table 5: Parameters used for optimization of growth condition by full factorial with center point.

Factor	Name	Units	Type	Low Actual	High Actual	Low Coded	High Coded
A	Temperature	°C	Numeric	24	32	-1	+1
B	pH	-	Numeric	6	8	-1	+1
C	Concentration	ppm	Numeric	400	1200	-1	+1
D	Agitation	rpm	Numeric	160	240	-1	+1

Table 6: Experimental design by full factorial method with center point

Runs	Treatment Combination Level of factors				High level (+) Low level (-)				Results			
	A	B	C	D	A	B	C	D	i	ii	iii	Average
1	24	6	400	160	-	-	-	-	30.7	27.4	26.8	28.3
2	32	6	400	160	+	-	-	-	38.4	33.7	33.5	35.2
3	24	8	400	160	-	+	-	-	31.4	28.6	32.1	30.7
4	32	8	400	160	+	+	-	-	40.1	29.7	27.7	32.5
5	24	6	1200	160	-	-	+	-	27.1	25.5	25.7	26.1
6	32	6	1200	160	+	-	+	-	30.2	25.8	32.2	29.4
7	24	8	1200	160	-	+	+	-	15	19.2	22.5	18.9
8	32	8	1200	160	+	+	+	-	22.8	19.7	18.7	20.4
9	24	6	400	240	-	-	-	+	25.6	27.3	32.9	28.6
10	32	6	400	240	+	-	-	+	37.2	35.1	36.6	36.3
11	24	8	400	240	-	+	-	+	26.1	29.6	25.9	27.2
12	32	8	400	240	+	+	-	+	33.5	31.6	30.9	32
13	24	6	1200	240	-	-	+	+	34.6	27.2	27.6	29.8
14	32	6	1200	240	+	-	+	+	38.3	29.7	34	34
15	24	8	1200	240	-	+	+	+	34.3	29.8	22.9	29
16	32	8	1200	240	+	+	+	+	37.2	32.8	29.6	33.2
17	28	7	800	200	-	0	0	0	29.5		30.7	30.8

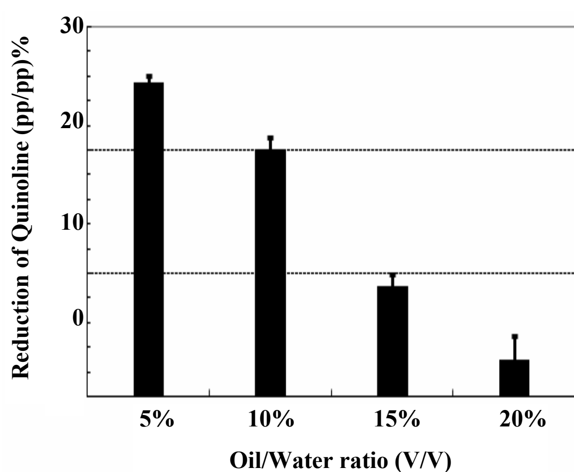

Fig. 5: Influence of Bacillus licheniformis on reduction of the total nitrogen of crude oil in stationary phase.

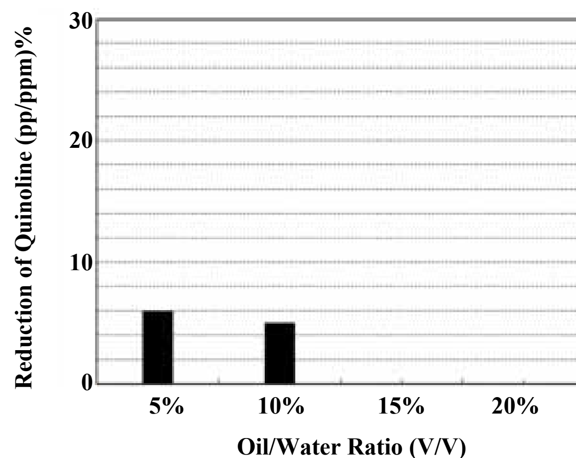

Fig. 6: Influence of Bacillus licheniformis on reduction of the total nitrogen of crude oil in growth phase.

Table 7: Optimum condition for quinoline reduction.

Temperature	pH	Concentration	Agitation	Pridiction of Quinoline Reduction by Design Expert 6.0	Quinoline Reduction In actual test
32	6.00	458.92	240.00	34.5594	35

used 1200 ppm in these shake flask enrichment culture experiments. Among the isolated bacteria pure culture CRC-75 was chosen because of having better ability to reduce quinoline and to withstand environmental changes such as temperature and pH. This culture was identified as *Bacillus licheniformis* by 16S rRNA technique with 100% homology. Optimization of the experimental culture conditions indicated that *B.licheniformis* could efficiently reduce 35% of quinoline in aqueous media. The obtained pure culture was tested using the nitrogen bioavailability assay for detecting the ability of consuming quinoline as the sole source of nitrogen but not as carbon source. The results showed this bacterium utilize quinoline as nitrogen source. In continue two phase culture media with 5, 10, 15 and 20% (crude oil/ONFS medium) were examined to determine the ability of *B.licheniformis* to remove nitrogen from oil.

The results indicated that resting cell of *B.licheniformis* were capable of removing 25% total nitrogen from 5% oil which is higher than reported by *Kilbane* [20]. Also experimental data showed that *B.licheniformis* couldn't reduce considerable amount of nitrogen from petroleum in growth phase (logarithmic phase) as shown in Fig. 6 .

Therfore, the results confirmed that effective enzymes in breakage of C-N bonds transuded at stationary phase in presence of model composition quinoline as a single of nitrogen source.

A comparison of Figs. 5 and 6 reveals mentioned subject. Investigations have shown biodegradation of quinoline by immobilized cell is faster than free organism [22]. Also genetics and immobilization of bacteria are two conventional methods for augmenting efficiency of the bacteria strain [14-17, and 20-27].

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