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Using petrochemical wastewater for synthesis of cruxrhodopsin as an energy capturing nanoparticle by *Haloarcula* sp. IRU1

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Abstract

In this study, the feasibility of cruxrhodopsin (CR) production as a multifunctional nanoparticle was investigated and optimized by *Halorcula*sp. IRU1, a novel halophile Archaea isolated from Urmia Lake, Iran in batch experiments. In this case, Taguchi method was used for effect measurement of three important factors (petrochemical wastewater, yeast extract and KH_2PO_4) on CR production. Results illustrated that the petrochemical wastewater concentration was the meaningful factor in CR synthesis. The optimum factor levels for petrochemical wastewater concentration, yeast extract and KH_2PO_4 were 2% (w/v), 0.2% (w/v) and 0.004% (w/v), respectively. Also, under these conditions, the predicted value for CR production was about 44.24%. Therefore, this investigation demonstrated that *Haloarcula* sp. IRU1 has a high potential for synthesis of CR from petrochemical wastewater.

Keywords: Urmia Lake; Multifunctional nanoparticles; Taguchi method; Biotechnology; Bioremediation

Introduction

Petrochemical industries and petroleum refineries produce large amounts of oily pollutants (1). A wide range of chemical substances such as environmental carcinogens can be resulted from this pollutants (2). Petrochemical wastewater is an emulsion of water, oil, organic compounds, fats, and metals and solid particles (3). Phenols, nitrobenzene, alkanes, cycloalkanes, toluene, xylenes, and polycyclic aromatic hydrocarbons are common organic compounds of petrochemical wastewater (4-6). The extensive use of petrochemical products leads to the contamination of almost all environmental resources (7). In the regions of petrochemical production, the topsoil and aquatic environments are specifically exposed to contaminations through the products of the factories (8).

The biological treatment, particularly by the acticeated sludge process has been widely used for removal of organic compounds from petrochemical

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wastewater (9). The microbial composition and activity of the activated sludge depend on the nature and availability of petroleum hydrocarbons, nutrient composition, and other environmental conditions (pH, temperature, dissolved oxygen, mixing system, plant configuration) (10-12). Hydrocarbons degradation ability are known in numerous microorganisms, predominantly aerobics including yeasts, bacteria and fungi (13).

Aerobic haloarchaea (the halophilic archeae) are heterotroph archaea that live in very salty environment with arid climates such as Urmia Lake in NW Iran (14). These ecosystems have a variety range of niches, which is related to the physiological characteristics of haloarchaea. Some haloarchaea can ferment argenin, while others can utilize sulfur, nitrate, DMSO as alternative electron acceptors (15). In this case, the ability to produce a proton gradient by the applying of photo-reactive rhodopsin proteins is common property of haloarchaea (16).

There are four functional groups of haloarchaeal rhodopsins. In order to create a proton electrochemical gradient for ATP production, the H^+ pump bacteriorhodopsin (BR) uses light energy to flagellar rotation and other energy requiring processes (17). BR is an integral membrane protein with seven transmembrane alpha-helices and a chromophore group, retinal (18). This energy capturing macromolecule has been recently suggested to have several industrial applications such as in solar cell, design of molecular electron devices, in holographic films and optical computers (19-21).

Cruxrhodopsin (CR) is a homologue of bacteriorhodopsin found in the species of genus *Haloarcula* (Halophilicarchaeon) (22). In the present study we report the ability of *Haloarcula* sp. IRU1 isolated from hypersaline Urmia lake, Iran in production of CR from petrochemical wastewater as carbon source.

Materials and Methods

Microorganism and growth conditions

Haloarcula sp. IRU1 (identified based on comparison of 16S rRNA gene sequences) isolated from hypersaline Urmia lake, Iran was provided from Alzahra University. *Haloarcula* sp. IRU1 isolated from hypersaline Urmia Lake, NW Iran was cultivated in 100 mL Erlenmeyer flasks containing 20 mL of a basal medium and incubated in a shaker at 42°C and 140 rpm for 7 days under aerobic conditions. The basal medium for culture consisted of (g/L): NaCl, 250; $MgCl_2·6H_2O$, 34.6; $MgSO_4·7H_2O$, 49.4; $CaCl_2·2H_2O$, 0.92; NaBr, 0.058; KCl, 0.5 and NaH_2CO_3, 0.17. The growth medium was supplemented with various nutrient compositions by varying petrochemical wastewater as carbon source (collected from Bisotoon Petrochemical Company, Kermanshah, Iran) at 0.25–2% (v/v), phosphorus source [KH_2PO_4, at 0.001–0.016% (w/v)], nitrogen source concentrations [yeast extract at 0.05–0.4% (w/v)] according to the details following experiment design (Table 1).

Table 1. Factors and their levels employed in the Taguchi experimental design for cruxrhodopsin (CR) production from petrochemical wastewater

Factor	Parameter	Level 1	Level 2	Level 3	Level 4
A	petrochemical wastewater % (v/v)	0.25	0.5	1	2
B	yeast extract %(w/v)	0.025	0.05	0.1	0.2
C	KH_2PO_4 %(w/v)	0.005	0.001	0.002	0.004

Measurement of cell growth

For determination of cell dry weight (CDW), the optical density (OD) of the culture broth using a spectrophotometer at 520nm was converted to CDW using a calibration curve, where one OD unit was equivalent to 0.3028 CDW (g/L).

Determination of CR production

Cells from 10 ml culture broth were harvested by centrifugation, and were lysed by re-suspending in equal volume (10 ml) of deionized water containing (0.01 mg) DNase (Fermentase). The lysate was homogenized and mixed with 4 M NaOH and 4 M NH_4OH

in the ratio of (9:0.5:0.5, v/v) in the dark. The absorbance at 568 nm (A_{568}) was first measured in the dark (A^0_{568}). The mixture was exposed to light for 24 h to remove retinal from membrane, and again the absorbance was measured (A^{24}_{568}). As the molecular weight of CR is 27.5 kDa and the molar extinction coefficient is 58,000 M^{-1} cm^{-1}, the concentration of CR was determined by the following equation (9; 17).

$$CR\ (g/l) = 27{,}500 \times A^0_{568} - A^{24}_{568} / 58{,}000.$$

2.4. Experiment design based on Taguchi method

All the combination experiments using the assigned parameter values were conducted with the aim to obtain the final optimum conditions. The Qualitek-4 software was use to design and analysis of Taguchi experiments (18).

Results

The utilization of petrochemical wastewater as a cheap source for production of biological compounds such as CR is very important these days because of petrochemical wastewater environmental issues and public health problems, and industrial application of CR.

The incisive factors (petrochemical wastewater, yeast extract and KH_2PO_4) affecting CR production were surveyed during the optimizing process. The results illustrated in Table 2 indicate that *Haloarcula* sp. IRU1 give the highest CR (15.01% of CDW) in the presence of petrochemical wastewater 0.5%, yeast extract 0.025% and KH_2PO_4 0.001%. Increasing the concentration of petrochemical wastewater decreases the growth rate of the microorganism for all experiments because petrochemical wastewater contains toxic substances for many microorganisms in high concentrations (19). Table 3 demonstrates the major impacts of factors on CR production by *Haloarcula* sp. IRU1. There is difference between the major impacts at level 1 and 2 in the last column labeled (L2-L1). Based on L1-L2 of the investigated factors (Table 3), petrochemical wastewater shows stronger impact on CR production followed by yeast extract and KH_2PO_4. Petrochemical wastewater had very impact at level 2 on CR production among different levels (Figure 1).

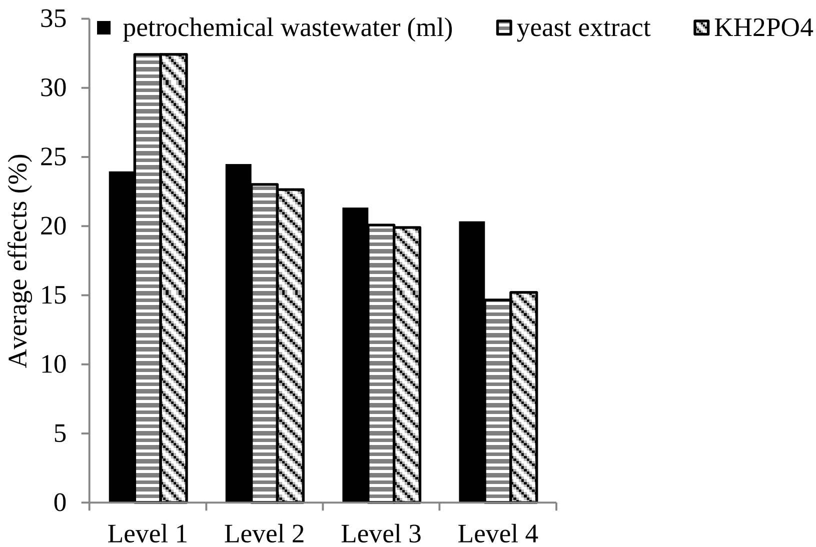

Figure 1. Effect of petrochemical wastewater, yeast extract and KH_2PO_4 in CR production gained by Qualitek-4 (W32b) software.

At a petrochemical wastewater amount of 0.5%, CR/CDW increased and reached optimum level. The effects of yeast extract and KH_2PO_4 were higher in level 1 (0.025% and 0.0005%, respectively) on CR/CDW. Table 4 demonstrates the results of the ANOVA. The objective of ANOVA through Taguchi approach is analyzing the results of the orthogonal array experiments and assessment how much variation that each factor has contributed. This analysis was carried out for a level of confidence of 95%. The analysis of this experimental information demonstrated that yeast extract play considerable role in CR production by *Haloarcula* sp. IRU1. This factor give maximum variance, sum of squares and percentage influence (221.201, 603.617 and 41.187, respectively) but petrochemical wastewater and KH_2PO_4 with low variance (16.172, 211.147), sum of squares (0, 573.458) and percentage influence (0, 39.129) respectively, have no significant effect on CR production. In Table 5 (based on Severity Index SI) three interactions between two factors were calculated by Qualitek-4 software. The highest interaction (SI 35.52) is observed between petrochemical wastewater and KH_2PO_4 (with most and least impact factor, respectively) but interaction between petrochemical wastewater and yeast extract (with more impact factor than KH_2PO_4) is low (SI24.38). These results suggest that the influence of petrochemical wastewater on CR production is dependent on levels of yeast extract and KH_2PO_4.

The optimum conditions for each factor in terms of achieving higher CR yield were summarized as shown in Table 6. These results show KH_2PO_4 and yeast extract are more important than petrochemical wastewater for CR production by *Haloarcula* sp. IRU1. Therefore, KH_2PO_4 and yeast extract concentrations have significant role in CR production than petrochemical wastewater and its levels. Also, the results illustrate that the total contribution from all factors and the current grand average of performance are 21.71 and 22.53%, respectively. These indicate the importance of Taguchi experimental methodology in optimizing production of CR from petrochemical wastewater in different conditions.

Table 2. The orthogonal array of Taguchi experimental design and corresponding CR/CDW

Trial	Factor A	Factor B	Factor C	(CDW) (mg/L)	CR (mg/l)	(CR/CDW)%
1	1	1	1	327.93	41.83	12.75
2	1	2	2	235.57	23.48	9.97
3	1	3	3	221.64	16.31	7.36
4	1	4	4	206.812	14.21	6.87
5	2	1	2	251.92	37.82	15.01
6	2	2	1	246.78	34.41	13.94
7	2	3	4	267.06	11.93	4.47
8	2	4	3	198.03	13.8	6.97
9	3	1	3	299.46	27.73	9.26
10	3	2	4	256.77	12.41	4.83
11	3	3	1	306.13	34.51	11.27
12	3	4	2	231.03	11.2	4.83
13	4	1	4	211.35	22.23	10.52
14	4	2	3	192.58	21.71	11.27
15	4	3	2	216.199	18.01	8.33
16	4	4	1	181.98	19.38	10.65

Table 3. The main effect of different factors on CR production

Factors	Level 1	Level 2	Level 3	Level 4	L1-L2
1 petrochemical wastewater (ml)	23.957	24.489	21.337	20.332	0.532
2 yeast extract	32.402	23.002	20.064	14.647	-9.401
3 KH_2PO_4	32.407	22.627	19.887	15.194	-9.78

Table 4. ANOVA for CR production by *Haloarcula* sp. IRU1 in different conditions

Factor	DOF (f)	Sum of squares (S)	Variance (V)	F-Ratio (F)	Pure Sum (S)	Percent P(%)
1 petrochemical wastewater	3	48.518	16.172	0.808	0	0
2 yeast extract	3	663.603	221.201	11.062	603.617	41.187
3 KH_2PO_4	3	633.443	211.147	10.559	573.458	39.129
Other Error	6	119.97	19.995			19.684
Total	15	1465.536				100.00%

Table 5. Estimated interaction of severity index (SI) for different parameters

#	Interacting Factor Pairs (Oder based on SI)	Columns	SI (%)	Column
1	Petrochemicalwastewater. KH_2PO_4	1×3	35.52	2
2	Petrochemicalwastewater. yeast extract	1×2	24.38	3
3	Yeastextract. KH_2PO_4	2×3	11.29	1

Table 6. Point prediction for optimum conditions of CR production by *Haloarcula* sp. IRU1

Factor	Level description	Level	Contribution
Petrochemical wastewater	Fact. A-lev2	2	1.96
Yeast extract	Fact. B-lev1	1	9.873
KH_2PO_4	Fact. C-lev1	1	9.878
Total contribution from all factors			21.71
Current grand average of performance			22.529
Expected result at optimum condition			44.24

Discussion

An efficient process for wastewater treatment due to mitigate free nitrous acid generation and its inhalation on biological phosphorous removal was already reported (23). Results have shown higher phosphorous removal efficiency with 41% than 30% by the conventional four steps biological nutrient removal process (13, 23). Treatment of petrochemical wastewater was carried out by rotting biological contactor (RBC) system. In this case, the attached biomass was acclimatized by gradually augmenting cyanide concentration from 5 to 40 mg l(-1) with simultaneous increase in the concentration of ammonia nitrogen (NH^{4+}-N) and chemical oxygen demand (COD) (24). Also, wastewater containing Cl_2 residue was treated by packed cage RBC system (25).

Taran (2011) reported using petrochemical wastewater for production of poly(3-hydroxybutyrate) (PHB) by *Haloarcula* sp. IRU1 (26). Results presented there illustrated that the optimum conditions for the maximum production of PHB were petrochemical wastewater 2% (as carbon source), tryptone 0.8% (as nitrogen source), KH_2PO_4 0.001% (as phosphorus source) and temperature 47ºC.

Inorganic and organic and pollution can be resulted from releasing inorganic and organic materials of agriculture, domestic and industrial water activities into environment (27). The sources of pollution include industrial water and domestic agricultural. Ion exchange, carbon adsorption, chemical precipitation, membrane processes and evaporations are conventional methods in wastewater treatment (28). Nowadays, biological treatments have obtained amicability for removing of harmful and toxic materials (29, 30).

The optimization of CR production by *Haloarcula* sp. IRU1 in different conditions was successfully preformed here using Taguchi experimental design. The influences of three factors (petrochemical wastewater, yeast extract and KH_2PO_4) were evaluated on the yield. The optimized conditions were petrochemical wastewater 2% (v/v), yeast extract 0.2% (w/v) and KH_2PO_4 0.004% (w/v). It is obvious that KH_2PO_4 concentration is the most considerable process factor affecting the CR production. This research lays the foundation for further investigation in producing energy capturing nanoparticle from cheap carbon source such as petrochemical wastewater.

References

1. Cerqueira, V.S., Hollenbach, E.B., Maboni, F., Vainstein, M.H., Camargo, F.A., Peralba, Md.C.R. and Bento, F.M. (2011) Biodegradation potential of oily sludge by pure and mixed bacterial cultures. *Bioresour. Technol.*, **102**, 11003-11010.
2. Chen, Y.M., Lin, W.Y., and Chan, C.C. (2014) The impact of petrochemical industrialisation on life expectancy and per capita income in Taiwan: an 11-year longitudinal study. *BMC Public Health.*, **14**, 247.
3. De Almeida, D.G., Soares Da Silva, R.D.C.F., Luna, J.M., Rufino, R.D., Santos, V.A., Banat, I.M., Ibrahim, M., Sarubbo, and Leonie, A. (2016) Biosurfactants: Promising Molecules for Petroleum Biotechnology Advances. *Front. Microbio.*, **7**, 1707-1718.
4. Jing, G., Chen, T., and Luan, M. (2016) Studying oily sludge treatment by thermo chemistry. *Arabian J. Chem.*, **1**, 457-460.
5. Zhang, J., Li, J., Thring, R.W., Hu, X., and Song, X. (2012) Oil recovery from refinery oily sludge via ultrasound and freeze/thaw. *J. Hazard Mater*., **204**, 195-203.
6. Tahhan, R.A., Ammari, T.G., Goussous, S.J., and Al-Shdaifat, H.I. (2011) Enhancing the biodegradation of total petroleum hydrocarbons in oily sludge by a modified bioaugmentation strategy. *Int. Biodeterior. Biodegrad.*, **65**, 130-134.
7. Genuis, S.J., Schwalfenberg, G., Siy, A-KJ., and Rodushkin, I. (2012) Toxic Element Contamination of Natural Health Products and Pharmaceutical Preparations. *PLoS ONE*, **7**, 49676.
8. García-Pérez, J., Boldo, E., Ramis, R., Pollán, M., Pérez-Gómez, B., Aragonés, N., López-Abente, G. (2007) Description of industrial pollution in Spain. *BMC Public Health*, **70**, 40.
9. Silva, C.C., Hayden, H., Sawbridge, T., Mele, P., Kruger, R.H., Rodrigues, M.V.N. C., Gustavo, G.L., Vidal, R.O., Sousa, M.P., Torres, A.P.R., Santiago, V.M.J., and Oliveira, V.M. (2012) Phylogenetic and functional diversity of metagenomic libraries of phenol degrading sludge from petroleum refinery wastewater treatment system. *AMB Exp.*, **2**, 18.
10. Porwal, H.J., Mane, A.V., and Velhal, S.G. (2015) Biodegradation of dairy effluent by using microbial isolates obtained from activated sludge. *Water Res. Indust.*, **9**, 1-15.
11. Sarkar, J., Kazy, S.K., Gupta, A., Dutta, A., Mohapatra, B., Roy, A., Bera, P., Mitra, A., and Sar, P. (2016) Biostimulation of indigenous microbial community for bioremediation of petroleum refinery sludge. *Front. Microbio*., **7**, 1407.
12. Lu, L., Huggins, T., Jin, S., Zuo, Y., and Ren, Z.J. (2014) Microbial metabolism and community structure in response to bioelectrochemically enhanced remediation of petroleum hydrocarbon-contaminated soil. *Environ. Sci. Technol.*, **48**, 4021-4029.
13. Rajasulochana, P., and Preethy, V. (2016) Comparison on efficiency of various techniques in treatment of waste and sewage water – A comprehensive review. *Res. Effic. Technol.*, **2**, 175-184.
14. Rodriguez-Valera, F. (1993) Introduction to saline environments. *Biol. Halo. Bact*., **1**, 1-23.
15. Spudich, J.L., and Jung, K.H. (2005) *Microbial rhodopsins: phylogenetic and functional diversity. Handbook of Photosensory Receptors*. Wiley-VCH, Pp. 1-23.
16. Frigaard, N.U., Martinez, A., Mincer, T.J., and DeLong, E.F. (2006) Proteorhodopsin lateral gene transfer between marine planktonic Bacteria and Archaea. *Nature*, **7078,** 847-50.
17. Oesterhelt, D., and Stoeckenius, W. (1973) Functions of a new photoreceptor membrane. *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 2853-2857.

18. Curnow, P., and Booth, P.J. (2009) The transition state for integral membrane protein folding. *Proc. Natl. Acad. Sci. U.S.A.*, **106**, 773-778.
19. Balu, R., Zhang, H., Zukowski, E., Chen, J.Y., Markelz, A.G., and Gregurick, S.K. (2008) Terahertz spectroscopy of bacteriorhodopsin and rhodopsin: similarities and differences. *Biophys. J.*, **94**, 3217-3226.
20. Trivedi, S., Choudhary, O.P., and Gharu, J. (2011) Different proposed applications of bacteriorhodopsin. *Recent Pat. DNA Gene Seq.*, **5**, 35-40.
21. Saeedi, P., Moosaabadi, J.M,, Sebtahmadi, S.S., Mehrabadi, J.F., Behmanesh, M., and Mekhilef, S. (2012) Potential applications of bacteriorhodopsin mutants. *Bioeng.*, **3**, 326-8.
22. Yang, Y., Yatsunami, R., Ando, A., Miyoko, N., Fukui, T., Takaichi, S., Nakamura, and S. (2015) Complete biosynthetic pathway of the C(50) carotenoid bacterioruberin from lycopene in the extremely halophilic archaeon *Haloarcula japonica*. *J. Bacteriol.*, **197**, 1614-1623.
23. Zhao, J., Wang, D., Li, X., Yang, Q., Chen, H., Zhong, Y., An, H., and Zeng, G. (2015) An efficient process for wastewater treatment to mitigate free nitrous acid generation and its inhibition on biological phosphorus removal. *Sci. Rep.*, **5**, 8602.
24. Kubsad, V., Gupta, S.K., and Chaudhari, S. (2005) Treatment of petrochemical wastewater by rotating biological contactor. *Environ. Technol.*, **26**, 1317-1326.
25. Sirianuntapiboon, S. (2006) Treatment of wastewater containing Cl2 residue by packed cage rotating biological contactor (RBC) system. *Bioresour. Technol.*, **97**, 1735-1744.
26. Taran, M. (2011) Utilization of petrochemical wastewater for the production of poly(3-hydroxybutyrate) by *Haloarcula* sp. IRU1. *J. Hazard. Mater.*, **188**, 26-8.
27. Zhou, G.J., Ying, G.G., Liu, S., Zhou, L.J., Chen, Z.F., and Peng, F.Q. (2014) Simultaneous removal of inorganic and organic compounds in wastewater by freshwater green microalgae. *Env. Sci. Process Im.*, **16**, 2018-1027.
28. Abdel Salam, O.E., Reiad, N.A., and El-Shafei, M.M. (2011) A study of the removal characteristics of heavy metals from wastewater by low-cost adsorbents. *J. Adv. Res.*, **2**, 297-303.
29. Ray, P.C., Yu, H., and Fu, P.P. (2009) Toxicity and environmental risks of nanomaterials: challenges and future needs. *J. Environ. Sci. Health C Environ. Carcinog. Ecotoxicol. Rev.*, **27**, 1-35.
30. Baker, D. (2004) Civilian exposure to toxic agents: emergency medical response. *Prehosp Disaster Med.*, **19**, 174-178.