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Microbial Degradation of Various Organic Pollutants Using Bacteria Isolated From Petroleum Sludge

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MICROBIAL DEGRADATION OF VARIOUS ORGANIC POLLUTANTS USING BACTERIA ISOLATED FROM PETROLEUM SLUDGE

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This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Science in Chemistry

Under the Supervision of Professor Syed Salman Ashraf

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Declaration of Original Work

I, Manal Ali Hassan Saeed Alhefeiti, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Microbial Degradation of Various Organic Pollutants using Bacteria Isolated from Petroleum Sludge*", hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Professor Syed Salman Ashraf, in the College of Science at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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Abstract

Intensification of agriculture and manufacturing industries has resulted in increased release of a wide range of xenobiotic compounds to the environment. The extensive discharge of hazardous waste in industrial wastewater and the recalcitrant nature of some of these organic pollutants have fueled a strong interest in exploring efficient and environmentally friendly approaches for wastewater remediation. Bioremediation approaches can provide efficient, inexpensive and environmentally safe cleanup tools. In the present study, our main objective was to isolate novel bacterial strains from UAE petroleum sludge and to examine their abilities to degrade various aromatic pollutants, including azo dyes and emerging pollutants. We report here on the isolation and purification of novel bacterial strains from petroleum sludge that were capable of efficiently degrading various classes of aromatic dyes. Of these 12 isolates, extensive biochemical and optimization studies were carried out with the most promising strain, MA1. The optimum culture conditions of MA1 strain were found to be at pH 7, with 100 ppm dye concentration, and under aerobic condition. DNA sequencing of the 16S rRNA gene was carried out for the 12 bacterial strains and the data showed that the isolates belonged to two different bacterial species: *Bacillus cereus* and *Pseudomonas guariconensis.* Confirmation of the degradation of the aromatic compounds by the chosen bacterial strains was done using HPLC and LC-MS/MS analyses. This novel strain, MA1, was able to efficiently degrade aromatic dyes (e.g. Toluidine Blue, Ponceau BS, Reactive Black 5 and Congo Red) and more importantly various emerging pollutants of human concern such as sulfamethoxazole, prometryn, and fluometuron.

Keywords: Bioremediation, pollutant degradation, emerging pollutants, aromatic dyes, 16S rRNA.

Title and Abstract (in Arabic)

التحلل الميكروبي لمختلف المركبات الملوثة بواسطة بكتريا مستخلصة من الرواسب البترولية

الملخص

توظيف الزراعة والصناعات التحويلية أدى إلى إنتاج العديد من المركبات الجديده على البيئة. كما أدى التفريغ الواسع النطاق للنفايات الخطرة في مياه الصرف الصناعي وتمرد الملوثات العضوية إلى الاهتمام الشديد باستكشاف طرق فعالة وصديقة للبيئة لمعالجة المياه المستعملة. إن توظيف نهج المعالجة البيولوجية يؤدي إلى توفير سبل تنظيف فعالة وغير مكلفه وآمنة بيئياً. في هذه الدراسة، هدفنا الرئيسي كان عزل سلالات بكتيرية جديدة من الرواسب النفطية في الإمارات ودراسة مدى قدرتها على تحلل الملوثات المختلفة، بما في ذلك أصباغ الأزو والملوثات الناشئة. كما قمنا في هذا البحث بعزل وتنقية سلالات بكتيرية جديدة من الرواسب النفطية والتي أثبتت قدرتها وكفاءتها على تحليل فئات مختلفة من الأصباغ الأروماتية. جدير بالذكر أنه من بين 12 نوع من البكتيريا والتي تم عزلها ، أجريت دراسات بيوكيميائية واسعة النطاق ودراسات أخرى لمعرفة الظروف الأمثل، ليتبين أن MA1 هي السلالة الواعدة من بين السلالات الأخرى. بالإضافة إلى ذلك، تبين بأن الظروف الأمثل لكفاءة هذه البكثيريا تحدث عند الرقم الهيدروجيني 7 مع تركيز 100 (جزء من المليون) وأيضا تحت الظروف الهوائيه أي بوجود الأكسجين . تم أيضا بحث 16S rRNA تسلسل الجينات ل 12 نوع من السلالات البكتيرية ليتبين أن البكتيريا التي قمنا بعزلها تنتمي إلى أجناس بكتيرية مختلفة (*Bacillus cereus, Pseudomonas guariconensis*). يجدر الإشارة أيضا بأنه تم تأكيد تحلل الرواسب من قبل البكتيريا باستخدام التقنية التحليلية LC-MS/MS. هذه السلالة الجديدة MA1، كانت قادرة على تحليل الأصباغ الأروماتية بكفاءة مثال (Toluidine Blue and, Ponceau BS, Reactive Black 5) وأيضا مختلف الأصباغ الجديدة ذات الإهتمام الإنساني مثال (sulfamethoxazole, prometryn, and fluometuron).

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مفاهيم البحث الرئيسية: المعالجة البولوجية، الرواسب النفطية، الأصبغ الأروماتية.

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Dedication

To my beloved parents and family

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List of Abbreviations

CP6R	Crystal Ponceau 6R
EPs	Emerging Pollutants
HOBT	1-Hydroxybenzotriazole
LB	Lysogeny Broth
MRM	Multiple Reaction Monitoring
NB-E	Nutrient Broth-E
PBS	Ponceau BS
PPCPs	Pharmaceuticals and Personal Care Products
ppm	Parts per million
RM	Redox Mediator

Chapter 1: Introduction

There is a pressing need to protect our natural resources and find novel and innovative approaches to degrade/remove various types of pollutants from our environment. Research studies regarding the use of efficient processes to clean and minimise pollution of water bodies are increasing (Gu et al. 2016; Petrie et al. 2014; Rodrigues et al. 2013; Wang et al. 2015) Extraction of natural resources, as well as industrialisation, have caused a wide range of environmental contamination and pollution. Toxic waste is being released in large amount at thousands of sites contaminating these places across the nation. Thus, each one of us is exposed to various xenobiotic/contaminants from the past due to the prevailing industrial practices, emission of pollutants and disposal of waste affecting natural resources (like air, water, and soil) both in cities and remote regions (Martins et al. 2015; Richardson et al. 2007; Alam et al. 2010). The risk to human health and environment is rising, and it is evident that the cocktail of pollutants is a contributor to the global epidemic of cancer and other degenerative diseases (Figure 1) (Access 2014).

Usually, aquatic pollution occurs by several sources including discharge of industrial effluents, illicit dumping of untreated wastewater, and agricultural fertilizer runoffs. All of these play a role in the contamination of water bodies with large quantities of organic pollutants, inorganic chemicals, as well as heavy metals (Richardson et al. 2007).

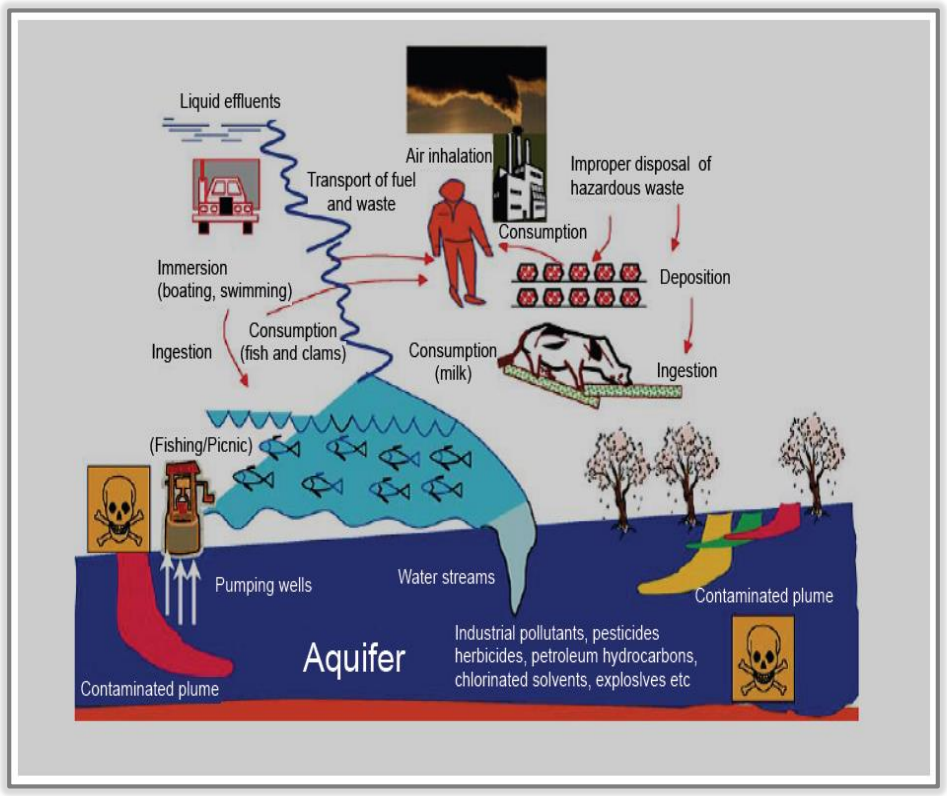


Figure 1: Fate and transport of organic/inorganic contaminants/pollutants and their harmful effects (Access 2014)

1.1 Aromatic compounds in the environment

Any organic molecule that comprise one or more aromatic rings, especially benzene rings, for example, are known as aromatic compounds (Seo et al. 2009). Aromatic compounds can be classified into three major groups: polycyclic aromatic hydrocarbons (PAHs), heterocyclics, and substituted aromatics. Out of the three categories, PAHs are known to be widely spread in the environment. PAHs can be defined as chemicals that have two or more fused aromatic rings in linear, cluster, or angular arrangements (Cheung & Kinkle 2001). Environmental Protection Agency (EPA) in the US has recognised 16 PAHs as major pollutants. The distribution of these compounds (PAHs) in the environment and probable exposure to humans are causing

a serious concern as they are possible human carcinogens. These PAHs are widely produced in the environment from several sources such as fuel combustion, automobiles, spillage of petroleum products, and waste incinerators (Seo et al. 2009).

Persistent Organic Pollutants (POPs) are considered as one of the most concerned environmental contaminants due to their presence in the environment, bioaccumulation through the food web, and they pose a threat of causing adverse effects to the environment and human health (Khodjaniyazov et al. 2012). POPs involve DDT, chlordane, aldrin, dieldrin, endrin, mirex, brominated flame retardants, and organometallic compounds such as tributyltin, PAHs, hexachlorobenzene, heptachlor, polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated biphenyls (PCBs), polychlorinated dibenzofurans (PCDFs), and toxaphene. PCDD/Fs are released accidentally from human activities. One of the major sources of POP is municipal waste incinerators (Diez 2010)

1.2 Emerging pollutants

Previously, organic compounds were not well-known to be present in water bodies, in terms of distribution or concentration but are now becoming more widely discovered as analytical techniques develop (Farré et al. 2012). These compounds, which have the ability to cause familiar or suspected adverse ecological or human health effects, are often called as emerging pollutants (EPs) or contaminants of emerging concern. EPs can be defined as any synthetic chemicals or microbes present in the environment and are normally not monitored but have the ability to affect several living organisms adversely. Emerging pollutants involve newly synthesised substances, and substances that were present in the environment since a long time but their existence and significance are only being clarified now (Daughton 2004). They

comprise an extensive array of various compounds and their transformation products: pharmaceuticals (e.g. nonsteroidal anti-inflammatory drugs (NSAIDs), analgesics, antibiotics, textile dyes, hormones, and personal care products pesticides (Lapworth et al. 2012). Although emerging pollutants may be degraded by numerous treatment systems of wastewater, it is noticed that these emerging pollutants have been detected in relatively high concentrations in different water resources (Ángel et al. 2014; Na et al. 2014; Sorensen et al. 2015). For example, recent studies have reported the detection of 10 ppm of acetaminophen, 17 b-estradiol, and up to 0.2 ppm of the reproductive hormone in the US water streams (Loos et al. 2007). Subsequently, the appearance of these physiologically active chemicals with high a concentration in the water supply is attracting a lot of attention by environment scientists. An interesting analysis has been conducted to find out physiologically active concentrations of various hormones, antibiotics, and other EPs in the water bodies in several countries (Table 1).

Even though various studies have reported sources, occurrence, and environmental behaviour of emerging pollutants (Stuart et al. 2012; Lapworth & Gooddy 2006; Pal et al. 2010; Deblonde et al. 2015), the pathway of these EPs from sources to receptors remains a subject for advanced research. This is due to the lack of information, mainly because of the problems created by physicochemical properties of target compounds, as well as the complexity of environmental characteristics among others, which may determine an unexpected behaviour of the emerging pollutants in air, water, or soil (Stuart et al. 2012; Pal et al. 2010). In order to clarify this condition, a study has provided a schematic pathway by which some EPs enter different receptors (like groundwater, consumers, and surface water) (Figure 2) (Aamand et al. 2014).

1.3 Degradation of organic pollutants by various methods

Several traditional techniques have been successfully used to treat polluted wastewater and remove various contaminating organic pollutants, such as chemical and physical methods, yet most of them have several limitations, such as overall cost, inefficiency, and difficulty in complete mineralisation of the pollutants.

A wide range of approaches has been developed for the removal of various synthetic aromatic pollutants from water bodies, as well as wastewaters thus, reducing their impact on the environment. Various chemical and physical methods have been used for the treatment of contaminated wastewater such as membrane filtration, precipitation, flocculation, irradiation, adsorption, and chemical oxidation such as Fenton's oxidation (Figure 3) (Franciscon et al. 2012). Although these methods can work effectively, they have several potential limitations, such as overall high cost, inefficiency, production of high sludge, and formation of toxic side products (Figure 4) (Alhassani et al. 2007).

Table 1: Summary of some pharmaceuticals and emerging contaminates detected in drinking water supply in various countries

PPC		Representative concentration, ng/L	Reference
NSAIDs and analgesics	Paracetamol	10000	(Kolpin et al. 2002)
	Diclofenac	15000	(Jux et al. 2002)
	Ibuprofen	1000	(Lapworth et al. 2012)
	Valsartan	1300	(Huerta-fontela et al. 2011)
Antibiotics	Sulfamethoxazole	700	(Vanderford & Snyder 2006)
	Lincomycin	750	(Lapworth et al. 2012)
	Erythromycin	450	(Managaki et al., 2007)
Hormones	Estrone (E1)	1	(Huerta-fontela et al. 2011)
	Diethylstilbestrol	5.3	(Caban et al., 2015)
	Estriol (E3)	72	(Huerta-fontela et al. 2011)
Pesticides	Carbofuran	25	(Papadakis et al., 2015)
	Lindane	34	(Papadakis et al., 2015)
Personal care product	Caffeine	14.60	(García-Vaquero et al., 2014)
	Bisphenol A	4500	(Erickson et al., 2014)
	Triclosan	35	(Azzouz and Ballesteros, 2013)
	Cotinine	88.5	(Sun et al., 2015)

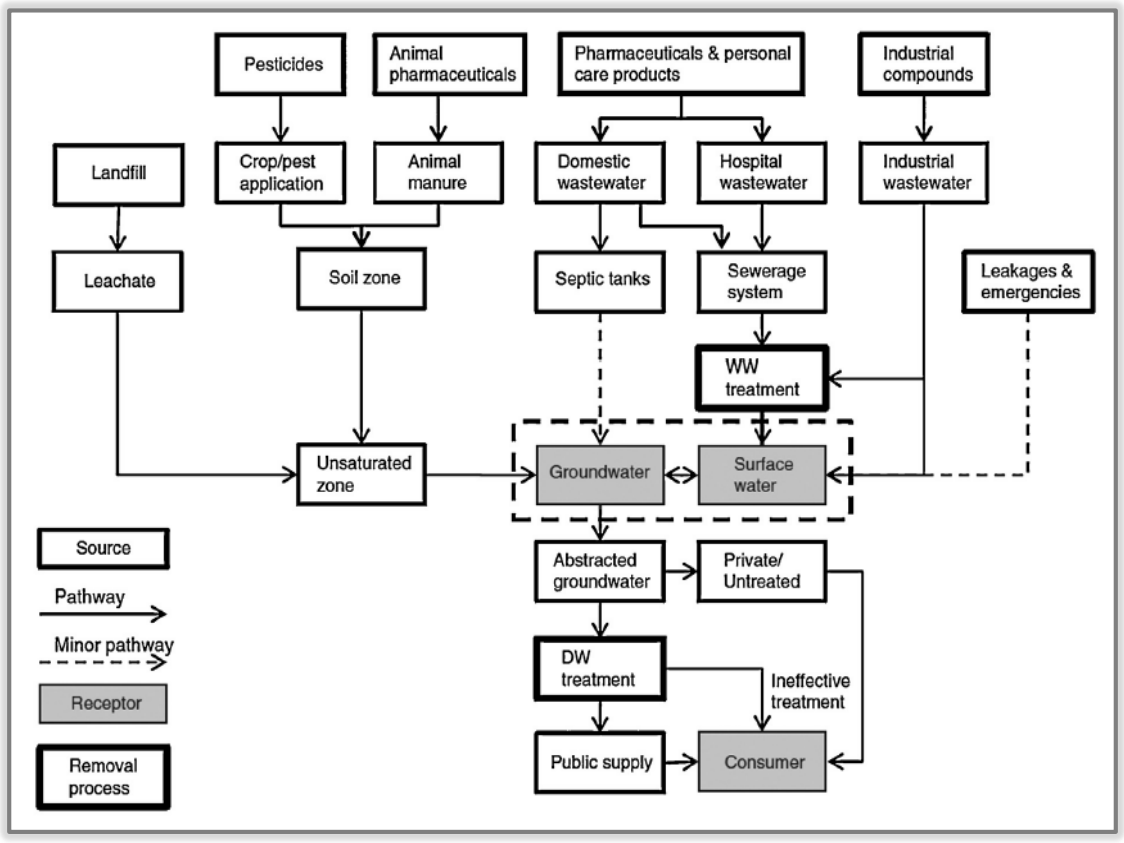


Figure 2: Schematic diagram of various EPs from sources to receptors (Aamand et al. 2014)

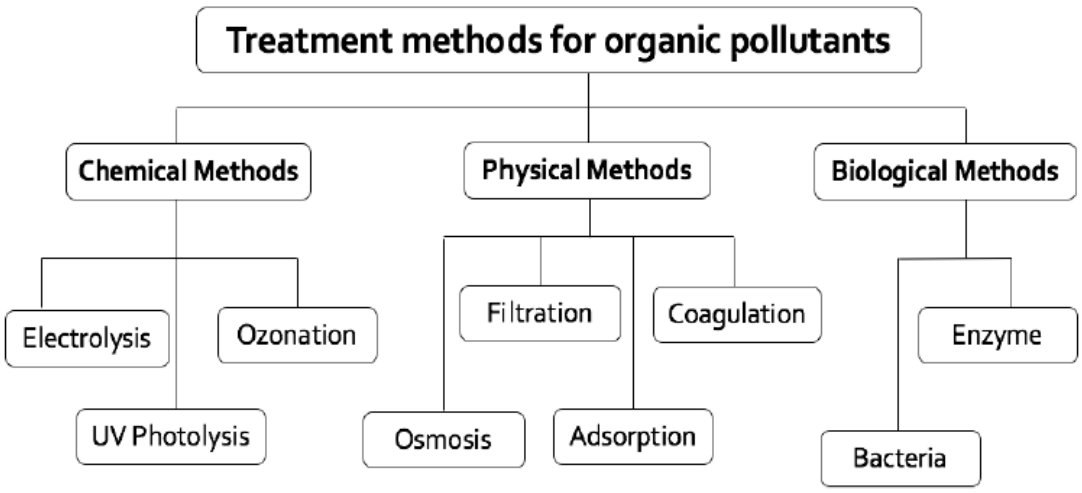


Figure 3: Treatment methods for the removal of organic pollutants from wastewater effluent

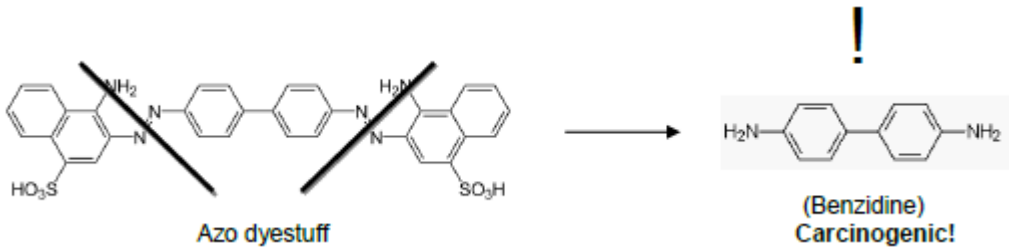


Figure 4: An example of organic aromatic pollutants (azo dye) breaking down into carcinogenic amines after chemical treatment (taken from http://garmentstech.com/why-need-to-be-sure-your-gament-textile-fibre-apparel-toys-leather-paper-and-plastic-product-are-azo-dyes-free/, April 09, 2016)

Bioremediation of emerging pollutants using bacteria or other microorganisms possessing degrading abilities is a highly attractive strategy that offers distinct advantages over the traditional techniques of treatment. The process of bioremediation is defined as the use of microorganisms to detoxify or remove pollutants owing to their diverse metabolic capabilities is an evolving method for the removal and degradation of many environmental pollutants including the products of petroleum industry (Rauf & Salman 2012). Microbial biodegradation is more eco-friendly, economical, and produces less amount of sludge. In most cases, they convert the organic pollutants to less harmful compounds that are not deadly to life forms (Tripathi & Srivastava 2011). Among this biological mode of treatment, bacterial degradation has shown amazing ability to decolourise dyes quickly and efficiently. Recent studies have shown that bacteria such as *Bacillus subtilis, Phanerochaete chrysosporium, Aeromonas hydrophila, Penicillium sp., Klebsiella pneumoniae, Proteus mirabilis, and Pseudomonas cepacia* can degrade various classes of toxic organic pollutants (Ali et al. 2014).

1.3.1 Physical methods

Physical techniques including filtration, adsorption, specific coagulation, and chemical flocculation have been widely utilised in the removal of organic pollutants. Various types of membrane filtration showed potential in the treatment of environmental pollutants. For example, Reverse Osmosis (RO) membranes showed a retention rate of 90% for the reactive organic aromatic pollutants (like dyes) and other chemical compounds (Ciardelli & Ranieri 2001). It has been indicated that RO membrane is capable of removal of hydrolyzed reactive dyes, all mineral salts, as well as chemical auxiliaries, but high energy consumption is its major limitation. Moreover, ultrafiltration can be utilised as a pre-treatment, followed by any other treatment process. Microfiltration has the ability to treat dye baths having pigment dyes, and it is used as a pre-treatment for nanofiltration or RO (Bruggen et al. 2005). Despite the effectiveness of these techniques, membranes have some considerable drawbacks including the formation of secondary waste streams, which requires further treatment, high investment costs, as well as potential membrane fouling are considered as major drawbacks in using membranes filtration in this field (Santos et al. 2007; Robinson et al. 2001).

Adsorption techniques have also attracted significant interest in the treatment of contaminated water because of their effectiveness in the removal of an extensive range of organic pollutants. Choosing the right adsorbent is based on various characteristics like capacity for target compounds, pore size, and regeneration of adsorbent (Sadhasivam et al. 2009). Various adsorbent materials have been used such as Activated Carbon (AC), which is used effectively for the removal of dyes. However, AC has relatively high cost, and therefore, it is not widely used. The other common

materials, which are economically feasible, are peat, wood chips, fly ash, polymeric resins, and others biological materials including maize stalks, corn cobs, and wheat straw for the removal of coloured wastewater (Robinson et al. 2001). On the other hand, adsorption techniques showed several limitations regarding the disposal of potential sludge and regeneration of the adsorbents. Physical methods based on Coagulation Sedimentation and Flocculation are another group of effective means for the removal of organic pollutants and disperse dyes. These techniques are known for their selectivity towards the category of contaminants existing in wastewater (Gautami & Khanam 2012). Several disadvantages have been noticed regarding these methods including the production of high sludge, their disposal or regeneration, low effectiveness with regard to a wide range of dyes, as well as their high cost (Karcher et al. 2001).

1.3.2 Chemical methods

Chemical approaches involve Advanced Oxidation Processes (AOP's), use of NaOCl, and electrochemical destruction (Särkkä et al. 2015; Ahmed et al. 2015; Brillas & Martinez-Huitle 2015; Rojas et al. 2016). The Advanced Oxidation Processes (AOP's) appear to be a promising approach, which have been effectively used for the removal of various organic contaminants from water and soil. Various review papers have reported various AOPs to be efficient for the treatment of wastewater (Robinson et al. 2001). AOP method is based on the generation of OH radicals for a sequence of reactions with organic pollutants, thereafter, to degrade the molecules into less harmful substances. However, AOPs have been noticed to be costly, since the process requires a continuous addition of expensive chemicals, as well as large consumption of electricity. In addition, advanced oxidation processes are not capable of treating large

amounts of wastewater.

Electrochemical process has also been successfully used for the treatment of wastewater. It has been reported that combining both UV with sonication has also worked to remove several organic pollutants (Kiai et al. 2014; Furgal et al. 2014). However, this technique is relatively resource-intensive and expensive. Table 2 summarised the benefits and drawbacks of the current physical and chemical methods of organic pollutants removal from industrial effluents (Robinson et al. 2001).

Table 2: Advantages and disadvantages of the current approaches of organic pollutants removal

Treatment methods	Advantages	Disadvantages
Physical methods		
Activated carbon	Good removal of wide variety of dyes	Very expensive and secondary waste
Wood chips	Good sorption capacity for acid dyes	Requires long retention times
Peat	Good adsorbent due to cellular structure	Specific surface areas for adsorption are lower than activated carbon
Membrane filtration	Removes all dye types	Concentrated sludge production
Electrokinetic coagulation	Economically feasible	High sludge production
Chemical methods		

Fenton's reagent	Effective decolorization of both soluble and insoluble dyes	Sludge generation
Ozonation	Applied in gaseous state: no alteration of volume	Short half-life
NaOCl	Initiates and accelerates azo-bond cleavage	Release of aromatic amines
Ion exchange	Regeneration: no adsorbent loss	Not effective for all dyes
Irradiation	Effective oxidation at lab scale	Requires a lot of dissolved O_2

1.4 Bioremediation/biodegradation

Biological treatment is considered as the most economical alternative compared to the physical and chemical techniques (Archna et al. 2013). The process by which pollutants are removed from the environment using biological methods, which exploits the metabolic potential of microorganisms to break down a wide range of compounds, is called as bioremediation or biodegradation process (Rauf & Salman 2012). The use of bioremediation processes for the removal of toxic compounds from water bodies is gaining considerable attention, and it is counted as a huge hope for a cleaner and healthier environment. Biodegradation of recalcitrant compounds relies on the presence of the biotransformation enzymes thus they are capable of degrading various types of pollutants (Rauf & Salman 2012; Saratale et al. 2011; Schückel et al. 2011). Several biotechnological techniques have attracted attention regarding the treatment of organic pollutants in an eco-efficient way, usually with the use of bacteria or in integration with physicochemical methods. Organic pollutants are xenobiotic

present in nature and recalcitrant to degradation. Thus, the use of microbial treatment process for complete degradation of organic pollutants from wastewater has various advantages such as being environment-friendly, cost-competitive, producing less sludge, forming non-toxic end products, as well as less water consuming (Saratale et al. 2011). The bioremediation methods can also be performed with a lower concentration of contaminants compared to the physical and chemical techniques, which would not be feasible.

The efficiency of microbial degradation is based on the adaptability and activity of microorganisms. Accordingly, many microorganisms have been tested for their ability in degrading and mineralising various organic pollutants in recent years (Pandey et al. 2007). Isolating the potent microorganisms and testing their degradation is one of the commonly biological aspects of wastewater treatment (Chen et al. 2008). A wide range of microorganisms are efficient in degrading wide variety of organic pollutants including bacteria (Saratale et al. 2017; Dawkar et al. 2008; Jadhav et al. 2007; Telke et al. 2008; Tripathi & Srivastava 2011), fungi (Table 3) (Fournier et al. 2004; Saratale et al. 2006), plants (phytoremediation) (Aubert & Schwitzgue 2004; Kagalkar et al. 2009), yeasts (Jadhav et al. 2007; Lucas et al. 2006; Saratale et al. 2017) actinomycetes (Machado et al. 2006), and algae (Acuner & Dilek 2004; Daneshvar et al. 2007; Gupta et al. 2006; Yan & Pan 2004). It has been reported that these species are also able to mineralize various organic pollutants under certain environmental conditions completely.

Many factors might affect microorganisms to use organic pollutants as substrates or metabolising them. Thus, it is important to understand catabolic pathways, mechanisms, and responsible enzymes to identify important factors for

effective removal of organic pollutants. A number of researches have been conducted to understand bioremediation process for various environmental pollutants like aromatic compounds that are considered as the most widespread and persistent environmental pollutants (Seo et al. 2009).

Table 3: Microbial method (bacterial & fungal) of organic pollutants degradation (Archna et al. 2013)

Microbial type(s)	Degrading dye(s)	Reference(s)
(I) Bacteria		
Citrobacter sp. CK3	Reactive Red 180	Hui Wang et al 2009
Listeria Sp	Red B5 and Black HFGR	Kuberan.et al 2011
Bacillus subtilis	Acidblue113	Gurulakshmi et al 2008
Klebsiella sp.	Orange 3R	Ponraj1 et al 2011
Salmonella sp.		
Pseudomonas sp.		
Enterococcus faecalis strain YZ66	C.I. reactive yellow 145	Sahasrabudhe et al. 2011
(II) Fungi		
Penicillium chrysogenum, Aspergillus niger Cladosporium sp.	Azo dye- Red 3BN	Kumar Praveen 2012
P. ostreatus (IE8)	Acid black 194,	Elizabeth Rodrı´guez
P. ostreatus (IE8)	Orisol blue BH	et al. 1999
T. hispida (8260)		
Bjerkandera sp.	Amaranth, Remazol	Swamy and
BOS55 P. chrysosporium	Black B, Reactive Blue15,	Ramsay 1999
P. ostreatus	RemazolOrange ,	
T. hirsuta	Tropaeolin O	
T. versicolor		

1.4.1 Bacterial degradation of organic pollutants

Generally, the degradation of organic pollutants (for example dyes) may occurs the condition of anaerobic and aerobic by different groups of bacteria. Several current studies emphasis on the use of microbial biocatalysts to eliminate dye from the effluent (Chang et al. 2004). Wide-range studies have been done to define the role of varied groups of bacteria in the degradation of azo dyes (Pandey et al. 2007). The bacterial decolorization of these dyes has been of significant interest since it can attain an

advanced degree of biodegradation and mineralization, is appropriate to a wide range of azo dyes, is low cost and eco-friendly, and produces less sludge (Khehra et al. 2006; Saratale et al. 2014).

1.4.1.1 Enzyme system involved in bacterial degradation

The bacterial degradation of aromatic pollutants (e.g. azo dyes) is mainly required the involvement of various oxidoreductive enzymes, like tyrosinase, DCIP reductase, lignin peroxidase, laccase, manganese peroxidase, veratryl alcohol oxidase, amino pyrine N-demethylase, and azo reductase. These oxidoreductase enzymes working on transferring electrons from a substrate to an acceptor (azo dye) (Fu & Viraraghavan 2001; Kaushik & Malik 2009; Zille et al. 2005; Souza et al. 2007; Joshi et al. 2010; Kurade et al. 2011). According to earlier studies, it has been noticed that initial reduction of azo group done by reductive enzymes, such as NADH-DCIP reductase, azo reductase, and the produced intermediates are mineralized by oxidative enzymes. Generally, azo dyes are poor electron compounds due to the existence of the azo group (-N=N-) and other electron withdrawing groups, like sulphonic (SO_3^-) group in the azo dyes (Karigar & Rao 2011).

1.4.1.2 Mechanisms of dye degradation by bacteria

As reviewed above, bioremediation using bacterial degradation system shows an efficient degradation of a wide range of organic pollutants. Interestingly, different bacterial species were extensively used to degrade various classes of dyes. However, very few published studies have proposed the exact mechanism of dye degradation using bacteria. For example, in the case of azo dyes, almost all the microbial degradation involves azo bonds (–N=N–) cleavage (Pandey et al. 2007). It was

proposed that this cleavage is mediated by various mechanisms, such as low molecular weight redox mediators and chemical reduction by biogenic resultants like sulphide. However, most recent studies reported that oxidoreductase enzymes, such as of peroxidase, laccase, tyrosinase, and reductase are the main factor in this process (Karigar & Rao 2011).

Generally, the cleavage of azo bonds (–N=N–) is mediated by azoreductase enzyme under anaerobic conditions that involve the transformation of four electrons. These electrons were further transferred to the azo dye, which is considered as the final electron acceptor leading to the formation of colourless intermediates (Khehra et al. 2005). These intermediates will be further degraded by these enzymes either aerobically or anaerobically (Chang et al. 2004). The decolorization of azo dyes under anaerobic conditions is considered as a non-specific and simple process (Pandey et al. 2007). Interestingly, it has been suggested that the effectiveness of anaerobic conditions is mainly because it has low redox potential (50 mV) and the absence of the oxygen facilitate electron transformation (Bromley-Challenor et al. 2000). Alternatively, nonspecific extracellular reactions that occur between reduced compounds, which produced by the anaerobic biomass, might lead to the degradation(Saratale et al. 2011).

On the other hand, aerobic treatment of azo dye found to be less efficient compared to anaerobic degradation in which these aerobic microbes lack the ability to reduce azo dye linkages. However, several bacterial strains found to be able to decolourize azo dyes successfully (Pandey et al. 2007). Although these strains require other organic carbon sources as they cannot utilise dye as the growth substrate (Stolz, 2001), there are very few strain that can use azo compounds as a source of carbon,

such as *Xenophilus azovorans* KF 46 (previously *Pseudomonas sp.* KF46) and *Pigmentiphaga kullae* K24 (previously *Pseudomonas sp.* K24). These strains cleave azo bonds reductively and use amines as the source of carbon and energy for their growth. It has been found that these microbes require azoreductase, as well as, a specific transport system that allow dye uptake (Russ et al. 2000).

1.5 Factors affecting bacterial degradation of aromatic dyes

The degradation of aromatic pollutants by bacteria is very sensitive to the difference in physico-chemical operational factors, such as pH, temperature, structure and concentration of organic pollutant, supplementation of different carbon and nitrogen sources, electron donor, redox mediators, aeration and agitation (Rauf & Salman 2012). Consequently, adapted bacteria, isolated from organic contaminated sites, are very effective in degradation process because of their adaption to various extreme environmental condition. Optimization of such abiotic conditions will help in the improvement of industrial scale bioreactors for bioremediation treatment. Following factors affecting the aromatic pollutants degradation by bacteria have been explained in detail (Pearce 2003a).

1.5.1 Oxygen

It is well-known that oxygen has a significant effect on the microbial degradation. It plays an important role in cell growth where it influences the physiological characteristics of the cells. On the other hand, it might influence the process of dye reduction where excess oxygen may inhibit this process. This is because it can act as electron acceptor in which it will reduce to water rather than azo dye (Adrian 2001). Furthermore, some of the intermediates produced, such as the

hydrazine form of the dye and azo anion free radical form of the dye, can be easily reoxidized by oxygen. Additionally, in the case of anaerobic bacteria, oxygen is considered toxic as it might directly inhibit azoreductase enzyme (Pearce 2003b). For instance, a study studied have tested the ability of *Escherichia coli* to decolorize 100 mg/l Reactive Red 22 dye under aerobic conditions, and they found that the bacterial degradation was significantly inhibited due to the dissolved oxygen level (Işik & Sponza 2003). In anaerobic conditions, the azo bond will be cleaved, but no further degradation will be observed where aerobic conditions are required for efficient degradation of the reactive azo dye molecule. This is mainly because that hydroxylation and ring-opening processes require the presence of oxygen. Consequently, the most effective treatment is found to be a two-stage process in which oxygen is introduced after the initial anaerobic reduction of the azo bond has taken place (Pandey et al. 2007).

1.5.2 pH

Often, the optimum pH for aromatic dye degradation is at neutral pH values (6 – 9) or at pH value of slight alkaline and the rate of aromatic dye decrease quickly at strongly acid or strongly alkaline pH values. As a result, the colored wastewater is often buffered to enhance the color removal performance of the cell culture (Chen et al. 2017; Kilic et al. 2007; Guo et al. 2007). Microbial reduction of azo bond cause an increase in the pH value because of the formation of aromatic amine metabolites, which have a basic pH more than the original azo compound. Changing the pH condition within a range of ~7.0 to 9.5 has shown a very slight effect on the dye reduction method. Previous studies have reported that the dye degradation rate raised nearly 2.5-fold as the pH was increased from value 5.0 to 7.0, while within pH values

of 7.0–9.5, no significant change was noticed on the rate of dye degradation (Chang et al. 2001).

1.5.3 Dye concentration

Scientific investigations discussed the presence of inverse relationship between the dye concentration and the decolorization rate. Results have shown that as the dye concentration increases the decolorization rate decreases. This may attributed to several reasons such that the increase in dye concentration may lead to a deleterious effects in bacteria, insufficient biomass concentration and the disturbance of azoreductase active sites by the dye particles with various structures (Chang 2014; Devassy et al. 2009; Jadhav et al. 2008). Parallel outcomes were noticed in the bacterial decolorization of different reactive azo dyes (Saratale et al. 2009; Kalyani et al. 2009). In addition, those azo dyes characterized by the presence of sulfonic acid groups (SO_3H) on their aromatic rings significantly led to the disruption of microorganisms growth at higher dye concentration (Chen et al. 2017; Kalyani et al. 2009). However, Saratale et al., (2009) revealed that using bacterial co-culture as an alternative to a pure culture led to a reduction in the increasing concentration associated effect and this may attributed to the synergistic effect of both microorganisms. Furthermore, other studies have proved the insensitivity of dye concentration on the decolorization rate. For example, a study on the reduction of food azo dyes in cultures of *Proteus vulgaris* have reported the absence of any effect of dye concentration on the decolorization rate (Pearce 2003a). This observation is well-matched with the mechanism of non-enzymatic reduction which is regulated by processes that doesn't depend on the dye concentration (Chang & Kuo 2000; Pearce 2003b; Telke et al. 2008).

1.5.4 Temperature

Temperature is a significant environmental factor for all processes associated with microbial vitality, including the biodegradation organic pollutants by bacteria. Bacteria are able to adapt changes in the environmental temperature via biochemical or enzymatic mechanisms (Chang & Kuo 2000; Santos et al. 2007). It was detected that maximum rate of bacterial decolorization tends to be at optimum temperature required for cell culture growth, reproduction and enzyme activities. Consequently, the deduction in the rate of the bacterial degradation can be found because of the loss of cell viability, reduction in reproduction, and denaturation of enzymes which are responsible for degradation (e.g. azo reductase enzyme) (Solís et al. 2012; Saratale et al. 2011). However, it has been detected that with some whole bacterial cells, the azo reductase enzyme is relatively thermostable and can stay active up to temperatures of 60 ˚C, over short time. In many systems, results have shown that optimum temperature for bacterial decolorization of aromatic dyes occur at a range of 25 ± 2 ˚C (Pearce 2003b).

1.6 Examples of bacterial degradation of selected organic environmental contaminants

1.6.1 Polycyclic aromatic hydrocarbons (PAHs)

In general, microbial degradation of organic compounds, like PAHs, can be divided into groups according to whether microorganisms gain energy from the degradation processes or not. Biodegradation process is a growth associated procedure where chemoorganotrophic microorganisms convert part of the carbon atoms in the organic substrate into their cell constituents, while another part of the carbon atoms are degraded to produce energy (Seo et al. 2009). It is reported that PAH with low

molecular mass (less than three benzene rings) are easily degraded by various aerobic chemoorganotrophic bacteria. On the other hand, only limited number of bacterial species were able to degrade PAHs with high molecular mass (four or more benzene rings) such as *Mycobacterium spp*. or *Sphingomonas spp.* (Samanta et al. 2002).

1.6.1.1 Degradation pathways of Naphthalene by *Comamonas testosteroni*

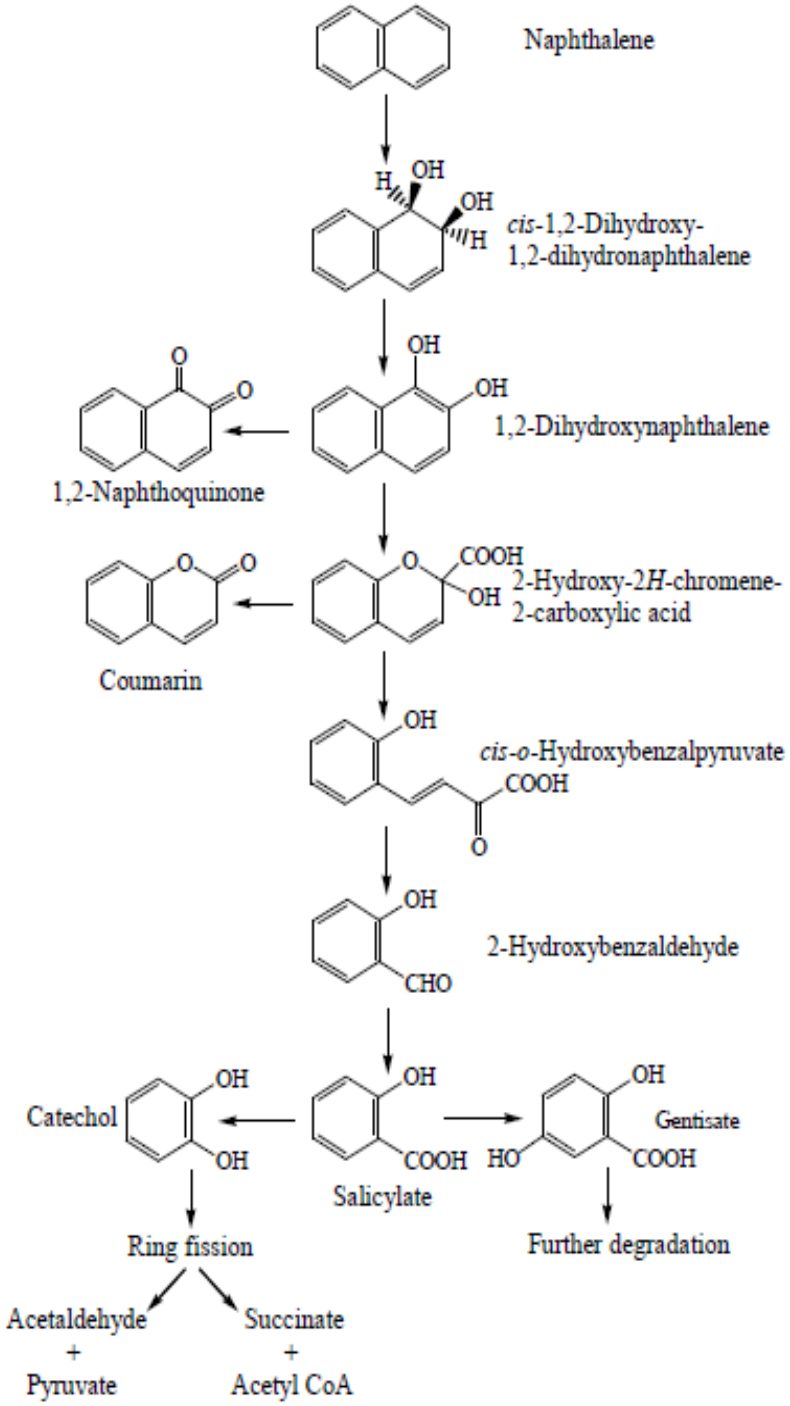
Naphthalene is commonly used as a model compound to examine the ability of various microorganisms to degrade PAHs, due to its simplicity and solubility among PAHs (Mollea et al. 2005). As a result, to understand the pathways of degrading three- or more ring of PAHs by bacteria, numerous studies of bacterial degradation were carried out on naphthalene. Many isolated bacteria showed efficient degradation on naphthalene (used naphthalene as a source of carbon and energy), such as *Alcaligenes*, *Burkholderia*, *Mycobacterium*, *Polaromonas*, *Pseudomonas*, *Ralstonia*, *Rhodococcus*, *Sphingomonas*, and *Streptomyces*. Scheme 1 summarizes the pathways of naphthalene degradation by bacteria (Pumphrey et al. 2017; Baird et al. 2002; Seo et al. 2009).

1.6.1.2 Bacterial degradation of polychlorinated phenols

Chlorinated phenols are widely released in the environment because of their extensive use as dyes synthesis, pesticides in agriculture and pharmaceuticals. The first known antiseptic compound was phenol, a subsequent developments were done to obtain a higher antimicrobial activity and acidity than phenol compounds which known currently as polychlorinated phenols (Bae et al. 2017). Chlorophenols, particularly the fully substituted pentachlorophenol (PCP), are used as preservatives for timber and textiles towards fungal rot and damage by insects. The toxic nature of PCP is based on its ability to uncouple oxidative phosphorylation and to alter the

electrical conductivity of cell membranes (Kurola 2006). Due to release, accidents and spills during the production and application of chlorophenols pollution by PCP has been reported in air, water, soil and sediments. For example, in Finland the widespread use of PCP containing wood preservative had led to contamination of soil around nearly 800 former saw mill sites. Even though PCP is toxic to microbes, variety of bacteria and fungi has been isolated with an ability to mineralize PCP. Both Gram-positive and Gram-negative bacteria capable of degrading PCP has been described, species of *Alcaligenes*, *Pseudomonas*, *Mycobacterium*, *Sphingomonas* and *Streptomyces* representing the most commonly reported taxa (Seo et al. 2009).

Scheme 1: Proposed catabolic pathways of naphthalene by *Comamonas testosterone* (Seo et al. 2009)



1.6.2 Degradation of antibiotics by various isolated bacteria

The antibiotics in the environment are generally released from various sources, which include effluent from manufacturing plants of pharmaceutical and facilities treating wastewater collected from hospitals, sewage, and veterinary clinics (Karthikeyan & Meyer 2006). The release of antibiotics in the environment generally gets accumulated in the ground and surface water. It has been observed by varied researchers that antibiotics exhibits biodegradable characteristics on making alterations in certain conditions (Drillia et al. 2005; Gartiser et al. 2007).

Two antibiotics ciprofloxacin, which is particularly effective against gram negative bacteria, and erythromycin, which shows an effective results against gram positive bacteria, were used to test the ability of bacterial degradation (Nnenna et al. 2011). Various reports have indicated that these antibiotics usually found in wastewater treatment facilities (Karthikeyan & Meyer 2006) The biodegradation tests using various bacterial isolates, such as *Micrococcus sp*., *Bacillus sp.*, *Pseudomonas sp*., and *Shigella sp*., showed that biodegradation of the selected antibiotics was achieved. Besides, the study showed that variation in pH had no significant effect on the biodegradation of the antibiotics. Thus, pH consider as unimportant factor compared to the other parameters (e.g. moisture, oxygen, absence of alternative sources of carbon and nitrogen) which have been proved to be essential for an effective biodegradation of antibiotics to take place (Nnenna et al. 2011).

1.6.3 Plastics degradation by various common isolated bacteria

Interestingly, some of the famous bacteria exposed their ability to degrade plastic polymers into their respective simple monomeric (Ghosh et al. 2013). Among

them, *Pseudomonas* is dominant, since several studies have reported its ability in degrading polyvinyl chloride (PVC), polythene, poly (3-hydroxybutyrate-co-3-mercaptopropionate), and poly (3-droxypropionate). However, *Bacillus brevis* was capable of degrading only polycaprolactone whereas *Streptomyces* have degraded PHB, poly (3-hydoxybutyarate-co-3-hydroxyvalerate), and starch or polyester. It has been stated that 39 bacterial isolates of the classes Proteobacteria and Firmicutes were able to degrade various types of plastics such as polyhydroxybutyrate (PHB), polycaprolactone (PCL), and polybutylene succinate (PBS) (Shimao M., 2001). Other bacterial species recognized having the ability of degrading plastics were *Bacillus* sp., *Staphylococcus* sp., *Streptococcus* sp., *Diplococcus* sp., *Micrococc us* sp., *Pseudomonas* sp., and *Moraxella* sp. (Kumar et al. 2007).

1.6.4 Decolorization of various azo dyes by bacteria

Various number of new processes has been developed for decolorization of azo dye. The decolorization of azo dyes using microbes is one of the best strategies, as this process involves biodegradation of azo dyes and thus, it is an environmentally friendly process. (Pandey et al. 2007). Recently, number of diverse bacteria have exhibited the ability to reduce azo dyes in both aerobic and anaerobic conditions. The obligate anaerobes that includes *Eubacterium sp* and *Clostridium sp* facultative anaerobes, which includes *Escherichia coli*, *Enterobacter agglomerans*, and *Bacillus cereus*; and several aerobes such as *Pseudomonas aeruginosa* are also found to possess the ability to reduce azo dyes in both aerobic and anaerobic conditions (Saratale et al., 2011). Two different mechanisms can be summarised for bacterial decolorization of azo dye. In the presence of azo compounds, aerobic bacteria are required to be acclimatised for an extended duration for inducing azoreductase expression as they

generally exhibit a high particularity to structures of dyes. On the contrary, decolorization completed with the use of anaerobic process is generally not specific, and the efficiency of anaerobic dye removal is far better in comparison to decolorization conducted under aerobic process. The result of these researches indicates that the decolorization of large range of wastewater azo dyes can be carried out more effectively under anaerobic process (Adrian 2001).

1.6.4.1 Removal of navy blue using an isolated bacterial strain *Bacillus pumilus* HKG212

Among the various azo dyes, Remazol Navy Blue (RNB), an extensively used azo dye in textile industries was reported to be decolorized by microbial degradation.(Saratale et al. 2017) .A recent study showed that *Bacillus pumilus* HKG212, a bacteria isolated from textile industry effluent waste water, is capable to degrade the textile azo dye, Remazol Navy Blue effectively. The optimal conditions for the decolorizing activity of *Bacillus pumilus* HKG212 were anaerobic culture environment with beef extract as nitrogen source supplementation, at pH 8.0, and 30 °C. *Bacillus pumilus* HKG212 showed decolorizing activity through a degradation mechanism rather than adsorption and it could tolerate high concentrations (up to 1500 mg L^{-1}) of RNB dye. Different analytical techniques like spectrophotometric, HPLC and FTIR analysis confirmed the degradation of azo bond of the dye RNB by the isolated bacterial strain (Das et al. 2016).

1.7 Objectives

The main objectives of the current work are summarized below:

1- Isolate and purify 12 different bacteria from petroleum sludge capable of degrading organic pollutants.
2- Study the ability of the bacterial isolates from petroleum sludge in degrading various azo dyes.
3- Identify the most potent bacteria that could degrade a wide-range of organic pollutants using 16S rRNA.
4- Develop a sensitive and fast LC-MSMS method for analyzing nine different emerging pollutants,
5- Examine the ability of the isolated bacterial isolate for degrading these 10 emerging pollutants.

Chapter 2: Materials and Methods

2.1 Chemicals and organic pollutants

All the chemicals and reagents used were of the highest purity and of analytical grade and were obtained from Sigma-Aldrich (USA). Toluidine Blue, Amido Black, Crystal Ponceau 6R, Trypan Blue, Methyl Blue, Orange G, Acid Red 40, Reactive Black 5 and Ponceau BS were obtained from Sigma-Aldrich (USA). Eriochrome Black T, Thiazole Yellow G, Naphthol Green B and Congo Red were purchased from Fluka chemical and Malachite Green from BDH. Drimarene Red CL 4BN was obtained from Clariant chemicals. The chemical structures, dye class and the λmax (maximum absorbance wavelength) of all dyes are listed in Table 4. All the culture media, nutrient broth and nutrient agar used were obtained from Sigma-Aldrich (USA). Nutrient Broth composition (Sigma-Aldrich) was as follows: 1 g/L D (+)-glucose, 15 g/L peptone, 6 g/L sodium chloride, 3 g/L yeast extract, 3 g/L, final pH 7.5 ± 0.2. All organic solvents (HPLC grade acetonitrile and methanol) were from Fisher Scientific (UK). LC-MS grade of acetonitrile, methanol, ammonium formate and formic acid were obtained from Sigma-Aldrich (USA).

2.2 Microorganisms and growth conditions

The bacterial strains were initially isolated from petroleum sludge, as described in the previous study (Ali et al. 2014) . However, for the current work, they were re-isolated and re-purified by multiple rounds of streaking. Isolation of bacteria was carried out by using the standard procedures using Nutrient Agar (NA) containing 1g/L D (+)-glucose, 15 g/L peptone, 6 g/L sodium chloride, 3 g/L yeast extract, 3 g/L, final pH 7.5 ± 0.2. The isolates were cultured and grown in a shaker-incubator at 37 °C.

2.3 Isolation and screening and identification of dye decolorizing strain

Pour plate technique was used for the isolation of the selected dye decolorizing bacteria. Well grown bacterial colonies were picked and further purified by streaking. The isolated strains were frozen in 20% glycerol at -80 ^{o}C and grown and maintained in Nutrient Broth at 4°C for no more than 1 week. Identification of the bacterial isolates was carried out using partial 16S rRNA sequencing of the crude DNA on a 3500 Genetic Analyzer by Macrogen sequencing services (South Korea). The phylogenetic analyses were performed using the online site http://www.phylogeny.fr which produced the phylogenetic tree.

2.4 Dye degradation

A loopful of bacteria culture from glycerol stock was inoculated in a 50 mL sterile tube containing 15 ml nutrient broth and incubated at 37°C under shaking condition (200 rpm) for 24 h. For sampling, all dyes from stock solution (2000 ppm) were sterilized by filtering through 0.45 μm nylon filter, before being added at 20 ppm in 15 mL nutrient broth and 0.15 mL freshly grown (overnight) bacterial culture, incubated for 24 h while shaking. One ml of sample was withdrawn at different time intervals and each time it was centrifuged at 13,000 rpm for 10 minutes to separate the bacterial cell mass. Decolorization of dye was determined by measuring the absorbance of the cleared supernatant at the absorbance maxima of the respective dyes. All experiments were performed in triplicates. Nitrogen purging (blowing nitrogen gas over the surface of the culture to displace the air and immediately tightly closing the tube cap) followed by static incubation were used to obtain anaerobic conditions in the experiments. Percentage of decolorization was calculated as follows:

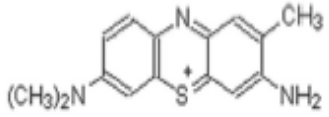
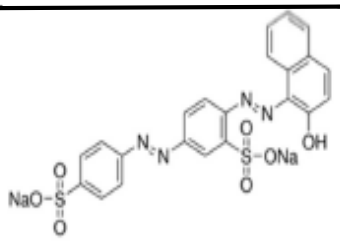
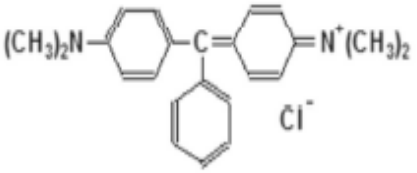
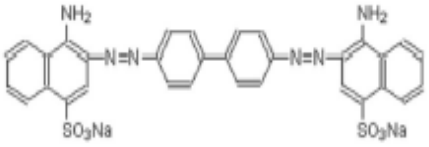
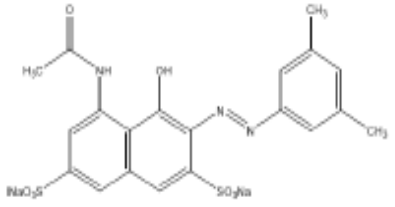
$$\% \text{ decolorization} = \frac{A0 - At}{A0} \times 100$$

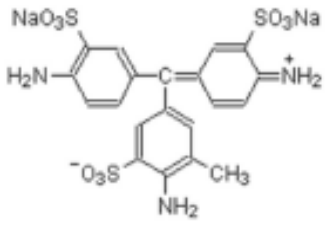
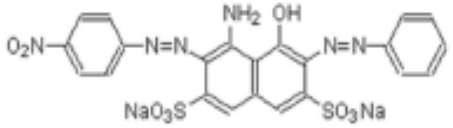
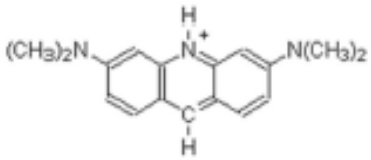
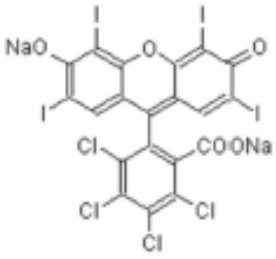
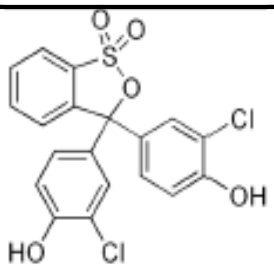
Where A0 is the initial absorbance and At is the final absorbance value.

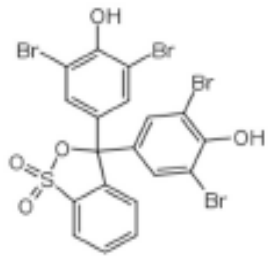
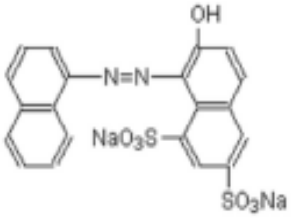
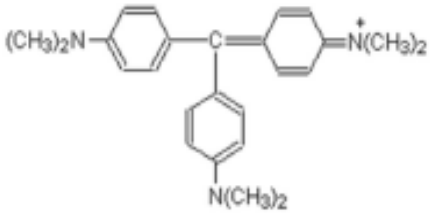
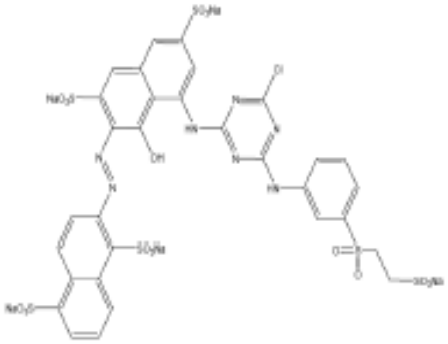
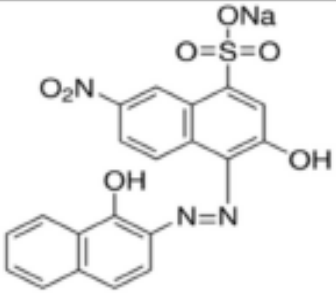
2.5 Optimization

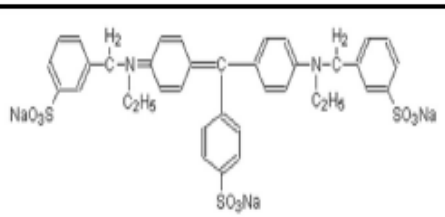
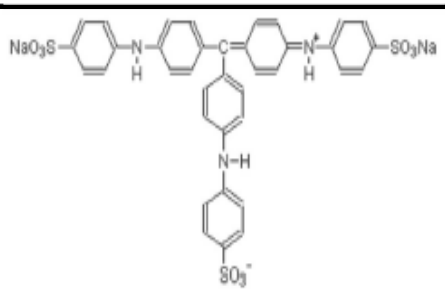
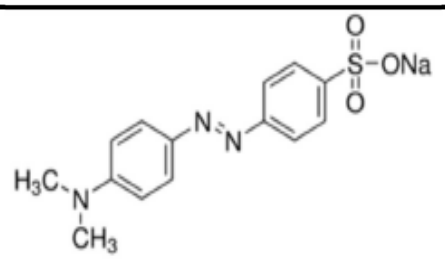
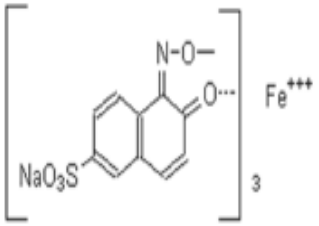
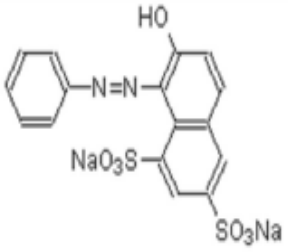
The effect of pH, concentration and carbon sources were studied and optimized using the standard "one parameter at a time" approach. For the pH optimization study, the decolorization of dye was observed in buffered Nutrient Broth media at pH values of 3, 5, 7, and 9 (after 24 hours of incubation with the bacterial strain). For the dye concentration experiments, Nutrient Broth (pH 7) containing increasing concentrations (20, 100, 200, 400, 800 and 1000 ppm) of dyes was used. The effect of carbon and nitrogen sources was examined by using different nutrient media such as Lysogeny Broth (LB). Nutrient Broth-E (NB-E), Nutrient Broth-2 (NB-2), 2xYT, Yeast Extract, Tryptone, and Peptone. All measurements were made by measuring the dye decolorization after 24 hours of bacterial strain incubation, and the experiments were carried out in triplicates.

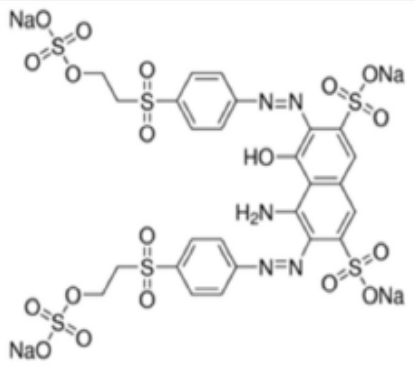
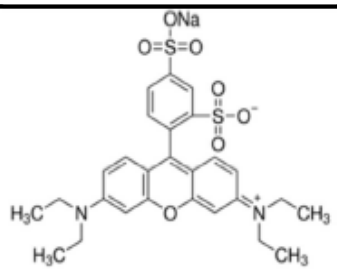
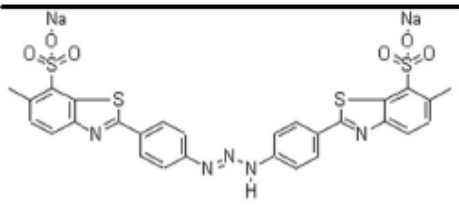
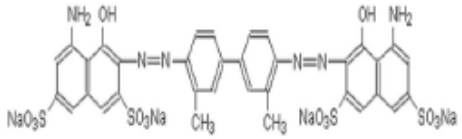
Table 4: Classification of dyes and their structures

Name of Dye	Class	λ_{max} (nm)	Structure
Toludine Blue	Thiazin	626	
Ponceau BS	Azo (di)	505	
Malachite Green	Triarylmethane	618	
Congo Red	Azo (di)	498	
Acid Red 40	Azo (mono)	506	

Acid Fuchsin	Triarylmethane	550	
Amido Black	Azo (di)	618	
Acridine Orange	Acridine	467	
Bengal Rose	Fluorone	547	
Chlorophenol Red	Phenol	572	

Bromophenol Blue	Phenol	598	
Crystal Ponceau 6R	Azo (mono)	510	
Crystal Violet	Triarylmethane	593	
Drimarene Red CL 4BN	Azo (mono)	532	
Eriochrome Black T	Azo (mono)	560	

Light green SF	Triarylmethane	630	
Methyl Blue	Triarylmethane	607	
Methyl Orange	Azo (mono)	507	
Naphthol Green B	Nitroso	714	
Orange G	Azo (mono)	475	

Reactive Black 5	Azo (di)	597	
Sulforhodamine B	Triphenyl-methane	565	
Thiazole Yellow G	Azo (mono)	402	
Trypan Blue	Azo (di)	607	

2.6 HPLC and LCMS analyses

The chromatographic experiments using HPLC–MS/MS system were carried out on 1260 Infinity HPLC and 6420 LC/MS (Agilent, USA) coupled with a solvent gradient pump and an automatic injector. The dye degradation products were separated using Agilent Zorbax SB-C18 column 150 mm × 4.6 mm packed with 5 μm particle size. The detection system was a diode array detector (Agilent, USA) with detection range between 200 and 780 nm. The signal acquired from the detector was recorded by HP Chemstation software. The mobile phase consisted of two solutions namely A and B. Solution A was made from 0.1 M ammonium formate (pH 6.7), whereas solution B was made from a mixture of acetonitrile + methanol (1:1). The gradient elution was from 0% to 80% in 40 min; the flow rate was 1 mL min^{-1} and the injection volume was 100 μL. The total run time was changed to 21 minutes for LCMS runs for some later samples to make the runs shorter and the analyses faster, but this did not change the resolution or area under the peaks of the emerging pollutants.

The mass spectrometer was equipped with an electrospray ionization (ESI) source and operated in positive polarity. The ESI conditions were as follows: capillary voltage: 3.5 kV, endplate offset was fixed at 500 V; skimmer at 40 V; trap drive at 53 V; the nebulizer pressure was 483 kPa; drying gas flow was 12 L min^{-1} and drying temperature was 350 °C. The mass range was from 50 to 700 Da. Tandem MS experiment was done using the Auto MSn mode wherein Helium gas was used as a collision gas.

Chapter 3: Results and Discussion

3.1 Dye degradation

As mentioned in the objectives, one of the main goals of the present work was to isolate a novel bacterial strain that would be capable of efficiently degrading various emerging pollutants. In order to rapidly identify the possible pollutant-degrading isolates, we tested the abilities of our 12 bacterial isolates to degrade 4 diverse classes of aromatic dyes under aerobic condition.

Figure 5 & 6 shows the degradation of Ponceau BS as well as Malachite green, respectively, by the 12 bacterial isolates. The results show that after just one day of incubation, strains MA1 and MA10, and day six, 7 out of 12 bacterial strains were capable of degrading Ponceau BS effectively under gentle shaking at 37 ^{o}C. Similarly, most of the strains started to show Malachite Green degradation after one day, and most dramatically by day 3 and 6. However, the results looked very different than that observed for Ponceau BS, as all the strains (except MA1) showed an efficient decolorization of Malachite Green. These 12 strains were also tested on 2 additional dyes, namely Congo Red and Toluidine Blue, and the initial screening results for these 4 dyes are summarized in Table 5. All dyes showed reasonable degradation when exposed to various bacterial strains, while Congo Red showed the poorest degradation the 12 bacterial isolates. Based on the results from this initial screening, isolates MA1, MA3, MA6 and MA12 showed most promising results in degrading various classes of dyes and were chosen for further study.

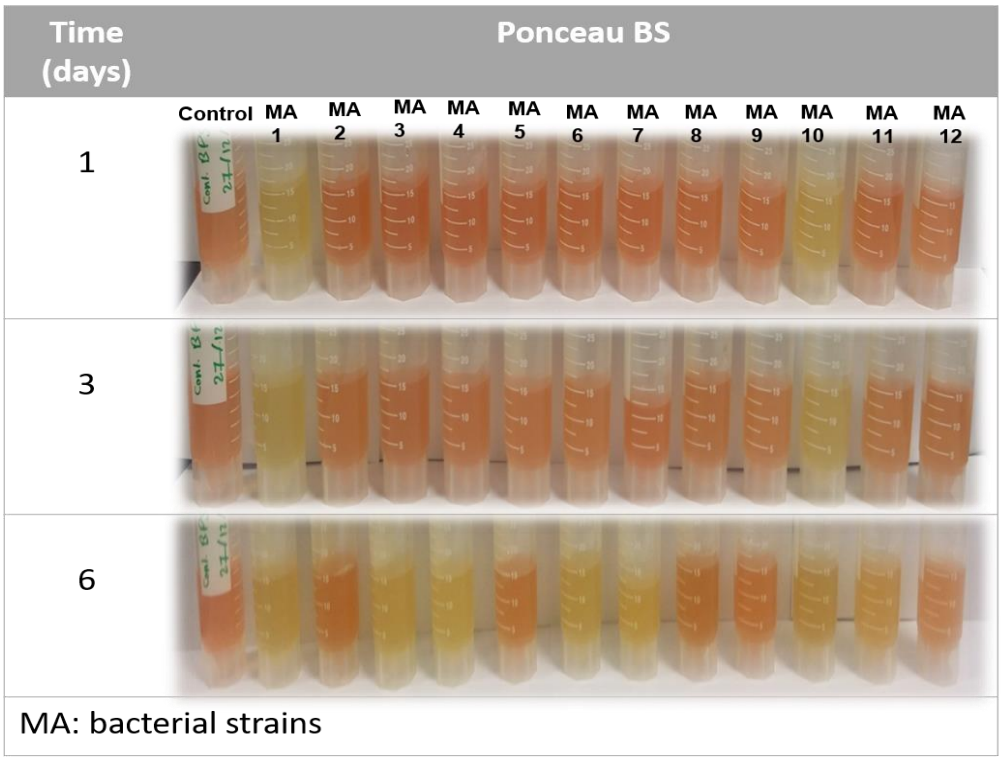


Figure 5: Aerobic bacterial degradation of Ponceau BS

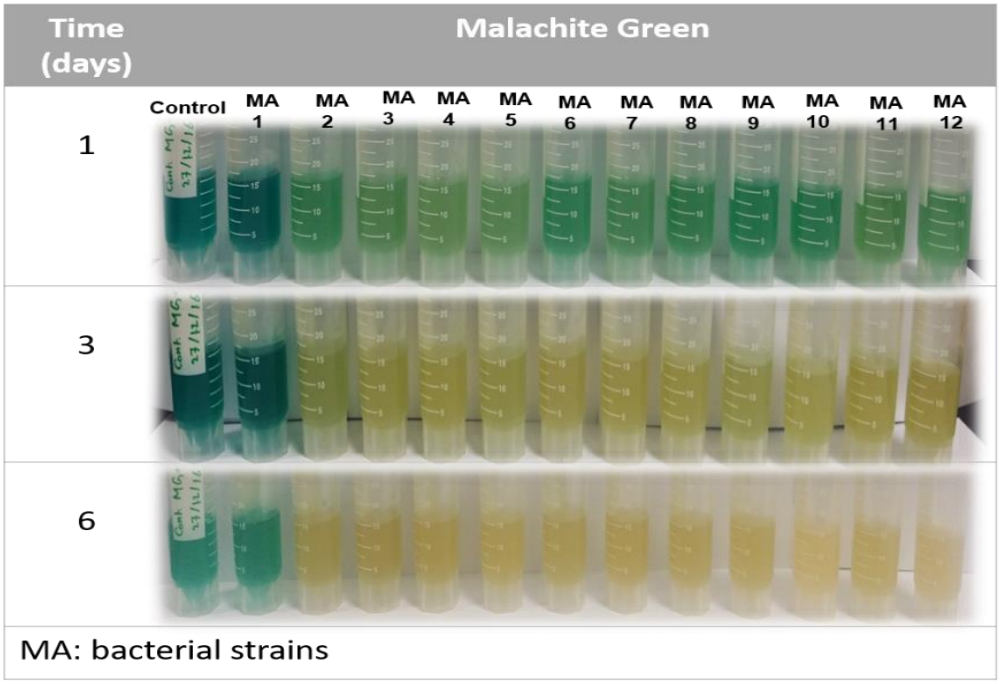


Figure 6: Aerobic bacterial degradation of Malachite Green

Table 5: Aerobic degradation of 4 dyes by all twelve bacterial strains

	Time (days)	MA 1	MA 2	MA 3	MA 4	MA 5	MA 6	MA 7	MA 8	MA 9	MA 10	MA 11	MA 12
Ponceau BS	1	***	-	-	-	-	-	-	-	-	***	-	*
	3	***	-	*	*	*	*	-	-	*	***	-	***
	6	***	**	**	*	**	*	*	-	*	***	*	***
Malachite Green	1	-	*	*	*	*	*	**	***	***	*	**	**
	3	-	**	**	**	**	**	**	***	**	*	**	***
	6	-	**	**	**	**	***	**	***	**	**	**	***
Toudine Blue	1	**	-	*	-	***	*	-	-	-	*	-	-
	3	**	-	*	-	***	*	-	-	-	*	-	-
	6	**	-	*	-	***	*	-	-	-	*	-	-
Congo Red	1	*	-	*	-	-	-	-	-	-	-	-	*
	3	*	-	*	-	-	-	-	-	-	*	-	*
	6	*	-	*	*	*	*	*	*	*	*	*	*

MA: Bacterial strains, (-) 0-20%,(*) 21-40%, (**) 41-75%, (***) 76-100%,

Although the dye-decoloration abilities of these strains were measured to help with the selection of specific strains for the degradation of emerging pollutants, nevertheless, these strains can also be used for treating dye-contaminated textile industrial effluents. In fact, many other groups have identified and published on dye-decolorizing bacteria. For example, Jadhav and Kagalkar have isolated *Galactomyces geotrichum* and *Bacillus sp.* strain of bacteria that could efficiently degrade Brilliant Blue G dye (Jadhav et al. 2008). In addition, by a newly isolated *Brevibacterium sp*. strain VN-15 have been found to degrade reactive sulfonated azo dyes effectively (Franciscon et al. 2012).

3.2 16S rRNA-based identification of the isolate

In order to identify the species and possible genus of the bacterial isolates as well as to see how these 12 isolates were related to each other, 16S rRNA gene sequencing was carried out. The sequencing data showed that based on 16S rRNA analysis, the 12 bacterial strains belonged to two different bacterial species: *Bacillus*

cereus (MA1), and *Pseudomonas guariconensis* (MA2 - MA12). Table 6 shows the individual bacterial strains, species name, the accession number of the closest GenBank match, and their identity (based on 16S rRNA sequence similarity to published sequences). The phylogenetic tree of these twelve strains is also shown in Figures 7-18. There were slight changes in the position of the isolted strains, as shown in their phylogenic tree (Figure 7-18), which are slightly defictual amd this may due to the program used by Macrogen. However, the main point of the 16S rRNA results, as described previously, were that MA1 seems to belong to *Bacillus cereus* while MA2 – MA12 seems to belong to *Pseudomonas guariconensis*.

Although MA2 to MA12 strains showed they were identical, based on the closest match GenBank sequence (Table 6), as well as their phylogenetic trees (Figures 7-18), the dye degradation data (Table 5) showed that they are not identical, as some of them show very different dye remediation profiles. Perhaps, the 16S rRNA analysis is not able to detect small minor differences in other parts of these bacterial genomes. This seems to be indicated in Figure 19, where only the forward sequences of these 12 strains are analyzed and displayed in a phylogenetic tree. We noticed a relative difference in the phylogenetic tree of the 12 isolated bacterial strains (Figure 19), this is might be due to the use of forward primer (partial sequence) instead of using the whole sequence.

Moreover, it is interesting to note that of the two species of bacterial strains that we have been isolated from petroleum sludge, one (*Bacillus cereus*) has been previously shown to be able to degrade various classes of organic pollutants. These studies have showed that the isolated *Bacillus cereus* was capable of degrading and detoxifying red, green, black, and yellow textile dyes as well as various aromatic compounds like phenol (Mahmood et al. 2015). Surprisingly, we were not able to find

any published references where *Pseudomonas guariconensis* or related bacteria could be used for dye degradation or other organic pollutant degradation. This is a very interesting observation that perhaps could be further explored in a separate project.

Table 6: Bacterial strain identification using 16S rRNA sequencing

Bacterial Isolate	**Identities**		**Species name**
	Match/Total	**Percentage (%)**	
MA 1	1490/1494	99	*Bacillus cereus* NR_115714.1
MA 2	1467/1474	99	*Pseudomonas guariconensis* HF674459.1
MA 3	1486/1498	99	*Pseudomonas guariconensis* HF674459.1
MA 4	1470/1479	99	*Pseudomonas guariconensis* HF674459.1
MA 5	1473/1483	99	*Pseudomonas guariconensis* HF674459.1
MA 6	1456/1465	99	*Pseudomonas guariconensis* HF674459.1
MA 7	1476/1486	99	*Pseudomonas guariconensis* HF674459.1
MA 8	1472/1481	99	*Pseudomonas guariconensis* HF674459.1
MA 9	1466/1474	99	*Pseudomonas guariconensis* HF674459.1
MA 10	1472/1481	99	*Pseudomonas guariconensis* HF674459.1
MA 11	1471/1480	99	*Pseudomonas guariconensis* HF674459.1
MA 12	1468/1477	99	*Pseudomonas guariconensis* HF674459.1

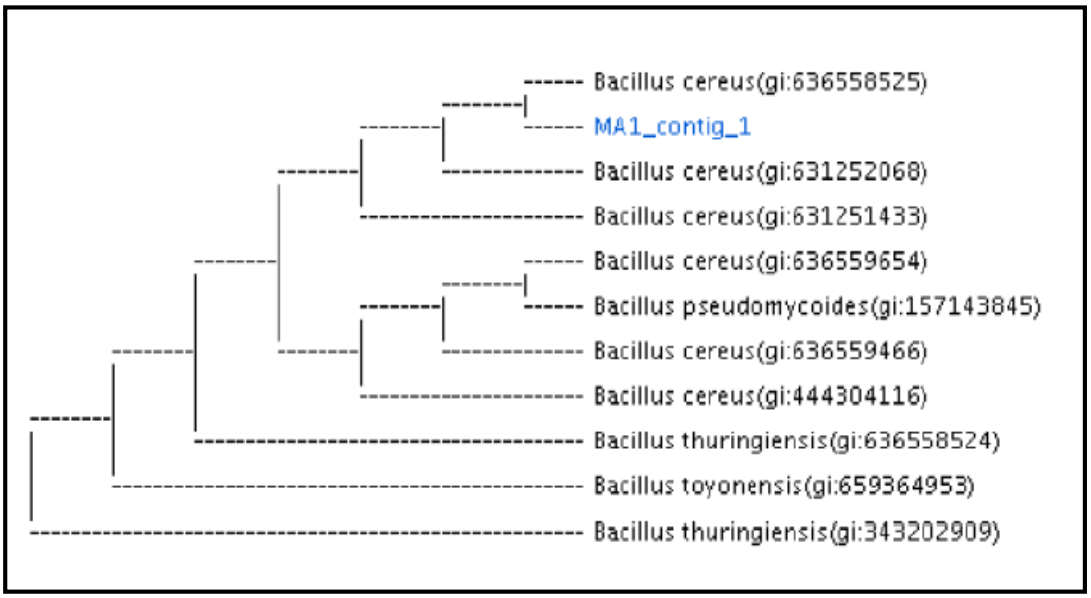


Figure 7: Phylogenetic tree of MA1 strain

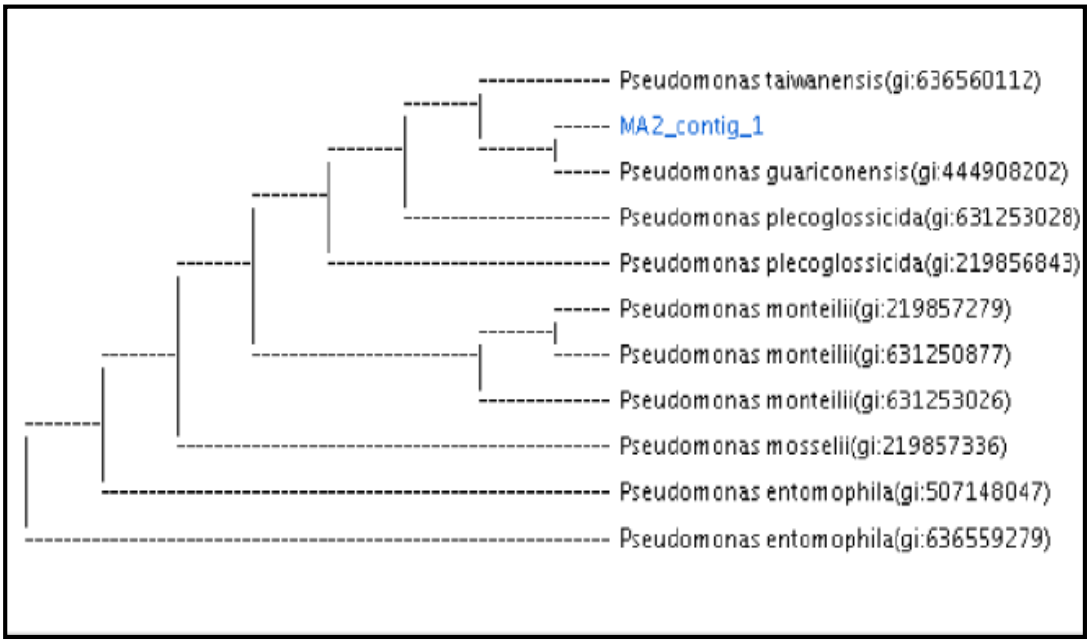


Figure 8: Phylogenetic tree of MA2 strain

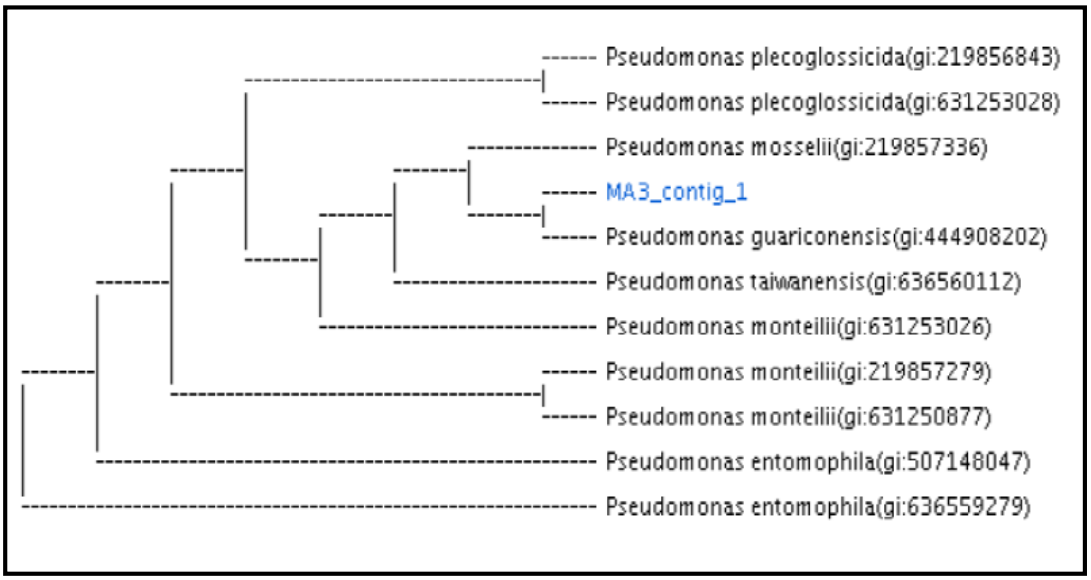


Figure 9: Phylogenetic tree of MA3 strain

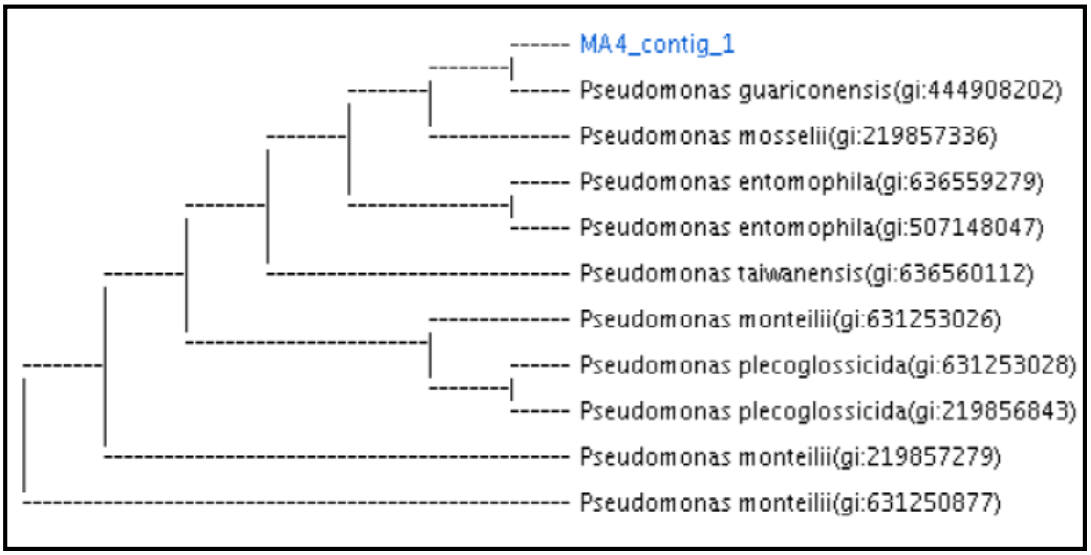


Figure 10: Phylogenetic tree of MA4 strain

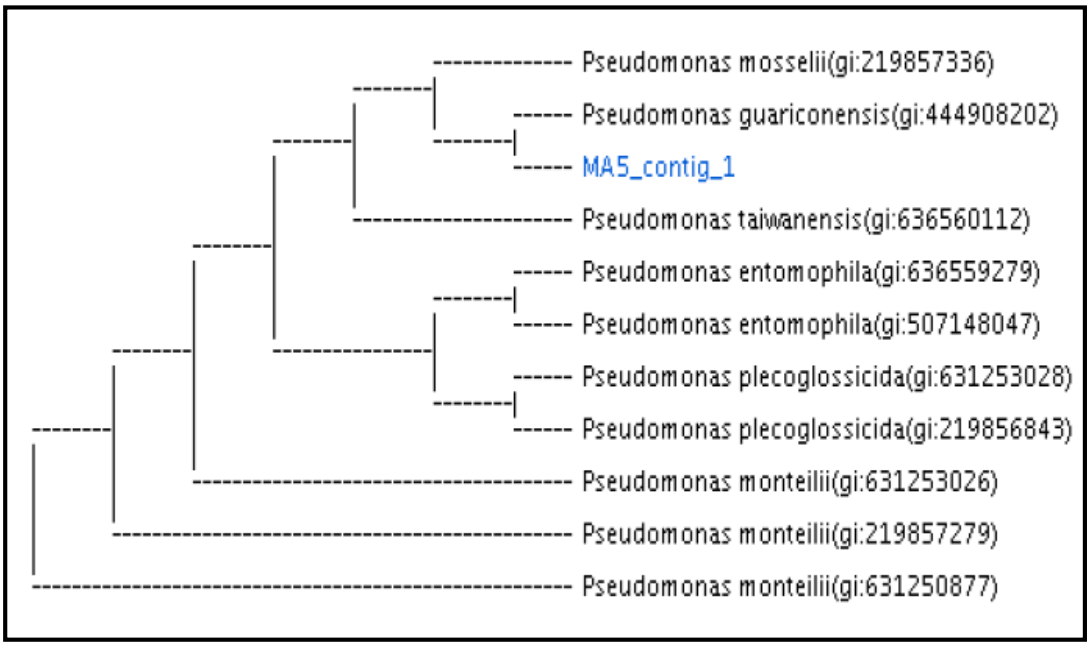


Figure 11: Phylogenetic tree of MA5 strain

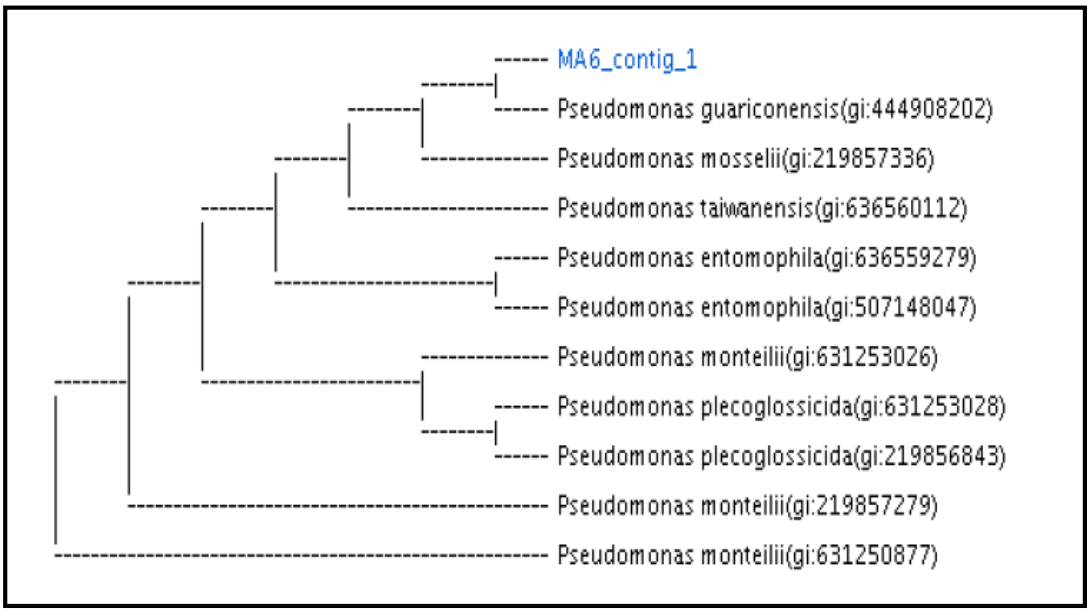


Figure 12: Phylogenetic tree of MA6 strain

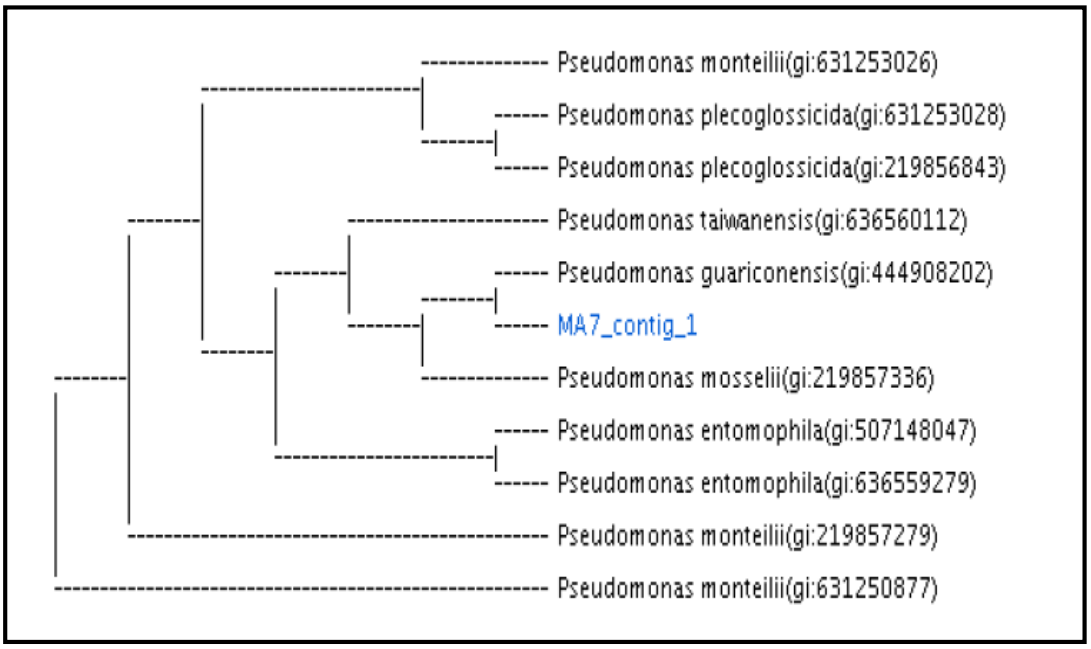


Figure 13: Phylogenetic tree of MA7 strain

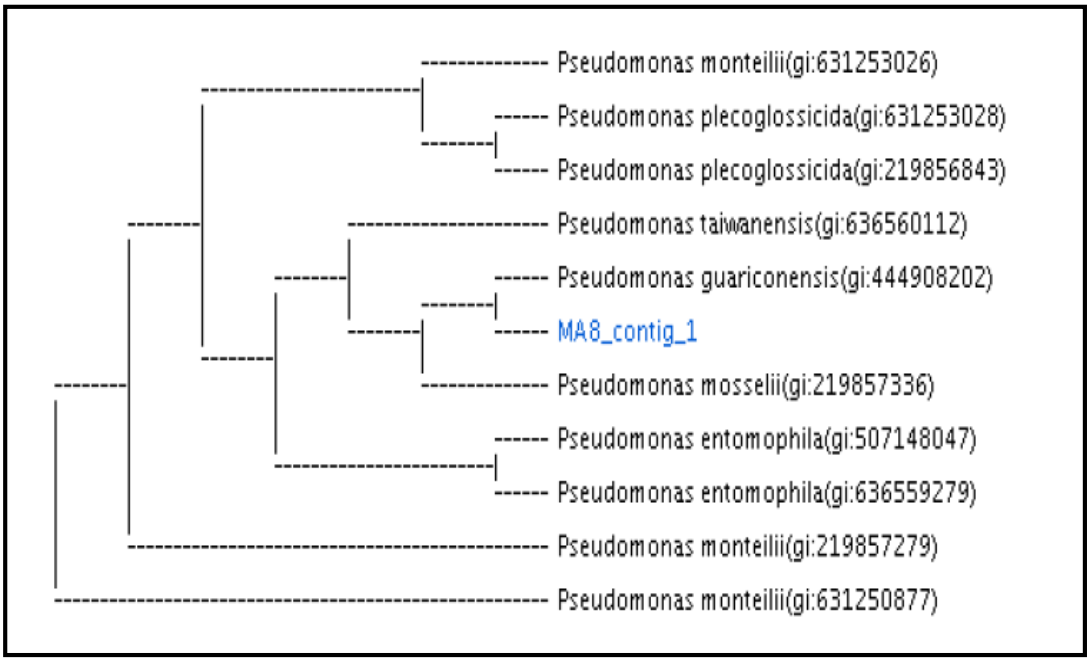


Figure 14: Phylogenetic tree of MA8 strain

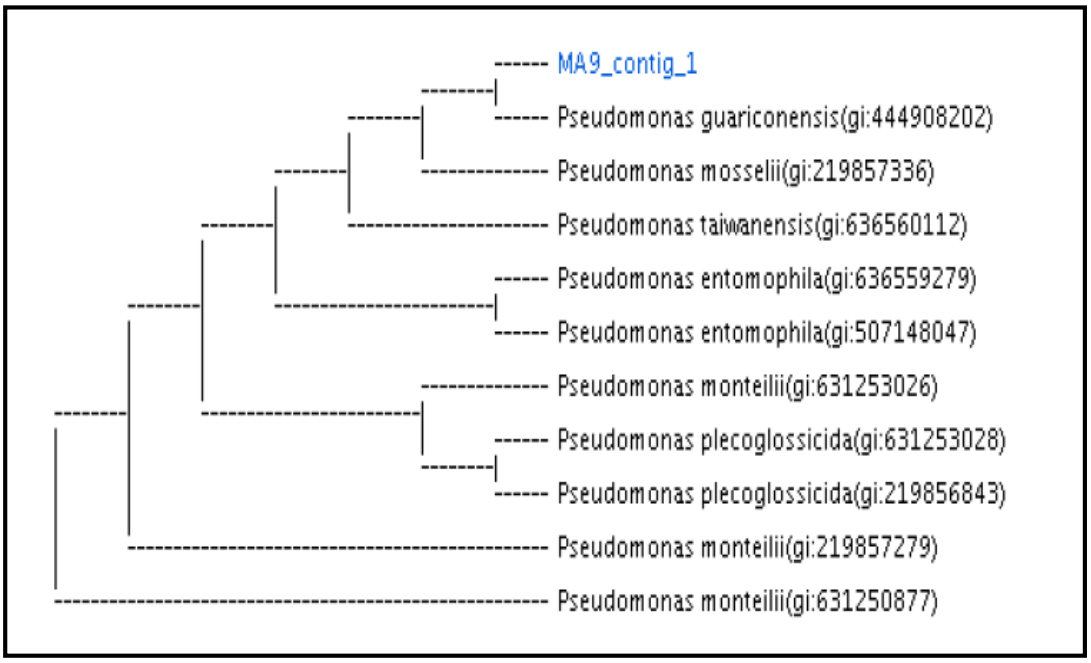


Figure 15: Phylogenetic tree of MA9 strain

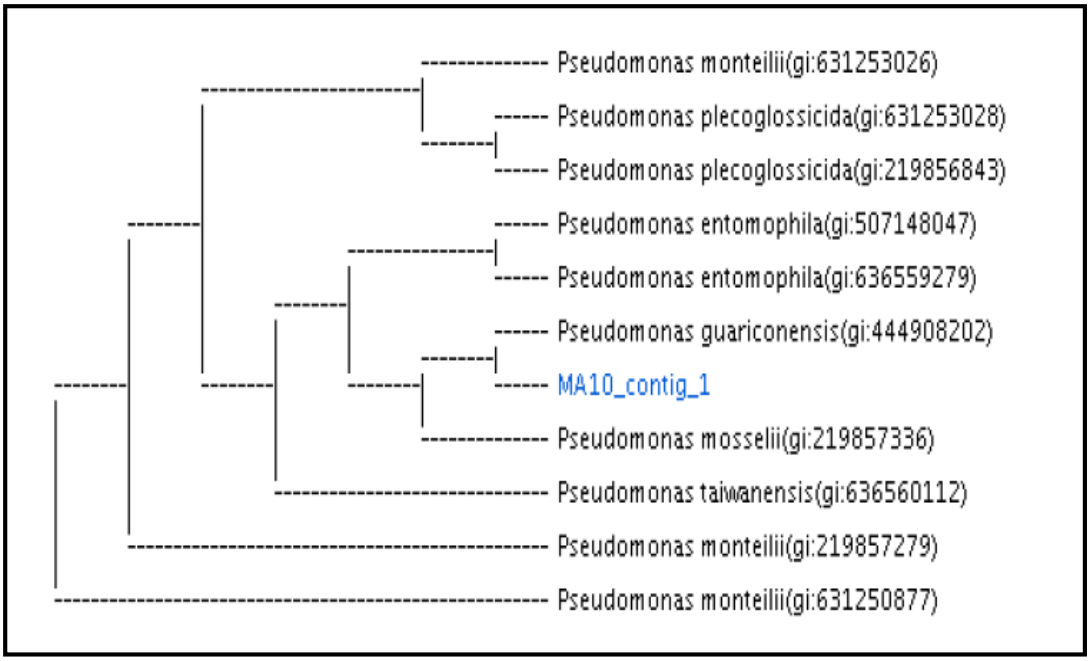


Figure 16: Phylogenetic tree of MA10 strain

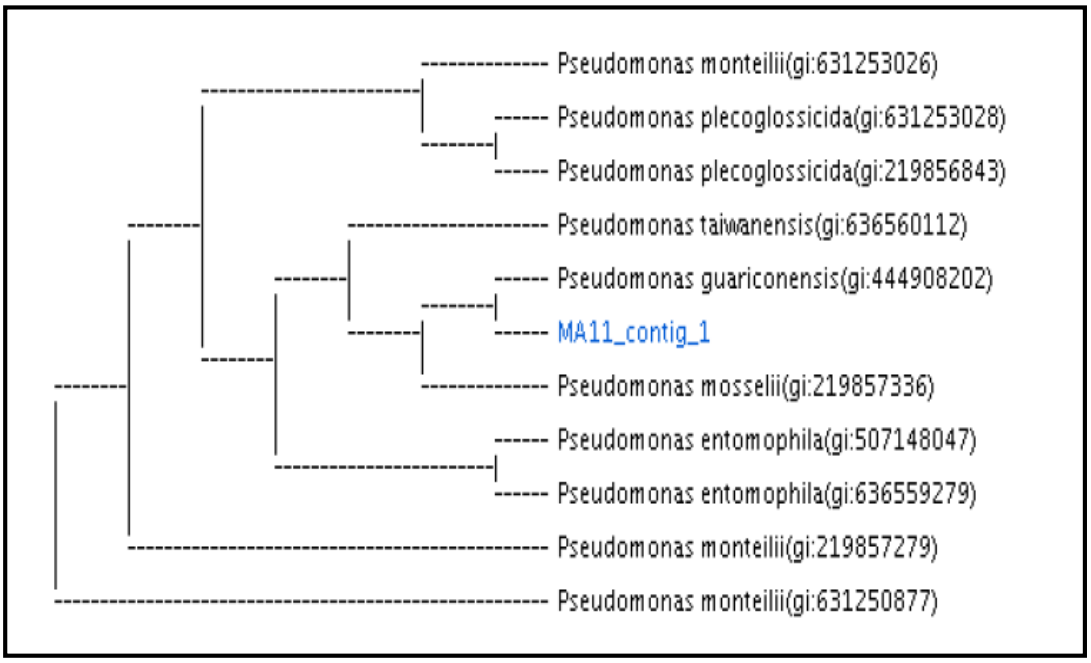


Figure 17: Phylogenetic tree of MA11 strain

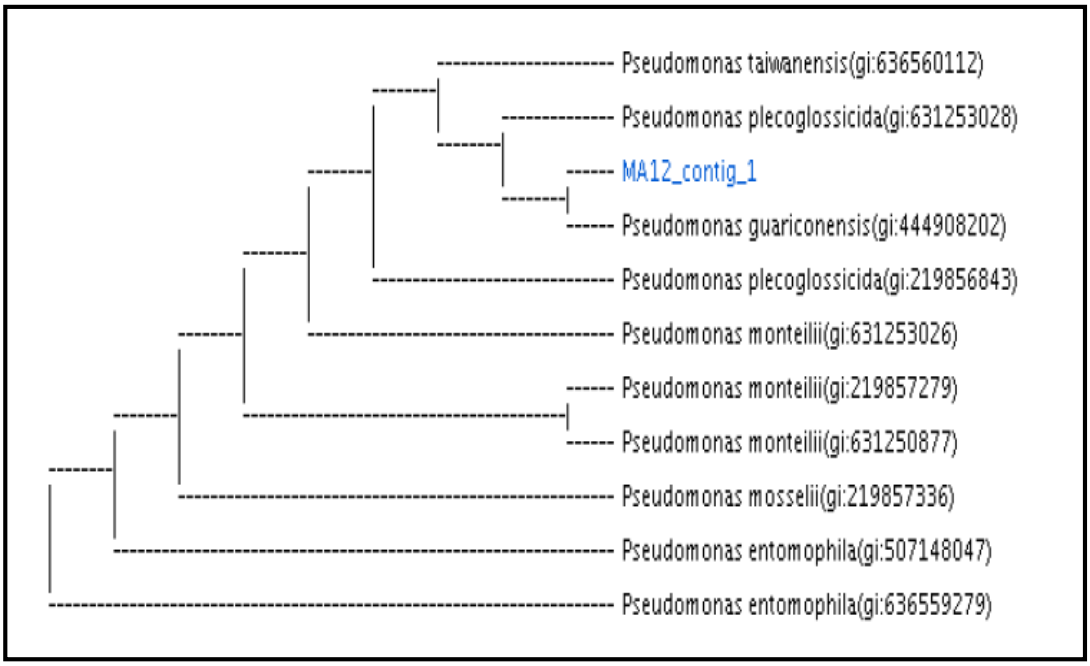


Figure 18: Phylogenetic tree of MA12 strain

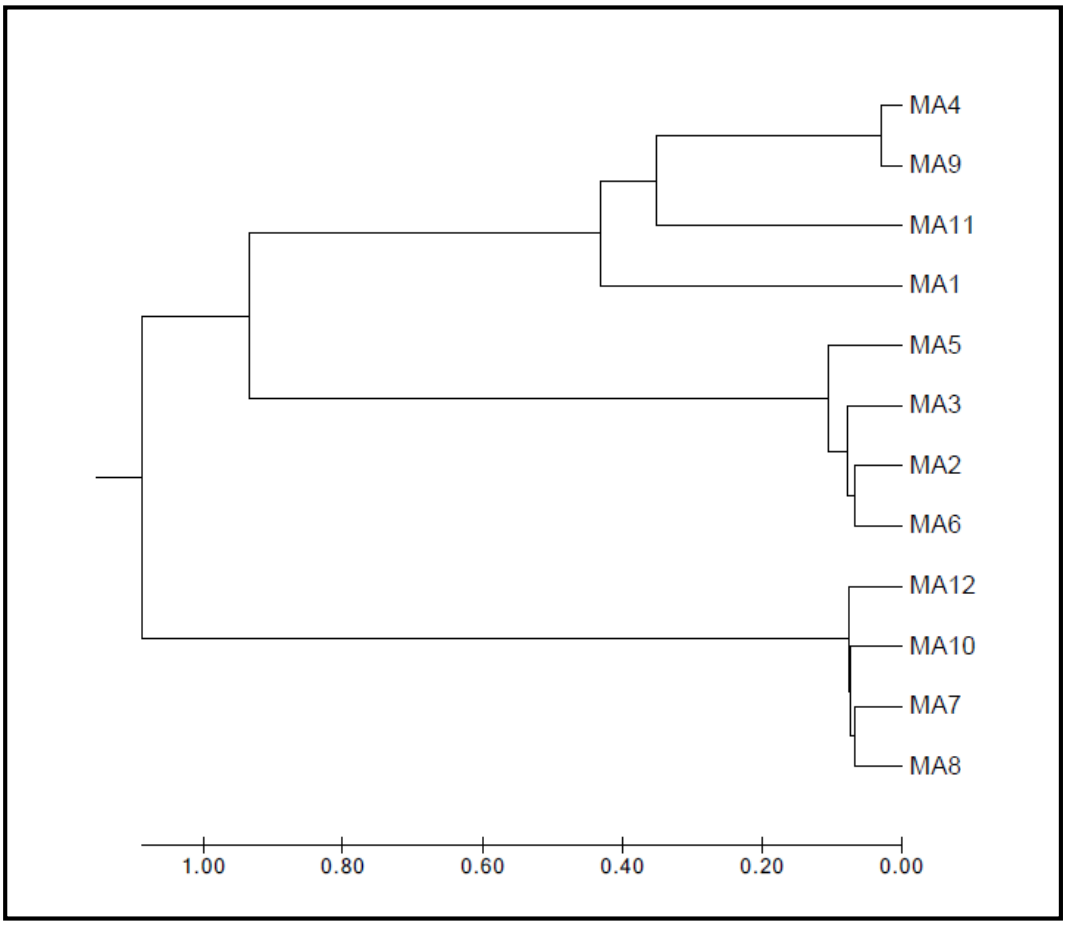


Figure 19: Phylogenetic tree of the 12 isolated bacterial strains based on 16S RNA partial sequences (using the forward primer)

Based on the dye degradation profiles (Table 5) as well as the phylogenetic analysis using partial 16S rRNA sequencing (forward primer sequencing only), we decided to choose the four most distinct bacterial isolates for further screening.

Therefore, additional experiments were carried out with additional dyes (12 dyes) with the four selected strains (MA1, MA3, MA6 and MA12) under aerobic conditions. These experiments were carried out in 2 mL deep 96-well blocks and the results are shown in Figure 20. Based on this secondary screening, one can notice that MA1 showed a very rapid and an efficient degradation of many diverse dyes as compared to the other selected strains. Impressively, MA1 was capable of degrading 7 dyes out of the 12 tested dyes. The other three strains, MA3, MA6 and MA12 showed almost similar degradation results for all the 12 dyes (able to degrade Reactive Black 5, Malachite Green and CP6R dyes). Based on this second screening, MA1 strain was chosen for further analysis as it showed to be the most effective strain for the efficient decolorization of many dyes.

The ability of MA1 to effectively degrade organic pollutants was further examined by a testing it on a wide range of aromatic dyes. Figure 21 shows the degradation of 24 structurally diverse aromatic dyes by the bacterial isolate MA1 under aerobic condition. The results showed that MA1 was able to degrade almost half of the selected dyes within 3 days, such as Acid Red 40, Acid Fuchsin, Crystal Ponceau 6R (CP6R), Drimarene Red CL 4BN, Light Green SF, Methyl Blue, Methyl Orange, Ponceau BS, Reactive Black 5 and Toludine Blue. Out of these dyes, 4 of them (CP6R, Methyl Orange, Ponceau BS and Toludine Blue) were chosen to further characterize and optimize the bioremediation ability of MA1. These specific dyes were chosen as they showed rapid and more than 75% decolorization within 3 days of incubation with MA1.

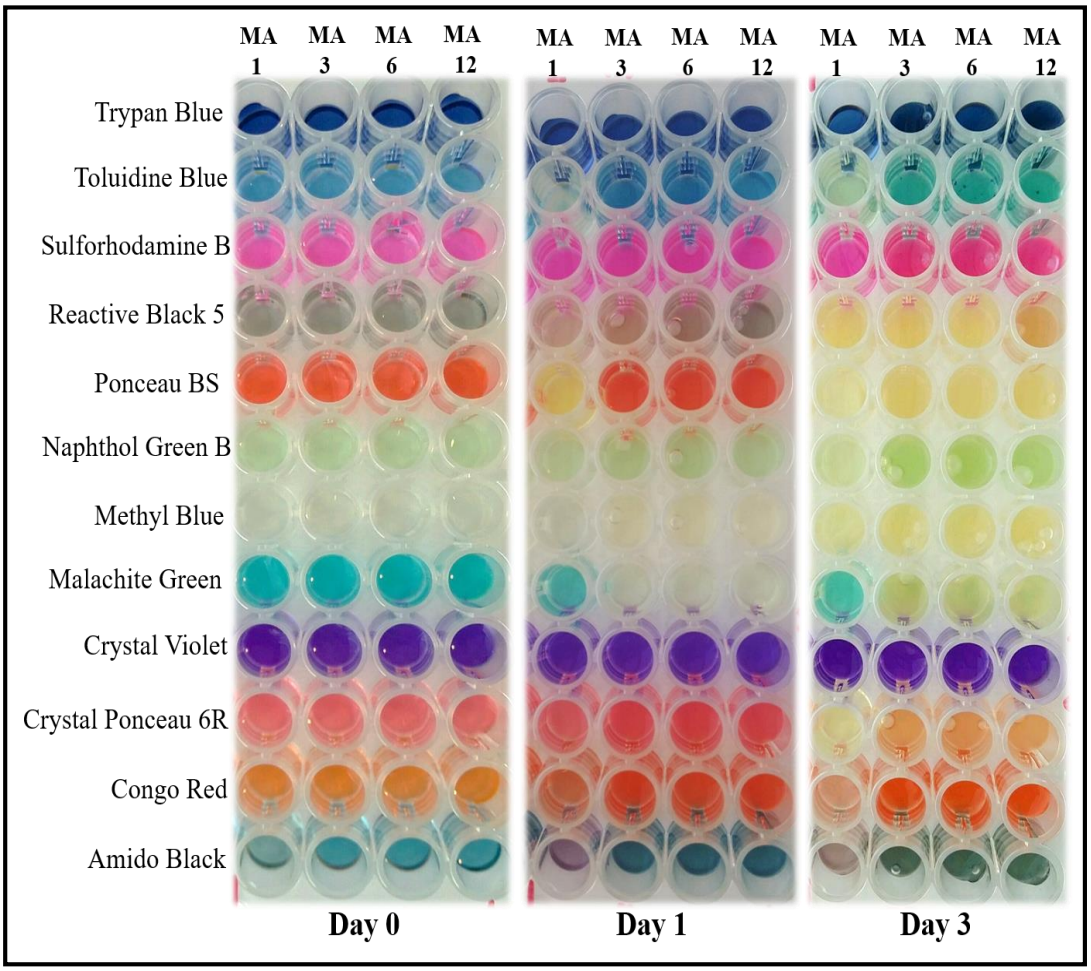


Figure 20: Aerobic degradation of 12 dyes by four bacterial strains

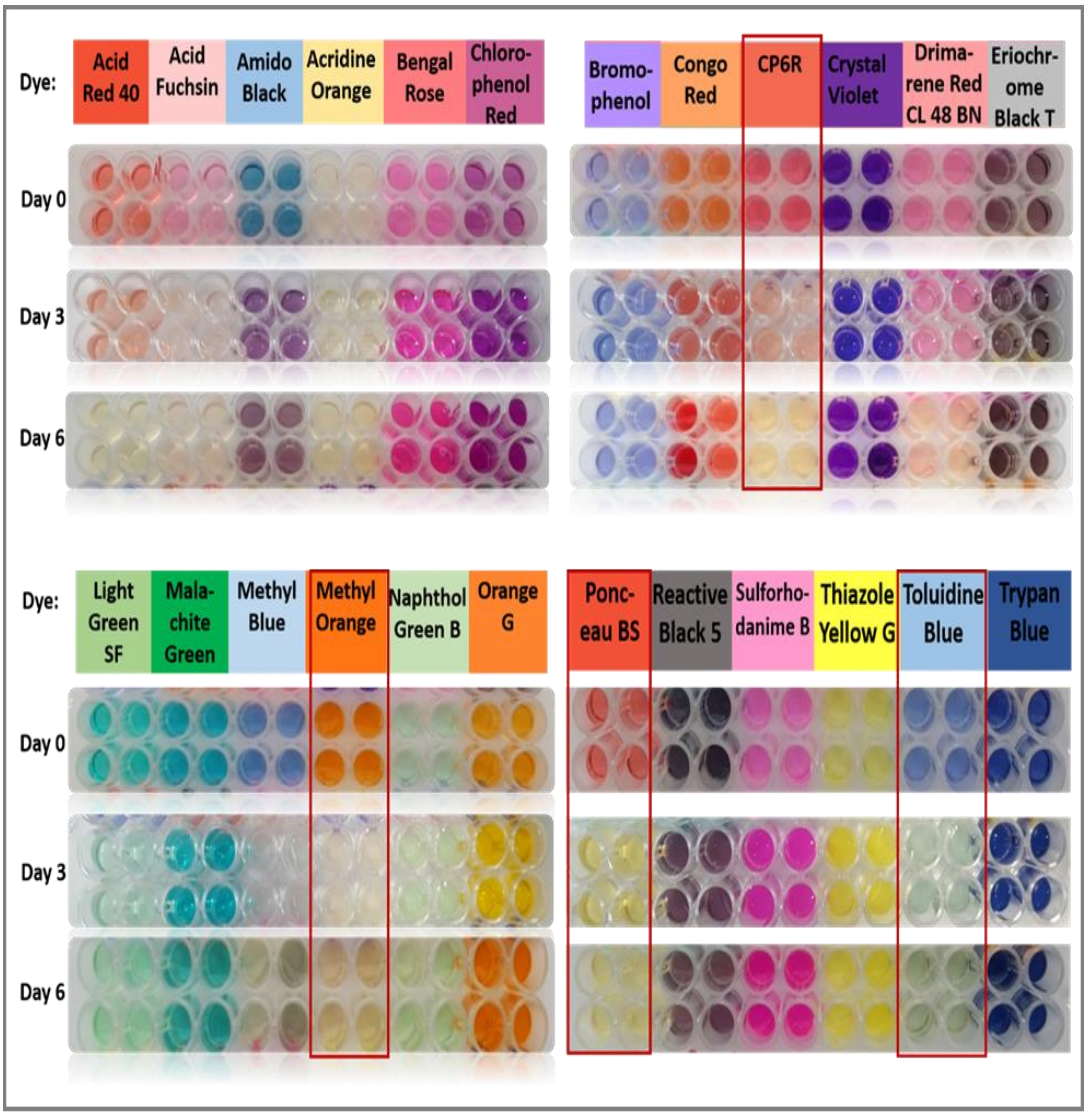


Figure 21: Aerobic degradation of 24 dyes by MA1 strains

3.3 Effect of physicochemical parameters on the decolorization performance

3.3.1 Effect of aerobic and anaerobic condition

Several studies have showed that bacterial degradation is better under aerobic and shaking conditions (Alhassani et al. 2007). Besides, aeration condition has a tremendous influence on dye decolorization capacity of the bacterial strain (Das et al. 2016). In order to examine this on the isolate (MA1), a study was done with the selected 4 dyes, which showed different profiles and were efficiently degraded by MA1 strain, under aerobic and anaerobic conditions. After incubating the dye in the culture medium for 48 hrs, MA1 showed >75% decolorization of Methyl Orange and Ponceau BS dyes at anaerobic condition which was similar (> 82%) under aerobic environment (Figure 22 & 23). Interestingly, Toluidine Blue showed much better degradation under aerobic condition (67 %), while it reduced to 4.5% under anaerobic environment. Taken together, it appears that these three dyes were more efficiently degraded under aerobic conditions when the contents were agitated as compared to anaerobic (static) condition. This indicates that when the contents are shaken, the dissolved oxygen content in the sample increased thereby increasing the % degradation of the dye solution.

On the other hand, for Crystal Ponceau 6R (CP6R), it appeared that this dye was better degraded by MA1 under anaerobic conditions (~ 56%) than aerobic conditions (~ 25%). This is similar to some published bacterial decolorization studies, where maximum decolorization was obtained under anaerobic condition (Chen, Chang, & Liu, 2015). This result implies that the isolated bacterial strain (MA1) was also able to grow in the lack of oxygen and that some dyes and compounds (like CP6R dye) could be better degraded under anaerobic conditions. It is known that under

anaerobic conditions azo dyes can act as electron acceptors and take electrons from the reduced electron carriers like NADH, quinones etc. and can get reduced. However, the reduced products of azo dyes are normally aromatic amines, which can be quite toxic. However, if the extra cellular environment is aerobic, the presence of oxygen may inhibit the azo dye reduction mechanism, due to the oxidation of the reduced redox mediator by oxygen rather than by the azo dye (Pearce 2003b). Therefore, the possible reason for reduced decolorization at shaking condition might be the competition between oxygen and the azo compounds for the reduced electron carriers under aerobic environment (Chang et al. 2001; Zee & Villaverde 2005).

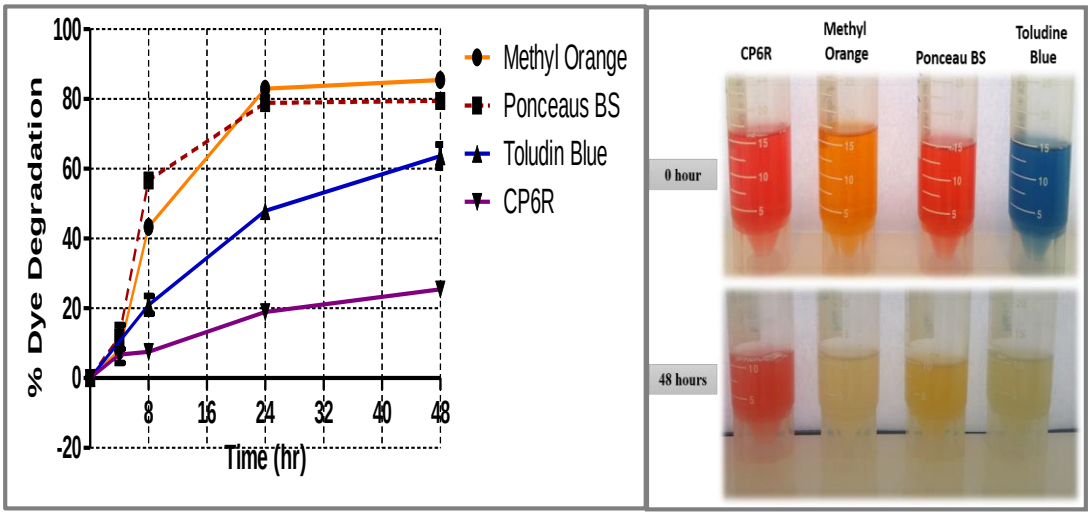


Figure 22: Aerobic degradation of 6 dyes by MA1 strains

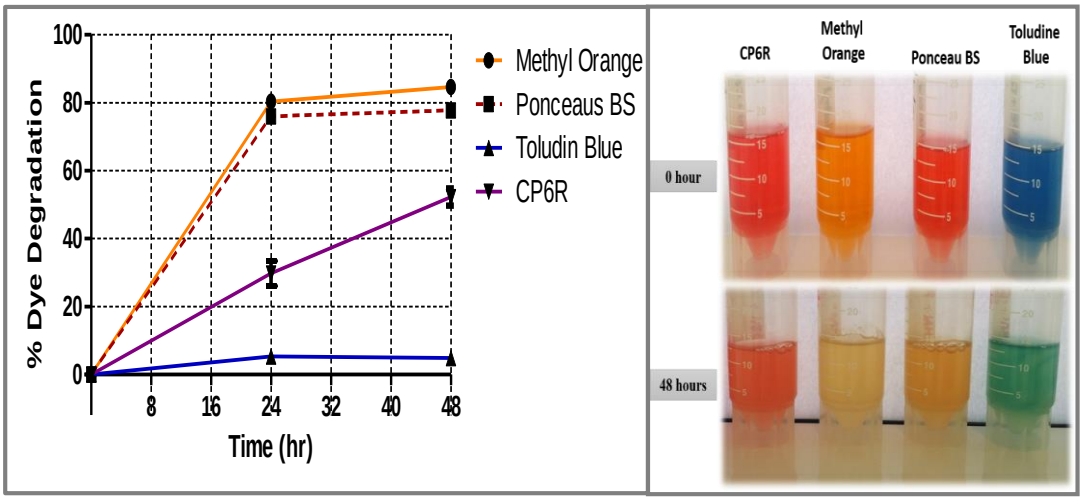


Figure 23: Anaerobic degradation of 6 dyes by MA1 strains

3.3.2 Effect of dye concentration

Organic pollutant concentration is another factor that play a significant role in bacterial-based degradation. In addition, it's important to find if the bacterial isolate is capable of decolorizing a relatively high concentration of dyes or other organic pollutants (which would normally be present in industrial effluents). Decolorization studies with different concentration ranging of Ponceau BS from 20 ppm to 1000 ppm was carried out under aerobic conditions (Figure 24). At initial dye concentrations of 20, 100, and 200 ppm, % of degradation were shown to be 79%, 88.5% and 72.3% respectively, within 24 hrs of incubation. As the dye concentration was increased to 400 ppm, 45.8 % decolorization was achieved after 24 hrs of incubation which increased to 87.7% after 48 hrs of incubation. At a subsequently increased dye concentration of 800 ppm, 62% decolorization was obtained after 48 hrs. Impressively, even at a Ponceau BS concentration of 1000 ppm (1 mg/L) *Bacillus cereus* (MA1) strain was able to degrade 50% of the dye by 48 hours. This clearly illustrates that, as the concentration of dye increases, the bacterial strain would take longer time for decolorization. Similar results have been stated in literatures where a reduction in the efficiency of degradation was detected with increase in dye concentration (Chang et al 2001; Shah et al 2013). As the dye concentration increase a toxic effect of dye and its metabolites became dominant, causing an inhibition in degradation. This inhibition can be explained by the toxic effect of dye and insufficient amount of biomass to uptake this higher concentration of dye and the capability of the enzyme to distinguish the substrate effectively at the very low concentrations (Jadhav et al 2007; Pearce et al 2003).

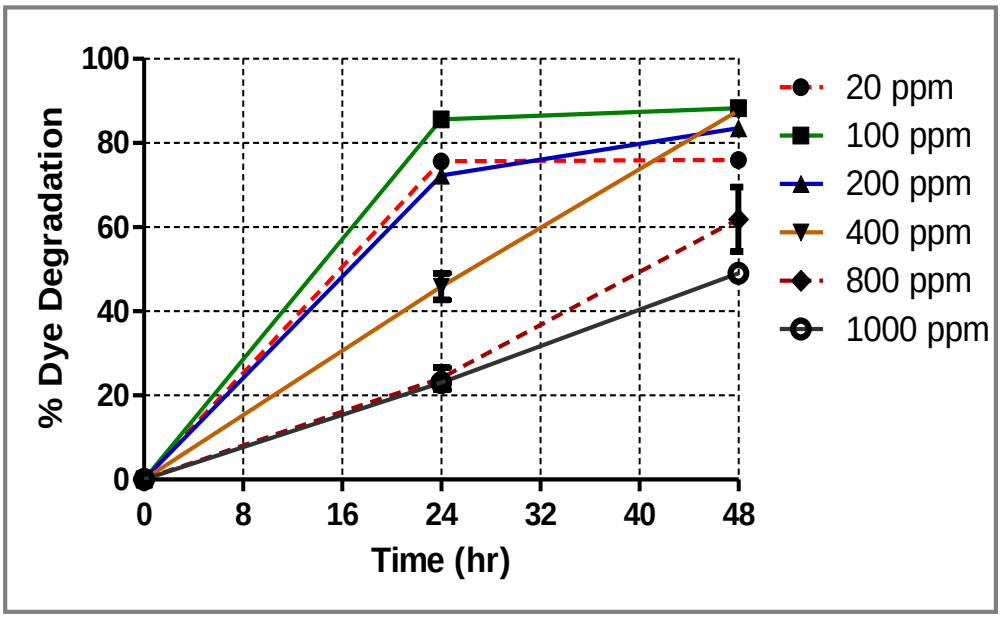


Figure 24: Effect of dye concentration on % decolorization of Ponceau BS by *Bacillus cereus*

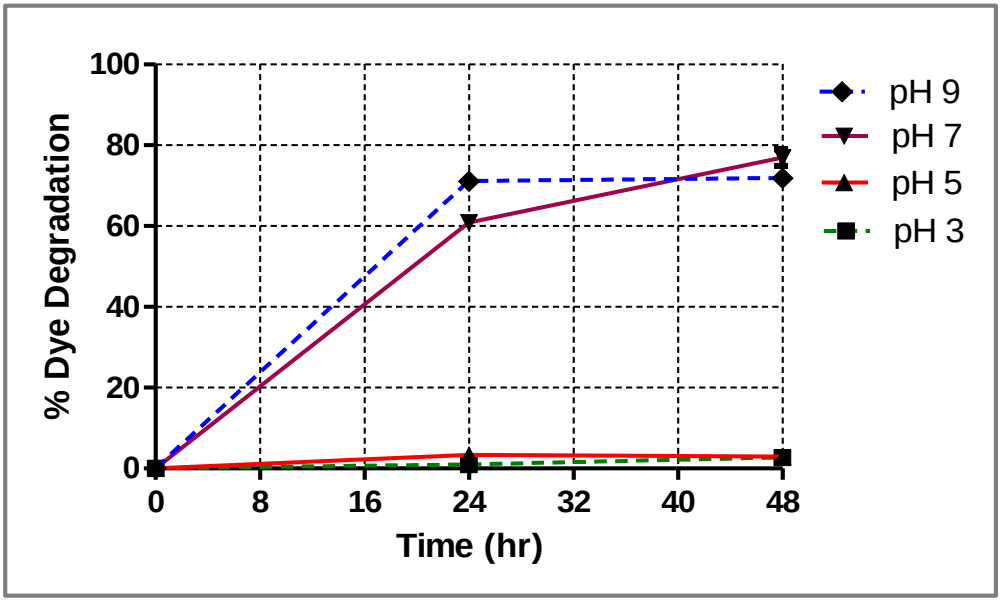


Figure 25: Effect of pH on % decolorization of Ponceau BS dye by *Bacillus cereus*

3.3.3 Effect of pH

The bacterial growth relies on the pH of culture media, which has been previously shown to directly affect dye decoloration (Walters et al 2005). Thus, the ability of *Bacillus cereus* (MA1) strain to degrade Ponceau BS at different range of pH 3, 5 and 9 (control was pH 7) was carried out. The isolated strain MA1 showed similar efficient decolorization efficiency at pH 7 and at pH 9. A maximum decolorization of around 70% was achieved under both pH values 7 and 9) within 48 hours of incubation (Figure 25). The % of decolorization was found to decrease dramatically at pH 5 and pH 3 with 2.28% and 2.39% respectively. The results showed no significant amount of decolorization in acidic media (pH 3 and 5) is most probably due to the inability of bacteria to grow sufficiently in highly acidic or alkaline media. Previously published studies have been found that optimum pH range of textile dye decolorization generally lies between 6.0 and 10.0 (Chen et al 2003; Guo et al 2007; Kılıç et al 2007). A similar study was depicted for the removal of various dyes (e.g. red, green, black, yellow, and mixed dyes), where the researchers found that *Bacillus cereus* was capable of decolorizing dyes within a broad range of pH 6 - 8.5 and optimum pH was obtained at pH 7.5 (Mahmood et al. 2015).

3.3.4 Effect of nutrients

Azo dyes may have poor carbon sources, hence to biodegrade such aromatic dyes, pure culture with supplement of nitrogen or carbon sources are required (Alhassani, 2014). Figure 26 shows the effect of various supplements (9 different culture media) on the decolorization of Ponceau BS. Although the *Bacillus cereus* strain was able to decolorize PBS in all media (except in water and NaCl), maximum decolorization was achieved with Lysogeny Broth (LB). Nutrient Broth-E (NB-E)

gave almost similar results as LB. Consecutively, Nutrient Broth-2 (NB-2), peptone and yeast extract showed a high and similar decolorization value of 75%, within 24 hrs. According to other reports, the best bacterial decolorization was obtained with the yeast extract. Yeast extract metabolism is important for regeneration of NADH, which is the electron donor for azo bond reduction. Moreover, it's been noticed that effectiveness of decolorization increased with increasing the concentration of yeast extract (from 1 to 5 g l^{-1}) (Asad et al. 2007).Whereas, tryptone and 2XYT displayed a moderate decolorization value of 66.7% and 64.8% respectively, within 24 hrs. Negligible decolorization was observed for supplements H_2O and NaCl.

3.3.5 Effect of redox mediator (RM)

It has been noticed that some of the organic pollutants cannot be readily degraded by enzymatic mechanism alone, rather, an additional diffusible, readily and small oxidizable molecules which namely redox mediators are needed. These molecules consider as "middle-men" in enzyme-based degradation of organic compounds (Zee & Cervantes 2009). As a result, we have decided to study this idea in the bacterial-based degradation. However, as can be seen in Figure 27, the addition of 1-hydroxybenzotrizole (HOBT) to the reaction mixture showed no significant difference or rapid degradation between control (no HOBT) and the mixture with HOBT. Interestingly, the reaction proceeded very rapidly without any need for redox mediator, actually, addition of HOBT resulted a slight reduction in the reaction rate. Apparently, this might occurs as a result of the competition between redox mediators, which seems also to act as substrates for bacterial enzymes, and dye molecules, hence leading to a lower degradation rate. These results and other previous studied showed that redox mediators don't play a key role for bacterial-based degradation reactions.

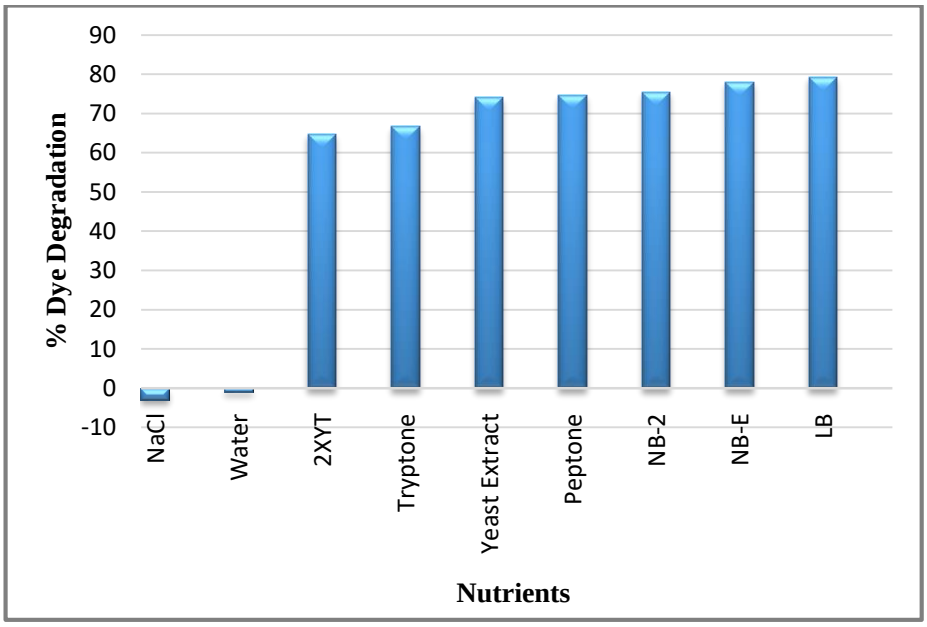


Figure 26: Effect of nitrogen source on the ability of bacterial isolate *Bacillus cereus* to degrade PBS dye

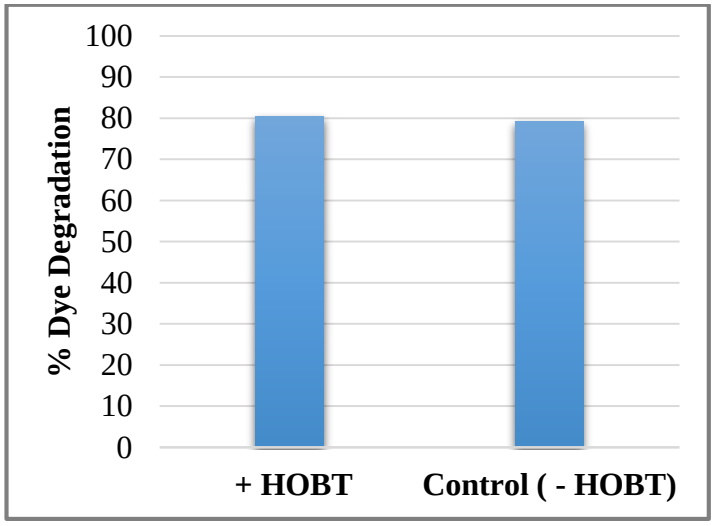


Figure 27: Effect of HOBT on the ability of bacterial stain *Bacillus cereus* (MA1) to degrade PBS dye

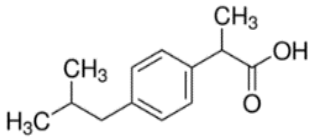
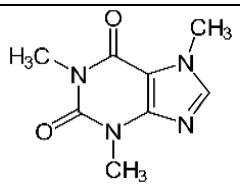
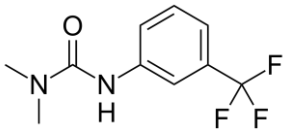
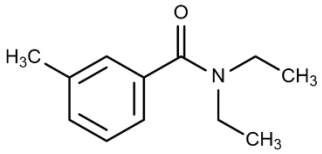
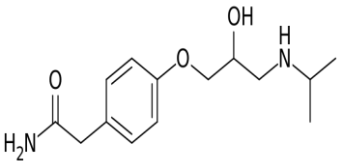
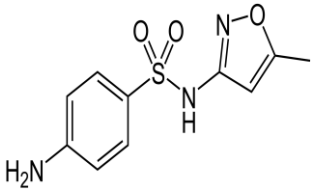
3.4 Degradation of emerging pollutants

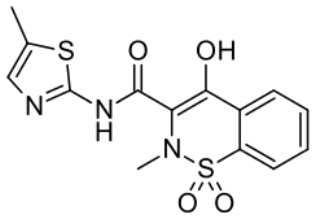
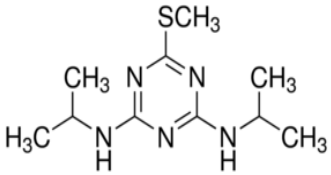
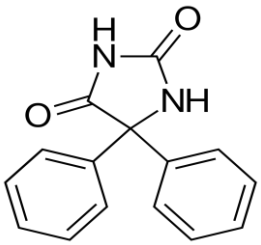
As mentioned earlier, one of the main objectives of the present work was to isolate novel bacterial isolates capable of degrading various classes of organic pollutants, including dyes and emerging pollutants. The preceding section showed that MA1 (and to a lesser extent MA3, MA6 and MA12) was capable of very efficiently degrading various classes of aromatic dyes, and therefore we wanted to next test the ability of this strain (MA1) to degrade a range of emerging pollutants, also known as contaminants of emerging concern in the environment (Table 7).

3.4.1 LC-MS/MS Method development

In order to examine the ability of our bacterial isolates to degrade these emerging pollutants, we first had to develop a sensitive and robust LC-MSMS based assay to quantitate these compounds. Table 7 shows the nine emerging pollutants that were chosen for this part of the work. As can be seen these pollutants include antibiotics, anti-inflammatory agents, pesticides, as well as other pharmaceuticals.

Table 7: Summary of the selected emerging contaminates and their detection in wastewater, surface water, and drinking water

Emerging Pollutant	Structure	Concentration	Reference
1. Ibuprofen **(Anti-inflammatory)**		12000 ng/L	(Lapworth et al. 2012)
2. Caffeine **(Stimulant)**		6.0 µg/L	(Vanderford & Snyder 2006)
3. Fluometuron **(Pesticides)**		317.604 µg/L	(Papadakis et al. 2015)
4. DEET **(Insect repellent)**		6500 ng/L	(Lapworth et al. 2012)
5. Atenolol **(Pharmaceutical: Beta blocker)**		900 ng/L	(Huerta-fontela et al. 2011)
6. Sulfamethoxazole **(Antibiotic)**		1.9 µg/L	(Vanderford & Snyder 2006)

7. Meloxicam **(Anti-inflammatory)**		218 ng/L	(Dasenaki & Thomaidis 2015)
8. Prometryn **(Pesticide)**		0.483 µg/L	(Papadakis et al. 2015)
9. Phenytoin **(Pharmaceutical: Psychiatrics)**		140 ng/L	(Huerta-fontela et al. 2011)

The overall scheme that was employed for the LC-MSMS method development is summarized in Figure 28, which shows the results we obtained for caffeine. Basically, analytical grade standards of these 9 emerging pollutants were used to prepare stocks, which were then analyzed in the MS2 mode of the LC-MSMS as described under Materials and Methods. The MS2 mode shows the "total ion chromatogram" for compound (showing a major peak around 14 minutes retention time), from which an "extracted ion chromatogram" was generated, which conformed the expected molecular mass of the compound (195 Da for caffeine). After this step, the "daughter ions" of this compound were generated by successively increasing the collision energy (volts) in the MS, and looking at the fragments (daughter ions) being generated. As can be seen in Fig. 28, gradually increasing the collision energy from 0V to 30V led to the generation of more and more daughter ions, with 30V producing the most number of daughter ions and completely breaking down all of the caffeine peak (195 m/z). The validity of daughter ions generated in the MSMS was confirmed manually by using Chem Office software to analyze all possible fragments of the compound (as shown in Fig. 28). Lastly, the daughter ion which had the highest intensity was used to generate "parent to daughter" ion pairs (195 → 138, in the case of caffeine), and this was then used to analyze the samples in the MRM (Multiple Reaction Monitoring) mode of the LC-MSMS, which produced a very specific, accurate and sensitive peak for the emerging pollutants. Table 8 shows the summary of the MRM method development for all the 9 emerging pollutants, which allowed for simultaneous and sensitive detection and analysis of these 9 emerging pollutants in a mixture within 30 minutes (Figure 28, "control" panel).

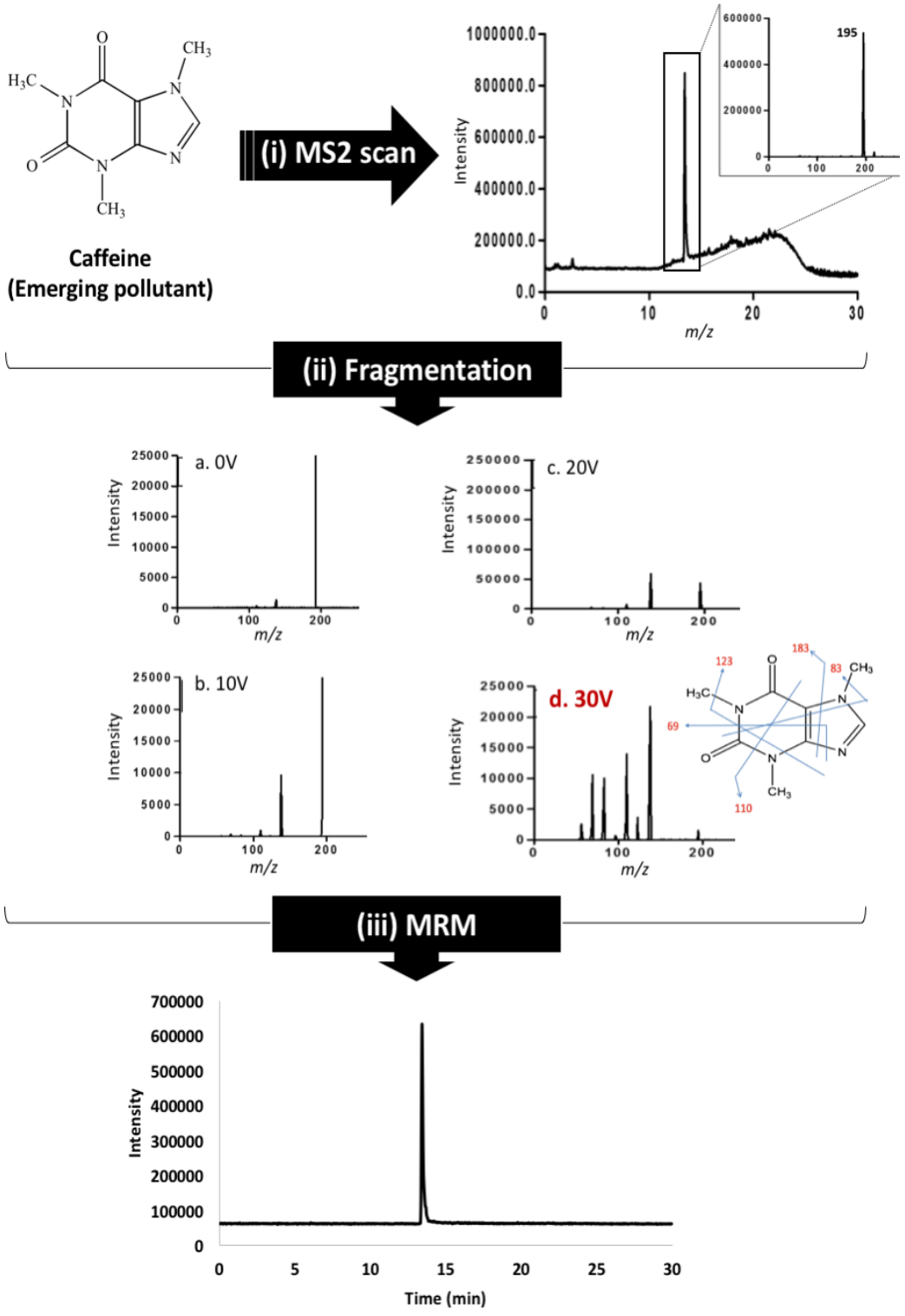


Figure 28: Schematic diagram of LC-MS method development of Caffeine

Table 8: Summary of the MRM mode for emerging pollutants

Emerging Pollutant	**Parent ion (m/z)**	**Daughter ion (m/z)**	**Polarity**	**Collision Energy (V)**
1. Ibuprofen	207	161	Positive	20
2. Caffeine	195	138	Positive	30
3. Fluometuron	233	72	Positive	30
4. DEET	192	119	Positive	30
5. Atenolol	267	190	Positive	20
6. Sulfamethoxazole	254	156	Positive	20
7. Meloxicam	352	115	Positive	6
8. Prometryn	242	158	Positive	30
9. Phenytoin	253	182	Positive	10

Incubation of this mixture of 9 emerging pollutants with MA1 bacterial isolate resulted significant decrease in the peak intensity of some of the emerging pollutants. As can be seen in Figure 29, MA1 appears to be able to significantly degrade Ibuprofen, Meloxicam, as well as Prometryn, as early as 3 days, and most pronounced by day 6. Interestingly, other emerging pollutants appeared not be degraded by MA1. Since, we only saw 3 of the 9 emerging pollutants degraded by our chosen bacterial isolate (MA1), we decided to test the other 3 "best dye-degrading" bacterial isolates as well, namely MA3, MA6 and MA12.

Figures 30, 31 and 32 show the results of emerging pollutants degradation by MA3, MA6, and MA12, respectively. It is interesting that a similar selective degradation of some (and not ALL) emerging pollutants can be seen in these chromatograms.

The results of these analyses are summarized in Table 9, which also shows (by gray shading) the emerging pollutants that showed > 75% degradation by any of the bacterial isolates. The cut-off of >75% degradation was chosen to account for significant error (~ 20%) in the emerging pollutant quantitation, due to the nature of the experimental setup. It is interesting to note the 4 bacterial isolates showed differential degradation abilities, with MA12 showing the most interesting and significant emerging pollutant degradation profile, as summarized below:

MA1: Able to degrade Meloxicam, Prometryn, and Ibuprofen.

MA3: Able to degrade Meloxicam, Atenolol, Sulfamethoxazole, Prometryn and Caffeine.

MA6: Able to degrade Meloxicam, Sulfamethoxazole, Prometryn and Caffeine.

MA12: Able to degrade Meloxicam, Sulfamethoxazole, Phenytoin, Prometryn, Fluometuron, and Caffeine.

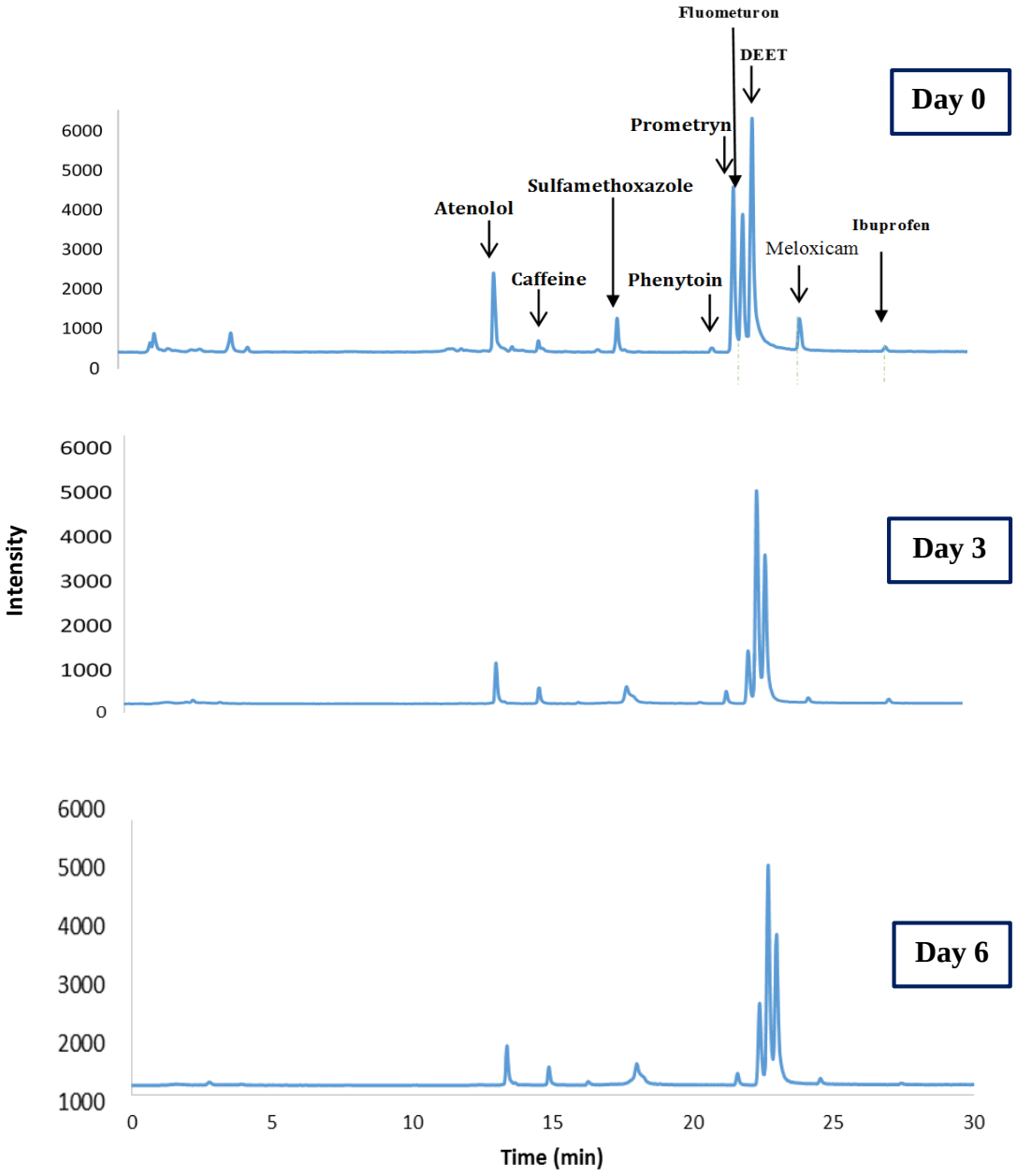


Figure 29: LC-MS chromatograms showing the degradation of various emerging pollutants by *Bacillus cereus* (MA1)

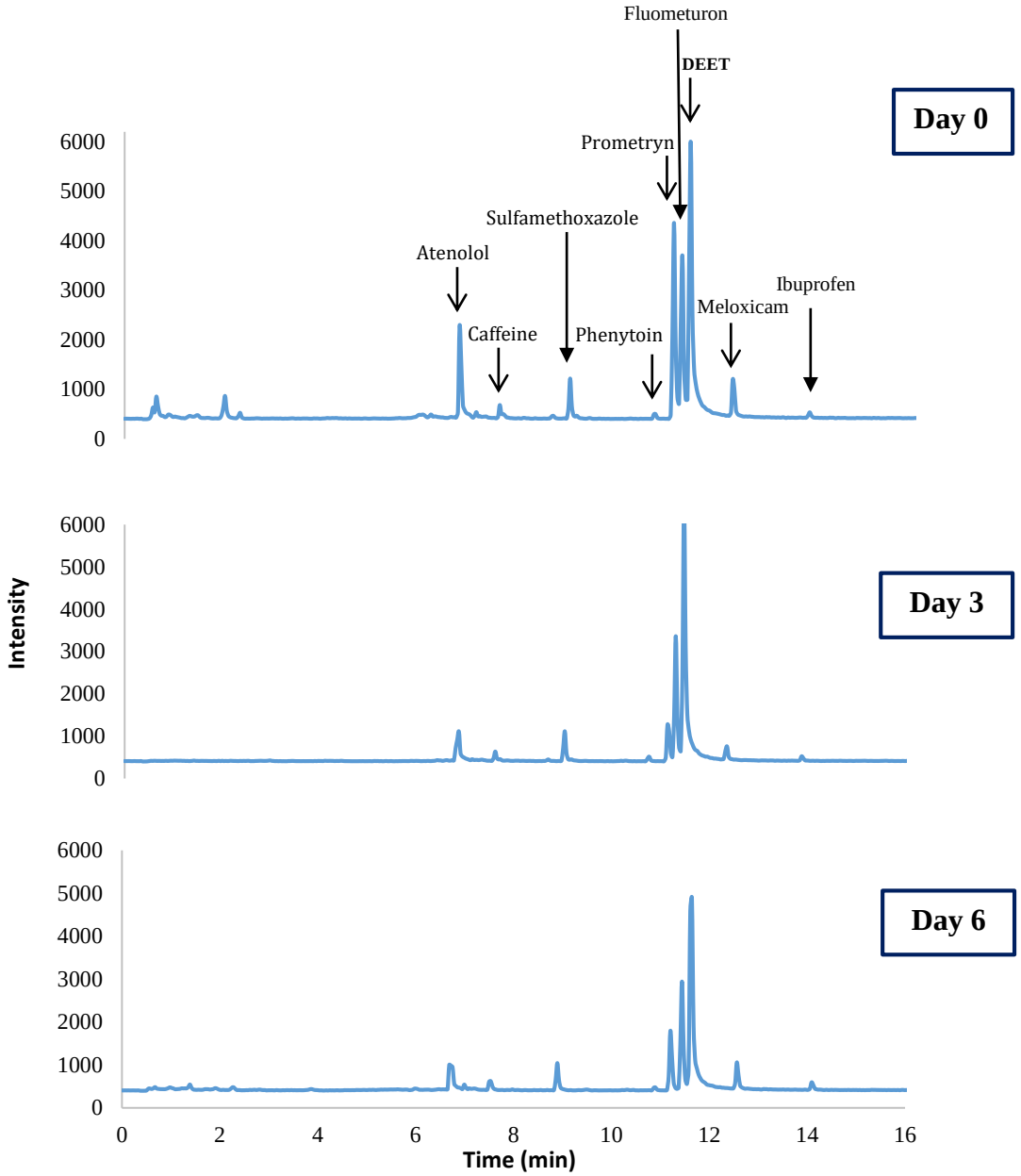


Figure 30: LC-MS chromatograms showing the degradation of various emerging pollutants by *Pseudomonas guariconensis* (MA3)

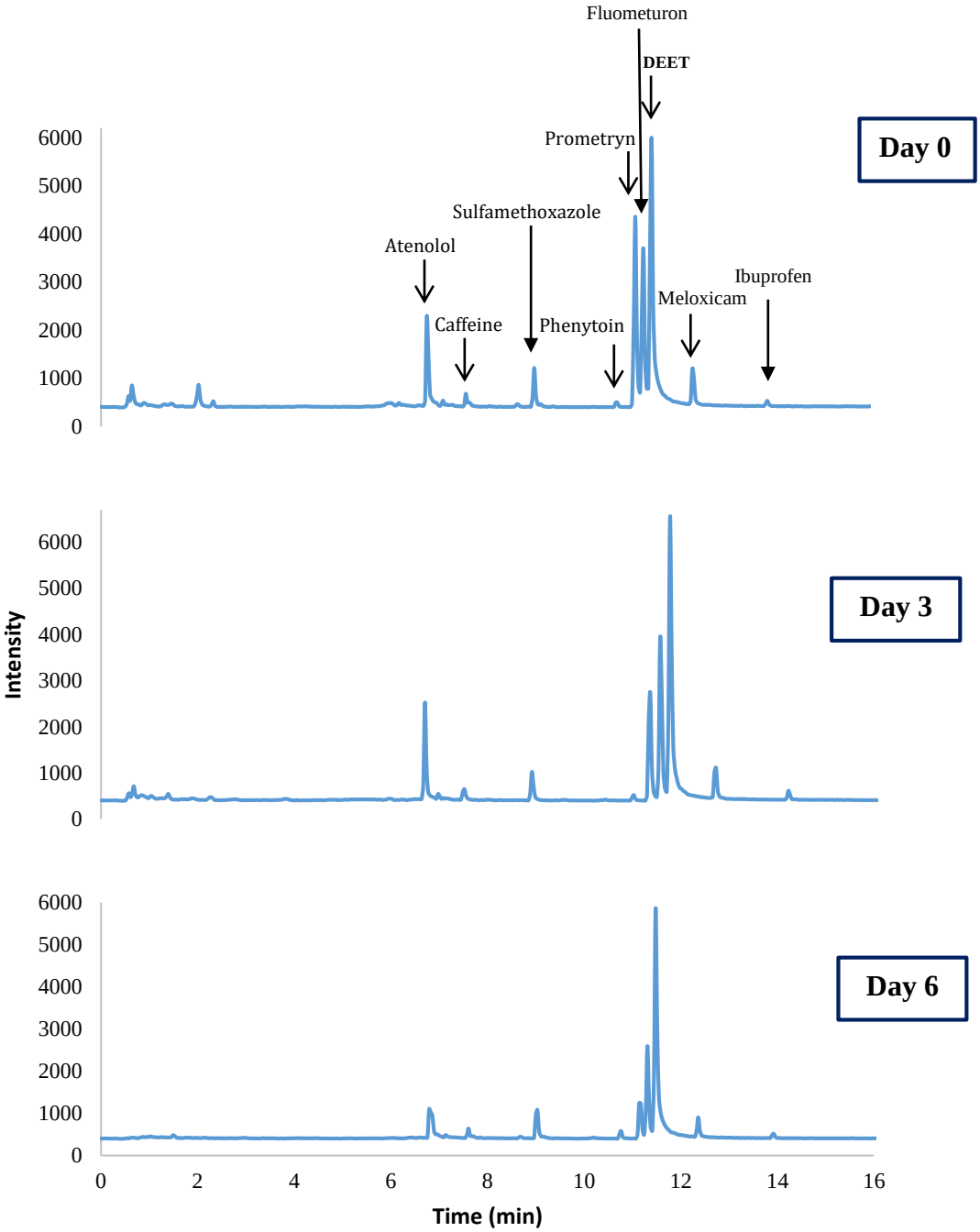


Figure 31: LC-MS chromatograms showing the degradation of various emerging pollutants by *Pseudomonas guariconensis* (MA6)

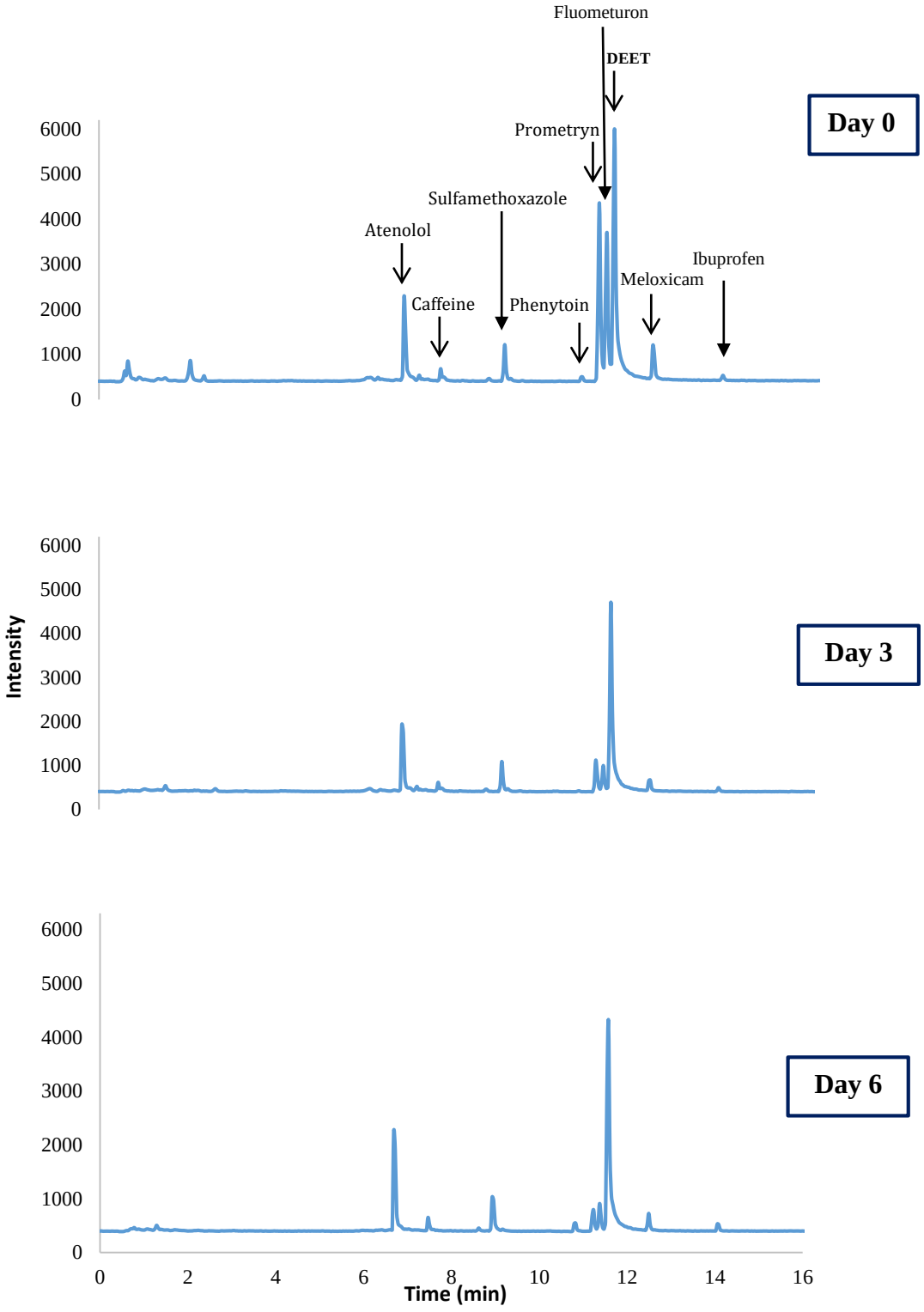


Figure 32: LC-MS chromatograms showing the degradation of various emerging pollutants by *Pseudomonas guariconensis* (MA12)

Table 9: Summary of emerging pollutant degradation (% compound remaining relative to control) by bacterial isolates MA1, MA3, MA6, and MA12

	MA 1			MA 3			MA 6			MA 12		
Emerging Pollutant	Day 1	Day 3	Day 6	Day 1	Day 3	Day 6	Day 1	Day 3	Day 6	Day 1	Day 3	Day 6
Meloxicam	100	95	50	96	88	47	84	69	48	54	49	40
Atenolol	99	93	84	94	65	56	97	96	92	96	96	92
Sulfamethoxazole	83	81	79	89	79	73	84	70	67	80	73	68
Phenytoin	95	93	84	76	67	76	94	78	76	92	94	75
Prometryn	83	70	65	77	62	57	66	65	59	66	63	49
Fluometuron	75	91	88	91	83	80	96	92	77	80	82	63
Ibuprofen	63	63	64	86	82	82	91	88	85	91	89	88
Caffeine	97	85	84	87	73	57	87	63	60	90	63	58
DEET	73	92	88	92	86	82	90	88	87	88	87	78

These results presented here are very interesting and appear to validate our hypothesis that dye-degradation based screening could be used to identify and isolate novel organic pollutant degrading bacteria. Furthermore, it is also interesting to note that some of our bacterial isolates could degrade some emerging pollutants that other isolates could not – for example, of the four isolates MA1 was the only one capable of degrading Ibuprofen and Meloxicam. Conversely, MA1 was not able to degrade Sulfamethoxazole, Phenytoin, Fluometuron, and Caffeine. These results underscore the need to employ not just single bacterial isolates in bioremediation approaches, rather, a consortium of many bacterial species should be used, which hopefully would provide the widest coverage of pollutant degradation ability.

Chapter 4: Conclusion

In summary, the data obtained here showed that that petroleum sludge is a rich source of microbes that could be used to degrade various classes of organic compounds. Our isolated bacterial strains seem to be a versatile group of bacteria that have tremendous potential for organic pollutants degradation and wastewater remediation. However, the bacterial-based pollutant degradation is sensitive to various in physico-chemical operational factors, such as pH, concentration of organic pollutant, bacteria nutrients, as well as aerobic and anaerobic condition, and they must be optimized for efficient degradation. Interestingly, our isolated bacterial strains were able to degrade a wide variety of aromatic dyes (such as Ponceau BS, Toluidine Blue and CP6R). Furthermore, an efficient LC-MS/MS method was developed to detect nine different emerging pollutants, which was further used to investigate their bacterial degradation by 4 isolated bacterial strains. The LC-MS/MS results showed a significant degradation of various emerging pollutants, such as Ibuprofen, Prometryn and Phenytoin, by our isolated bacteria, an interesting finding which needs to be further investigated and exploited.

4.1 Further Studies

The data presented here shows the power and the limitations of bacteria strains in degrading organic pollutants. Though our results are quite significant and very promising, it is obvious that additional research is needed to further enhance our understanding of how these versatile and powerful bacterial strains can be used for efficient environmental and wastewater bioremediation. A few suggestions for further studies in this project are presented below:

1. Targeted genome sequencing for the isolate bacteria strains will be useful to identify the various enzymes (e.g. peroxidases, laccases) that are most likely involved in the degradation of the aromatic pollutants and to come up with metabolic pathways involved in pollutant degradation.

2. Creating a consortium of isolated bacterial strains and testing them for the degradation of these 9 emerging pollutants would be very interest. In a microbial consortium, the individual strains may be able to degrade different organic pollutants and may even utilize metabolites produced by the co-existing strains for further decomposition.

All of these might enhance our understanding of how these and other bacterial strains could be used efficiently for organic pollutants degradation.

References

Aamand, J. et al., 2014. Emerging pollutants in the environment : present and future challenges in biomonitoring , ecological risks and bioremediation. *New Biotechnology*, 00(00), pp.1–10.

Access, O., 2014. Environmental Bioremediation : A Low Cost Nature ' s Natural Biotechnology for Environmental Clean-up. *Petroleum & Environmental Biotechnology*, 5(4), pp.1–12.

Acuner, E. & Dilek, F.B., 2004. Treatment of tectilon yellow 2G by *Chlorella vulgaris*. *Process Biochemistry*, 39(5), pp.623–631.

Adrian, L., 2001. Mechanism of Dye Reduction of Azo Dyes in Anaerobic Mixed Culture. *Journal of Environmental Engineering*, 127(9), pp.844–849.

Ahmed, M., Ngo, H. & Guo, W., 2015. Adsorptive removal of antibiotics from water and wastewater : Progress and challenges. *Science of the Total Environment*, 532, pp.112–126.

Alam, M. et al., 2010. Mutagenicity and genotoxicity of tannery effluents used for irrigation at Kanpur, India. *Ecotoxicology and Environmental Safety*, 73(7), pp.1620–1628.

Alhassani, H.A., Rauf, M.A. & Ashraf, S.S., 2007. Efficient microbial degradation of Toluidine Blue dye by *Brevibacillus sp*. *Dyes and Pigments*, 75(2), pp.395–400.

Ali, L. et al., 2014. Efficient Aerobic Degradation of Various Azo Dyes by a *Sphingomonas sp* Isolated from Petroleum Sludge. *Journal of Bioremediation & Biodegradation*, 05(03).

Ángel, M. et al., 2014. Antimicrobial resistance determinants in antibiotic and biocide- resistant gram-negative bacteria from organic foods. *Food Control*, 37, pp.9–14.

Archna, Lokesh, K.N. & Kiran, S., 2013. Biological methods of dye removal from textile effluents - A review. *Journal of Biochemical Technology*, 3(5), pp.177–180.

Asad, S. et al., 2007. Decolorization of textile azo dyes by newly isolated halophilic and halotolerant bacteria. *Bioresource Technology*, 98(11), pp.2082–2088.

Aubert, S. & Schwitzgue, J., 2004. Screening of plant species for the phytotreatment of wastewater containing sulphonated anthraquinones. *Water Research*, 38(16), pp.3569–3575.

Bae, H., Yamagishi, T. & Suwa, Y., 2017. Evidence for degradation of 2-chlorophenol by enrichment cultures under denitrifying conditions.

Microbiology, 148(1), pp.221–227.

Baird, M.S. et al., 2002. Salicylate 5-Hydroxylase from Ralstonia sp . Strain U2 : a Monooxygenase with Close Relationships to and Shared Electron Transport Proteins with Naphthalene Dioxygenase. *Journal of Bacteriology*, 184(6), pp.1547–1555.

Brillas, E. & Martinez-Huitle, C., 2015. Decontamination of wastewaters containing synthetic organic dyes by electrochemical methods. An updated review. *Applied Catalysis B: Environmental journal*, 166-167, pp.603–643.

Bromley-Challenor, K. et al., 2000. Decolorization of an azo dye by unacclimated activated sludg under anaerobic condition. *Water Research*, 34(18), pp.4410–4418.

Bruggen, B. et al., 2005. Fouling of nanofiltration and ultrafiltration membranes applied for wastewater regeneration in the textile industry. *Desalination*, 175(1), pp.111–119.

Chang, J., 2014. Decolorization and biodegradation of reactive dyes and dye wastewater by a developed bacterial consortium. *Bioresource Technology*, 100(9), pp.2493–2500. Available at: http://dx.doi.org/10.1016/j.biortech.2008.12.013.

Chang, J. et al., 2001. Kinetic characteristics of bacterial azo-dye decolorization by *Pseudomonas luteola*. *Water Research*, 35(12), pp.2841–2850.

Chang, J., Chen, B. & Lin, Y.S., 2004. Stimulation of bacterial decolorization of an azo dye by extracellular metabolites from Escherichia coli strain NO_3. *Bioresource Technology*, 91(3), pp.243–248.

Chang, J. & Kuo, T., 2000. Kinetics of bacterial decolorization of azo dye with Escherichia coli NO3. *Bioresource Technology*, 75(2), pp.107–111.

Chen, B. et al., 2008. Revealing azo-dye decolorization of indigenous Aeromonas hydrophila from fountain spring in Northeast Taiwan Revealing azo-dye decolorization of indigenous Aeromonas hydrophila from fountain spring in Northeast Taiwan. *Journal of the Chinese Institute of Chemical Engineers*, 39(5), pp.495–501.

Chen, C., Chang, C. & Liu, S., 2015. Partial Degradation Mechanisms of Malachite Green and Methyl Violet B by *Shewanella*. *Journal of Hazardous Materials*, 177(1-3), pp.281–289. Available at: http://dx.doi.org/10.1016/j.jhazmat.2009.12.030.

Chen, K. et al., 2017. Decolorization of the Textile Azo Dyes by Newly Isolated Bacterial Strains bacterial strains. *Journal of Biotechnology*, 101(1), pp.57–68.

Cheung, P. & Kinkle, B., 2001. Mycobacterium Diversity and Pyrene Mineralization

in Petroleum-Contaminated Soils Texture. *Applied and Environmental Microbiology*, 67(5), pp.2222–2229.

Ciardelli, G. & Ranieri, N., 2001. The treatment and reuse of wastewater in the textile industry by means of ozonation and electroflocculation. *Water Research*, 35(2), pp.567–572.

Daneshvar, N. et al., 2007. Biological decolorization of dye solution containing Malachite Green by microalgae *Cosmarium sp* . *Bioresource Technology*, 98(6), pp.1176–1182.

Das, A., Mishra, S. & Verma, V.K., 2016. Enhanced biodecolorization of textile dye remazol navy blue using an isolated bacterial strain *Bacillus pumilus* HKG212 under improved culture conditions. *Journal of Biochemical Technology*, 6(3), pp.962–969.

Dasenaki, M. & Thomaidis, N., 2015. Multianalyte method for the determination of pharmaceuticals in wastewater samples using solid-phase extraction and liquid chromatography – tandem mass spectrometry. *Analytical and Bioanalytical Chemistry*, 407(15), pp.4229–4245.

Daughton, C., 2004. Non-regulated water contaminants : emerging research. *Environmental Impact Assessment Review*, 24(7-8), pp.711–732.

Dawkar, V. et al., 2008. Biodegradation of disperse textile dye Brown 3REL by newly isolated *Bacillus sp*. VUS. *Journal of Applied Microbiology*, 105(1), pp.14–24.

Deblonde, T., Cossu-leguille, C. & Hartemann, P., 2015. Emerging pollutants in wastewater : A review of the literature. *International Journal of Hygiene and Environmental Health*, 214(6), pp.442–448.

Devassy, B., Goyal, D. & Khanna, S., 2009. International Biodeterioration & Biodegradation Decolorization of textile azo dyes by aerobic bacterial consortium. *International Biodeterioration & Biodegradation*, 63(4), pp.462–469.

Diez, M., 2010. Biological Aspects Involved In The Degradation Of Organic Pollutants. *Journal of soil science and plant nutrition*, 10(3), pp.244–267.

Drillia, P. et al., 2005. On the occasional biodegradation of pharmaceuticals in the activated sludge process : The example of the antibiotic sulfamethoxazole. *Journal of Hazardous Materials*, 122(3), pp.259–265.

Farré, M. et al., 2012. Achievements and future trends in the analysis of emerging organic contaminants in environmental samples by mass spectrometry and bioanalytical techniques. *Journal of Chromatography A*, 1259, pp.86–99.

Fournier, D. et al., 2004. Biodegradation of Octahydro-1,3,5,7- tetranitro-1,3,5,7-

tetrazocine (HMX) by Phanerochaete chrysosporium : New Insight into the Degradation Pathway. *Environmental Science & Technology*, 38(15), pp.4130–4133.

Franciscon, E. et al., 2012. Decolorization and biodegradation of reactive sulfonated azo dyes by a newly isolated *Brevibacterium sp*. strain VN-15. *SpringerPlus*, 1(1), p.37.

Fu, Y. & Viraraghavan, T., 2001. Fungal decolorization of dye wastewaters : a review. *Bioresource Technology*, 79(3), pp.251–262.

Furgal, K., Meyer, R. & Bester, K., 2014. Removing selected steroid hormones, biocides and pharmaceuticals from water by means of biogenic manganese oxide nanoparticles in situ at ppb levels. *Chemosphere*, 136, pp.321–326.

Gartiser, S. et al., 2007. Ultimate biodegradation and elimination of antibiotics in inherent tests. *Chemosphere*, 67(3), pp.604–613.

Gautami, G. & Khanam, S., 2012. Selection of optimum configuration for multiple effect evaporator system. *Desalination*, 288, pp.16–23.

Ghosh, S., Pal, S. & Ray, S., 2013. Study of microbes having potentiality for biodegradation of plastics. *Environmental Science and Pollution Research*, 20(7), pp.4339–4355.

Gu, Y. et al., 2016. Quantification of the water , energy and carbon footprints of wastewater treatment plants in China considering a water – energy nexus perspective. *Ecological Indicators*, 60, pp.402–409.

Guo, J. et al., 2007. Biocalalyst effects of immobilized anthraquinone on the anaerobic reduction of azo dyes by the salt-tolerant bacteria. *Water Research*, 41(2), pp.426–432.

Gupta, V. et al., 2006. Biosorption of copper (II) from aqueous solutions by *Spirogyra* species. *Journal of Colloid and Interface Science*, 296(1), pp.59–63.

Huerta-fontela, M., Galceran, M. & Ventura, F., 2011. Occurrence and Removal of Pharmaceuticals and Hormones Through Drinking Water Treatment. *Water Research*, 45(3), pp.1432–1442.

Işik, M. & Sponza, D.T., 2003. Effect of oxygen on decolorization of azo dyes by *Escherichia coli* and *Pseudomonas sp*. and fate of aromatic amines. *Process Biochemistry*, 38(8), pp.1183–1192.

Jadhav, J.. et al., 2007. Decolourization of azo dye methyl red by *Saccharomyces cerevisiae* MTCC 463. *Chemosphere*, 68(2), pp.394–400.

Jadhav, S. et al., 2008. Decolorization of Brilliant Blue G dye mediated by degradation of the microbial consortium of *Galactomyces geotrichum* and

Bacillus sp . Journal of the Chinese Institute of Chemical Engineers, 39(6), pp.563–570.

Joshi, S. et al., 2010. International Biodeterioration & Biodegradation Exploring the potential of natural bacterial consortium to degrade mixture of dyes and textile effluent. *International Biodeterioration & Biodegradation*, 64(7), pp.622–628.

Jux, U. et al., 2002. Short communication Detection of pharmaceutical contaminations of river, pond, and tap water from Cologne (Germany) and surroundings. *International Journal of Hygiene and Environmental Health*, 205(5), pp.393–398.

Kagalkar, A. et al., 2009. Bioresource Technology Biotechnological strategies for phytoremediation of the sulfonated azo dye Direct Red 5B using *Blumea malcolmii* Hook. *Bioresource Technology*, 100(18), pp.4104–4110.

Kalyani, D. et al., 2009. Ecofriendly biodegradation and detoxification of Reactive Red 2 textile dye by newly isolated *Pseudomonas sp*. SUK1. *Journal of Hazardous Materials*, 163(2-3), pp.735–742.

Karcher, S., Kornmu, A. & Jekel, M., 2001. Screening of commercial sorbents for the removal of reactive dyes. *Dyes and Pigments*, 51(2-3), pp.111–125.

Karigar, C. & Rao, S., 2011. Role of Microbial Enzymes in the Bioremediation of Pollutants : A Review. *Enzyme Research*, p.11.

Karthikeyan, K. & Meyer, M.T., 2006. Occurrence of antibiotics in wastewater treatment facilities in Wisconsin , USA. *Science of the Total Environment*, 361(1-3), pp.196–207.

Kaushik, P. & Malik, A., 2009. Fungal dye decolourization : Recent advances and future potential. *Environment International*, 35(1), pp.127–141.

Khehra, M. et al., 2006. Biodegradation of azo dye C . I . Acid Red 88 by an anoxic-aerobic sequential bioreactor. *Dyes and Pigments*, 70(1), pp.1–7.

Khehra, M. et al., 2005. Comparative studies on potential of consortium and constituent pure bacterial isolates to decolorize azo dyes. *Water Research*, 39(20), pp.5135–5141.

Khodjaniyazov, K. et al., 2012. Degradation and Detoxification of Persistent Organic Pollutants in Soils by Plant Alkaloid Anabasine. *Journal of Environmental Protection*, 03(01), pp.97–106.

Kiai, H. et al., 2014. Application of membrane distillation technology in the treatment of table olive wastewaters for phenolic compounds concentration and high quality water production. *Chemical Engineering and Processing : Process Intensification*, 86, pp.153–161.

Kilic, N. et al., 2007. Characterization of a Simple Bacterial Consortium for Effective Treatment of Wastewaters with Reactive Dyes and Cr(VI). *Chemosphere*, 67, pp.826–831.

Kolpin, D. et al., 2002. Pharmaceuticals, Hormones, and Other Organic Wastewater Contaminants in U . S . Streams, 1999-2000 : A National Reconnaissance. *Environmental Science & Technology*, 36(6), pp.1202–1211.

Kumar, S., Hatha, A. & Christi, K., 2007. Diversity and effectiveness of tropical mangrove soil microflora on the degradation of polythene carry bags. *International journal of tropical biology*, 55(3-4), pp.777–786.

Kurade, M., Waghmode, T. & Govindwar, S., 2011. Preferential biodegradation of structurally dissimilar dyes from a mixture by *Brevibacillus laterosporus*. *Journal of Hazardous Materials*, 192(3), pp.1746–1755.

Kurola, J., 2006. Microbial activities in boreal soils : Biodegradation of organic contaminants at low temperature and ammonia oxidation. pp. 1-53.

Lapworth, D. et al., 2012. Emerging organic contaminants in groundwater : A review of sources, fate and occurrence. *Environmental Pollution*, 163, pp.287–303.

Lapworth, D. & Gooddy, D., 2006. Source and persistence of pesticides in a semi-confined chalk aquifer of southeast England. *Environmental Pollution*, 144(3), pp.1031–1044.

Loos, R. et al., 2007. Polar herbicides, pharmaceutical products, perfluorooctanesulfonate (PFOS), perfluorooctanoate (PFOA), and nonylphenol and its carboxylates and ethoxylates in surface and tap waters around Lake Maggiore in Northern Italy. *Analytical and Bioanalytical Chemistry*, 387(4), pp.1469–1478.

Lucas, M. et al., 2006. Biodegradation of the diazo dye Reactive Black 5 by a wild isolate of Candida oleophila Biodegradation of the diazo dye Reactive Black 5 by a wild isolate of *Candida oleophila*. *Enzyme and Microbial Technology*, 39(1), pp.51–55.

Machado, K. et al., 2006. Biodegradation of reactive textile dyes by basidiomycetous fungi from brazilian ecosystems. *Brazilian Journal of Microbiology*, 37(4), pp.481–487.

Mahmood, R. et al., 2015. Enhancing the Decolorizing and Degradation Ability of Bacterial Consortium Isolated from Textile Effluent Affected Area and Its Application on Seed Germination. *The Scientific World Journal*, 2015, pp.1– 9.

Martins, L. et al., 2015. Application of cellulose-immobilized riboflavin as a redox mediator for anaerobic degradation of a model azo dye Remazol Golden Yellow RNL. *Industrial Crops & Products*, 65, pp.454–462.

Mollea, C., Bosco, F. & Ruggeri, B., 2005. Fungal biodegradation of naphthalene : microcosms studies. *Chemosphere*, 60(5), pp.636–643.

Na, G. et al., 2014. Sulfonamide antibiotics in the Northern Yellow Sea are related to resistant bacteria : Implications for antibiotic resistance genes. *Marine Pollution Bulletin*, 84(1-2), pp.70–75.

Nnenna, F., Lekiah, P. & Obemeata, O., 2011. Degradation of antibiotics by bacteria and fungi from the aquatic environment. *Journal of Toxicology and Environmental Health Sciences*, 3(10), pp.275–285.

Pal, A. et al., 2010. Impacts of emerging organic contaminants on freshwater resources : Review of recent occurrences, sources, fate and effects. *Science of the Total Environment*, 408(24), pp.6062–6069.

Pandey, A., Singh, P. & Iyengar, L., 2007. Bacterial decolorization and degradation of azo dyes. *International Biodeterioration and Biodegradation*, 59(2), pp.73–84.

Papadakis, E.-N. et al., 2015. Pesticides in the Surface Waters of Lake Vistonis Basin, Greece : Occurrence and Environmental Risk Assessment. *Science of the Total Environment, The*, 536, pp.793–802.

Pearce, C., 2003a. The Removal of Colour From Textile Wastewater Using Whole Bacterial Cells : A Review. *Dyes and Pigments*, 58(3), pp.179–196.

Pearce, C., 2003b. The removal of colour from textile wastewater using whole bacterial cells: A review. *Dyes and Pigments*, 58(3), pp.179–196.

Petrie, B., Barden, R. & Kasprzyk-Hordern, B., 2014. A review on emerging contaminants in wastewaters and the environment : Current knowledge, understudied areas and recommendations for future monitoring. *Water Research*, 72, pp.3–27.

Pumphrey, G., Madsen, E. & Madsen, P., 2017. Naphthalene metabolism and growth inhibition by naphthalene in *Polaromonas naphthalenivorans* strain CJ2. *journal of Microbiology*, 153, pp.3730–3738.

Rauf, M. & Salman, A., 2012. Survey of recent trends in biochemically assisted degradation of dyes. *Chemical Engineering Journal*, 209, pp.520–530.

Richardson, S. et al., 2007. Occurrence, genotoxicity, and carcinogenicity of regulated and emerging disinfection by-products in drinking water : A review and roadmap for research. *Mutation Research*, 636(1-3), pp.178–242.

Robinson, T. et al., 2001. Remediation of dyes in textile effluent: A critical review on current treatment technologies with a proposed alternative. *Bioresource Technology*, 77(3), pp.247–255.

Rodrigues, D. et al., 2013. Strategies based on silica monoliths for removing pollutants from wastewater effluents : A review. *Science of the Total Environment*, 461-462, pp.126–138.

Rojas, R. et al., 2016. Adsorption study of low-cost and locally available organic substances and a soil to remove pesticides from aqueous solutions and a soil to remove pesticides from aqueous solutions. *Journal of Hydrology*, 520, pp.461–472.

Russ, R., Rau, J. & Stolz, A., 2000. The Function of Cytoplasmic Flavin Reductases in the Reduction of Azo Dyes by Bacteria. *Applied and Environmental Microbiology*, 66(4), pp.1429–1434.

Sadhasivam, S. et al., 2009. Metabolically inactive Trichoderma harzianum mediated adsorption of synthetic dyes : Equilibrium and kinetic studies. *Journal of the Taiwan Institute of Chemical Engineers*, 40, pp.394–402.

Samanta, S., Singh, O. & Jain, R., 2002. Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation. *Trends in Biotechnology*, 20(6), pp.243–248.

Santos, A., Cervantes, F. & Lier, J., 2007. Review paper on current technologies for decolourisation of textile wastewaters: Perspectives for anaerobic biotechnology. *Bioresource Technology*, 98(12), pp.2369–2385.

Saratale, G.D., Kalme, S.D. & Govindwar, S.P., 2006. Decolorisation of textile dyes by *Aspergillus ochraceus* (NCIM-1146). *Indian Journal of Biotechnology*, 5, pp.407–410.

Saratale, R. et al., 2011. Bacterial decolorization and degradation of azo dyes: A review. *Journal of the Taiwan Institute of Chemical Engineers*, 42(1), pp.138–157.

Saratale, R. et al., 2017. Decolorization and biodegradation of textile dye Navy Blue HER by *Trichosporon beigelii* NCIM-3326. *Journal of Hazardous Materials*, 166(2-3), pp.1421–1428.

Saratale, R. et al., 2014. Ecofriendly degradation of sulfonated diazo dye CI Reactive Green 19A using *Micrococcus glutamicus* NCIM-2168. *Bioresource Technology*, 100(17), pp.3897–3905.

Saratale, R. et al., 2009. Enhanced decolorization and biodegradation of textile azo dye Scarlet R by using developed microbial consortium-GR. *Bioresource Technology*, 100(9), pp.2493–2500.

Särkkä, H., Vepsäläinen, M. & Sillanpää, M., 2015. Natural Organic Matter (NOM) Removal by Electrochemical Methods — A review. *Journal of Electroanalytical Chemistry*, 755, pp.100–108.

Schückel, J., Matura, A. & Pée, K. Van, 2011. One-copper laccase-related enzyme from *Marasmius sp.*: Purification, characterization and bleaching of textile dyes. *Enzyme and Microbial Technology*, 48(3), pp.278–284.

Seo, J.-S., Keum, Y.-S. & Li, Q., 2009. Bacterial Degradation of Aromatic Compounds. *International Journal of Environmental Research and Public Health*, 6, pp.278–309.

Shimao, M., 2001. Biodegradation of plastics Masayuki Shimao. *Current Opinion in Biotechnology*, 12(3), pp.242–247.

Solís, M. et al., 2012. Microbial decolouration of azo dyes : A review. *Process Biochemistry*, 47(12), pp.1723–1748.

Sorensen, J. et al., 2015. Emerging contaminants in urban groundwater sources in Africa. *Water Research*, 72, pp.51–63.

Souza, S., Forgiarini, E. & Souza, A., 2007. Toxicity of textile dyes and their degradation by the enzyme horseradish peroxidase (HRP). *Journal of Hazardous Materials*, 147(3), pp.1073–1078.

Stuart, M. et al., 2012. Review of risk from potential emerging contaminants in UK groundwater. *Science of the Total Environment*, 416, pp.1–21.

Telke, A. et al., 2008. Kinetics and Mechanism of Reactive Red 141 Degradation by a Bacterial Isolate *Rhizobium radiobacter* MTCC 8161. *Journal of Basic Microbiology*, 55, pp.320–329.

Tripathi, A. & Srivastava, S., 2011. Ecofriendly Treatment of Azo Dyes: Biodecolorization using Bacterial Strains. *International Journal of Bioscience, Biochemistry and Bioinformatics*, 1(1), pp.37–40.

Vanderford, B. & Snyder, S., 2006. Analysis of Pharmaceuticals in Water by Isotope Dilution Liquid Chromatography/Tandem Mass Spectrometry. *Environmental Science & Technology*, 40(23), pp.7312–7320.

Wang, H. et al., 2015. Bioelectrochemical system platform for sustainable environmental remediation and energy generation. *Biotechnology Advances*, 33(3-4), pp.317–334.

Yan, H. & Pan, G., 2004. Increase in biodegradation of dimethyl phthalate by *Closterium lunula* using inorganic carbon. *Chemosphere*, 55(9), pp.1281–1285.

Zee, F. & Cervantes, F., 2009. Impact and application of electron shuttles on the redox (bio) transformation of contaminants : A review. *Biotechnology Advances*, 27(3), pp.256–277.

Zee, F.P. Van Der & Villaverde, S., 2005. Combined anaerobic – aerobic treatment of azo dyes — A short review of bioreactor studies. , 39, pp.1425–1440.

Zille, A. et al., 2005. Degradation of Azo Dyes by *Trametes villosa* Laccase over Long Periods of Oxidative Conditions. *Applied and Environmental Microbiology*, 71(11), pp.6711–6718.