A COMPARATIVE STUDY OF OZONATED AND NON-OZONATED EFFLUENTS TO SCREEN NOVEL PARANITROPHENOL DEGRADERS

JAYASHREE PAWAR, DILEEP KAMAT, SHEELA KAMAT

Abstract: Secondary treatment of industrial effluent with ozonation has been used to treat organic pollutants. In this study, the effect of bioremediation and ozonation on the effluent of a chemical industry with high concentration of nitro phenolic compounds was analysed.

Molecular techniques have made it clear that a large proportion of bacterial diversity in natural habitats remains uncultured and therefore unexplored. There has also been evidence of bacteria that grow only in dilute nutrient media and form microscopic colonies. This study studied unculturable bacteria involved in degradation of paranitrophenol from ozonated and non-ozonated effluent, through the 16S rDNA analysis. Dilute nutrient media containing the industrial effluent were used to isolate bacteria from the sludge sample that could tolerate 100 ppm of paranitrophenol. Identification of these cultures was performed by 16S rDNA analysis.

Bioremediation followed by ozonation was found to be an effective way of reducing BOD and COD of the paranitrophenol containing industrial effluent. Isolates from non-ozonated and ozonated effluent indicated that three isolates out of fourteen possibly represented new species. While Bacillus species predominated in ozonated effluent, non-ozonated effluent showed presence of diverse genera of organisms including *Pseudomonas, Micrococcus*, *Arthrobacter* and *Bacillus*. Our study revealed that dilute culture media simulating the natural environment was helpful in isolation of three possible new species of bacteria involved in degradation of paranitrophenol.

Keywords: PNP; dilute media; uncultured bacteria; ozonation; industrial effluent.

Introduction: Nitroaromatic compounds are used in many industrial processes, including the preparation of pesticides, explosives, textiles and paper. Paranitrophenol is an important intermediate formed during the manufacturing of analgesic and antipyretic drugs [3]. As a result of these widespread applications, wastewaters and water resources, including groundwater and surface waters, have become contaminated with this compound. P-Nitrophenol is toxic and thereby harmful to public health and aquatic life and has been listed by the USEPA as a priority pollutant [8]. Sometimes conventional wastewater treatment methods have failed to treat persistent organic pollutants effectively. Therefore, these compounds have been often detected as water pollutants. Remediation of wastewaters containing these pollutants is difficult, since they are usually resistant to biological degradation [5].

Chemical oxidation with ozone has been used as secondary effluent treatment to treat organic pollutants [14]. Ozone is an allotrope of oxygen and is more powerful than chlorine and other oxidants and reacts with many compounds via direct or indirect reactions. The term “The great plate count anomaly” was coined by Staley and Konopka in 1985 [9] to describe the difference in orders of magnitude between the numbers of cells from natural environments that form

colonies on agar media and the numbers countable by microscopic examination [4]. One explanation for the "great plate count anomaly" is that many of the microbial species that dominate in natural settings are not adapted for growth in media containing high concentrations of complex organic carbon. Many microorganisms may need oligotrophic or other fastidious conditions to be successfully cultured. There are many examples of microbial strains that are common in nature, but can only be cultivated by specialized techniques [13].

This study involved analyzing the effect of bioremediation and ozonation on BOD and COD of industrial effluent with high concentration of nitro phenolic compounds, whether the treatment has been successful in reducing these parameters for safe discharge of the effluent in a water body. The study also aimed at analysis of bacterial diversity of the ozonated and non-ozonated effluent samples and isolation of novel, yet uncultured paranitrophenol degrading bacteria in the sludge sample.

Materials and methods:

Ozonation of effluent: The ozone was generated at the rate of 1gm/hour using an ozone generator (A M Ozonic Pvt. Ltd).

Determination of BOD and COD: The effluent was tested for Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) using standard procedures (APHA 2007).The experiment was performed in triplicates and average values have been stated in the results.

Isolation of PNP degrading cultures:

For isolation of novel paranitro phenol degrading bacteria, sludge sample was inoculated on media plates containing 0.01% nutrient agar or 0.01 % casein along with 10% effluent and 100 ppm PNP in the media. Isolates on the plate showing PNP decolourization were maintained in pure culture on both dilute media as well as nutrient agar.

Molecular identification of cultures:

- DNA Extraction: Genomic DNA was extracted from the isolated cultures. The protocol by Watanabe et al [11] was scaled down to 1.5 ml culture and used for DNA extraction.
- Polymerase Chain Reaction: Fifty ng of the extracted DNA was added to a master mix prepared in PCR grade distilled water containing 10X PCR buffer without $MgCl_2$, 1.5 mM $MgCl_2$, 250µM of each of the dNTPs, 0.5µM of each of the universal 16S rDNA primers (Forward primer: 5'AGAGTTTGATCCTGGCTC3' and reverse primer: 5'GCTCGTTGCGGGACTTAA3') and 2 units of *Taq* DNA polymerase (Merck). PCR was carried out according to the following thermo cycling parameters: "initial denaturation at 94˚C for 5 mins, 25 cycles each with denaturation at 94˚C for 1 min, primer annealing for 1 min at 55˚C, and primer extension 72˚C for 1 min with 5 min final extension at 72˚C". Appropriate positive and negative controls were also set up. The amplification products were run on ethidium bromide stained 1% agarose gel along with a molecular weight marker (Merck).
- Identification of isolates: Amplification products were sequenced (Merck) and further analysed by BLASTn and Ribosomal Database Project (RDP).

Results& discussion:

Determination of effect of bioremediation and ozonation on BOD and COD of raw effluent:

In the present study, bioremediation and ozonation were used to reduce the BOD and COD of the effluent. A consortium of isolates from the sludge sample was prepared and used for bioremediation. In one set of experiments, the effluent was treated first with consortia of microorganisms (bioremediation) for 72 hours

followed by ozonation for 10 mins. In the other set of experiment, the effluent was treated first with ozonation for 10 mins followed by bioremediation. Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) of the effluent before and after treatment were used as criteria to analyse the effect of these treatments on the effluent. Raw effluent was found to have BOD and COD of 1760 ppm and 4200 ppm respectively. It was found that bioremediation reduced BOD by 95% in 72 hours followed by ozonation for 10 mins which further reduced BOD by 62%. Similarly, COD was reduced by 90% after bioremediation and further by 71% after ozonation. In the second set, ozonation for 10 mins reduced BOD by 40% followed by bioremediation for 72 hours which further reduced it to 90%. Similarly, COD was reduced after ozonation by 38% and then further by bioremediation to 90% (Table 1).

Table 1: Effect of Bioremediation and ozonation on effluent

Sample	BOD	COD
Effluent before ozonation / bioremediation	1760	4200
After bioremediation	88	420
After bioremediation followed by ozonation	33	122
After ozonation	1056	2606
After ozonation followed by bioremediation	106	260

Bioremediation is expected to breakdown compounds to smaller components which would be further saturated by oxidation using ozone, resulting in BOD andCOD reduction. In contrast, if the effluent is first ozonated, the xenobiotic compounds formed are difficult to be further saturated by bioremediation. Hence, bioremediation for 72 hours followed by ozonation for 10 mins was found to be more effective in BOD, COD reduction than ozonation and then bioremediation.

Isolation of novel PNP degrading cultures: High concentrations of nutrients used in conventional cultivation attempts may inhibit a large number of microorganisms, as many natural microbial communities flourish in oligotrophic conditions. Indeed, reports have indicated that the use of low concentrations of nutrients has increased microbial recovery [2], [12]. Simulation of natural environment has been another approach to culture the previouslyuncultured [10]. Inclusion of effluent in the medium is expected to provide nutrients and/ or signalling molecules and help culture the uncultured microorganisms. Both these approaches have been used in our study to isolate yet uncultured bacteria that can degrade 100 ppm paranitrophenol.

Dilute nutrient media containing effluent were used to isolate bacteria that could tolerate 100 ppm of paranitrophenol. Sludge from the same effluent sample was used as inoculum. Micro colonies on agar media that could show visible decolourization of PNP either on lines of

streaking or overall decolourization on the plate were selected. Seven cultures from ozonated and seven from non ozonated samples were isolated having ability to decolorize PNP, which was also confirmed by inoculation in broth. Decolourization of PNP containing broth was observed within 72 hours.

Molecular identification of cultures: In order to characterize the isolated cultures, DNA was extracted from these cultures and subjected to PCR amplification using universal 16S rDNA primers. Amplification products were sequenced and further analysed by BLASTn and RDP.

Because of universal distribution of 16S rDNA and the presence of species specific variable regions, this molecular approach have been extensively used for bacterial phylogeny leading to the establishment of large public domain databases, and its application to bacterial identification [1], [7]. BLASTn analysis and the Ribosomal Database Project (RDP) have been widely used in analysis of 16S rRNA gene sequences. It has been demonstrated that 16S rRNA gene sequence data on an individual strain with a nearest neighbour exhibiting a similarity score of <97% in BLASTn analysis represents a new species, the meaning of similarity scores of >97% is not as clear [6]. This latter value can represent a new species, or, alternatively, indicate clustering within a previously defined taxon.

Sequence match of RDP is used to identify the most similar sequences in the RDP to search query 16S rRNA gene sequence. The similarity between search sequence and sequences in the database is represented in terms of S_ab value. S_ab is a complex similarity score. Two identical sequences will have a score of 1.0, and the closer the score is to 1.0, the more similar the sequences are. Results of BLASTn and RDP analysis of 16S rDNA sequences of isolates are given in table 2 and 3.

Sequence analysis of isolates from ozonated samples revealed that out of seven cultures, five belonged to genus *Bacillus* which showed BLASTn similarity score of more than 97%. Cultures O3a and O12b belonging to genus *Bacillus* and *Pseudomonas* respectively had BLASTn score of less than 97%, thus may represent new species (Table 2).

Table 2: Sequence analysis of isolates from ozonated effluent

Culture	BLASTn analysis	RDP analysis
O3a	*Bacillus* 95%	*Bacterium F112MS/ B subtilis*0.692
O3b	*Bacillus* 97%	*Bacillus sp. NRRL B* 0.738
O6b	*Bacillus* 97/98%	*Firmicutes bacterium Jbg1/B pumilus*0.9
O11	*B.spps/safensis/ pumilus* 99%	*B. pumilus* 0.9
O13	*B. pps/safensis /pumilus* 99%	*Bacillus sp. Pc-SA2 1.000*
O14	*Bacillus/uc bacillus* 98%	*Bacillus 0.78*
O12b	Uncultured *Pseudomonas sp.* 91%	*Pseudomonas/ uc 0.3*

(UC: uncultured bacteria)

Sequence analysis of isolates from non ozonated samples revealed that out of seven cultures, three belonged to genus *Pseudomonas*, one culture to genus *Microbacterium*, two to genus *Arthrobacter* and one was *Bacillus*. All cultures except culture A20 showed BLASTn similarity score of more than 97%. Molecular identification of culture A20 revealed that it was *Pseudomonas* with BLASTn similarity score of 96% and may indicate a new species (Table 3).

Table 3: Sequence analysis of isolates from non-ozonated effluent

Culture	BLASTn analysis	RDP analysis
A3b	*Microbacterium* 99%	*Microbacterium* 1.00
A9	*Arthrobacter* 98%	*Arthrobacter* 0.94
A10	*Arthrobacter* 98%	*Arthrobacter* 0.889
A16b	*Pseudomonas* 99%	*Pseudomonas* 0.93
A18	*Pseudomonas* 99%	*Pseudomonas* 0.895
A20	*Pseudomonas* 96%	*Pseudomonas* 0.789
A21	*Bacillus* 93%	*Bacillus* 0.5

Conclusion: This study indicates that use of dilute media simulating the natural environment has been helpful in isolating three new species, two of *Pseudomonas* and one of *Bacillus* having ability to degrade 100 ppm paranitrophenol.

Comparison of bacterial diversity of ozonated and non-ozonated samples revealed that while the non-ozonated effluent showed presence of diverse bacterial flora, the ozonated one predominantly contained *Bacillus* and *Pseudomonas*.

Bioremediation for 72 hours followed by ozonation for 10 mins was found to be an effective way of reducing the BOD COD of paranitrophenol containing industrial effluent as compared to ozonation of the effluent for 10 mins & then bioremediation.

References:

1. Amann, R. I., Ludwig, W. and Schleifer, K-H., "Phylogenetic identification and in situ detection of individual microbial cells without cultivation", Microbiological Reviews, Vol. 59, pp.143-169, 1995.
2. Aslam Z, Yasir M, Khaliq A, Matsui K & Chung Y, "Too much bacteria still unculturable", Crop and environment, Vol. 1, No.1, pp.59-60, 2010.
3. Chang, Y.C., and Chen, D.H., "Catalytic reduction of 4-nitrophenol by magnetically recoverable Au nanocatalyst", Journal of Hazardous materials. Vol. 165, pp 664-669, 2009.
4. Jannasch, H. W., and G. E. Jones, "Bacterial populations in seawater as determined by different methods of enumeration", Limnology and Oceanography, Vol.4, pp.128–139, 1959.
5. O'Connor, O.A., Young, L.Y., "Toxicity and

anaerobic biodegradability of substituted phenols under methanogenic conditions", Environmental Toxicology and Chemistry Vol. 8, pp.853–862, 1989.

6. Petti C.A., "Detection and identification of microorganisms by gene amplification and sequencing", Clinical Infectious Diseases, Vol. 44, pp.1108–1114, 2007.
7. Rappe, M & Giovannoni, S. "The uncultured microbial majority", Annual Review of Microbiology, Vol. 57, pp 369-394, 2003.
8. Shi, H.x., Xu, X.W., Xu, X.H.,Wang, D.H.,Wang, Q.D, "Mechanistic study of ozonation of p-nitrophenol in aqueous solution", Journal Of Environmental science., Vol.17, pp.926-929, 2005.
9. Staley, J. T., and A. Konopka, "Measurements of *in situ* activities of non photosynthetic microorganisms in aquatic and terrestrial habitats", Annual Review of Microbiology, Vol. 39, pp.321–346, 1985.
10. Stewart E., "Growing unculturable bacteria", Journal of bacteriology, Vol. 16, pp.4151-4160, 2012.
11. Watanabe K., Yamamoto S., Hino S., & Harayama S., "Population Dynamics of Phenol-Degrading Bacteria in Activated Sludge Determined by gyrB-Targeted Quantitative PCR", Applied and Environmental Microbiology, Vol. 64, No.4, pp 1203-1209, 1998.
12. Watve M. V., Shejval C., Sonawane M., Rahalkar A., Matapurkar Y., Shouche M., Patole N., Phadnis A., Champhenkar K., Damle S., Karandikar V., Kshirsagar& Jog M., "The 'K' selected oligophilic bacteria: a key to uncultured diversity?", Current Science, Vol.78, pp. 1535-1542, 2000.
13. Wirsen, C. O., S. M. Sievert, C. M. Cavanaugh, S. J. Molyneaux, A. Ahmad, L. T. Taylor, and E. F. DeLong, "Characterization of an autotrophic sulfide-oxidizing marine Arcobacter spps. that produces filamentous sulphur", Applied and Environmental Microbiology, Vol. 68, pp 316–325, 2002.
14. Yogeswary, P., Rashid Mohd, M., "Degradation of phenol by catalytic ozonation." Journal of Chemical and Natural Resources Engineering, Vol. 2, pp 34-46, 2007.

***

Jayashree Pawar/Dileep Kamat/Sheela Kamat/

Department of Microbiology/Mithibai College of Arts/

Commerce & Science /kamats2@hotmail.com