Microbial demetallization of crude oil using *Aspergillus* sp.: vanadium oxide octaethyl porphyrin (VOOEP) as a model of metallic petroporphyrins

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Abstract
An isolate from polluted soil identified as Aspergillus sp. MS-100 was able to consume vanadium oxide octaethyl porphyrin as a model for protoporphyrins in crude oil. The isolate degrades about 55% of vanadium oxide octaethyl porphyrin (VOOEP) under optimum conditions during 7 days. The release of more than 0.96 mgl^{-1} of free vanadium into the aqueous phase was confirmed using atomic absorption. By using the Taguchi experimental design method, the optimum values of pH, temperature and initial concentration of VOOEP were determined as 5.5, 30ºC, and 20 mg/l, respectively. The reduction of VOOEP in the culture medium was accelerated by Ag^{+} and inhibited by Zn^{2+} and EDTA. The Sn^{2+} and Pb^{2+} ions showed a stimulatory effect at 0.1 mM and an inhibitory effect at 1 mM.
Keywords: Aspergillus; Crude oil; Demetallization; Metalloporphyrin; Vanadium.

INTRODUCTION

Oil is a mixture of hydrocarbons containing a variety of organic compounds. The presence of vanadium in crude oil has an adverse effect on the refinery processes and acts as a poison on catalysts used in catalytic cracking, hydrogenation and hydro-desulphurization processes. This leads to a significant decrease in the yield of cracked products. Metals in crude oil increase the production of coke and gases and cause a marked shortening in the catalyst's life (Adarme *et al.,* 1990; Premuzic *et al.,* 1997; Premuzic and Lin, 1999). The process involving the oxidation of sulphur dioxide to sulphur trioxide is catalyzed by vanadium in fuel oil combustion, leading to corrosion and formation of acid rain. In addition, metal porphyrins are relatively volatile and when crude oil is vacuum-distilled they tend to carry over the heavier fraction of the distillated liquids. Hence, traces of vanadium are usually found in vacuum gas oil (Kukes and Aldag, 1985; Reynolds, 2001; Savastano, 1991; Xu, 1992, 1998).

Among strategies for removing metals from crude oil, chemical processes have been commonly used because of their effectiveness. Many chemical processes such as metal removal with solvent (Savastano, 1991), and the hydrodemetallizing process (Adarme *et al*., 1990; Bartholdy and Hannerup, 1990) are used for this purpose. These are often expensive and produce secondary pollution in the environment (Hernandez *et al*., 1998). Because of these concerns, the use of microbial methods is expected to increase. Although some researchers have already reported microorganisms with the ability to degrade nickel protoporphyrins (Arellano *et al*., 2003; Dedeles *et al*., 2000; Eckemann and Vogelpohl, 1990; Mogollen *et al*., 1998), research is currently being carried out on the demetallization of VOOEP as a model of metallic petroporphyrins in the crude oil (Fig. 1).

This research reports the characteristics of an isolate obtained from polluted soil with a good ability to degrade metallic porphyrin rings such as VOOEP; optimization of VOOEP degradation, and the antagonistic and synergistic effects of some cations and chemicals on the microbial demetallization of vanadium.

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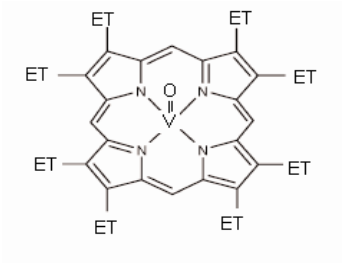

Figure 1. Molecular structure of vanadium oxide octaethyl porphyrin (VOOEP).

MATERIALS AND METHODS

Isolation and screening of microorganisms: Microorganisms were isolated from polluted soil at the Isfahan refinery, Isfahan. One gram of polluted soil was diluted in distilled water and a loopful of the diluted sample was streaked on the plates containing nutrient potato dextrose agar (PDA, 4% (w/v)). Pure colonies were obtained by serial plating. Incubation was carried out at 27ºC, with shaking at 140 rpm for 7 days. The screening medium (pH 5.5) included KH_2PO_4, 2.44 g; Na_2HPO_4, 2.57 g; NH_4Cl, 2 g; $MgCl_2.2H_2O$, 0.2 g; $CaCl_2.6H_2O$, 0.001 g; $FeCl_2.6H_2O$, 0.001 g; $MnCl_2.4H_2O$, 0.004 g, and VOOEP, 20 mg/l. VOOEP was purchased from Aldrich Chemical Company. Other reagents were prepared from Merk Chemical Company, Germany.

Spore preparation and culture conditions: Fungal spores were harvested according to the method by Rapper and Fennell, (1977), after growing on Potato dextrose agar (PDA) medium at 27ºC for 30 days. A sample containing 3.36×10^7 spores per ml was inoculated into 100 ml of culture medium in a 250 ml flask under sterile conditions and incubated for 7 days at 30ºC, with shaking at 140 rpm. The culture medium had the same composition as the screening medium. In order to form a culture medium with a homogenous consistency, VOOEP was dissolved in methylene chloride and then added to the above mineral medium as carbon source yielding VOOEP with an initial concentration of 20 mg/l.

Identification of the microorganism: Identification of the isolate was carried out using macroscopic and microscopic characteristics. Morphological evaluations included observations of colony diameter, color and texture, *etc*., after 7 days of growth. The major microscopic characteristics studied were conidial head, conidia size, color of conidia, etc. Finally, the examined characteristics were compared with those of the standard tests (Rapper and Fennell, 1977).

Optimization of VOOEP degradation using the Taguchi methodology: In this study, we optimized the microbial demetallization of VOOEP by *Aspergillus* sp. using the Taguchi methodology. A standard orthogonal array was used to examine three factors at three levels (Table 1). The results were analyzed to determine the main effects of the factors. The analysis of variance was applied to determine which factors are statistically significant. All calculations were performed using the Taguchi experimental design software (Qualitek4, Demo version) downloaded from the internet (http://www.pqm.cz/Engpqm/frsoftware.htm).

Effect of cations and EDTA on VOOEP biodegradation: In order to evaluate the influence of cations and chelators on VOOEP degradation, concentrated metal salt stock solutions of AgCl, $SnCl_2$, $PbCl_2$, $ZnCl_2$ and EDTA were prepared with sterile distilled water and added to the medium to give concentrations of 0.1 and 1 mM. The percentage of degraded VOOEP in the culture was measured after addition of metal salts and EDTA using a UV-Visible spectrophotometer (V-570, Jasco-Japan).

Analytical methods: In order to estimate the degradation activity of the isolate, culture broth was centrifuged and residual VOOEP was extracted from the supernatant using methylene chloride as solvent. The organic phase was separated from the aqueous phase by centrifugation at 2,610 *xg* for 15 min. The aqueous phase obtained was then examined for the presence of the released vanadium using an atomic absorption spectrophotometer (AA220 model, Varian-USA). The percentage of degraded VOOEP in the organic phase was determined by the UV-visible spectrophotometer, at 407 nm (Dedeles *et al*., 2000). HPLC analyses of degraded VOOEP were carried out using a C18 column (Nova-Pack, 3.9×150 mm) with methanol/acetone buffer (15:5:1) as elutant at a flow rate of 1 ml/min. HPLC system was equipped with Waters 1525 binary HPLC pump and Waters 2487 model UV-visible absorbance detector. Detection wavelength was 254 nm (Flynn and Freeman, 1987).

RESULTS

Isolation and identification of microorganism: A strain identified as *Aspergillus* sp. MS-100 was isolat-

Table 1. Variables and their levels used in the Taguchi experimental design method.

Factors	Level 1	Level 2	Level 3
Temperature (ºC)	20	30	40
pH	4	5.5	7
VOOEP* Concentration (mg/l).	20	40	80

* Vanadium oxide octaethyl porphyrin (VOOEP).

ed from polluted soils of the Isfahan refinery. It was selected out of 19 other isolates, based on the ability to degrade VOOEP, after growth on screening medium containing VOOEP as sole carbon source (initial percentage degradability obtained in the screening medium was 37% at 27ºC after 7 days). Macroscopic and microscopic characteristics of the isolate observed were as follows: globular shape, smooth walled surface, biseriate large sized vesicle serration, metulae covering the entire vesicle, and very rough and irregular conidia surface.

Optimization using the Taguchi method: The growth of the strain on VOOEP was optimized using the Taguchi method of statistical experimental design involving pH, temperature, and initial VOOEP concentration. In this research, L9 orthogonal array was selected and 9 experimental runs were carried out (Table 2). The influence of each variable is shown in the last column of Analysis of variance (ANOVA) (Table 3). The contribution of temperature, pH, and initial VOOEP concentration on growth of the strain was determined as 61.197, 13.113 and 22.634, respectively. Temperature was the most significant factor in

Table 2 . L9 orthogonal array of Taguchi experimental design method and corresponding VOOEP degradation.

Trial No.	Column			VOOEP degradation%	
	Temperature (ºC)	pH	VOOEP* concentration (mg/l)	Assay 1**	Assay 2**
1	1	1	1	11.3	10.8
2	1	2	2	15.2	14.7
3	1	3	3	8.1	7.9
4	2	1	2	30.3	29.6
5	2	2	3	37.5	36.9
6	2	3	1	55.1	54.6
7	3	1	3	6.2	6.0
8	3	2	1	37.0	36.7
9	3	3	2	13.3	12.7

*VOOEP: vanadium oxide octaethyl porphyrin **VOOEP degradation were measured twice and shown as assay 1 and 2. In order to estimate the degradation activity of the isolate, culture broth was centrifuged and residual VOOEP was extracted from the supernatant using methylene chloride as solvent. The organic phase was separated from the aqueous phase by centrifugation at 2,610xg for 15 min. The percentage of degraded VOOEP in the organic phase was determined by the UV-visible spectrophotometer at 407 nm.

Table 3. Analysis of variance (ANOVA).

No.	Factors	DOF*	Sums of Squares	Variance	F-ratio**	Pure sum	Percent (%)
1	Temperature pH	2	2797.423	1398.711	171.282	2781.091	61.197
2	VOOEP	2	612.243	306.121	37.486	595.910	13.113
3	Concentration (mg/l)	2	1044.93	522.465	63.979	1028.598	22.634
	Other/Error	11					3.056
	Total	17	4544.424	8.166			100.00

*DOF: Degree of freedom

**F-value shows that the selected factors are significantat more than 90% confidence limit.

Table 4. Main effects of factors at assigned levels on VOOEP degradation.

	Factor	Level 1	Level 2	Level 3	L2-L1
1	Temperature (ºC)	11.333	40.666	18.649	29.332
2	pH	15.699	29.666	25.283	13.967
3	VOOEP Conc entration (mg/l)	34.25	19.3	17.1	-14.95

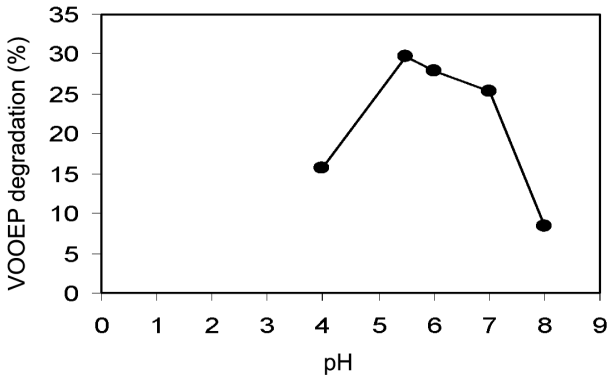

Figure 2. Effect of pH on VOOEP degradation at 30ºC and initial VOOEP concentration 20 mg/l. (Y-axis shows the average of VOOEP degradation (%) estimated by Taguchi method in different pH's).

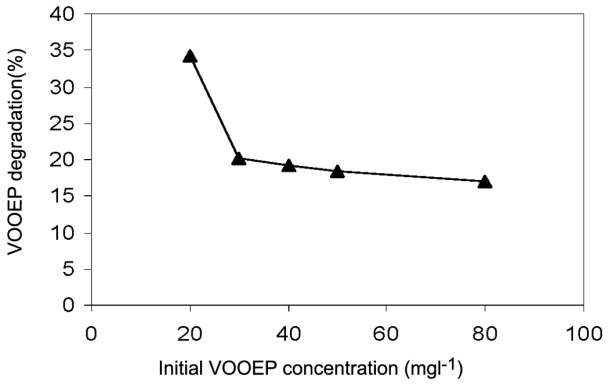

Figure 4. Effect of initial vanadium concentration on VOOEP degradation at pH 5.5 and 30ºC. (Y-axis shows the average value of VOOEP degradation (%) estimated by Taguchi method in different initial VOOEP concentrations).

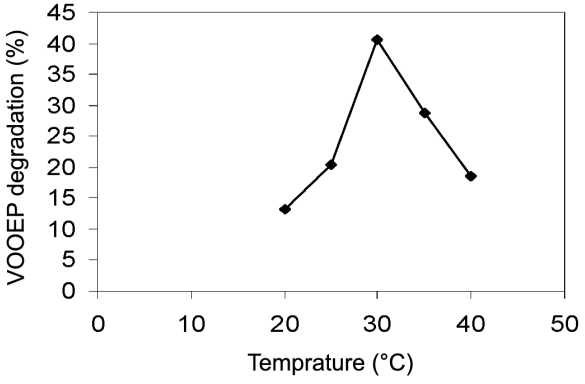

Figure 3. Effect of temperature on VOOEP degradation at pH 5.5 and initial VOOEP concentration 20 mg/l. (Y-axis shows the average of VOOEP degradation (%) estimated by Taguchi method in different temperatures).

VOOEP degradation. From the obtained F-ratio, the selected factors considered in the experimental design are significant at more than 90% confidence limit. The main effects of factors were depicted in Table 4. The difference between L2 and L1 for each factor describes the relative influence of the effect. The sign of difference (+ or -) indicates whether the change from level 1 to level 2 increased or decreased (Table 4). An analysis of data showed that temperature and VOOEP concentration have strong positive and negative effects, respectively. The optimum values of pH, temperature, and initial VOOEP concentration obtained were 5.5, 30ºC and 20 mg/l. The optimum pH value of 5.5 was obtained (Fig. 2). The degradation of VOOEP was measured optimally at 30ºC (Fig. 3) and initial VOOEP concentration of 20 mg/l (Fig. 4). Under optimum conditions, the secreted enzyme(s) produced by the strain had the highest activity and led to the highest percentage of VOOEP degradation (Dedeles *et al.,* 2000). As shown in Figure 4, the use of a higher concentration of substrate (>20 mg/l) did not result in

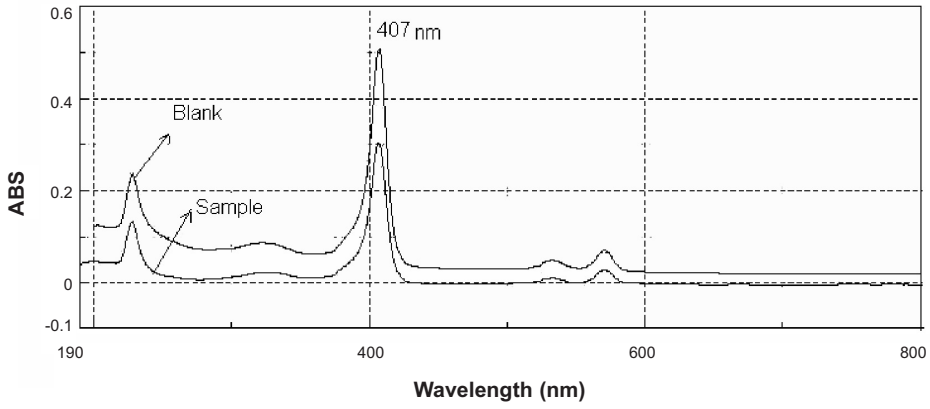

Figure 5. The UV-visible absorption spectra of the culture containing partly degraded VOOEP after 7 days. Y-axis shows the measured absorbance. Peaks at 407 nm indicate VOOEP variation in medium. Sample solution was an organic phase containing residual VOOEP extracted from the supernatant by methylene chloride as solvent. Control solution used was medium before inoculation solved in methylene chloride solution.

extensive consumption and degradation of VOOEP. To validate the proposed optimum conditions, experiments were repeatedly performed for VOOEP degradation under optimum conditions. Under such optimal conditions, VOOEP degradation of approximately 55% was obtained, which corresponded with the expected percentage degradation in the range of 57.482 ± 3.334 with confidence level of 95% and confidence interval of ± 3.334.

The UV-visible absorption spectra of the culture containing partly degraded VOOEP after 7 days and before inoculation are presented in Figure 5. The difference observed between the sample and control peaks at 407 nm proved the degradability potential of the isolate. The results of atomic absorption measurements under optimum growth conditions confirmed the presence of approximately 0.96 mg/l of vanadium (representing approximately 55% released vanadium) in the aqueous phase. It is evident that there has been a transfer of free vanadium from the organic phase to the aqueous phase after degradation.

The potential of the *Aspergillus* strain to degrade

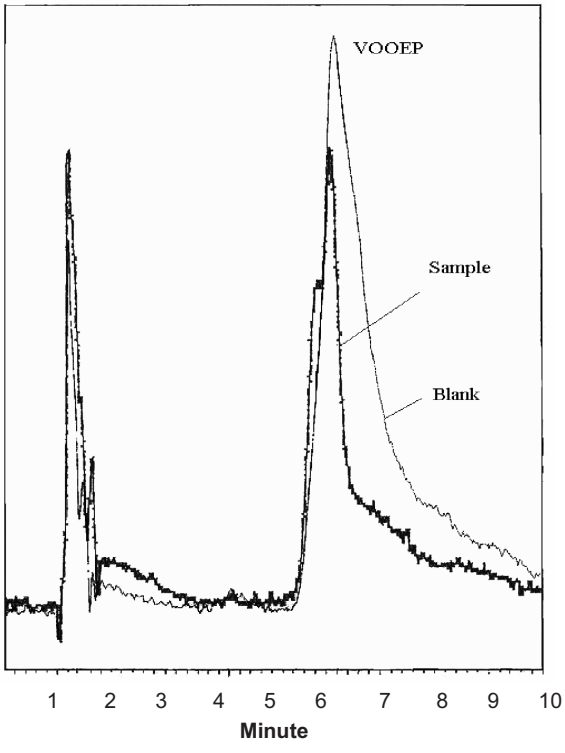

Figure 6. Chromatogram of VOOEP degradation using HPLC. Y-axis shows the absorbance measured with Waters 2487 model UV-visible absorbance detector (Detection wavelength, 254 nm). HPLC analyses were carried out using a C18 packed column (Nova-Pack, 3.9×150 mm) with an elutant containing ethanol/acetone/buffer (15:5:1) at a flow rate of 1 ml/min. Sample solution was an organic phase containing residual VOOEP extracted from the supernatant by methylene chloride as solvent. Control solution used was medium before inoculation solved in methylene chloride solution.

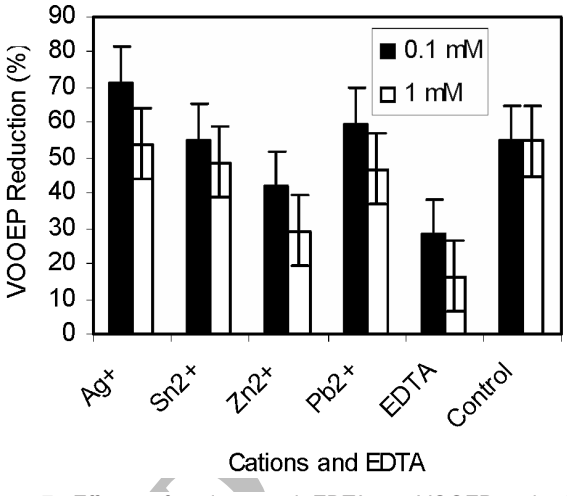

Figure 7. Effects of cations and EDTA on VOOEP reduction. Control: culture broth without adding the mentioned cations and chemicals was centrifuged and residual VOOEP extracted from the supernatant using methylene chloride as solvent at the same conditions with samples.

VOOEP was determined by HPLC under optimum growth conditions (Fig. 6). The HPLC chromatogram confirmed that the VOOEP complex was consumed and two peaks were observed at approximately 1 and 6 min, respectively. The smaller peak was related to methylene chloride used for extracting organic compounds and the bigger peak attributed to VOOEP. The decrease in the sample curve area (by approximately 50%) in comparison with the blank area indicated that VOOEP degradation occurred.

Effects of cations and EDTA: The Effects of some inorganic salts on the degradation of VOOEP were evaluated. The strongest stimulation was observed by Ag^{+}. A strong inhibitory effect resulted from the addition of Zn^{2+} and EDTA (Fig. 7). This figure shows that Ag^{+} has a stimulatory effect at both concentrations whereas Sn^{2+} and Pb^{2+} have an inhibitory effect at 0.1 mM and a stimulatory effect at 1 mM. These stimulatory effects of such ions on VOOEP biodegradation can likely be attributed to further secretions of porphyrinase into the medium. The inhibitory effect of some ions is related to the tendency to compete with the substrate since protoporphyrinase is an electron donor (Dedeles *et al*., 2000)**.**

DISCUSSION

Vanadium present as an organometallic compound is an important constituent of crude oil. The recovery of vanadium from crude oil is most necessarily due to the

environmental and economical aspects (Hernandez *et al*., 1998). In this research, an isolate belonging to the *Aspergillus* species, with an ability to consume VOOEP as a sole carbon source was obtained. The results of VOOEP analysis showed the possibility of VOOEP degradation and release of vanadium into the aqueous phase. This microorganism can reduce trace quantities of vanadium in VOOEP as a model of metallic petroprophyrins in crude oil. The spectrophotometric experiments and HPLC analyses confirmed the degradability potential of *Aspergillus* sp. MS-100 with respect to demetallization of crude oil. Cultivation conditions and VOOEP degradation in falsk cultures were studied using the Taguchi experimental design method. Optimum conditions proposed were pH 5.5, 30ºC and 20 mg/l of VOOEP. The experimental results were corresponding to optimum conditions predicted by the Taguchi method (Figs. 2-4).

Among optimized factors, temperature had a higher contribution. Under optimum conditions, VOOEP degradation of approximately 55% was obtained. Biological degradation of VOOEP was stimulated by adding Ag^{+} and inhibited using Zn^{2+} and EDTA. Sn^{2+} and Pb^{2+} ions showed a stimulatory effect at low levels and an inhibitory effect at high concentrations. The stimulatory and inhibitory mechanisms of VOOEP degradation are yet unclear. The antagonistic and synergistic effects of some cations and chemicals on the demetallization of VOOEP have been attributed to the stimulatory and inhibitory effects of cations on the active sites of porphyrinase enzyme(s). They are also caused by competition between the substrate and cations because protoporphyrinase is an electron donor (Arellano *et al*., 2003; Dedeles *et al*., 2000; Fedorak *et al*., 1993; Mogollen *et al*., 1998). The ability of this isolate to utilize VOOEP as a sole carbon source promises a potential biotechnological application in the oil industries for reducing vanadium levels in crude oil, in future research.

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