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Metagenomic analysis of oxygenases from activated sludge

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HIGHLIGHTS

- Metagenome of activated biomass was mined for genes encoding oxygenases.
- 191 Oxygenases were annotated in the metagenome.
- Genes from the central meta cleavage pathway are most abundant.
- Genes encoding for oxygenases compared across four metagenome datasets.

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ABSTRACT

Oxygenases play a key role in degradation of the aromatic compounds in the wastewater. This study explores the oxygenase coding gene sequences from the metagenome of activated biomass. Based on these results, the catabolic capacity of the activated sludge was assessed towards degradation of naphthalene, anthracene, phenol, biphenyl and o-toluidine. Oxygenases found in this study were compared with oxygenases from three other metagenome datasets. Results demonstrate that despite different geographical locations and source, many genes coding for oxygenases were common between treatment plants. 1, 2 Homogentisate dioxygenase and phenylacetate CoA oxygenases were present in all four metagenomes. Metagenomics provides a vast amount of data that needs to be mined with specific targets to harness the potential of the microbial world.

1. Introduction

Industrial wastewater contains a diverse array of toxic compounds that cannot be released directly into the environment. Their degradation is mostly carried out by the activated sludge process (ASP) either at Effluent Treatment Plants (ETPs), treating wastewater generated by large-scale industries or Common Effluent Treatment Plants (CETPs) that treat wastewater generated by a cluster of small-scale industries (Kapley and Purohit, 2009). The last decade has seen a number of reports that are trying to understand the activated sludge system in order to improve treatment efficiency (Martín et al., 2006; Moosvi and Madamwar, 2007; Rani et al., 2008; Sivaprakasam et al., 2008; Sentchilo et al., 2013; Chang et al., 2014). Answers lie in the microbial metagenome of the activated sludge, since it is the largest reservoir of genes responsible for enzymatic reactions involved in degradation (Galvão et al., 2005). An inventory of all bacterial species present in active biomass can be obtained by taxonomy studies which in turn help understand the functional aspects of the system (Díaz et al., 2006; Kapley et al., 2007; Hill et al., 2010). Access to the prokaryotic genetic information has been made easier after the advent of next generation sequencing technology that allows us an insight of the microbial world that was previously hidden due to culture-dependent biases. Analysis of CETP metagenome allows us to understand the taxonomic and catabolic diversity; improves chances of discovering novel pathway/gene that are involved in the degradation of a complex range of effluents entering the CETP (More et al., 2014).

Wastewater treatment plants mostly follow the aerobic route of degradation and hence, oxygenases play a crucial role in the ASP. Degradation is commenced by oxidizing the aromatic ring, making them more susceptible to cleavage by ring-cleaving dioxygenases (Phale et al., 2007). Compounds bearing two hydroxyl groups on two adjacent carbon atoms serve as substrates to this oxygenase class of enzymes. (Eltis and Bolin, 1996). The oxygenase can be grouped into two types; enzymes that catalyse insertion of a single oxygenase atom, monooxygenase and enzymes that catalyse insertion of a both oxygenase atoms, dioxygenases. Diverse classes of oxygenases are studied and characterized using tools like PCR, cloning, metagenomic libraries etc. (Kulakov et al., 1998; Kapley

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and Purohit, 2001; Moharikar et al., 2003; Baldwin et al., 2003; Selvakumaran et al., 2011; Sharma et al., 2012).

Since oxygenases play such an important role in biodegradation, this study explores and documents their actual abundance in a target CETP. This group has already reported the metagenome sequence of the target CETP (National Center for Biotechnology Information (NCBI) accession number AERA00000000.1) and this sequence data was used to identify oxygenases in the biomass. The distribution of various oxygenases in the metagenome was studied using KRONA extension, of MG-RAST (MetaGenomics Rapid Annotation using Subsystem Technology). Based on the different types of oxygenases present in the sample niche, five model compounds were selected and the efficiency of their degradation by activated biomass was evaluated. Presence of different oxygenases across three additional metagenome datasets was analyzed and oxygenases common to all four metagenome datasets were observed using a Venn diagram. Metagenomics unlocks the black-box that was the activated biomass and offers enormous scope and potential for improving treatment processes.

2. Methods

2.1. Metagenome datasets from diverse locations

We have earlier reported sequence data of a metagenome prepared from activated biomass of a CETP (NCBI accession number AERA00000000.1). This sequence data was used to identify different oxygenases present in the activated biomass. Results were compared with oxygenases found in three other metagenome data sets across diverse wastewater composition and geographical locations; viz., metagenomes of Sewage Effluent Treatment Plant (ETP, MG-RAST ID: 4467420.3), Enhanced Biological Phosphorus Removal treatment plant (EBPR, MG-RAST ID: 4463936.3), and Tannery waste metagenomes (Tannery, MG-RAST ID: 4494888.3) located at Hong Kong, Denmark and China, respectively (Albertsen et al., 2011; Yu and Zhang, 2012; Wang et al., 2013). The detail geographical locations of the metagenome sampling sites are depicted in Supplementary Fig. 1.

2.2. Bioinformatics pipeline used in analysis

2.2.1. Oxygenase analysis in CETP metagenome

In order to obtain catabolic oxygenases abundance information, CETP metagenome was explored with SEED subsystems in MG-RAST (Meyer et al., 2008). We referred SEED subsystem as well as GenBank database with parameters of a maximum e-value of 1e-5, a minimum identity of 60%, and a minimum alignment length of 15 amino acids for proteins, and visualized using Krona hierarchical data browser (an internal plug-in of MG-RAST) (Ondov et al., 2011). Further, for evaluation of the diverse oxygenase annotations, the abundant hits grouped under the subsystem of 'Metabolism of Aromatic Compounds' using KEGG (Kanehisa and Goto, 2000) were selected for comparison with other metagenomes.

2.2.2. Comparative analysis with other metagenomes

For all possible oxygenase relations such as union, intersection and exclusive enzymes hits between four metagenome sets, Venn diagram was generated by web-based Lucidchart tool (https://www.lucidchart.com). The abundance of all diverse oxygenases involved in degradation of aromatic compound pathways, present in all four metagenomes, were determined by heatmap. The result is displayed in tabular format, with heatmap-style color coding which highlights the varying abundance of all oxygenases. To study the abundance of oxygenases of all four metagenomes, the heatmap clustering using MG-RAST with bray-curtis distance metric was performed where the oxygenase hits were grouped by function using normalized values. The pipeline used has been illustrated in Fig. 1.

2.3. Degradation potential of activated biomass

Activated biomass samples were collected from the ASP (activated sludge process) unit of the CETP as reported earlier (Kapley and Purohit, 2009). The biomass was maintained in the laboratory at 30 °C, with continuously air sparging and addition of wastewater collected from the inlet of the CETP. Sludge sample volume to account for 3000 mg/L MLSS, was harvested by centrifugation and inoculated in 50 ml M9 media (Kapley et al., 2007) in a 250 ml Erlenmeyer flask supplemented with 2 mM substrate and incubated at 30 °C and 120 rpm. Degradation of five substrates; anthracene, biphenyl, naphthalene, phenol and o-toluidine were analysed independently in triplicate ($5 \times 3 = 15$ flasks). Degradation of each substrate by the activated biomass was analysed by GC–MS at two time points; 0 h, to represent the initial substrate concentration, and 48 h, to monitor degradation.

For GC–MS analysis, samples were extracted in ethyl acetate (1:1 ratio v/v). GC–MS analysis was done using GC-FID (Perkin–Elmer Clarus 500) and DB-5 column with an oven temperature program ramped from 60 °C at 10 °C min up to 280 °C and held at 280 °C for 3 min. The injector and detector temperature was 280 °C and 300 °C respectively. Percent degradation of compounds was calculated as reported earlier (Chhatre et al., 1996). To check the degradation profile, both the samples (0 h samples and 48 h samples) were analyzed by GC–MS (Varian 3800, model walnut, Greek, USA) using DB-5 column.

3. Results and discussion

3.1. Oxygenases from CETP metagenome

Degradation of wastewater in the CETP occurs due to the catabolic genes housed in the microbial population of the biomass. Since aerobic degradation is channelled via oxygenases, this study analyses their abundance, diversity and distribution in the metagenome. 191 oxygenases were annotated by homology of metagenomic contigs with GenBank and are listed in Table 1. Approximately 50% of these sequences coded for oxygenase genes involved in metabolism of aromatic compound degradation, which

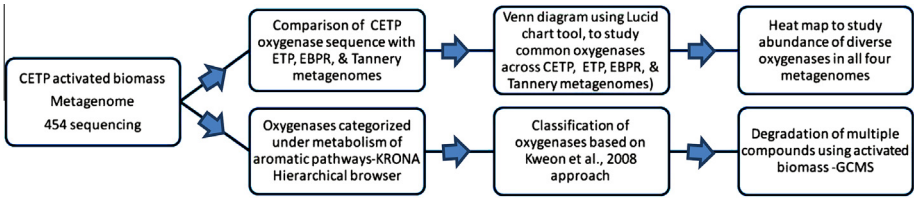

Fig. 1. Pipleline demonstrating the *insilico* analysis used to characterize the CETP metagenome that was sequenced using the 454 platform (Roche). Sequence data available at NCBI Genbank NCBI accession number AERA00000000.1.

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