CHEMICAL ENGINEERING TRANSACTIONS

VOL. 27, 2012

A publication of AIDIC
The Italian Association of Chemical Engineering
Online at: www.aidic.it/cet

Guest Editors: Enrico Bardone, Alberto Brucato, Tajalli Keshavarz
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ISBN 978-88-95608-18-1; **ISSN** 1974-9791

DOI: 10.3303/CET1227065

Identification and Characterization of Aromatic Degrading *Halomonas* in Hypersaline Produced Water and COD Reduction by Bioremediation by the Indigenous Microbial Population Using Nutrient Addition

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Hypersaline produced water is a high volume waste stream that is typically contaminated by toxic low molecular weight aromatic compounds exemplified by phenol. Aromatic compound degrading *Halomonas* were isolated from hypersaline produced water obtained from offshore operations in Brazil, which had a chemical oxygen demand (COD) of 4300 mg/L. These isolates were able to aerobically degrade the oxygenated aromatics phenol, benzoic acid, para-hydroxybenzoic acid and some isolates were shown to produce aromatic dioxygenase activities associated with highly conserved aromatic degradation pathways utilized by a broad range of aromatic degrading bacteria. The presence of aromatic degrading bacteria in the hypersaline produced water suggested that the COD content could be reduced by bioremediation using the indigenous microbial population by the addition of nutrients. Using this approach a variety of nitrogen, phosphorous and carbons sources were identified that individually or in combination significantly improved the reduction in COD after aerobic incubation. These results demonstrate the potential of applying bioremediation to undiluted hypersaline produced water for COD reduction and aromatic compound removal.

1. Introduction

During production of crude oil large amounts of hypersaline wastewater is also produced, particularly as the wells decline in oil production (Speight, 2007). For every bbl of oil produced, approximately 10 bbl of water is also generated, which is frequently hypersaline. For example, produced water volume estimates in the United States in 2009 were in the range of 15 to 20 billion barrels (Clark and Veil J.A., 2009).
In addition to salinity, which ranges from 100 mg/L to 400,000 mg/L, produced water also contains traces of oil, H_2S, heavy metals and soluble organic compounds. The soluble organic components consist largely of phenolics and other oxygenated aromatics and low molecular weight aromatic hydrocarbons (Veil et al., 2004). If discharged into the environment without prior treatment it can cause severe hazards by contamination of soil, and surface and groundwater. Thus, the treatment and disposal of produced waters is a critical need within the oil industry and it is also a major component of

Please cite this article as: Piubeli F., Grossman M., Fantinatti-Garboggini F. and Durrant L., 2012, Identification and characterization of aromatic degrading halomonas in hypersaline produced water and cod reduction by bioremediation by the indigenous microbial population using nutrient addition., Chemical Engineering Transactions, 27, 385-390 DOI: 10.3303/CET1227065

the cost of producing oil and gas. The ability to efficiently and economically dispose of this water is critical to the success in the oil production business. Current technologies are fairly well developed for removing oil and solids from produced water but dissolved organics are more problematic (Arnold et al., 2004). One option for treatment of produced water dissolved aromatics is to develop robust bioremediation systems that can remove the toxic organic content under the hypersaline conditions. Conventional microbiological treatment processes do not function well at high salt concentrations indicating that microbial bioremediation of undiluted hypersaline produced water requires halophiles (Lefebvre and Moletta, 2006). However, little is known about their degradative capabilities, degradation pathways and aromatic biodegradation in hypersaline environments.

The aim of this work was to investigate the aromatic degradation potential of bacteria present in hypersaline produced water and to evaluate the ability to reduce the carbon oxygen demand (COD) and aromatic content of the produced water by stimulating the indigenous microbial community through the addition of limiting nutrients.

2. Materials and Methods

2.1 Produced water

Produced water from offshore Brazilian oil production in the Campos Basin, was obtained from the Petrobras (Petróleo Brasileiro S.A.) terminal (Almirante Barroso Maritime Terminal) in São Sebastião, SP, Brazil. The produced water contained approximately 100 g/L of NaCl and a COD of 4300 mg/L.

2.2 Isolation of bacteria from produced water

A 2 mM aqueous solution of either phenol, benzoic acid, or hydroxybenzoic acid was sprayed on the surface of Halophile Growth Medium containing 10 % NaCl (HGM 10 % NaCl) agar plates as described by Fairley et al. (2002). The plates were inoculated by spreading 100 µL of produced water on the surface and incubated at 38 ºC. Isolates were purified twice and stock cultures of each isolate were prepared from individual colonies selected from this second purification step.

2.3 Determination of salt tolerance

The isolates were evaluated for growth in HGM plus 7.5 g/l yeast extract containing 0.5 %, 1 %, 2 %, 3 %, 5 %, 7.5 %, 15 %, 20 % or 25 % (w / v) NaCl.

2.4 Growth of halophilic bacteria isolates on monoaromatic compounds

Fifty mL of HGM 10 % NaCl medium supplemented with 0.1 % of the vitamin solution (Wolin et al., 1963), with or without 0.05 % yeast extract, and containing 2 mM of the indicated aromatic compound, were inoculated to an OD at 600 nm (OD_{600}) of 0.01. Cultures were incubated for 12 days at 38 ºC with shaking at 130 rpm and samples were taken as indicated for measurement of OD_{600}. Controls without inoculum were incubated under the same conditions. Growth was determined by monitoring the increase in OD_{600}. Cultures were run in triplicate for each time point and the value given is the average of the three measurements. Results are given as the maximum OD_{600} achieved over the 12-day incubation period.

2.5 Degradation of monoaromatic compounds by halophilic bacteria

Degradation of aromatic compounds present in the growth experiments was determined at the time of inoculation (T0) and after 12 days (T12). Quantitative analysis of aromatic compounds in cell free supernatants was performed by high performance liquid chromatography (HPLC) using a Shimadzu liquid chromatograph, model LC-6A, equipped with a Varian reverse phase C18 column (5 mm x 150 mm x 46 mm) and UV-Vis detector. Separation was achieved by isocratic elution with acetonitrile:water (70:30) as mobile phase, with a flow rate of 0.6 mL/min and UV absorbance at 260 nm.

2.6 Enzymatic activity

Enzyme activities were measured as the volume (ml) of enzyme required to convert 1 mmol of substrate per minute. Catechol 1,2-dioxygenase (1,2-CD) activity was determined according to the method of Ornston, et al. (1966). Catechol 2,3-dioxygenase (2,3-CD) activity was determined according to the method of Masai, et al. (1995). Enzyme activity is presented qualitatively as positive or

negative using a cutoff of 0.05, which represents an unambiguous difference in absorbance values between controls receiving no cell extract and cell extract samples.

2.7 Evaluation of nutrients and carbon sources to stimulate COD reduction in produced water by the indigenous microbial population

Several nutrients were tested for their effect on stimulating the indigenous microbial community present in the produced water to reduce COD. The additives used are summarized in Table 1. Additives were evaluated for their effect on COD reduction, in duplicate, in flasks containing 50 mL of produced water incubated at 38 °C with shaking at 150 rpm. After four days the contents of each flask were centrifuged at 3000 x ***g*** for 15 min at 5 ºC and the supernatant used for COD and HPLC analysis. HPLC analysis was performed as described above. COD was determined by absorbance using a Hach model DR-2000 spectrophotometer (Hach Company, USA) according to manufactures instructions.

Table 1: Matrix of nutrient additions

Addition		Alanine (0.2 g/L)	Histidine (0.2 g/L)	GA* (0.2 g/L)	Lactose (0.2 g/L)
Vitamins[#]	+	+	+	+	+
KH_2PO_4 (0.1 g/L) NH_4NO_3 (0.5 g/L)	+	+	+	+	+
KNO_3 (1.0 g/L)	+	+	+	+	+
NH_4NO_3 (1.0 g/L)	+	+	+	+	+
KH_2PO_4 (0.1 g/L)	+	+	+	+	+
*YE (0.01 g/L)	+	+	+	+	+
Tryptone (0.02 g/L)	+	+	+	+	+
Histidine (0.2 g/L)	+	-	-	-	-
GA (0.2 g/L)	+	-	-	-	-
Lactose (0.2 g/L)	+	-	-	-	-
Alanine (0.2 g/L)	+	-	-	-	-

*GA = Glucuronic acid, YE = yeast extract. All concentrations are final concentrations. [#]1 % vitamin solution (Wolin et al., 1963). + = added, - = not added.

2.8 Isolation of total genomic DNA and sequencing of PCR amplified 16S ribosomal RNA genes

Genomic DNA was extracted using the CTAB (Cetyl trimethylammonium bromide) methodology (Ausubel et al., 1989). Bacterial 16S rDNA was amplified using the bacteria domain specific primer 1100R (5'-AGGGTTGCGCTCGTTG-3') and the universal primer 27F (5'-AGAGTTTGATCC TGGCTCAG-3'). All PCR amplification reaction where performed in a BioRad model iCycler. PCR products were purified using mini-columns (GFX PCR DNA and Gel Band Purification Kit, GE Health Care) and submitted to sequencing in an automated sequencer (MegaBace, GE Health Care). The sequencing reactions were performed with the DYEnamic ET Dye Terminator Cycle Sequencing Kit MegaBace DNA Analysis Systems (GE Health Care) using the internal primers 16SBac10f, 16SBac 765f, 16SBac 782r and 1100r.The sequences were compared against the nucleotide collection (nr/nt) database, using the nucleotide blast (blastn) Basic Local Alignment Search Tool available through the National Center for Biotechnology Information website.

3. Results and Discussion

3.1 Isolation of bacteria from produced water

Six isolates demonstrating growth on at least one of the tested aromatic compounds were selected. Comparative DNA sequence analysis of the 16S ribosomal RNA genes indicated they are members of the genus *Halomonas* and most closely associated with the species *H. alimentaria*. *H. alimentaria* has previously been identified by 16S rRNA gene analysis in a consortium that degraded phenanthrene at 5 %, 10 %, and 15 % salinity (Zhao et al., 2009). Although all of the isolates obtained from the produced water are phylogenetically most related to this genus species, their salt tolerance, aromatic degradation patterns and aromatic dioxygenase profiles were different, as described below. All strains grew well in this medium over the range of 0.5 to at least 20 % NaCl, with the exceptions of BA1 that did not grow above 15 % and of DP3, which grew poorly between 0.5 and 2 % NaCl and also at 15 % and above. *Halomonas* are a genus of moderately halophilic bacteria that have been shown in recent years to degrade a wide range of aromatic compounds under hypersaline conditions (Zhuang et al., 2010; Yang et al., 2010; Oie et al., 2007; García et al., 2005), including benzoic acid, p-hydroxybenzoic acid, cinnamic acid, salicylic acid, phenylacetic acid, phenylpropionic acid, phenol, p-coumaric acid, ferulic acid and p-aminosalicylic acid, benzoate, salicylate, guaiacol, vanillin, dibenzo-p-dioxin, biphenyl and fluorene.

3.2 Growth, degradation and aromatic dioxygenase activity of isolates grown on aromatic compounds

In general all isolates exhibited very little to no growth in the media containing phenol in the absence of yeast extract (data not shown). However, with the addition of 0.5 g/L yeast extract, isolate DP2 demonstrated good growth followed by DP3, PA1 and to lesser extent DP1. Isolate PA2 grew only slightly above initial OD_{600} of 0.01, reaching an OD at 600 nm of only 0.11. Comparison of the extent of degradation for the different isolates shows that the maximum OD_{600} correlated with the percent degradation qualitatively but was not a linear predictor of the quantitative extent of degradation. For example, DP2 and DP3, which had the highest maximum OD values of 0.57 and 0.25, respectively, also degraded phenol to the greatest extent, 81 and 90%, respectively. With regard to aromatic dioxygenase activity, 1,2-CD and 2,3-CD activity was observed for DP1 and DP2, consistent with two broadly distributed aromatic oxidation pathways for phenol degradation, involving intradiol cleavage and extradiol cleavage, respectively. Isolate PA2, which grew only slightly, produced virtually no degradation, but did produce detectable 1,2-CD activity.

Isolate DP2, DP3, and BA1 grew well in media containing benzoic acid and yeast extract, all reaching a similar maximum OD of approximately 0.3. Isolates BA1 and DP2 degraded 99% of the benzoic acid, while DP3 degraded 45 %. Isolated DP2 produced detectable levels of 1,2-CD and 2,3-CD, suggesting that benzoic acid was being converted to catechol. DP1 grew poorly, only reaching an OD_{600} nm of 0.1, but did produce detectable levels of 1,2-CD and a 70 % reduction in the level of benzoic acid.

When grown in the presence of PHBA, DP2 reached the highest OD (0.3), resulting in 100% removal of the compound. Both 1,2-CD and 2,3-CD activity were detected suggesting that PHBA was converted to catechol and further oxidized through both the intradiol and extradiol routes. Isolates PA1 and PA2 reached a lower OD than DP2 and produced less degradation, 42 % and 25 % respectively. Isolate PA1 also produced detectable levels of 1,2-CD 3,4-PCD activity, suggesting PHBA was oxidized via conversion to catechol and protocatechuate.

3.3. Bioremediation of produced water COD by indigenous microorganisms

A variety of nitrogen, phosphate, carbon and vitamin amendments and combinations thereof (Table 1) were added to produced water in 96 well plates and visually screened for stimulation of cell growth as indicated by increased turbidity (data not shown). From this initial screen those compounds that produced a noticeable increase in cell growth were chosen as bioremediation additives for evaluation of their effect on reducing the COD of produced water in 50 mL cultures. Figure 1 shows the percent COD reduction in the nutrient amended produced water cultures that significantly improved COD removal. In the absence of any nutrient addition the COD was reduced by 20 % either due to loss via the volatilization of compounds and/or by biodegradation by the indigenous organisms.

The results indicate that the addition of nitrogen and phosphorous and readily assimilated carbon sources stimulated the indigenous microbial population resulting in an increased overall reduction in COD. The addition of a combination of KH_2PO_4 and NH_4NO_3 to a final concentration of 0.1 and 0.5 g/L, respectively, more than doubled the COD reduction, increasing COD reduction from 20 % to approximately 50 %. The introduction of either KH_2PO_4 or NH_4NO_3 individually, to a final concentration of 0.1 and 1 g/L, respectively, achieved similar levels of COD reduction (50 – 60 %). The addition of the 0.2 g/L of alanine, histidine, glucuronic acid, tryptone or lactose alone did not improve COD reduction. However, when these carbon and nitrogen sources were added in combination with other amendments significant increases in COD were observed (Figure 1). Most notably, the combination of histidine with 1.0 g/L KNO3, glucuronic acid and 0.1 g/L KH_2PO_4, alanine with 0.1 g/L KH_2PO_4, and glucuronic acid and 0.2 g/L tryptone, which increased the level of COD reduction from 20 % to 63 %, 68 %, 72 % and 89 %, respectively. Glucuronic acid is a component of polysaccharides produced by marine phytoplankton and therefore represents a common nutrient in marine waters and may explain the strong stimulation that glucuronic acid produced in combination with tryptone. These results indicate that a carbon source alone is not sufficient to provide the growth limiting nutrients required for improved COD reduction.

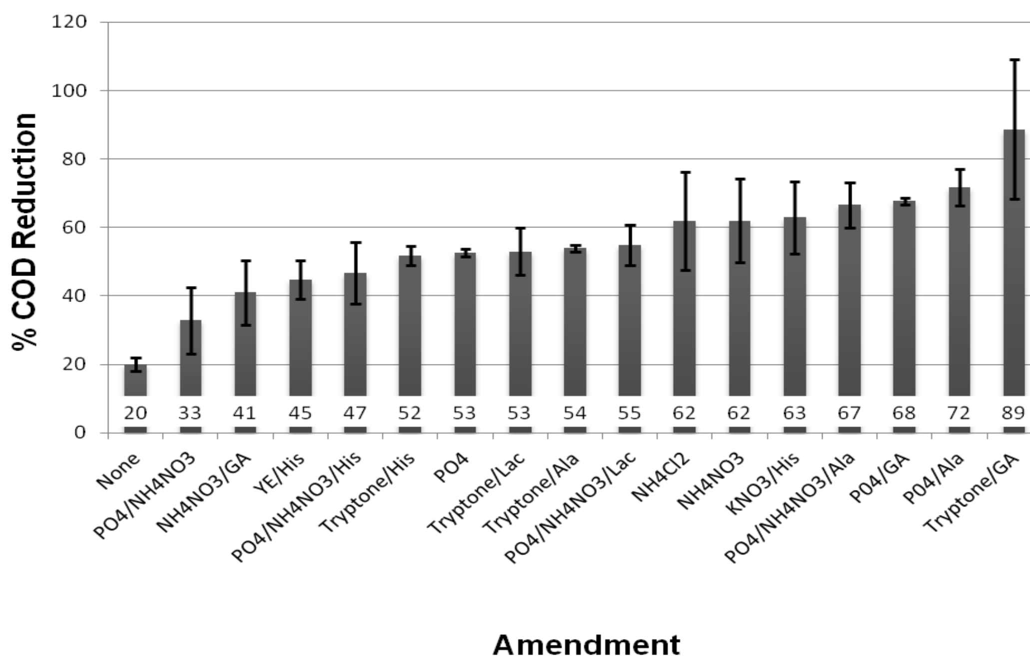

Figure 1. COD reduction in the nutrient amended produced water cultures after 4 days of incubation. Ala = alanine , His = histidine, Lac = lactose, GA = Glucuronic acid, YE = yeast extract. The average COD reduction value is shown at the bottom of the graph. Error bars indicate the standard deviation of the COD reduction values obtained

The produced water studied in this work was treated with hydrogen peroxide at the storage facility, and although this treatment represses hydrogen sulfide production for a period of time and results in some reduction in COD, a significant amount of COD remains and is a persistent concern with regard to its toxicity and the potential effect on local dissolved oxygen levels when disposed of offshore. We isolated bacteria from the genus *Halomonas* from the produced water that are capable of degrading one or more of the aromatic compounds, phenol, benzoic acid and PHBA. The activity of the aromatic dioxygenases catechol 1,2 dioxygenase (1,2-CD) and catechol 2,3 dioxygenase (2,3-CD) were also observed for some of the isolates when grown on these compounds and the presence of these activities largely correlated with degradation. The presence of aromatic degrading halophiles in the

produced water suggested that bioaugmentation by addition of nutrients could stimulate the metabolic activity of the indigenous microbial community to further metabolize the remaining organic compounds and correspondingly reduce the COD content. These results show that bioremediation of hypersaline produced water for the reduction of COD and aromatics is feasible without dilution, however, the addition of nutrients and the presence of aromatic degrading halophiles is likely to be required.

Acknowledgment

We thank FAPESP and CNPq for financial support.

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