BIOREMEDIATION OF RDX AND HMX CONTAMINATED SOIL AND SEDIMENTS USING JANIBACTER CREMEUS IMMOBILIZED IN CALCITE AND EGG SHELL BASED BIOFORMULATIONS

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2020

Dedicated
to
My Father

CERTIFICATE

This is to certify that this thesis entitled **"Bioremediation of RDX and HMX contaminated soil and sediments using *Janibacter cremeus* immobilized in calcite and egg shell based bioformulations"** submitted to the Delhi Technological University, Delhi, for the award of the degree of Doctor of Philosophy, is based on the original research work carried out by me under the supervision of **Prof. Jai Gopal Sharma,** Department of Biotechnology, Delhi Technological University, Delhi and co- supervision of **Dr. S. Mary Celin** (Scientist F), Centre for fire, explosive and environment safety (CFEES), Defence Research and Development Organization, Delhi. It is further certified that the work embodied in this thesis has neither partially nor fully been submitted to any other university or institution for the award of any degree or diploma

27/8/20

Anchita Kalsi
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This is to certify that the above statement made by the candidate is correct to the best of our knowledge.

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DECLARATION

I, Anchita Kalsi, certify that the work embodied in this Ph.D. thesis is my own bonafide work carried out under the supervision of **Prof. Jaigopal Sharma,** Department of Biotechnology, Delhi Technological University, Delhi and co-supervision of **Dr. S. Mary Celin** (Scientist F), Centre for fire, explosive and environment safety (CFEES), Defence Research and Development Organization, Delhi for a period of July 2016 to August 2020 at Centre for fire, explosives and environment safety (CFEES), Defence Research and Development Organization, Delhi. The matter embodied in this Ph.D. thesis has not been submitted for the award of any other degree/diploma.

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Date: 27/8/20

Anchita Kalsi

Place: Delhi

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ABSTRACT

Explosives are toxic compounds predominantly found in the military areas. High explosives, Hexahydro-1,3,5- trinitro-1,3,5-triazine (RDX) and Octahydro-1,3,5,7-tetranitro-1,3,5,7-tatrazocine (HMX) are the most commonly used secondary explosives. They are heterocyclic nitramine compounds which can contaminate both soil and water. They are classified as a potential human carcinogen by United States Environment Protection Agency (USEPA) based on animal studies. Because of their detrimental effects on human and environmental health, the treatment of RDX and HMX contaminated soils is of utmost importance. Many physical and chemical methods have been developed for the treatment of contaminated soil. But, these approaches are expensive, non-specific and can lead to secondary pollution. Hence, a need for eco-friendly approach to mitigate the toxic effects of RDX and HMX has been paved leading to a shift to bioremediation as a possible treatment technology.

This study focuses on the use of indigenous bacterial isolates (obtained from explosive contaminated sites) immobilized to develop two novel bioformulations, for achieving the remediation goals. Three isolates, namely, *Janibacter cremeus, Pseudomonas mosselii* and *Pseudomonas entomophila* from explosive contaminated sites were subjected to remediation of RDX/ HMX in aqueous phase to confirm their efficacy of degradation. All three isolates were evaluated for their degradation efficiency of 60 mg/L RDX and 6 mg/L HMX in minimal salt medium. Bacterial growth, nitrite released and residual explosive concentrations were monitored throughout the study of 30 days. Also, the first order degradation kinetics were studied for the three isolates and the respective half-lives of both RDX and HMX were calculated. The three isolates exhibited positive growth in presence of RDX/ HMX. Degradation of RDX and HMX by *J. cremeus* and *P. entomophila* were accompanied by substantial release of nitrite, whereas, *P. mosselii* exhibited negligible release of nitrite. RDX degradation was observed to be maximum for *J. cremeus* (88 %) followed by *P. entomophila* (83 %) and *P. mosselii* (80 %). HMX degradation was also observed to be highest for *J. cremeus* (92 %) followed by *P.*

entomophila (89 %) and *P. mosselii* (76 %). Based on the results obtained, *J. cremeus* was selected for evaluation of RDX/ HMX degradation in soil and sediments.

J. cremeus was immobilized to prepare two novel bioformulations for delivery of microbe to soil/ sediment. Also, the carriers in the bioformulations played a major role in assisting the remediation process. Bioformulation 1 (BF1) was prepared using calcite and cocopeat as carriers. Bioformulation 2 (BF2) was prepared using egg shell powder, cocopeat, tween and sodium bi carbonate as carriers. Both the bioformulations were observed to be viable for six months under storage at 4 °C. The bioformulations were tested for its remediation potential in soils contaminated with 65 mg/Kg RDX / 3000 mg/Kg of HMX. The remediation experiments were conducted under saturated as well as unsaturated moisture conditions at 35 °C for 35 days. RDX was observed to be degraded by 75 and 60 % under saturated and unsaturated conditions respectively. The saturated treatment sets exhibited better microbial growth during the study in terms of live cell count and total enzyme activity. The bacteria, *J. cremeus* was observed to exhibit significant release of nitrite under both unsaturated as well as saturated conditions. Mass spectrometric studies showed that, both the conditions lead to the formation of nitroso-derivatives of RDX. But under saturated condition, an intermediate, 5-hydroxy-4-nitro-2,4-diazapentanal was observed which is a precursor to 4-nitro-2,4-diazabuatnal ultimately leading to mineralization to formaldehyde, carbon di oxide and other simpler compounds.

HMX on the other hand was observed to be degraded only under saturated conditions by 40 %, The unsaturated conditions exhibited negligible reduction in HMX concentration. Moreover, the microbial activity in the unsaturated treatment sets was observed to decrease continuously. Mass spectrometric (MS) analysis was performed to identify the intermediates formed during HMX degradation. Nitroso derivatives of HMX were observed during the anoxic degradation of HMX. Also, observed was the presence of 5-hydroxy-4-nitro-2,4-diazapentanal, a precursor of 4- nitro-2,4-diazabutanal, which eventually could get mineralized to formaldehyde and other simpler compounds.

Sediments from explosive manufacturing facility were remediated using the developed BF 1 and 2. Sediment A and B were acidic and highly contaminated with nitramine explosives. Sediment A was characterized by the presence of RDX. The RDX was observed to be degraded by 87-88 % in 150 days in sediment A. Sediment B was co-contaminated with RDX and HMX. RDX was degraded by 53-55 %, whereas, HMX was degraded by 47-49 % in 90 days. The degradation was observed to be accompanied by release of nitrite. Also, applied bioformulations (BF 1 and 2) lead to an increase in the pH of both the sediments, thereby enhancing the microbial activity.

The study demonstrates successful development and application of eco-friendly, economical and highly efficient bioformulations that can play a crucial role in remediation of hazardous nitramine explosive compounds present in soil/ sediments.

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ABBREVIATIONS

RDX	Royal Demolition Explosive
HMX	High Melting Exploisve
TNT	Trinitrotoluene
UXO	Unexploded Ordnance
HPLC	High Performance Liquid Chromatography
TSA	Trypticase Soy Agar
MSM	Minimal Salt Medium
CFU	Colony Forming Units
SEM	Scanning Electronic Microscopy
E.C	Electrical Conductivity
TOM	Total Organic Matter
USDA	United States Department of Agriculture
WHC	Water Holding Capacity
FDA	Fluorescein Di Acetate
BF	Bioformulation
MS	Mass Spectrometry
NEDD	N-(1-naphthyl) ethylene diamine dihydrochloride
PTFE	Polytetrafluorethylene
USEPA	United States Environment Protection Agency
ATSDR	Agency for Toxic Substances and Disease Registry
EPA	Environment Protection Agency
SSL	Soil Screening Limit
OB/OD	Open Burning/ Open Detonation
ZVI	Zero Valent Iron
nm	Nanometer
□m	Micrometer

cm	Centimeter
mm	Millimeter
mg/L	Milligrams/ liter
mg/Kg	Milligrams/ kilogram
mL/ min	Milliliter/ minute
□ C	Degree Celsius
%	Percentage
w/w	Weight/ weight
v/v	Volume/ volume
rpm	Rotations per minute
mS/ m	Milli siemens/ meter

Chapter 1
Introduction

CHAPTER 1

INTRODUCTION

1.1 General

Explosive contamination of land and water is a one of the major environmental concerns. With an increased use of explosive chemicals in various fields, their penetration into the soil as well as the aquatic environment has become inexorable. Explosives being recalcitrant in nature, tend to persist in the environment for long time. The fate of these compounds in soil has been well studied (Yamamoto et al. 2004; Kalderis et al. 2011; Clausen and Korte 2011). Managing the sites contaminated with explosives has become a global problem. New advancements in treatment technologies are attempted to combat this problem. With high priority to the environment safety in place, it has become a daunting task to achieve remediation using eco-friendly methods. The major challenges in application of bioremediation is the limited bioavailability of explosives and parameter optimization to achieve high treatment efficiency. With these factors in mind, this work has been taken up to optimize and develop a biological treatment strategy for treatment of explosive contaminated soil/ sediments.

1.2 Explosives

Explosives are energetic compounds which contain nitrogen and oxygen rich chemicals, having the potential of self-oxidation. The oxidation leads to generation of small

gaseous molecules, viz, N_2, H_2O and CO_2. Explosives on detonation create shock waves in the surroundings leading to an explosion, with the release of toxic compounds in the environment (Kalderis et al. 2011; Singh et al. 2012). Explosives are majorly used in industries and military operations. Improper handling and disposal of waste from manufacturing, loading, assembly and packaging (LAP) activities are few of the causes of explosives finding their way into the environment. Contamination in the live firing ranges, open burning/ detonation sites as well as in the washout lagoon soils of former manufacturing plants and also military bases are few of the well documented sites with explosive contamination worldwide (Thorn et al. 2004). Many countries have also been reported to dump old explosives into the sea. Burying of outdated explosives have also been observed. Another major factor in soil contamination is the disposal of explosive wastewaters (Kalderis et al. 2011). Explosives are also used in mining operations. The blasting process for exploration of minerals leads to release of fumes and residues of explosives (Juhasz and Naidu 2007). The explosive residues deposited on the soil surface pose a major threat to the soil ecosystems.

Many episodes of soil contamination by explosives have been reported all over the world. The Pantex plant used by the US Army during the World War II have been reported to be highly contaminated with Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), Octogen (HMX) and Trinitrotoluene (TNT). Whereas in Australia, a former explosive manufacturing facility have been reported to be highly contaminated with TNT. A pure TNT layer of about 3 cm thickness has been found to be located 10-15 cm below the soil surface (Martel et al. 2007). Similarly, a former ammunition site in Germany has been found to be highly polluted with explosives, their metabolites, heavy metals and also polycyclic aromatic hydrocarbons

(Eisentraeger et al. 2007). Other than the manufacturing facilities, unexploded ordnance (UXO) also remains a major problem. The Vietnam Ministry of Defense in 2002 estimated that approximately 7-8 % of the land in the country was affected by UXO. 1,215 sites have been identified to be affected by UXO in Australia. Whereas, in US, 1400 sites have been identified to be possible UXO sites. This accounts for 10 million acres of land (Pichtel, 2012). Jenkins et al., in 2001 and Pennington et al., in 2002 have also reported the presence of RDX in water leached from live fire hand grenade ranges. They also reported the presence of RDX in groundwater at the range.

Soil and water pollution due to explosives is a major concern. Explosives are not only hazardous to environment but also to human health. United States environment protection agency (USEPA) has classified explosives as potential human carcinogen. (RDX) is one of the most common explosives used worldwide. Ingestion of RDX, can lead to neurological damage. Also, it is characterized by a low sorption coefficient; hence, it is not retained in the soils and easily migrates to the groundwater (ATSDR 2012; EPA 2005). Octogen (HMX), another commonly used explosive has been reported to be harmful to plants and animals. Toxicological studies have showed that, HMX ingestion can lead to central nervous system and liver damages. Most of these explosives are stable in soil due to their chemical structure and also due to their capability to bind to soil organic matter. These factors render them to be resistant to soil remediation strategies (Rylott et al. 2011). Though plethora of remediation strategies already exist for the cleanup of explosive contaminated soils, it's important to adopt technologies which are not only efficient but also environment friendly.

EPA has put forth soil screening levels (SSL) for different explosives based on their risk assessment. SSL for Trinitro toluene (TNT) has been calculated as 19 mg/Kg in residential whereas, 79 mg/Kg in industrial soils (EPA 2013). SSL for perchlorate which is a major constituent in solid rocket propellants has been calculated as 55 mg/kg for residential and 820 mg/Kg for industrial areas (EPA 2017a). Similarly, the SSL for RDX has been calculated to be 6.1 and 28 mg/Kg for residential and industrial soils respectively (EPA 2017b). Dinitrotoluene (DNT) exist in six isomeric forms, of which, 2,4- and 2,6-DNT are the most commonly existing ones. EPA has calculated SSL for 2,6-DNT as 0.36 and 1.5 mg/Kg in residential and industrial sites respectively. The SSL for a mixture of 2,4 and 2,6-DNT have also been calculated for residential as well as industrial soils as 0.8 and 3.4 mg/kg respectively (EPA 2017c). Also, according to EPA (1993) soils with contamination of more than 10% (on the dry weight basis) of secondary explosive contamination are found to be susceptible to initiation and propagation.

1.3 Classification of Explosives

Explosives can be classified into different types based on:

1. Properties
2. Chemical composition

1.3.1 Classification based on properties

Explosives can be classified as high explosives, low explosives and pyrotechnics (Agrawal 2015). Figure 1.1 gives an overview of the classification.

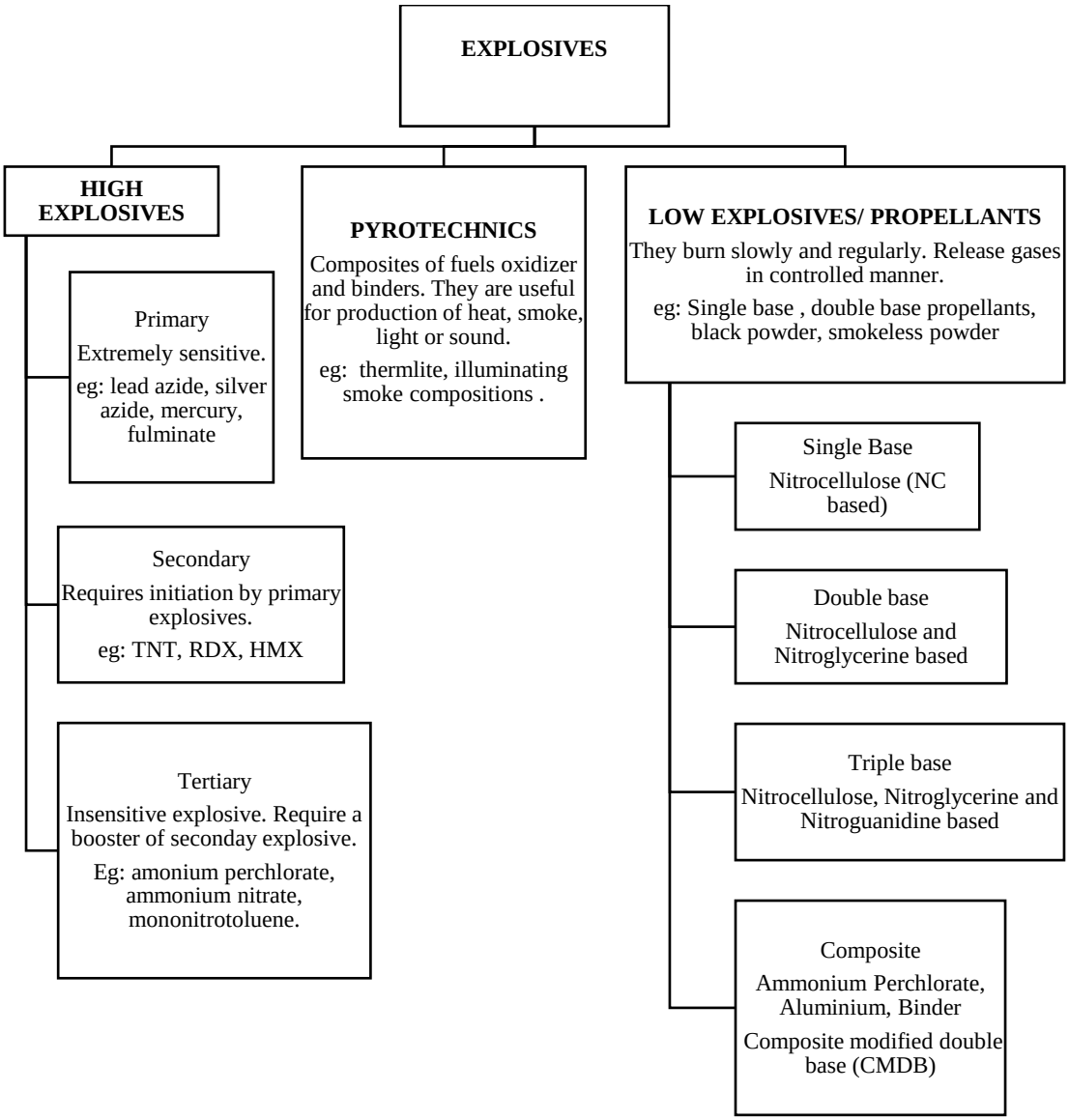


Figure 1.1: Classification of explosives based on properties

1.3.2 Classification based on chemical composition

Explosives can also be classified on the basis of their chemical composition (Singh et al. 2012):

a. Nitrate esters

b. Nitroaromatics

c. Nitramines

Table 1.1 classifies the explosives based on chemical composition with examples and health effects. Structures of some common explosives have been given in figure 1.2

Table 1.1: Classes of explosives based on chemical composition

Sl. No.	Class	Examples	Toxic Effects
1	Nitrate esters	Glycerol trinitrate (GTN; propane-1,2,3-triyl trinitrate) Pentaerythritol tetran- itrate (PETN; 2,2-bis[nitrooxymethyl]-propane-1,3-diyl dinitrate)	Methaemoglobinaemia
2	Nitroaromatics	2,4,6-trinitrophenol (TNP) 2,4,6-trinitrotoluene (TNT) 2,4- dinitroanisole (DNAN)	Haemolytic anaemia and testicular toxicity
3	Nitramines	RDX (Royal Demolition Explosive or Research Department Explosive)-hexahydro-1,3,5-trinitro 1,3,5-triazine HMX (High melting explosive)- octa-hydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine CL-20- 2,4,6,8,10,12-Hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane	CNS damage, convulsions, liver damages

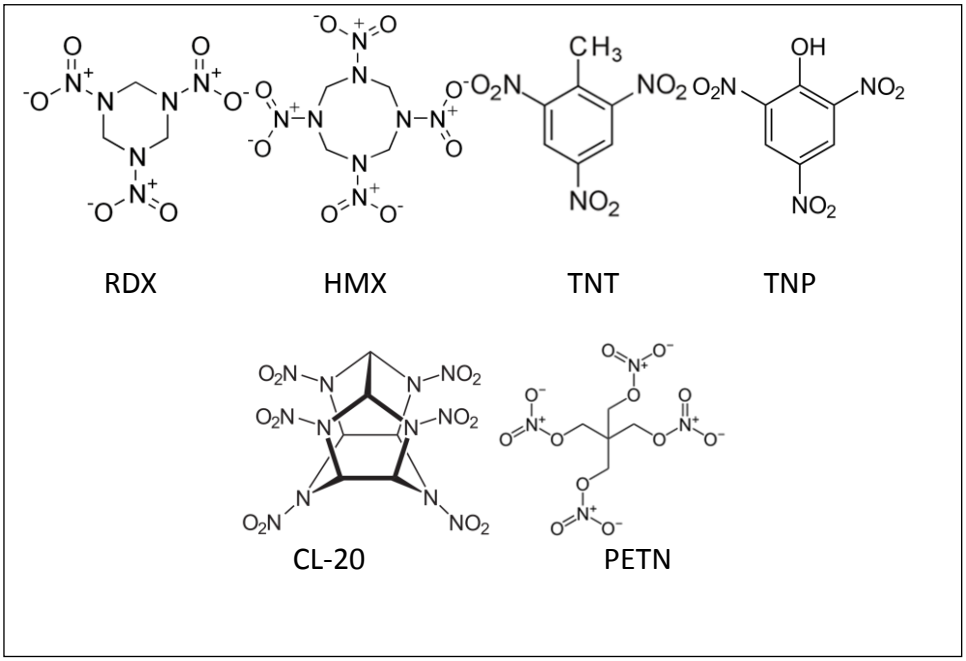


Figure 1.2: Structures of some commonly used explosives

1.4 Nitramine Explosives

1.4.1 RDX

Royal Demolition Explosive or cyclotrimethylenetrinitramine or cyclonite or hexogen or T_4 is a very powerful explosive. It was first prepared by Hennig in 1899. It found application during the World War II. Today, RDX is one of the most commonly used military explosive. It belongs to the class of explosive nitramines (ATSDR 2012). It is white crystalline powder, sparingly soluble in water. The properties of RDX have been discussed in table 1.2 (USEPA, 2014).

Table 1.2: Physico- chemical properties of RDX

Property	Value
Chemical Abstract Service (CAS) number	121-82-4
Physical appearance	White crystalline powder
Molecular weight (g/mol)	222.26
Water solubility (at 25°C in mg/L)	59.7
Octanol- water partition coefficient (Log K_{OW})	0.87
Soil organic carbon-water coefficient (Log K_{oc})	1.80
Vapor pressure at 20°C (mm Hg)	1.0×10^{-9}
Henry's Law Constant at 25°C (atm-m^3/mol)	2.0×10^{-11}

1.4.1.1Manufacture of RDX

RDX is manufactured by Bachmann process (Bachmann and Sheehan, 1949). The conventional process for RDX preparation utilized hexamethylenetetramine, nitric acid and formaldehyde as raw materials (Hale, 1925). The major disadvantages were use of large amount of nitric acid for the reaction to proceed and loss of considerable amount

of formaldehyde during oxidation by nitric acid. Another major drawback included the low yield of RDX even under optimum conditions. The Bachmann process for RDX preparation involves the use of a mixture of hexamethylenetetramine and ammonium nitrate, to which acetic anhydride-nitric acid mixture was added at a temperature of 70-75°C. Use of acetic anhydride led to anhydrous medium preventing dangerous fume off. The reactants are continuously stirred at the specific temperature for 15 min. The reaction led to the precipitation of RDX crystals. The mixture was filtered and washed with acetic acid and water, cooled and dried, giving a yield of 61-63 %. When the mixture was cooled before filtration, yield obtained was 70-73.5%. Drowning the reaction mixture with warm water led to the production of colorless solid which was further purified by heating in a solution of sodium acetate and concentrated aqueous ammonia. The product so obtained was further warmed with 70 % nitric acid to produce RDX. RDX production led to the release of HMX as a byproduct, which was present in the solution and was decanted. This method led to the synthesis of 2 moles of RDX from 1 mole of hexamethylenetetramine (Bachmann and Sheehan, 1949).

1.4.2 HMX

HMX is also known as Octogen or high melting explosive. It is also known as cyclotetramethylene- tetranitramine. It differs from RDX in having an extra CH_2NNO_2group.It is white crystalline solid, practically insoluble in water. It finds application in nuclear devices, rocket fuels and plastic explosives. The properties of HMX have been tabulated below (table 1.3) (USEPA, 2014).

Table 1.3: Physico-chemical properties of HMX

Property	Value
Chemical Abstract Service (CAS) number	2691-41-0
Physical appearance	White crystalline powder
Molecular weight (g/mol)	296.16
Water solubility (at 25°C in mg/L)	5
Octanol- water partition coefficient (Log K_{OW})	0.16
Soil organic carbon-water coefficient (Log K_{oc})	1.80
Vapor pressure at 20°C (mm Hg)	2.41×10^{-8}
Henry's Law Constant at 25°C (atm-m^3/mol)	8.7×10^{-10}

1.4.2.1 Manufacture of HMX

HMX was originally produced as a byproduct of RDX manufacture by Bachmann process. It is a superior explosive than RDX due to its high velocity of detonation, higher melting point and better stability to acids and alkali (Das et al. 2006). Even with such advantages, the use of HMX is limited by its higher production cost. Modified Bachman process relies in inhibition of formation of RDX and other nitro bodies.Raw materials used are hexamine, acetic acid, ammonium nitrate and nitric acid. They are continuously mixed at a temperature of 44°C, leading to the formation of an intermediate precursor; DPT. DPT is further converted to HMX on reaction with acetic anhydride with paraformaldehyde as a catalyst. The HMX produced was simmered at 96° C to precipitate out the crude HMX. Recrystallization of HMX was done using acetone. Pure HMX is sieved and dried to produce β HMX.

1.5 Remediation of explosive contaminated soil

Remediation of soils contaminated with explosives need to be dealt on the site basis. It depends on various environmental factors as well as the extent of explosive contamination (Rodgers and Bunce 2001). Soil remediation strategies can be broadly classified into physical, chemical and biological. Figure 1.3 outlines the various treatment strategies associated with soil remediation. Physical and chemical methods, though faster are generally costly and energy intensive. Also, they suffer from a major drawback of transferring the contaminant from one phase to the other (Ward and Singh 2004). Hence, emphasis is being laid on use of biological remediation strategies owing to their eco-friendly nature.

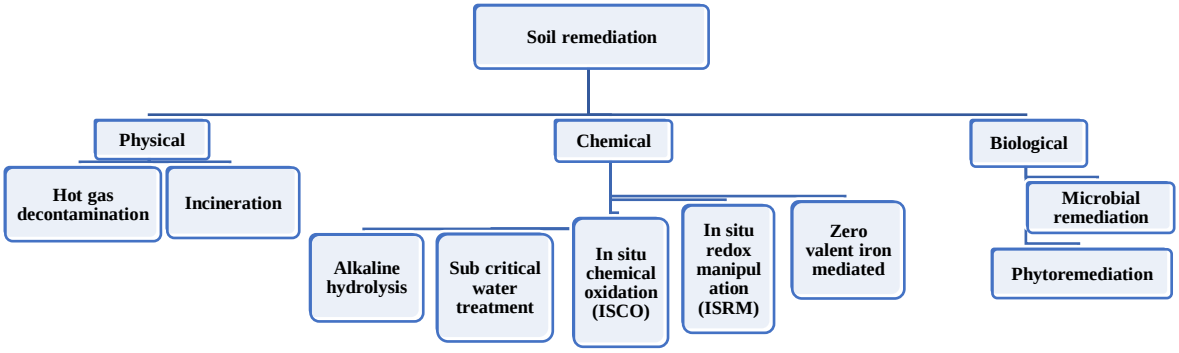


Figure 1.3: Treatment strategies for remediation of soils contaminated with explosives

1.6 Treatment strategies

Physical and chemical treatment technologies for decontamination of explosives in soil are generally faster and can help in decontamination of high concentrations of explosives. Also, they are not dependent on the environmental conditions for their performance in remediation. These technologies generally lead to a phase transfer leading to secondary pollution (Kalderis et al. 2011).

1.6.1 Physical treatment technologies

1.6.1.1 Incineration

Incineration employs the use of very high temperatures (860-1200° C) for destruction of explosive contaminated soils or debris, unexploded explosives and bulk explosive waste (Van Ham1998). USEPA in 1991 had declared incineration as one of the best technologies for treatment of explosive contaminated sludges. The process may take place in a controlled environment like the enclosed ovens, or it can be accomplished by open burning/ open detonation (OB/OD). OB/OD being uncontrolled reaction, it leads to the generation of toxic air emissions. With strict regulations in place, OB/OD is not recommended. Another major drawback is the equipment and personnel safety. Since explosives are highly sensitive to impact and friction, the process commands greater regulations and safety precautions when under progress (Garg et al.1991). Though these methods help in faster decontamination of explosive contaminated sites, the materials decontaminated cannot be reused and must be scrapped or landfilled. Other disadvantages of this technique include high cost incurred for equipment and its maintenance, fuel costs and also additional treatments for discharge systems. Another major problem associated with incineration is the incomplete combustion leading to release of particulate matter and air pollutants (Garg et al. 1991).

1.6.1.2 Hot gas decontamination:

Hot gas decontamination is majorly utilized for decontamination of explosive contaminated soil, structures and equipment. Though the operational conditions are site specific, it generally involves heating with hot gas at 260° C for one hour which leads to the volatilization of the explosives. The gas effluent released is then treated in an

afterburner. This technique is suitable for destruction of explosive sediments in stockpile which needs to be discarded as hazardous material (Raghavan et al. 1989; Topfer 1995; Hyman and Dupont 2001; Hewitt 2001). The major concerns related to this technology are the atmospheric emissions from the thermal oxidizer. Constant monitoring is of utmost importance so as to keep a check on hazardous emissions. Another major limitation associated with this technology is the design of the furnace. Special care must be taken to consider any possible explosions due to the presence of explosives (Hewitt 2001).

1.6.2 Chemical treatment technologies

1.6.2.1 Alkaline hydrolysis

Alkaline hydrolysis is one of the alternative technologies available for treatment of chemical weapons. The process leads to decomposition of explosive compounds to organic and inorganic salts, soluble organic compounds and gases (Balakrishnan et al. 2003). The transformation of explosive compounds has been long studied. Janowsky, in 1891, first established use of base for transformation of the explosive compound, TNT. Various substances that can be used to raise the pH of soil include, metal oxides, hydroxides and carbonates. Such materials, find applications in wastewater treatment, acid mine drainage and agricultural soil treatment (Larson et al. 2008). Lime in various forms has been investigated for its ability to increase pH and also degrade explosive compounds present in soil. Calcium hydroxide has been found to be one of the most promising bases (Brooks et al. 2003) and has also been adapted for field applications (Thorne et al. 2004; Martin et al. 2013). This technology has been demonstrated for successful removal of both nitroaromatics and nitramines present in soil (Hansen et al. 2003; Brooks et al. 2003).

Efficiency of alkaline hydrolysis to remove explosive greatly depends on the contact of explosive compound and the base in soil pore water. The transportation of hydroxyl ion through the soil plays a major role in the process. Since, the reaction of alkaline hydrolysis occurs in the aqueous phase, it is important for the munition compound and the base applied to be dissolved in soil pore water. Soil chemistry also plays a crucial role in alkaline hydrolysis. The cation species may undergo exchange with other cations present on the soil sites. These may also be exchanged with H^+ ions present in soils with low pH, leading to a reaction with OH^- ions, causing the buffering of the soil system, thereby, inhibiting the hydrolysis process. The base cations could also form insoluble hydroxides, removing them the hydrolysis reaction. Also, the hydrogen ions on functional groups of humic matter may dissociate under high pH, hampering the hydrolysis reaction (Larson et al. 2008). Much research has been intended to investigate the efficiency of alkaline hydrolysis process for soil treatment. Emmrich in 2001, investigated alkaline hydrolysis for treatment of TNT contaminated soils of two former ammunition plants located in Germany. Hansen et al., in 2003, studied the remediation of TNT contaminated soil using lime treatment. Davis et al., in 2006 conducted microcosm studies for treatment of soils from munition plants and active firing ranges. Davis et al., in 2007a, studied the mechanism of alkaline hydrolysis of TNT and RDX, the final products and the biodegradability of the reaction products. Davis et al., in 2007b conducted mesocosm studies for treatment of TNT, RDX and HMX contaminated soils using lime. Coyle et al., in 2017, performed laboratory studies using novel reactive gas process for treatment of soils contaminated with explosives. This process involves the use of ammonia in air mixture to raise the soil pH and hence degrade explosives by alkaline hydrolysis.

The major drawback of this technology is the high pH (12-13) that has to be maintained in the soil for its effectiveness. This high pH lowers the soil quality and affects its physical, chemical and biological properties. Soils with high alkalinity have been found to be detrimental to plants. It has also been observed the retardation in organic matter mineralization as the bacterial activity is hindered. Another disadvantage of alkaline hydrolysis includes, high moisture content required to be maintained in soils for the reaction to occur. Though this is helpful for effectiveness of reaction, it may also lead to transportation of the explosive compound through the soil profile to the groundwater table. This could pose a serious threat for groundwater contamination at polluted sites (Anderson and Ventola, 2015).

1.6.2.2 Sub critical water treatment

Subcritical water (SCW) is water held at subcritical condition, that is, temperature above 100 °C and below its critical temperature of 374 °C and a pressure of 22.1 MPa. The high pressure helps maintain water in the liquid state (Carr et al.2011). The subcritical water can act as an effective solvent, catalyst and reactant for hydrolytic conversions (Brunner 2009). Under subcritical conditions, water can help in explosive removal by acting like other organic solvents, aiding in solubilization and separation (Islam et al. 2015). Also, under such conditions, self-ionization of water molecules lead to increase in concentration of $H^+$and OH^- ions, which can lead to removal of organic explosive compounds by oxidation (Kuhlmann et al. 1994; Oh et al. 2011; Islam et al. 2015).Hawthorne et al., in 2000, studied the use of SCW process for treatment of soils contaminated with TNT, RDX and HMX. They used water at subcritical conditions in static mode rather than flowing. Islam et al. (2015) studied the use of subcritical water

treatment for removal of explosive in soils co contaminated with explosive and heavy metals as generally found in firing ranges. The explosives studied were RDX and TNT.

1.6.2.3 Insitu chemical oxidation (ISCO)

ISCO is the use of chemical oxidants for the treatment of explosive contaminants. This requires the delivery of the oxidizing agents in the subsurface of the soil. It can be performed using four different types of oxidizing agents, viz, modified Fenton's reagent or catalyzed hydrogen peroxide propagation (CHP), ozone sparging, persulfate and permanganate treatment (Watts et al. 2006). The success of the process depends on the delivery of the oxidant to the subsurface as well as its effective reaction with the explosive contaminant. Delivery techniques include: deep soil mixing, hydraulic fracturing, multi- point vertical lancing, horizontal well recirculation, and vertical well recirculation (U.S. Department of Energy report 1999).

ISCO using permanganate generally can be performed in aqueous medium only (Petri et al. 2010). Hence, much work has been done for remediation of explosives in aqueous phase (Adam et al. 2004; Waldemer and Tratnyek 2006).

Use of modified Fenton is also a well-established technique for ISCO. It involves the use of ferrous ion along with H_2O_2. The process leads to release of hydroxyl radicals, which are extremely potent oxidizing agents. Majority of work related to Fenton has been carried out for aqueous phase, but it has been demonstrated for soils too (Watts et al.1991; Bier et al. 1999; Shermata and Hawari 2000; Yardin and Chiron2006).

Ozone is a highly reactive gas. The electronic configuration of O_3 contributes to its reactivity. It has dual electrophilic and nucleophilic character O_3 gas can be purged into the soil profile for remediation of unsaturated soils. The O_3 reacts with iron oxides and

organic matter to produce hydroxyl radicals which can lead to the oxidation of explosives in soil (Adam et al. 2006).

Another potent chemical oxidant is persulfate. Persulfate has higher stability than H_2O_2. It forms sulfate radicals which are stronger oxidizing agent than H_2O_2, O_3 and permanganate. Persulfate can be activated by heat, alkaline pH, permanganate or ferrous ions. Soil contaminated with explosives have also been remediated using this technology (Waisner and Hoag 2006; Waisner et al. 2008).

1.6.2.4 Insitu redox manipulation (ISRM)

ISRM involves the use of a reducing agent for transformation of explosives (Szecsody et al.2001; Adam et al. 2005; Boparai et al. 2008). Most commonly used chemical reductant is sodium dithionite. Sodium dithionite reacts with naturally occurring iron to form ferrous ions, which lead to formation of a chemically reduced zone. The ferrous ions, then act as reactants to transform the high explosives (Clayton et al. 2005). Boparai et al., in 2008 evaluated the efficacy of dithionite reduced sediments for the degradation of RDX, HMX and TNT. They found that, all three explosives were reduced and on analysis of the intermediates, they found that RDX was transformed to nitroso derivatives. Adam et al., in 2005 studied the biodegradability of the intermediates formed by ISRM of RDX. They found that, the intermediates could be successfully biodegraded aerobically.

1.6.2.5 Zero valent iron mediated remediation

Iron typically exists as ferrous or ferric iron in nature. Zero valent iron (ZVI/Fe^0) is synthesized. Application of ZVI has been focused on its electron donating capacity. ZVI is fairly reactive with water and can act as an electron donor, hence is a promising

candidate for site remediation (Stumm and Morgan 1996).Application of zerovalent iron (Fe^0) for remediation of explosive contaminated soils is well researched and documented (Singh et al. 1998; Singh et al. 1999; Oh et al. 2001; Oh et al. 2002; Comfort et al. 2003; Park et al. 2004; Jiamjitrpanich et al. 2010). ZVI in presence of water replaces the oxygen of nitro group with hydrogen making it further easier to break down. Oh et al., in 2001 conducted integrated studies using ZVI and anaerobic sludge for the remediation of RDX contaminated soil. They concluded that bioaugmentation along with ZVI could help in effective degradation of RDX.

Comfort et al., in 2003, conducted laboratory and pilot scale experiments for remediation of soils contaminated with RDX. In yet another study, Park et al., in 2004, studied the potential of ZVI for remediation of soils co contaminated with RDX, HMX and TNT. Their results showed that ZVI was effective for RDX and TNT, but not HMX. In presence of RDX, ZVI acted on it rather than HMX. This was attributed to its low solubility. Hence, they proposed the use of cationic surfactants for increasing HMX solubility there by increasing the efficacy of ZVI.

Though ZVI is very successful in treatment of explosive compounds, its application in field scale level is mainly limited by the requirement of proper mixing. Under unsaturated conditions, mixing is crucial so that the reactants are well in contact with ZVI. And hence, in static conditions it's very necessary to employ high speed mixers (Comfort et al. 2003). When applied in slurries, the equipment required for continuous mixing adds to the cost of treatment. Also, such slurries further require dewatering adding an extra step in the treatment process (Kalderis et al. 2011).

1.6.3 Bioremediation

Sustainability utilizes the finite resources judiciously to provide for the present as well as future generations. With sustainability gaining importance, green and sustainable remediation approaches are being encouraged to optimize all phases of remediation. Sustainable remediation promotes renewable energy, material recycling, minimizes the waste and energy consumed. The above-mentioned physicochemical approaches do not include the criteria of sustainability (Megharaj and Naidu 2017). Bioremediation involves the use of biological agents (plants and microbes) for the remediation of explosive contaminated soils. It is a green and sustainable solution for the remediation of such sites. The major drawback associated with bioremediation is its slow speed of action as well as its success in field application.

1.6.3.1 Phytoremediation

Phytoremediation is an eco-friendly, low cost alternative for the physical and chemical treatment methods. It utilizes the ability of plants to grow in presence of the explosive contaminants, tolerate their toxicity and remediate the soils with the help of plant specific enzymes. Phytoremediation can be based on phytoextraction, phytostabilization, phytodegradation or phytovolatilization (Kalderis et al. 2011). Phytoremediation is an extensively studied field with much available literature on remediation of explosive contaminated soil (Palazzo and Leget1986; Schneider et al. 1996; Burken et al. 2000; Adamia et al. 2006; Vila et al. 2007; Gandia-Herrero et al. 2008; Brentner et al. 2008; Gunning et al. 2014). Studies on phytoremediation of explosives have been reported using different plant species, viz, Eurasian water milfoil, *Myriophyllum spicatum* and vetiver grass, *Chrysopogon zizanioides* for TNT and reed canary grass, fox sedge, and rice for RDX treatment (Kiiskila et al. 2015). Guinea grass

(*Panicum maximum*) was reported to possess ability to phytoremediate RDX and HMX in soils. In the same study it was also observed that addition of a carbon substrate like molasses improve the phytoremediation efficiency (Lamichhane et al. 2012). Beta vulgaris have been reported to take up and transform GTN and PETN (Goel et al. 1997). Though this technique has been successfully evaluated for explosive contamination, factors such as its suitability being limited to shallow levels of contamination, selection of plant species, maintenance issues, phytotoxicity of contaminants, slower rate of degradation, unknown effects of the by-products formed during the process and danger of contaminants entering the food chain limits its application for onsite remediation.

1.6.3.2 Microbial remediation

Microbial remediation involves the use of microorganisms for the destruction of explosive contaminated soils. Microbes being ubiquitous in nature have the ability to survive even in soils highly contaminated with explosives. The microbes having the ability to degrade explosives specifically express different types of enzymes which trigger the use of explosive compound as a substrate (C or N source) for its survival and growth, hence convert the explosive to innocuous form. It is a slow process that depends on the growth rate of the microbe. The microbe involved in the remediation process may be indigenously present or may be introduced into the contaminated site from elsewhere (Chaudhary and Kim2019). Microbes degrade the explosive pollutant by either of two ways: aerobic or anaerobic. Figure 1.4 gives an overview of both the processes. Figure 1.5 represents the general mechanism of microbial remediation of explosives.

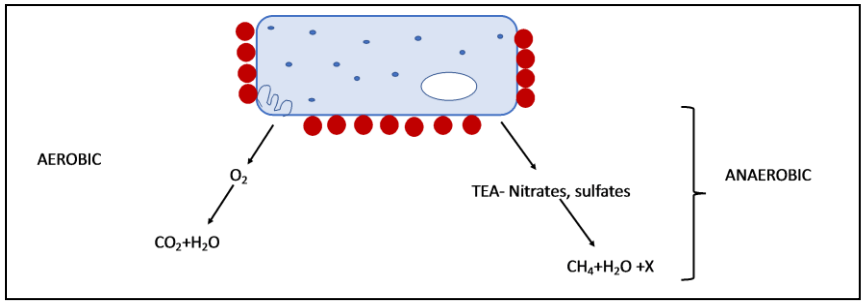


Figure 1.4: Aerobic and anaerobic microbial metabolism

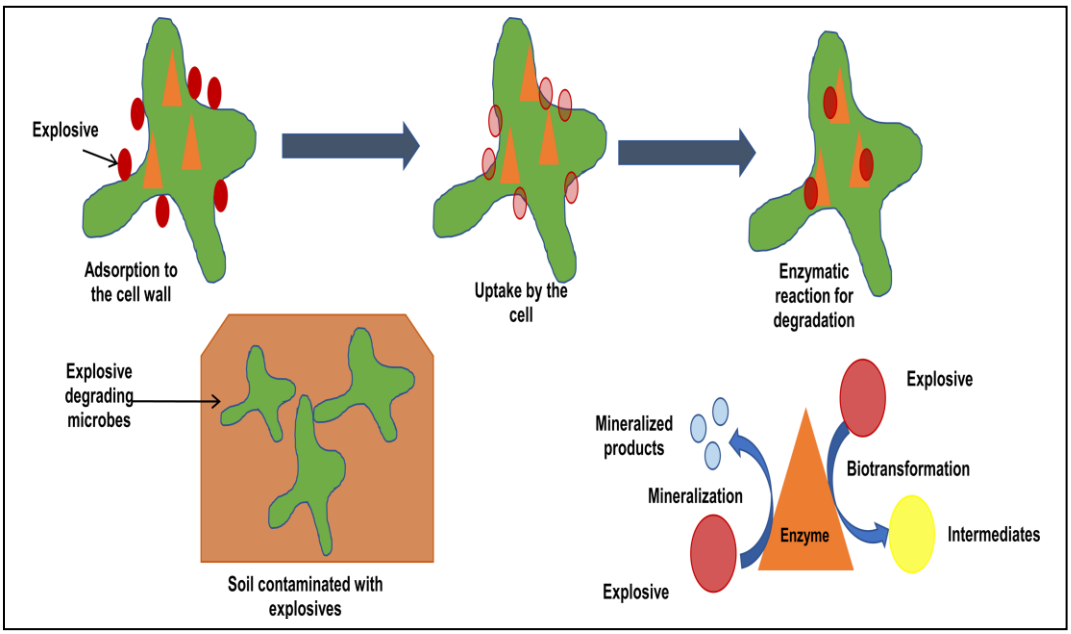


Figure 1.5: Mechanism of explosive microbial remediation

Keeping in the mind the various drawbacks of physical and chemical methods for treatment of explosive contaminated soil, the present work proposes the use of eco-friendly and economical microbial remediation approach with the following objective and scope.

1.7 Objective

Bioremediation of explosive contaminated soil and sediment using immobilized bacteria.

1.8 Scope

1. Evaluation of explosive degrading ability of indigenous microorganisms isolated from contaminated sites.
2. Development of two novel bioformulation using two different natural carriers with *Janibacter cremeus* as the active ingredient.
3. Viability testing of the developed bioformulations
4. Application of the developed bioformulations in the bioremediation of soils contaminated with RDX and HMX.
5. Application of the developed bioformulations in the bioremediation of explosive contaminated sediments obtained from manufacturing industries.

Chapter 2
Review of Literature

CHAPTER 2

REVIEW OF LITERATURE

2.1 Bioremediation of Explosives

Remediation of soils contaminated with hazardous high explosive, RDX and HMX is one of the major challenges faced by the defence sector. Due to their recalcitrant nature, they persist in the environment for a long time. Thus, its treatment is of much importance. Soils contaminated with RDX and HMX can be treated using various physical, chemical and biological methods. RDX and HMX in general are resistant to various chemical treatments. Also, due to their low bioavailability, bioremediation of explosives is a major challenge. Because of these reasons, not much work is available on treatment of RDX and HMX contaminated soils. Therefore, this work for *in situ* bioremediation of RDX and HMX is an important step to mitigate the existing problem.

2.2 Role of Microbes in Bioremediation

Microbes are ubiquitous in nature, hence find application in treatment of hazardous chemicals. Use of microbial remediation strategies for treatment holds an upper hand in comparison to other techniques, as they are economically feasible, eco-friendly and with appropriate optimization can lead to complete mineralization of the target contaminant.

Sites contaminated with explosives are generally inhabited by microbial species that are resistant to the toxic effects of the explosive compounds and also can contribute in the remediation of such siteswhen appropriate growth conditions are provided. Plethora of work is available for remediation of explosive *ex situ* (Hawari et al., 2001; Khan et al.

2012; Singh et al. 2012) but the major challenge lies in its transformation to field level, as most isolate fail to survive in actual contaminated sites. The major advantage of using an isolate from the contaminated site lies in its ability to survive *in situ* (Cupples, 2013).

2.3 Microbial remediation of explosive contaminated soils

Remediation of soils contaminated with explosives need to be dealt on the site basis. It depends on various environmental factors as well as the extent of explosive contamination (Rodgers and Bunce 2001). Microbial remediation technologies can be classified under two groups: *in situ* and *ex situ* (Chaudhary and Kim2019). Table 2.1 discusses the various advantages and disadvantages of the microbial treatment technologies.

2.3.1 *In situ* remediation

These are the technologies that are executed on site and include natural attenuation, bioventing, biostimulation, bio augmentation and combined biostimulation and augmentation.

2.3.1.1 Natural attenuation

Natural attenuation or intrinsic remediation is the use of microorganisms present naturally in the soils contaminated with explosives to remediate the site. These microbes have an inherent capability to utilize the contaminant as a nutrient source for its growth (Muter et al. 2008). USEPA defines natural attenuation as the use of natural processes to contain and limit the spread of contaminants present in a site. Natural processes can be biological (microbial) or physical as during sorption, volatilization, dilution etc (Mulligan and Yong 2004). Microbes are the major players during the natural attenuation process. Many factors contribute to the rate of biodegradation, viz,

availability of oxygen and other electron acceptors like nitrate, presence of water and minerals, rate of aerobic and anaerobic degradations (Mulligan and Yong 2004). Many explosive contaminated sites have been attempted to be remediated by natural attenuation. Kundu et al., in 2016 studied the natural attenuation of 4-nitrotoluene (4-NT), 2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6- DNT) in artificially spiked soils. They found degradation of 20-25%. Mark et al., in 2016, evaluated the natural attenuation potential of insensitive munition compound NTO (3-nitro-1,2,4-triazol-5-one). These studies suggest that since natural attenuation is a slow process, it may be combined with other biological processes like bioaugmentation and biostimulation to increase the degradation efficiency.

2.3.1.2 Bioventing

The unsaturated vadose zone soils are characterized by low microbial count and lower catabolic activity. Bioventing is the supply of electron donors to unsaturated soils to aid in biodegradation by the naturally occurring microorganisms. It can be either aerobic or anaerobic. Aerobic bioventing requires the supply of oxygen to the soil using an injection, whereas, anaerobic bioventing requires the addition of other electron donors like nitrogen. The electron donors injected decide the fate of the contaminant. Aerobic bioventing leads to oxidative transformation whereas, anaerobic bioventing leads to reductive transformation (Hohener and Ponsin, 2014; Solid Waste and Emergency Response 5203P 2006). The first *in situ* bioventing field demonstration was performed at Pantex facility in Texas in 2001 by Rainwater et al. the ability of indigenous microbes to reduce RDX, TNB and TNT was enhanced by injection of nitrogen gas for 333 days. Their results showed increased reductive metabolic activity of the microbes leading to substantial decrease in RDX and TNB concentrations.

2.3.1.3 Biostimulation

Remediation of any contaminant in soil generally depends on various factor, viz, nutrients, pH, temperature, moisture content, soil characteristics, available oxygen and the concentration of contaminant (Bundy et al. 2002; Atagana, 2008). Remediation potential of indigenous microorganisms present in the contaminated soil can be enhanced by biostimulation, which involves the addition of limiting nutrients and electron acceptors like nitrogen, phosphorus or carbon to stimulate the growth of the indigenous microbes. Extensive literature survey has suggested that, biostimulation is the majorly used bioremediation technique for remediation of explosive contaminated soils (Boopathy 2000; Bruns-Nagel 2000; Payne et al. 2013; Xin et al. 2013). Molasses is the most commonly used carbon source for biostimulation. Other common carbon amendments include, cheese whey and waste glycerol. Boopathy (2000), used various carbon compounds as co-substrates during the insitu remediation of TNT contaminated soil from the Joliet Army Ammunition Plant. He used, molasses and succinate for biostimulation of soils columns. He observed that, treatment controls without any carbon amendment showed no decrease in TNT concentration and those amended with succinate were not found to be very successful either. His results concluded that, molasses and not succinate was a good source of carbon for the indigenous microbes to utilize and breakdown TNT.

2.3.1.4 Bioaugmentation

Bioaugmentation is the process of inoculating explosive degrading microbes in to the contaminated soil. A single microbe or a consortium can be used for this purpose. The rationale for this technology is that the indigenous microbes may lack the ability to degrade explosives present or that they are stressed due to the exposure to high

concentrations of explosives. Hence, to speed up the remediation, bioaugmentation may be employed to reduce the lag phase of the organisms and in turn reduce explosive concentration. The microorganism introduced during bioaugmentation can be isolated from the contaminated site, or historical site, or isolated by careful screening or genetically modified to support the remediation of explosive in soil (Adams et al. 2015). Labidi et al. in 2001, used *Rhizobium trifolii* isolated from atrazine contaminated soil for remediation of TNT contaminated soils. The bacteria were first carefully screened for its explosive degrading ability in liquid medium. The results showed that, bioaugmentation lead to 60% degradation of TNT in 2 days.

2.3.1.5 Combination of bioaugmentation and biostimuation

Researchers have also tried the simultaneous application of bioaugmentation and stimulation for better remediation of explosive contaminated soils. Many studies have also been done to compare the efficiency of these technologies alone as well as in combination (Labidi et al. 2001; Muter et al. 2012). The results have proven that, if the nutrients supplied are not toxic to the microbes, remediation of explosives is more efficient and faster. Nõlvak et al., in 2013 studied the remediation of TNT contaminated soils using combination technology. The soils were inoculated with a consortium predominated by *Pseudomonas* and *Stenotrophomonas* species and amended with molasses and cabbage leaf extracts. the results showed much greater degradation when compared to the bioaugmentation or stimulation alone. Karakaya et al. (2009) studied the bioremediation of a high explosive CL-20 using *Phanerocheate chrysosporium* along with supplementary carbon (glycerol) and nitrogen (ammonium sulfate, yeast extract) sources. CL-20 was found to be mineralized by up to 51% during the study. Also, Muter et al., in 2012 established that, bioaugmentation with addition of nutrients

like molasses and cabbage leaf extract gave a much better remediation efficiency in highly TNT contaminated soils. Kao et al., in 2016 also suggested the use of biostimulation and bioaugmentation together for the treatment of TNT and PETN contaminated soil as bioaugmentation was found to be unsuccessful.

2.3.2 *Ex situ* bioremediation

These technologies involve excavation of the contaminated soil followed by its treatment and then replacement of the treated soil. The various technologies include, bioslurry reactors, composting, biopile and landfarming.

2.3.2.1 Bioslurry reactors

Bioslurry reactors are highly engineered systems for treatment of explosive contaminated soils. They are extremely powerful systems which have been successfully implemented for remediation of soil contaminated with explosives in feasibility studies (Boopathy et al. 1998; Fuller et al. 2003; Park et al. 2003; Boopathy, 2005) as well as field scale studies (Manning et al. 1996). In a bioslurry reactor, soil is excavated, pretreated and loaded into the reactor, where it is mixed with water or wastewater to maintain a uniform consistency. The process can by aerobic, anoxic or anaerobic. It can be operated in batch, semi continuous or continuous modes. Pretreatment is an important step for better performance of the system. This involves crushing and screening for finer textures (Tomei and Daugulis 2012). Slurry bioreactors also find application in determining the actual potential of a biological strategy for remediation of contaminated site (Robles-González et al. 2008). Boopathy in 2001 studied the remediation of HMX contaminated soil using a batch slurry reactor, with the addition of molasses as a carbon substrate for the native microorganisms to degrade HMX. The results showed 97% degradation in HMX concentration in 4 months. Zhang et al. in 2001 performed a pilot scale test using a

slurry reactor for the remediation of 2,4-DNT and 2,6-DNT contaminated soil. They emphasized on soil washing to obtain soil fines contaminated with explosives. Aerobic slurries were maintained for a period of 3 months.

2.3.2.2 Composting

Composting is the process of conversion of organic matter into humus like substances which are non toxic. The process involves mixing of the soil with bulking agents (wood chips, hay, straw) and organic amendments (cattle manure, vegetable waste). The resulting mixture of the soil and amendments can be spread in the form of piles or windrows. The role of the bulking agent is to provide porosity, aeration and nutrition, whereas, the organic amendment helps in maintaining an optimum carbon to nitrogen ratio to promote biological activity. Another important feature of composting is the elevated temperatures that help in the catabolic activities of the microorganisms, to degrade the explosive compound (Chaudhary and Kim 2019). Many sites worldwide have been remediated by this technology. A few of the examples include, the Pueblo Chemical Depot contaminated with TNT, DNT and RDX, was treated using composting, the explosives were found to be degraded in a time period of 15- 30 days. Windrow composting was performed at Umatilla Army Depot to treat TNT, RDX and HMX. After 40 days, the explosive concentrations were below the clean up goals. Hawthorne Army Depot also employed composting for remediation of TNT, RDX, HMX and ammonium picrate. The duration of 28 days was found to be very effective to reach the clean up goals (Williams et al. 1992; Goetz and Brenner 2002).

2.3.2.3 Biopile

Biopiles involves the heaping of excavated contaminated soil above ground and mixing with suitable amendments (nutrients, chemical for pH adjustments, bulking agents). It

also requires the use of forced aeration for tilling and reducing the space requirements. The goal of this treatment is to convert the explosive contaminants into harmless products, making the soil safe for disposal and other uses. The heaps of contaminated soil in the form of free standing piles are called biopiles, whereas, those supported by walls or sides are called as biocells. Pueblo Chemical Depot set up biopile installation for treatment of TNT contaminated soils. TNT reduced from 3800 mg/Kg to 10 mg/Kg in 50 days. A biopile was also successfully used at Joliet Army Ammo Plant for treatment of TNT (3000 mg/Kg) and tetryl (7500 mg/Kg) contaminated soil. In a duration of 4 months the TNT and tetryl reduced to 50 and 250 mg/Kg respectively. In another biopile installation at Yorktown Naval Weapons Station, TNT, RDX and HMX were targeted. In a duration of 3 months, TNT reduced from1329 to 2.9 ppm, RDX reduced from 319 ppm to 13.5 ppm whereas, HMX reduced from 98 ppm to not detectable range (Goetz and Brenner 2002).

2.3.2.4 Land farming

Land farming involves the excavation of soil to be treated and spreading into high-density polyethylene (HDPE) lined beds. These are periodically tilled for better aeration and mixing (Tomei et al. 2012). Recently, the integration with other biological approaches, viz, bioaugmentation and biostimulation have improved the functioning of landfarming (Jeong et al. 2015). Clark and Boopathy in 2007 evaluated the efficacy of landfarming for the remediation of explosive contaminated soil from Louisiana Army Ammunition Plant. They targeted the explosives, TNT, RDX and HMX. 82% of TNT was reduced in 182 days, whereas, RDX and HMX were not reduced to much extent which was attributed to the complexity of the molecules.

Table 2.1: Microbial remediation technologies (*in situ* and *ex situ*): advantages and disadvantages

Sl. No.	Technology	Mechanism of action	Advantages	Disadvantages	Examples
1	Biological Natural Attenuation	Explosives reduced by naturally existing microbes that utilize the contaminant as a source to derive energy and for growth.	Ease of use. Lower costs. Can be combined with other techniques.	Longer remediation times. Lack of knowledge on the complex mechanisms involved. Not very efficient technology for highly contaminated sites.	Zhao et al. 2004, Ronen et al. 2008, Monteil-Rivera et al. 2009, Pennington et al. 2001, Crocker et al. 2005
2	Bioventing	Supply of electron donors (aerobic or anerobic) in to the soils in a controlled manner to stimulate the catabolic activities of the indigenous microbes	Cost effective. Enhances microbial activity	Time taking process. Requires specific instrument. Success depends on soil characteristics , permeability and respiration rate	Kuroda, 1997, Sagi-Ben Moshe et al. 2010, Wijker et al. 2013
3	Biostimulation	Supply of limiting nutrients like, carbon, nitrogen and phosphorus to the soil.	Cost effective Improves the nutrient content of the soil. Improves the bioavailability of the explosive t the microbial community.	Elongated periods of treatment.	Funk et al. 1993, Boopathy et al. 1997, Drzyzga et al. 1999, O'Niell and Nzengung 2003 Lamichhane et al. 2012, Won and Borden, 2016, Jugnia et al. 2017
4	Bioaugmentation	Introduction of explosive degrading microbe or consortia into the contaminated soil.	Co-metabolism. Indigenous microbe's activity supported by inoculated microbes. Lesser time duration in comparison to natural attenuation.	Introduced microbes may or may not perform in field conditions. Natural environmental conditions may affect the growth of the introduced microbes.	Van Dillewijn et al. 2007, Muter et al. 2012, Nõlvak et al. 2013, Anasonye et al. 2015, Fournier et al. 2004, Price et al. 2011, Xu et al. 2019

Sl. No.	Technology	Mechanism of action	Advantages	Disadvantages	Examples
5	Bioslurry reactors	Contaminated soil excavated and loaded into a reactor, where its mixed with water to make a slurry. Microorganisms are also added into the reactor for remediation.	Controlled process. Can be run in aerobic, anaerobic or anoxic conditions. Contaminant bioavailability increased.	Higher costs. Need for excavation of soil. Time consuming Laborious.	Guiot et al. 1999, Rocheleau et al. 1999, Shen et al. 2000, Knicker et al. 2001, Weeks et al. 2003, Newcombe and Crawford, 2007, Sheibani et al. 2011, Xin et al. 2013.
6	Composting	Soils are mixed with bulking agents and organic amendments for composting and are arranged in piles or windrows for the thermophilic process to occur. Intermittent mixing is generally provided.	End product is a humus rich substance which can be further used for filling or other purposes.	Large space requirements. Laborious and time consuming. Lack of knowledge of bacteria or fungi involved in the process.	Williams et al. 1992, Griest et al. 1995, Gunderson et al. 1997, Jarvis et al. 1998.
7	Biopile	Contaminated soils piled in heaps above ground. Temperature, pH and nutrients are also supplied along with forced aeration.	Cost effective. Requires less space.	Soil drying. Forced aeration requires specific set ups	Dubois et al. 1997
8	Landfarming	Contaminated soils are excavated and spread on lined beds	Low technology footprint, easy, less laborious.	Volatilization may occur, time consuming, leaching of contaminant may occur	Clark and Boopathy, 2007

Weighing the pros and cons of various microbial remediation treatment strategies and also considering their impact on the environment helps in making the choice of ideal remediation strategy. Such a strategy needs to reach the remediation goal as well as be eco-friendly. *In situ* microbial remediation can provide wholesome results for

remediation of explosives contaminated soil. Specifically, combined process of bioaugmentation and biostimulation can help reach the remediation goal faster with minimal impact on the environment. It not only is cost effective, faster, simpler but also cuts down the need for any type of soil transportation and replacement, making it an ideal choice for remediation. Though the field application of such a treatment strategy is yet to be implemented, we feel that, at the field level the remediation goal would be easily reached as the native microbial population of contaminated sites would complement the microbial species introduced. Also the biostimulation will help the microbes in better survival in the harsh environmental conditions.

The present work focusses on the bioaugmentation for remediation of explosive contaminated soil and sediments. Bioaugmentation here has been achieved by the use of carriers for delivery of microbes into the soil/ sediments.

2.4 Use of carriers in delivering inoculants into soil

There are different approaches for delivering organisms to the soil. Some use the bacteria in liquid culture stage. But, the disadvantages of the approach include improper distribution in soil profile, low shelf life and activity. Hence, new techniques were developed. The most promising being the use of carriers for delivery.

The support material on to which the microbial cell is immobilized is called the carrier. Selection of the carrier plays a crucial role in the whole process as this can affect the objective of the task (Martins et. al.2013).

The criteria for selection of carrier for development of immobilized systems and bioformulations include the following: (Zacheus et. al. 2000; Martins et. al. 2013; Bayat et. al. 2015).

1 Non-toxic and non-polluting

2 High cell mass loading capacity

3 High mechanical, biological and chemical stability

4 Long shelf life

5 Adequate functional groups

6 Low cost

7 Optimum diffusion distance from flowing media to centre of carrier

8 Easy separation of cells and carrier from media

9 Easy to handle and regenerate

The biocarriers can be classified as natural (zeolite, clay, ceramic, porous glass etc.) or synthetic (polymers like polyurethane, polyvinyl and other resins). They can be organic or inorganic in nature (Verma et. al. 2006). Figure 2.1 represents a schematic diagram depicting the classification of biocarriers.

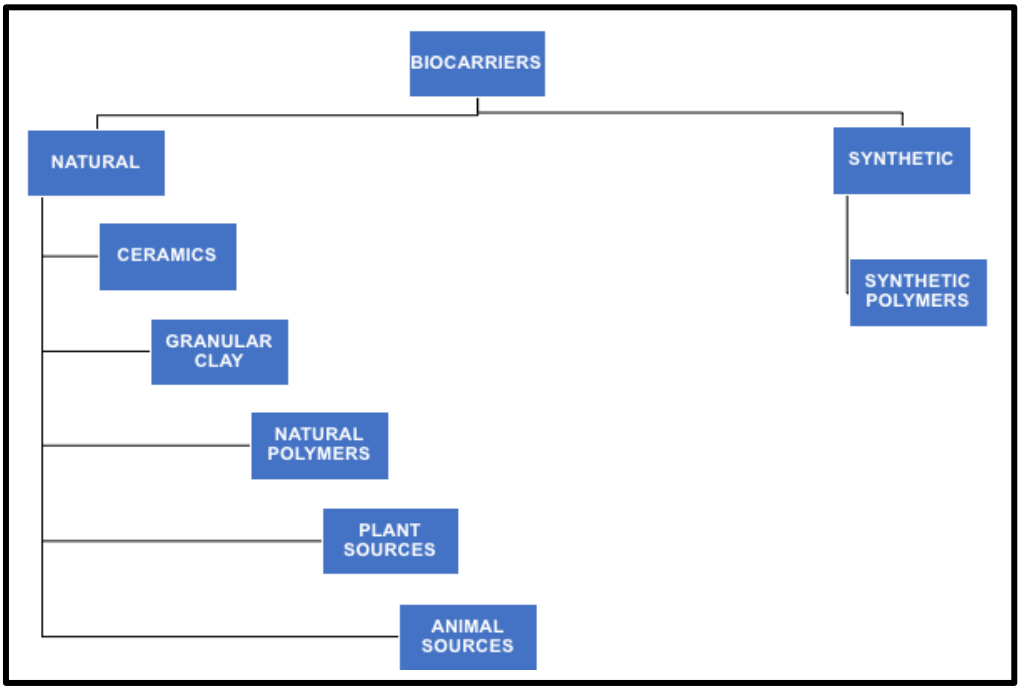


Figure 2.1: Classification of biocarriers

The immobilization of organisms on the carrier support material helps in the formation of bioformulations. These developed formulations mainly serve the following purposes: (Brar et al. 2006)

1. Stabilization of the microbes during application and storage
2. Ease of delivery of the microbial agent to the desired area.
3. Protection of the microbes from adverse environmental conditions.
4. Increased activity of the selected microbe.

The general components of bioformulations include carrier, enrichment additive and the active ingredient (A.I). These may be applied singly or in combinations for application (Schisler et al. 2004). Figure 2.2 represents the steps in formulation development and application, figure 2.3 represents the different classes of formulations (Brar et al. 2006) that can be developed for the purpose.

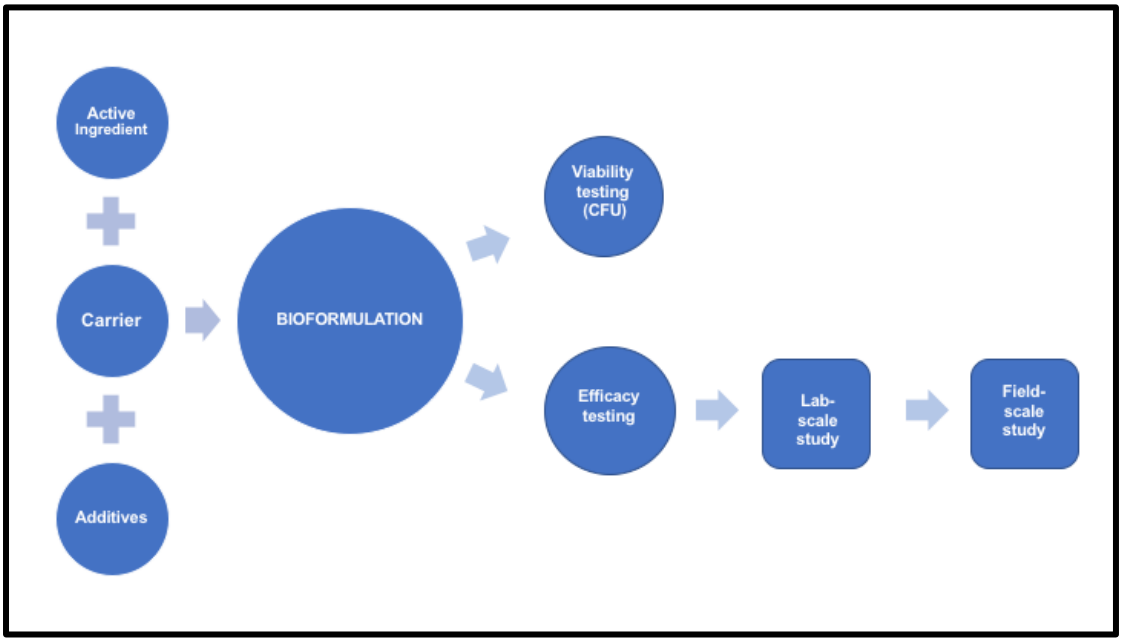


Figure 2.2: Schematic representation of bioformulation development, testing, lab scale studies and field application

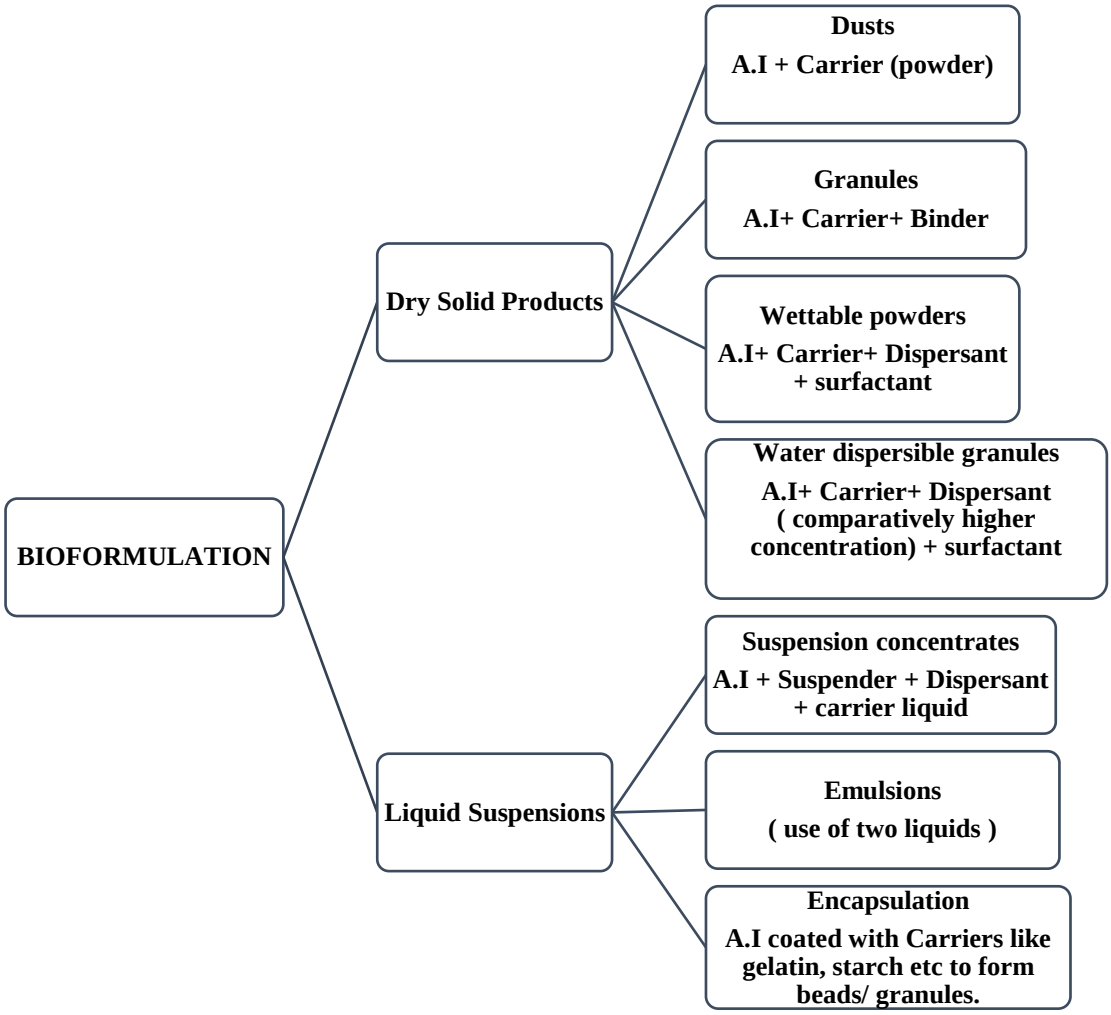


Figure 2.3: Schematic representation of different types of bioformulations

Bioformulations have been commonly used in agricultural applications (Pandey and Maheshwari, 2007; Omer, 2010; Jorjani et al. 2011). But, recently due to their eco-friendly nature and ease of application, bioformulations are being developed for treatment of soils contaminated with hazardous waste. Presently, lab scale studies mainly use the simple dust and granular formulations for bioremediation. Many literatures have cited the use of such formulations for successful delivery of microbes into the soil and have also reported this method to be more efficient in the degradation

process. Xu and Lu (2010) used peanut hull powder for the remediation of crude oil contaminated soil. Peanut hull powder exhibited porous structure, large surface area and strong adsorption capacity aiding to better immobilization of microbes and better oil removal. Similarly, Lawniczak et al. in 2011 immobilized bacterial consortia by biofilm formation for the degradation of phenol. They carried out the immobilization on various supports including clay pellets, paperboard, polypropylene and polyvinyl chloride rings. The clay pellets were concluded suitable for immobilization of phenol high degraders. There has also been literature citing the principle of encapsulation to develop bioformulation. One such example is the formulation developed by Li et al. in 2005. They studied the degradation of phenanthrene and pyrene in soil slurry reactors. They used Zoogloea sp. for the degradation process. This organism was immobilized on a biocarrier suitable for its colonization. The biocarrier was composed of polyvinyl alcohol (PVA) 10%, sodium alginate (SA) 0.5%, and powdered activated carbon (PAC) 5%. They didn't observe any toxicity due to the carrier on the immobilized cells. Also, they conducted studies to compare the degradation of free cultures and immobilized cells. Their results showed that the immobilized cells showed twice the degradation capacity than free cultures. These studies prove that the formulations are an effective microbe delivery system. Table 2.2 summarizes the application of carriers in the treatment of various soil contaminated by hazardous compounds.

Table 2.2: Carriers used in bioremediation of soil contaminated with organic hazardous compounds

Sl. No.	Hazardous contaminant	Biocarrier	Reference
1	High molecular weight hydrocarbons	Vermiculite	Straube et al. 2003
2	Petroleum	Wheat bran	Obuekwe and Al-Muttawa, 2001
		Saw dust	
		Styrofoam	Agamuthu et al. 2013
		Cow dung	
		Sodium alginate and diatomite	Maqbool et al. 2012
		Biochar and wood chips	Zhang et al. 2016
3	Diesel	Poly Vinyl Alcohol	Cunningham et al. 2004
4	PAH	Plant residues and biochar from wood chip, orange peel and pine needle.	Chen et al. 2012
		Corn cobs	Wang et al. 2012
		Rabbit food	Sayara et al. 2011
5	Crude oil	Chicken manure and rice husk	Adams et al. 2017
6	Toluene	Clay based material- bentonite and kaolin	Ting et al. 2010
7	Penta chloro phenol	Organomineralo complex derived from humid acid bound to zeolite	Dercova et al. 2007
8	Cypermethrin	Maize biochar	Liu et al. 2017
9	Mesotrione	Sugarcane baggase	Liu et al. 2015
10	Lindane	Cloth bags	Saez et al. 2014
11	Hexachlorohexane (HCH)	Corncob	Raina et al. 2008
12	Carbofuran	Corncob and sugarcane bagasse	Plangklang and Reungsang, 2009
13	Lead	Straw	Huang et al. 2006
		Agar beads	Hassiba et al. 2014
14	Mercury	Xanthan gum-based biopolymer	McCarthy et al. 2017
15	Copper	Alginate beads	Ozdemir et al. 2005a
16	Cadmium and cobalt	Alginate beads	Ozdemir et al. 2005b

For complete understanding of the remediation process, it is necessary to understand the various steps and enzymes involved. Hence, a detailed study of the degradation pathway is imperative to improve the process. The transformation and mineralization aspects of cyclic nitramines, viz, RDX and HMX have been widely studied in various microbial species. The various transformation pathways followed by microbes have been described briefly here. The bioremediation mechanism involved can include the loss or reduction of nitro functional group, enzymatic cleavage, α- hydroxylation or transfer of hydride ion. Generally, the intermediates formed during the pathways decompose instantaneously in water to produce nitrite, nitrous oxide and formaldehyde as common end products.

2.5 Biotransformation pathways of RDX

RDX biotransformation can occur by any of the three mechanisms, viz, two- electron reduction, denitration and direct enzymatic cleavage (Crocker et al., 2006).

2.5.1 Two electron reductive pathway

In this pathway, two or more redox equivalents, $2e^-/2H^+$ are added to RDX. It was originally proposed by McCormick et al., in 1981. It leads to the sequential formation of mono, di- and tri- nitroso derivatives of RDX, viz, MNX, DNX and TNX. Further they postulated the formation of hydroxylamino derivatives that may lead to the formation of hydrazines, methanol and formaldehyde. Some literature shows only the formation of methanol and formaldehyde as the products of pathway (Hawari et al. 2000, Oh et al. 2001, Adrian and Chow, 2001, Halasz et al. 2002, Adrian et al. 2003). Few bacterial species like, *Klebsiella pneumoniae* strain SCZ-1, *Shewanella sp.* HAW-EB5, *Clostridium bifermentans* strain HAW-1 have exhibited this as a minor pathway (Zhao

et al. 2002, 2003). *Methylobacterium spp.* and *enterobacteria* on the other hand have exhibited it as a major route for RDX degradation. Oxygen insensitive type I nitroreductases have been found to initiate two electron reduction pathways (Kitts et al. 2000). Another alternative is the formation of MNX, hydroxylamino, amino intermediates which led to the formation of 1,3,5-triamino-1,3,5-triazine. Such a pathway was observed by Zhang and Hughes in 2003. Bhushan et al. in 2002 observed the formation of MNX in *Aspergillus niger*. Instead of the formation of DNX and TNX, another intermediate methylenedinitramine (MDNA), a ring cleavage product was observed.

2.5.2 Direct enzymatic cleavage

In this pathway direct cleavage occurs at the C-N, N-NO_2 and C-H bonds leading to the formation of MDNA or bis-(hydroxymethyl) nitramine (BHNA). Such a transformation was observed by Hawari et al. 2000 and Halasz et al. 2002 during the anaerobic transformation of RDX by sewage sludge. It was postulated that, cleavage of C-N by hydrolase or α- hydroxylation of C-H can lead to the formation of MDNA. MDNA and BHNA on spontaneous decomposition in water led to the formation of nitrous oxide and formaldehyde.

2.5.3 Single electron transfer/ denitration

This is the most common pathway for RDX degradation. It is also a thermodynamically favorable route (Qasim et al. 2005). Both aerobic and anaerobic denitration occurs with different routes. During the anaerobic denitration, oxygen sensitive type II nitroreductases lead to the generation of a free radical anion (RDX^{-}) followed by the release of nitro group. This leads to the destabilization of the molecule in turn causing

ring cleavage with the formation of MDNA. MDNA further decomposes to produce nitrous oxide and formaldehyde (Zhao et al. 2002, 2003, Bhushan et al. 2002). During aerobic denitration, two single electron transfers occur leading to the release of two nitro groups. In such a scenario, 4-nitro-2,4-diazabutanal (NDAB), nitrous oxide, ammonium, formaldehyde and carbon dioxide are produced (Bhushan et al. 2003, Fournier et al. 2002). Bacterial species like, *Rhodococcus rhodochrous* strain 11Y, *Gordonia sp*. strain KTR 9, *Williamsia sp.* KTR4 (Seth-Smith et al. 2002, Thompson et al. 2005) have been postulated to follow this pathway for RDX degradation. But it has been found that these strains could not further metabolize NDAB, but *Methylobacterium sp.* strain JS178 has been found to degrade NDAB to nitrous oxide and carbon dioxide (Fournier et al. 2005).Table 2.3 shows the different bacterial isolates and the intermediates generated during the bioremediation of RDX.

Table 2.3: Selected microbial species that degrade RDX under aerobic/ anaerobic conditions and the intermediates formed

Sl. No	Organism	Metabolic path	Intermediates formed	References
1	Mixed culture	Anaerobic	MNX, DNX, TNX, Hydroxylamino-DNX	Adrian and Chow, 2001
2	*Acetobacterium malicum* strain HAAP-1	Anaerobic	MNX,formaldehyde, MDNA, N_2O	Adrian and Arnett, 2004
3	Granular sludge	Anaerobic	MNX, DNX, TNX	An et al., 2010
4	*Desulfovibrio sp.*	Anaerobic	MNX, DNX	Arnett and Adrian, 2009
5	Mixed culture	Anaerobic	MNX, DNX, TNX, acetate, hydrazine, 1,1-dimethylhydrazine, 1,2-dimethylhydrazine, MDNA, BHNA , formaldehyde, methanol	Beller, 2002
6	Mixed culture	Anaerobic	Trace amounts of MNX in sulfate unamended conditions.	Arnett et al., 2009

Sl. No	Organism	Metabolic path	Intermediates formed	References
7	*Rhodococcus sp.*	Aerobic	NDAB (under aerobic conditions) MDNA (under microaerophilic conditions)	Bernstein et al., 2011
8	*Rhodococcus sp*. strain DN22	Aerobic Microaerophilic	NDAB, Formaldehyde, ammonium and Nitrite ions, N_2O, CO_2 MEDINA	Coleman et al., 1998; Fournier et al., 2002; Bhushan et al., 2003; Fuller et al., 2010; Halasz et al., 2010
9	*Stenotrophomonas maltophilia* PB1	Aerobic	C3H9N3O5 M/Z 167, C3H8N3O4 M/Z 136	Binks et al., 1995
10	Microbial consortia containing 16 RDX degraders	Aerobic	MNX, DNX, TNX (analyzed) MDNA, BHNA, NDAB (Proposed)	Cho et al., 2013
11	Microbial consortia	Anaerobic	MNX, DNX, TNX	Freedman and Sutherland, 1998
12	*Pseudomonas fluorescens I-C* *Pseudomonas putida II-B*	Anaerobic Aerobic (not successful)	MDNA, formaldehyde, NDAB (minor amount)	Fuller et al., 2009
13	*R. rhodochrous* 11Y *Rhodococcus sp.* Strain A	Aerobic, Microaerophilic, Anaerobic (Not successful)	NDAB (Microaerophilic and aerobic), MDNA (aerobic)	Fuller et al., 2010
14	Sludge	Anaerobic	MDNA, nitrous oxide, formaldehyde and carbon di oxide	Halasz et al., 2002
15	Mixed consortia	Anaerobic	Pathway 1: MNX, DNX Pathway 2: MEDINA, BHNA Both pathways led to the production of nitrous oxide, formaldehyde, methanol, formic acid, methane and carbon di oxide	Hawari et al., 2000
16	*Rhodococcus sp*. Cytochrome P450	Aerobic Anaerobic	NDAB, nitrite, formaldehyde MDNA, nitrite, formaldehyde	Jackson et al., 2007

Sl. No	Organism	Metabolic path	Intermediates formed	References
17	*Clostridium bifermentans*	Anaerobic	MNX, DNX, N2O, HCHO,CH3OH	Zhao et al., 2003
18	*Clostridium sp.* EDB2	Anaerobic	NO2., N2O, HCHO, HCOOH,CO2	Bhushan et al., 2004
19	*Geobacte rmetallireducens* strain GS-15	Anaerobic	MNX, DNX, TNX, MDNA, NO2, HCHO	Kwon and Finneran, 2008
20	*Gordonia TR4 /Williamsia KTR9*	Aerobic	NDAB, NO2., HCHO, CO2	Thompson et al., 2005; Indest et al., 2010
21	*Klebsiella pneumoniae* strain SCZ-1	Anaerobic	MDNA, N2O, HCHO,CH3OH, CO2	Zhao et al., 2002
22	*Phanerochaete chrysosporium*		MNX, N2O, CO2	Shermata and Hawari, 2000
23	*R. rhodochrous* strain 11Y	Aerobic Anaerobic	NDAB, NO2, HCHO, Formate, formaldehyde MDNA, NO2, HCHO	Jackson et al., 2007; Seth-Smith et al., 2002 and 2008
24	*Rhizobium rhizogenes* BL, *Burkholderia sp.* BL	Aerobic	NO2, NO3, N2O, HCHO,CO2	Lee and Brodman, 2004
25	*Rhodococcus sp*. strain YH1	Anaerobic	MNX, DNX, TNX, HCHO	Hawari et al., 2000; Nejidat et al., 2007
26	*Shewanella halifaxensis* HAW-EB4	Anaerobic	MNX, DNX, TNX, MDNA, NDAB, HCHO, N2O	Zhao et al., 2003
27	*Shewanella oneidensis* MR-1	Anaerobic	MNX, DNX, TNX, MDNA, NDAB, HCHO, N2O	Perreault et al., 2012
28	*Acremonium sp*. HAW-OCF3	Aerobic	MDNA,MNX, DNX, TNX, HCHO,N2O	Bhatt et al., 2006
29	*Aspergillus niger*	Anaerobic	MNX, DNX, TNX, HCHO,N2O	Bhushan et al., 2002
30	*Cladosporium cladosporioides*	Aerobic	NO2, NO3, N2O, HCHO,CO2	Lee and Brodman, 2004

Sl. No	Organism	Metabolic path	Intermediates formed	References
31	*Phanerochaete chrysosporium*	Aerobic	CO2,N2O	Fournier et al., 2004; Sheremata and Hawari, 2000; Stahl et al., 2001
32	*Geobacter metallireducens strai*n GS-15 *Geobacter sulfurreducens strain* PCA	Anaerobic	MNX, DNX, TNX	Kwon and Finneran, 2006
33	*Providencia retgeri* B1, *Morganella morganii* B2, and *Citrobacter freundii* NS2	Aerobic followed by Oxygen depleted conditions	MNX, DNX, TNX,	Kitts et al., 1994
34	Mixed consortia	Anaerobic	MNX, DNX, TNX, 1,1-dimethylhydrazine,and1,2-dimethylhydrazine HCHO, CH_3OH	McCormick et al., 1981
35	Mixed consortia	Anaerobic	MNX, DNX, TNX, Nitrous oxide, MDNA, carbon di oxide,	Oh et al., 2001
36	Mixed consortia	Anaerobic	MNX, DNX, TNX, MEDINA	Perumbakkam and Craig, 2012
37	Mixed consortia	Anaerobic	MNX, DNX, TNX	Roh et al., 2009
38	Mixed consortia	Aerobic/ Anaerobic	MNX, DNX, TNX, NDAB, nitrite	Ronen et al., 2008
39	*Acetobacterium paludosum*	Anaerobic	Formate, N_2O	Sherburne et al., 2005
40	Mixed consortia	Aerobic	N_2O, CO_2	Sheremata et al., 2001
41	*Clostridium acetobutylicum*	Anaerobic	mononitroso-(II), monohydroxyl- amino-(III), mononitroso-monohydroxylamino-(IV), monoamino-(V), diamino-(VI), and triamino-(VIII) compounds	Zhang and Hughes, 2003

2.6 Biotransformation pathways of HMX

Because of its structural similarity to RDX, HMX has been found to follow similar mechanism in its transformation. Though it's more difficult to degrade HMX than RDX, microbes generally causing transformation of RDX can also transform HMX (Boopathy 2001, Bhushan et al. 2004, Van Aken et al. 2004). Biotransformation of HMX has been reported by the following pathways.

2.6.1 Two electron reductive pathway

Like RDX, HMX also undergoes two electron reductive pathway with the formation of nitroso derivatives. This route has generally been proposed in case of anaerobic degradation of HMX. The nitroso derivatives detected include; Octahydro-1-nitroso-3,5,7-trinitro-1,3,5,7 tetrazocine (1NO-HMX),Octahydro-1,3-dinitroso-5,7-dinitro-1,3,5,7 tetrazocine or Octahydro-1,5-dinitroso-3,7-dinitro-1,3,5,7 tetrazocine (2NO-HMX), Octahydro-1,3,5-trinitroso-7-nitro-1,3,5,7-tetrazocine (3NO-HMX), octahydro-1,3,5,7-tetranitroso-1,3,5,7-tetrazocine (4NO-HMX) (Zhao et al. 2004a, 2004b, 2004c, Monteil- Rivera et al. 2003).

2.6.2 Single electron transfer/denitration

The single electron transfer led to the release of nitrite accompanied by formation of ring cleavage products MDNA and NDAB. The end products of such a pathway were observed to be nitrous oxide, formic acid, ammonim and formaldehyde (Bhushan et al. 2003). Such a mechanism was observed in Clostridium bifermentans HAW1 and *Phanercheate chrysosporium* (Zhao et al. 2004b).

2.6.3 Alternate pathway for HMX transformation

Yet another pathway by which HMX can be transformed was elucidated by Hawari et al (2001). This pathway postulates the formation of ring cleavage intermediates MDNA

and BHNA, which further led to the formation of formaldehyde, formic acid and nitrous oxide as end products. Table 2.4 discusses the various pathways involved in HMX

Table 2.4: Selected microbial species that degrade HMX under aerobic/ anaerobic conditions and the intermediates formed

Sl. No	Organism	Metabolic path	Intermediates formed	References
1	Methanogenic mixed culture	Anaerobic	Nitroso-HMX intermediates	Adrian et al., 2003
2	*Clostridium sp*. strain EDB2	Anaerobic	NO2 , N2O, HCHO, and HCOOH	Bhushan et al., 2004
3	*Citrobacter freundii* NS2	Anaerobic	Mononitroso-HMX, Dinitroso-HMX	Kitts et al., 1994
4	*Morganella morganii*	Anaerobic	Mononitroso-HMX, Dinitroso-HMX	Kitts et al., 1994
5	*Providencia retgeri*	Anaerobic	Mononitroso-HMX, Dinitroso-HMX	Kitts et al., 1994
6	*Klebsiella pneumoniae* SCZ1	Anaerobic	NDAB, MDNA, Formaldehyde, nitrous oxide	Zhao et al., 2004b
7	*Clostridium bifermentans* HAW-1	Anaerobic	NDAB, MDNA, Formaldehyde, nitrous oxide	Zhao et al., 2004b
8	*Clostridium sp*. HAW-G4	Anaerobic	NDAB, MDNA, Formaldehyde, nitrous oxide	Zhao et al., 2004b
9	*Clostridium sp*. HAW-E3	Anaerobic	NDAB, MDNA, Formaldehyde, nitrous oxide	Zhao et al., 2004b
10	*Clostridium sp*. HAW-HC1	Anaerobic	NDAB, MDNA, Formaldehyde, nitrous oxide	Zhao et al., 2004b
11	*Clostridium sp*. HAW-EB17	Anaerobic	Mono, di and tri-nitroso-HMX	Zhao et al., 2004c
12	*Desulfovibrio sp*. HAW-EB18	Anaerobic	Mono, di and tri-nitroso-HMX	Zhao et al., 2004c
13	*Fusobacteria* isolate HAW-EB21		Mono, di and tri-nitroso-HMX, NDAB, MDNA, Formaldehyde, nitrous oxide	Zhao et al., 2004c, Zhao et al., 2004b
14	*Methylobacterium sp*. BJ001	Aerobic	Mono, di and tri-nitroso-HMX	Van Aken et al., 2004
15	*Phanerochaetechrysosporium*	Anaerobic	Mono, di and tri-nitroso-HMX, NDAB, MDNA, Formaldehyde, nitrous oxide	Fournier et al., 2004
16	Mixed culture	Anaerobic	Mono, di and tri-nitroso-HMX	Shen et al., 2000

Though many established abiotic technologies (physical and chemical) are available, bioremediation has emerged as an ecofriendly alternative. Successful implementation of bioremediation at a particular site depends majorly on the physico-chemical characterization and type of pollutant. This helps in deciding the type of technique to be applied (*ex situ* or *in situ*) for successful decontamination. *Ex situ* technologies are mainly associated with excavation and replacement, thereby making them energy intensive as well as expensive processes. On the other hand, insitu technologies suffer from the drawbacks of being slow, involve uncertainty of the conditions under the soil surface. It has become imperative to adopt technologies that are not only environment friendly, but also, cost effective with high efficiencies that can be transformed into the field. With many emerging technologies in place, a plethora of options have opened up to clean up explosive contaminated soils. Research on developing and optimizing microbial remediation technologies with eco-friendly carriers for field scale application is the need of the hour.

Critical assessment of the available literature on bioremediation of explosive contaminated soils revealed that factors such as retention and bioavailability of the target contaminant is essential for enabling maximum contact of pollutant with the potential microorganism. This could be achieved by loading the microorganism on a suitable biocarrier. Carrier chosen for immobilization of microbe should be ecofriendly, economic and sustain microbial growth and maintenance.

Bioremediation of RDX and HMX contaminated soil using natural carriers for immobilization of explosive degrading microbes has not been reported yet. Hence, this study aims to utilize natural carriers that not only contribute in delivery of the microbe but also aid in the remediation process, so as to achieve sustainable remediation goals.

Chapter 3
Materials and Methods

CHAPTER 3

MATERIALS AND METHODS

Bioremediation studies of explosives were carried out in aqueous, soil and sediment phases. Aqueous phase studies were carried out to evaluate the efficiency of three explosive degrading microbes isolated from explosive contaminated sites. *Janibacter cremeus* was observed to exhibit highest degradation potential of RDX and HMX in 30 days in comparison to *Pseudomonas mosselii* and *Pseudomonas entomophila*. Due to its high degradation potential and it being a facultative anaerobe, *J. cremeus* was selected for soil remediation studies.

Two novel eco- friendly bioformulations were prepared using *J. cremeus* for the remediation process. Soil remediation studies were carried out at microcosm level for 35 days under unsaturated and saturated conditions. The remediation was monitored by analyzing the live cell count, bulk enzyme activity, nitrite released and residual explosive concentration. The intermediates produced during the remediation process were also observed.

Sediments contaminated with RDX/ HMX were remediated using the bioformulations at mesocosm level. Sediment A was collected from an RDX manufacturing facility in India. It was observed to be acidic and contaminated with high concentration of RDX. The mesocosm remediation was carried out at room temperature for 5 months. Sediment B was collected from an HMX manufacturing facility in India. It was observed to be highly acidic and contaminated with RDX and HMX. The mesocosm remediation was carried out at room temperature for 3 months. The change in pH, bulk

enzyme activity, nitrite concentration and residual explosive concentrations were monitored

The major analytical instruments used for parameter analysis include UV-visible spectrophotometer, Scanning electron microscopy (SEM), Fourier Transmission Infrared Spectroscopy (FTIR), High Performance Liquid Chromatography (HPLC), Mass spectroscopy (MS).

3.1 Chemicals

RDX and HMX (>99.9 % purity) was obtained from a manufacturing facility in India. All other chemicals, microbial growth media and reagents used throughout were of analytical grade. All the solvents used for quantitative explosive analysis were of High-Performance Liquid Chromatography (HPLC) grade. LC-MS grade solvents were used for LC-MS/MS analysis.

3.2 Media composition

The primary cultures of the selected bacterial strainswere maintained on Tryptic Soy Agar (TSA) [(g/L of distilled water): casein peptone (17), soya peptone (3), NaCl (5), di-potassium phosphate (2.5), dextrose (2.5) and agar (15)] plates.

The aqueous phase remediation studies were carried out in Minimal Salt Medium (MSM) [(g/L of distilled water): K_2HPO_4 (1.7), KH_2PO_4 (1.3), $MgSO_4.7H_2O$ (0.0616), Glucose (0.9), Succinic acid (0.59), Glycerol (9.2) and trace elements (mg/L of distilled water) $FeSO_4 \cdot 7H_2O$ (2), $CaCl_2.2H_2O$ (0.03), $MnCl_2.4H_2O$ (0.5), H_3BO_3 (0.05), $ZnCl_2$ (0.05), $CuCl_2$ (0.03), $Na_2MoO_4 \cdot 2H_2O$ (0.01), $CoCl_2 \cdot 6H_2O$ (0.5), $NiCl_2 \cdot 6H_2O$ (0.05)] (Thompson et al. 2005).

Bioformulations were prepared by cultures grown in Luria-Bertani broth [(g/L of distilled water): Caesin enzymic hydrolysate (10), yeast extract (5), sodium chloride (10), pH 7.5±0.2].

The live cell counts were monitored on Nutrient agar plates[(g/L of distilled water): peptone (5), NaCl (5), beef extract (1.5), yeast extract (1.5), Agar agar (15), pH 7.4±0.2].

3.3 Microbial culture characterization and growth conditions

Three distinct isolates: Pseudomonas mosselli, *Pseudomonas entomophila* and *Janibacter cremeus* were used in the study. *Pseudomonas mosselii* and *Pseudomonas entomophila* were isolated from explosive contaminated effluent whereas, *Janibacter cremeus* was isolated from explosive contaminated soil. These microbes were identified using 16s rRNA sequencing and the lyophilized culture of the same were obtained from Institute of Microbial Technology, (IMTECH),

Chandigarh, India. The classification and characterization of the bacterial isolates have been given in table 3.1 and 3.2 respectively.

Table 3.1: Scientific classification of the bacterial isolates screened (Dabboussi et al. 2002, Hamada et al. 2013, Dieppois et al. 2015)

Classification	***P. mosselii***	***P. entomophila***	***J. cremeus***
Kingdom	Bacteria	Bacteria	Bacteria
Phylum	Proteobacteria	Proteobacteria	Actinobacteria
Class	Gamma Proteobacteria	Gamma Proteobacteria	Actinobacteria
Order	Pseudomonadales	Pseudomonadales	Actinomycetales
Family	Pseudomonadaceae	Pseudomonadaceae	Intrasporangiaceae
Genus	Pseudomonas	Pseudomonas	Janibacter
Species	*P. mosselii*	*P. entomophila*	*J. cremeus*

Table 3.2: Characterization of the bacterial isolates screened

Characterization	***P. mosselii***	***P. entomophila***	***J. cremeus***
MTCC No.	12871	12852	12869
Gram staining	Negative	Negative	Positive
Motility	Motile	Motile	Non- motile
Shape	Rods	Rods	Rods- coccoid
Colony morphology (on nutrient agar)	Circular and non-pigmented	Fluorescent colonies due to pigment produced	Circular and creamy
Aerobic/ anaerobic	Aerobic	Aerobic	Facultative anaerobe

The cultures were revived on TSA plates and were then sub cultured to obtain pure cultures (figure 3.1) which were then grown for three generations in MSM.

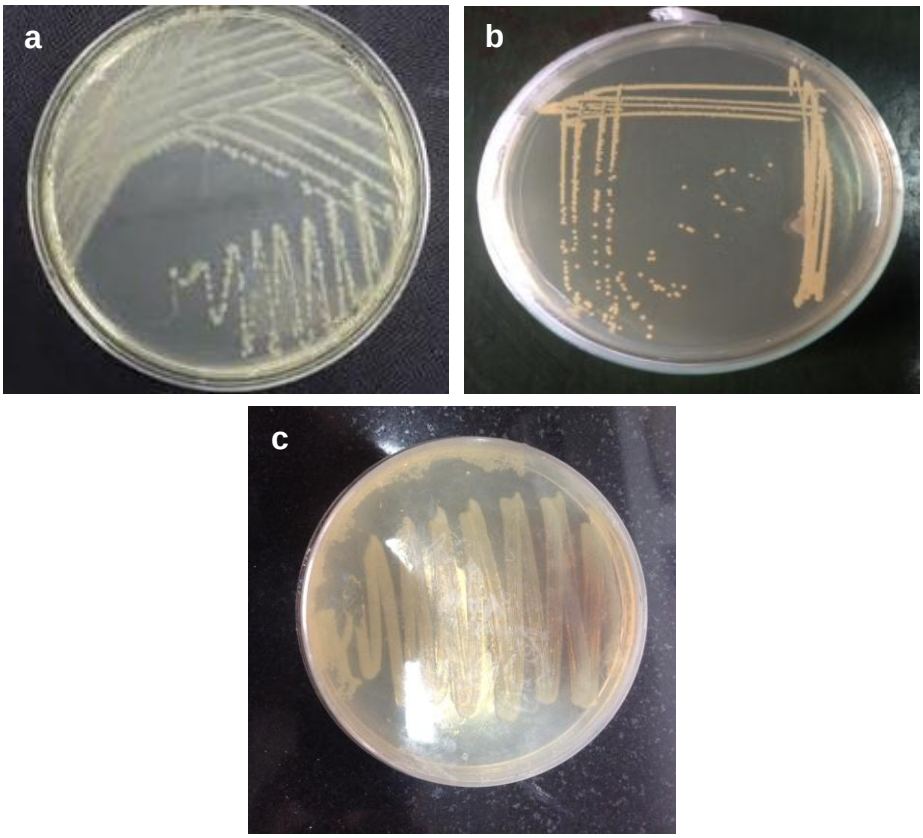


Figure 3.1: Pure cultures of a) *Janibacter cremeus* b) *Pseudomonas mosselii* c) *Pseudomonas entomophila*

The growth of the culture was monitored by observing its absorbance at 600 nm using Perkin Elmer UV- Visible spectrophotometer (Model Lambda 650S). The third generation culture acclimatised to growth in minimal medium was then used in the experiments in its log phase with an absorbance of greater than 0.8 at 600 nm.

3.4 Aqueous phase bioremediation studies

The bacterial isolates, in their log phase were inoculated in the minimal medium (MSM) spiked with 60 mg/L of RDX or 6mg/L of HMX as sole source of nitrogen under sterile conditions. The bacteria were inoculated at 5% (v/v) in the medium containing explosive. Two control experiments were also set up, viz, MSM with the bacterial inoculation but without RDX/HMX and MSM with RDX/HMX but without inoculation. The study was conducted for a period of 30 days under sterile conditions on an incubator shaker at 35° C and 120 rpm. The samples were withdrawn on regular intervals to monitor the residual explosive concentrations. The growth of the bacteria was monitored at 600 nm using UV-Visible spectrophotometer. Nitrite released was also monitored. The bacterial species reported to have exhibited best degradation rates was selected for soil studies.

3.4.1 Growth of bacterium

The growth of bacterium in the MSM was monitored using a Perkin Elmer, Model Lambda 650S UV-Visible Recording Spectrophotometer. 2 mL of sample aliquots were withdrawn at regular intervals. The samples were directly analysed for bacterial growth by observing the absorbance at 600 nm.

3.4.2 Monitoring of the nitrite ion released during bioremediation

3.4.2.1 Preparation of aqueous phase samples

1 mL of sample aliquot was withdrawn at regular intervals. The bacterial cells were removed by centrifugation at 10000 rpm for 10 min at 4 °C. The supernatant was used for analysis.

3.4.2.2 Analysis

To 600 µL of the supernatant, 150 µL of sulphanilamide solution was added. The reaction mixture was incubated at room temperature for 5 minutes. This was followed by the addition of 150 µL of N-(1-naphthyl) ethylene diamine dihydrochloride (NEDD) solution. The reaction was allowed to proceed at room temperature for 20 minutes. The volume of the reaction mix was raised to 3 mL by addition of distilled water (2.1 mL). The absorbance of the resulting mix was recorded at 540 nm. The concentration of nitrite present was measured by preparing sodium nitrite standard curve (Mercimek et al. 2013). The sodium nitrite standard curve has been given in figure 3.2.

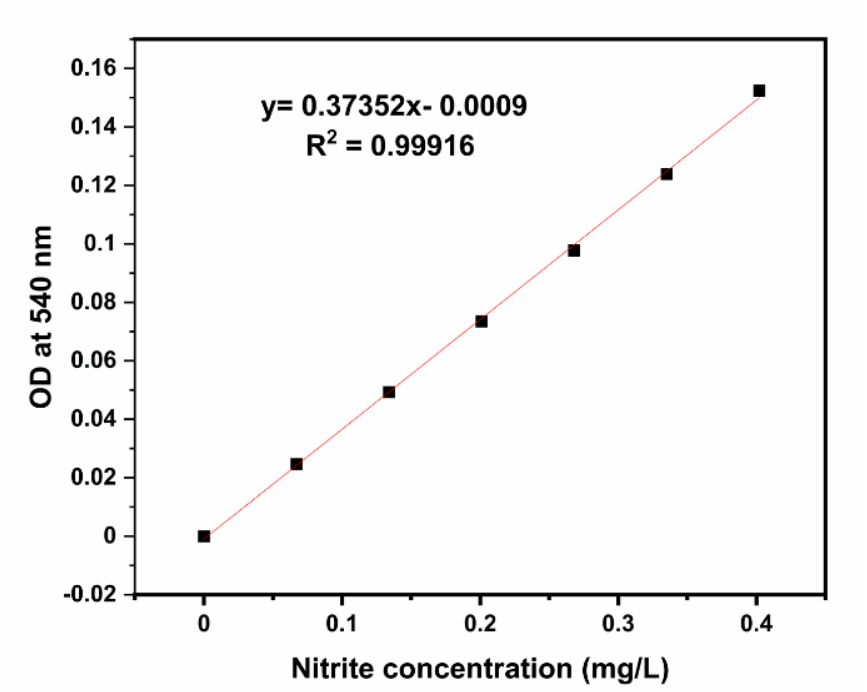


Figure 3.2: Standard graph for nitrite estimation

3.4.3 Residual explosive concentration

3.4.3.1 Preparation of aqueous phase samples

2 mL of sample aliquot was centrifuged at 10000 rpm for 10 min at 4 °C to obtain cell free supernatant. To 1 mL of the supernatant, 1 mL of acetonitrile was added. The

sample was then filtered through 0.22 µm polytetrafluorethylene (PTFE) Millex filters (USEPA Method 8330 B).

3.4.3.2 Analysis

A High Performance Liquid Chromatograph (HPLC) equipped with a diode array detector (Dionex Ultimate 3000, Thermo Fisher, United States) was used to determine the residual explosive (RDX/HMX) concentration. Reversed phase C-18 column (3 µm, 150 x 4.6 mm) was used as the stationary phase. Separation was obtained using acetonitrile: water (50:50) as mobile phase, with a flow rate of 1mL/ min. RDX and HMX were detected at 254 nm wavelength (USEPA Method 8330 B). The concentration of explosives was calculated from the standard graph obtained using known concentrations of explosives. Figures 3.3 and 3.4 shows the calibration curves for RDX and HMX respectively.

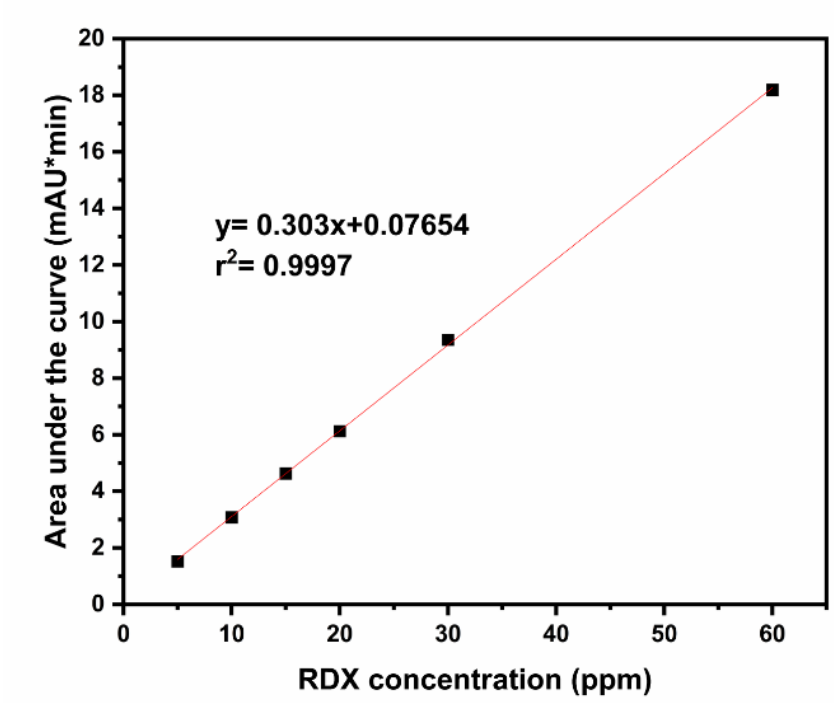


Figure 3.3: Standard graph estimation of RDX concentration

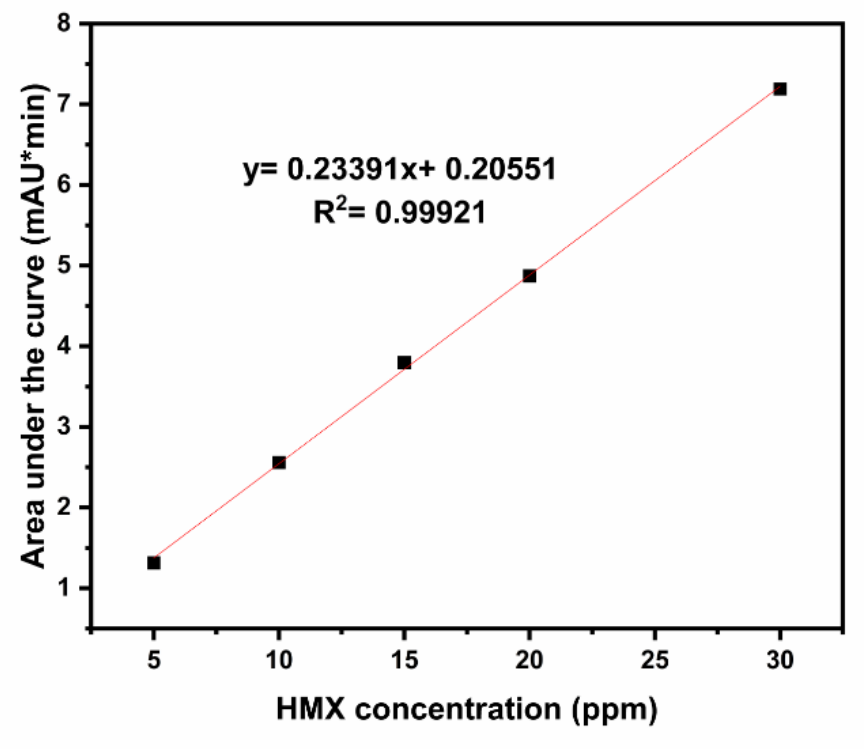


Figure 3.4: Standard graph for estimation of HMX concentration

3.5 Soil phase Bioremediation Studies

3.5.1 Preparation of bioformulations

Two distinct bioformulations were prepared for the remediation of explosive contaminated soil and sediments. Bioformulation 1 (BF1) was a powder formulation, whereas, bioformulation 2 (BF 2) was a wettable powder formulation.

3.5.1.1 Preparation of carriers for bioformulations

Two bioformulations were prepared using different carrier mixes. For the preparation of bioformulation 1 (BF 1), calcite (>90% purity) and cocopeat was used. Calcite was chosen as the inorganic clay carrier because of its high surface area, availability in plenty, ease of use and mild alkaline nature. Cocopeat was selected as the organic amendment because of its eco-friendly nature, rich nutrient and organic content and

adsorptive properties. Cocopeat was air dried and sieved through 600 μm sieve to get uniform surface area. Calcite powder was used as such for the experiments.

Bioformulation 2 was prepared in the form of wettable powder, with egg shell, cocopeat, a surfactant and a dispersing agent as the constituents. Egg shells were obtained from a local egg vendor. It was first washed thoroughly, air dried and then finely powdered before use. Cocopeat was procured from a local farm vendor. It was passed through a 600 μm sieve for uniform size distribution. Tween, a very commonly used detergent was used as a surfactant. Sodium bi carbonate functioned as a dispersing agent as well as a buffering agent.

All the carrier components for both the formulations were sterilized by autoclaving twice at 121 °C, 15 lbs pressure for 15 min with an intermittent incubation of 24 h.

3.5.1.2 Enrichment of *J. cremeus*

The explosive degrading microbe with the highest degradation potential (*J. cremeus*) from the three cultures evaluated, was selected for the development of bioformulation for the remediation of explosive contaminated soil and sediments. The bacterium was first acclimatized to increasing concentrations of explosive. For this, *J. cremeus* was inoculated in MSM spiked with 1 and 10 mg/L HMX and RDX respectively. It was then incubated at 35 °C and 120 rpm till it reached its log phase. Once in its log phase, the bacterial culture was transferred to MSM containing 2 and 20 mg/L of HMX and RDX respectively. The same process was repeated to acclimatize the bacteria to a mixed concentration of 6 and 60 mg/L HMX and RDX respectively. This enriched bacterial culture was used for the preparation of the bioformulations.

3.5.1.3 Preparation of the bacterial suspension

The enriched bacterial culture of *J. cremeus* was cultured in Luria- Bertani broth at 35 °C for 48 h in an orbital incubator shaker at 120 rpm. The culture was grown to a minimum cell count of 10^9 before being used as an inoculum for development of formulation (IS: 8268 1976, Pandey and Maheshwari, 2007). The cells were then harvested by centrifugation at 10000 rpm for 15 min and resuspended in sterile distilled water and stored at 4°C until further use.

3.5.1.4 Development of BF1 of *J. cremeus*

The harvested cultures with a high CFU of about 10^{14} were used to prepare the bioformulation (1 Kg). The carriers, calcite and cocopeat were added in the ratio 8:2 (w/w). To 400 mL bacterial suspension of *J. cremeus*, 800 g of calcite and 200 g of cocopeat was added in sterile conditions (figure 3.5). It was mixed thoroughly to ensure even distribution of bacterial cells.

3.5.1.5 Development of BF2 of *J. cremeus*

The harvested cultures with a high CFU of about 10^{14} were used to prepare the bioformulation (1 Kg). The carriers, egg shell powder, cocopeat, tween, sodium bi carbonate was added in the ratio 8:1:0.6:0.4 (w/w). To 400 mL bacterial suspension of *J. cremeus*, 800 g of egg shell powder, 100 g of cocopeat, 60 g of tween and 40 g of sodium bi carbonate was added in sterile conditions (figure 3.5). The mixture was mixed thoroughly for uniform distribution of bacterial cells.

The prepared formulations were dried in aseptic conditions to remove excess moisture for 24 h and then sealed in sterile polyethylene bags. The bags were sealed such that, there was a head space of at least 75% to provide proper aeration to the immobilized bacteria and was stored at 4 °C (Pandey and Maheshwari, 2007).

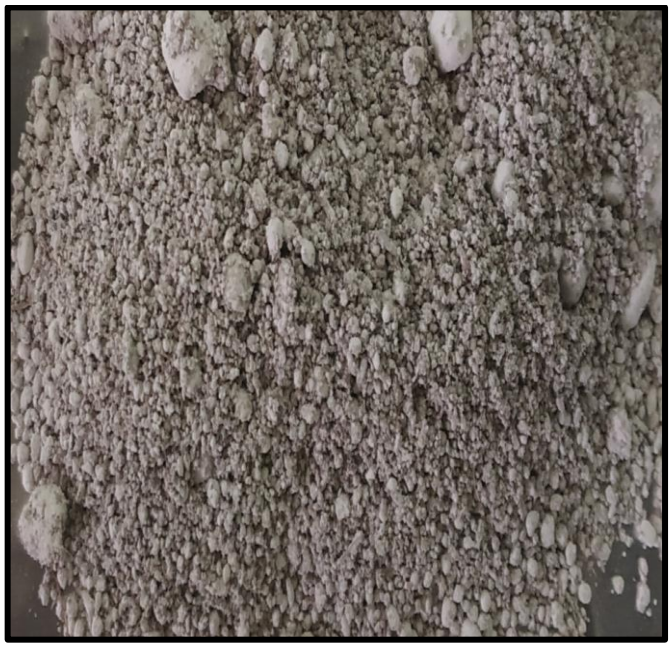


Figure 3.5: Bioformulation 1 developed by immobilization of *J. cremeus* on calcite and cocopeat mix.

Figure 3.6: Bioformulation 2 developed by immobilization of *J. cremeus* on egg shells, cocopeat, tween and sodium bi carbonate

3.5.2 Viability of the immobilized microbes

The developed bioformulations was tested for its viability when stored at 4°C for 6 months according to the Indian standards (IS: 8268, 1976). The viability was monitored

by observing the Colony Forming Units (CFU) of the developed formulation Also, the developed formulation was viewed under EVO18 Zeiss Scanning Electron Microscope (SEM) to confirm the immobilization of the bacteria onto the carrier mix.

3.5.2.1 Colony forming units (CFU)

The colony forming unit (CFU) was used as an indicator of the viability of microbial cells in the developed bioformulation. It was calculated using the plate count method. Briefly, 1 g of BF was suspended in 9 mL autoclaved distilled water. Serial dilution was performed using autoclaved distilled water upto 10^{10} dilutions. 0.1 mL of the dilution was plated on nutrient agar plates, and spread well using an L-spreader. The plates were then incubated at 35° C for 24-48 h. The colonies were then counted using a colony counter. The CFU was calculated using the formula:

$$CFU = \frac{No.of\ colonies \times\ dilution\ factor}{volume\ plated}$$

3.5.2.2 Scanning Electron Microscopy (SEM)

The solid samples (bioformulations) were air dried and coated with Au-Pd by sputter coating and then viewed under EVO18 Zeiss scanning electron microscope.

3.5.3 Soil microcosm studies for bioremediation of RDX and HMX

Uncontaminated background soils from explosive manufacturing facilities were subjected to remediation with two different prepared bioformulations. The RDX/HMX degradation efficiency of the bioformulations (BF 1/ 2) were evaluated under two different moisture conditions and the degradation pathways were elucidated.

3.5.4 Soil preparation

Uncontaminated sandy loam soils from an RDX/HMX manufacturing facility in India were used in the study. Soil samples collected from top layer (0-20 cm depth) were air-dried, ground with a pestle to break the aggregates, passed through 2 mm sieve. The soils were characterized (Table 3.3) for their texture, pH, electrical conductivity (E.C), bulk density, moisture content, water holding capacity and total organic matter content (TOM).

Table 3.3: Physico-chemical characterization of soils used in RDX (soil A) and HMX (soil B) bioremediation studies

Sl. No.	Physico- chemical property	Soil A	Soil B
1	Texture	Sandy loam	Sandy loam
2	pH	6.91	7.09
3	Electrical conductivity (mS/ m)	0.9	0.5
4	Bulk density (g/ cm^3)	1.15	1.12
5	Moisture content (%)	1.007	3.9345
6	Water holding capacity (%)	46.8	65.813
7	Total organic carbon (%)	0.0611	0.0963

3.5.4.1 Soil texture analysis

The texture of soils used in the study (soil A/B) was analysed using the standard USDA (United States Department of Agriculture) method as adapted from Thien et al. 1979 as described in the classification triangle (figure 3.7).

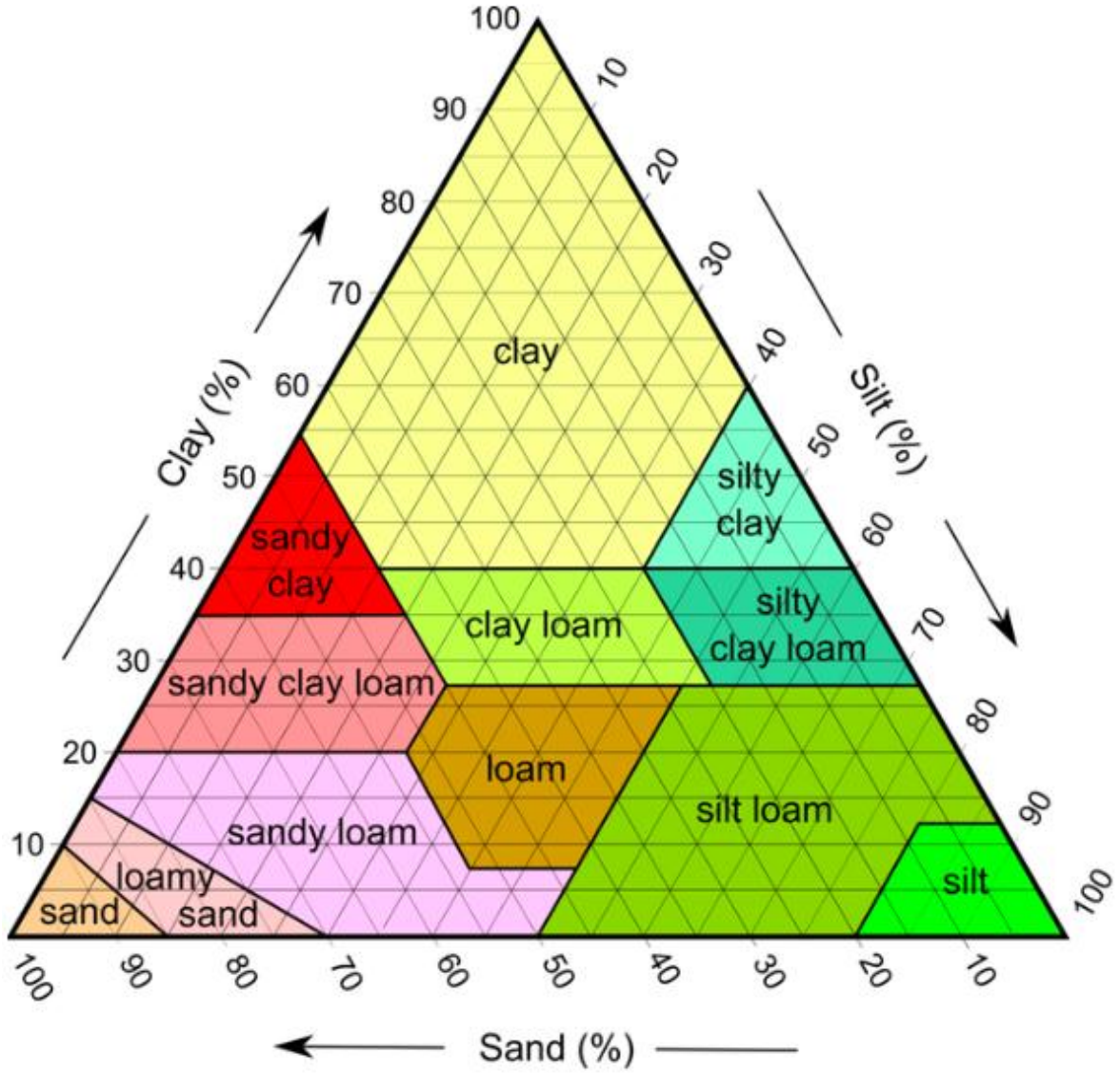


Figure 3.7: Classification triangle for soil texture determination

3.5.4.2 Soil pH

The pH soil samples were measured using method given by Rhoades (1992). 10 g of soil was weighed in a flask containing 20 mL of distilled water. Mixture was stirred for 10 min and then kept undisturbed for 30 min. This mixture was filtered using Whatman paper 42. pH of the filtrate was measured using pH meter (EUTECH instruments PS510).

3.5.4.3 Electrical conductivity (EC)

Electrical conductivity of soil was estimated by saturated paste extract method (Jackson, 1967). Soil and water suspension were prepared by adding 10 g of soil in 50 ml of distilled water. Mixture was allowed to stand, to permit the water imbibition by soil. After 1h conductivity of the filtrate was estimated by directly dipping the conductivity cell into the suspension within one hour of preparation of soil- water suspension using instrument E.C meter (HQ 14D, Hach instruments). E.C was expressed in milli Siemen (mS)/meter (m).

3.5.4.4 Soil moisture content

Soil moisture content was measured using method given by Anderson and Ingram (1993). 10 g of soil was weighed in a dry glass petri plate. Weight of petri plate (W_1) and total weight of soil plus petri plate (W_2) was recorded. Then Soil was oven dried at 105 ˚C until weight stabilized. Petri plate containing soil was reweighed (W_3). Percentage of moisture content was calculated using following formula given below:

$$Moisture\ content\ (\%) = \frac{(Weight\ of\ moist\ soil - Weight\ of\ oven\ dry\ soil) \times 100}{Weight\ of\ oven\ dry\ soil}$$

Where,

Weight of moist soil (g) = W_2- W_1

Weight of oven dry soil (g) = W_2- W_3

3.5.4.5 Soil water holding capacity

1 g of soil was taken on a dry glass crucible with a porous base. The weight of the crucible without and with soil was recorded. The crucible was placed in a petriplate with water just in level with its base. The set up was left overnight. The crucible was

then left to dry to drain excess of water. The weight of the crucible with wet soil was recorded. The water holding capacity was then calculated using the given formula.

$$Water\ holding\ capacity\ (\%) = \frac{Weight\ of\ dry\ soil \times 100}{Weight\ of\ saturated\ soil}$$

3.5.4.6 Soil bulk density

The soil replacement method was used to determine the bulk density. A 100 mL dry measuring cylinder was taken. The soil was packed into the cylinder by gradual tapping and compression. The soil was then removed from the cylinder and weighed. The bulk density was calculated using the formula:

$$Bulk\ density = \frac{Dry\ weigt\ of\ soil\ filled\ in\ the\ cylinder}{Volume\ filled}$$

The result was expressed in g/cm^3

3.5.4.7 Total organic matter

Total organic matter (TOM) was determined gravimetrically. 1 g of soil sample was taken in a crucible. It was heated at 105 °C to determine the dry weight. The soil was then heated in by a muffle furnace at 550° C for 4 h (Drozd et al.1997). The TOM was calculated using the formula:

$$Total\ Organic\ Matter\ (\%) = \frac{(W_1 - W_2) \times 100}{W_1}$$

Where,

W_1 is weight of the soil before heating in muffle furnace

W_2 is the weight of soil after heating in muffle furnace

3.5.5 Microcosm studies

Microcosm studies were conducted for 35 days duration to check the remediation ability of the developed formulation. 25 mL amber colored Erlenmeyer flasks were used as microcosm reactors. Two treatments were set up with different moisture content. Moisture levels in the treatment set 1 (unsaturated set) were maintained to 50-60% of the water holding capacity (WHC), whereas, treatment set 2 (saturated set) were maintained in water flooded conditions. Soil were inoculated with the prepared bioformulation at 10 % (w/w) (IS: 8268, 1976). The inoculated soils were viewed using EVO18 Zeiss Scanning Electron Microscope (SEM) to confirm the inoculation of the bacteria in the soil medium.

5g of soil was weighed in the amber colored flasks. The prepared flasks were then sterilized by autoclaving twice at 121°C and 15 lbs pressure for 15 min with intermittent 24 h incubation period. Once cooled, the soils were spiked with RDX/ HMX. The concentration of explosive was decided based on the maximum concentration found at actual site soil. The RDX manufacturing facility showed a maximum RDX in the range of 60-70 mg/ Kg, whereas, HMX manufacturing facility showed a maximum HMX in the range of 2500-3000 mg/ Kg. Hence, soils were spiked with 65 mg/Kg RDX and 3000 mg/Kg HMX respectively under aseptic conditions. Then the developed bioformulation was inoculated into the soil at 10 % (w/w). The soils were thoroughly mixed to ensure homogeneity. Control experiments were also set up with uninoculated bioformulation (only carrier materials) and only soil for both treatment sets. Table 3.4 details the treatment sets in the experiment. The prepared treatment sets were incubated at 30°C for 35 days. Moisture content of the treatment

sets was regularly maintained under sterile conditions. Experiments were carried out in triplicates and mean values are reported. Samples were withdrawn at weekly intervals for further analysis.

Table 3.4: Treatment sets in the microcosm bioremediation experiment

Treatment set	SET 1 (unsaturated)	SET 2 (Saturated)
	Moisture Content: 50-60 % of WHC	**Moisture Content: Flooded conditions**
Sub-sets	Formulation (BF1/2 inoculated in soil, RDX/ HMX)	Formulation (BF1/2 inoculated in soil, RDX/ HMX)
	Uninoculated BF (BF without microbe inoculated in soil, RDX/ HMX)	Uninoculated BF (BF without microbe inoculated in soil, RDX/ HMX)
	Blank control (only soil with RDX/ HMX)	Blank control (only soil with RDX/ HMX)

3.5.5.1 Sampling and analysis

Samples for each treatment set were withdrawn at 0, 7, 14, 21, 28 and 35 days. At each sampling, the experimental set flask (in triplicates) were sacrificed for the analysis of various parameters viz., bacterial count, total catalase and esterase activity using fluorescein diacetate (FDA) hydrolysis, nitrite release and residual explosive concentration.

3.6 Bioremediation of Sediments from explosive manufacturing facilities

3.6.1 Sediment preparation

Sediments from two ordnance manufacturing facility in India were selected for the study. Sediment A was obtained from an RDX manufacturing facility, whereas, sediment B was obtained from an HMX manufacturing facility in India.The sediments were characterized for their pH, water holding capacity, total organic matter and

explosives present. Table 3.5 describes the physico-chemical characteristics of the sediments used in the study.

Table 3.5: Physico-chemical characterization of sediments A and B under study

Sl. No.	Physico- chemical property	Sediment A	Sediment B
1	pH	4.03	2.8
2	Water holding capacity (%)	45.6	62.5
3	Total organic matter (%)	2.488	3.3734
4	Explosive present	RDX	RDX, HMX
5	RDX (mg/Kg)	976601	82272.59
6	HMX (mg/Kg)	___	71807.42

3.6.2 Mesocosm experiments

300 g of sediments (A/B) was weighed in amber coloured reagent bottles. To maintain conditions similar to the actual site, no sterilization was performed. The developed bioformulations (BF1/ BF 2) were inoculated into the sediment at 20% (w/w). the sediments were thoroughly mixed to ensure homogeneity. Controls were also set up with uninoculated BF as amendment and blank sets without any amendments. All sets were maintained in saturated conditions, where in the sets were maintained in flooded conditions. Table 3.6 details the treatment sets in the experiment. The prepared treatment sets were incubated at room temperature. Moisture content of the treatment sets was regularly maintained. Experiments were carried out in triplicates and mean values are reported.

Table 3.6: Treatment sets in the mesocosm bioremediation experiment

Treatment set	SET (Saturated) Moisture Content: Flooded conditions
Sub-sets	Formulation (BF1/2 inoculated in sediment A/B)
	Uninoculated BF (Uninoculated BF1/2 without microbe inoculated in sediment A/B)
	Blank control (only sediment(A/B) with RDX/ HMX)

3.6.3 Sampling and analysis

Samples for each treatment set were withdrawn at regular intervals. At each sampling, the experimental set flask (in triplicates) were analyzed for various parameters viz., pH, total catalase and esterase activity using fluorescein diacetate (FDA) hydrolysis, nitrite release and residual explosive concentration.

3.7 Elucidation of Degradation Pathway

3.7.1 Resting cell incubation

The degradation of RDX and HMX by *J. cremeus* was evaluated by observing the enzyme responsible for degradation. As reported nitroreductase enzyme is a major enzyme system responsible for degradation of nitramine explosives.

J. cremeus was grown in presence of RDX. The cells were harvested in the late log phase, washed and resuspended in MSM containing RDX at 10 mg/L and increasing sodium azide (0, 0.1, 0.5, 1mM) as it's a reported inhibitor of nitroreductase enzyme (Villanueva 1959; Roldán et al. 2008).The RDX degradation was monitored using HPLC.

3.7.2 Monitoring the degradation products

The degradation intermediated produced during the degradation of RDX and HMX by *J. cremeus* in soil was qualitatively analyzed using mass spectroscopy (MS).

3.8 Analytical Techniques

The degradation process was monitored by analysing various parameters, viz, growth of the bacterium in liquid culture, live cell count in soil studies, bulk enzyme activity, nitrite released, residual explosive concentration using HPLC and the intermediates produced using MS.

3.8.1 Scanning Electron Microscopy (SEM)

The solid samples (soil) were air dried and coated with Au-Pd by sputter coating and then viewed under EVO18 Zeiss scanning electron microscope.

3.8.2 Microbial growth in terms of live cell count

The live cell count was used as an indicator of microbial growth during the degradation experiments with soil microcosms. The live cell count was calculated using the plate count method. Briefly, 1 g of soil was suspended in 9 mL autoclaved distilled water. Serial dilution was performed using autoclaved distilled water upto 10^{10} dilutions. 0.1 mL of the dilution was plated on nutrient agar plates, and spread well using an L-spreader. The plates were then incubated at 35° C for 24-48 h. The colonies were then counted using a colony counter. The CFU was calculated using the formula:

$$CFU = \frac{No.of\ colonies \times\ dilution\ factor}{volume\ plated}$$

3.8.3 Total enzyme activity

The total enzyme activity in terms of Flourescein diacetate hydrolysed was monitored as an indicator of the microbial growth and function. 0.5 g of soil was taken in a conical flask, followed by addition of 20 mL 0.06 M sodium phosphate buffer at pH 7.6 and 0.2-mL FDA solution (Chen et al., 1988, Adam and Duncan, 2001). The reaction was allowed to proceed for 20 min. The samples were then filtered through Whatman paper

no.1. The reaction was then stopped using equal volume of acetone. Samples were then analyzed spectrophotometrically at 500 nm using a Perkin Elmer, Model Lambda 650S UV- Visible spectrophotometer. The values were then determined using standard curve prepared using the specific soil/ sediment (Figures 3.8-3.11). Enzyme activity was expressed as FDA hydrolyzed in μg per gram of soil per 20 min.

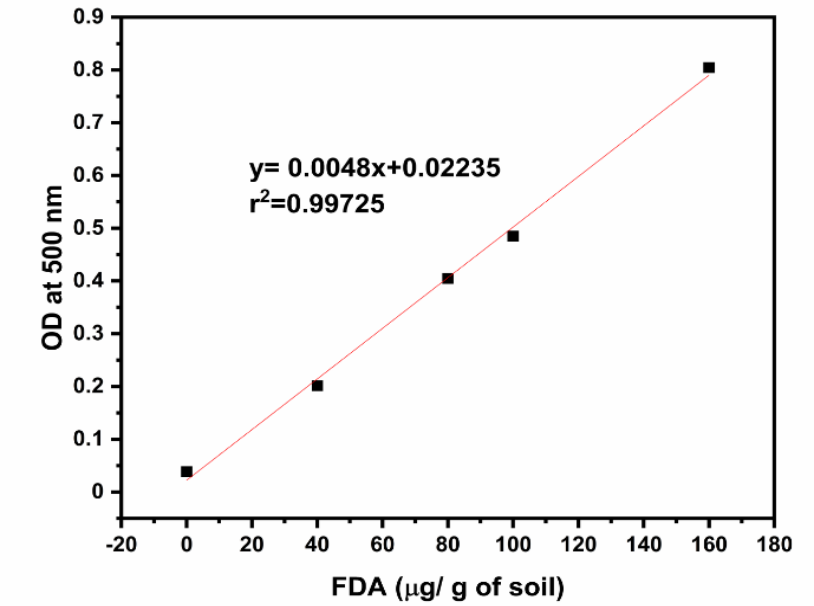


Figure 3.8: Standard graph for FDA hydrolysis in background soil used in RDX remediation study

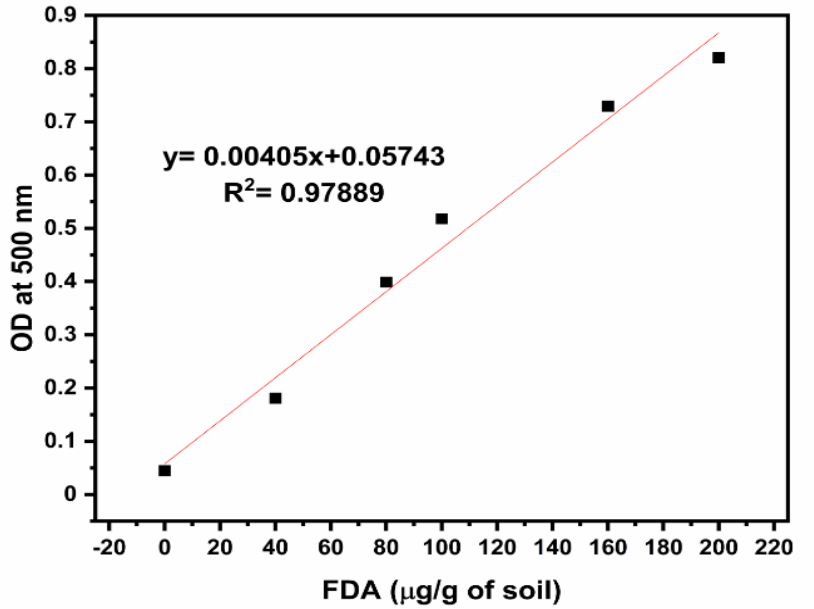


Figure 3.9: Standard graph for FDA hydrolysis in background soil used in HMX remediation study

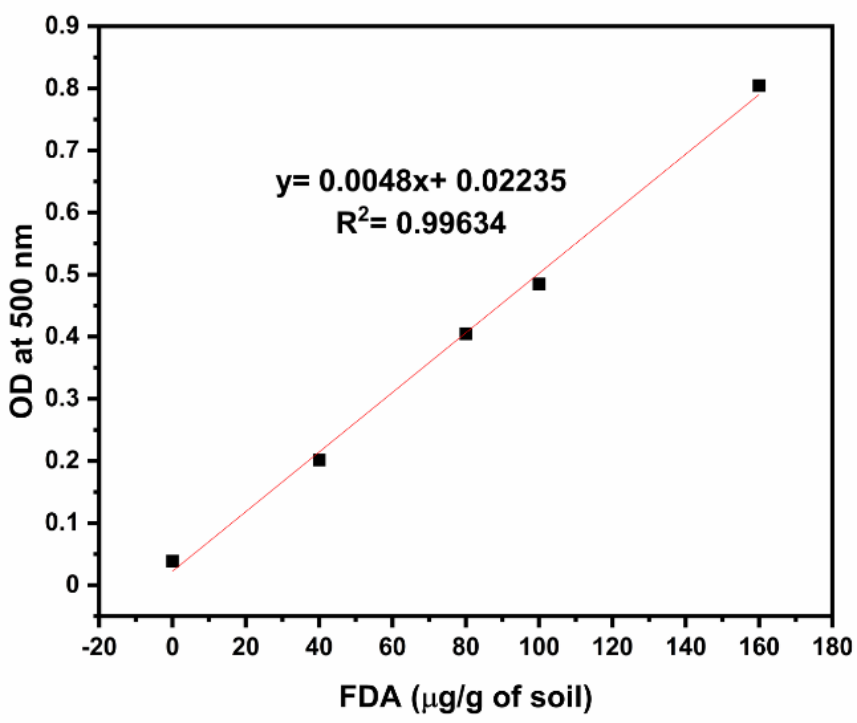


Figure 3.10: Standard graph for FDA hydrolysis in sediment A

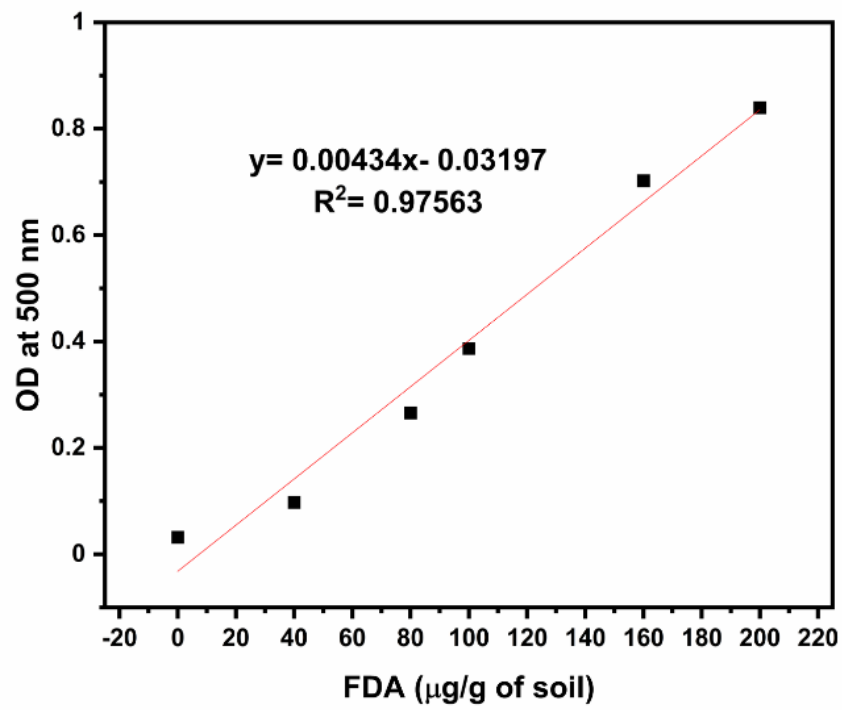


Figure 3.11: Standard graph for FDA hydrolysis in sediment B

3.8.4 Monitoring of the nitrite ion released during bioremediation

3.8.4.1 Preparation of soil phase samples

To 0.5 g of air-dried soil/sediment samples, 2.5 mL of 2M potassium chloride solution was added as described by Keeney and Nelson (1982). The extraction was carried out on an orbital shaker for 1 h at 100 rpm. The supernatant was then used for analysis.

3.8.4.2 Analysis

Refer section 3.4.2.2

3.8.5 Residual explosive concentration

3.8.5.1 Preparation of soil phase samples

2 g of air-dried soil/sediment sample was mixed with 10 mL of acetonitrile. The samples were shaken at 18°C for 18 h on an incubator shaker. The prepared samples were then filtered through 0.22 μm Teflon filters (Millex filters).

3.8.5.2 Analysis

Refer section 3.4.3.2

3.8.6 Mass spectrometry

The degradation pathways for treatment of soil with bioformulation were analysed using mass spectrometry. Mass spectrometric (MS) analysis of the acetonitrile extracted sample was performed using Synapt G2 (Waters ACQUITY QSM) MS system in positive ion mode (ES^+). RDX/ HMX and its degradation products were separated on an Acquity UPLC® BEH column (C18, 1.7 μm) at 0.75 mL/ min flow rate over a period of 20 min.

Chapter 4
Results and Discussion

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Aqueous phase Degradation Evaluation of RDX and HMX by Native Microbes

Explosives, RDX and HMX are characterized by high toxicity. Hence, their remediation by microbes can be a challenge. Microbial communities present in the contaminated sites (primarily soil, groundwater, aquifer) exhibit the inherent ability to thrive in presence of the contaminant (Cupples 2013). The use of such bacterial communities for remediation purpose provides an advantage to the approach. Many researchers have isolated explosive degrading microbes from contaminated sites and utilized them for the bioremediation experiments. Arnett and Adrian in 2009 isolated *Geobacter sp., Acetobacterium sp., Desulfovibrio sp.,* from an RDX waste water treatment plant. Roh et al., in 2009 also isolated bacteria belonging to *Pseudomonas, Enterobacter* and Azospirillum species from contaminated groundwater and aquifer. HAW-EB21 was isolated from the marine sediments of the dumpsite of a former ammunition plant (Zhao et al. 2004). Singh et al., in 2011 isolated *Bacillus cereus* strain PU isolated from firing range for biodegradation of 2,4,6- trinitrotoluene. Use of such resistant microbes in the bioremediation of explosive is being explored worldwide.

In the present assignment, I have carried out explosive degradation evaluation studies using 3 bacterial species, namely, *Pseudomonas mosselii*, *Pseudomonas entomophila* and *Janibacter cremeus* (culture details given in chapter 3) from various explosive manufacturing sites in India. Different explosive manufacturing facilities under

Ministry of Defence were selected for the purpose. The soil, water and sediment samples were collected from these sites. The collected samples were then subjected to isolation of various microbial species present. The isolated bacterial species were then screened for preliminary explosive degradation potential on plates with medium containing RDX/HMX. Griess assay was also used for screening of bacterial cultures. The positive degrading microbes were further characterized. 16S rRNA sequencing was performed to identify the bacterial species. Extensive biochemical characterization was also performed (figure 4.1), sequencing and bioinformatics studies were carried out at Institute of microbial technology (IMTECH), Chandigarh, India.

The selected bacterial species were then evaluated for explosive (RDX and HMX) degradation potential in aqueous phase.

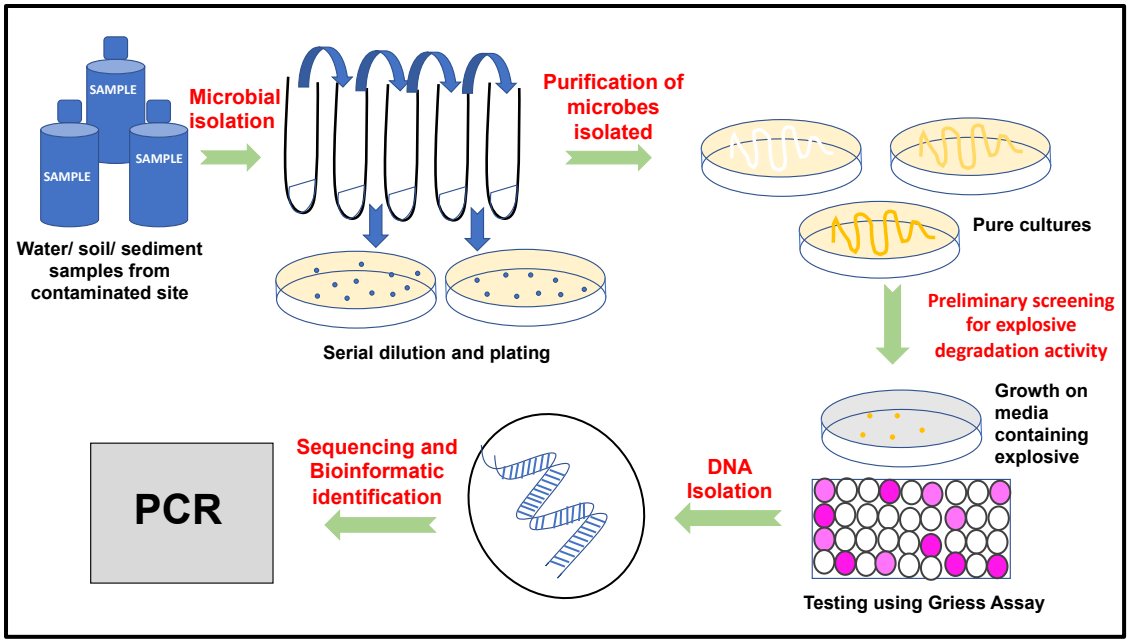


Figure 4.1: Isolation and identification of microbes from contaminated samples obtained from explosive manufacturing industries

The microbial species isolated from the contaminated sites were evaluated for their explosive degradation efficiencies. Both RDX and HMX degradation efficacies were

evaluated. Minimal Salt medium was spiked with explosive (RDX/HMX) and inoculated with *P. mosselii, P. entomophila* and *J. cremeus* separately. The flow chart detailing the experimental details has been given in figure 4.2.

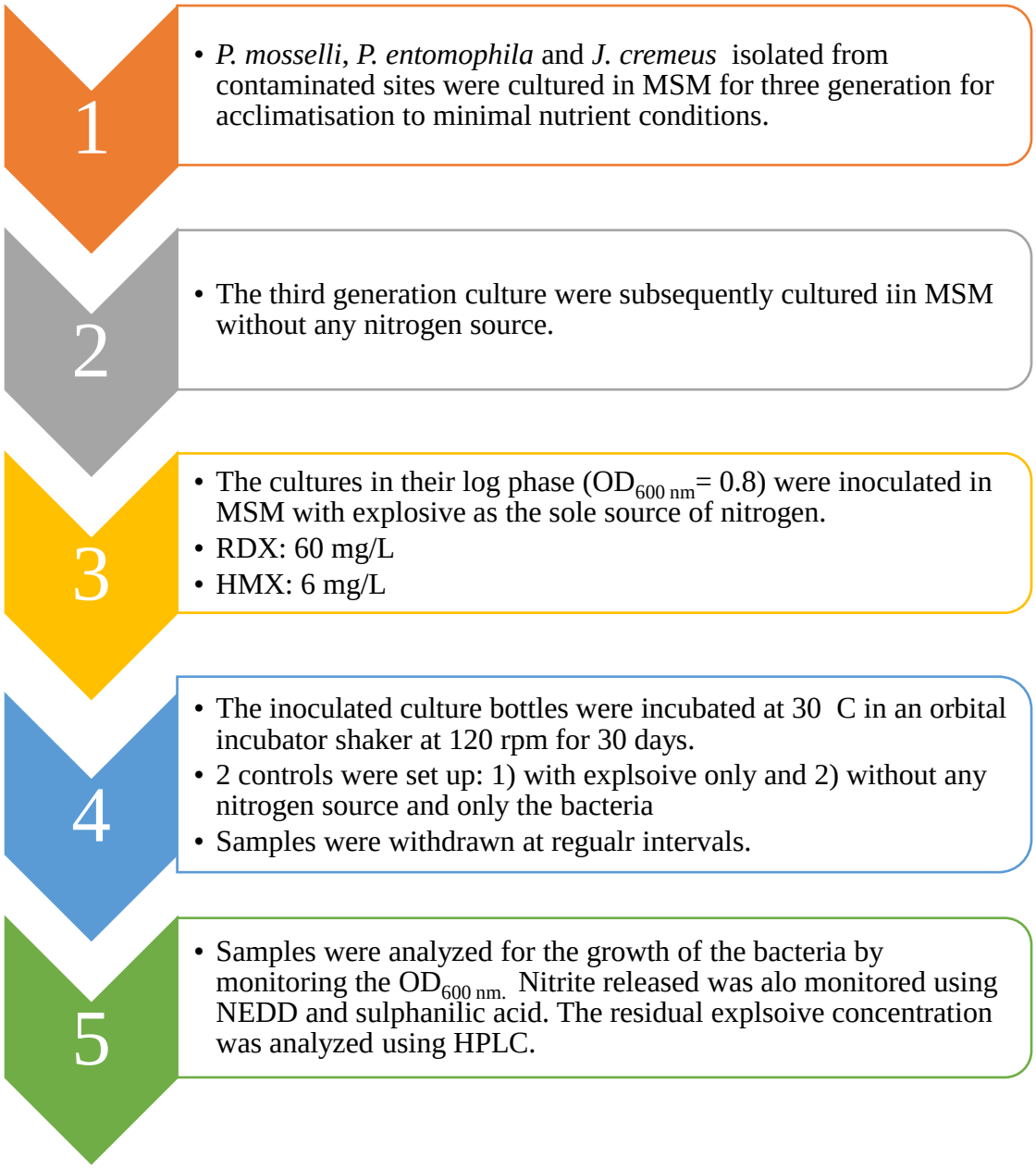


Figure 4.2: Experimental overview for aqueous phase degradation evaluation of RDX and HMX by native microbes.

4.1.1 Bioremediation of RDX

Growth of bacteria in presence of RDX

P. mosselii, P. entomophila and *J. cremeus* were evaluated for their growth in presence of 60 ppm RDX in aqueous medium throughout the 30 days study. It was observed that all three bacterial species could survive well in presence of RDX. Figure 4.3 shows the comparative growth of *P. mosselii, P. entomophila* and *J. cremeus* in presence and absence of RDX. As observed all three bacterial species follow a sigmoid growth pattern in both conditions. All three bacterial species exhibited a lag phase of 1 day in presence of explosive (RDX). *P. mosselii* showed a maximum growth at day 7 and 10 for without and with RDX respectively. It exhibited a stationary phase of about 1 week with RDX, showing a decline henceforth. *J. cremeus* on the other hand is characterized by a maximum growth of 1.241 and 1.123 for with and without RDX (respectively) on fifth day. *P. entomophila,* showed a maximum growth on 3rd day, with a stationary phase of 7 days. The proliferation of the isolates in presence of RDX proves that, the explosive compound doesn't negatively affect its growth. Growth of the isolates in presence and absence of RDX as well as degradation of the explosive compound later shows that all three isolates utilize RDX as a non-growth substrate (Van Aken et al. 2004; Solyanikova et al. 2011; Nagar et al. 2018). Studies by Arnett and Adrian, 2009 also showed similar results. They observed that, the bacterium Desulfovibrio sp. could grow on RDX as sole source of nitrogen and could metabolize it to simpler compounds. Bernstein et al. in 2011 also observed that *Rhodococcus sp.* grew on RDX as sole source of nitrogen for the degradation of RDX.

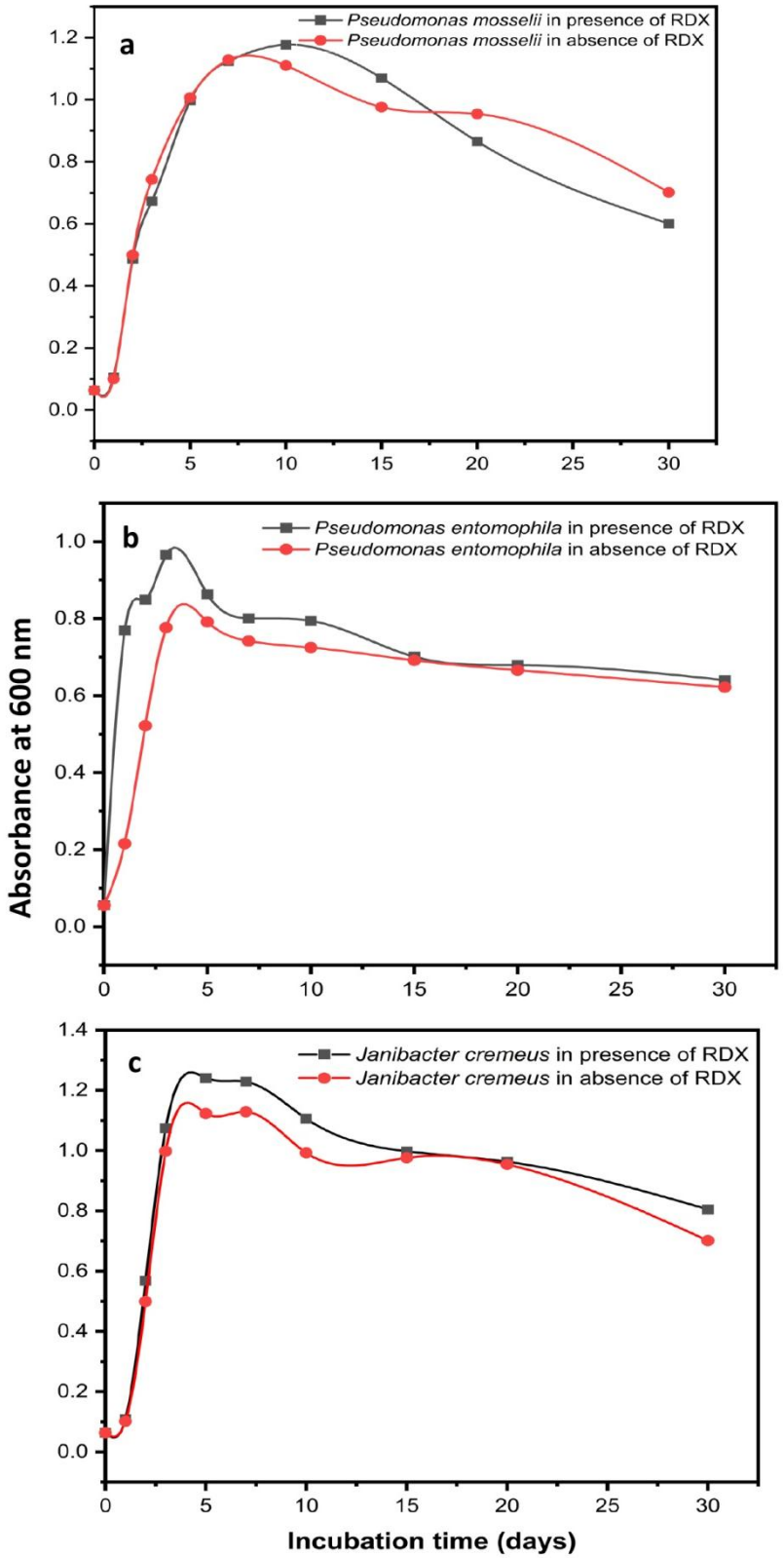


Figure 4.3: Comparative growth of a) *P. mosselii* b) *P. entomophila* c) *J. cremeus* in presence and absence of 60 mg/L RDX.

Release of nitrite during degradation of RDX in aqueous phase

Nitrite is a major metabolite released during the biodegradation of RDX (McCormick et al. 2006), hence monitoring of its release is an important parameter to be assessed. *P. mosselii*, *P. entomophila* and *J. cremeus* on incubation with 60 mg/L of RDX were monitored for the amount of nitrite released during the biodegradation process (figure 4.4). Briefly, to 600 μL of the supernatant, 150 μL of sulphanilamide solution was added. The reaction mixture was incubated at room temperature for 5 minutes. This was followed by the addition of 150 μL of N-(1-naphthyl) ethylene diamine dihydrochloride solution. The reaction was allowed to proceed at room temperature for 20 minutes. The volume of the reaction mix was raised to 3 mL by addition of distilled water (2.1 mL). The absorbance of the resulting mix was recorded at 540 nm. The concentration of nitrite present was measured by preparing sodium nitrite standard curve (Mercimek et al. 2013). *P.mosselii* showed a negligible release of 0.0824 mg/L nitrite ion after 30 days of the study.The negligible release of nitrite could be attributed to absence of denitrification during the degradation. Nitroreductase enzyme systems are generally responsible for conversion of nitrate to nitrite. *P. mosselii* is characterized by the absence of this enzyme system (Dabboussi et al. 2002), thereby leading insignificant release in nitrite. Howsoever, the little amount of nitrite released could be attributed to the effect of photodegradation of RDX in the medium. *P. entomophila* showed a release of 3.34 mg/L, whereas, *J. cremeus* exhibited a release of 10.16 mg/L of nitrite in 30 days. Nitrite is generally released as the first intermediate during the biotransformation of RDX, as proposed by Bhushan et al.(2003) and Zhao et al. (2003). Nitrite ion is released by two single electron transfers. The release of nitrite indicates that, RDX degradation in *P. entomophila* and *J. cremeus* follows the single electron transfer/ denitration pathway (Crocker et al. 2006). Denitration has been reported to be an essential pathway in ring cleavage (Figure 4.5).

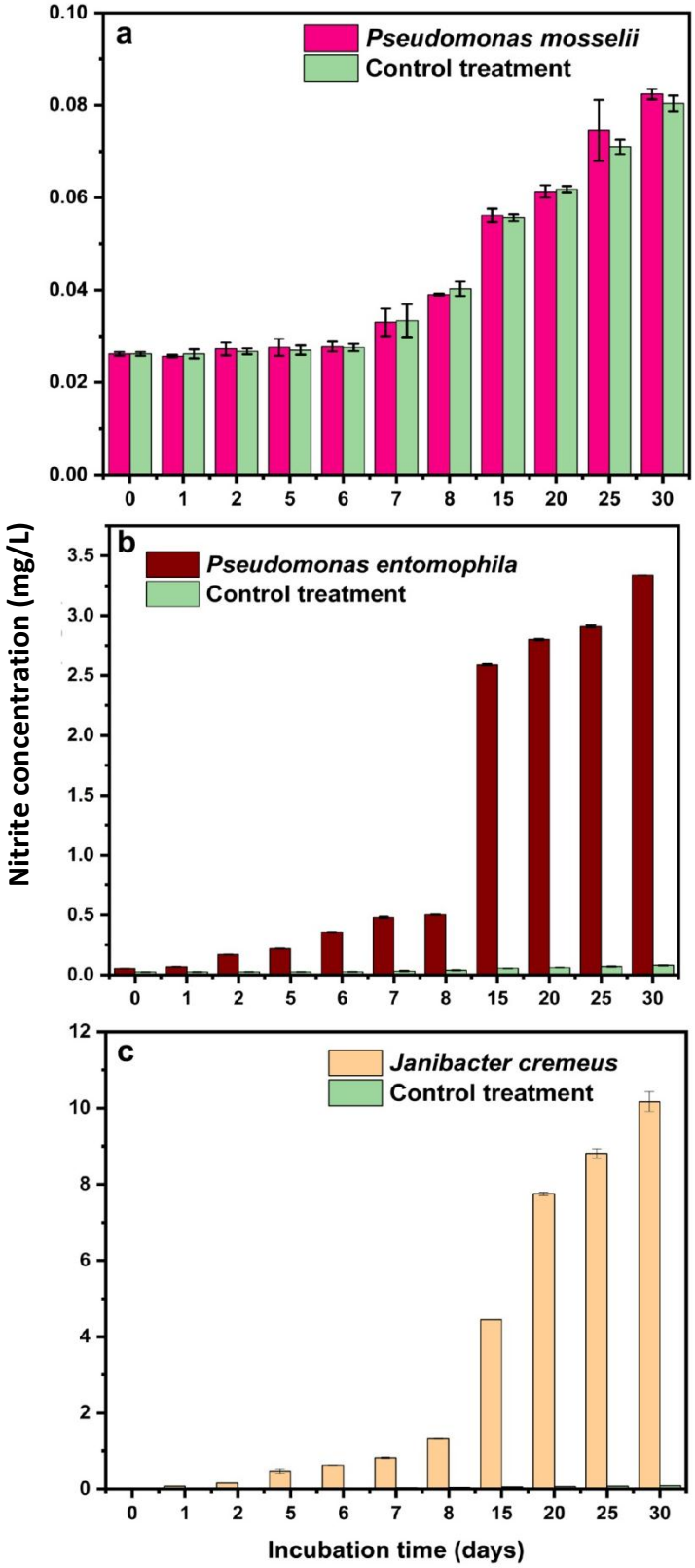


Figure 4.4: Release of nitrite ion during the bioremediation of 60 mg/L RDX by a) *P. mosselii* b) *P. entomophila* c) *J. cremeus* in MSM.

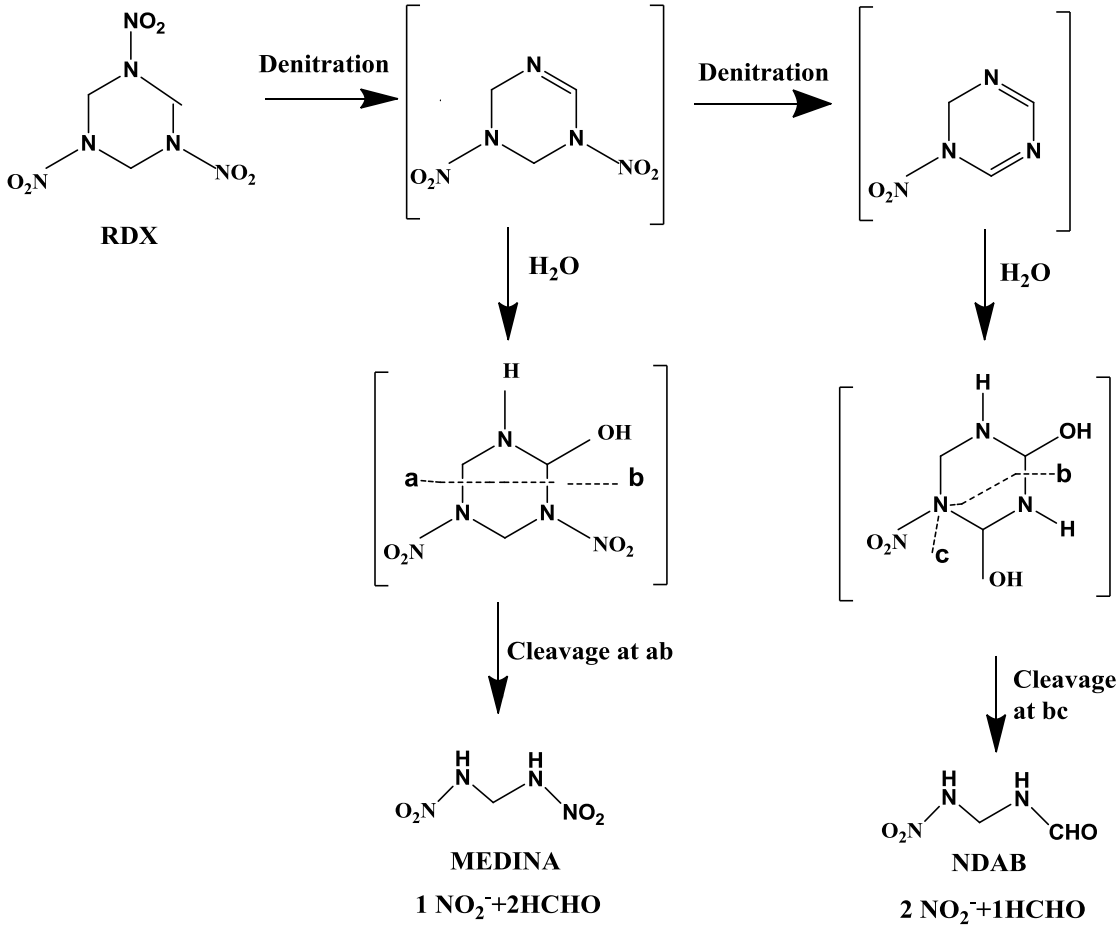


Figure 4.5: Mechanism of denitration leading to ring cleavage (Jackson et al. 2007)

Estimation of the residual RDX concentrations

Residual RDX concentration in the media was analyzed using HPLC (Dionex Ultimate 3000, USA). Acetonitrile: water (50:50 v/v) was used as the mobile phase. Separation was obtained on C18 column, with RDX being detected at 254 nm. It was observed that *J. cremeus* exhibited a maximum degradation efficiency of 88 % followed by *P. entomophila* (83 %) and *P. mosselii* (80%) for 60 mg/L RDX in MSM. The control experiments showed negligible decrease in RDX (Figure4.6). All three isolates could grow in presence and absence of RDX and coulddegrade the explosive compound. This indicates that, the isolates did not use RDX breakdown for its growth, showing co-metabolism. Similar co-metabolic relationship has been previously shown by Beller (2002), Van Aken et al.

(2004). Hence, proving that all the three isolates on exposure to RDX could breakdown the molecule to release mineralized products and did not depend on its presence for growth.

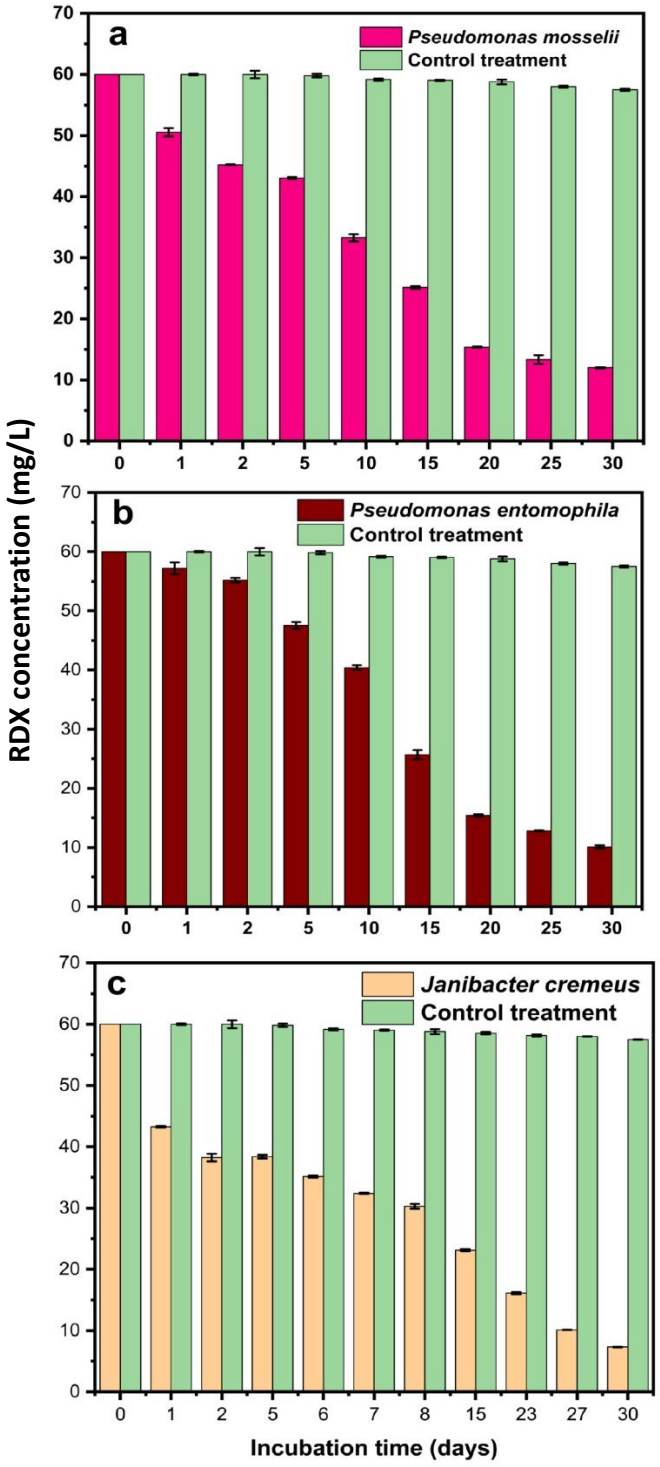


Figure 4.6: Degradation of 60 mg/L RDX by a) *P. mosselii* b) *P. entomophila* c) *J. cremeus* in MSM

The reaction kinetics for the biodegradation was also calculated to determine the rate of reaction. First order kinetic model as given by equation 1, was applied to the residual RDX concentrations (in treatment sets).

$$\ln A = -kt + \ln A_0 \qquad \text{Eq (1)}$$

Eq 1 represents the linear form of the first- order kinetic model, wherein,

A_0 represents initial RDX concentration (mg/L), A is the RDX concentration (mg/L) at time t (days), k is the RDX degradation rate constant. The half-life for RDX degradation was then calculated using the following equation (2)

$$t_{\frac{1}{2}} = \frac{0.693}{k} \qquad \text{Eq (2)}$$

where, $t_{1/2}$ is the half-life of RDX, that is the time required for degradation of RDX by half. The rate constant for degradation of RDX and the half-life of RDX for respective bacteria by *P. mosselii*, *P. entomophila*and *J. cremeus*was obtained from graph shown in figure 4.7, has been tabulated in table 4.1.

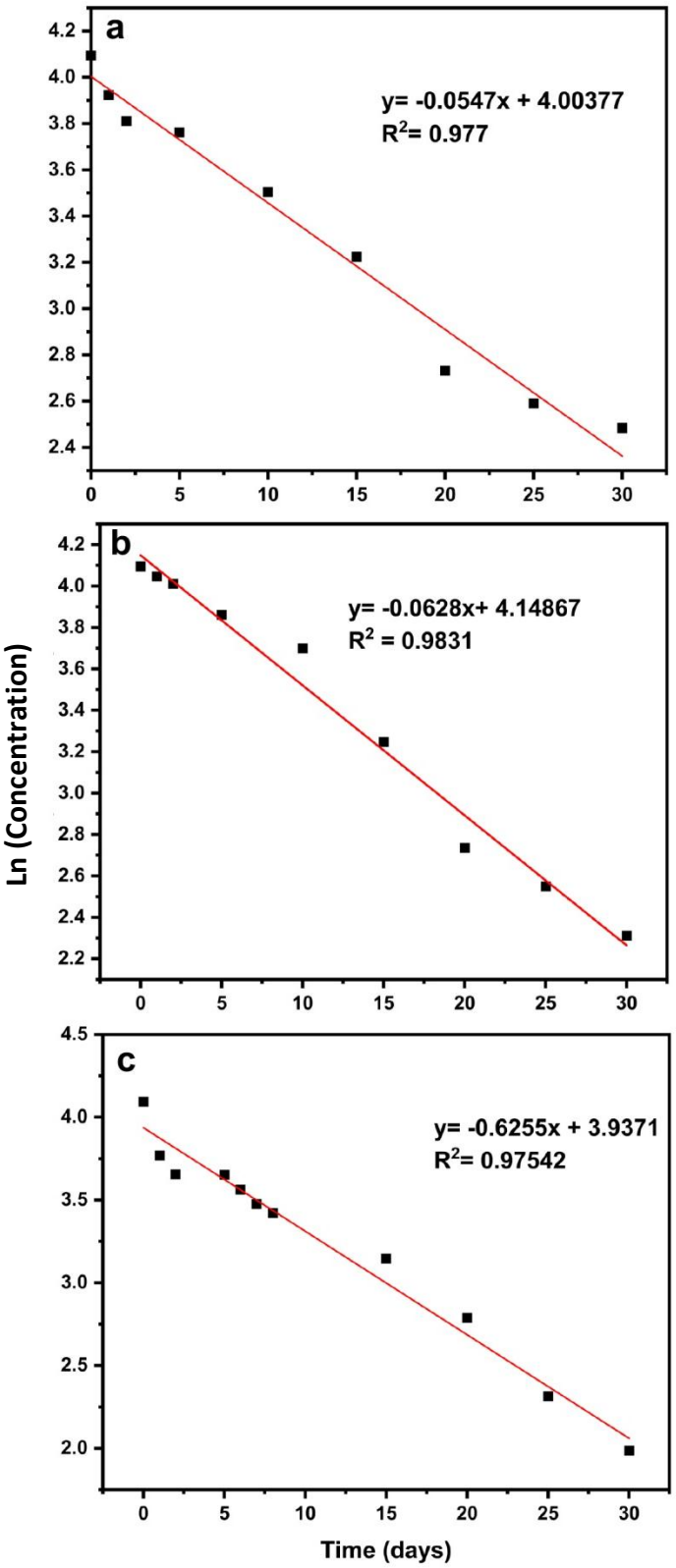


Figure 4.7: First order kinetics for RDX degradation by a) *P. mosselii* b) *P. entomophila* c) *J. cremeus* in MSM

Table 4.1: Rate constant and Half life of RDX on treatment with different explosive degrading isolates

Bacteria	Rate constant (/day)	Half-life of RDX (days)
P. mosselii	0.0547	12.669
P. entomophila	0.0628	11.035
J. cremeus	0.0625	11.088

4.1.2 Bioremediation of HMX

Growth of bacteria in presence of HMX

P. mosselii, *P. entomophila* and *J. cremeus* were evaluated for their growth in presence of 6 ppm HMX in aqueous medium throughout the 30 days study. It was observed that all three bacterial species could survive well in presence of HMX. Figure 4.8 shows the comparative growth of P. mosselli, *P. entomophila* and *J. cremeus* in presence and absence of HMX. As observed all three bacterial species follow a typical growth pattern in both conditions. This proves that, presence of HMX does not affect the growth of the bacteria. Similar results were obtained by Van Aken et al. in 2004, wherein, they studied the effect of various nitro substituted explosives on *Methylobacterium sp.* strain BJ001. In the present study, all three bacterial species exhibited a lag phase of 1 day in both presence of HMX. The short lag phase shows that all three isolates were able to adjust and adapt to the new physiological conditions (presence of HMX) in 1 day by synthesizing the requisite mRNA and proteins to survive in presence of HMX (Maier, 2009). *P. mosselii* showed a maximum growth at day 10 for both conditions. It exhibited a maximum growth of 1.18 in presence of HMX, whereas, 1.09 in absence of HMX. This proves that, presence of HMX did not have a degenerative effect on the bacterium.

P. entomophila, on the other hand showed a maximum growth of 0.97 absorbance (at 600 nm) in 3 days when exposed to HMX, whereas 0.78 in absence of HMX. It exhibited a long stationary phase of 1 week under both the conditions. *J. cremeus* was observed to be characterized by a maximum growth of 1.35 and 1.14 for with and without HMX (respectively) on the seventh day.The stationary phase is generally achieved due to the consumption of carbon and energy sources. But as we observe, the growth in the stationary phase is unbalanced, and some change the absorbance is seen. The increase in absorbance can be due to the lysis of dead cells that provide nutrients and energy to the existing cells (Maier, 2009). Also, another reason could be the accumulation of metabolites that may affect the growth and survival of microbes. In case of explosives, nitrite is one such metabolite. Also, the other intermediates formed during the process can also lead to the stationary phase of bacterial growth. Howsoever, the cells can still metabolize and degrade HMX in this state (Maier, 2009).

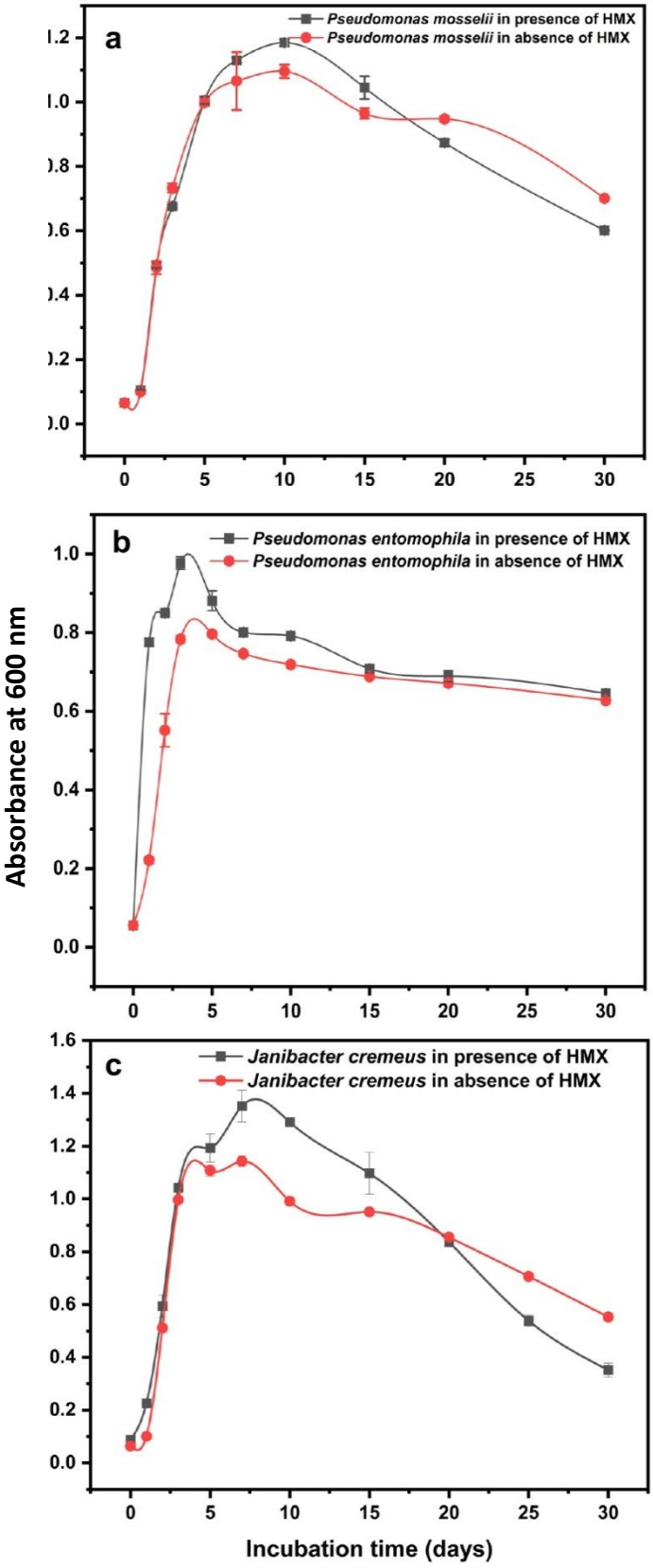


Figure 4.8: Comparative growth of a) *P. mosselii* b) *P. entomophila* c) *J. cremeus* in presence and absence of 6 mg/L HMX.

Release of nitrite during degradation of HMX in aqueous phase

Nitrite has been reported to be the first metabolite produced during the degradation of HMX (Crocker et al. 2006), hence monitoring of its release is an important parameter to be assessed. *P. mosselii, P. entomophila* and *J. cremeus* on incubation with 6 mg/L of HMX were monitored for the amount of nitrite released during the biodegradation process (figure 4.9). *P. mosselii* showed a negligible release of 0.0804 mg/L nitrite ion after 30 days of the study. As discussed earlier, *P. mosselii* lacks the activity to convert nitrate to nitrite hence the insignificant release of nitrite (Dabboussi et al. 2002). HMX, like other nitro explosives has been reported to be amenable to photodegradation, therefore, all the control experiments as well as treatments with *P. mosselii* lead to the release of small amount of nitrite ions (Imran et al. 2012). *P. entomophila* showed a release of 1.16 mg/L, whereas, *J. cremeus* exhibited a release of 3.3 mg/L of nitrite in 30 days. Both the isolates are characterized by the typical property of transformation of nitrate to nitrite (Hamada et al. 2013; Dieppois et al. 2015). Release of nitrite involves single electron transfer which destabilizes the HMX ring leading to the ring cleavage (Crocker et al. 2006). Such a reaction is thermodynamically favorable. Similar mechanism of nitrite release was observed by Zhao et al. 2004b in *Clostridium bifermentans* HAW1 and by Fournier et al (2004b) in *Phanerocheate chrysosporium*.

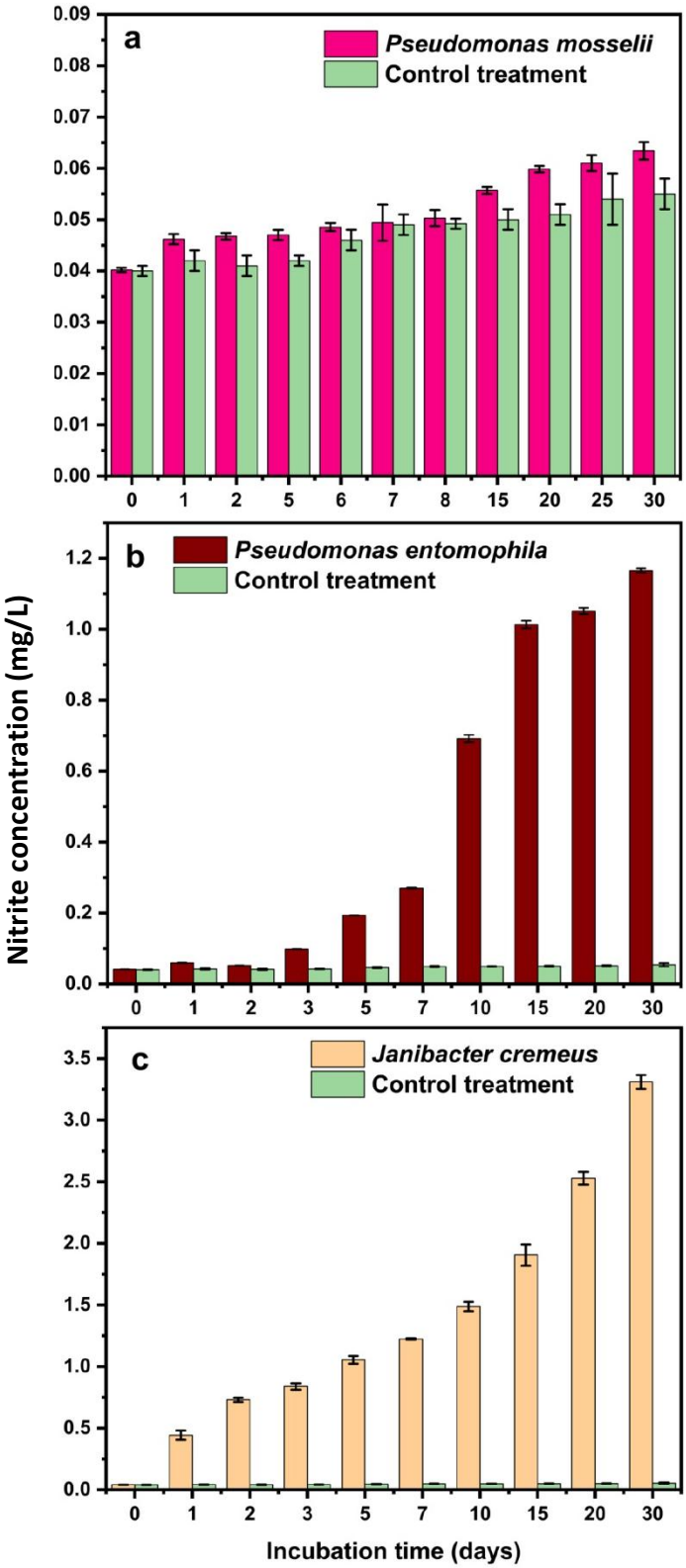


Figure 4.9: Release of nitrite ion during the bioremediation of 6mg/L HMX by a) *P. mosselii* b) *P. entomophila* c) *J. cremeus* in MSM

Estimation of the residual HMX concentrations

Residual HMX concentration in the media was analyzed using HPLC (Dionex Ultimate 3000, USA). Acetonitrile: water (50:50 v/v) was used as the mobile phase. Separation was obtained on C18 column, with HMX being detected at 254 nm. It was observed that *J. cremeus* exhibited a degradation efficiency of 92 % followed by *P. entomophila* (89 %) and *P. mosselii* (75.8 %) for 6 mg/L HMX in MSM. The control experiments showed negligible decrease in HMX (Figure 4.10). As observed all the three isolates grew in absence of HMX. Also, when HMX was present as the sole source of nitrogen, the bacterial isolates could grow and degrade the compound. This indicates that, the bacterial species did depend on the HMX degradation for its growth, proving co-metabolic degradation of HMX. Nagar et al. (2018) observed *Planomicrobium flavidum* proliferated in presence and absence of HMX under aerobic conditions and also could degrade the nitramine, proving co-metabolic degradation. The control without bacterial inoculation showed negligible decrease in HMX concentration.

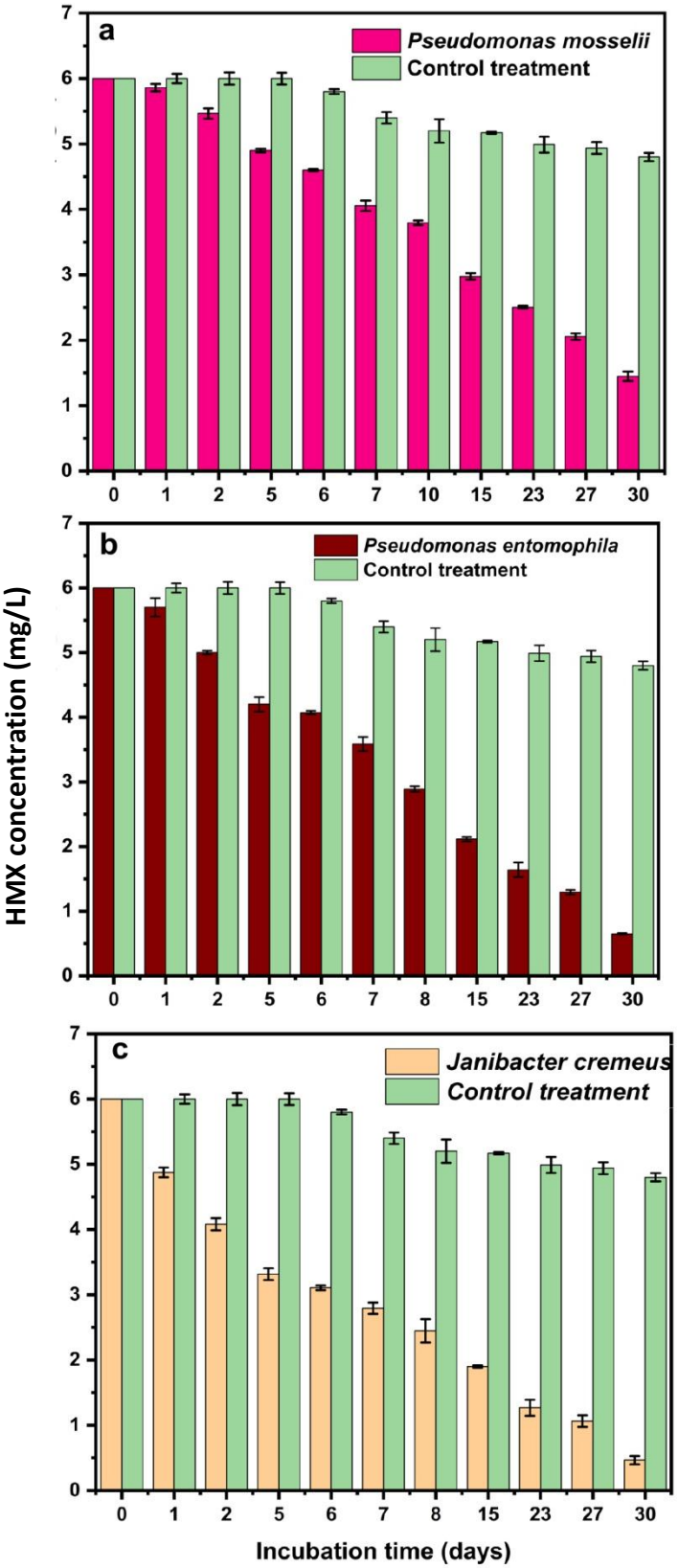


Figure 4.10: Degradation of 6mg/L HMX by a) *P. mosselii* b) *P. entomophila* c) *J. cremeus* in MSM

The reaction kinetics for the biodegradation was also calculated to determine the rate of reaction. First order kinetic model as given by equation 1, was applied to the residual HMX concentrations (in treatment sets).The half-life for HMX degradation was then calculated using the following equation 2 described previously.

The rate constant for degradation of HMX and the half-life of HMX for respective bacteria by *P. mosselii*, *P. entomophila* and *J. cremeus* was obtained from graph shown in figure 4.11, has been tabulated in table 4.2.

Table 4.2: Rate constant and Half life of HMX on treatment with different explosive degrading isolates

Bacteria	Rate constant (/day)	Half-life of HMX (days)
P. mosselii	0.0427	16.22
P. entomophila	0.0564	12.287
J. cremeus	0.0649	10.67

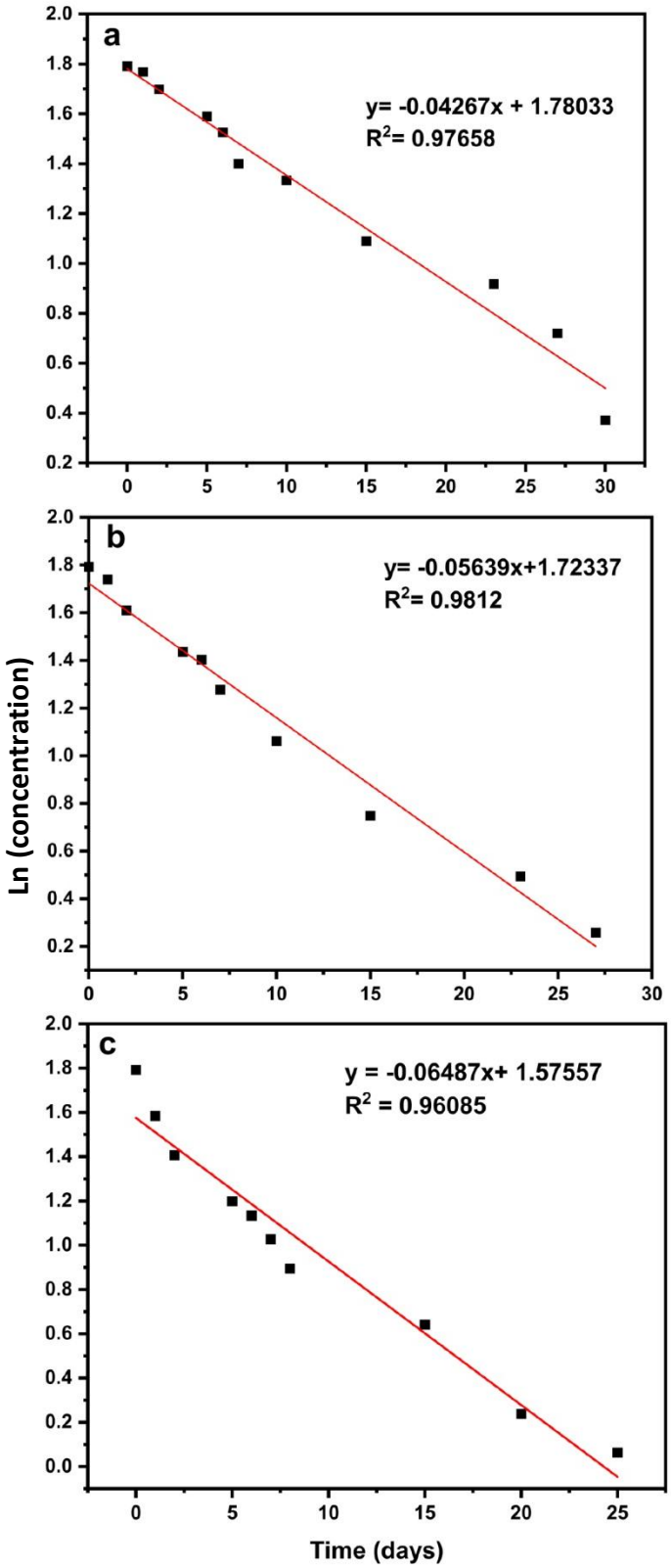


Figure 4.11: First order kinetics for HMX degradation by a) *P. mosselii* b) *P. entomophila* c) *J. cremeus* in MSM

4.1.3 Conclusion

Three native bacterial species isolated from explosive contaminated sites, viz, *P. mosselii*, *P. entomophila* and *J. cremeus* were evaluated for their explosive degradation efficiency in aqueous phase. The results obtained showed that, *J. cremeus* was the best explosive degrading bacteria with a degradation efficiency of 88 % for RDX and 92 % for HMX. All bacterial species except *P. mosselii* showed substantial release in nitrite proving denitrification to be a major step in ring cleavage. All the microbial species showed that explosive degradation followed a first order kinetics and the degradation was a co-metabolic process. The studies conclude *J. cremeus* to be a potential explosive degrading bacterium and hence was chosen for further soil phase studies.

4.2 Bioremediation of explosive contaminated soil and sediment using calcite based bioformulation (BF-1)

Effective in situ bioremediation of contaminated soil can be carried out by bioaugmentation (Kalsi et al. 2020). It involves the introduction of indigenous or allochthonous wide type or genetically engineered microbes to accelerate the remediation process. But the major hurdle lies in the delivery of the microorganism into the desired site and survival of the introduced strain or consortia (Kalsi et al. 2020). Hence, many researchers have applied encapsulated as well as immobilized microbial cells for various purposes (Liang et al. 2009; Xu and Lu, 2010; Chen et al. 2012). Though immobilized microbes have been successfully applied for treatments of many hazardous compounds, not much work is reported for remediation of explosive contaminated soil.

Carriers used in immobilization of microbes play a very crucial role in delivering as well as maintaining high cell density. The carrier used must be eco-friendly, so that it does not contribute to any more pollution at site (Cunningham et al. 2004). Also, immobilization offers protection to the introduced microbial species against unfavorable abiotic factors like pH and toxic compounds present in the site (Pritchard, 1992). This also helps in reducing any competition with the indigenous microbes present in the soil (Lin and Wang, 1991). The higher the affinity of the explosive to the immobilization mix, the higher the remediation rate, hence a novel immobilization carrier must aid in the remediation of the explosive compound. Many studies have exploited the use of natural carriers for delivery of the desired microbes or consortia to the contaminated sites (Chen et al. 2012; Xu and Lu, 2010; Cunningham et al. 2004).

Indigenous microbes at the contaminated sites generally have an ability to degrade various pollutants present in the site. Enrichment and delivery of such indigenous microbes along with a nutrient providing carrier support to the site can lead to faster rates of remediation and can also prevent leaching of the contaminant in the soil profile. The aim of this work is to develop a novel clay based bioformulation immobilized with *Janibacter cre*meus, for the *in situ* remediation of explosive contaminated soils and sediments.

Here, we use calcite as the inorganic carrier with cocopeat as an organic amendment to develop a wettablepowder based bioformulation (BF1) immobilized with *J. cremeus*, an indigenous isolate found to have a good degradation efficiency for both RDX and HMX. Figure 4.12 represents the work in brief.

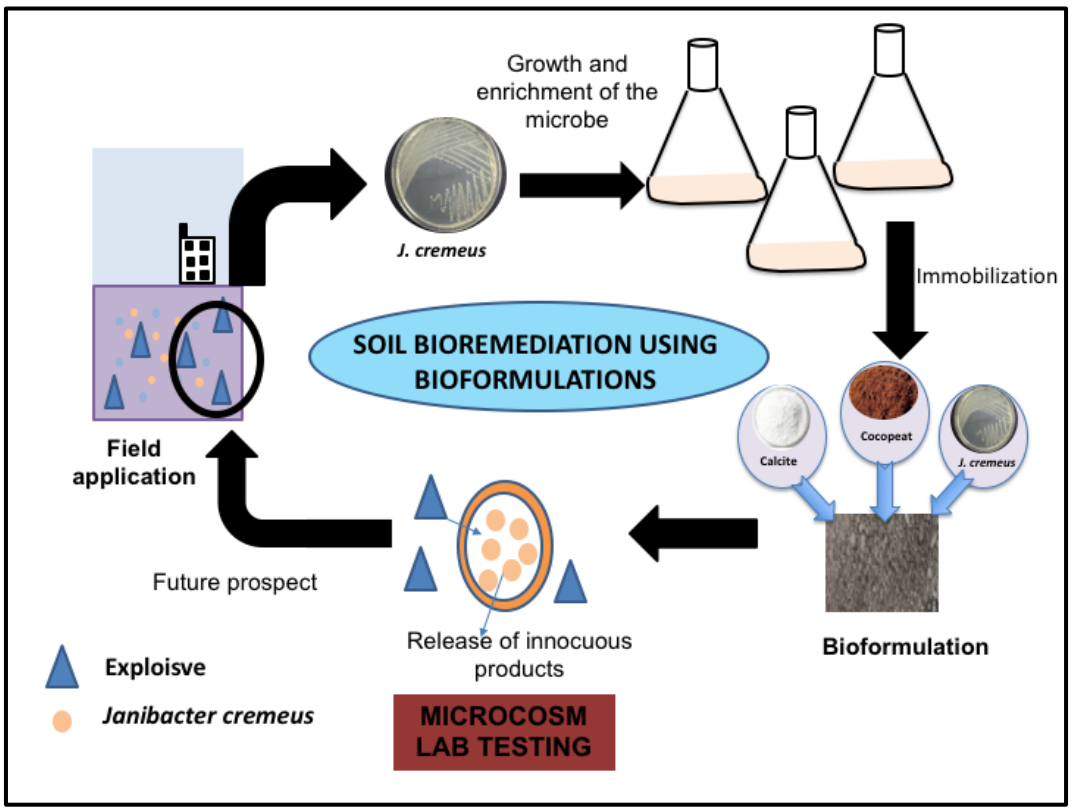


Figure 4.12: Steps involved in development and application of calcite based bioformulation (BF1)

Aqueous phase studies comparing the degradation efficiencies of three explosive degrading organisms showed, *J. cremeus* to be the most efficient. Moreover, it being a soil facultative anaerobe contributed to its selection for incorporation into formulations to remediate soil and sediments contaminated with explosives (RDX and HMX).

Janibacter cremeus was used for development of a powder bioformulation consisting of an inorganic carrier (calcite) and a plant-based material (cocopeat) as an organic amendment. This developed bioformulation was used for remediation of RDX and HMX in soils and sediments. Figure 4.13 shows the work flow of the experiment. The developed calcite based bioformulation was evaluated for its viability, shelf life and explosive degradation efficiency. Degradation evaluation was studied at microcosm level by monitoring various physico-chemical and biological parameters.

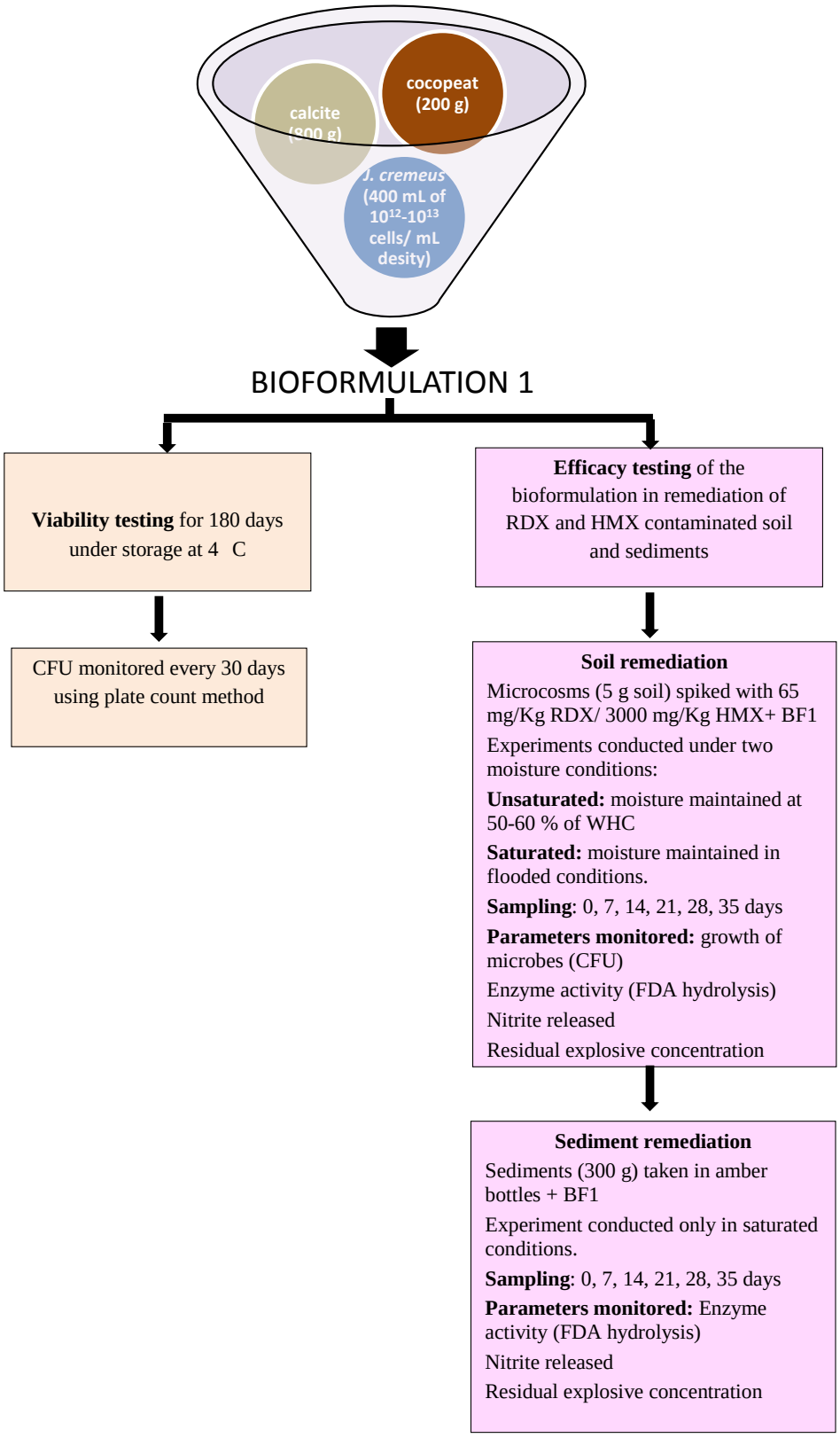


Figure 4.13: Experimental details of soil and sediment remediation using calcite based bioformulation (BF1)

4.2.1 Preparation of bioformulation 1

Selection of explosive degrader for bioformulation preparation

The aqueous phase studies conducted to evaluate the efficacy of the explosive degrading microbes, *Pseudomonas mosselii*, *Pseudomonas entomophila* and *Janibacter cremeus* isolated from explosive contaminated sites to remediate RDX and HMX. They were investigated for their comparative performance in degrading RDX and HMX. Bioformulation 1 was prepared by immobilizing enriched cultures of *J. cremeus* onto calcite and cocopeat. *J. cremeus* was selected due to its good degradation efficiency for both RDX and HMX, also because it was a facultative anaerobe which could survive in depleted oxygen conditions. The calcite based bioformulation thus developed finds application in soil remediation. Soil being a complex medium is characterized by local aerobic and anoxic pockets. These differences in the redox potential may lead to inactivity of strict aerobes/anaerobes. Hence, making *J. cremeus* a potential organism for explosive contaminated soil remediation (Sims and Kanissery, 2019).

Enriched cultures of *J. cremeus* were used for the bioformulation development. J. cemeus was grown in increasing concentrations of RDX and HMX so as to acclimatize the bacteria with the presence of the nitrogenous explosive compound as sole source of nitrogen. Seth-smith et al. in 2002 had observed that the bacteria, *Rhodococcus rhodochrous,* showed better and faster degradation of RDX with those cultures that were previously acclimatised to RDX as their sole nitrogen source. Hence, use of enriched culture would make the bacterial survival easier in the adverse soil environment.

Viability of the developed bioformulation 1

Bioformulation1 developed was stored in autoclaved polyethylene bags at 4 °C. The viability of the developed formulation (BF1) was monitored for 180 days using plate

count agar method. Figure 4.14 shows the viability of the formulation over the period of time. The graph indicates that the immobilized cells of *J. cremeus* were stable upto150 days on storage at 4° C, with only a loss of only 18 % by the end of 180 days. This proves that the prepared bioformulation was efficient in maintaining the culture for a long period of time, hence proving calcite and cocopeat as efficient carriers of bacterial cells. Gentili et al. (2006) observed immobilized cells of *Rhodococcus corynebacterioides* on chitosan flakes to be stable with a loss of only 2 log units when stored at 4°C. Also, the developed bioformulation was observed to meet the minimum requirement of cell count (10^8 g^{-1} of the carrier) as proposed by the IS specifications (IS: 8268, 1976). It is important to remember that, the initial CFU of the inoculum plays a major role in remediation of a compound, as higher CFU (cell population) would indicate smaller lag phase, thereby, better and faster removal (Maier and Pepper, 2015).

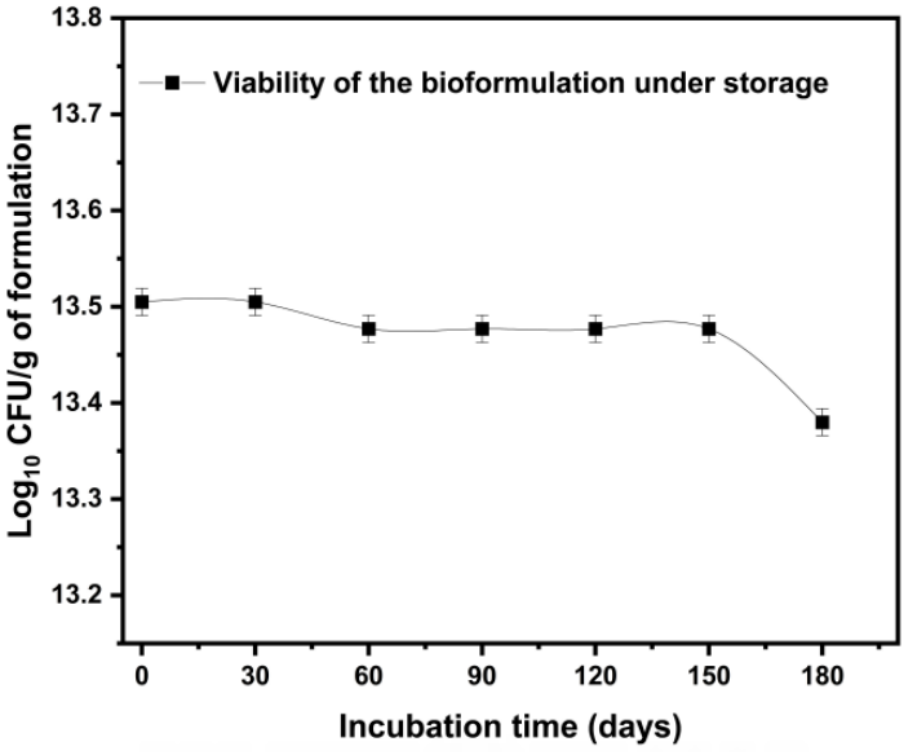


Figure 4.14: Viability of the developed calcite bioformulation (BF1) under storage at 4°C.

SEM analysis of the developed bioformulations

SEM analysis of the developed formulation (BF1) was also performed to confirm the immobilization of the bacteria, *J. cremeus* on the carrier mix. The SEM image (Figure 4.15a and b) shows the carrier mix before and after immobilization. The presence of ovoid shaped bacteria on the surface of carrier mixture (Figure 4.15 b) proves the cells were well immobilized.

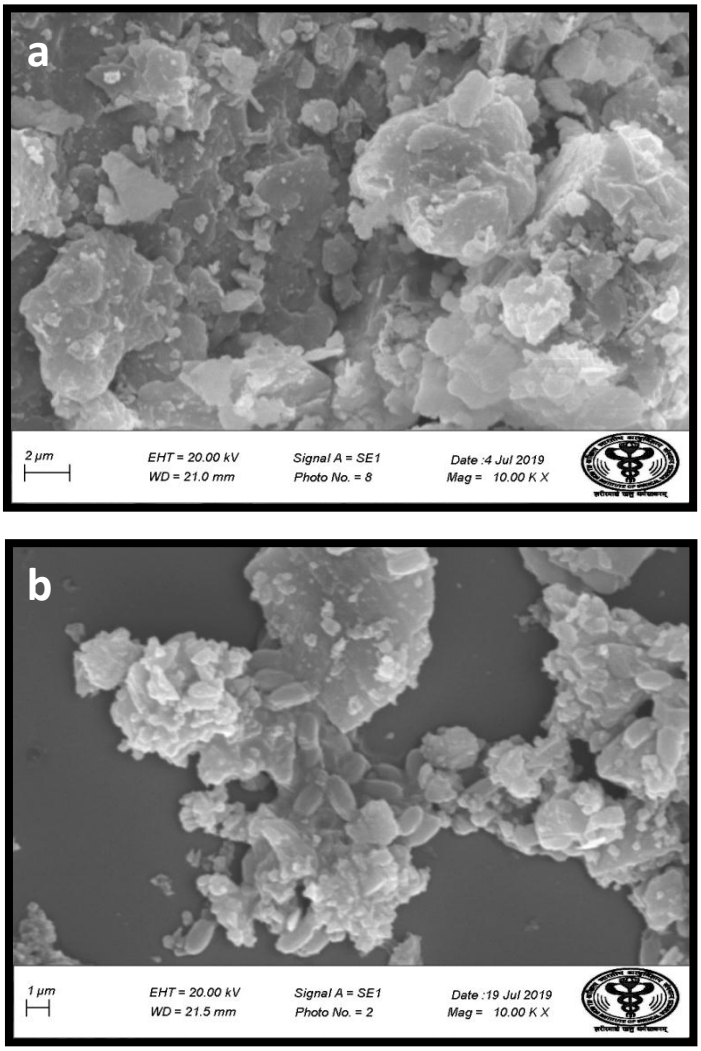


Figure 4.15: (a) Electron micrograph of carrier mix (calcite and cocopeat) before immobilization (b) Electron micrograph of the developed bioformulation

Role of carrier mix in bioformulation 1

Calcite, natural clay derived generally from limestone formations, is a rich and natural source of calcium carbonate. Clays are characterized by easy availability, environmental stability and high adsorptive properties (Biswas et al. 2015a). Clay minerals have been reported to act as support and also provide a protective environment for microbes. The use of clay with immobilized microbe for remediation of various organic contaminants has also been reported (Sarkar et al. in 2012). Clays aid in adsorption of the organic pollutant, which is then bioavailable for the microbial reduction. Remediation of polyaromatic hydrocarbons (PAH) and Volatile organic carbons (VOC) using clay- microbe interaction has been reported to be successful (Froehner et al. 2009; Quintelas et al. 2013; Biswas et al. 2015b). Cocopeat on the other hand served as an organic amendment, providing nutrient support to the bacteria (Nunal et al. 2014) as well as increasing the adsorption of organic compounds (Vijayaraghavan et al. 2015; Premkumar and Vijayaraghavan, 2014) thereby, increasing its bioavailability for the microbial interaction. Since explosives are characterized by low sorption coefficient and hence low bioavailability in soil (ATSDR 2012; EPA 2005), use of such carrier materials (calcite and cocopeat) aids in overcoming these problems, hence improving the efficiency of bioremediation.

4.2.2 Bioremediation of soils contaminated with high explosives: Microcosm studies

The efficacy of the developed bioformulation was tested by monitoring the bioremediation ability. RDX/HMX at the field scale soil concentrations were maintained in the synthetic microcosms for the study purpose. An RDX manufacturing facility and an HMX manufacturing facility were selected for the sampling. The background uncontaminated soils (0-20 cm depth) from these manufacturing units were used in the study. The soils were air dried, ground using mortar and pestle, passed

through 600-micron sieves for uniform particle size distribution and were utilized for the explosive degradation studies.

4.2.2.1 Bioremediation of RDX in soil using BF1

RDX bioremediation studies were performed in microcosm levels by spiking the background soils of an RDX manufacturing facility in India with 65 mg Kg^{-1} of RDX. Two treatment sets were analyzed for their remediation efficacy. The soils in set 1 were maintained at 50- 60 % of WHC (unsaturated) and set 2 soils were flooded with water (saturated). Sacrificial sampling was performed at regular intervals of 0, 7, 14, 21, 28 and 35 days. The samples were monitored for live cell count, total enzyme activity in terms of FDA hydrolyzed, nitrite released and residual RDX concentration for both the treatment sets.

4.2.2.1.1 Microbial activity assessment in the microcosms

SEM analysis

SEM analysis was performed on soils on inoculation with the formulation. The SEM images as shown in figure 4.16, shows that, the formulation was well mixed and the bacteria were present in the soil matrix.

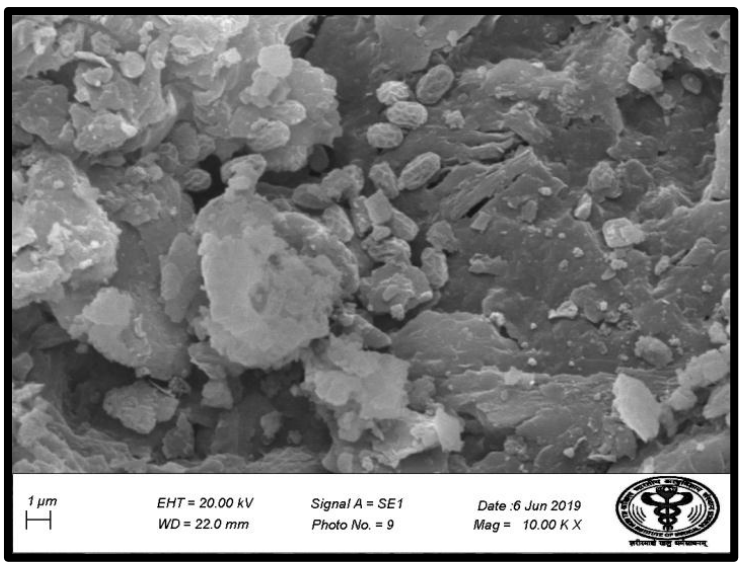


Figure 4.16: SEM image of the bioformulation inoculated in the contaminated soil

Growth of the immobilized bacterium in soils contaminated with RDX

The live cell count was monitored in the formulation set of Treatment 1 (unsaturated) and 2 (saturated) to assess the growth of the immobilized bacterium *J. cremeus* in the soils contaminated with RDX. Figure 4.17 shows the live cell count of the bacteria immobilized in the calcite bioformulation during the study. The bacteria on inoculation in the soil medium showed rapid growth with an increase in CFU. The maximum viable cells were obtained at day 21, with a decrease in bacterial growth after it. But the loss of bacterial cells was not lower than 10^7 g^{-1} of the soil sample, which is a requisite cell count for bioremediation (Pandey and Maheshwari, 2007). The rapid growth of the bacteria shows that *J. cremeus* was not adversely affected by the presence of RDX. The higher bacterial growth in saturated conditions can be attributed to higher moisture content in soils (Ringelberg et al. 2003).

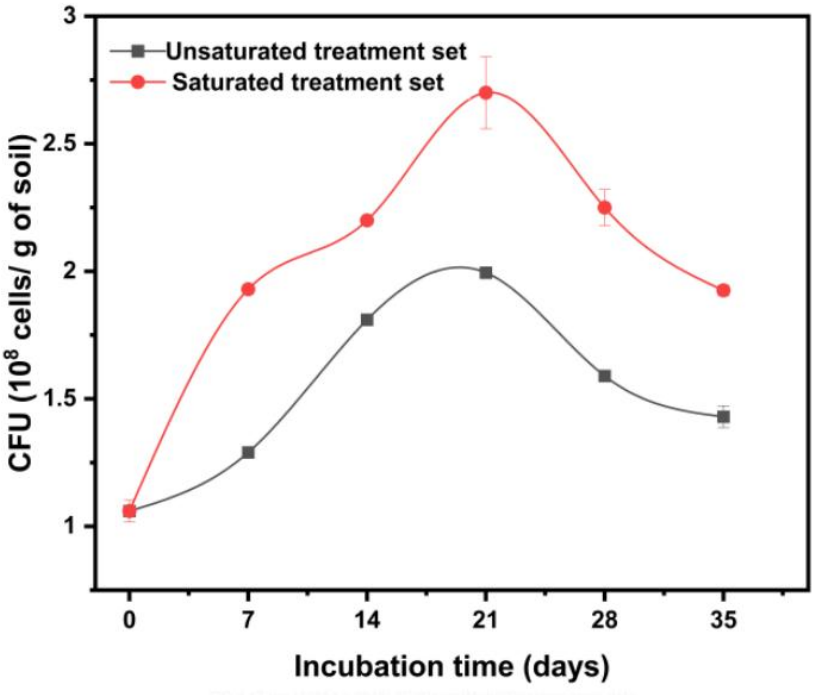


Figure 4.17: Comparison of the live cell count of *J. cremeus* embedded in the calcite bioformulation (BF1) during RDX degradation in unsaturated and saturated treatment sets.

At higher water content, the diffusion rates of oxygen are observed to be lowered, leading to anoxic conditions, promoting the growth of facultative/ strict anaerobes (Maier, 2009). Since, *J. cremeus*, a facultative anaerobe prefers anaerobic conditions, their growth under anoxic conditions is better than aerobic conditions.

Total enzyme activity of *J. cremeus* in soils contaminated with RDX

The total enzyme activity as a measure of cell viability was also performed by monitoring the FDA hydrolyzed. The soil samples were subjected to a reaction with FDA for 20 min. The enzyme activity was expressed as FDA hydrolysed per gram of soil per 20 min. Since the microcosm studies were carried out in strictly sterile conditions, the blank and calcite controls in both the treatment sets reported negligible hydrolysis of FDA. Whereas, the microcosms inoculated with the calcite bioformulation (BF1) exhibited significant hydrolysis of FDA in both sets. Figure 6 shows the enzyme activity for treatment sets 1 and 2. The positive growth of bacteria, *J. cremeus* present in the calcite bioformulation, during RDX degradation was reflected by the total enzyme activity. As seen in figure 4.18, the total enzyme activity of the bacterium in unsaturated condition though increasing with time, is far less than the activity expressed in saturated conditions. Pan et al. in 2016 observed a higher microbial activity in increased moisture conditions. Friedel et al., in 2000, had studied the effect of water content in soil on the microbial activity. They had also observed that, higher water content promoted microbial activity.

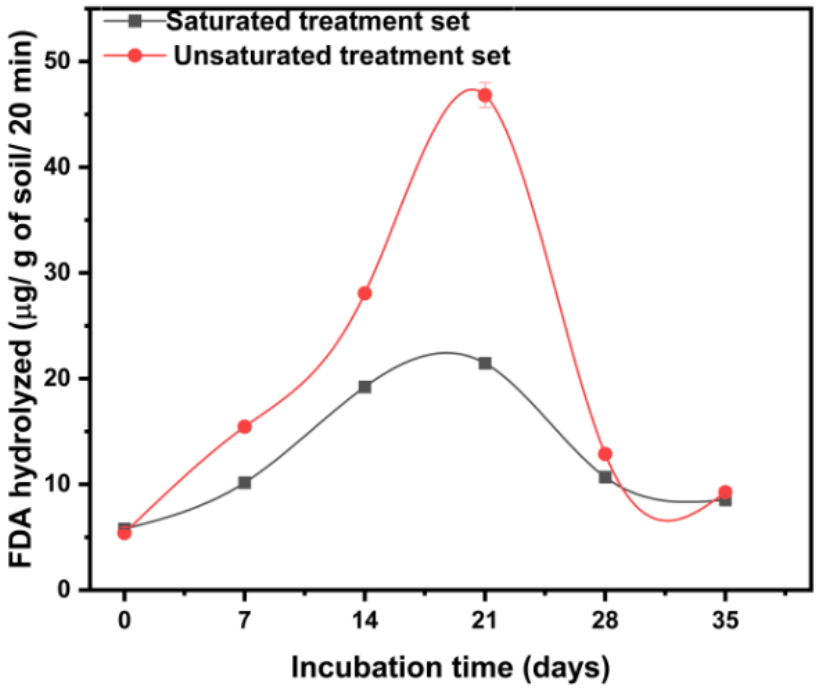


Figure 4.18: Comparison of total enzyme activity in terms of FDA hydrolyzed in soils incubated with calcite bioformulation (BF1) in saturated and unsaturated conditions.

4.2.2.1.2 Degradation analysis in microcosms

Nitrite released during remediation of RDX contaminated soil microcosms

Denitration is one of the major steps in the biodegradation of RDX (Crocker et al. 2006) leading to the release of the most common intermediate in the RDX degradation pathway, that is nitrite. Fournier et al., in 2002, studied the key metabolite in the biodegradation of RDX by *Rhodococcus sp*. strain DN22. They found that nitrite was the first metabolite released which they suggested to be produced by denitration prior to ring cleavage. Zhao et al. (2002 and 2003), Bhushan et al. in 2003, have also postulated pathways with the concomitant release of nitrite. The nitrite release was monitored spectrophotometrically by the method described by Mercimek et al., 2013. The results (Figure 4.19) showed that the nitrite released in microcosms inoculated with calcite bioformulation, increased up to 28 days with a peak at concentration of 10.63 and 8.38

mg Kg^{-1}in saturated and unsaturated treatment sets respectively, with a further decrease in concentration later. This decrease in nitrite concentrations in the later stages can be attributed to the uptake of nitrite ions by nitrite assimilatory enzymes. Denitrification leads to the assimilation of nitrites. These accumulated nitrites often have an inhibitory effect on the microbial growth, hence microbes switch on the nitrite reductase enzymes, that convert the assimilated nitrite to nitric oxide (Albina et al. 2019). Also, Coleman et al., in 1998, reported a similar pattern of nitrite release and uptake during the aerobic degradation of RDX by *Rhodococcus sp*. strain DN22. The higher concentration of nitrite in saturated sets indicate higher extent of denitrification occurring, hence more releaseof nitrite.Nitroreductases are the major enzyme system present in *J. cremeus* responsible for denitration of RDX. We can conclude that, saturated conditions can lead to a local anaerobic environment, hence promoting the activity of nitroreductases.

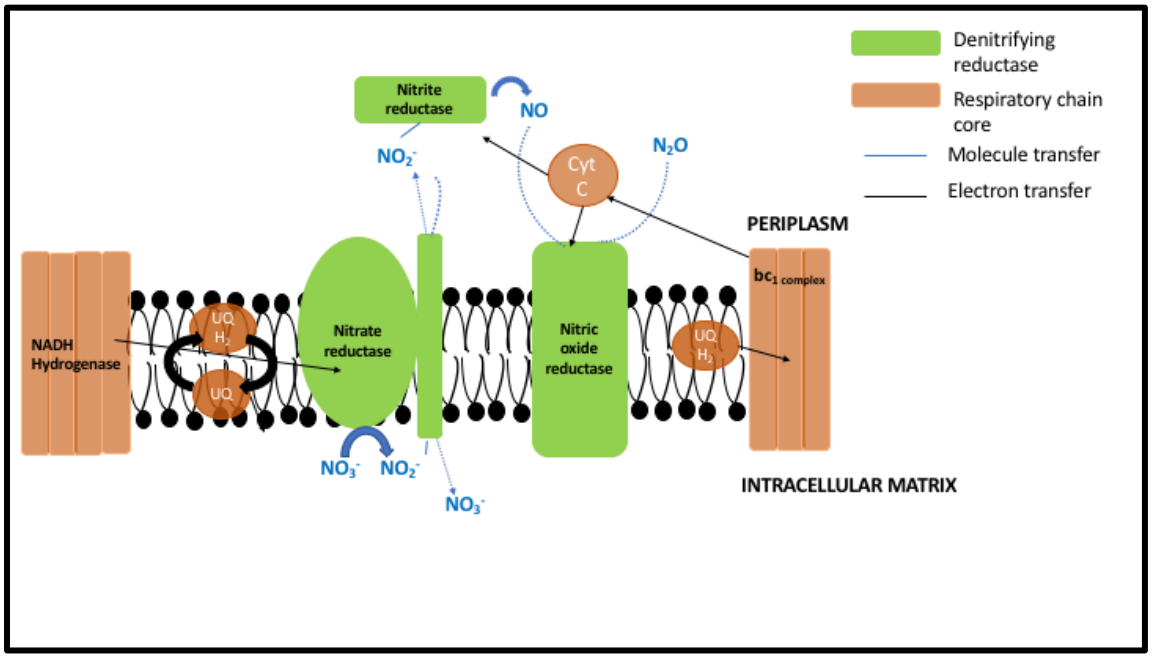


Figure 4.19: General denitrification path coupled with respiratory chain followed by bacteria

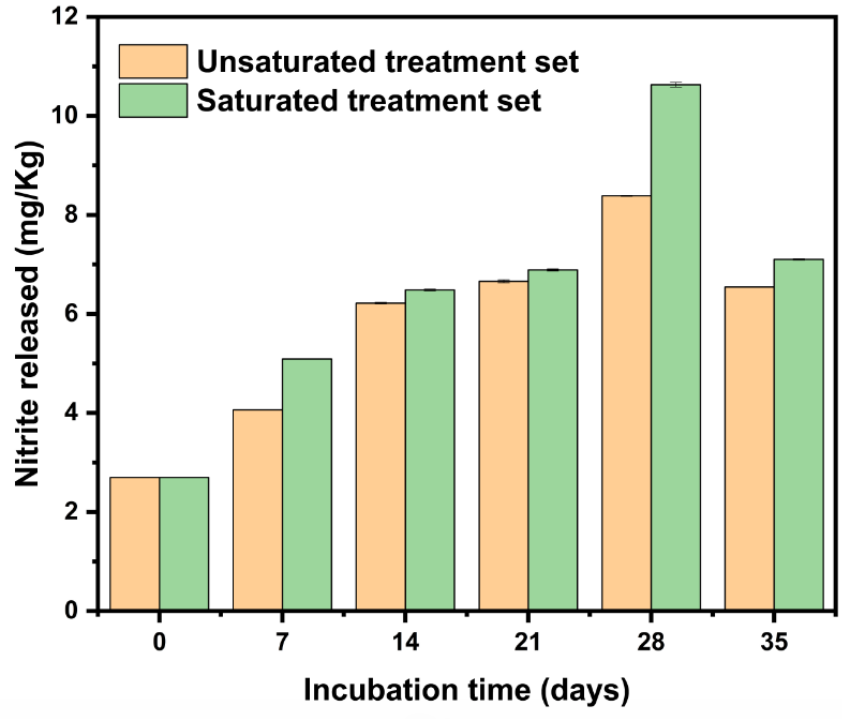


Figure 4.20: Comparison of the nitrite released during RDX bioremediation in unsaturated and saturated treatment sets.

Monitoring of residual RDX concentration

The residual RDX concentration was analyzed using HPLC. The unsaturated treatment set (set 1) showed lower degradation than saturated or well drained treatment set (set 2). The contaminated soils treated with uninoculated bioformulation, showed a 4.13 and 4.89 % degradation of RDX in unsaturated and saturated conditions respectively (Figure 4.21b). Whereas the treatment sets with BF1 as the inoculant showed 60.43 (for unsaturated) and 75.02 % (for saturated) reduction in RDX concentration (Figure 4.21c). The control experiments without any amendment showed negligible reduction in RDX concentration of 3.2 and 5.9% for unsaturated and saturated conditions respectively (Figure 4.21a). Though bioaugmentation hasn't been yet studied with different soil water content, bioremediation by natural attenuation has been studied previously. Under different moisture contents, Sagi-Ben Moshe et al., in 2012, found that increase in soil

water content led to better remediation of explosives RDX, HMX and TNT. They postulated that, higher soil water content led to reduction in redox potential thereby enhancing the remediation. Ringelberg et al., in 2003 had also found a similar pattern. They also observed higher RDX degradation with higher soil moisture content.

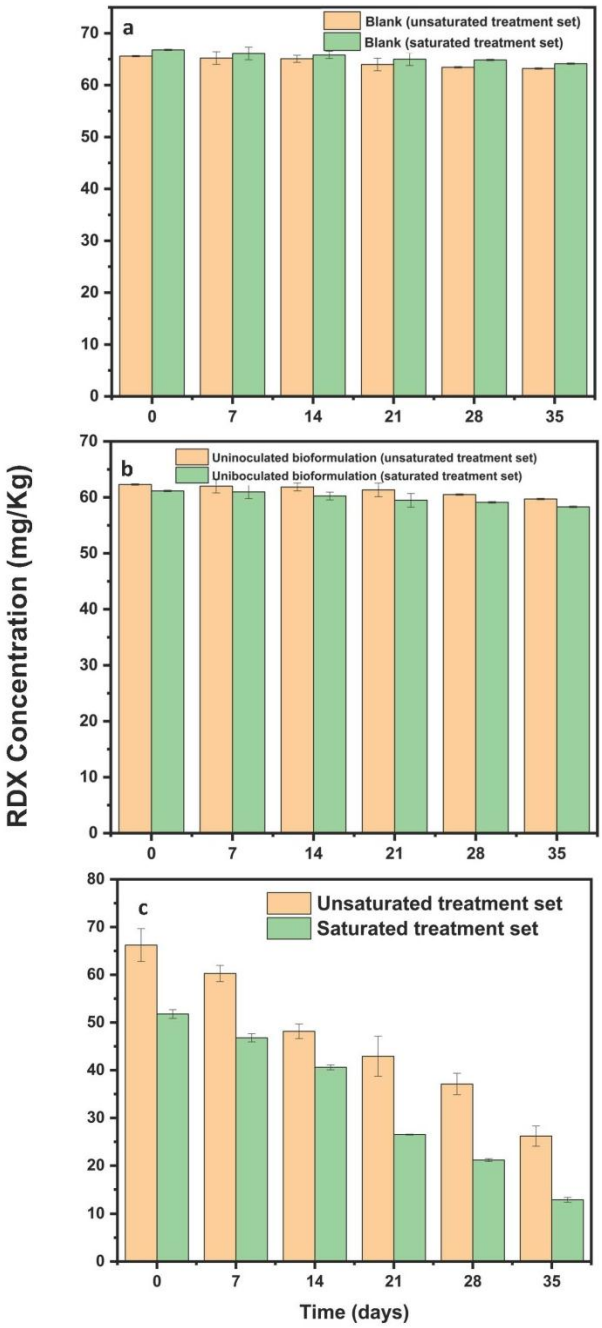


Figure 4.21: RDX degradation in different treatment sets under unsaturated and saturated conditions (a) Blank (b) uninoculated bioformulation (c) BF1

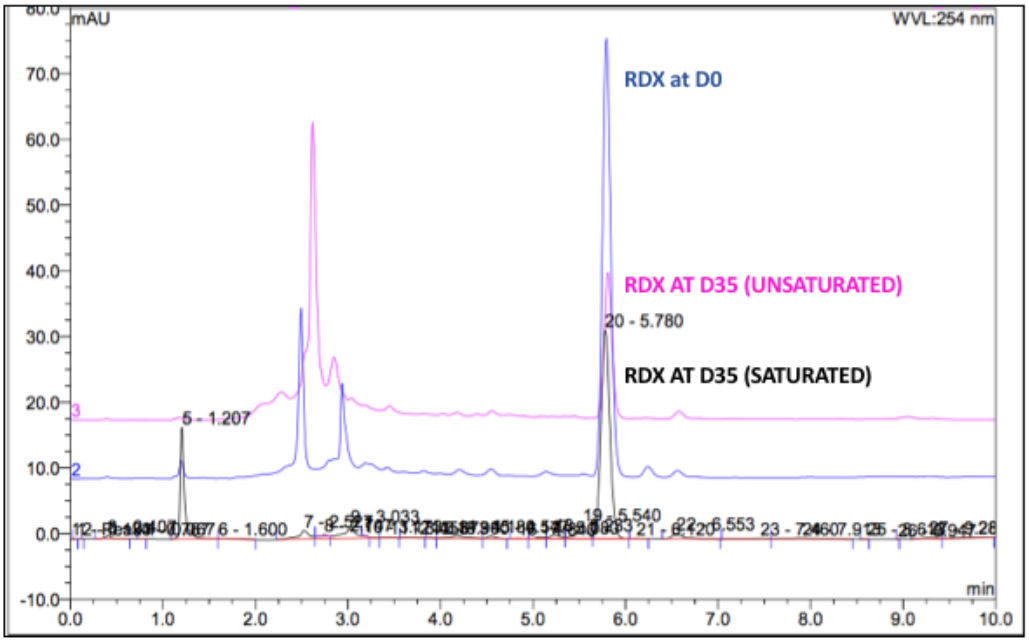


Figure 4.22: HPLC overlaid chromatogram showing RDX degradation using developed BF1 under unsaturated and saturated conditions.

4.2.2.2 Bioremediation of HMX in soil using BF1

HMX bioremediation studies were performed in microcosm levels by spiking the collected sandy loam soils with 3000 mg Kg^{-1} of HMX. Two treatment sets were analyzed for their remediation efficacy. The soils in set 1 were maintained at 50- 60 % of WHC (unsaturated) and set 2 soils were flooded with water (saturated). Sacrificial sampling was performed at regular intervals of 0, 7, 14, 21, 28 and 35 days. The samples were monitored for live cell count, total enzyme activity in terms of FDA releasedand residual explosive concentration for both the treatment sets.

4.2.2.2.1 Microbial activity assessment in the microcosms

Growth of the immobilized bacterium in soils contaminated with HMX

The bacterial growth in the treatment sets inoculated with the developed bioformulation (BF1) were monitored in terms of live cell count. This was used as parameter to assess

the effect of HMX on the growth and survival of the bacteria *J. cremeus* in the soil. Figure 4.23 compares the live cell count of the immobilized bacteria during HMX bioremediation study und saturated and unsaturated conditions. As observed, the CFU of the bacteria under saturated condition increased through the study, with a maximum bacterial growth observed at day 21. Thereafter, a decrease in the CFU was observed. But the decrease was not below 10^7 cells which is a prerequisite of bioremediation in soil (Pandey and Maheshwari, 2007). Thereby showing that, the bacterial growth and population was positive for remediation of HMX. Under unsaturated treatment conditions, there was a continuous decrease in the bacterial population throughout the study. The decrease in unsaturated treatment sets may be attributed to the fact that, high HMX concentration (3000 mg Kg^{-1}) was hazardous to bacteria under aerobic conditions.

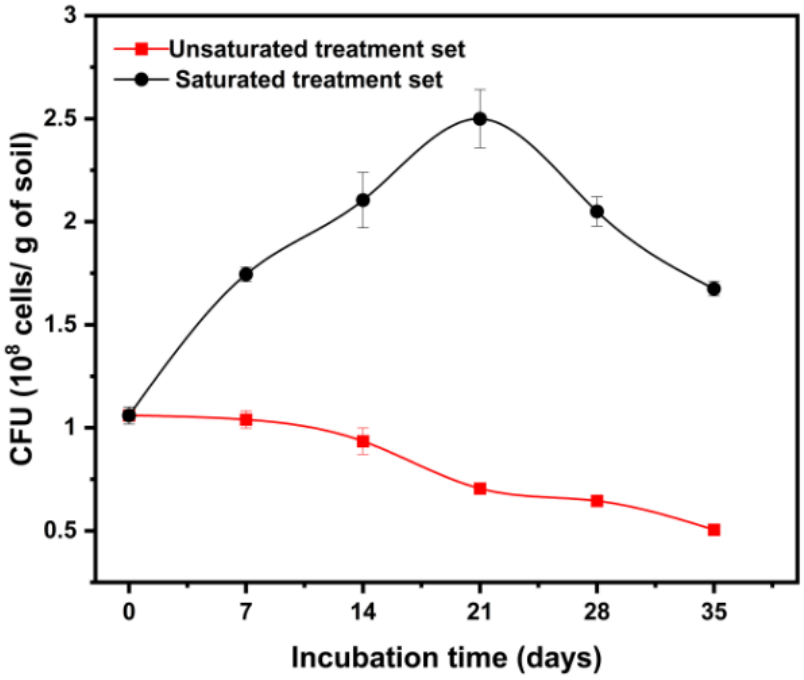


Figure 4.23: Growth of immobilized *J. cremeus* in HMX contaminated soil under unsaturated and saturated conditions.

Monitoring of total enzyme activity

Cell viability was tested as total enzyme activity measured in terms of FDA hydrolyzed by the living cells. The soil samples were analyzed for the FDA hydrolysis to predict the total enzyme activity. The treatment sets inoculated with the developed bioformulation were analyzed for the same. Figure 4.24 compares the total enzyme activity in both conditions (saturated and unsaturated). Under saturated conditions, the total enzyme activity increased with time, with a peak at day 21 which reported a 36μg FDA hydrolysed/ g of soil in 20 min. The enzyme activity was found to decrease thereafter. Under unsaturated conditions, treatment sets show decrease in the enzyme activity with time. This pattern was found to be similar to the microbial growth monitored. An important reason for higher enzyme activity under saturated conditions can be drawn to the anaerobic conditions prevailing in the treatment sets. Nitroreductase enzyme is a major enzyme system responsible for nitramine degradation. *J. cremeus* is known to possess this enzyme system (Hamada et al. 2013). Under anaerobic conditions, type I oxygen-insensitive nitroreductases are much more active and lead to the degradation of nitro-organics (Roldán et al. 2008). Thus, leading to higher enzyme activity.

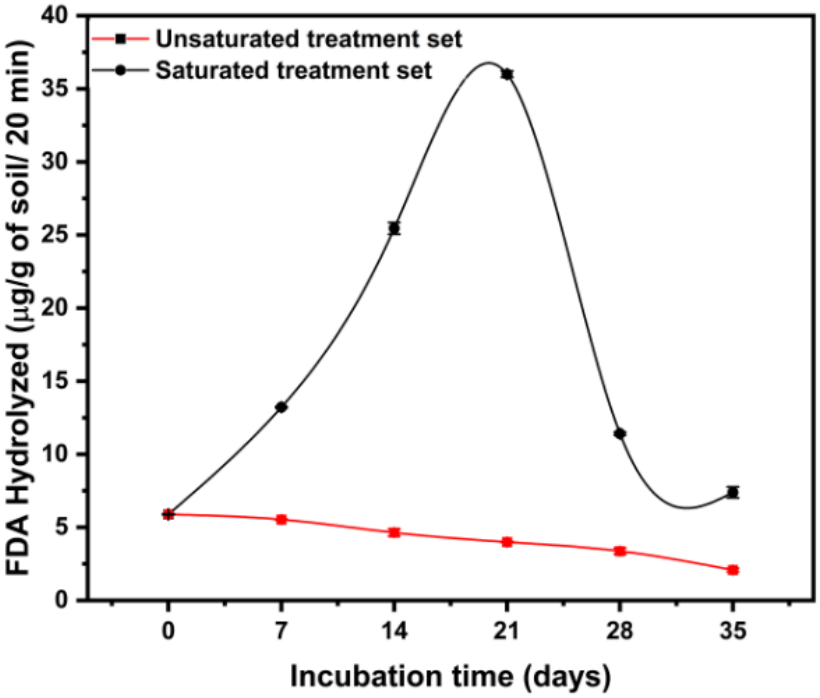


Figure 4.24: Comparison of the total enzyme activity during HMX remediation in the saturated and unsaturated treatment sets

4.2.2.2.2 Degradation analysis in microcosms

Monitoring the nitrite released

Nitrite being an important intermediate in the degradation of HMX was monitored throughout the study. Our results showed that, nitrite was released during saturated treatment of HMX with the developed bioformulation (BF1), but not with the unsaturated treatment sets (figure 4.25). The nitrite released in the saturated microcosms was found to increase continuously (highest concentration of 33.47 mg Kg^{-1} was observed at day 35). Formation of nitrite during bioremediation of HMX has been reported previously (Singh et al. in 2008; Nagar et al. in 2018).

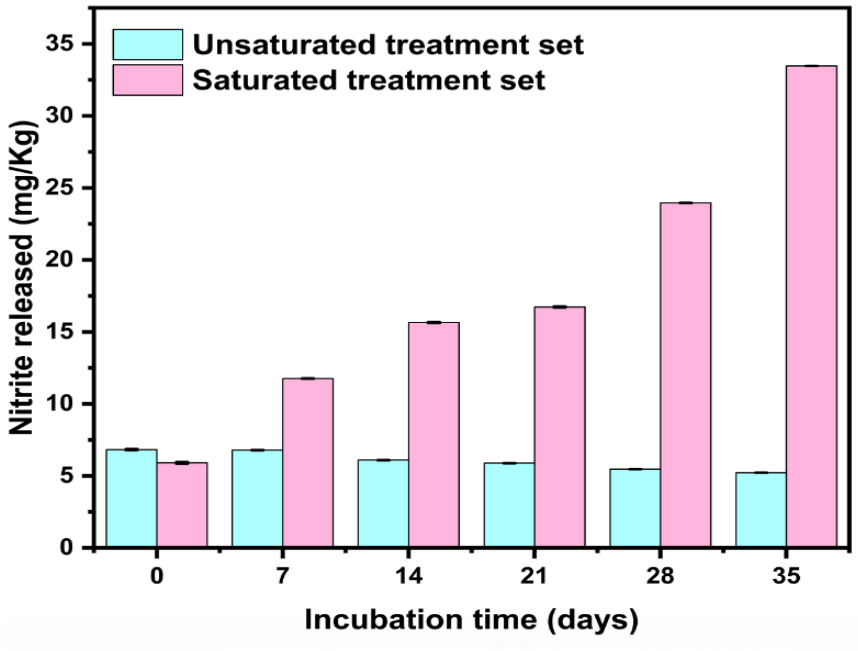


Figure 4.25: Release of nitrite during HMX remediation using BF1 under saturated and unsaturated conditions

Residual HMX concentration

The residual HMX concentration was analyzed for determining the HMX degradation potential of the developed bioformulation (BF1). The HMX degradation in uninoculated treatment sets was observed to be negligible (Figure 4.26 a, b). The treatment sets with the bioformulation as inoculant exhibited a negligible and 40 % degradation under unsaturated and saturated conditions respectively (Figure 4.26c). The decrease in the microbial growth and enzyme activity in the unsaturated treatment sets suggest a lack of bacterial activity resulting in absence of significant degradation of HMX under aerobic conditions. Under anoxic (saturated) conditions, 40% degradation was observed in overlaid chromatogram (Figure 4.27), positively corresponding to the microbial activity. Higher moisture content in soil led to reduction in redox potential thereby enhancing the remediation of explosives (Sagi-Ben Moshe et al. 2012). The reduced redox potential led to an anoxic environment promoting anaerobic degradation of HMX, which has been reported to be more efficient than aerobic process.

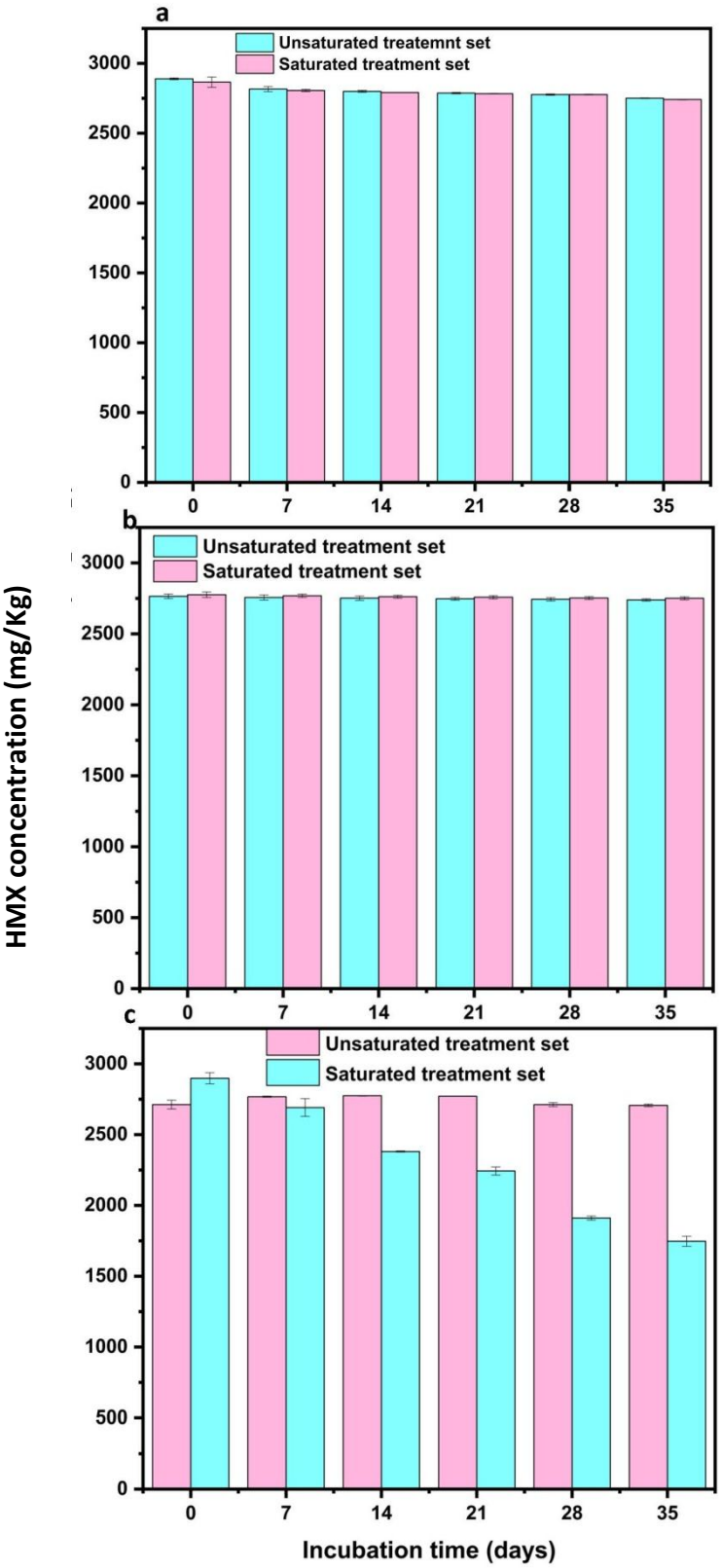


Figure 4.26: HMX degradation in different treatment sets under saturated and unsaturated conditions (a) blank (b) uninoculated bioformulation (c) BF1

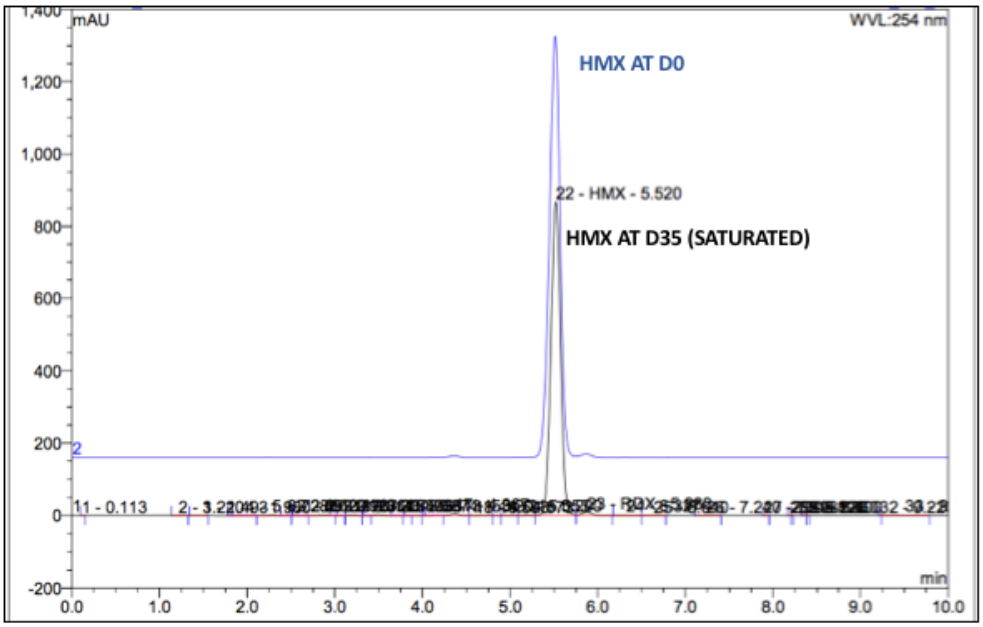


Figure 4.27: Overlaid HPLC chromatogram showing initial and final peaks for HMX under saturated treatment conditions on treatment with BF1.

4.2.3 Bioremediation of explosive contaminated sediments using BF1

The sediments present in the premises of explosive manufacturing industries are generally contaminated with high concentration of explosives (Figure 4.28). It has been observed that, the sediments obtained from HMX manufacturing facilities generally contain both HMX and RDX. This is due to the production process. As explained previously, HMX is manufactured by a modified Bachman's process. In this, RDX is produced as a by-product. Due to the presence of both RDX and HMX, these sediments are considered more toxic and also, are difficult to degrade. The sediments are also characterized by very low pH. The acids used in the manufacture of the explosive compound, viz, RDX and HMX contribute to the acidic nature. The major challenge in bioremediation of sediments in general is the low pH, as it hinders the microbial activity and survivability.

In this study, bioformulation (BF1) was tested for its efficacy in treatment of explosive contaminated acidic sediments obtained from explosive manufacturing facilities in India. Sediment A, obtained from an RDX manufacturing facility, was found to be contaminated with RDX in high concentrations (984000 mg/Kg). Figure 4.28 depicts the chromatograms for sediments from both explosive manufacturing facilities. Sediment B, obtained from an HMX manufacturing facility, was found to be co-contaminated with RDX (82000 mg/Kg) and HMX (71000 mg/Kg) in high concentrations. Both the sediments were characterized by very low pH, which can exhibit a detrimental effect on microbes. The high concentration of explosives as well as very low pH of the sediment could exhibit detrimental effects on the growth and survivability of the microbes and hence, effect the bioremediation potential of the same.

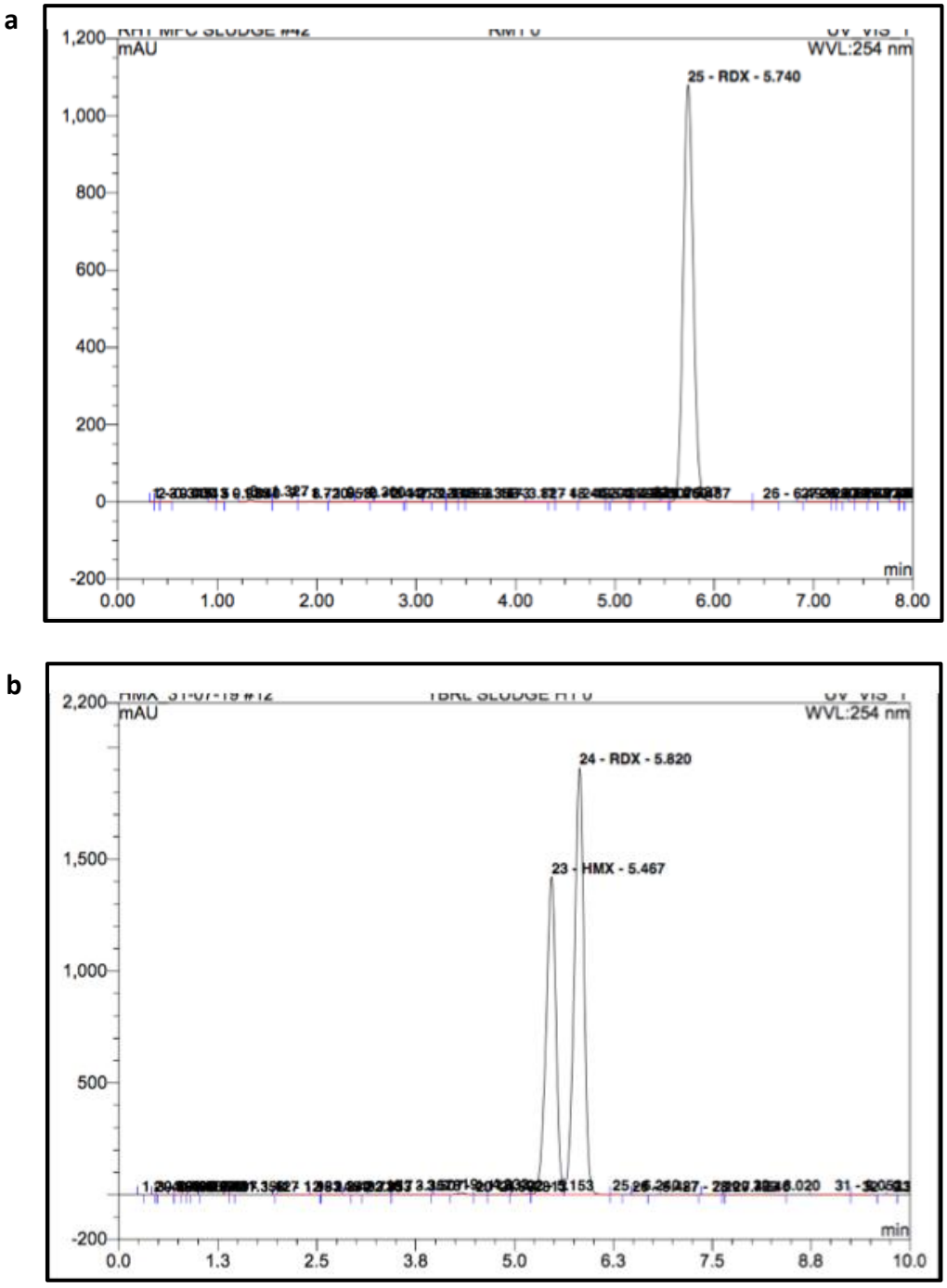


Figure 4.28: Chromatograms depicting explosives present in (a) Sediment A (b) Sediment B

The sediments obtained from RDX (Sediment A) and HMX (sediment B) manufacturing facilities were subjected to bioremediation using the bioformulation, BF1 at mesocosm levels. 300 g of sediment was weighed (moisture content corrected)

in amber colored 1000 mL bottles. They were then inoculated with 20 % (w/w)of BF1. Control experiments with only the sediment as well as sediment amended with uninoculated bioformulation at 20 % (w/w) were also set up. Experiments were conducted in saturated conditions at room temperature for 5 months, with samples withdrawn weekly for the first month, followed by monthly samples.

4.2.3.1 pH monitoring

The sediment A is characterized by a low pH of 4. On addition of uninoculated bioformulation and BF1 the pH of the sediment was observed to increase to 6.7instantaneously. Sediment B is characterized by a further low pH of 2.8. On addition of uninoculated bioformulation and BF1, its pH was observed to be 5.7. Increase in the pH on addition of BF1 and uninoculated bioformulation could be attributed to calcite present in the carrier mix. As discussed earlier, calcite is essentially calcium carbonate which is a mild alkali. Also, calcium carbonate is a known amendment in remediation and revitalization of soil contaminated with hazardous compounds. It's use has also been reported in stabilization of acidic soils (EPA 542-R-07-013, 2007). Presence of excess water in the remediation system as well as presence of calcium carbonate can bring about mild hydrolysis.

Liming, that is the addition of lime to soils, has been reported to stimulate biological activity. It has also been observed to increase the cycling of nitrogen, phosphorus and sulphur thereby promoting its bioavailability. The pH increase in the study does not cross 7.2, thereby, not leading to the harmful effects of alkali addition.

Calcium carbonate (present in calcite) dissolves in the soil- water slowly to produce bi carbonate ions.The bicarbonate ions released in the process neutralize the hydronium

ions present in the soil solution. The calcium ions formed on the other hand displaces any hydronium ions present on the clay and humus particles, thereby increasing the pH of the system (figure 4.29). As the study progressed, a gradual increase in the pH of the sediments was observed (figure 4.30 a and b), which could be attributed to this slow and continuous action of calcium carbonate.

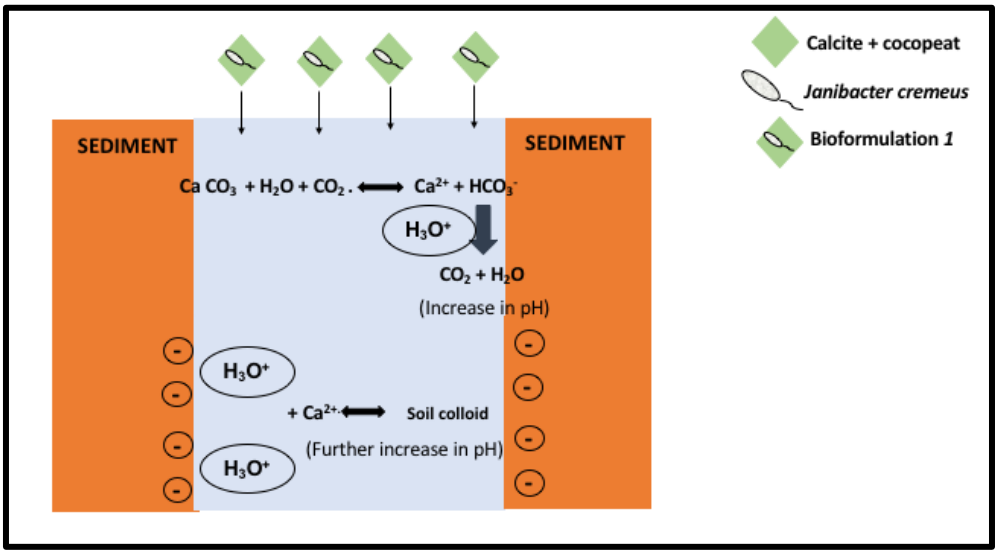


Figure 4.29: Mechanism of increase in pH of sediment on addition of BF1

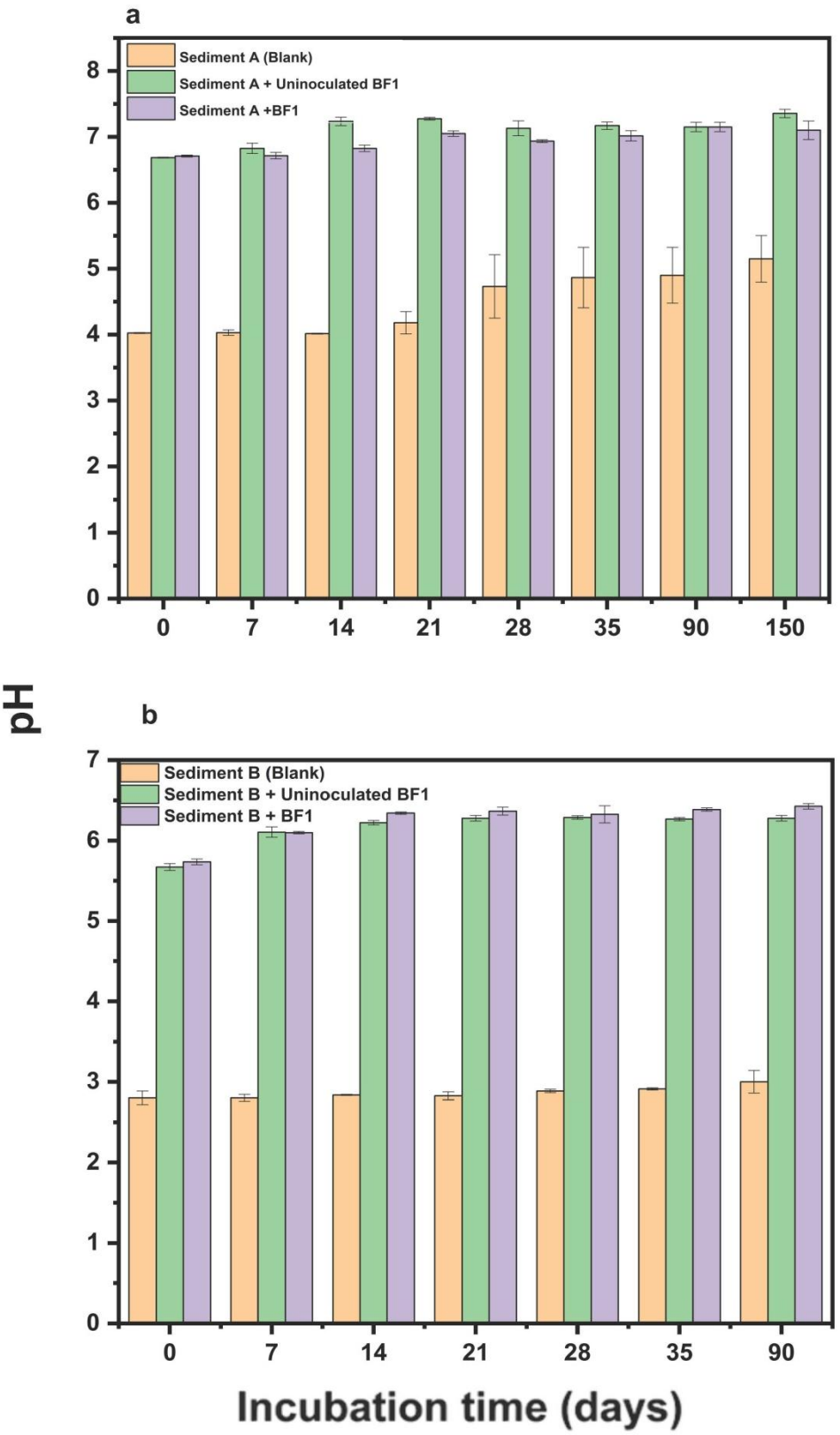


Figure 4.30: Comparison of pH change on addition of BF1 during the remediation of (a) Sediment A (b) Sediment B

4.2.3.2 Total enzyme activity of the heterotrophic microbes in treatment study

The total enzyme activity in terms of FDA hydrolyzed, was used as a measure of the live bacterial population in the treatment sets. Fluorescein diacetate is a fluorescein conjugated with two acetate moieties. On hydrolysis (by both free and membrane bound enzymes) followed by dehydration, the colorless FDA is converted to colored Fluorescein, which can be spectrophotometrically analyzed at 500 nm. The enzymes responsible for FDA hydrolysis are generally ubiquitous, viz, esterase, proteases and lipases (Adam and Duncan, 2001). This technique has been well documented as a measure of microbial biomass in sediments (Mahu et al. 2018). Since, the treatments were not sterile; the total enzyme activity was attributed to all the microbes active in the sediment. The total enzyme activity of treatment sets of sediment A and B with the BF1 as inoculant was found to be higher than uninoculated control followed by the blank controls (Figure 4.31). Since the sediment is characterized by a very low pH, the diversity of microorganisms active is lower than those found at neutral pH (Uyttebroek et al., 2007).The increase in pH due to calcite in treatment sets (uninoculated and inoculated bioformulations), led to an increase in the respective enzyme activity due to activation of otherwise dormant microbes. Also, the treatment sets with BF1 as amendment, exhibited much higher enzyme activity, which could be reasoned to the growth and proliferation of *J. cremeus* and other ubiquitous microbes. The increase in the total enzyme activity in blank controls could be reasoned with the activation of anaerobic microbes, due to the water saturation conditions maintained.

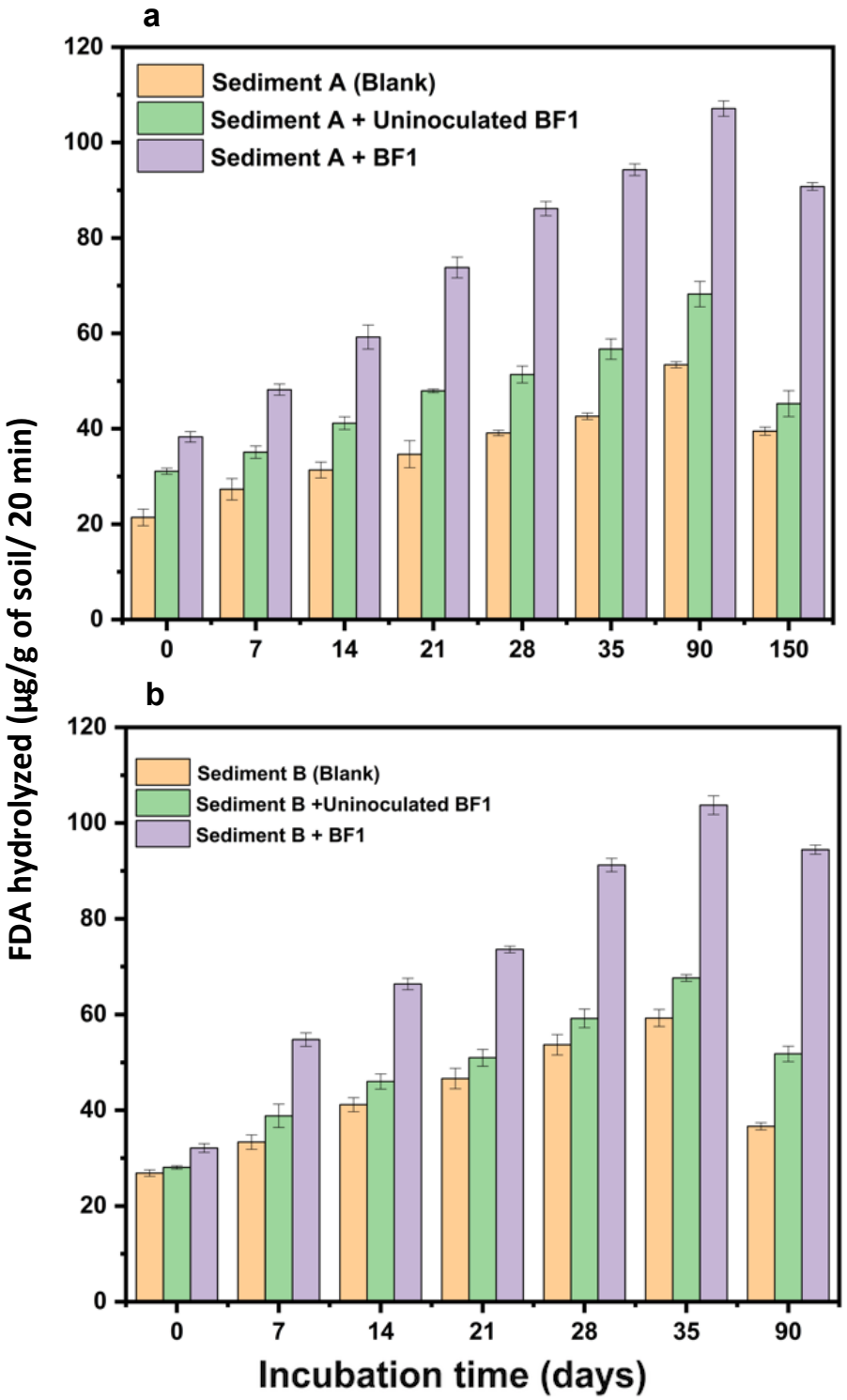


Figure 4.31: Total enzyme activity of the heterotrophic microbes during the remediation study in (a) Sediment A (b) Sediment B

4.2.3.3 Nitrite release during bioremediation of sediments

The treatment of explosive contaminated sediments (A and B) was carried out under saturated conditions. The saturated conditions lead to reduction in the redox potential, making the system anoxic/ anaerobic (Sims and Kanissery, 2019). RDX and HMX catabolism is characterized by release of nitrite under both aerobic as well as anaerobic conditions (Fuller et al. 2016; Zhao et al. 2004 a and b).The controls without any amendments under saturation showed an increase in nitrite content. This could be due to the activation of anaerobic microorganisms present in the sediment due to reduced conditions leading to denitration. The controls with uninoculated bioformulationand BF1 as amendment showed an increase in nitrite higher than compared to blank treatment. This could be attributed to thedenitration due to microbes as well as mild chemical hydrolysis reaction occurring due to the presence of calcium carbonate.The trend in change of nitrite concentration has been depicted in figure 4.32. Balakrishnan et al. in 2003, had studied the process and intermediates formed during the hydrolysis of nitramine explosives, RDX and HMX. Their results corresponded to the action of alkali. They had suggested nitrite to be the first intermediateformed during the process. This study utilizes a mild alkali, calcium carbonate in form of calcite. As observed, calcite not only acts as a buffering agent leading to an increase in pH of the acidic sediments to neutral range, but also contributes in the hydrolysis reaction of the explosives.

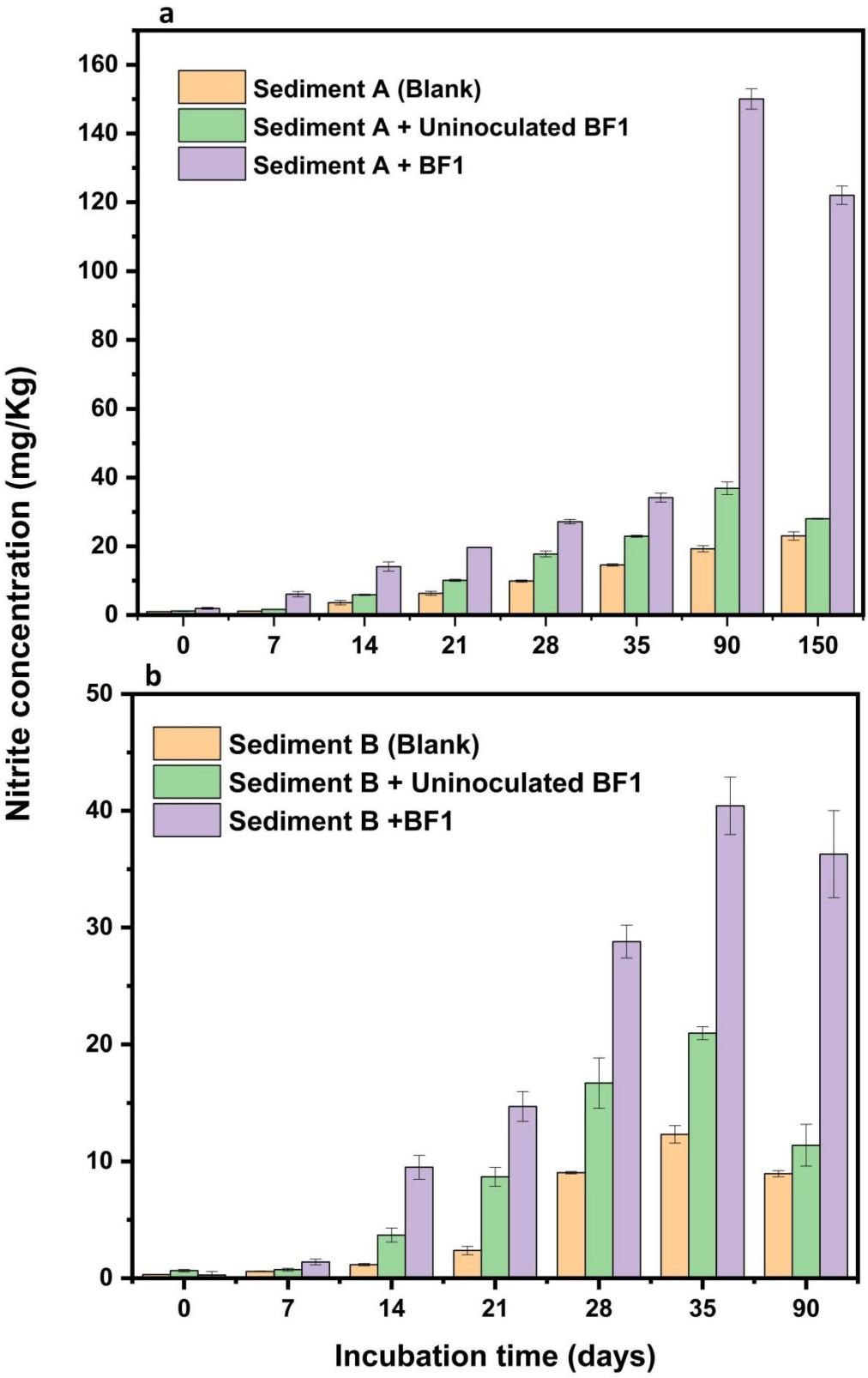


Figure 4.32: Change in nitrite concentration during remediation of (a) Sediment A (b) Sediment B

4.2.3.4 Residual explosive concentration

The sediments were found to be highly contaminated with explosive compounds (RDX and HMX). Based on the performance of developed bioformulation BF1 in soil microcosm studies, the bioremediation of sediments was carried out under saturated conditions. Moreover, it has been reported that, biodegradation is generally enhanced under saturated conditions (Price et al. 2001; Speitel et al. 2001; Ringelberg et al. 2003). The bioformulation BF1 contains calcite and cocopeat as the carrier. Both calcite and cocopeat have high surface area that can help in adsorption of the bacterium, *J. cremeus*, thereby leading to its immobilization. Also, both calcite and cocopeat have been reported to be adsorbents for organic pollutants (Biswas et al. 2015b; Vijayaraghavan et al. 2015; Premkumar and Vijayaraghavan, 2014). This process thereby increases the bioavailability of the explosive compound, leading to its better transformation (Figure4.33).

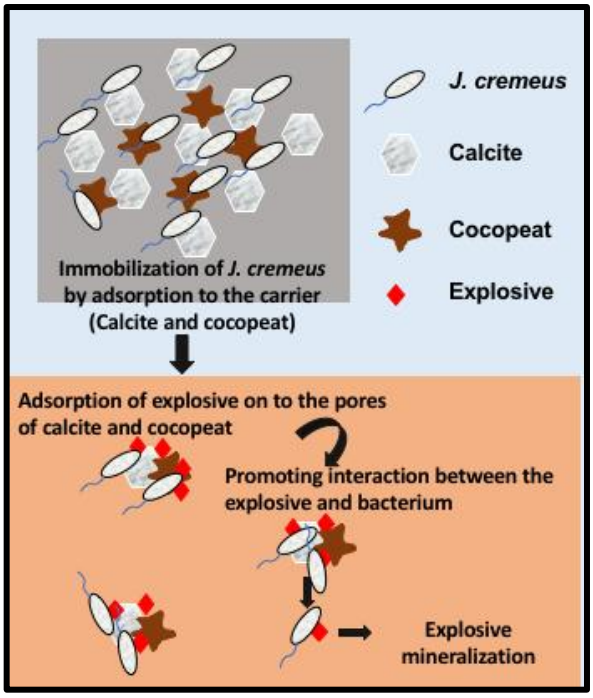


Figure 4.33: Role of calcite and cocopeat in remediation of explosive remediation

Sediment A was characterized by a very high concentration of RDX. The residual RDX concentration in control without any amendments was found to decrease by 30 % in 5 months. The uninoculated bioformulation amended control showed a degradation of 40 % and the treatment sets inoculated with the developed BF1, showed a 90% decrease in RDX concentration in 5 months (figure 4.34). The reduction inexplosive concentration in formulation treated sets (BF1) were in agreement with the limits described by EPA (1993), which renders sediments (less than 10 % on dry weight basis) unsusceptible to initiation and propagation.

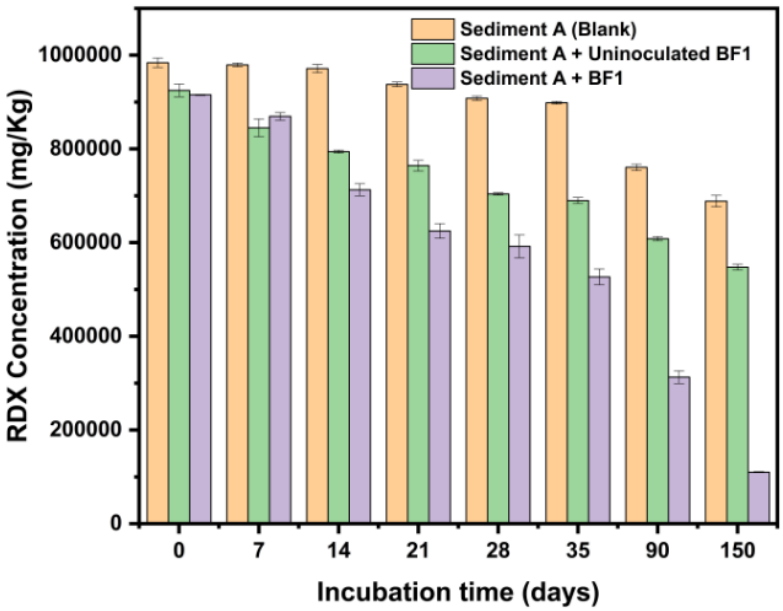


Figure 4.34: Change in RDX concentration in Sediment A during remediation with BF1

Sediment B on the other hand was characterized by a co-contamination of RDX and HMX. The control treatment sets showed a 30 and 26 % decrease in RDX and HMX respectively. On the other hand, the treatment sets with BF1 as amendment showed 55 and 47 % reduction in RDX and HMX respectively (figure 4.35 and 4.36). Figure 4.37 shows the overlaid chromatograms of the blank and BF1 treated sets. The reduced degradation of RDX in comparison to sediment A could be attributed to the inhibitory

effect due to the coexistence of HMX. It has been reported that, high concentration of an explosive can inhibit the degradation of a coexisting explosive (Berstein et al. 2011). Jackson et al in 2007 reported the inhibition of RDX degradation by the presence of TNT due to the inhibition of cytochrome P450, an important enzyme system responsible for RDX degradation. Fuller et al. in 2009 observed that, high concentrations of RDX led to an inhibition in HMX degradation.

It is important to understand is that the developed bioformulation (BF1) helps in bioremediation of the acidic sediments by 1) raising the pH so as to help survival of microbes 2) immobilized *J. cremeus* works in tandem with other indigenous microbes that may possess the ability to degrade explosives, for remediation of the sediments 3) the calcite present also acts as a mild hydrolysis agent 4) pores on the surface of calcite and cocopeat offer adsorption sites for both *J. cremeus* and explosives, thereby, increasing the bioavailability of explosive compound.

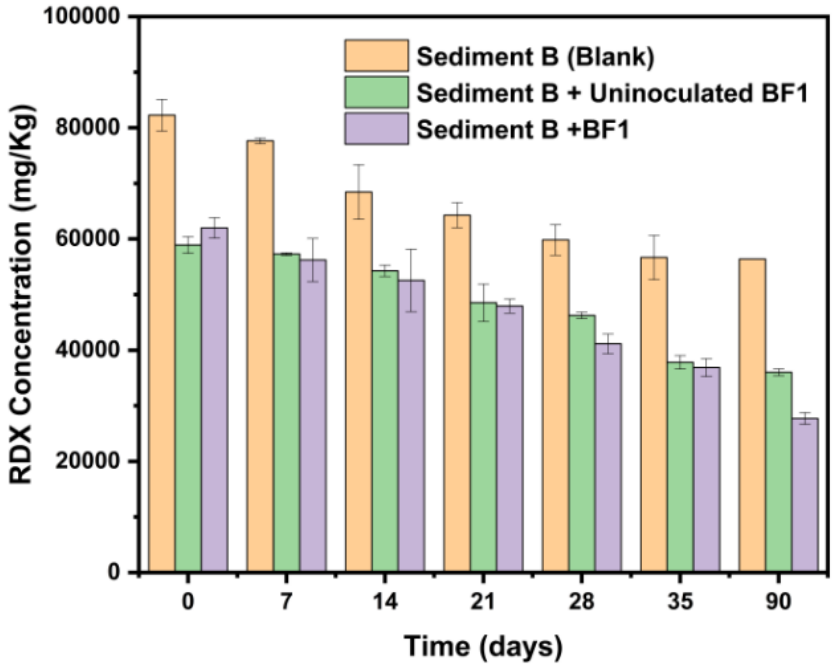


Figure 4.35: Change in RDX concentration in Sediment B during remediation with BF1

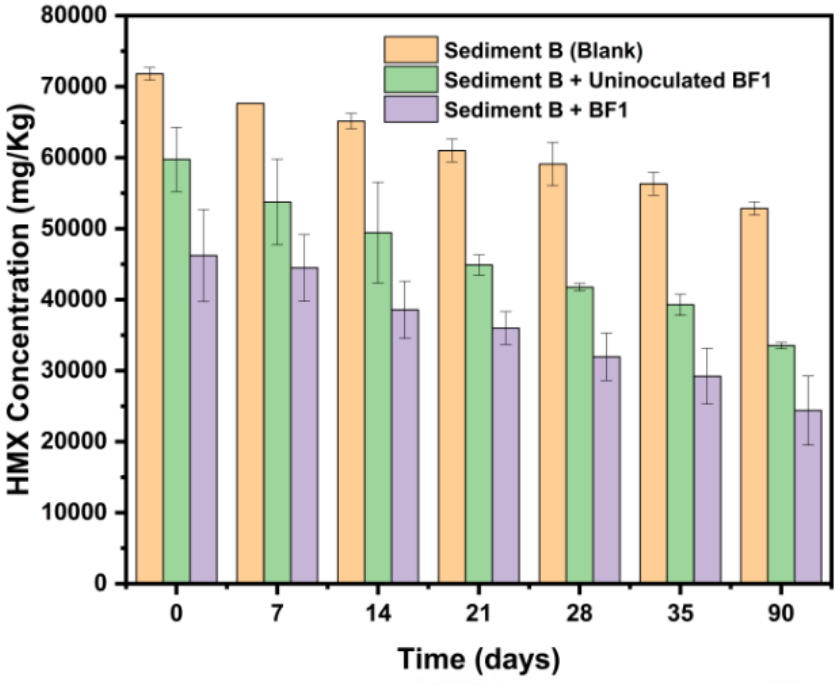


Figure 4.36: Change in HMX concentration in Sediment B during remediation with BF1

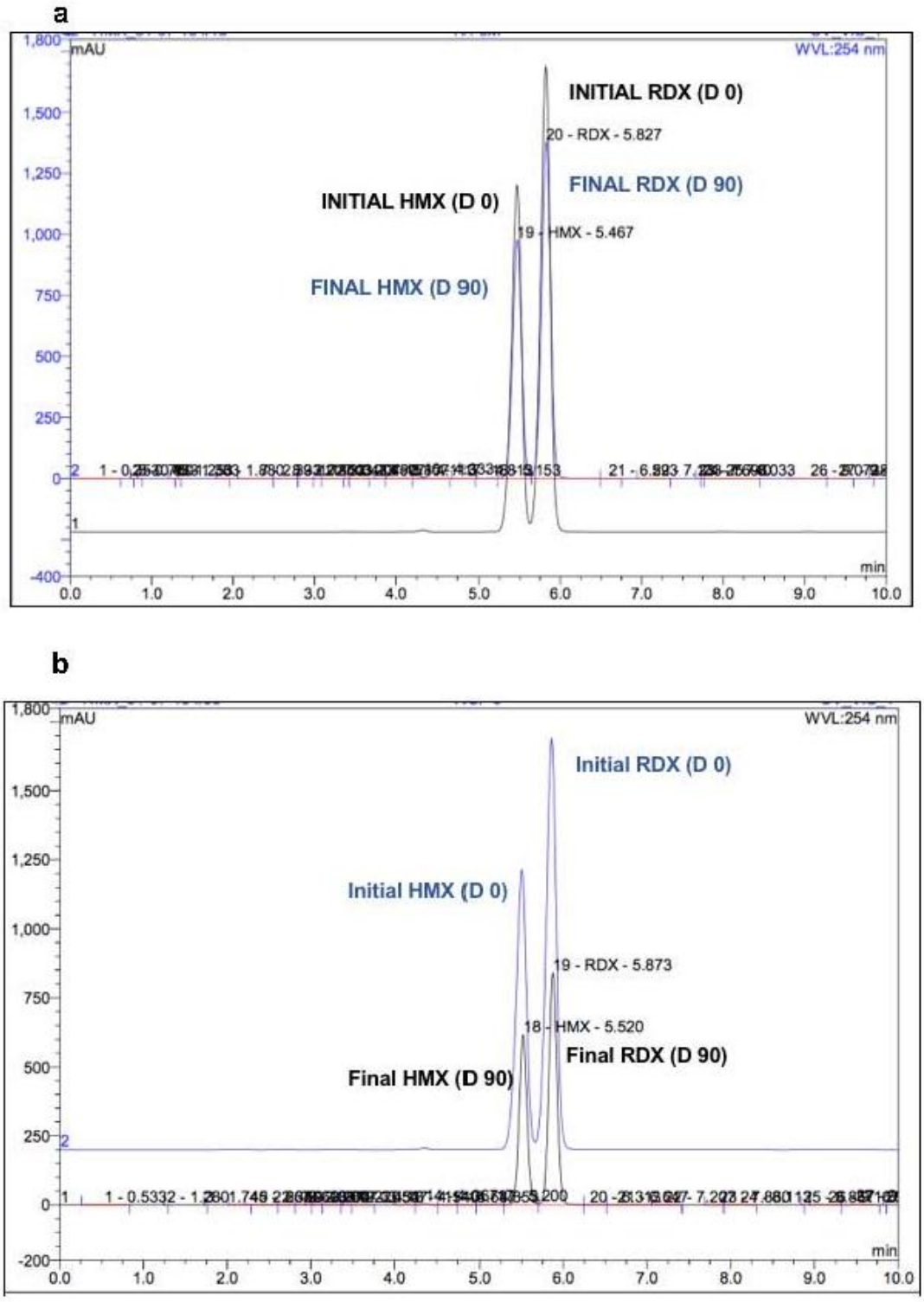


Figure 4.37: Overlaid chromatogram comparing initial and final explosive concentrations in (a) blank (b) treatment with BF1

4.2.4 Conclusion

The explosive degrading soil bacterium *J. cremeus* was immobilized on a carrier mix of calcite and cocopeat to develop a powder formulation (BF1). The developed formulation, BF1 was tested for its viability and efficacy. The viability studies showed the carrier to be supportive of cell population for 150 days with 18% loss by the end of 180 days. The BF1 was tested for its efficacy to degrade RDX and HMX in soil under saturated and unsaturated conditions. RDX (65 mg/Kg) was degraded by 60 and 75 % under unsaturated and saturated conditions respectively. HMX (3000 mg/Kg) was observed to be degraded (40 %) under saturated conditions only. The BF1 was then tested to treat acidic sediments (A and B) obtained from explosive manufacturing facilities in India. The RDX in sediment A was successfully degraded by 90 % in 150 days. Sediment B was characterized by a co-contamination of RDX and HMX. The effective degradation of 55 % for RDX and 47 % for HMX was obtained in 90 days. The developed BF1 has proven to have potential for minimizing the explosion hazard of the contaminated sediments and soils. Constituents of the BF -1 viz, calcite and cocopeat, played a significant role in treating the hazardous explosive contaminant present in soil and sediments. The results obtained demonstrate that, the developed bioformulation (BF1) can find application in site remediation of explosive handling and manufacturing facilities.

4.3 Bioremediation of explosive contaminated soil and sediment using eggshell based bioformulation (BF-2)

Many technologies have been known for the remediation of explosive contaminated soil, viz, physical, chemical and biological. Physical and chemical technologies are associated with use of high temperature or chemicals for achievement of treatment goal. These technologies are not successful as they majorly lead to phase transfer of explosive pollutant, rather than mineralization, thereby leading to secondary pollution. To overcome these drawbacks, focus has been laid on bioremediation, as an eco-friendly tool to manage explosive contaminated soils (Kalderis et al. 2011). Bioremediation techniques have been in place for some time now, and can be broadly classified as microbial or plant mediated remediation. Microbial remediation utilizes the ubiquitous nature of microbes. These methods are sustainable and inexpensive alternative to physical and chemical methods. But, the process is associated with few drawbacks, viz, longer treatment durations, lower efficiencies and dependence on environmental factors (Xin et al. 2013). Hence, research needs to be focused on increasing the efficiency of the process.

Recently, bioaugmentation and/ or biostimulation for remediation of soils has gained much importance. But the major drawback lies in the limited survivability of the augmented microbe. Hence, use of carriers for delivery of microbes into the soil has been proposed for remediation of hazardous compounds (Wang et al. 2019; Zhang et al. 2019; Kalsi et al. 2020). Carriers that aid in increasing the bioavailability as well as provide nutrients to microbe help in better degradation. Therefore, selection of carrier materials based on these criteria help in better remediation of the explosive compound.

Explosives are characterized by low bioavailabilty due to their low sorption in soil (Monteil-Rivera et al. 2003). Use of carriers that can aid in increasing the bioavailability by increasing the sorption of explosive to soil can aid in better and rapid degradation by the immobilized microbe. Also, the carrier can help provide nutrients for survival of the bacteria, as well as provide conditions to help its growth in adverse conditions present in the contaminated soil. Many types of natural and synthetic materials have found application in remediation of various hazardous contaminants. With a shift in focus towards cost effectiveness, environmental and social sustainability, use of natural materials as a source of biochar or catalyst has already been studied in detail and many natural sources have been experimented with, for degradation of various contaminants (Ye et al. 2019 a,b; Ye et al. 2020). With the present scenario, exploring use of otherwise waste materials as carriers is also gaining acceptance (Simons et al. 2012). Examples include, egg shells and coco peat derived from coconut husks. Egg shells are very easy to source and inexpensive source of calcium carbonate. Calcium carbonate is a known amendment used in reclamation and remediation of soils contaminated with hazardous compounds (EPA 542-R-07-013, 2007). It has been reported as an amendment in treatment of military range soils contaminated with heavy metals (Siebielec and Chaney, 2012). Egg shells are also known to contain other essential nutrients that may contribute to survival of microbes (Antwi-Akomeah et al. 2018). Cocopeat is rich in organic matter and a very good adsorbent. It is known that increased organic carbon may increase the sorption of organic contaminants (Tucker et al. 2002) and make it bioavailable to microbe. Cocopeat has already been reported in sorption of dyes like, crystal violet, methylene blue, malachite green (Vijayaraghavan et al. 2015; Premkumar and Vijayaraghavan, 2014). It can also provide nutrients for

microbial survival and growth (Nunal et al. 2013). Use of eggshells and cocopeat as carriers for explosive degrading microbes hasn't been explored yet. The current study aims at development of a sustainable waste management approach wherein a bioformulation consisting of egg shellsas major constituent wasimmobilised with a native explosive degrading microorganism thatwas evaluated for in situ remediation of high explosive contaminated soils and sediments. Figure 4.38 represents the overview of the work carried out.

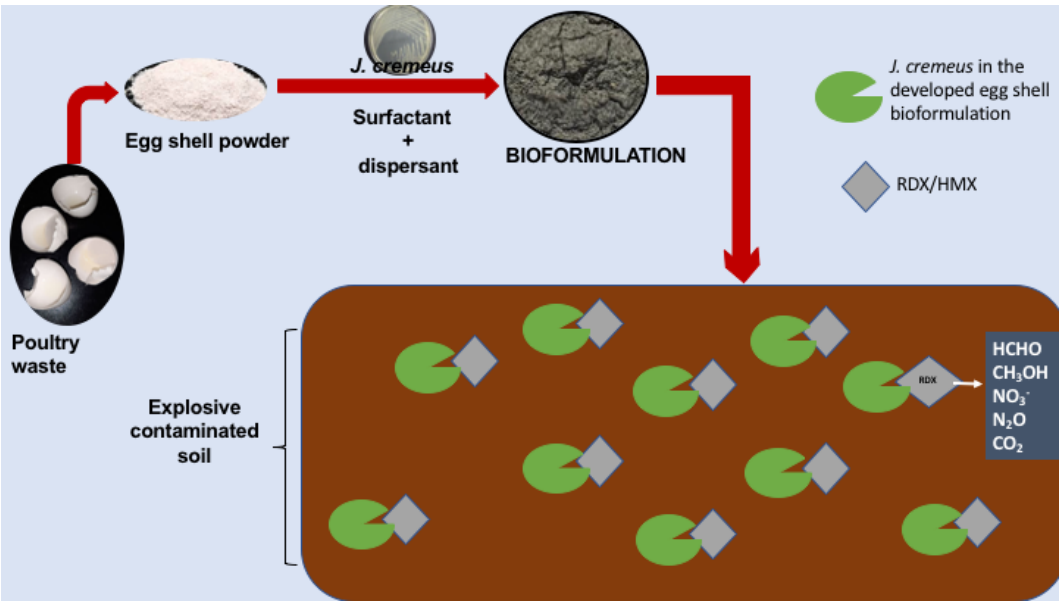


Figure 4.38: Steps involved in development and application of eggshell based bioformulation (BF- 2)

Janibacter cremeus was selected for the development of a novel bioformulation based on its performance in the aqueous phase studies. The bioformulation was prepared using egg shell powder as carrier. Other components of the water dispersible powder formulation included cocopeat as the organic amendment, Tween as the surfactant and sodium bi carbonate as the dispersant. The novel formulation developed was tested for its viability under storage at 4 °C for 180 days. Also, the formulation was tested for its

efficacy in treatment of explosives RDX and HMX present in soil and sediments. Figure 4.39 gives the experimental details brief.

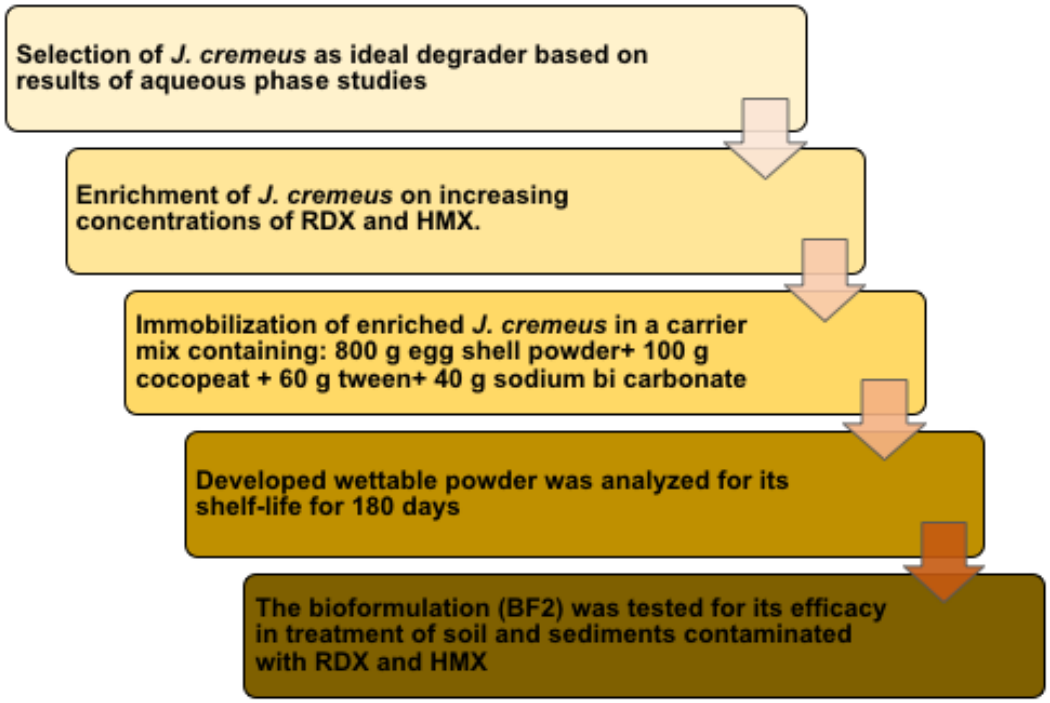


Figure 4.39: Experimental details of soil and sediment remediation using egg shell based bioformulation (BF2)

4.3.1 Preparation of Bioformulation 2

Selection of explosive degrading microorganism for bioformulation preparation

The aqueous phase studies conducted to evaluate the efficacy of the explosive degrading microbes, *Pseudomonas moselii*, *Pseudomonas entomophila* and *Janibacter cremeus* isolated from explosive contaminated sites to remediate RDX and HMX, were used to select the potential organism. Bioformulation 2 was prepared by immobilizing enriched cultures of *J. cremeus* onto powdered egg shells (carrier), cocopeat (organic amendment), tween (surfactant) and sodium bi carbonate (dispersant). *J. cremeus* was selected due to its good degradation efficiency for both RDX and HMX, also because it was a facultative anaerobe which could survive in depleted oxygen conditions. The bioformulation thus

developed finds application in soil and sediment remediation. Soil is a complex medium with oxic and anoxic interfaces. Respective differences in redox potential of the soil medium may lead to inactivity of strict aerobes/anaerobes. Hence, making *J. cremeus* a potential organism for explosive remediation (Sims and Kanissery, 2019).

Enriched cultures of *J. cremeus* were used for the bioformulation development. J. cemeus was grown in increasing concentrations of RDX and HMX so as to acclimatize the bacteria with the presence of the nitrogenous explosive compound as sole source of nitrogen.Use of acclimatized culture has already been reported in bioremediation of many hazardous pollutants (Madeira et al. 2019; Gupta et al. 2016; Patel et al. 2020).

Gunnison et al., in 1997 had observed that aerobic or microaerophilic mixed cultures that were acclimated to explosives could lead to better mineralization of explosive compounds at site. Hence, use of enriched culture would make the bacterial survival easier in the adverse soil environment and also aid in better remediation of explosive compounds.

Viability of the developed bioformulation 2

The novel wettable powder Bioformulation (BF2) developed was stored in autoclaved polyethylene bags at 4 °C. The viability of the developed formulation (BF2) was monitored for 180 days using plate count agar method. Figure 4.40 shows the viability of the formulation over the period of time. The viability of the developed bioformulation was found to be stable with a loss of only 2 log units at the end of 180 days. The cell counts in terms of CFU (Colony forming unit) at the time of preparation was observed to be in the order of 10^{13} per gram of formulation, which is much higher than the recommended cell count as prescribed by the Indian standards (IS: 8268, 1976; Pandey and Maheshwari, 2007) for effective bioremediation. The presence of live cells

at the end of six months, prove that the carrier material is successfully able to provide nutrition for growth and maintain the cells under storage.

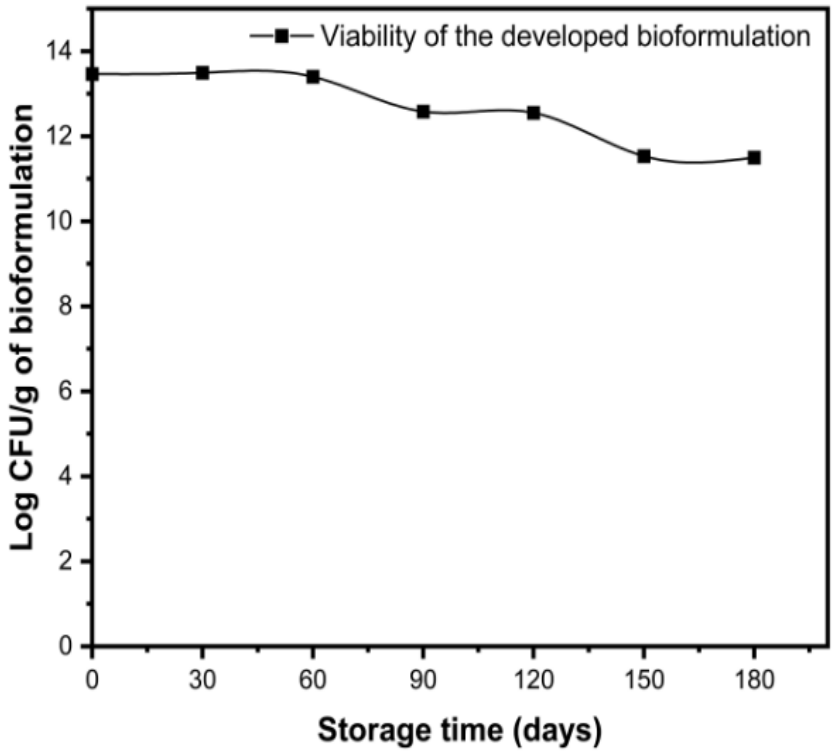


Figure 4.40: Viability of the developed bioformulation (BF2) under storage at 4 °C

SEM analysis of the developed bioformulations

SEM analysis of the developed wettable formulation (BF2) was also performed to confirm the immobilization of the bacteria, *J. cremeus* on the carrier mix. The SEM image (Figure 4.41) showed that, the bacteria were well immobilized on to the surface of the carrier mix.

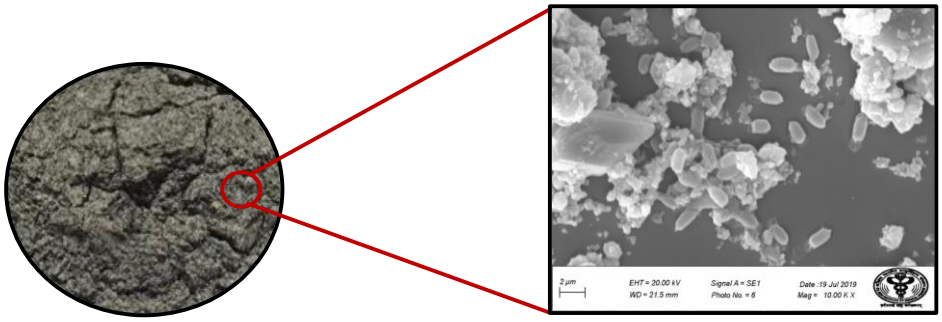


Figure 4.41: Electron micrograph image of the eggshell based bioformulation (BF2)

Role of carrier mix in bioformulation 2

The main carrier, egg shell was selected due to the following criteria: 1) a sustainable bio resource obtained from poultry waste 2) economically viable 3) derived from waste material that has no application elsewhere. Egg shells are mainly composed of the inorganic calcium carbonate (94 %), magnesium carbonate and calcium phosphate (1 % each). It also contains organic matter (4 %) and other insoluble proteins (Abdulrahman et al. 2014). They are also a rich source of essential nutrients like, N, P, K, Na and C, which are necessary for microbial growth and survival (Antwi-Akomeah et al. 2018).Calcium carbonate has been reported as a common soil amendment in reclamation and remediation of soils contaminated with hazardous compounds (EPA 542-R-07-013, 2007). The presence of organic content in the egg shells can also contribute in the maintenance of bacterial cells immobilized. The bacterium immobilize to egg shells by adsorption. The microbe could be trapped in the abundant pores in the egg shells (Li et al. 2019). Cocopeat, a plant based bioresource derived from coconut husk is a rich source of organic matter, is economical and easy to source. It helps in increasing the adsorption of explosives, the moisture holding capacity of soil and is also

a rich source of carbon, phosphorous and other nutrients, thereby providing favourable environment for microbial growth (Nunal et al. 2013). The surfactant, tween is a nature friendly detergent that functions as a wetting agent. It enhances the emulsifying, dispersing, spreading and wetting properties of the developed bioformulation. Sodium bi carbonate acts as a dispersing agent as well as a buffer for pH adjustment. It improves the dispersion of the developed bioformulation (Brar et al. 2006).

4.3.2 Bioremediation of soils contaminated with high explosives: Microcosm studies

The efficacy of the developed bioformulation was tested by monitoring the bioremediation ability. RDX/HMX at the field scale soil concentrations were maintained in the synthetic microcosms for the study purpose. An RDX manufacturing facility and an HMX manufacturing facility in India were selected for the sampling. The background uncontaminated soils (0-20 cm depth) from these manufacturing units were used in the study. The soils were ground using mortar and pestle, passed through 600 micron sieves for uniform particle size distribution and were analyzed for their physico-chemical characteristics.

4.3.2.1 Bioremediation of RDX in soil

RDX bioremediation studies were performed in microcosm levels by spiking the background soils of an RDX manufacturing facility with 65 mg Kg^{-1} of RDX. Two treatment sets were analyzed for their remediation efficacy. The soils in set 1 were maintained at 50- 60 % of WHC (unsaturated) and set 2 soils were flooded with water (saturated). Sacrificial sampling was performed at regular intervals of 0, 7, 14, 21, 28 and 35 days. The samples were monitored for live cell count, total enzyme activity in terms of FDA released, nitrite released and residual RDX concentration for both the treatment sets.

4.3.2.1.1 Microbial activity assessment in the microcosms

Growth of the immobilized bacterium in soils contaminated with RDX

The growth of *J. cremeus* immobilized in the developed bioformulation was monitored for its growth during RDX degradation study under unsaturated and saturated conditions (figure 4.42). *J. cremeus* showed growth in presence of RDX under unsaturated as well as saturated conditions. The highest CFU was observed on day 21 (for both unsaturated as well as saturated conditions), followed by decline in growth. The loss of bacterial cells was not lower than 10^7 g^{-1} of the soil sample, which is a requisite cell count for bioremediation (Pandey and Maheshwari, 2007). The saturated treatment sets showed better growth than unsaturated sets. The rapid growth of the bacteria shows that *J. cremeus* could utilize RDX as a nitrogen source and was not adversely affected by the presence of RDX.

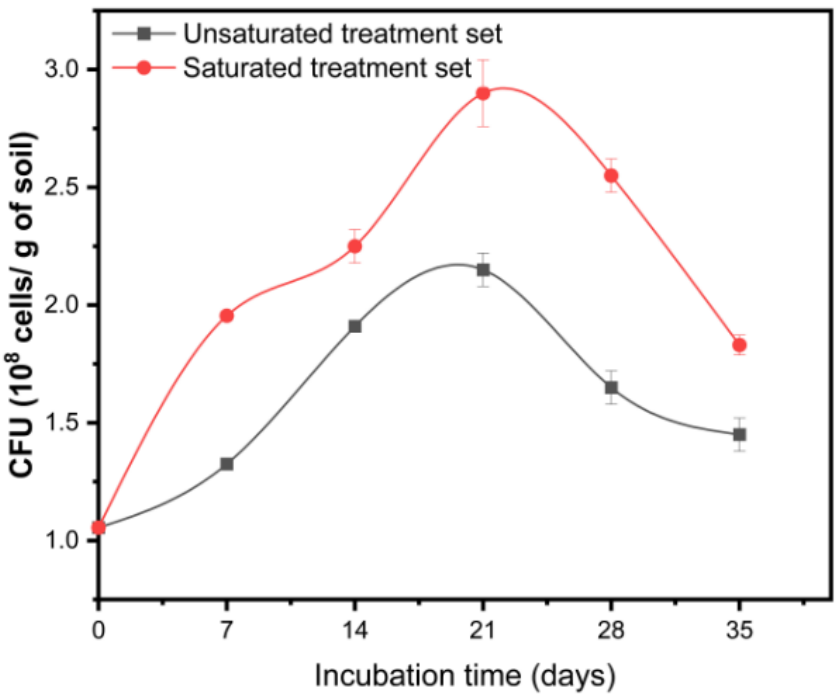


Figure 4.4.2: Growth of immobilized *J. cremeus* in RDX contaminated soil under unsaturated and saturated conditions using BF2

Total enzyme activity of *J. cremeus* in soils contaminated with RDX

Another parameter monitored to assess the growth and function of immobilized *J. cremeus* was estimation of the total enzyme activity in terms of FDA hydrolysed per gram of soil in 20 min. Day 21 showed the highest enzyme activity in both unsaturated and saturated treatment sets inoculated with the developed bioformulation (figure 4. 43). The unsaturated sets exhibited a maximum enzyme activity of 23 μg FDA hydrolysed/g of soil, whereas, saturated sets exhibited a maximum of 45 μg FDA hydrolysed/g of soil. The results obtained correlates with the bacterial live cell count monitored. The total enzyme activity of the bacterium in unsaturated condition though increasing with time is far less than the activity expressed in saturated conditions. Enzyme activity of denitryifying bacteria were found to be higher under saturated conditions (anoxic condition) as observed by Stres et al. 2008.

Liang et al., in 2003, had also observed a similar pattern. They observed that increasing moisture content led to a significant increase the microbial activity. Moreno-Espindola et al., in 2018, had studied the effect of water content in soil on the enzyme activity of the bacterial community. They had also observed that, higher water content promoted enzyme activity.

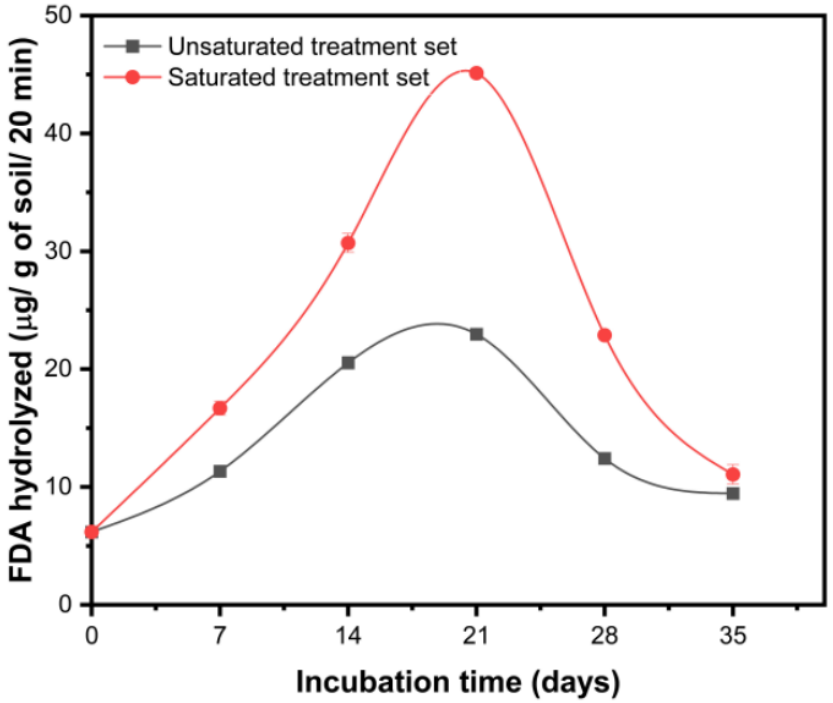


Figure 4.43: Total enzyme activity in the treatment sets with bioformulation (BF2) under unsaturated and saturated conditions during RDX remediation

4.3.2.1.2 Degradation analysis in microcosms

Nitrite released during remediation of RDX contaminated soil microcosms

The RDX degradation pathway followed by *J. cremeus*, the active ingredient in the developed novel bioformulation was by evaluating nitrite released. Nitrite being an important metabolite, is generally the first released intermediate during degradation (Crocker et al. 2006). Hence, monitoring of the nitrite release is an important step. The nitrite release was measured spectrophotometrically (Mercimeck et al. 2013). Fournier et al., in 2002, studied the key metabolite in the biodegradation of RDX by *Rhodococcus sp.* strain DN22. They found that nitrite was the first metabolite released which they suggested to be produced by denitration prior to ring cleavage. Zhao et al. (2002 and 2003), Bhushan et al. in 2003, have also postulated pathways with the concomitant release of nitrite. Our results (figure 4.44) showed that the nitrite released

in unsaturated RDX microcosms inoculated with the developed bioformulation, increased up to 21 days with a peak at concentration of 12.47 mg/ Kg with a further decrease in concentration later. This decrease in nitrite concentrations in the later stages can be attributed to the uptake of nitrite ions by nitrite assimilatory enzymes as reported by Lenke et al., in 1992. Sadani et al., in 2017 also monitored nitrite changes during bioremediation of Pentaerythritol Tetranitrate (PETN) contaminated soils. They observed a similar pattern with an increase in nitrite concentration in the initial stages followed by its decrease later. The saturated treatment sets showed continuous increase in the nitrite release, with a concentration of 14.85 mg/Kg at day 35. Jackson et al. in 2007 also observed a similar pattern, wherein anaerobic degradation of RDX leads to continuous release of nitrite.

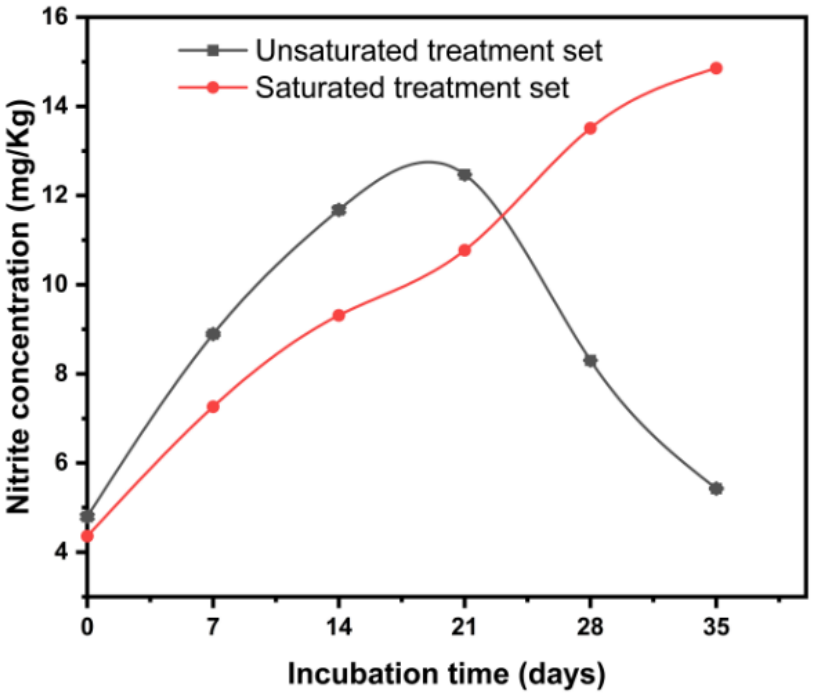


Figure 4.44: Nitrite released during the bioremediation of RDX using bioformulation (BF2) under unsaturated and saturated conditions.

RDX degradation efficiency of bioformulation -2

The developed novel egg shell based bioformulation was used for the degradation of RDX contaminated soil under unsaturated and saturated conditions. HPLC was used to determine the residual RDX concentration at different sampling times. The control treatment sets under unsaturated as well as saturated conditions exhibited negligible degradation. The treatments with bioformulation, under unsaturated conditions, exhibited 62 % degradation whereas, under saturated conditions, 73 % of RDX degradation was observed (Figure 4.45). Figure 4.46 depicts the overlaid chromatogram comparing RDX degradation under unsaturated and saturated conditions. Though bioaugmentation hasn't been yet studied with different soil water content, bioremediation (of explosives) by natural attenuation has been studied previously. Under different moisture contents, Sagi-Ben Moshe et al., in 2012, found that increase in soil water content led to better remediation of explosives viz., RDX, HMX and TNT. They postulated that, higher soil water content led to reduction in redox potential thereby enhancing the remediation. Ringelberg et al., in 2003 had also found a similar pattern. They also observed higher RDX degradation with higher soil moisture content. The goal of any treatment set up is to attain the soil screening level (SSL) as put forth by EPA. EPA in 2017, stated the SSL for RDX to be 28 mg/Kg for industrial soils. Our results of both the treatments indicate that, the SSL as prescribed was achieved in 35 days.

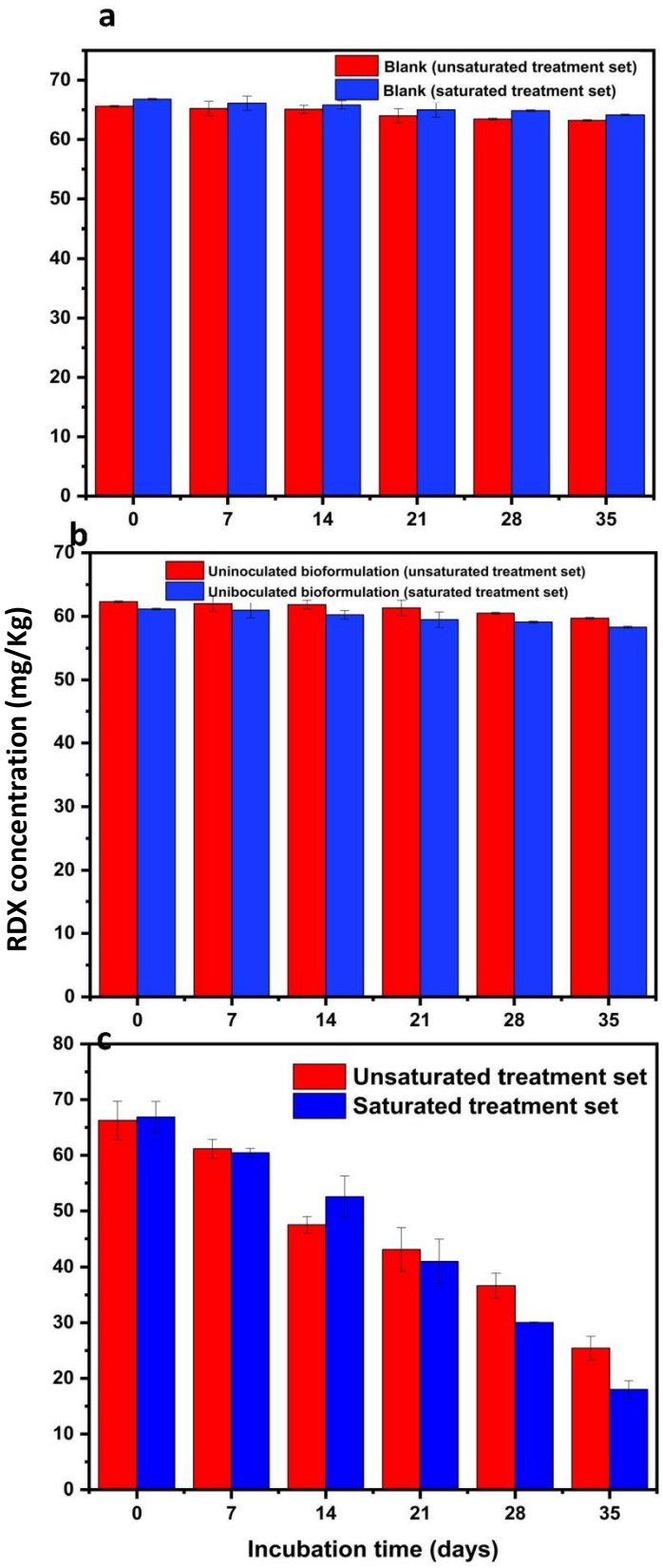


Figure 4.45: RDX degradation under unsaturated and saturated conditionsin (a) Blank (b) Uninoculated bioformulation (c) BF2

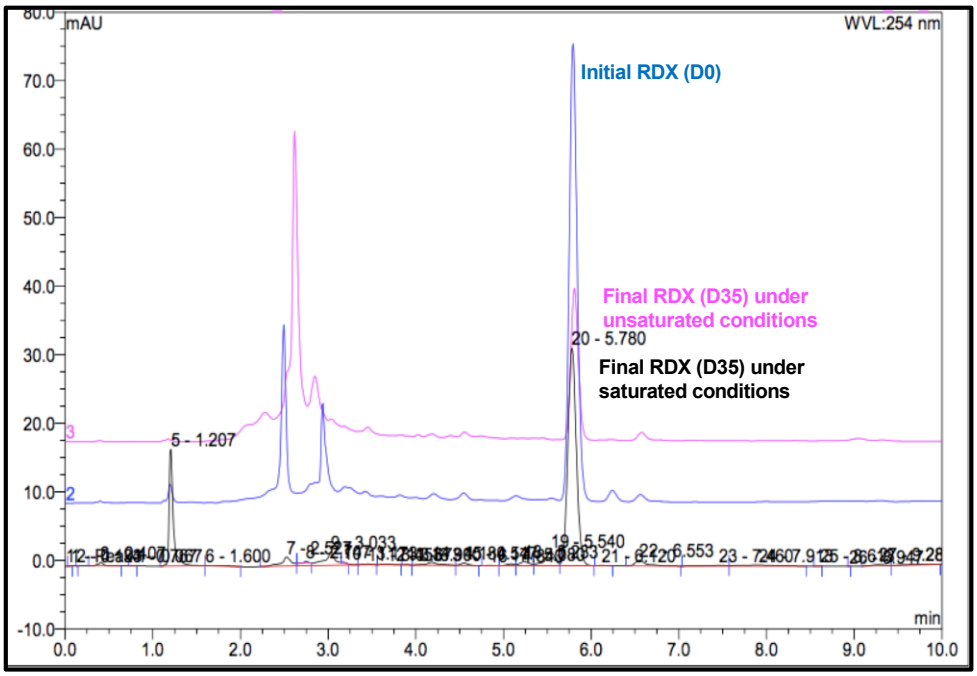


Figure 4.46: Overlaid HPLC chromatogram comparing RDX degradation under unsaturated and saturated conditions using BF2

4.3.2.2 Bioremediation of HMX in soil using BF2

HMX bioremediation studies were performed in microcosm levels by spiking the collected sandy loam soils with 3000 mg Kg^{-1} of HMX. Two treatment sets were analyzed for their remediation efficacy. The soils in set 1 were maintained at 50- 60 % of WHC (unsaturated) and set 2 soils were flooded with water (saturated). Sacrificial sampling was performed at regular intervals of 0, 7, 14, 21, 28 and 35 days. The samples were monitored for live cell count, total enzyme activity in terms of FDA released, nitrite and residual explosive concentration for both the treatment sets.

4.3.2.2.1 Microbial activity assessment in the microcosms

Growth of the immobilized bacterium in soils contaminated with HMX

The bacterial growth in the treatment sets inoculated with the developed bioformulation (BF2) was monitored in terms of live cell count. This was used as parameter to assess

the effect of HMX on the growth and survival of the bacteria *J. cremeus* in the soil. Figure 4.47 compares the live cell count of the immobilized bacteria during HMX bioremediation study und saturated and unsaturated conditions. As observed, the CFU of the bacteria under saturated condition increased up to day 21, followed by a decrease in live cell count. Whereas, under unsaturated conditions, there was a continuous decrease in the live cell count throughout the study. The use of acclimatized bacterium played a crucial role in growth. As the bacteria, *J. cremeus* was already acclimatized to HMX, the presence of HMX in soil environment didn't affect its growth and it could easily proliferate (Maier and Pepper 2015). Another important factor that plays a crucial role in growth of the bacteria is the moisture. Ringelberg et al. (2003) had observed that, microbial mass was higher in microcosms with saturated in comparison to unsaturated conditions.Also, as *J. cremeus* is a facultative anaerobe, it is characterized by a better growth under anoxic/ anaerobic (saturated) conditions.

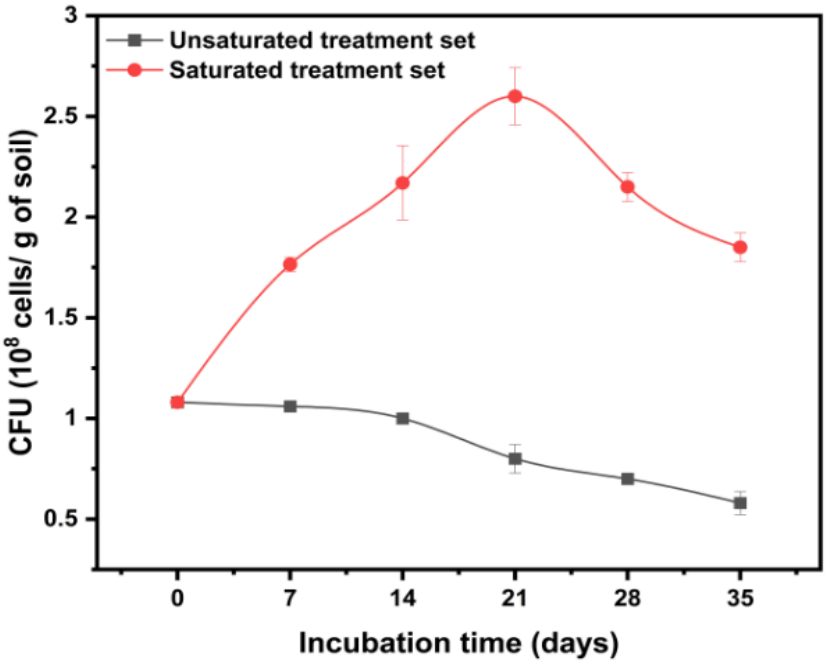


Figure 4.47: Comparison of the live cell count of *J. cremeus* embedded in BF2 during the treatment of HMX under unsaturated and saturated conditions.

Monitoring of total enzyme activity

The total enzyme activity, a measure of microbial metabolism was monitored by Fluorescein diacetate (FDA) hydrolysis. FDA is generally hydrolyzed by proteases, lipases, esterases and other enzymes produced by microorganisms. The BF2 treatment sets in the study were analyzed for the total enzyme activity under unsaturated and saturated conditions (figure 4.48). As observed in the results, the enzyme activity under saturated conditions increased to a peak of 36 μg / g of soil/ 20 min, followed by its decrease. Also, under unsaturated conditions, there was observed a continuous fall in the enzyme activity. The results obtained arein agreement with the results for live cell countmonitored during the study. As the live cell count was observed to increase a corresponding increase in the enzyme activity was also observed and vice versa. An important reason for higher enzyme activity under saturated conditions can be drawn to the anaerobic conditions prevailing in the treatment sets. Nitroreductase enzyme is a major enzyme system responsible for nitramine degradation. *J. cremeus* is known to possess this enzyme system (Hamada et al. 2013). Under anaerobic conditions, type I oxygen- insensitive nitroreductases are much more active and lead to the degradation of nitro-organics (Roldan et al. 2008). Thus, leading to higher enzyme activity.

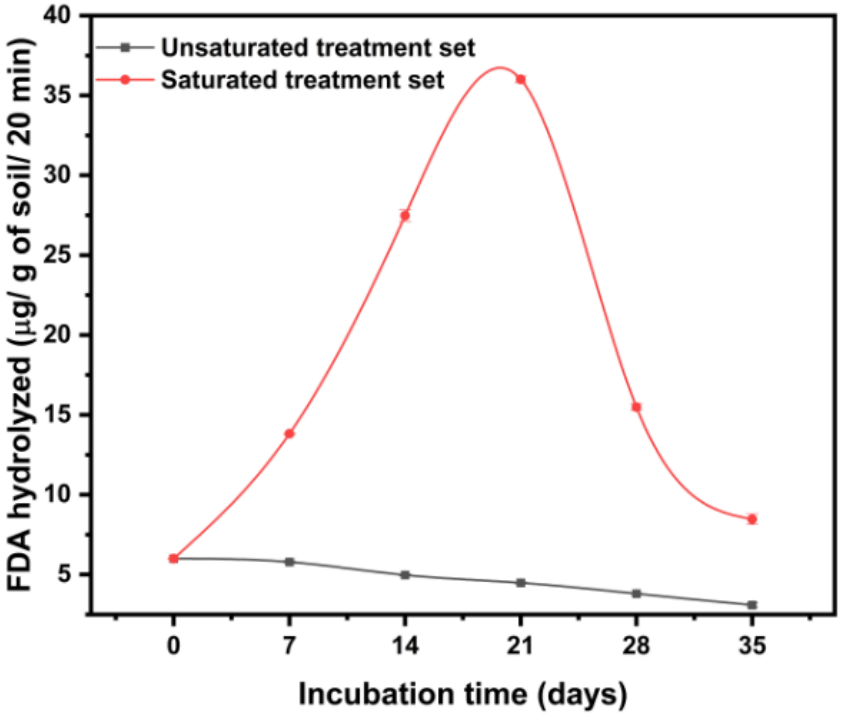


Figure 4.48: Comparison of the total enzyme activity in the saturated and unsaturated treatment sets subjected to bioremediation by BF-2

4.3.2.2.2 HMX degradation monitoring in microcosms

Nitrite release analysis

Breakdown of HMX is generally linked to concomitant release of nitrite (Crocker et al. 2006). The results observed showed that treatment sets with unsaturated conditions didn't exhibit a production of nitrite, whereas, there was observed an accumulation of nitrite in the saturated treatment sets (figure 4.49). Day 35 recorded a high nitrite concentration of 38.6 mg/Kg. Nitrite formation during remediation of HMX has been previously reported by Singh et al. in 2008 and also by Nagar et al. in 2018. The lack of nitrite formation in the unsaturated treatment sets could be attributed to lack of denitration due to absence of growth of microbes.

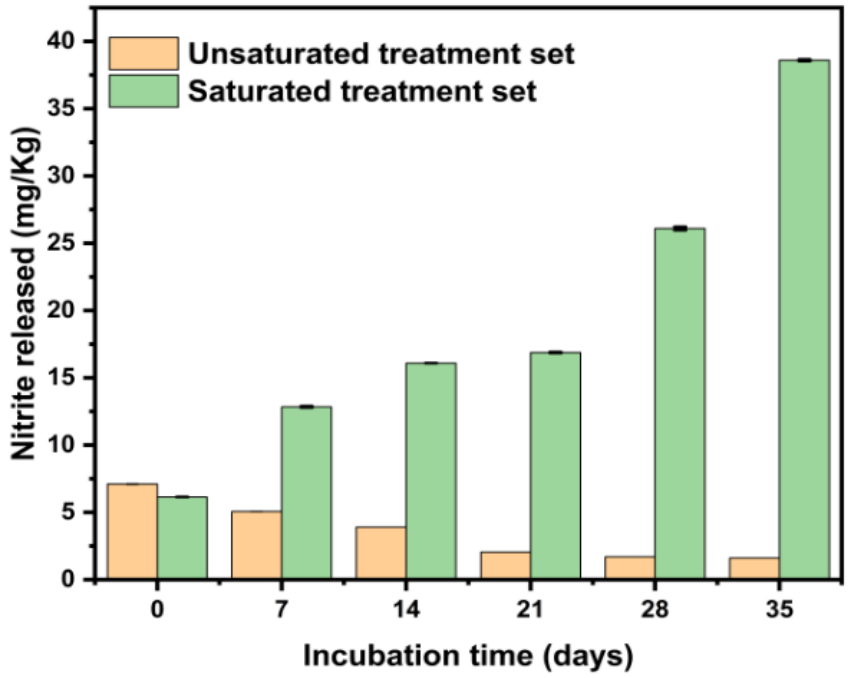


Figure 4.49: Release of nitrite during HMX remediation using BF2 under saturated and unsaturated conditions.

Residual HMX concentration

HMX degradation efficiency of the developed BF2 was analyzed based ion the residual HMX concentrations in the microcosms. HPLC was used to monitor the HMX concentration in each of the samples. The blank and control of uninoculated bioformulation was observed to be negligible (figure 4.50 a and b). The HMX concentration in the unsaturated treatment sets with BF2 was also observed to be negligible. Whereas, the saturated treatment sets with BF2 exhibited 40.2 % degradation in HMX at the end of the study (figure 4.50 c). Figure 4.51 shows the overlaid chromatogram for HMX degradation using BF2 under saturated conditions. The degradation pattern is in concurrence with the other findings of the study. The lack of microbial activity in the unsaturated sets led to lack of degradation of HMX. HMX has been documented to be effectively degraded under anaerobic conditions (Adrian et al. 2003; Bhushan et al. 2004; Zhao et al. 2004 b and c; Van Aken et al. 2004). The saturated conditions in the treatment sets lead to lowering of the

redox potential, thereby leading to an anoxic/ anaerobic condition aiding in the degradation of HMX (Sagi-Ben Moshe et al 2012).

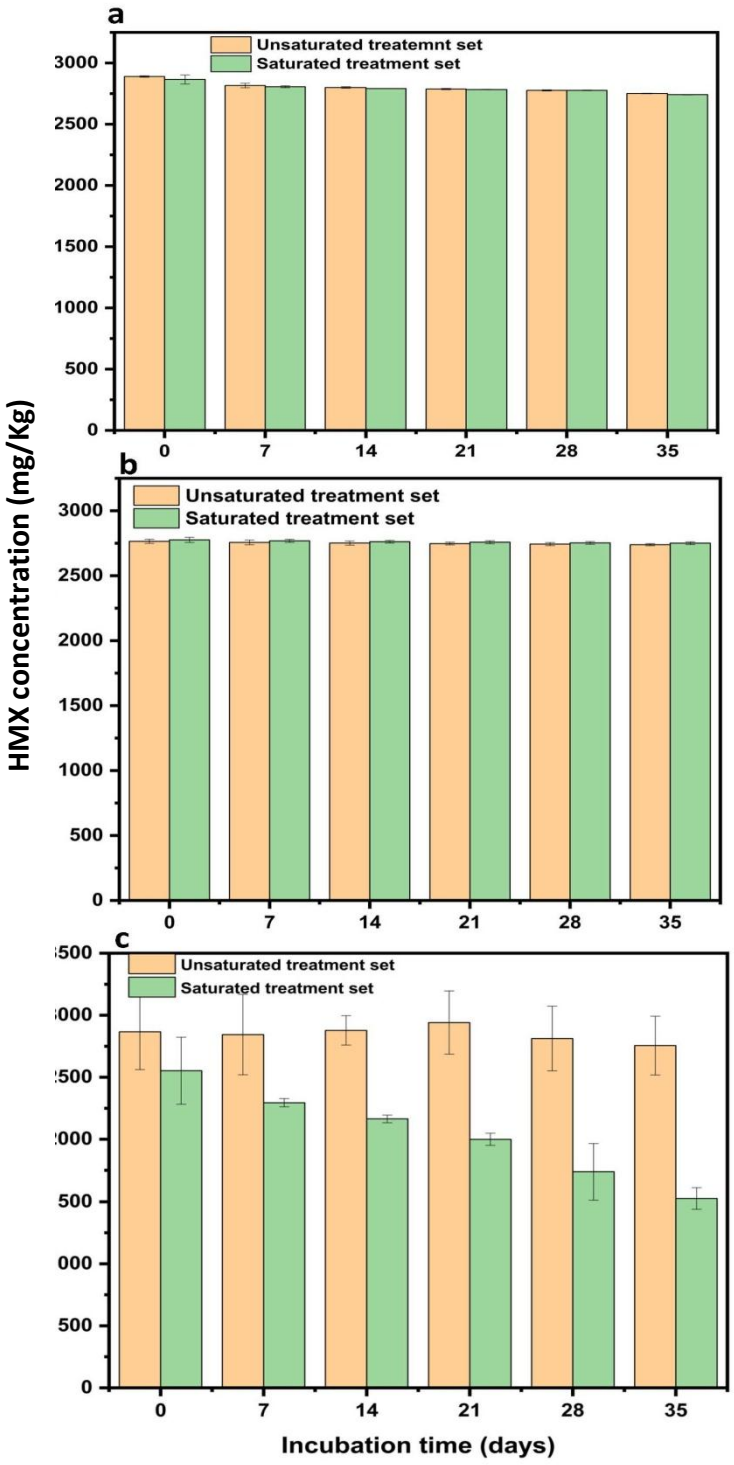


Figure 4.50: HMX degradation under saturated and unsaturated conditions in (a) Blank (b) Uninoculated Bioformulation (C) BF2

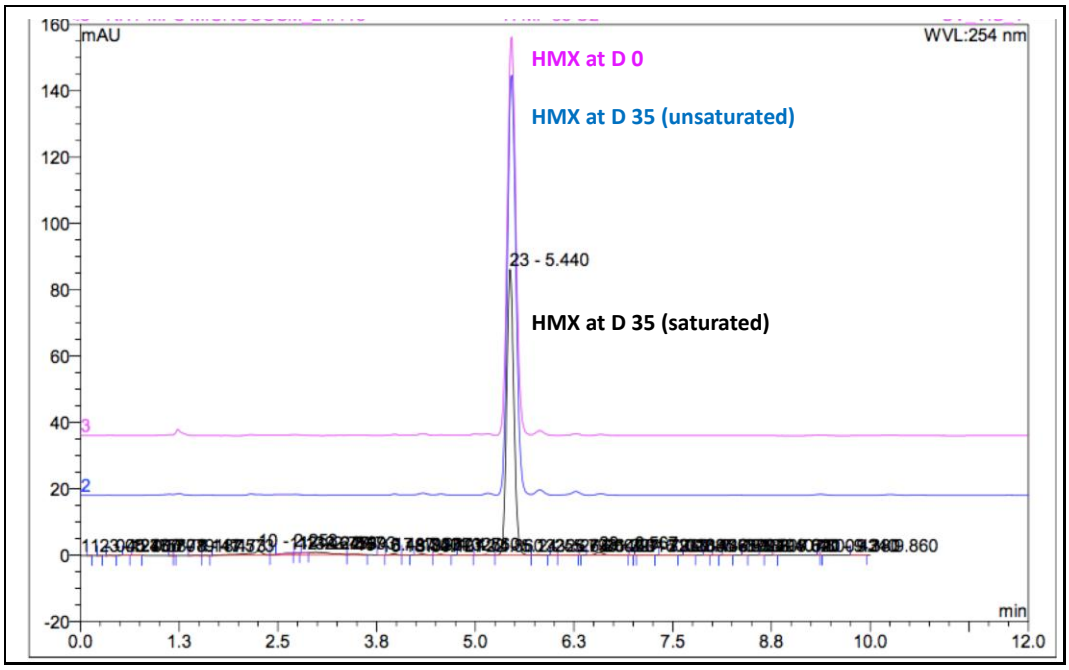


Figure 4.51: Overlaid HPLC chromatogram comparing degradation under unsaturated and saturated conditions

4.3.3 Bioremediation of explosive contaminated sediments

The developed bioformulation (BF2) was tested for its efficacy in treatment of explosive contaminated sediments obtained from explosive manufacturing facilities in India. Two sediments, A and B obtained from RDX and HMX manufacturing facility respectively were chosen for the study. Both the sediments were characterized by a low pH and high explosive concentrations, making them a very difficult target for the microbes. Sediment A was characterized by presence of high concentrations of RDX (984000 mg/Kg). Sediment B on the other hand was observed to be co-contaminated with RDX (82000 mg/Kg) and HMX (71000 mg/Kg).

The sediments obtained from RDX (Sediment A) and HMX (sediment B) manufacturing facilities were subjected to bioremediation using the developed bioformulation, BF2 at mesocosm levels. 300 g of sediment was weighed (moisture

content corrected) in amber colored 1000 mL bottles. They were then inoculated with 20 % (w/w) formulation BF2. Control experiments with only the sediment (blank) as well as sediment amended with uninoculated bioformulation alone at 20 % (w/w) were also set up. Experiments were conducted in saturated conditions at room temperature, with samples withdrawn regularly.

4.3.3.1 pH change

The sediment A is characterized by a low pH of 4. On addition of the uninoculated bioformulation and BF2, the pH of the sediment was found to increase to 7.2. Sediment B is characterized by a further low pH of 2.8. On addition of the uninoculated bioformulation and BF2, its pH was found to be 7.6 (Figure 4.52). As discussed earlier, egg shells are rich in calcium carbonate, thereby can lead to increase of pH. Also, presence of sodium bi carbonate as an important constituent helps in the buffering further. Presence of excess water in the remediation system as well as presence of calcium carbonate and sodium bi carbonate can bring about mild hydrolysis.

Liming has been reported as an effective method for remediation (Martin et al. 2012). It involves the use of hydrated lime, ie, calcium hydroxide to increase the pH to around 10 to bring about effective alkaline hydrolysis. The increased pH of the soil leads to harmful effects, viz, decreased nutrient cycling, toxic levels of certain metals and many more (Filipek, 2011). Though the addition of BF2 led to a substantial increase in the pH, the increase didn't make the soil alkaline.

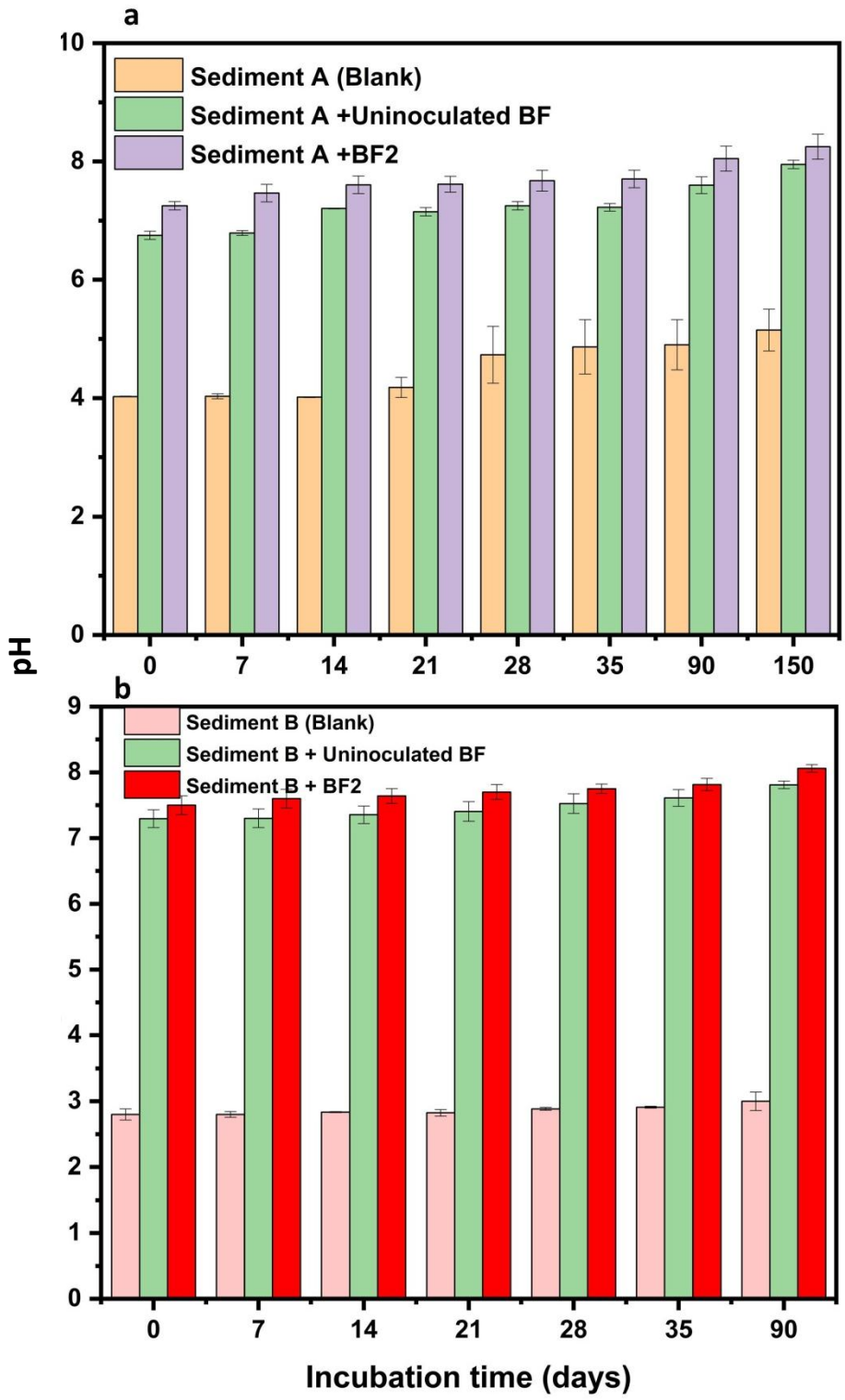


Figure 4.52: Change in pH of (a) Sediment A (b) Sediment B on treatment with BF2

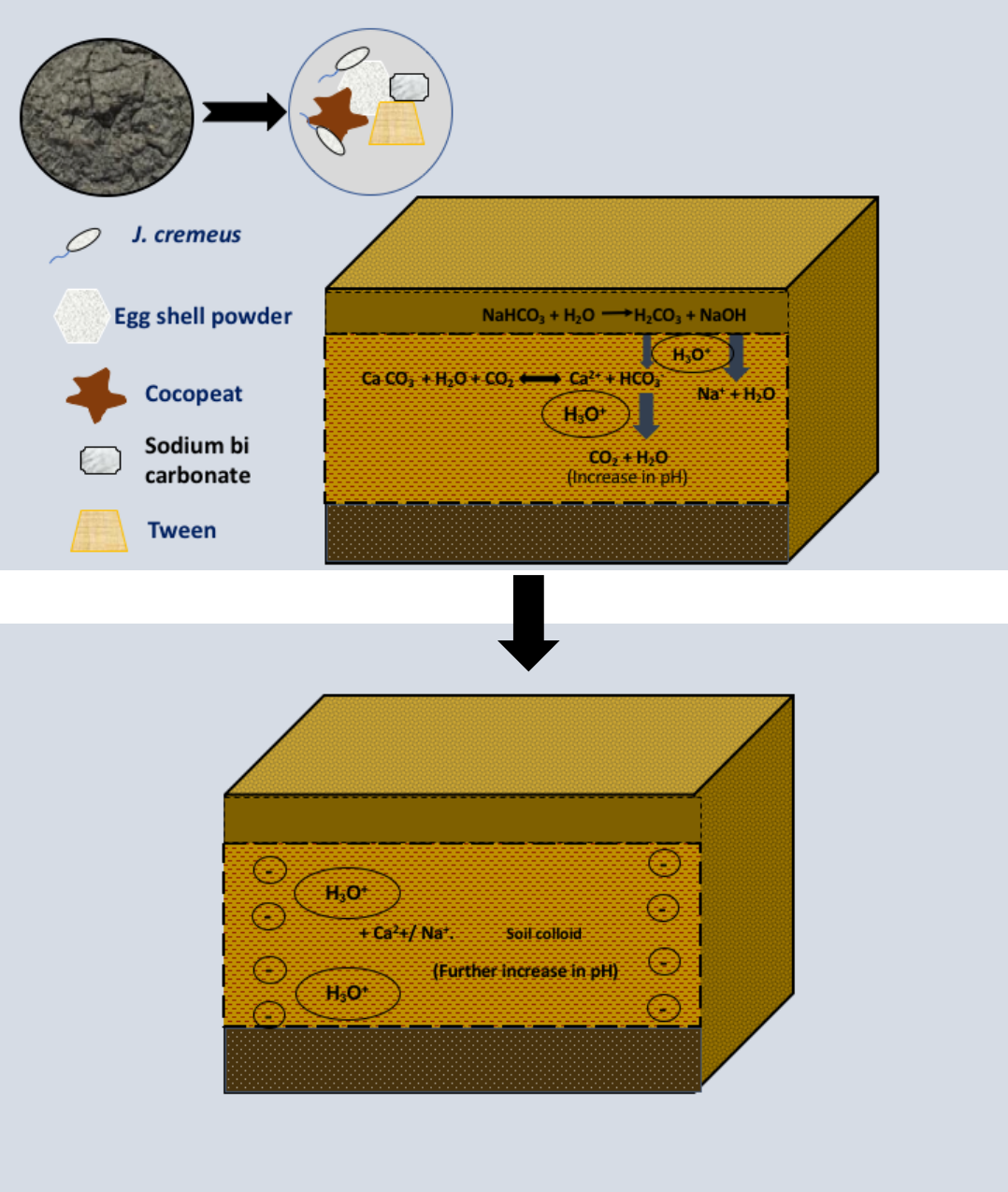


Figure 4.53: Action of BF2 in pH regulation of the sediments

The calcium carbonate in the egg shell powder dissolves in the soil- water slowly to produce bi carbonate ions. Also, the sodium bi carbonate dissociates in water to form bicarbonate ions. These ions neutralize the hydronium ions in the sediment solution, thereby increasing the pH of the system. As observed in the result (figure 4.52), there is a continuous increase in the pH of the system throughout the study. This increase can be

attributed to the resultant calcium and sodium ions which displace the hydronium ions present on the clay and humus particles.

4.3.3.2 Total enzyme activity of the heterotrophic microbes in treatment study

The total enzyme activity in terms of FDA hydrolyzed, was used as an indicative measure of the bacterial metabolism in the treatment sets. Fluorescein diacetate is a fluorescein conjugated with two acetate moieties. It is non-polar, non-fluorescent compound that penetrates the cell membrane. It gets hydrolyzed by various nonspecific enzymes (esterases, acylases and lipases) leading to the release of the fluorescent fluorescein (Mahu et al. 2018). The amount of fluorescein produced is directly proportional to the live microbial activity in soil and sediments (Gumprecht et al. 1995; Koster et al. 1991)

The bioremediation of sediments was conducted under non-sterile conditions. Hence, the total enzyme activity obtained represents the total live microbial activity of all the heterotrophic microbes present in the sets. Figure 4.54 represents the results obtained in the study during the remediation of the sediments A and B. As observed, blank control exhibited far less microbial activity in comparison to the treatment sets with BF2 as amendment.

Since the sediment is characterized by a very low pH, the diversity of active microorganisms is lower than those found at neutral pH. Fernández-Calviñoet al. in 2011, had also demonstrated that soil pH had a direct influence on the soil bacterial community. The total enzyme activity in treatment sets with higher pH was better than blank because of the activation of microbial species that may have been inactive due to the acidic pH. Also, it was observed that, though the pH of the control sets was in the acidic range, there was an increase in the FDA hydrolyzed, which could be due to the growth of anaerobic microbes present in the sediments due to the saturated/ anoxic conditions.

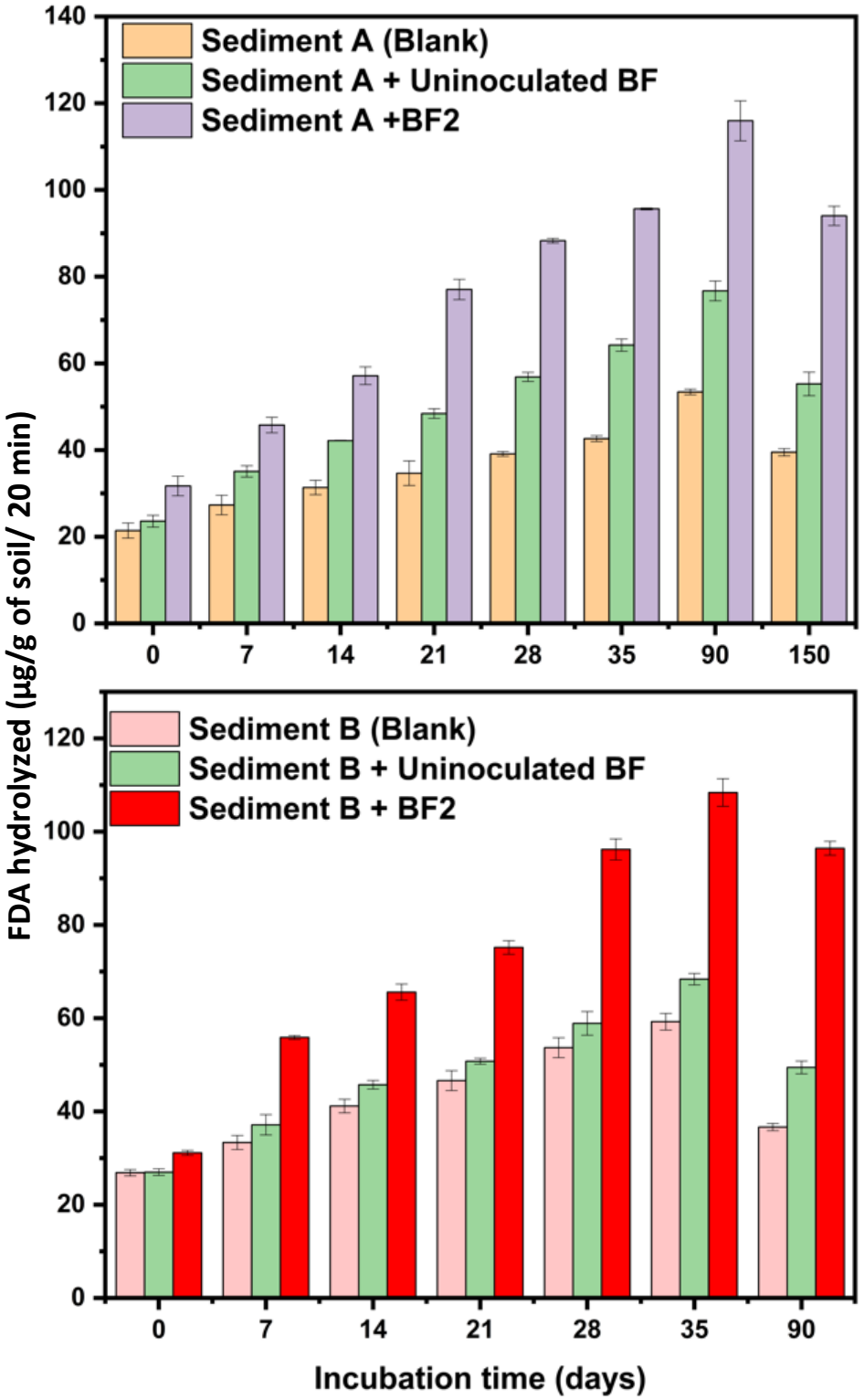


Figure 4.54: Change in the total enzyme activity in terms of FDA hydrolyzed during remediation of (a) Sediment A (b) Sediment B when treated with BF2.

4.3.3.3 Nitrite release during bioremediation of sediments

RDX and HMX degradation is characterized by release of nitrite under aerobic as well as anaerobic conditions (Crocker et al. 2006). The remediation studies of the sediments were carried out under saturated/ anoxic conditions. Figure 4.55 represents the nitrite released during the study. As observed the blank treatment sets showed the least amount of nitrite in comparison to the other treatment sets (uninoculated bioformulation and BF2). The higher nitrite content of the other treatment sets could be attributed to mild hydrolysis action due to increased pH as observed by Balakrishnan et al. in 2003.The presence of excess water and alkalis may have contributed to mild hydrolysis of both RDX and HMX present in the sediments.

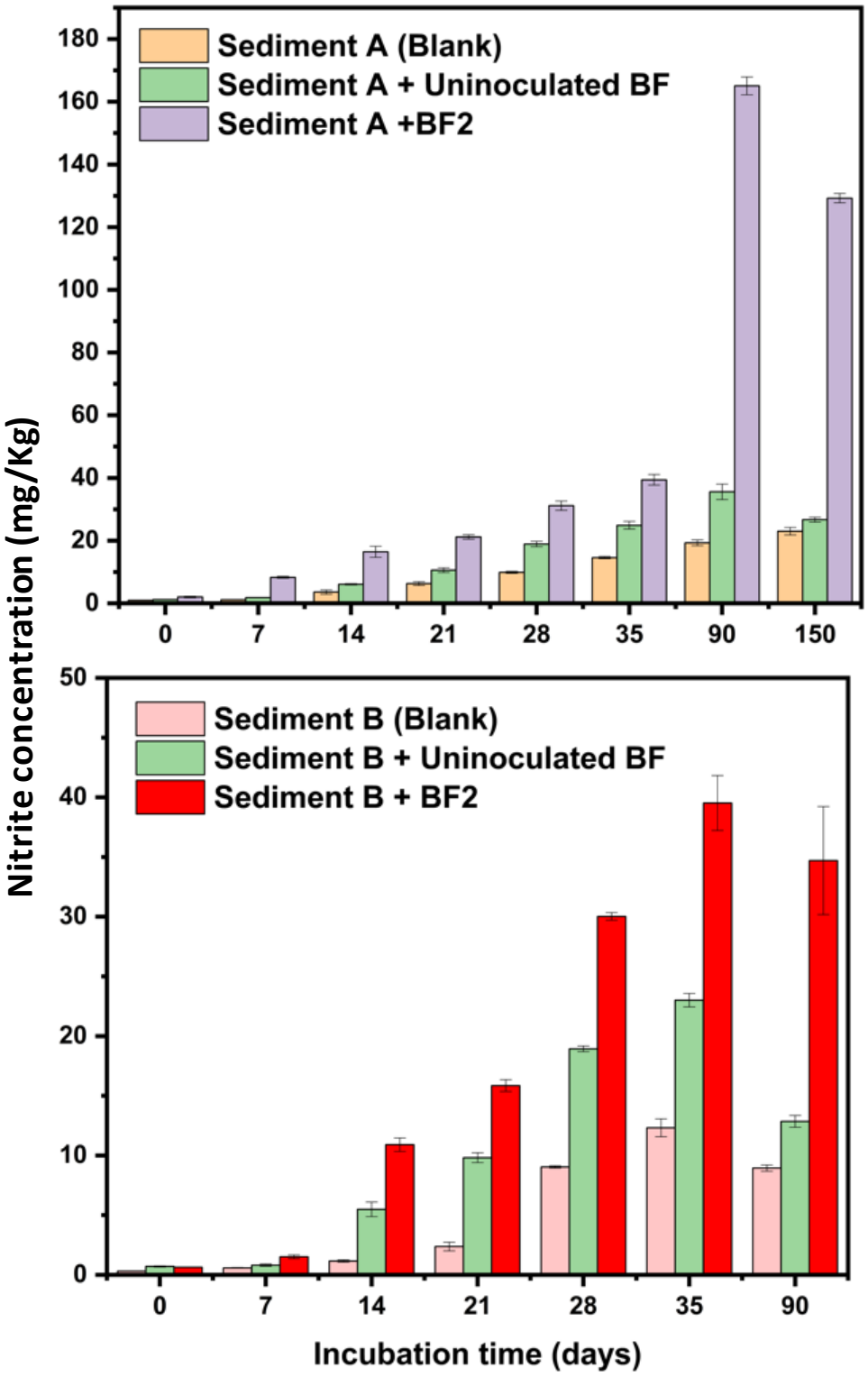


Figure 4.55: Release of nitrite during remediation of (a) Sediment A (b) Sediment B on treatment with BF2.

4.3.3.4 Residual explosive concentration

The remediation studies for sediments A and B was carried out under anoxic/saturated conditions. The high concentration of explosives in the sediments could be toxic to microbes, hence lead to inactivity of the microbes. Also, it is well documented that, nitramine explosives are better transformed and mineralized under anoxic/ anaerobic conditions. Hence, the experiments were carried out under saturated conditions for effective remediation (Price et al. 2011; Speitel et al. 2001; Ringelberg et al. 2003).

Another important factor that effected the remediation of explosives in sediment is the presence of various constituents of BF2, which play a specific role in remediation of the sediments (figure 4.56). As discussed earlier, cocopeat helped in adsorption of both explosives as well as the microbes on its surface by adsorption. It is well documented that, cocopeat can help in adsorption of microbes (Nunal et al. 2013) and can also adsorb organic compounds (Vijayaraghavan et al. 2015), thereby increasing the bioavailability of the nitramines. Egg shell powder not only helped in increasing the pH of the sediment, it also played a crucial role in adsorption of both microbes and explosives, by adsorption thereby contributing to remediation. This was confirmed by the FTIR spectrum obtained (figure 4.57). The FTIR spectrum of egg shell powder shows strong peak at 1423 cm^{-1}, 875 cm^{-1} and 712 cm^{-1} .The presence of these peaks prove that calcium carbonate is the major inorganic component of egg shell powder (Iram et al. 2019). Broadening of peak at 1423 cm^{-1} on FTIR for egg shell powder immobilized with *J. cremeus* and RDX (separately) suggests interaction due to hydrogen bonding (Coates, 2000). This confirms that, egg shell powder can adsorb both the bacterial culture as well as explosive chemical, increasing the bioavailability of explosive for bacteria.Moreover, the presence of other essential nutrients, proteins and organic content can also contribute in the growth of bacterial cells. Cocopeat is also a rich source of carbon, phosphorous and other nutrients, thereby providing favourable environment

for microbial growth (Nunal et al. 2013). The surfactant, tween is a nature friendly detergent that functions as a wetting agent. It enhances the emulsifying, dispersing, spreading and wetting properties of the developed bioformulation, helping it remain in suspension. Sodium bi carbonate acts as a dispersing agent as well as a buffer for pH adjustment. It also improves the dispersion of the developed bioformulation (Brar et al. 2006).

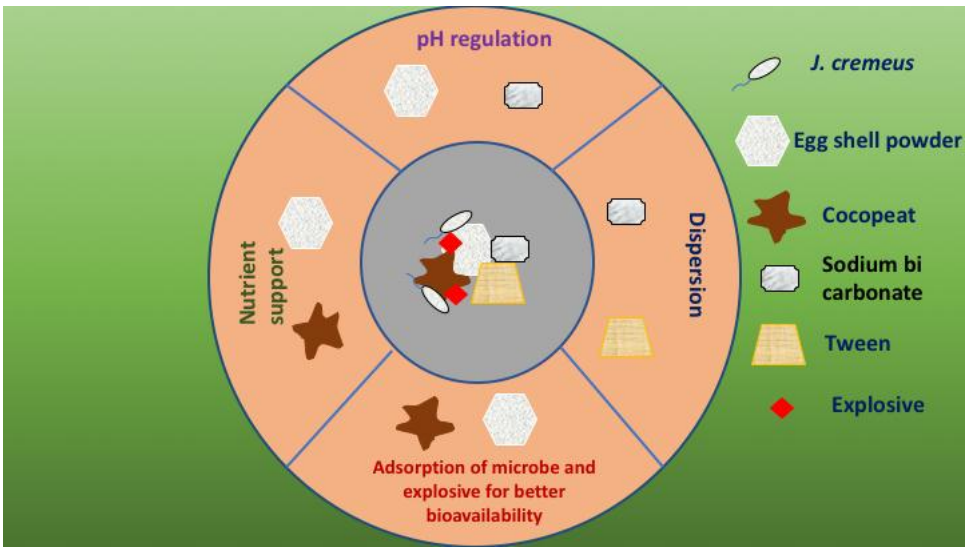


Figure 4.56: The role of various constituents of BF2 in remediation of sediments

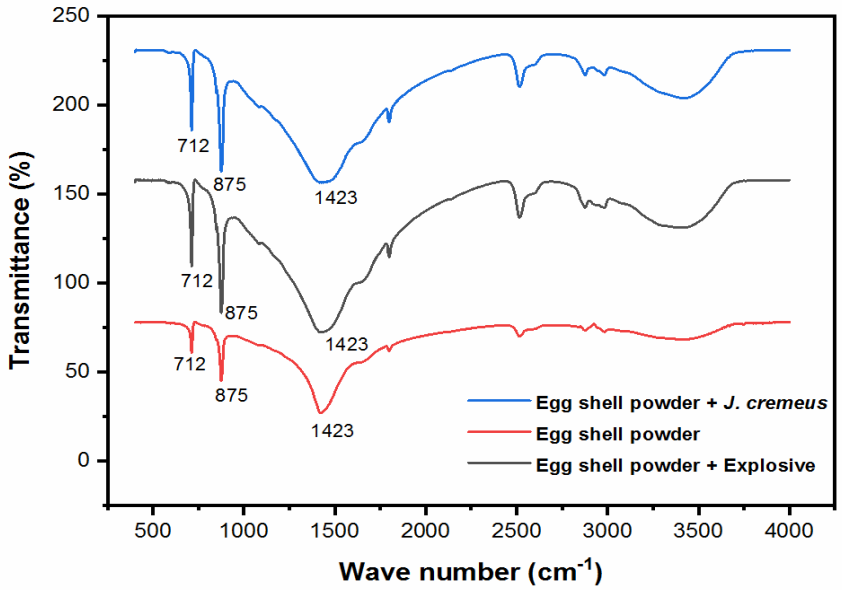


Figure 4.57: FTIR spectrum of the egg shell powder, egg shell power immobilized with *Janibacter cremeus* and egg shell powder with explosive

Sediment A exhibited RDX degradation of 87.4 % with BF2 as the amendment in 5 months (Figure 4.58). This led to a decrease in RDX concentration to 108783 mg/Kg in sediments. This concentration was in agreement with the limit described by EPA (1993), which indicates that the sediment was rendered unsusceptible to initiation and propagation.

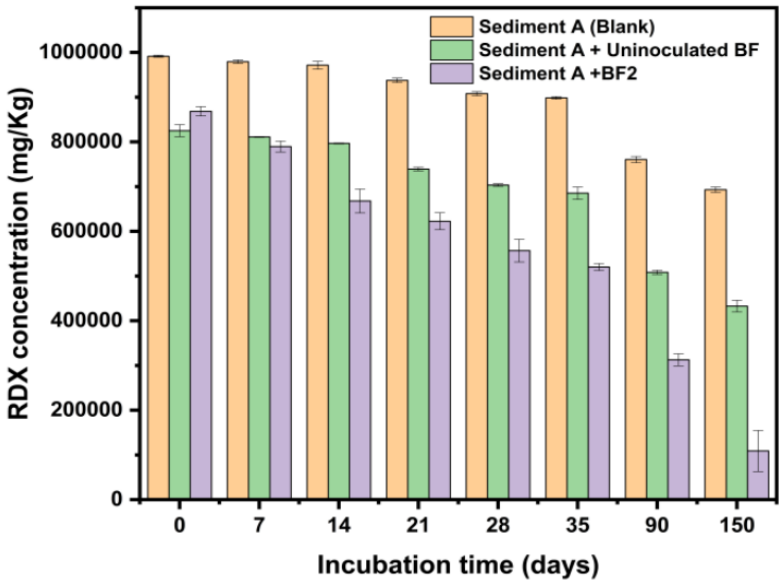


Figure 4.58: RDX degradation in sediment A on treatment with BF2

Sediment B on the other hand was characterized by a co-contamination of RDX and HMX. The treatment sets with BF2, showed 53 and 49% reduction in RDX and HMX respectively (Figure 4.59 and 4.60). Figure 4.61 represents the HPLC overlaid chromatogram representing RDX and HMX before and after remediation with BF2. HMX is generally less susceptible to degradation in comparison to RDX. Hence, the reduced degradation of HMX was observed. But when we compare both the sediments (A and B), we observe that the degradation of RDX in sediment B is slower in comparison to sediment A even though it is characterized by much less initial concentration. This could be attributed to co-contamination of RDX and HMX. It has been reported that, high concentration of an explosive can inhibit the degradation of a

coexisting explosive (Bernstein et al. 2011). Jackson et al in 2007 reported the inhibition of RDX degradation by the presence of TNT due to the inhibition of cytochrome P450, an important enzyme system responsible for RDX degradation. Fuller et al. in 2009 observed that, high concentrations of RDX led to an inhibition in HMX degradation.

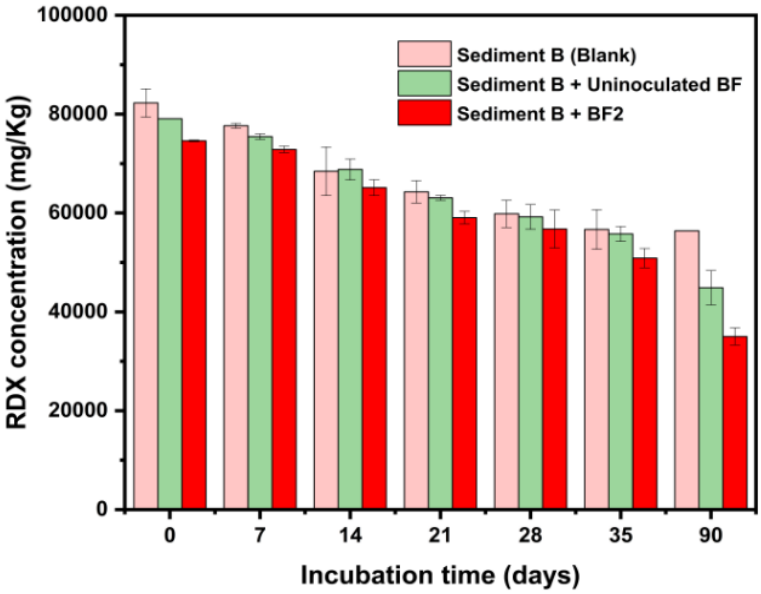


Figure 4.59: Degradation of RDX in Sediment B using BF2

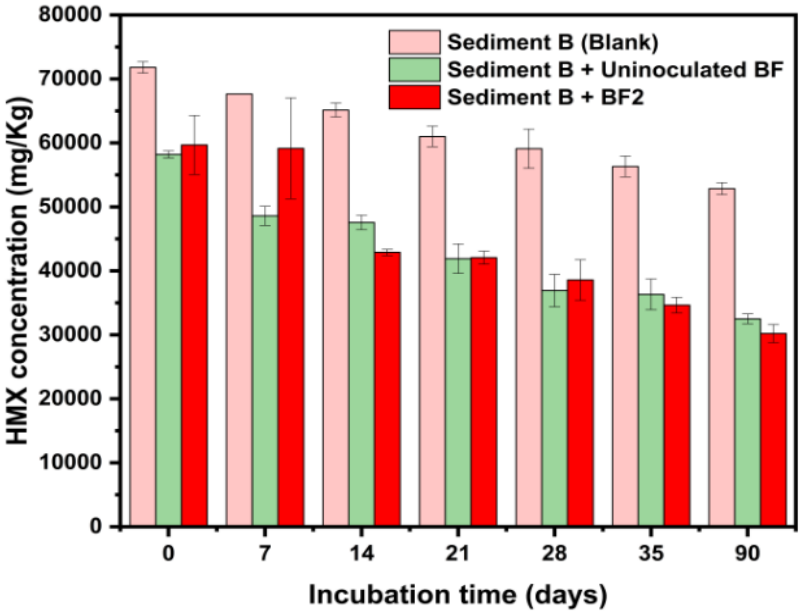


Figure 4.60: Degradation of HMX in sediment B using BF2

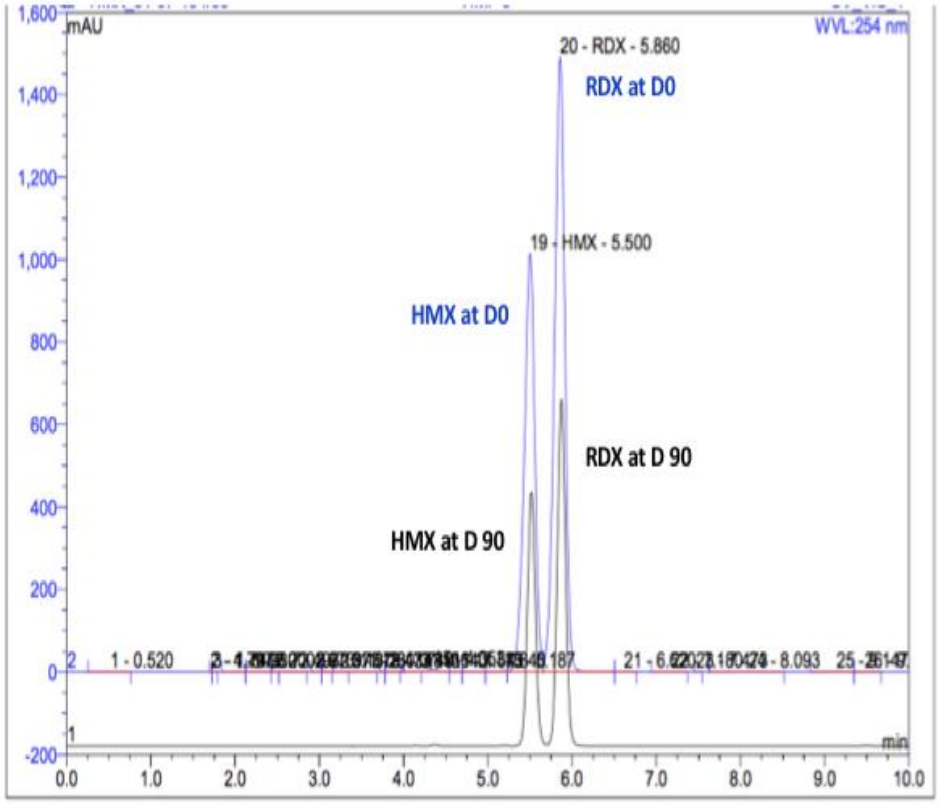


Figure 4.61: HPLC overlaid chromatogram of sediment B representing degradation of RDX and HMX on treatment with BF2

4.3.4 Conclusion

The explosive degrading soil bacterium *J. cremeus* was immobilized on a carrier mix of egg shell powder, cocopeat, tween and sodium bi carbonate to develop a wettable powder formulation (BF2). The developed formulation, BF2 was tested for its viability and efficacy. The viability of the developed bioformulation was found to be stable with a loss of only 2 log units at the end of 180 days. The BF2 was tested for its efficacy to degrade RDX and HMX in soil under saturated and unsaturated conditions. RDX (65 mg/Kg) was degraded by 62 and 73 % under unsaturated and saturated conditions respectively. HMX (3000 mg/Kg) was observed to be degraded (40 %) under saturated conditions only. The BF2 was then tested to treat acidic sediments (A and B) obtained from explosive manufacturing facilities in India. The RDX in sediment A was

successfully degraded by 87.4 % in 150 days. Sediment B was characterized by a co-contamination of RDX and HMX. The effective degradation of 53 % for RDX and 49 % for HMX was obtained in 90 days. The developed BF2 has proven to have potential for minimizing the explosion hazard of the contaminated sediments and soils. Constituents of the BF -2 viz, egg shell powder, cocopeat, tween, sodium bi carbonate played a significant role in treating the hazardous explosive contaminant present in soil and sediments. The results obtained demonstrates that, the developed novel bioformulation (BF2) was found to be a successful eco-friendly tool for in situ bioremediation of explosive contaminated industrial sites

4.4 Elucidation of Explosive Degradation Pathway

Various surveys have shown that, explosives are heterogeneously dispersed in soil. Though they are present in smaller concentrations as observed from global data (Crocker et al. 2006), the manufacturing sites as well as fire and testing ranges have been documented to be highly contaminated (Pichtel, 2012). Nitramine explosives, viz, RDX and HMX are characterized by low sorption in soil; thereby they find their way to the ground water table. This contributes to their persistence in the soil environment. USEPA has classified RDX as priority pollutant and HMX as contaminant of concern.Many indigenous and non-indigenous microbial species have been identified and studied for the explosive degradation potential (Chaudhary and Kim, 2019). Since microbes are ubiquitous and can adapt to harsh environmental conditions, they play major role in remediation of contaminated soil and water. Even with all the advantages the major issues on application of microbial site remediation lies in longer duration and also lower efficacy. Hence, there is a need for enhancement of bioremediation approaches for on-site applications. This goal can be achieved by better understanding of the microbial degradation processes.

The metabolic profiling of explosives is a very useful technology for elucidation of explosive degradation pathway during bioremediation. Microbes possess specific enzymes, viz, nitroreducatses and cytochrome P450, which catalyse the reduction of explosives. Metabolomics/ metabolite profiling is a major tool that aids in identification and quantitation of the major explosive intermediates, thereby, helping in prediction of the enzymes responsible for the transformation. Also, it is a much less intensive and easier process than direct identification of enzymes (proteomics).The metabolite analysis also helps in determining the impact of the intermediates or the end products

formed during the explosive degradation. It can also help in understanding the toxic effects of intermediates on the microbial population which can impact the efficacy of the process. Hence, the use of omic- sciences, especially, metabolomics plays a crucial role in optimization of biodegradation process.

'Omics' sciences give a holistic view of a biological entity. It describes the genes (genomics), mRNA (transcriptomics), proteins (proteomics) and metabolites (metabolomics) (figure 4.62). Omics technology helps in better understanding of various biological and physiological processes and find application in various fields viz, disease processes, drug metabolism and xenobiotic degradation (Horgan and Kenny, 2011). In xenobiotic degradation, of all the 'omics' technologies, metabolomics plays a crucial role in understanding the remediation process better. Metabolomics basically refers to all the low molecular weight entities (metabolites) produced or modified by a living system. It entails the identification and quantification of all the intracellular and extracellular metabolites. It directly refers to the biochemical activity of the cells (Villas-Bôas et al., 2005). Metabolites are result of biological and environmental factors, hence its profiling can build the knowledge of its genotype and phenotype.

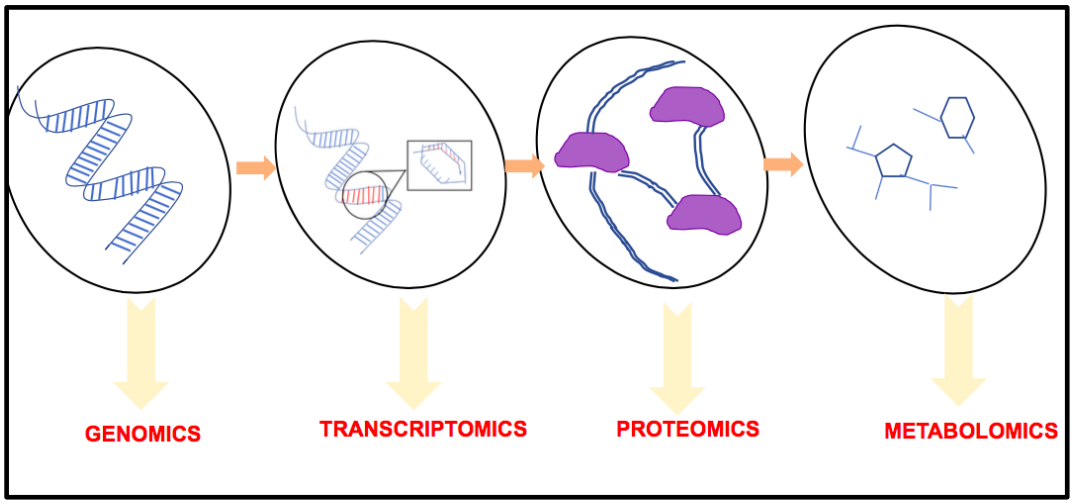


Figure 4.62: An overview of the 'Omics' science

Metabolomics can be categorized into two approaches, viz, targeted and non-targeted. Targeted approach is hypothesis driven, wherein a specific class of metabolite are identified and quantified. It has proven useful in assessing the response of an organism on exposure to a xenobiotic stress as reported by Bando et al. 2011, Huang et al. 2016 and 2017. The major advantage of a targeted approach is that the chemical properties of the metabolite is known, therefore the sample preparation and processing can be tailor- made to minimize matrix effects and other background interferences. The non-targeted approach leads to the generation of a new hypothesis that needs to be further tested. This approach involves the analysis of all the metabolites in a biological system (Zhou et al. 2012). These identified molecules can be mapped to networks and pathways, hence, help in identification process. But the major lacuna here lies in the lack of complete databases unlike for proteins (Schrimpe-Rutledge et al. 2016). There are many tools available for metabolomic studies of explosive biodegradation. Ultraviolet- visible (UV-Vis) spectroscopy, Fourier transform infrared spectroscopy (FTIR), stable isotope probing (SIP), High Performance liquid chromatography (HPLC), Gas chromatography (GC), Nuclear magnetic resonance (NMR), and mass spectroscopy (MS) are the major ones.

Mass spectrometry can be used in conjunction with either liquid chromatography or Gas chromatography. Both LC and GC can be used for the separation and identification of polar and non-polar compounds. As explosives are thermally labile compounds, LC based methods are more suitable that GC based methods. Due to its high sensitivity, LC-MS has been widely used for metabolite profiling. Figure 4.63 represents a basic work flow for metabolite detection using LC-MS.

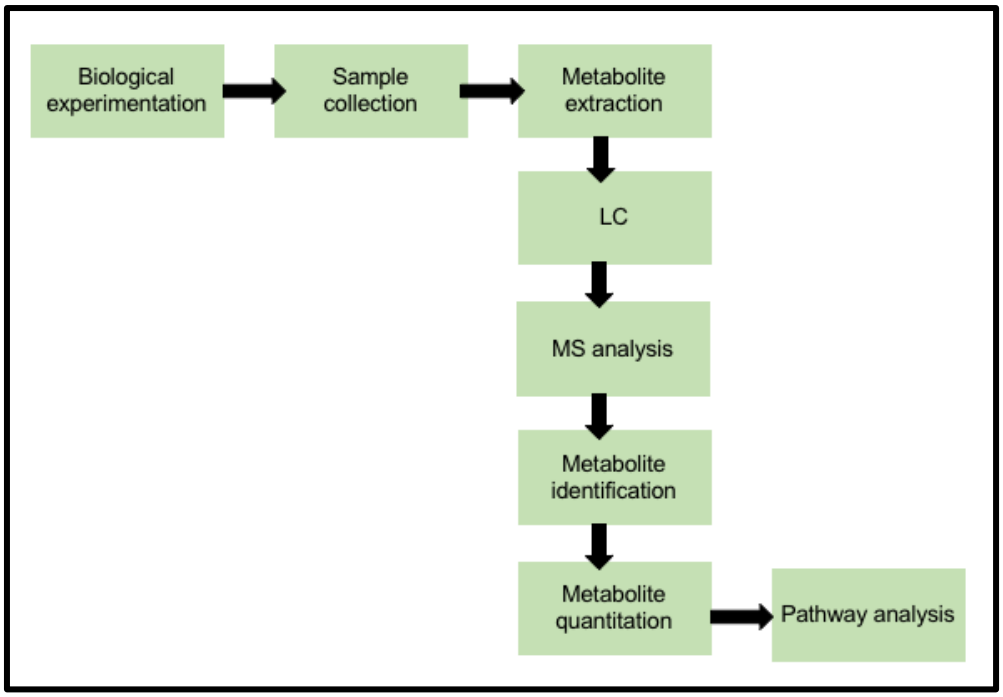


Figure 4.63: A typical work flow of LC-MS based metabolomics

Janibactercremeus, was evaluated for the intermediates formed during the bioremediation of both RDX and HMX. Mass spectroscopy was used to identify the various intermediates formed so as to elucidate the degradation pathway.

The bacterium *J. cremeus* was evaluated for the various intermediates formed during the degradation of explosives RDX and HMX using mass spectroscopy. Also the role of the enzyme, nitroreductase was confirmed in degradation of the nitramine explosives by using resting cell incubations with sodium azide, a specific inhibitor of nitroreductases. The samples for MS were extracted using acetonitrile. Mass spectrometric analysis (Synapt G2 Waters ACQUITY QSM, USA) of the samples were performed in positive ion mode. RDX and its degradation products were separated on aC18 column (130 A, 1.7 µm, 2.1mm* 100 mm) at 0.75 mL/ min flow rate over a period of 20 minutes.

4.4.1 Evaluation of enzyme responsible for explosive degradation

RDX and HMX are nitramine explosives which are degraded by the same enzyme systems, Cytochrome P450 and Nitroreductase. To observe the role of nitroreductase in RDX degradation, its activity was inhibited using specific inhibitor, sodium azide (Villanueva 1959; Roldán et al. 2008). Sodium azide at different concentrations, viz, 0.1 mM, 0.5 mM, 1 mM, was used to study the effect on RDX degradation. *J. cremeus* grown in presence of RDX as sole nitrogen source was harvested in its late log phase and resuspended in MSM with RDX at 10 mg/L concentration. The RDX degradation was monitored using HPLC. Figure 4.64 shows the comparative RDX degradation at different inhibitor concentrations. With no inhibitor, RDX degradation was found to be 61.13 % in 7 days. It was observed that, with increasing concentration of sodium azide, RDX degradation decreased, showing that, nitroreductase was a key player in RDX degradation pathway in *J. cremeus*. Type I nitroreductases (oxygen insensitive) may be responsible for the two electron transfer process leading to the formation of nitroso derivatives of RDX, denitration and ring cleavage leading to the formation of bis-(hydroxymethyl) nitramine (BHNA) and MDNA (methylenedinatramine) (Kitts et al. 1994, 2000; Young et al. 1997a and b).

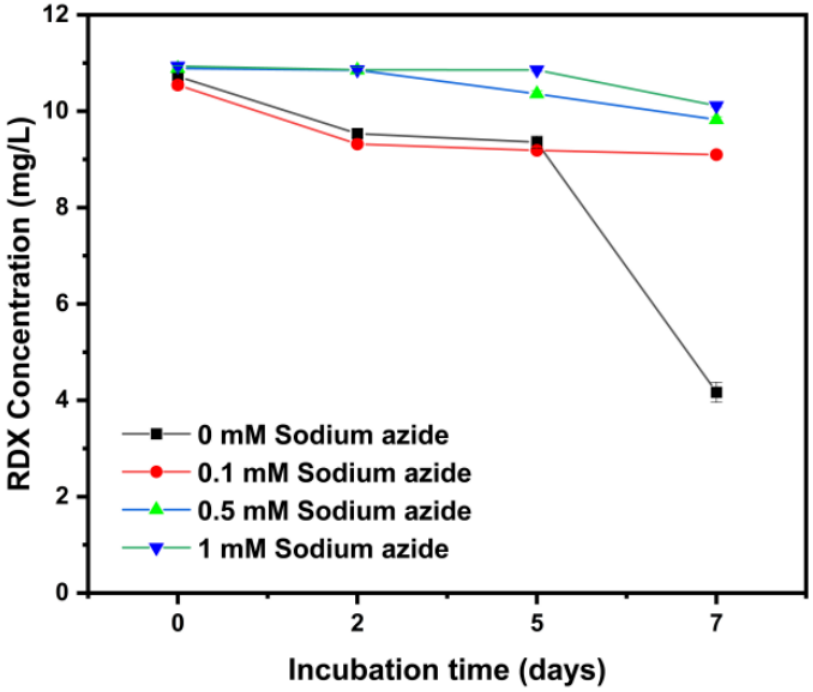


Figure 4.64: Effect of increasing concentrations of sodium azide, a specific inhibitor of nitroreductase enzyme on RDX degradation

4.4.2 Elucidation of RDX degradation pathway by *J. cremeus*

Mass spectrometry (MS) using positive electron spray ionization (ESI) was used to determine the intermediatesformed during the degradation of RDX. The intermediates were then used to deduce the proposed pathway. The ring cleavage products of RDX degradation under unsaturated and saturated conditions were monitored (Table 4.3).

Table 4.3: Intermediates obtained during RDX remediation under unsaturated as well as saturated conditions

Unsaturated treatment set		**Saturated treatment set**	
Intermediate	**m/z**	**Intermediate**	**m/z**
Hexahydo-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX)	207	Hexahydro-1,3-dinitroso-5-mononitro-1,3,5-triazine	191
Hexahydro-3,5-dintroso-1,3,5-triazine (TNX-NO)	145	5-hydroxy-4-nitro-diazapentanal	149
Hexahydro-1,3,5-triamino-1,3,5-triazine	133	Hexahydro-3,5-dintroso-1,3,5-triazine (TNX-NO)	145
____	_____	Hexahydro-1,3,5-triamino-1,3,5-triazine	133

The mass spectra (figures 4.65 a and b) reveal the presence of peak at m/z 207 in the unsaturated treatment sets, indicating the presence of mono nitroso RDX (MNX). Another major peak was observed at m/z 145 (M-NO), which signifies the presence of trinitroso derivative of RDX (TNX) (Florián et al. 2007; Naja et al. 2008). This shows that, *J. cremeus* exhibited the two-electron reduction pathway as proposed by McCormick et al. (1981). Another intermediate peak was observed at m/z 133. This corresponds to the triamino RDX derivative as observed by Zhang and Hughes (2003) in *Clostridium acetobutylicum* under anaerobic conditions. Though the unsaturated soil conditions are thought to be aerobic, it is very important to remember that in soil, different sites and pockets may exhibit different redox potential. Therefore, some sites may exhibit a local anoxic environment leading to the formation of an anaerobic pathway intermediate (Sims and Kanissery, 2019). In this pathway, MNX is further converted to a hydroxylamino derivative which is further converted to an amino derivative followed by a triamino derivative. Though no other denitration intermediates were observed, the nitrite released under unsaturated conditions could be attributed to the denitration of MNX as observed by Zhao et al in 2002 and 2003. *Clostridium bifermentans* HAW1 and *Klebsiella pneumoniae* SZC1 exhibited similar pathway of denitration of RDX via the MNX route, which led to ring cleavage and mineralization of RDX to formaldehyde, methanol and nitrous oxide.

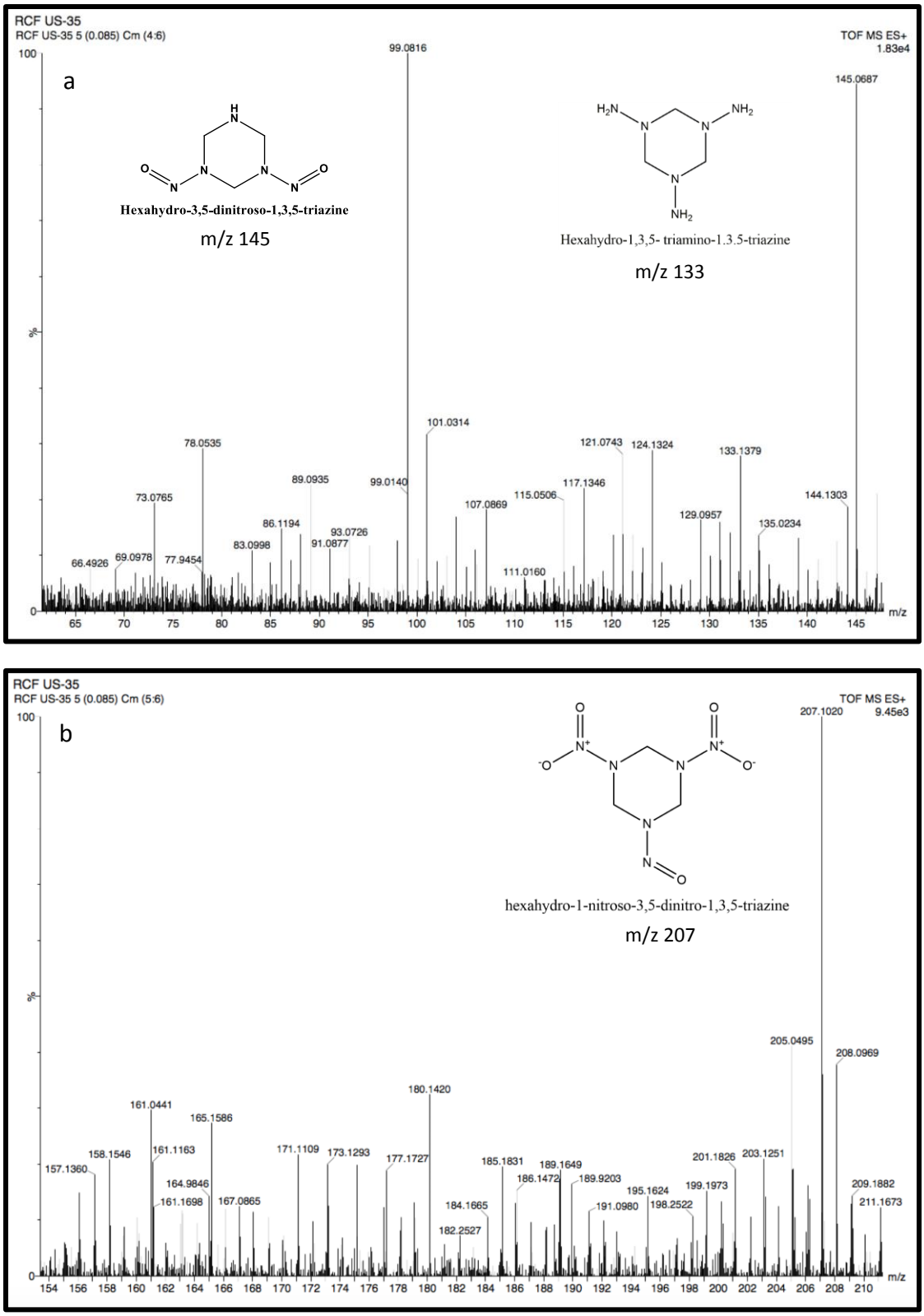


Figure 4.65: MS chromatogram for RDX degradation under (a &b) unsaturated conditions

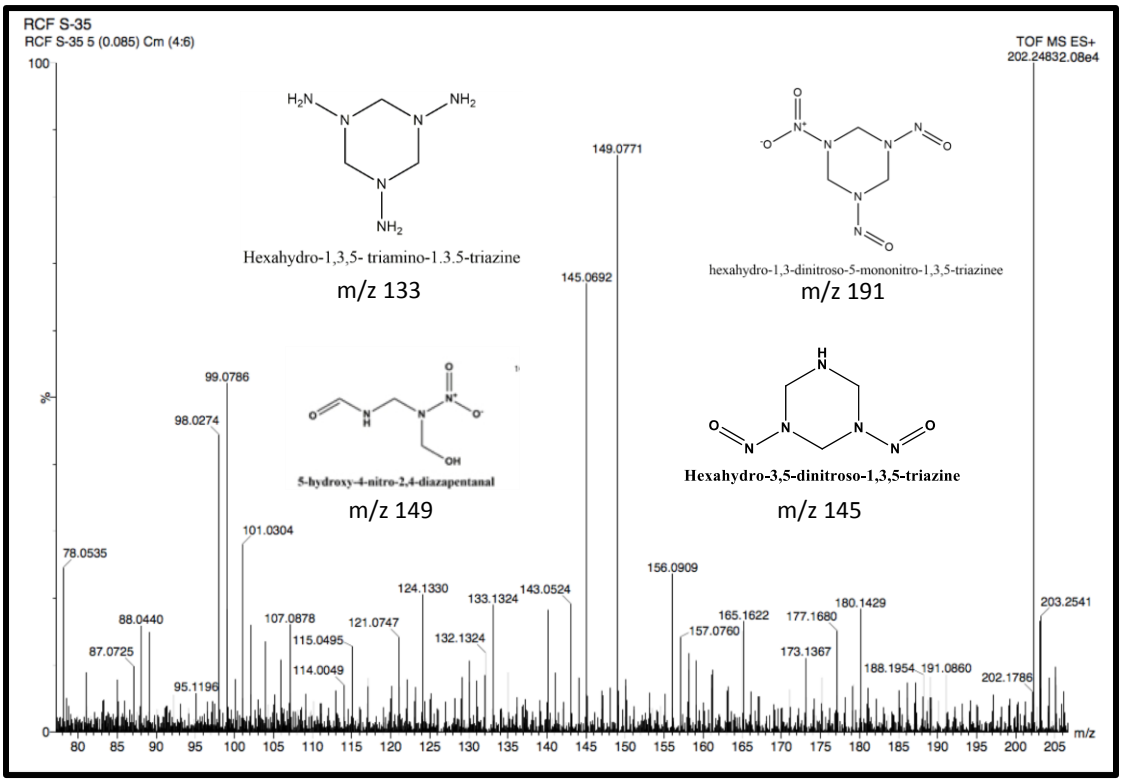
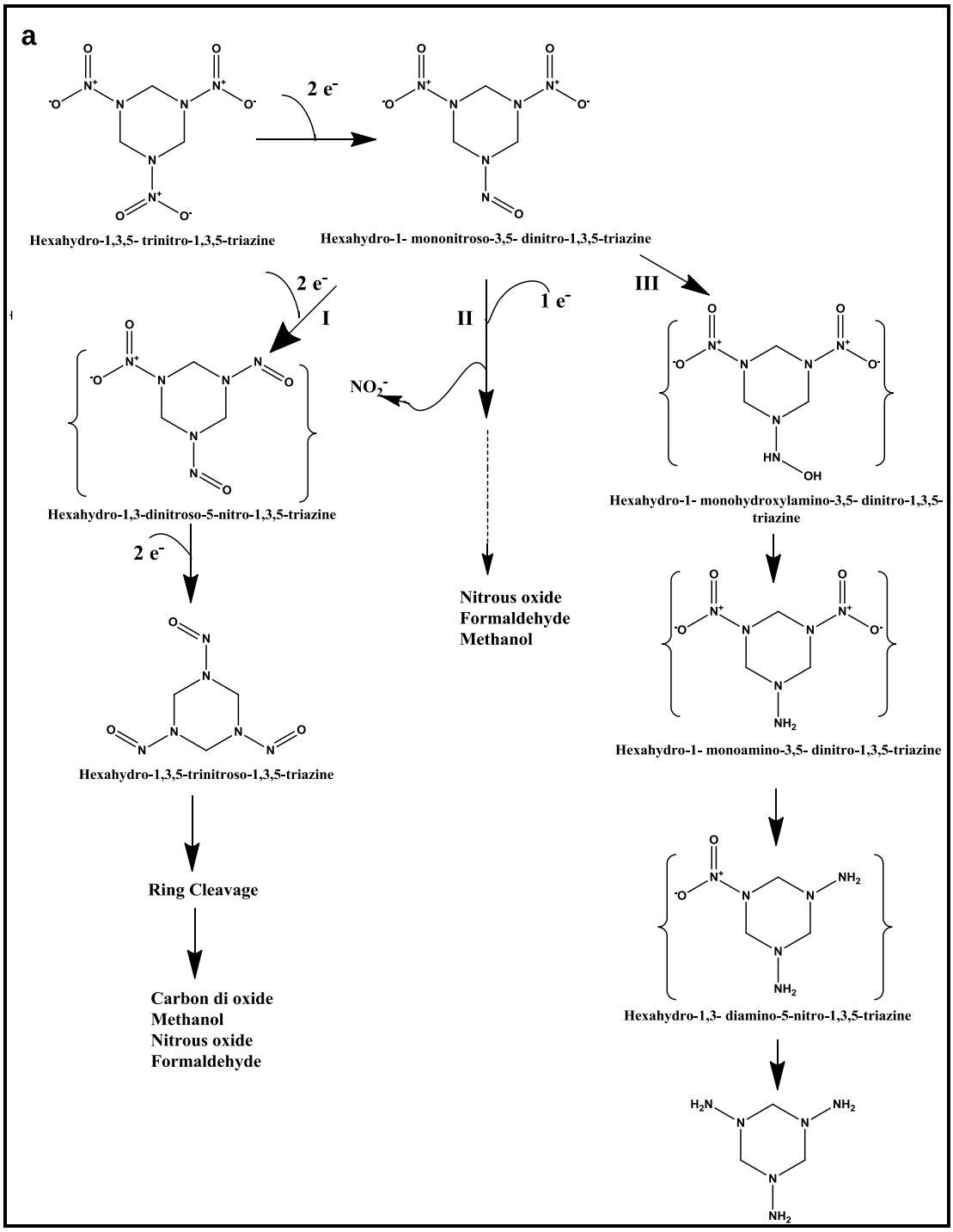


Figure 4.66: MS chromatogram for RDX degradation under saturated conditions

Mass spectrum for RDX biodegradation under saturated treatment (figure 4.66) reveals the presence of a peak at m/z 191, which corresponds to dinitroso RDX (DNX). Similar to the unsaturated system, a peak at m/z 145 correspond to TNX (M-NO). This shows that, under saturated conditions, the bacteria, *J. cremeus* breaks RDX by two electron reductive pathway to MNX, which on further reduction is converted to DNX and then to TNX. Such a pathway is typical in case of anaerobic RDX degradation as observed by Kwon and Finneran in 2008 in the bacterium *Geobacter metallireducens* strain GS-15. A similar path was observed by Perreault et al in 2012 in *Shewanella oneidensis* MR1 and by Kwon and Finneran (2006) in *Geobacter sulfurreducens* strain PCA. Another intermediate peak was observed at m/z 133 (triamino RDX) as observed by Zhang and Hughes in 2003 during anaerobic degradation of RDX. The nitrite release during RDX degradation could be attributed to the presence of an intermediate at m/z 149 which

corresponds to 5-hydroxy-4-nitro-2,4-diazapentanal. This intermediate was observed by Balakrishnan et al in 2003. This pathway led to the release of 4NDAB (4-nitro-2,4-diazabuatnal) ultimately leading to mineralized product, formaldehyde. Figure 4.67 a and b shows the proposed pathway under unsaturated and saturated conditions respectively.



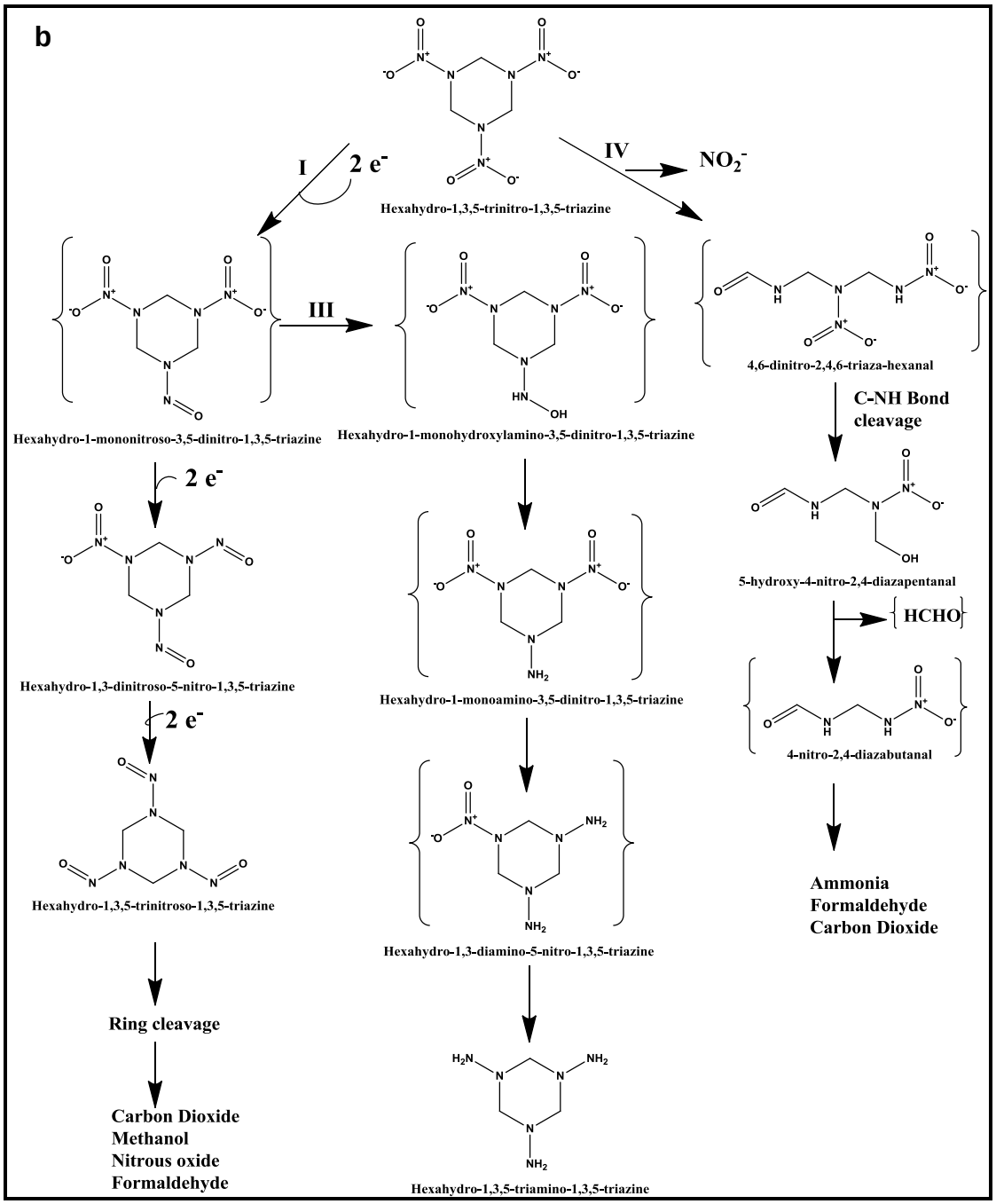


Figure 4.67: Proposed degradation pathway for RDX under (a) Unsaturated/ aerobic conditions and (b) saturated/ anoxic conditions. Path I: McCormick et al. 1981; Kwon and Finneran, 2008, Path II: Zhao et al. 2003; Path III: Zhang and Hughes, 2003. Path IV: Balakrishnan et al. 2003. The compounds shown in brackets { } weren't observed/ analyzed in this study.

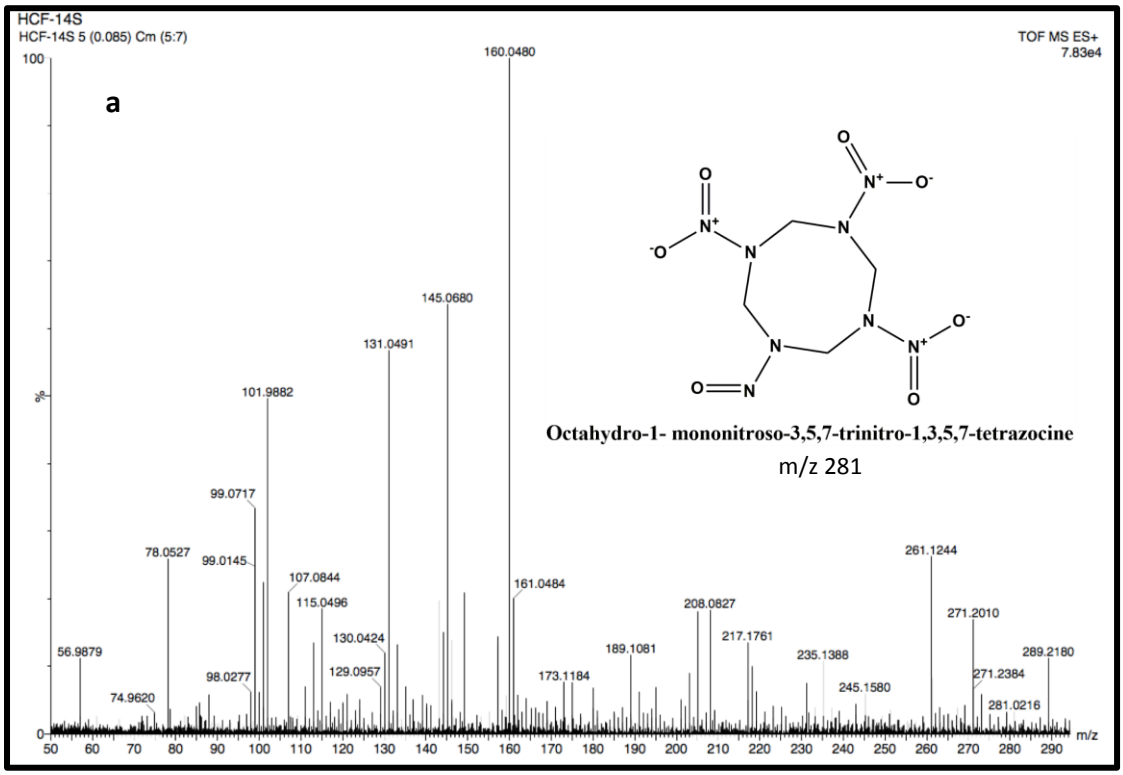
4.4.3 Elucidation of HMX degradation pathway by *J. cremeus*

HMX degradation by *J. cremeus* was carried out under unsaturated and saturated conditions. The unsaturated conditions lead to negligible degradation of HMX. The

intermediates formed during HMX degradation under saturated conditions were monitored (Figure 4.68 a and b).All the intermediates have been tabulated in table 4.4.

Table 4.4: Intermediates formed during HMX remediation under saturated conditions

Intermediate	m/z
Octahydro-1-mononitroso-3,5,7-trinitro-1,3,5,7-tetrazocine	281
Octahydro-1,3,5,7-tetranitroso-1,3,5,7-tetrazocine	231
5-hydroxy-4-nitro-diazapentanal	149



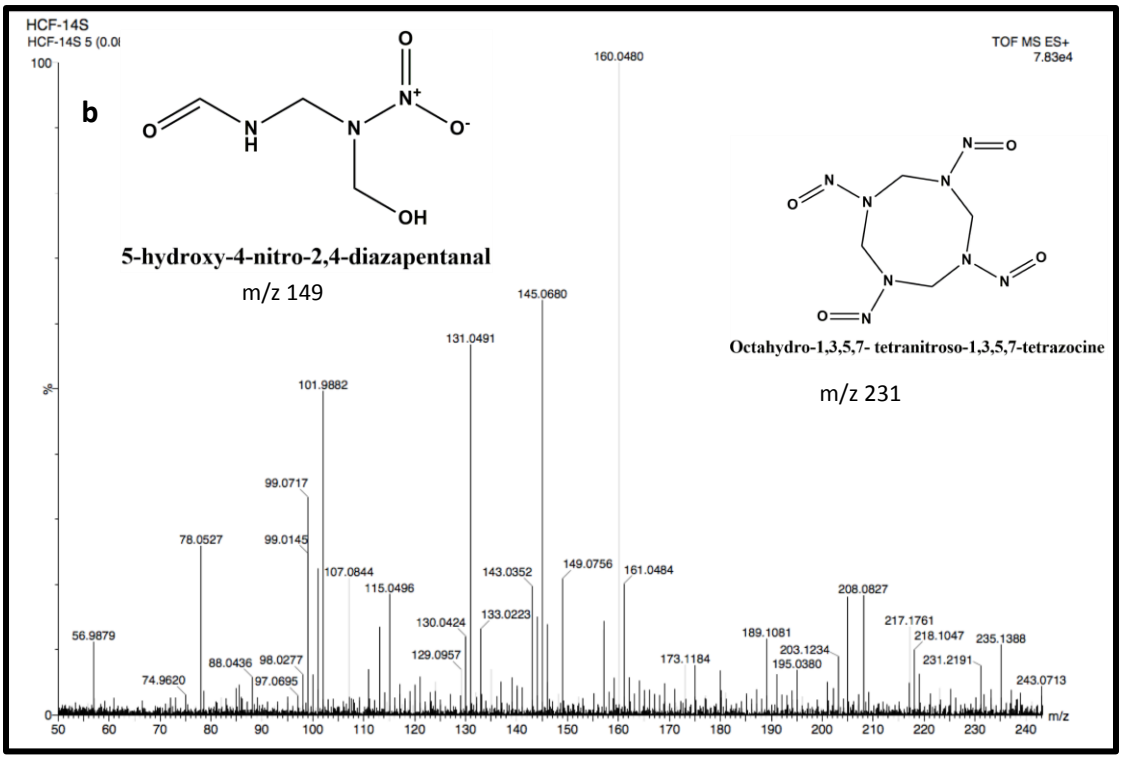


Figure4.68 (a) & (b): MS spectrum for HMX contaminated soil under saturated conditions on treatment with the developed bioformulation.

The mass spectrum revealed the presence of a peak at m/z 281, corresponding to mononitroso derivative of HMX (1 NO-HMX). Another peak was observed at m/z 231 corresponding to the formation of tetranitroso derivative of HMX (4 NO-HMX). The presence of mononitroso derivatives of HMX indicates the two electron reduction pathway (McCormick et al. 1981). Though, the intermediates dinitroso-HMX (2 NO-HMX) and tri-nitroso-HMX (3 NO-HMX) weren't observed, the presence of 4NO-HMX shows the completion of reduction pathway. This proves that, HMX under saturated conditions with *J. cremeus* underwent anaerobic degradation, similar to that observed by Zhao et al (2004a). Similar formation of nitroso- HMX derivatives has been reported during the anaerobic degradation of HMX using *Clostridium bifermentans* strain HAW1 (Zhao et al. 2004 (b); Monteil-Rivera et al. 2003).

MS of the samples also revealed the presence of 5-hydroxy-4-nitro-2,4-diazapentanal at m/z 149. Substantial release of nitrite indicates the denitration as an important step in the ring cleavage of HMX. The presence of 5-hydroxy-4-nitro-2,4-diazapentanal confirms the ring cleavage. 5-hydroxy-4-nitro-2,4-diazapentanal is an intermediate postulated to be formed during the formation of 4-nitro-2,4-diazabutanal (4-NDAB). HMX transforms to a series of intermediates with concomitant release of nitrite to ultimately produce NDAB, which further breaks down to formaldehyde attaining mineralization of HMX in soil (Balakrishnan et al. 2003). Hence, the presence of 5-hydroxy-4-nitro-2,4-diazapentanal in this study shows that, HMX undergoes denitration leading to its mineralization.

The intermediates revealed two pathways which could lead to the degradation of HMX by *J. cremeus* under anoxic (saturated) conditions. Figure 4.69 shows the proposed degradation pathway for HMX remediation under saturated conditions using the developed bioformulation.

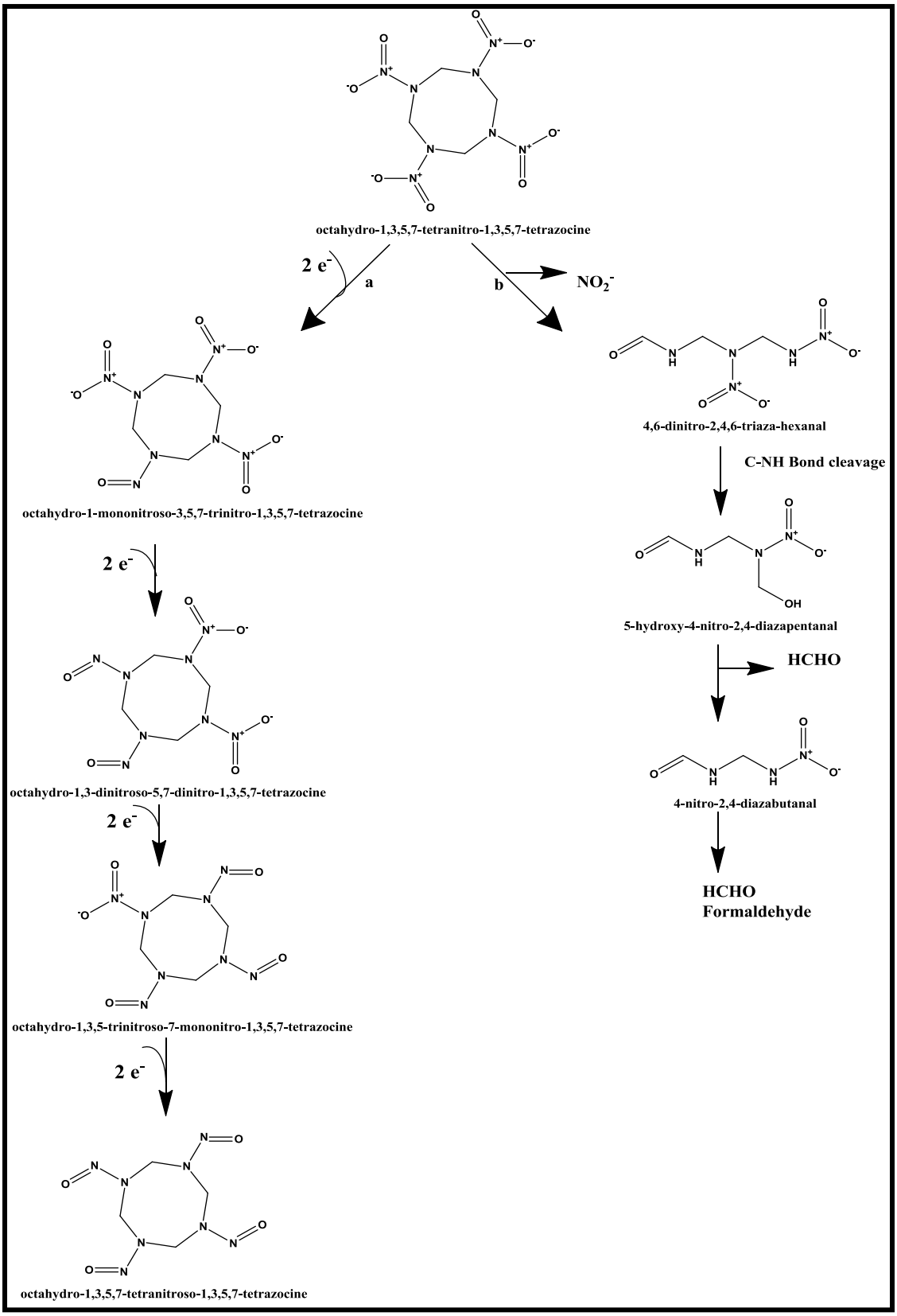


Figure 4.69: Proposed degradation pathway of HMX under saturated conditions on treatment with the developed clay based bioformulation. Path a: Two electron reduction pathwayMonteil-Rivera et al. 2003); Path b: Denitration pathway (Balakrishnan et al. 2003).

4.4.4 Conclusion

Janibacter cremeus was evaluated for the intermediates formed during the remediation of explosives, RDX and HMX to determine the degradation pathways. Nitroreductase enzyme was observed to be one of the important enzyme systems responsible for the degradation of the nitramine explosives. The degradation pathway for RDX was proposed for saturated as well as unsaturated conditions.Mass spectrometric studies showed that, both the conditions lead to the formation of nitroso-derivatives of RDX. But under saturated condition, an intermediate, 5-hydroxy-4-nitro-2,4-diazapentanal was observed which is a precursor to 4-nitro-2,4-diazabuatnal ultimately leading to mineralization. HMX on the other hand was observed to be degraded only under saturated/ anoxic conditions. Mass spectrometric (MS) analysis was performed to identify the intermediates formed during HMX degradation. Nitroso derivatives of HMX were observed during the anoxic degradation of HMX. Also, observed was the presence of 5-hydroxy-4-nitro-2,4-diazapentanal, a precursor of 4- nitro-2,4-diazabutanal, which eventually could get mineralized to formaldehyde. The results obtained clearly indicate that, *J. cremeus* degraded RDX and HMX by multiple pathways and lead to mineralization of the explosive compounds.

Chapter 5
Conclusion

CHAPTER 5

CONCLUSION

Owing to the hazardous nature of explosives, their treatment is of utmost importance. Due to the various drawbacks of physical and chemical treatment methods, a great deal of emphasis has been laid on eco- friendly and cost- effective biological means. The present study explores the use of indigenous bacterium immobilized in two novel eco-friendly bioformulations for the remediation of two most commonly used nitramine explosives, RDX and HMX. The isolates were first evaluated for their degradation potential in aqueous medium. The bacterial isolate exhibiting the best degradation potential was then selected for preparation of two novel bioformulations which were analyzed for its viability. The prepared bioformulations were then evaluated for its efficacy to degrade RDX and HMX in soil and sediments.

5.1 Aqueous Phase Remediation of Explosives

- Three bacterial species isolated from explosive contaminated sites, namely, *Janibacter cremeus*, *Pseudomonas entomophila* and *Pseudomonas mosselii*, were evaluated for their explosive degradation efficiency of 60 mg/L RDX and 6 mg/L HMX.
- *Janibacter cremeus* exhibited the highest degradation of RDX (88 %) and HMX (90 %), followed by *P. entomophila* and *P. mosselii*. All three bacterial isolates exhibited growth in presence and absence of explosive, indicating that the explosive was utilized as a non-growth substrate and that the degradation was due to co-metabolism.
- For soil remediation studies, *J. cremeus* was selected as:

It exhibited the highest degradation efficiency for both RDX and HMX and it was a facultative anaerobe indicating that it could survive in aerobic as well as anoxic conditions also.

5.2 Preparation of Bioformulations

- *J. cremeus* was immobilized on to a carrier support. Two bioformulations were prepared which were eco-friendly and cost effective.
- Bioformulation 1 (BF1), a powdered formulation was prepared using calcite, a natural clay mineral and cocopeat, a natural agricultural waste.
- Bioformulation 2 (BF2), a wettable powder formulation comprised of egg shell powder, cocopeat, a surfactant (Tween) and a dispersant (sodium bi carbonate.
- Both, BF1 and 2 exhibited good viability of the bacterial cells even after 180 days of storage at 4°C.

5.3 Soil remediation studies

The developed bioformulation were evaluated for their explosive degradation ability in soil. RDX and HMX degradation were carried out under unsaturated/aerobic as well as saturated/ anoxic moisture conditions.

5.3.1 Remediation of RDX in soil

- RDX degradation was observed to be 60 % under aerobic conditions whereas, 75 % under anoxic conditions.
- The RDX concentration at end of the study with both the bioformulations individually resulted in meeting the USEPA proposed soil screening limit (SSL) of 28 mg/ Kg, rendering the soil safe.

- The degradation pathway for remediation was also elucidated using mass spectroscopy (MS). The mass spectrum revealed the presence of mono and trinitroso derivatives under aerobic as well as anoxic conditions. Another major intermediate observed was triamino derivative of RDX under both the conditions. Anoxic conditions exhibited the presence of 5-hydroxy-4-nitro-2,4-diazapentanal which is a precursor of 4-nitro diazabutanal (NDAB) ultimately leading to mineralization to formaldehyde and other simpler products. The pathway proposed showed mineralization of RDX under both aerobic as well as anoxic conditions.

5.3.2 Remediation of HMX in soil

- HMX degradation was monitored at a high concentration of 3000 mg/ Kg as observed in actual field conditions.
- Both BF1 and BF2 exhibited negligible degradation under unsaturated/ anoxic conditions.
- Under anoxic/ saturated conditions, BF1 and 2 exhibited 40 % degradation of HMX in 35 days.
- The degradation pathway was also elucidated under saturated moisture conditions. The observed intermediates were, mononitroso and tetranitroso- derivatives of HMX. Also observed was 5-hydroxy-4-nitro-2,4-diazapentanal. The pathway proved mineralization of HMX to simpler compounds under anoxic conditions.

5.4 Sediment remediation studies

- The developed bioformulations were also applied for remediation of two explosive contaminated acidic sediments obtained from explosive manufacturing facilities in India.

- Sediment A was observed to be acidic (pH 4) and highly contaminated with RDX (9,84,000 mg/Kg) was obtained from an RDX manufacturing facility.
- The BF1 lead to an increase in pH to 7.5 whereas, BF2 lead to a n increase to pH 8. The buffering action was attributed to calcium carbonate present in both calcite and egg shell powder. About 87-88 % degradation of RDX was observed in 150days with both BF1 and 2.
- Sediment B was characterized by a very low pH of 2.08 and co-contamination with both RDX (82000 mg/Kg) and HMX (71000 mg/Kg). It was obtained from HMX manufacturing facility in India.
- RDX exhibited a degradation of 53- 55 % whereas HMX was observed to be degraded by 47- 49 %.

The results obtained prove that BF1 and 2 play a crucial role in delivering the microbe to the contaminated site. They can also aid in degradation of nitramine compounds so as to meet the international regulatory limits, to declare a site safe.

5.5 Future research directions

The novel bioformulations could be utilized as a sustainable remediation tool for treatment of nitroorganic contaminated sites. The method adopted for preparation is economical and eco-friendly and the constituents are sustainable in nature. Moreover, the application process is also simple.

The studies reported here are of lab scale level. The results obtained in the studies show that with proper upscaling in place, it can be a potential solution for on-site remediation of explosive contaminated soil/ sediments/ sludge.

The bioformulations can find following applications:

a. The developed bioformulations can be utilized for remediation of not only RDX and HMX, but also other nitro organic compounds at site.
b. The present study has been designed keeping in mind the manufacturing facilities. The bioformulations can also find application in remediation of firing and testing ranges, which have been documented to be highly contaminated with explosives.
c. The bioformulations developed can also be used in treatment of pure chemicals left in the unexploded munition shells.
d. The bioformulations can also be used as an ingredient in composting of explosives.
e. The effect of bioformulations on fate and transport of the nitroorganic explosives can also be tested to evaluate the migration potential of explosives from soil to ground water.
f. The mild alkaline nature of the developed bioformulations can also aid in reclamation of acidic soils.

In conclusion, the developed bioformulations are much more efficient than natural attenuation, which is widely practiced for field scale remediation. Also, they could be successful replacement for other treatment technologies (physical and chemical) in place. They can also aid in attaining the goals of circular economy and sustainable waste management.

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List of Publications

LIST OF PUBLICATIONS

Journal Publications

1. **Kalsi, A.,** Mary Celin, S., Bhanot, P., Sahai, S., & Sharma, J. G. (2020). Microbial remediation approaches for explosive contaminated soil: Critical assessment of available technologies, Recent innovations and Future prospects. Environmental Technology & Innovation, 100721.doi:10.1016/j.eti.2020.100721 (Impact factor: 3.356)

2. **Kalsi, A.,** Celin, S. M., & Sharma, J. G. (2020). Aerobic biodegradation of high explosive hexahydro-1,3,5- trinitro-1,3,5-triazine by *Janibacter cremeus* isolated from contaminated soil. Biotechnology Letters. doi:10.1007/s10529-020-02946-6 (Impact factor: 1.977)

3. **Kalsi, A.,** Celin, S. M., Bhanot, P., Sahai, S., & Sharma, J. G. (2020). A novel egg shell-based bio formulation for remediation of RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) contaminated soil. Journal of Hazardous Materials, 123346. doi:10.1016/j.jhazmat.2020.123346 (Impact factor: 9.038)

Conference Presentations

1. **Anchita Kalsi,** S. Mary Celin, Sandeep Sahai, Jai Gopal Sharma (2018). Bioremediation of RDX contaminated soil using a novel bioformulation prepared with indigenous RDX degrading bacteria, presented at 59th Annual Conference of Association of Microbiologist of India and International Symposium on Host-Pathogen Interactions at University of Hyderabad.

2. **Anchita Kalsi,** S. Mary Celin, Sandeep Sahai, Jai Gopal Sharma (2019). Microbial bioformulation for the remediation of soil polluted with Hexahydro- 1,3,5- trinitro-1,3,5- triazine (RDX) - A mesocosm study, presented at ESDACON at Jawaharlal Nehru University, Delhi.

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Microbial remediation approaches for explosive contaminated soil: Critical assessment of available technologies, Recent innovations and Future prospects

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ABSTRACT

Soils contaminated with explosives are a major threat to the environment. Explosives in soil can migrate to the ground water leading to harmful effects to human life and the environment. Several technologies have been proposed for the remediation of explosive contaminated soil, including, physical, chemical and biological methods. Physical and chemical treatment technologies, though fast, suffer from a major drawback of being environmentally unsafe. Hence, with environment in center stage, biological treatment methods have gained importance. Microbial remediation, a type of biological treatment technology plays a major role in bioremediation of explosive contaminated sites. Microbes, being ubiquitous find application in various forms to serve the purpose of remediation. This review critically assesses the various microbial treatment technologies, both *in situ* and *ex situ* available for remediation of explosive contaminated soil. Also, it discusses the environmental impact of these technologies along with the various emerging trends in the field of microbial remediation in the past decade. And finally, we explore the future prospects of microbial remediation that can provide a sustainable solution for soil explosive contamination.

Contents

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1. Introduction

1.1. General

Explosive contamination of land and water is a major environmental concern. With an increased use of explosive chemicals in various fields, their penetration into the soil as well as the aquatic environment has become inexorable. Explosives being recalcitrant in nature, tend to persist in the environment for long time. Enough studies have been conducted to assess the fate of these compounds in soil (Yamamoto et al., 2004; Kalderis et al., 2011; Clausen and Korte, 2011). Managing sites contaminated with explosives has become a global problem. Many countries are trying to test new advancements in treatment technologies to combat this problem. With high priority to the environment safety in place, it has become a daunting task to achieve remediation using eco-friendly methods. The major hurdle lies in the toxicity of the explosive compounds to living organism (microbes and plants) that can provide a suitable eco-friendly solution to this problem. This review critically assesses the available microbial treatment technologies for remediation of explosives, also discusses the emerging trends in microbial remediation in the past decade and future prospects of this technology.

1.2. Explosives

Explosives are energetic chemicals which are highly reactive, have potential of self-oxidation and are classified as hazardous. The oxidation leads to generation of small gaseous molecules, viz, N_2, H_2O and CO_2. Explosives on detonation create shock waves in the surroundings leading to an explosion, with the release of toxic compounds in the environment (Kalderis et al., 2011; Singh et al., 2012). Explosives are majorly used in industries and military operations. Improper handling and disposal of waste from manufacturing, loading, assembly and packaging (LAP) activities are few of the causes of explosives finding their way into the environment. Contamination in the live firing ranges, open burning/ detonation sites as well as in the washout lagoon soils of former manufacturing plants and also military bases are few of the well documented sites with explosive contamination worldwide (Thorn et al., 2004). Many countries have also been reported

to dump old explosives into the sea. Burying of outdated explosives have also been observed. Another major factor in soil contamination is the disposal of explosive wastewaters (Kalderis et al., 2011). Explosives are also used in mining operations. The blasting process for exploration of minerals leads to release of fumes and residues of explosives (Juhasz and Naidu, 2007). The explosive residues deposited on the soil surface pose a major threat to the soil ecosystems.

Many episodes of soil contamination by explosives have been reported all over the world. The Pantex plant used by the US Army during the World War II have been reported to be highly contaminated with Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), Octogen (HMX) and Trinitro toluene (TNT). Whereas, in Australia, a former explosive manufacturing facility have been reported to be highly contaminated with TNT. A pure TNT layer of about 3 cm thickness have been found to be located 10–15 cm below the soil surface (Martel et al., 2007). Similarly, a former ammunition site in Germany have been found to be highly polluted with explosives, their metabolites, heavy metals and also polycyclic aromatic hydrocarbons (Eisentraeger et al., 2007). Other than the manufacturing facilities, unexploded ordnance (UXO) also remains a major problem. The Vietnam Ministry of Defense in 2002, estimated that approximately 7%–8% of the land in the country was affected by UXO. 1215 sites have been identified to be affected by UXO in Australia. Whereas, in US, 1400 sites have been identified to be possible UXO sites, which accounts for 10 million acres of land (Pichtel, 2012). Jenkins et al. in 2001 and Pennington et al. in 2002 have also reported the presence of RDX in water leached from live fire hand grenade ranges. They also reported the presence of RDX in groundwater at the range.

Explosives are not only hazardous to environment but also human health. United States environment protection agency (USEPA) has classified explosives as potential human carcinogen. RDX is one of the most common explosives used worldwide. Ingestion of RDX, can lead to neurological damage. Also, it is characterized by a low sorption coefficient, hence, it is not retained in the soils and easily migrates to the groundwater (Agency for Toxic Substances and Disease Registry, 2012; EPA, 2005). Octogen (HMX), another commonly used explosive has been reported to be harmful to plants and animals. Lab testing have showed that, HMX ingestion can lead to central nervous system and liver damages. Most of these explosives are stable in soil due to their chemical structure and also due to their capability to bind to soil organic matter. These factors render them to be resistant to soil remediation strategies (Rylott et al., 2011). Though plethora of remediation strategies already exist for the cleanup of explosive contaminated soils, it is important to adopt technologies which are not only efficient but also environment friendly.

EPA has put forth soil screening levels (SSL) for different explosives based on their risk assessment. SSL for Trinitro toluene (TNT) has been calculated as 19 mg/kg in residential whereas, 79 mg/kg in industrial soils (EPA, 2013). SSL for perchlorate which is a major constituent in solid rocket propellants has been calculated as 55 mg/kg for residential and 820 mg/kg for industrial areas (EPA, 2017a). Similarly, the SSL for RDX has been calculated to be 6.1 and 28 mg/kg for residential and industrial soils respectively (EPA, 2017b). Dinitrotoluene (DNT) exist in six isomeric forms, of which, 2,4- and 2,6-DNT are the most commonly existing ones. EPA has calculated SSL for 2,6-DNT as 0.36 and 1.5 mg/kg in residential and industrial sites respectively. The SSL for a mixture of 2,4 and 2,6-DNT have also been calculated for residential as well as industrial soils as 0.8 and 3.4 mg/kg respectively (EPA, 2017c). Also, according to EPA (1993) soils with contamination of more than 10% (on the dry weight basis) of secondary explosive contamination are found to be susceptible to initiation and propagation.

1.3. Classification of explosives

Explosives can be classified into different types based on:

1. Properties
2. Chemical composition

Classification based on properties:

Explosives can be classified as high explosives, low explosives and pyrotechnics (Agrawal, 2015). Fig. 1 gives an overview of the classification.

Classification based on chemical composition:

Explosives can also be classified on the basis of their chemical composition (Singh et al., 2012):

a. Nitrate esters
b. Nitroaromatics
c. Nitramines

Table 1 classifies the explosives based on chemical composition with examples and health effects.
Structures of common explosives have been given in Fig. 2.

This review describes briefly the various available treatment technologies for remediation of explosive contaminated soil, laying focus on microbial remediation technologies. The paper critically examines the advantages and disadvantages of the techniques as well as the life cycle of these microbial remediation technologies. Though many reviews are available that describe the existing technologies for remediation of explosive contaminated soils, this review discusses the various emerging trends in microbial remediation in the past decade and future prospects for treatment which have not yet been reviewed.

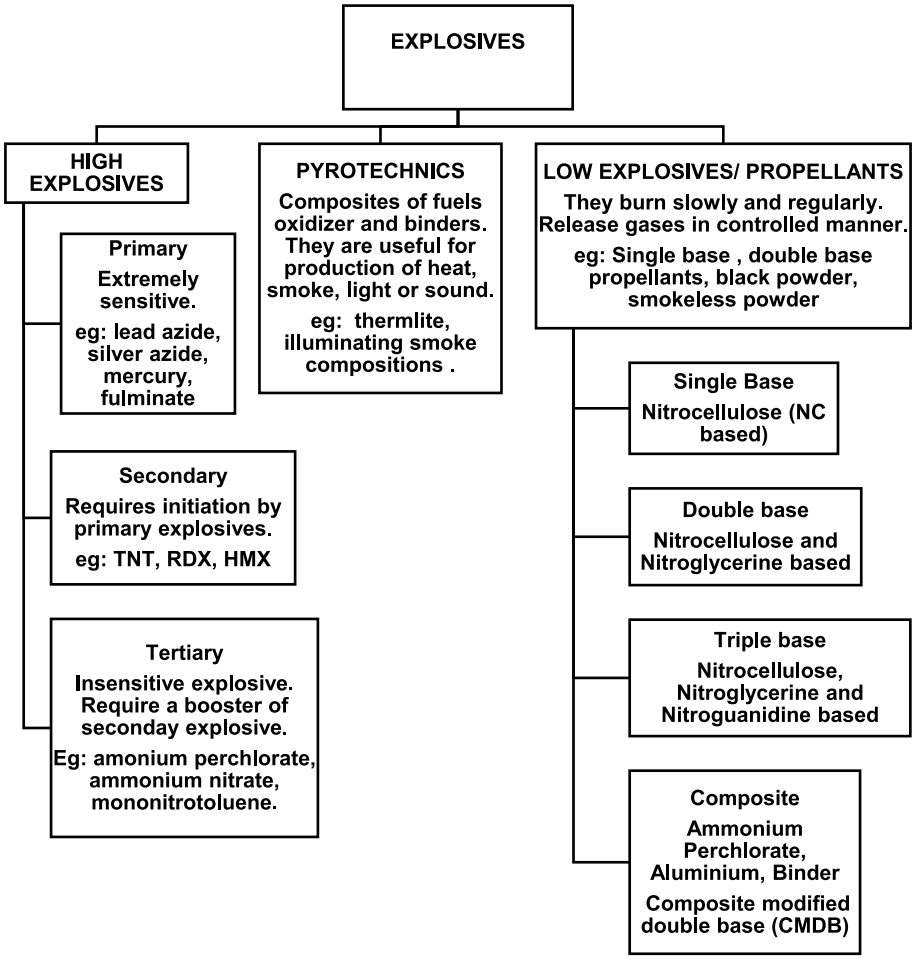

Fig. 1. Classification of explosives based on properties.

Table 1
Classes of explosives based on chemical composition.

Sl. No.	Class	Examples	Effects
1	Nitrate esters	Glycerol trinitrate (GTN; propane-1,2,3-triyl trinitrate) Pentaerythritol tetra nitrate (PETN; 2,2-bis[nitrooxymethyl]-propane-1,3-diyl dinitrate)	Methaemoglobinaemia
2	Nitroaromatics	2,4,6-trinitrophenol (TNP) 2,4,6-trinitrotoluene (TNT) 2,4- dinitroanisole (DNAN)	Haemolytic anaemia and testicular toxicity
3	Nitramines	RDX (Royal Demolition Explosive or Research Department Explosive)- hexahydro-1,3,5-trinitro 1,3,5-triazine HMX (High melting explosive)- octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine CL-20- 2,4,6,8,10,12-Hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane	CNS damage, convulsions, liver damages

2. Available treatment technologies

Remediation of soils contaminated with explosives need to be dealt on the site basis. It depends on various environmental factors as well as the extent of explosive contamination (Rodgers and Bunce, 2001). Soil remediation strategies can be broadly classified into physical, chemical and biological. Fig. 3 outlines the various treatment strategies associated with soil remediation. Physical and chemical methods, though faster are generally costly and energy intensive. Also, they

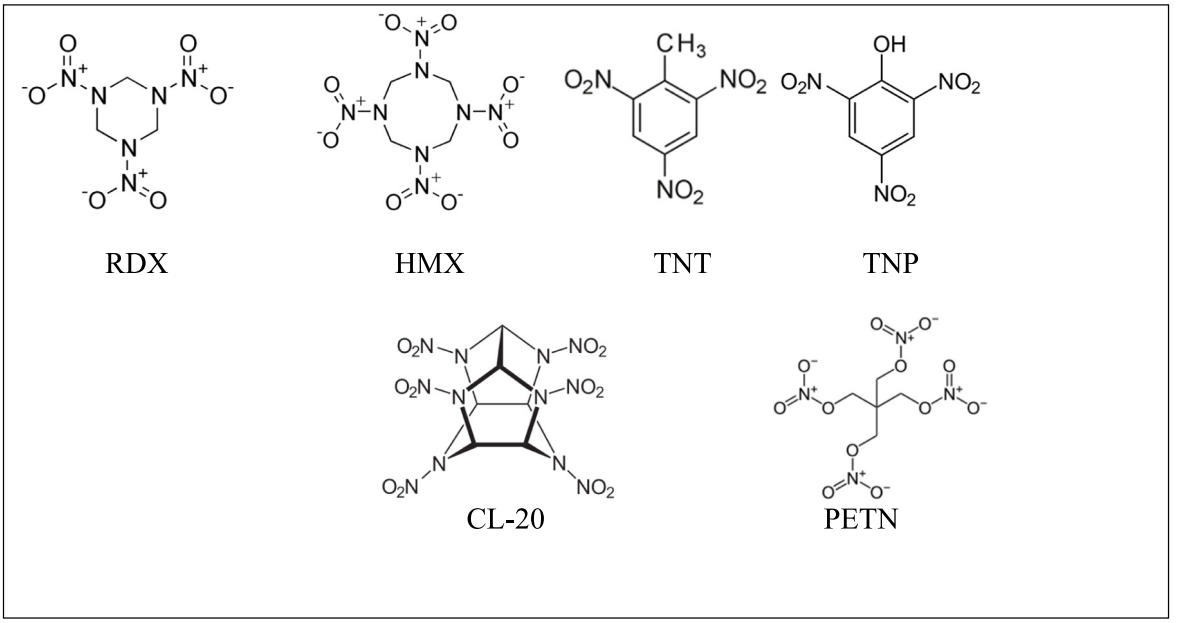

Fig. 2. Structures of some common explosives.

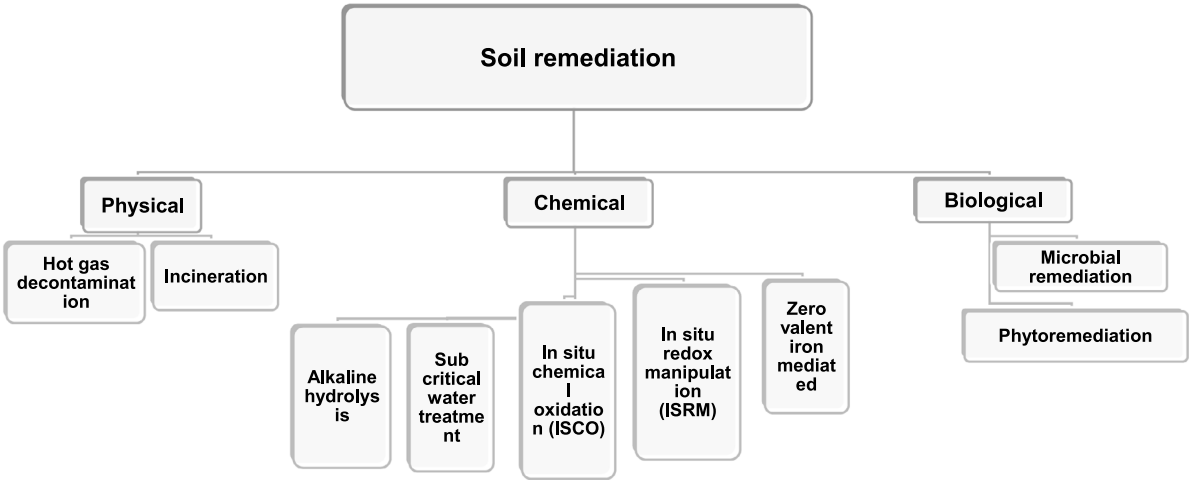

Fig. 3. Treatment strategies for remediation of soils contaminated with explosives.

suffer from a major drawback of transferring the contaminant from one phase to the other (Ward and Singh, 2004). Hence, emphasis is being laid on use of biological remediation strategies owing to their eco-friendly nature.

2.1. Physical and chemical treatment technologies

Physical and chemical treatment technologies for decontamination of explosives in soil are generally faster, can help in decontamination of high concentrations of explosives. Also, they are not dependent on the environmental conditions for their performance in remediation. These technologies generally lead to a phase transfer leading to secondary pollution (Kalderis et al., 2011).

2.2. Physical treatment technologies

2.2.1. Incineration

Incineration employs the use of high temperatures (860–1200 °C) for destruction of explosive contaminated soils or debris, unexploded explosives and bulk explosive waste (Garg et al., 1991; Van Ham, 1998). USEPA in 1991, had declared

incineration as one of the best technologies for treatment of explosive contaminated sludge. The process may take place in a controlled environment like the enclosed ovens, or it can be accomplished by open burning/ open detonation (OB/OD). OB/OD being uncontrolled reaction, it leads to the generation of toxic air emissions. With strict regulations in place, OB/OD is not recommended. Another major drawback, is the equipment and personnel safety. Since explosives are highly sensitive to impact and friction, the process commands greater regulations and safety precautions when under progress (Garg et al., 1991). Though these methods help in faster decontamination of explosive contaminated sites, the materials decontaminated cannot be reused and must be scrapped or landfilled. Other disadvantages of this technique include high cost incurred for equipment and its maintenance, fuel costs and also additional treatments for discharge systems. Another major problem associated with incineration is the incomplete combustion leading to release of particulate matter and air pollutants (Garg et al., 1991).

2.2.2. Hot gas decontamination

Hot gas decontamination is majorly utilized for decontamination of explosive contaminated soil, structures and equipment. Though the operational conditions are site specific, it generally involves heating with hot gas at 260 °C for one hour which leads to the volatilization of the explosives. The gas effluent released is then treated in an after burner. This technique is suitable for destruction of explosive sediments in stockpile which needs to be discarded as hazardous material (Raghavan et al., 1989; Topfer, 1995; Hyman and Dupont, 2001; Hewitt, 2001). The major concerns related to this technology are the atmospheric emissions from the thermal oxidizer. Constant monitoring is of utmost importance so as to keep a check on hazardous emissions. Another major limitation associated with this technology is the design of the furnace. Special care must be taken to consider any possible explosions due to the presence of explosives (Hewitt, 2001).

2.3. Chemical treatment technologies

2.3.1. Alkaline hydrolysis

Alkaline hydrolysis is one of the alternative technology available for treatment of chemical weapons. The process leads to decomposition of explosive compounds to organic and inorganic salts, soluble organic compounds and gases (Balakrishnan et al., 2003). The transformation of explosive compounds has been extensively studied. Janowsky in 1891, first established use of base for transformation of the explosive compound, TNT. Various substances that can be used to raise the pH of soil include, metal oxides, hydroxides and carbonates. Such materials, find applications in wastewater treatment, acid mine drainage and agricultural soil treatment (Larson et al., 2008). Lime in various forms have been investigated for its ability to increase pH and also degrade explosive compounds present in soil. Calcium hydroxide has been found to be one of the most promising base (Brooks et al., 2003) and has also been adapted for field applications (Thorne et al., 2004; Martin et al., 2013). This technology has been demonstrated for successful removal of both nitroaromatics and nitramines present in soil (Hansen et al., 2003; Brooks et al., 2003).

Efficiency of alkaline hydrolysis to remove explosive greatly depends on the contact of explosive compound and the base in soil pore water. The transportation of hydroxyl ion through the soil plays a major role in the process. Since, the reaction of alkaline hydrolysis occurs in the aqueous phase, it is important for the munition compound and the base applied to be dissolved in soil pore water. Soil chemistry also plays a crucial role in alkaline hydrolysis. The cation species may undergo exchange with other cations present on the soil sites. These may also be exchanged with H^+ ions present in soils with low pH, leading to a reaction with OH^- ions, causing the buffering of the soil system, thereby, inhibiting the hydrolysis process. The base cations could also form insoluble hydroxides, removing them the hydrolysis reaction. Also, the hydrogen ions on functional groups of humic matter may dissociate under high pH, hampering the hydrolysis reaction (Larson et al., 2008). Much research has been intended to investigate the efficiency of alkaline hydrolysis process for soil treatment. Emmrich in 2001, investigated alkaline hydrolysis for treatment of TNT contaminated soils of two former ammunition plants located in Germany. Hansen et al. in 2003, studied the remediation of TNT contaminated soil using lime treatment. Davis et al. in 2006 conducted microcosm studies for treatment of soils from munition plants and active firing ranges. Davis et al. in 2007a, studied the mechanism of alkaline hydrolysis of TNT and RDX, the final products and the biodegradability of the reaction products. Davis et al. in 2007b conducted mesocosm studies for treatment of TNT, RDX and HMX contaminated soils using lime. Coyle et al. in 2017, performed laboratory studies using novel reactive gas process for treatment of soils contaminated with explosives. This process involves the use of ammonia in air mixture to raise the soil pH and hence degrade explosives by alkaline hydrolysis.

The major drawback of this technology is the high pH (12–13) that has to be maintained in the soil for its effectiveness. This high pH lowers the soil quality and effects its physical, chemical and biological properties. Soils with high alkalinity have been found to be detrimental to plants. It has also been observed the retardation in organic matter mineralization as the bacterial activity is hindered. Another disadvantage of alkaline hydrolysis includes, high moisture content required to be maintained in soils for the reaction to occur. Though this is helpful for effectiveness of reaction, it may also lead to transportation of the explosive compound through the soil profile to the groundwater table. This could pose a serious threat for groundwater contamination at polluted sites (Anderson and Ventola, 2015).

2.3.2. Subcritical water treatment

Subcritical water (SCW) is water held at subcritical condition, that is, temperature above 100 °C and below its critical temperature of 374 °C and a pressure of 22.1 MPa. The high pressure helps maintain water in the liquid state (Carr et al., 2011). The subcritical water can act as an effective solvent, catalyst and reactant for hydrolytic conversions (Brunner, 2009). Under subcritical conditions, water can help in explosive removal by acting like other organic solvents, aiding in solubilization and separation (Islam et al., 2015). Also, under such conditions, self-ionization of water molecules lead to increase in concentration of H^+ and OH^- ions, which can lead to removal of organic explosive compounds by oxidation (Kuhlmann et al., 1994; Oh et al., 2011; Islam et al., 2015). Hawthorne et al. in 2000, studied the use of SCW process for treatment of soils contaminated with TNT, RDX and HMX. They used water at subcritical conditions in static mode rather than flowing. Islam et al. (2015) studied the use of subcritical water treatment for removal of explosive in soils co-contaminated with explosive (RDX and TNT) and heavy metals as generally found in firing ranges.

2.3.3. In situ chemical oxidation (ISCO)

ISCO is the use of chemical oxidants for the treatment of explosive contaminants. This requires the delivery of the oxidizing agents in the subsurface of the soil. It can be performed using four different types of oxidizing agents, viz, modified Fenton's reagent or catalyzed hydrogen peroxide propagation (CHP), ozone sparging, persulfate and permanganate treatment (Watts et al., 2006). The success of the process depends on the delivery of the oxidant to the subsurface as well as its effective reaction with the explosive contaminant. Delivery techniques include: deep soil mixing, hydraulic fracturing, multi-point vertical lancing, horizontal well recirculation, and vertical well recirculation (U.S. Department of Energy, 1999).

ISCO using permanganate generally can be performed in aqueous medium only (Petri et al., 2010). Hence, much work has been carried out for remediation of explosives in aqueous phase (Adam et al., 2004; Waldemer and Tratnyek, 2006).

Use of modified Fenton is also a well-established technique for ISCO. It involves the use of ferrous ion along with H_2O_2. The process leads to release of hydroxyl radicals, which are extremely potent oxidizing agents. Majority of work related to Fenton has been carried out for aqueous phase, but it has been demonstrated for soils too (Watts et al., 1991; Bier et al., 1999; Sheremata and Hawari, 2000; Yardin and Chiron, 2006).

Ozone is a highly reactive gas. The electronic configuration of O_3 contributes to its reactivity. It has dual electrophilic and nucleophilic character. O_3 gas can be purged into the soil profile for remediation of unsaturated soils. The O_3 reacts with iron oxides and organic matter to produce hydroxyl radicals which can lead to the oxidation of explosives in soil (Adam et al., 2006).

Another potent chemical oxidant is persulfate. Persulfate has higher stability than H_2O_2. It forms sulfate radicals which are stronger oxidizing agent than H_2O_2, O_3 and permanganate. Persulfate can be activated by heat, alkaline pH, permanganate or ferrous ions. Soil contaminated with explosives have also been remediated using this technology (Waisner and Hoag, 2006; Waisner et al., 2008).

2.3.4. In situ redox manipulation (ISRM)

ISRM involves the use of a reducing agent for transformation of explosives (Szecsody et al., 2001; Adam et al., 2005; Boparai et al., 2008). Most commonly used chemical reductant is sodium dithionite. Sodium dithionite reacts with naturally occurring iron to form ferrous ions, which lead to formation of a chemically reduced zone. The ferrous ions, then act as reactants to transform the high explosives (Clayton et al., 2005). Boparai et al. in 2008 evaluated the efficacy of dithionite reduced sediments for the degradation of RDX, HMX and TNT. They found that, all three explosives were reduced and on analysis of the intermediates, they found that RDX was transformed to nitroso derivatives. Adam et al. in 2005 studied the biodegradability of the intermediates formed by ISRM of RDX. They found that, the intermediates could be successfully biodegraded aerobically.

2.3.5. Zero valent iron mediated remediation

Iron typically exists as ferrous or ferric iron in nature. Zero valent iron (ZVI/Fe^0) is synthesized. Application of ZVI has been focused on its electron donating capacity. ZVI is fairly reactive with water and can act as an electron donor, hence is a promising candidate for site remediation (Stumm and Morgan, 1996). Application of zerovalent iron (Fe^0) for remediation of explosive contaminated soils is well researched and documented (Singh et al., 1998, 1999; Oh et al., 2001, 2002; Comfort et al., 2003; Park et al., 2004; Jiamjitrpanich et al., 2010). ZVI in presence of water replaces the oxygen of nitro group with hydrogen making it further easier to break down. Oh et al. in 2001 conducted integrated studies using ZVI and anaerobic sludge for the remediation of RDX contaminated soil. They concluded that bioaugmentation along with ZVI could help in effective degradation of RDX.

Comfort et al. in 2003, conducted laboratory and pilot scale experiments for remediation of soils contaminated with RDX. In yet another study, Park et al. in 2004, studied the potential of ZVI for remediation of soils co-contaminated with RDX, HMX and TNT. Their results showed that ZVI was effective for RDX and TNT, but not HMX. In presence of RDX, ZVI acted on it rather than HMX. This was attributed to its low solubility. Hence, they proposed the use of cationic surfactants for increasing HMX solubility, thereby, increasing the efficacy of ZVI.

Though ZVI is very successful in treatment of explosive compounds, its application in field scale level is mainly limited by the requirement of proper mixing. Under unsaturated conditions, mixing is crucial so that the reactants are well in contact with ZVI. And hence, in static conditions its very necessary to employ high speed mixers (Comfort et al., 2003). When applied in slurries, the equipment required for continuous mixing adds to the cost of treatment. Also, such slurries further require dewatering adding an extra step in the treatment process (Kalderis et al., 2011).

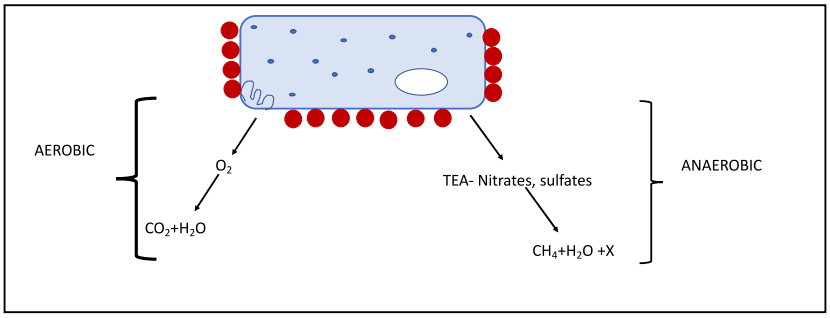

Fig. 4. Aerobic and anaerobic microbial metabolism.

2.4. Bioremediation

Sustainability utilizes the finite resources judiciously to provide for the present as well as future generations. With sustainability gaining importance, green and sustainable remediation approaches are being encouraged to optimize all phases of remediation. Sustainable remediation promotes renewable energy, material recycling, minimizes the waste and energy consumed. The above-mentioned physicochemical approaches do not include the criteria of sustainability (Megharaj and Naidu, 2017). Bioremediation involves the use of biological agents (plants and microbes) for the remediation of explosive contaminated soils. It is a green and sustainable solution for the remediation of such sites. The major drawbacks associated with bioremediation is its low speed of action as well as its limited success in field application.

2.4.1. Phytoremediation

Phytoremediation is an eco-friendly, low cost alternative for the physical and chemical treatment methods. It utilizes the ability of plants to grow in presence of the explosive contaminants, tolerate their toxicity and remediate the soils with the help of plant specific enzymes. Phytoremediation can be based on phytoextraction, phytostabilization, phytodegradation or phytovolatilization (Kalderis et al., 2011). Phytoremediation is an extensively studied field with much available literature on remediation of explosive contaminated soil (Palazzo and Leggett, 1986; Schneider et al., 1996; Burken et al., 2000; Adamia et al., 2006; Vila et al., 2007; Makris et al., 2007; Gandia-Herrero et al., 2008; Brentner et al., 2008; Gunning et al., 2014). Studies on phytoremediation of explosives have been reported using different plant species, viz, Eurasian water milfoil, *Myriophyllum spicatum* and vetiver grass, *Chrysopogon zizanioides* for TNT and reed canary grass, fox sedge, and rice for RDX (Kiiskila et al., 2015). Guinea grass (*Panicum maximum*) was reported to possess ability to phytoremediate RDX and HMX in soils. In the same study it was also observed that addition of a carbon substrate like molasses improved the phytoremediation efficiency (Lamichhane et al., 2012). *Beta vulgaris* have been reported to take up and transform GTN and PETN (Goel et al., 1997). Though this technique has been successfully evaluated for explosive contamination, factors such as its suitability being limited to shallow levels of contamination, selection of plant species, maintenance issues, phytotoxicity of contaminants, slower rate of degradation, unknown effects of the by-products formed during the process and danger of contaminants entering the food chain limits its application for onsite remediation (Hannink et al., 2002).

2.4.2. Microbial remediation

Microbial remediation involves the use of microorganisms for the destruction of explosive contaminated soils. Microbes being ubiquitous in nature, have the ability to survive even in soils highly contaminated with explosives. The microbes having the ability to degrade explosives specifically express different types of enzymes which trigger the use of explosive compound as a substrate (C or N source) for its survival and growth, hence convert the explosive to innocuous form. It is a slow process that depends on the growth rate of the microbe. The microbe involved in the remediation process may be indigenously present or may be introduced into the contaminated site from elsewhere (Chaudhary and Kim, 2019). Microbes degrade the explosive pollutant by either of two ways: aerobic or anaerobic. Fig. 4 gives an overview of both the processes. Fig. 5 represents the general mechanism of microbial remediation of explosives.

Microbial remediation technologies can be classified under two groups: *in situ* and *ex situ* (Chaudhary and Kim, 2019). Table 2 discusses the various advantages and disadvantages of the microbial treatment technologies.

3. *In situ* remediation

These are the technologies that are executed on site and include natural attenuation, bioventing, biostimulation, bioaugmentation and combined biostimulation and augmentation.

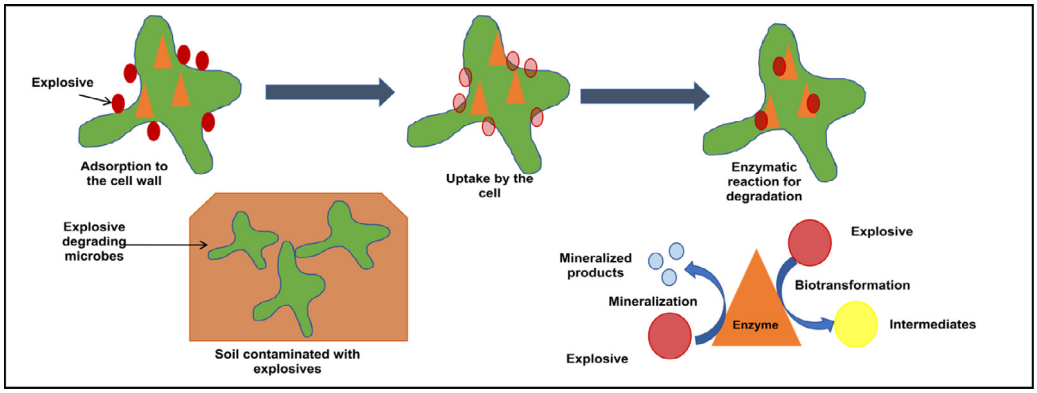

Fig. 5. Mechanism of explosive microbial remediation.

3.1. Natural attenuation

Natural attenuation or intrinsic remediation is the use of microorganisms present naturally in the soils contaminated with explosives to remediate the site. These microbes have an inherent capability to utilize the contaminant as a nutrient source for its growth (Muter et al., 2008). USEPA (1999) defines natural attenuation as the use of natural processes to contain and limit the spread of contaminants present in a site. Natural processes can be biological (microbial) or physical as during sorption, volatilization, dilution etc (Mulligan and Yong, 2004). Microbes are the major players during the natural attenuation process. Many factors contribute to the rate of biodegradation, viz, availability of oxygen and other electron acceptors like nitrate, presence of water and minerals, rate of aerobic and anaerobic degradation (Mulligan and Yong, 2004). Many explosive contaminated sites have been attempted to be remediated by natural attenuation. Kundu et al. in 2016 studied the natural attenuation of 4-nitrotoluene (4-NT), 2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT) in artificially spiked soils. They found degradation of 20%–25%. Mark et al. in 2016, evaluated the natural attenuation potential of insensitive munition compound NTO (3-nitro-1,2,4-triazol-5-one). These studies suggest that since natural attenuation is a slow process, it may be combined with other biological processes like bioaugmentation and biostimulation to increase the degradation efficiency.

3.2. Bioventing

The unsaturated vadose zone soils are characterized by low microbial count and lower catabolic activity. Bioventing is the supply of electron donors to unsaturated soils to aid in biodegradation by the naturally occurring microorganisms. It can be either aerobic or anaerobic. Aerobic bioventing requires the supply of oxygen to the soil using an injection, whereas, anaerobic bioventing requires the addition of other electron donors like nitrogen. The electron donors injected decide the fate of the contaminant. Aerobic bioventing leads to oxidative transformation whereas, anaerobic bioventing leads to reductive transformation (Höhener and Ponsin, 2014; Solid Waste and Emergency Response 5203P, 2006). The first *in situ* bioventing field demonstration was performed at Pantex facility in Texas in 2001 by Rainwater et al. The ability of indigenous microbes to reduce RDX, TNB and TNT was enhanced by injection of nitrogen gas for 333 days. Their results showed increased reductive metabolic activity of the microbes leading to substantial decrease in RDX and TNB concentrations.

3.3. Biostimulation

Remediation of any contaminant in soil generally depends on various factor, viz, nutrients, pH, temperature, moisture content, soil characteristics, available oxygen and the concentration of contaminant (Bundy et al., 2002; Atagana, 2008). Remediation potential of indigenous microorganisms present in the contaminated soil can be enhanced by biostimulation, which involves the addition of limiting nutrients and electron acceptors like nitrogen, phosphorus or carbon to stimulate the growth of the indigenous microbes. Extensive literature survey has suggested that, biostimulation is the majorly used bioremediation technique for remediation of explosive contaminated soils (Boopathy, 2000; Bruns-Nagel et al., 2000; Payne et al., 2013; Xin et al., 2013). Molasses is the most commonly used carbon source for biostimulation. Other common carbon amendments include, cheese whey and waste glycerol. Boopathy (2000), used various carbon compounds as co-substrates during the *in situ* remediation of TNT contaminated soil from the Joliet Army Ammunition Plant. He used, molasses and succinate for biostimulation of soil columns. He observed that, treatment controls without any carbon amendment showed no decrease in TNT concentration and those amended with succinate were not found to be very successful. His results concluded that, molasses and not succinate was a good source of carbon for the indigenous microbes to utilize and breakdown TNT.

Table 2
Microbial remediation technologies (*in situ* and *ex situ*): advantages and disadvantages.

Sl. No.	Technology	Mechanism of action	Advantages	Disadvantages	Examples
1	Biological Natural Attenuation	Explosives reduced by naturally existing microbes that utilize the contaminant as a source to derive energy and for growth.	Ease of use. Lower costs. Can be combined with other techniques.	Longer remediation times. Lack of knowledge on the complex mechanisms involved. Not very efficient technology for highly contaminated sites.	Zhao et al. (2004), Ronen et al. (2008), Monteil-Rivera et al. (2009), Pennington et al. (2001) and Crocker et al. (2005)
2	Bioventing	Supply of electron donors (aerobic or anaerobic) in to the soils in a controlled manner to stimulate the catabolic activities of the indigenous microbes	Cost effective. Enhances microbial activity	Time taking process. Requires specific instrument. Success depends on soil characteristics , permeability and respiration rate	Kuroda (1997), Sagi-Ben et al. (2010), Wijker et al. (2013)
3	Biostimulation	Supply of limiting nutrients like, carbon, nitrogen and phosphorus to the soil.	Cost effective Improves the nutrient content of the soil. Improves the bioavailability of the explosive t the microbial community.	Elongated periods of treatment.	Funk et al. (1993), Boopathy et al. (1997), Drzyzga et al. (1999), O'Niell and Nzengung (2003), Lamichhane et al. (2012), Won and Borden (2016) and Jugnia et al. (2017)
4	Bioaugmentation	Introduction of explosive degrading microbe or consortia into the contaminated soil.	Co-metabolism. Indigenous microbe's activity supported by inoculated microbes. Lesser time duration in comparison to natural attenuation.	Introduced microbes may or may not perform in field conditions. Natural environmental conditions may affect the growth of the introduced microbes.	Van Dillewijn et al. (2007), Muter et al. (2012), Nõlvak et al. (2013), Anasonye et al. (2015), Fournier et al. (2004), Price et al. (2011) and Xu et al. (2019)
5	Bioslurry reactors	Contaminated soil excavated and loaded into a reactor, where its mixed with water to make a slurry. Microorganisms are also added into the reactor for remediation.	Controlled process. Can be run in aerobic, anaerobic or anoxic conditions. Contaminant bioavailability increased.	Higher costs. Need for excavation of soil. Time consuming Laborious.	Guiot et al. (1999), Rocheleau et al. (1999), Shen et al. (2000), Knicker et al. (2001), Weeks et al. (2003), Newcombe and Crawford (2007), Sheibani et al. (2011) and Xin et al. (2013).
6	Composting	Soils are mixed with bulking agents and organic amendments for composting and are arranged in piles or windrows for the thermophilic process to occur. Intermittent mixing is generally provided.	End product is a humus rich substance which can be further used for filling or other purposes.	Large space requirements. Laborious and time consuming. Lack of knowledge of bacteria or fungi involved in the process.	Williams et al. (1992), Griest et al. (1995), Gunderson et al. (1997) and Jarvis et al. (1998).
7	Biopile	Contaminated soils piled in heaps above ground. Temperature, pH and nutrients are also supplied along with forced aeration.	Cost effective. Requires less space.	Soil drying. Forced aeration requires specific set ups	Dubois et al. (1997)
8	Landfarming	Contaminated soils are excavated and spread on lined beds	Low technology footprint, easy, less laborious.	Volatilization may occur, time consuming, leaching of contaminant may occur	Clark and Boopathy (2007)

3.4. Bioaugmentation

Bioaugmentation is the process of inoculating explosive degrading microbes in to the contaminated soil. A single microbe or a consortia can be used for this purpose. The rationale for this technology is that the indigenous microbes

may lack the ability to degrade explosives present or that they are stressed due to the exposure to high concentrations of explosives. Hence, to speed up the remediation, bioaugmentation may be employed to reduce the lag phase of the organisms and in turn reduce explosive concentration. The microorganism introduced during bioaugmentation can be isolated from the contaminated site, or historical site, or isolated by careful screening or genetically modified to support the remediation of explosive in soil (Adams et al., 2015). Labidi et al. in 2001, used *Rhizobium trifolii* isolated from atrazine contaminated soil for remediation of TNT contaminated soils. The bacteria was first carefully screened for its explosive degrading ability in liquid medium. The results showed that, bioaugmentation lead to 60% degradation of TNT in 2 days.

3.5. Combination of bioaugmentation and biostimulation

Researchers have also tried the simultaneous application of bioaugmentation and stimulation for better remediation of explosive contaminated soils. Many studies have also been reported to compare the efficiency of these technologies alone as well as in combination (Labidi et al., 2001; Muter et al., 2012). The results have proven that, if the nutrient supplied are not toxic to the microbes, remediation of explosives is more efficient and faster. Nõlvak et al. in 2013 studied the remediation of TNT contaminated soils using combination technology. The soils were inoculated with a consortia predominated by *Pseudomonas* and *Stenotrophomonas* species and amended with molasses and cabbage leaf extracts. the results showed much greater degradation when compared to the bioaugmentation or stimulation alone. Karakaya et al. (2009) studied the bioremediation of a high explosive CL-20 using *Phanerocheate chrysosporium* along with supplementary carbon (glycerol) and nitrogen sources (ammonium sulfate and yeast extract). CL-20 was found to be mineralized by up to 51% during the study. Also, Muter et al. in 2012 established that, bioaugmentation with addition of nutrients like molasses and cabbage leaf extract gave a much better remediation efficiency in highly TNT contaminated soils. Kao et al. in 2016 also suggested the use of biostimulation and bioaugmentation together for the treatment of TNT and PETN contaminated soil as bioaugmentation was found to be unsuccessful.

4. *Ex situ* bioremediation

These technologies involve excavation of the contaminated soil followed by its treatment and then replacement of the treated soil. The various technologies include, bioslurry reactors, composting, biopile and landfarming.

4.1. Bioslurry reactors

Bioslurry reactors are highly engineered systems for treatment of explosive contaminated soils. They are extremely powerful systems which have been successfully implemented for remediation of soil contaminated with explosives in feasibility studies (Boopathy et al., 1998; Fuller et al., 2003; Park et al., 2003; Boopathy, 2005) as well as field scale studies (Manning et al., 1996). In a bioslurry reactor, soil is excavated, pretreated and loaded into the reactor, where it is mixed with water or wastewater to maintain a uniform consistency. The process can by aerobic, anoxic or anaerobic. It can be operated in batch, semi continuous or continuous modes. Pretreatment is an important step for better performance of the system. This involves crushing and screening for finer textures (Tomei and Daugulis, 2012). Slurry bioreactors also find application in determining the actual potential of a biological strategy for remediation of contaminated site (Robles-González et al., 2008). Boopathy in 2001, studied the remediation of HMX contaminated soil using a batch slurry reactor, with the addition of molasses as a carbon substrate for the native microorganisms to degrade HMX. The results showed 97% degradation in HMX concentration in 4 months. Zhang et al. in 2001 performed a pilot scale test using a slurry reactor for the remediation of 2,4-DNT and 2,6-DNT contaminated soil. They emphasized on soil washing to obtain soil fines contaminated with explosives. Aerobic slurries were maintained for a period of 3 months.

4.2. Composting

Composting is the process of conversion of organic matter into humus like substances which are non-toxic. The process involves mixing of the soil with bulking agents (wood chips, hay, straw) and organic amendments (cattle manure, vegetable waste). The resulting mixture of the soil and amendments can be spread in the form of piles or windrows. The role of the bulking agent is to provide porosity, aeration and nutrition, whereas, the organic amendment helps in maintaining an optimum carbon to nitrogen ratio to promote biological activity. Another important feature of composting is the elevated temperatures that help in the catabolic activities of the microorganisms, to degrade the explosive compound (Chaudhary and Kim, 2019). Many sites worldwide have been remediated by this technology. A few of the examples include, the Pueblo Chemical Depot contaminated with TNT, DNT and RDX, was treated using composting, the explosives were found to be degraded in a time period of 15–30 days. Windrow composting was performed at Umatilla Army Depot to treat TNT, RDX and HMX. After 40 days, the explosive concentrations were below the clean-up goals (Weston, 1993). Hawthorne Army Depot also employed composting for remediation of TNT, RDX, HMX and ammonium picrate. The duration of 28 days was found to be very effective to reach the clean-up goals (Williams et al., 1992; Goetz and Brenner, 2002).

4.3. Biopile

Biopiles involves the heaping of excavated contaminated soil on a liner above the ground and mixing with suitable amendments (nutrients, chemical for pH adjustments, bulking agents). It also requires the use of forced aeration for tilling and reducing the space requirements. The goal of this treatment is to convert the explosive contaminants into harmless products, making the soil safe for disposal and other uses. The heaps of contaminated soil in the form of free standing piles are called biopiles, whereas, those supported by walls or sides are called as biocells. Pueblo Chemical Depot set up biopile installation for treatment of TNT contaminated soils. TNT reduced from 3800 mg/kg to 10 mg/kg in 50 days. A biopile was also successfully used at Joliet Army Ammo Plant for treatment of TNT (3000 mg/kg) and tetryl (7500 mg/kg) contaminated soil. In a duration of 4 months the TNT and tetryl reduced to 50 and 250 mg/kg respectively. In another biopile installation at Yorktown Naval Weapons Station, TNT, RDX and HMX were targeted. In a duration of 3 months, TNT reduced from 1329 to 2.9 ppm, RDX reduced from 319 ppm to 13.5 ppm whereas, HMX reduced from 98 ppm to not detectable range (Goetz and Brenner, 2002).

4.4. Land farming

Land farming involves the excavation of soil to be treated and spreading into high-density polyethylene (HDPE) lined beds. These are periodically tilled for better aeration and mixing (Tomei and Daugulis, 2012). Recently, the integration with other biological approaches, viz, bioaugmentation and biostimulation have improved the functioning of landfarming (Jeong et al., 2015). Clark and Boopathy in 2007, evaluated the efficacy of landfarming for the remediation of explosive contaminated soil from Louisiana Army Ammunition Plant. They targeted the explosives, TNT, RDX and HMX. 82% of TNT was reduced in 182 days, whereas, RDX and HMX were not reduced to much extent which was attributed to the complexity of the molecules.

5. Life cycle assessment (LCA)

Remediation of any site definitely leads to a cleaner environment, but it can have negative impacts on the environment generally at local or global scales. LCA is useful to evaluate all the environmental aspects of any treatment technique, product or services (Zhao et al., 2017). Site remediation, comes with some negative environmental impacts, viz, global warming, depletion of natural resources, noise and other forms of environmental pollution. LCA considers all the negative effects of a treatment strategy, hence helping in deciding how to treat a site for total health of the environment (Suèr et al., 2004). The major aspects considered for LCA of any technique is the space of treatment, time span and secondary processes taking place during the treatment (Bender et al., 1998; Diamond et al., 1999). Table 3 provides the LCA for various *ex situ* and *in situ* microbial treatment technologies applied for remediation of explosive contaminated soils.

Weighing the pros and cons of various microbial remediation treatment strategies and also considering the life cycle as well as their impact on the environment helps in making the choice of ideal remediation strategy. Such a strategy needs to reach the remediation goal as well as be eco-friendly. As Tables 2 and 3 elaborates the above parameters, we can conclude that, *in situ* microbial remediation can provide wholesome results for remediation of explosives contaminated soil. Specifically, combined process of bioaugmentation and biostimulation can help reach the remediation goal faster with minimal impact on the environment. It not only is cost effective, faster, simpler but also cuts down the need for any type of soil transportation and replacement, making it an ideal choice for remediation. Though the field application of such a treatment strategy is yet to be implemented, we feel that, at the field level the remediation goal would be easily reached as the native microbial population of contaminated sites would complement the microbial species introduced. Also the biostimulation will help the microbes in survivability in the harsh environmental conditions.

6. Emerging trends for the microbial remediation of explosive contaminated soil

As discussed above, extensive studies have been carried out to evaluate the remediation potential of microbes for explosive contaminated soils. The past decade (2009–2019) has seen some new innovative approaches applied in microbial remediation. This section discusses the various emerging trends that can be further optimized and used in full scale for remediation purposes.

6.1. Biosurfactant mediated microbial remediation

Surface active compounds or surfactants are generally used for increasing the bioavailability of compounds that are not easily accessible to the microbes. Explosives, due to their low solubility, generally have a low bioavailability. Hence, their remediation is a major task when opting for biological agents. Bina et al. in 2018, used rhamnolipid, a biosurfactant as an amendment to the soil for treatment of TNT. They used soil spiked with 1000 mg/kg of TNT for the study. Rhamnolipid was added at a rate of 60 mg/kg to the soil. They found that, those soils amended with rhamnolipid showed a 73% decrease in TNT, whereas, those without any amendments showed a 58% degradation in TNT in a span of 154 days. Karami et al. in 2017 studied the aerobic bioremediation of soils contaminated with a mixture of TNT and PETN. The microbial inoculum

Table 3
Life cycle assessment (LCA) for microbial treatment technologies.

Sl No.	Technology	Negative environmental impact	Positive environmental impact	Models	Reference
1	Natural attenuation	• Time span of treatment • Low treatment efficacy	• No energy consumption	LCI using Matlab and Excel	Suèr et al. (2004) and Cadotte et al. (2007)
2	Biostimulation Bioaugmentation And combined processes	• Use of finite resources • Land use • Soil quality • Chemical migration	• No transportation required	CML Method Ecoinvent database	Diamond et al. (1999) and Zhao et al. (2019)
3	Bioslurry reactors	• Off-site soil transport. • High energy consumption. • Source of electricity used could also contribute towards the environmental impact	• High cleaning effect	USES-LCA Nordic guidelines on LCA	Ribbenhed et al. (2002)
4	Composting	• Excavation of soil • Vessel manufacture for remediation process (in case of in-vessel)/ Land use (in case of windrow or pile form) • Gaseous emissions • Leachate disposal/ leakage	• Mature compost that can replace chemical fertilizers	Life cycle inventory (LCI) Sima Pro 7 software	Blengini (2008)
5	Biopile	• Excavation of soil • Considerable consumption of energy • Soil preparation • Barrier manufacture (in case of biocells)	• Re use of soil	LCI using Matlab and Excel	Inoue and Katayama (2007) and Cadotte et al. (2007)
6	Land farming	• Aquatic ecotoxicity • Terrestrial ecotoxicity • Aquatic eutrophication	• Comparatively low energy consumption	Sima Pro	Segovia et al. (2019) Besalatpour et al. (2011)

used was obtained from activated sludge of textile wastewater treatment plant. Addition of rhamnolipids increased the degradation of TNT and PETN from 53 and 57% to 98 and 91% respectively at the end of 22 weeks. Sadani et al. (2017) also used rhamnolipid as a biosurfactant to increase the bioremediation of soils contaminated with PETN. They used a combination of anaerobic and aerobic treatments for the study. Anaerobic treatment for 80 days showed 74% degradation of PETN and the subsequent aeration for 30 days allowed the removal of rest of the PETN present in the soil which resulted in 98% removal of PETN. Amin et al. in 2017, evaluated the effect of monorhamnolipid surfactant on the natural (anaerobic) attenuation of soils contaminated with TNT and PETN. They observed a major increase in the degradation of both the explosives. These studies observed that, biosurfactants like rhamnolipid helped the microbes (present naturally or external cultures) to use the nitroaromatic compounds as sole source of nitrogen, thereby, proving this strategy to be a feasible *in situ* remediation option.

6.2. Integrated treatment technologies

Combining microbial technologies with other physical, chemical or biological treatment technologies, aids in faster and efficient removal of explosives from soil. Such coupled reactions do not rely on single type of reactions, but instead, on set of reactions that occur simultaneously or in series for the same result.

6.2.1. Chemical–biological processes

Madeira et al. in 2019 combined chemical and microbial treatment for remediation of DNAN, DNT and NTO contaminated soils. They used bioreduction as a pretreatment to *In situ* chemical oxidation (ISCO). The soil microorganisms could reduce nitro groups of the explosives to amino groups. These reduced intermediates were found to be easily oxidized by potassium permanganate. Another chemical–biological coupled reaction involves combined iron and ferric reducing bacteria like *Shewanella oneidensis*, *Clostridium geopurificans*, *Geobacter metallireducens* for treatment of explosive contaminated soils. Degradation occurs by the following two strategies:

- Secondary chemical reactions stimulated by microorganism: Ferric reducing microorganisms produce ferrous iron which can directly reduce the explosive compound (Kwon et al., 2011; Perreault et al., 2012; Niedźwiecka et al.,

2017). Such a treatment strategy have many advantages over direct microbial remediation, like, the microbes do not need to have specialized enzymes to break down explosives, also, high concentrations of explosives do not exhibit an inhibitory effect of microbes.

- Chemical reactions with secondary biological reactions: chemical reactions rapidly decrease a large part of the explosive contaminant, making it a feasible environment for biological reactions to take over for long term attenuation (Kwon and Finneran, 2010).

6.2.2. Biological–biological:

Another important development in the field of bioremediation of explosive contaminated soil is the use of nitrogen fixing bacterium in combination with rhizoremediation. Generally, nitrogen limiting conditions, lead to aerobic degradation of the nitrogen containing explosive (Thompson et al., 2005). So, nitrogen fixing bacteria present can assimilate the nitrogen and aid in the growth of plants and in turn help in remediation. Khan et al. in 2015, used a novel nitrogen fixing consortium with an ability to degrade RDX with addition of starch as a co-substrate. They suggested that almost complete mineralization of RDX had occurred with the formation of formate, nitrite, nitrate and ammonia without accumulation of any secondary metabolites. They found *Rhizobium, Rhizobacter* and *Terrimonas* as the major players involved in the biodegradation process.

Integrating aerobic and anaerobic microbial remediation process for treatment of explosives like NTO is also catching up in the past decade. Aerobic remediation of NTO in soil by the naturally occurring bacteria leads to production of intermediates like 3- hydroxylamino-1,2,4-triazol-5-one (HTO) and 3-amino-1,2,4-triazol-5-one (ATO). ATO requires anaerobic conditions to then get mineralized to nitrate and nitrite (Krzmarzick et al., 2015).

Lamichhane et al. in 2012 used integrated treatment of biostimulation with phytoremediation for treatment of explosive contaminated Hawaiian soils. They studied the use of Guinea Grass (*Panicum maximum*) for remediation of RDX and HMX. Their results concluded that, biostimulation of site with molasses in integration with phytoremediation gave enhanced reduction rates, there by declaring this method as a potential strategy to remediate highly contaminated explosive sites.

6.3. Immobilized enzymes

Enzymes are biological catalyst, that lower the activation energy and help in the breakdown of substrates (in this case, explosives). Due to their smaller sizes in comparison to microbial cells, enzymes have better contact with the pollutant, mobility and facilitate faster and effective degradation of pollutants (Rao et al., 2010). Immobilization of enzymes for better delivery into the contaminated environment is an emerging strategy for remediation. Researchers have used this technique for delivery and remediation of explosive contaminated aqueous media (Wang et al., 2010; Zhang et al., 2015), but it has not been much explored in case of soil bioremediation. Karthikeyan et al. in 2016, immobilized a novel ether hydrolase enzyme, DNAN demethylase for remediation of the insensitive explosive, DNAN. The enzyme was encapsulated in biogenic silica for being used in bioreactors.

6.4. Novel approaches

The last decade has also seen many novel approaches being used for bioremediation of explosive contaminated soils. One such technique involves the incorporation of explosive degrading microbe in the explosive formulation itself, to help breakdown unexploded compounds. Nyanhongo et al. in 2009 incorporated *Pseudomonas putida* GG04 and *Bacillus* SF, known TNT degraders into TNT formulations to aid in bioremediation of TNT residues or unexploded explosives. They observed that, the presence of these microbes did not affect the properties of the explosive formulations. In another novel approach, Erkelens et al. in 2012, used previously bioremediated hydrocarbon contaminated soil (PBR) as an amendment for bioremediation of TNT chips. They found that, PBR had higher metabolic rates and nitroreductase genes. PBR showed a degradation efficiency of 70%. This strategy promotes sustainable waste management. Since, treated soil is being re used for treatment of another compound, it helped in increasing the life span of landfill sites. In yet another study, Avila-Arias et al. (2017), found that, amendment of cultures with a supplementary carbon source helped in better transformation of TNT. So, they isolated two bacteria, viz, *Achromobacter spanius S17* and *Pseudomonas veronii S94* which were biosurfactant producers. These organisms showed a high potential for TNT transformation. Their high efficiency to transform TNT even at high concentrations was attributed to the biosurfactant produced. Hence, isolation and application of microbes capable of producing surfactants can help in better remediation of explosive contaminated soils.

6.5. Genetic bioremediation of explosive compounds

Microbial gene locus responsible for their ability to remediate explosive compound is highly conserved. Hence, specific microorganisms exhibit this property. Many naturally occurring microbes lack the specific gene function, hence are not degraders of explosives. Many studies have been conducted to impart or enhance the degradation ability of microbes using tools of genetic engineering. Lee et al. in 2009, compared the activity of wild strain of *Stenotrophomonas maltophilia* OK-5 with genome shuffled strain *S. maltophilia* OK-5 mt-3. The wild strain was found to degrade 0.2 mM TNT in 6 days whereas

it could not tolerate a high concentration of 0.5 mM. The mutant strain on the other hand, could easily degrades 0.5 mM in 8 days and 1.2 mM in 24 days. They observed over expression of certain proteins responsible for TNT degradation in the genome shuffled strain, making this technology a promising candidate to increase the efficiency of explosive degrading microbes. Jung et al. in 2011, performed horizontal gene transfer (HGT) to transfer plasmid (exhibiting the conserved locus xplAB) pGKT2 from *Gordonia sp.* KTR9 to *Gordonia polyisoprenivorans, Rhodococcus jostii RHA1* and *Nocardia sp. TW2.* They observed a successful transfer of plasmid as well as RDX degradation capacity, making the organisms potential RDX degraders. Jung et al. (2019), studied the effect of the transconjugates developed (as mentioned previously) in RDX degradation in flow through column set ups. They concluded that the use of bacteria with transferable traits could lead to disseminating RDX degrading ability to native organisms thereby, enhancing bioremediation.

7. Future prospects

It is clear from all the research data, that bioremediation techniques are diverse and effective in successful treatment of explosive contaminated sites. A lot of potential still lies in this field that can be explored further for better efficacy and application of microbial remediation technology on site. Molecular techniques like 'Omics' (genomics, proteomics, metabolomics and transcriptomics) are crucial for better understanding of microbial identification, metabolism of explosives and genetic potential for the remediation purpose. More interestingly, integrative omics approaches can help in analyzing the population at a contaminated site. Metagenomics, metatranscriptomics, metaproteomics and metametabolomics address the whole of DNA, RNA, proteins and metabolites respectively in the environment. These techniques can help harvest the potential of the unculturable microorganisms for remediation of explosive contaminated soils (Bell et al., 2014; El Armani et al. 2015; Malla et al., 2018).

7.1. Immobilized bacteria for better delivery to soil

As discussed earlier, bioaugmentation is a successful technique for microbial remediation. But, the major drawback associated with it lies in the delivery of microbes to the contaminated soil. Even though immobilized enzymes have been utilized for remediation of DNAN contaminated soils (Karthikeyan et al., 2016), there is a need to use immobilized systems for delivery of microbes to soil. The explosive degrader could be immobilized onto a natural eco-friendly carrier for better delivery and survival of the microbe in the contaminated environment, increasing the efficiency of bioaugmentation process (Wang et al., 2019; Zhang et al., 2019).

7.2. Microbial fuel cells

Microbial fuel cells (MFC's) for the remediation of explosive contaminated water has been already researched (Liu et al., 2013; Xie et al., 2018). These can also be applied to soil remediation to derive a dual benefit of electricity generation and detoxification of the contaminated site, as in remediation of pesticides (Cao et al., 2015; Zhang et al., 2014) and petroleum hydrocarbons (Wang et al., 2011; Lu et al., 2014; Li et al., 2015). Yu et al. in 2017, remediated PAH contaminated soils by employing soil MFC's. They observed that by decreasing the electrode distance, the degradation as well as electricity generated increased. They also observed that, PAH's degraded based on their bioavailability and the electric stimulation led to the growth of microbes other than electrigens. Soil MFC's have advantages like simple configuration and set up, consumption of low energy, does not disturb the soil profile and lesser impact on the native soil microorganisms (Cao et al., 2015). These advantages make them a suitable candidate for application in explosive contaminated soil remediation.

7.3. Electrokinetic bioremediation

The major drawback of microbial remediation is its low speed, which can be due to low bioavailability or interaction of the explosive compound to the bacteria. This can also be overcome by applying non-uniform electric current. Application of electric current on the soil matrix leads to various transport mechanisms, viz, electroosmosis, electrophoresis, dielectrophoresis and electromigration. These processes cause movement of bacterial cells, organic molecules, nutrients and pore fluids in the matrix (Luo et al., 2006). This approach of leads to the enhancement of *in situ* bioremediation by increasing the mass transfer and interaction of the bacterial cells with organic compounds and nutrients. Such an integrated approach is termed as bio-electrokinetic remediation. This technique has been applied for remediation of many organic pollutants (Luo et al., 2006; Hassan et al., 2019; Wang et al., 2016). Application of this strategy could accelerate the *in situ* bioremediation of explosive contaminated sites.

7.4. Nanobioremediation

Nanobioremediation is another integrated remediation approach that can help in faster and efficient remediation of explosive compounds. It applies the principles of nanotechnology and bioremediation. Nanomaterials offer higher reactivity with pollutant, larger contact surfaces and better disposal capability. This remediation technique has been applied for organic contaminants, as demonstrated by Singh et al. (2013) and Han et al. (2017). Another aspect of this technology encompasses immobilization of enzymes on nanomaterials. Nanoenzymes have been reported for remediation of soils contaminated with aromatic hydrocarbons (Acevedo et al., 2010; Dai et al., 2011; O'Driscoll et al., 2013). Concerns over environmental safety of nanomaterials may limit its application and acceptance for remediation. Studies have shown that, nanoscale materials may undergo agglomeration leading to non-uniform distribution in subsurface soils (Tratnyek and Johnson, 2006; Phenrat et al., 2007). This self-aggregation may also lead to the entry of the nanomaterial into the food chain (Boxall et al., 2007). Another major concern lies in its safety to environment and living organisms. Kreyling et al. in 2006, suggested the bioaccumulation of nanomaterials in environmentally relevant species. Also, there have been reports on effects of nanomaterials on microbial communities (Klaine et al., 2008).

7.5. Clay minerals and natural composites

Natural clay minerals with or without modification hold great potential for bioremediation of soils. Natural clays are easily available, cheap, environmentally stable, highly adsorptive and exhibits ion exchange properties. The application of clays in treatment has been reported for various pollutants, viz, phenols, hydrocarbons, pesticides, dyes, herbicides, fungicides, metals etc. Few examples of commonly used clay minerals include, montmorillonite, vermiculite, kaolinite, bentonite, sepiolite etc. Biswas et al. (2015a). Clay minerals act as support and provide a protective environment for the growth of microbes. A new remediation approach of combining the adsorption of pollutant by the clays followed by their biotransformation using microbes has been explored recently (Sarkar et al., 2012). Much work has been reported in remediation of PAH and VOC based on clay–microbe interaction. (Singh et al., 2003; Froehner et al., 2009; Chen et al., 2009; Warr et al., 2009; Quintelas et al., 2013; Biswas et al., 2015b). This technique can find application in remediation of explosive contaminated sites.

8. Conclusion

Though many established abiotic technologies (physical and chemical) are available, bioremediation has emerged as an ecofriendly alternative. Successful implementation of bioremediation at a particular site depends majorly on the physicochemical characterization and type of pollutant. This helps in deciding the type of technique to be applied (*ex situ* or *in situ*) for successful decontamination. *Ex situ* technologies are mainly associated with excavation and replacement, thereby making them energy intensive as well as expensive processes. On the other hand, *in situ* technologies suffer from the drawbacks of being slow, involve uncertainty of the conditions under the soil surface. It has become imperative to adopt technologies that are not only environment friendly, but also, cost effective with high efficiencies that can be transformed into the field. With many emerging technologies in place, a plethora of options have opened up to clean up explosive contaminated soils. Integrated treatment technologies seem to be the most promising one, with bringing in the best of two different approaches (chemical/biological). Research on developing and optimizing microbial remediation technologies for field scale application is the need of the hour.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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ORIGINAL RESEARCH PAPER

Aerobic biodegradation of high explosive hexahydro-1,3,5-trinitro-1,3,5-triazine by *Janibacter cremeus* isolated from contaminated soil

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Abstract
Objective To evaluate the ability of *Janibacter cremeus* a soil bacterium isolated from explosive contaminated site in degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and to study enzyme responsible for degradation.
Results The isolate exhibited 88% degradation of RDX in 30 days of incubation. The biodegradation process followed the first order kinetics. The half- life of RDX was calculated to be 11.088 days. The RDX degradation process was complemented by concomitant release of nitrite ions with 0.78 mol of nitrite released per mole of RDX. The metabolites; Trinitroso- RDX, diamino-RDX, trimino-RDX, bis- (hydroxymethyl) nitramine and methylenedintramine derivative, viz, methylene- *N*- (hydroxy- methyl)-hydroxylamine- *N*-(hydroxymethyl) nitroamine corresponding to the molecular weights 174, 162, 132, 122 and 167 Da respectively were also detected. Nitroreductase enzyme was found to be responsible for RDX degradation.
Conclusion *J. cremeus* could degrade RDX as sole source of nitrogen, via three different pathways wherein, Nitroreductase enzyme was found to play a major role. The efficient degradation of RDX makes *J. cremeus* suitable in treatment of contaminated water and soil at field scale levels.

Keywords Bioremediation · Degradation pathway · Explosives · Nitroreductase enzyme

List of symbols
ln Natural logarithm
k Rate constant

Introduction

Explosives are toxic compounds predominantly found in the military areas. High explosive, Hexahydro-1,3,5- trinitro-1,3,5-triazine (RDX) is one of the most commonly used military explosive. It's a heterocyclic nitramine compound which finds its way into the soil and water generally through improper disposal of manufacturing and process waste, loading, assembly, packaging (LAP) and testing (Oh et al. 2003). RDX has been classified as a potential human carcinogen by United States Environment Protection Agency (USEPA 2011) based on animal studies. Because of

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its detrimental effects, the treatment of RDX contaminated waters is of utmost importance. Many physical methods such as adsorption, ozonation, exposure to ultraviolet radiation (UV) and chemical methods such as alkaline/ acid hydrolysis, catalyst mediated and advanced oxidation processes have been developed for the treatment of contaminated waters. But, these approaches are expensive, non-specific and can lead to secondary pollution (Singh et al. 2011a, b). Hence, a need for eco-friendly approach to mitigate the toxic effects of RDX has been paved leading to a shift to bioremediation as a possible treatment technology.

Microbial communities present in the contaminated sites (primarily soil, groundwater, aquifer) exhibit the inherent ability to thrive in presence of the contaminant (Cupples 2013). The use of such bacterial communities for remediation purpose provides an advantage to the approach. Many researchers have isolated explosive degraders from contaminated sites and utilized them for the bioremediation experiments. Arnett and Adrian (2009) isolated *Geobacter sp*., *Acetobacterium sp*., *Desulfovibrio sp.*, from an RDX waste water treatment plant. Roh et al. (2009) also isolated bacteria belonging to *Pseudomonas, Enterobacter* and *Azospirillum* species from contaminated groundwater and aquifer. HAW-EB21 was isolated from the marine sediments of the dumpsite of a former ammunition plant (Zhao et al. 2004). Singh et al. (2011a, b) isolated *Bacillus cereus* strain PU isolated from firing range for biodegradation of 2,4,6- trinitrotoluene. Use of such resistant microbes in the bioremediation of explosive is being explored worldwide.

The earliest work in RDX biodegradation cites the use of anaerobic process (McCormick et al. 1981). But, plethora of work since then have indicated the use of aerobic biodegradation process for the mineralization of RDX (Binks et al. 1995; Coleman et al. 1998; Seth-Smith et al. 2008; Fournier et al. 2002). Seth-Smith et al. (2008) elucidated the pathway responsible for aerobic degradation of RDX by *Rhodococcus rhodochrous* strain 11Y and also identified the enzyme responsible for the same. Kitts et al. (2000) studied the involvement of type I (oxygen insensitive) nitroreductases in degradation of RDX, thereby proving that this enzyme could also be responsible for RDX degradation under aerobic conditions.

Janibacter cremeus was first isolated by Hamada et al. (2013) from the foreshore of an island in Japan. It is one of the ten species under the genus *Janibacter*. *The other species includes, Janibacter limosus, Janibacter terrae, Janibacter brevis, Janibacter anopheles*, *Janibacter melonis, Janibacter corallicola*, *Janibacter hoylei, Janibacter alkaliphilus*, *Janibacter indicus.* Not much work has been reported so far in respect to degradation of hazardous compounds. Khessairi et al. (2014), used *Janibacter sp*. strain FAS23, in remediation of Pentachlorophenol. *Janibacter cremeus,* on the other hand hasn't been explored for its remediation capacity for any xenobiotics. This research was undertaken to evaluate the native microbe *Janibacter cremeus* in remediation of RDX under aerobic conditions. We, for the first time explore the capability of *Janibacter* sp. in remediation of RDX, thereby paving a path for its future applications in field scale remediation.

Material and methods

Chemicals and media components

RDX (> 99% purity) was provided by an RDX manufacturing facility in India. All other chemicals used throughout this study were of analytical and gradient grade and were obtained from standard manufacturers.

The primary cultures of *Janibacter cremeus* were maintained on Tryptic Soy Agar (TSA) plates. During the aqueous phase study the culture of *J. cremeus* was grown and maintained in Minimal Salt Medium (MSM) (media compositions have been detailed in Online Resource 1).

Microbial culture characterization and growth conditions

The organism *Janibacter cremeus*, was isolated from explosive contaminated soil and the lyophilized culture of the same (MTCC NO. 12869) was obtained from Institute of Microbial Technology, (IMTECH), Chandigarh, India. The culture was revived on TSA plates and were then sub cultured for three generations in MSM. The growth of the culture was monitored by observing its absorbance at 600 nm using Perkin Elmer UV- Visible spectrophotometer (Model Lambda 650S). The third generation culture acclimatised to growth in minimal medium was grown in MSM without any nitrogen source. The cultures in log

phase with an absorbance of 0.8 at 600 nm was used in the degradation experiments. The bacterium was assessed for its growth in different concentrations of RDX (20, 40 and 60 mg/L) in MSM, where RDX served as sole source of nitrogen. The degradation studies were undertaken at the water solubility limit of RDX (60 mg/L).

Aqueous phase RDX degradation experiments

The bacteria, *J. cremeus,* in its log phase was inoculated in the minimal medium (MSM) spiked with 60 mg/L of RDX (sole nitrogen source) under sterile conditions. The bacterium was inoculated at 5% (v/v) in the medium containing RDX. Two control experiments were also set up, viz, MSM (without any nitrogen source) with the bacterial inoculation and MSM with RDX as nitrogen source but without inoculation. The study was conducted for a period of 30 days under sterile conditions on an incubator shaker at 35 °C and 120 rpm. The samples were withdrawn on regular intervals to monitor the degradation capacity of the selected bacteria. The growth of the bacteria was monitored at 600 nm using UV–Visible spectrophotometer.

Monitoring the RDX degradation efficiency

The RDX degradation efficiency of the microbe was monitored by measuring the residual RDX concentrations in the treated samples. The residual RDX concentration was measured by USEPA 8330A (EPA 2006) method using High Performance Liquid Chromatography (HPLC). Briefly, 2 mL aliquot of samples withdrawn were centrifuged to discard the cellular content. The supernatant was then extracted for RDX by addition of equal volume of acetonitrile. The prepared sample was then filtered through 0.45 µm Teflon filters (Millex filters). Analysis of RDX was carried out using Dionex model HPLC (Thermo Fisher make). C18 column, (3 µm, 150 × 4.6 mm) functioned as the stationary phase. The mobile phase was a mixture of acetonitrile and water (50:50 v/v). Separation was performed at a flow rate of 1 mL/min. RDX was detected at 254 nm.

Analysis of metabolites

Nitrite, an important metabolite in the degradation of RDX was analysed spectrophotometrically at 540 nm (Mercimek et al. 2013) briefly described in online resource 2. 1 mL of aliquot was centrifuged at $9500\times g$ for 10 min to obtain cell free supernatant. The supernatant was used for the analysis of nitrite.

Mass spectrometric (MS) analysis of the acetonitrile extracted sample was performed using Synapt G2 (Waters ACQUITY QSM) MS system in positive ion mode (ES +). RDX and its degradation products were separated on an Acquity UPLC® BEH column (C18, 1.7 µm) at 0.75 mL/min flow rate over a period of 20 min.

Results and discussion

Janibacter cremeus was tested for its efficiency to degrade RDX in aqueous medium so as to predict its application in further studies for treatment of actual contaminated sites. The bacteria was inoculated in MSM containing RDX as lone nitrogen source and glycerol, glucose and succinate as carbon sources. The studies were carried out at 35 °C at 120 rpm in an orbital incubator shaker.

Growth of *J. cremeus* on RDX

Janibacter cremeus was assessed for its growth in different concentrations of RDX after it was acclimatised to MSM for three generations. Figure 1 compares the growth of the isolate in different concentrations of RDX (20, 40 and 60 mg/L) in MSM. The increasing concentration of RDX lead to a comparative lower growth of the bacterium. Maximum absorbance at 600 nm was recorded at day 5. The cultures at 20 mg/L showed maximum growth of 1.426, followed by 1.327 at 40 mg/L and lastly 1.241 at 60 mg/L concentration. The degradation efficacy of the bacteria was monitored at 60 mg/L of RDX. *J. cremeus* was found to grow well even in the control bottles without any nitrogen source. Figure 2 compares the growth of the bacteria in presence and absence of RDX (60 mg/L). As can be seen, the cells of the bacterium follow a sigmoid growth pattern in both conditions. The bacteria is characterized by a short lag phase of 2 days. The cells reach maximum

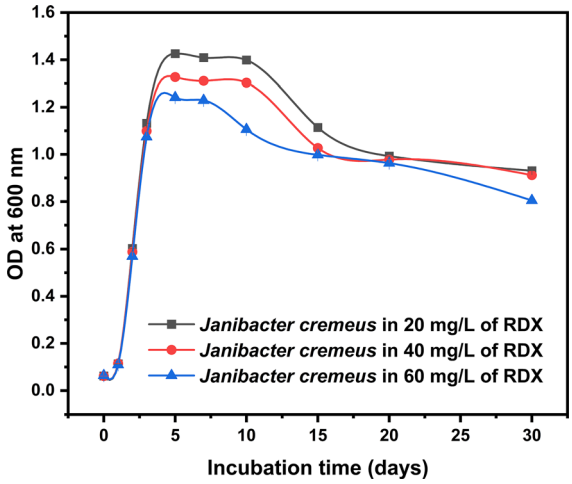


Fig. 1 Comparison of growth of *J. cremeus* in different concentrations of RDX. Error bars denote standard deviation of mean (n = 3) at a given time

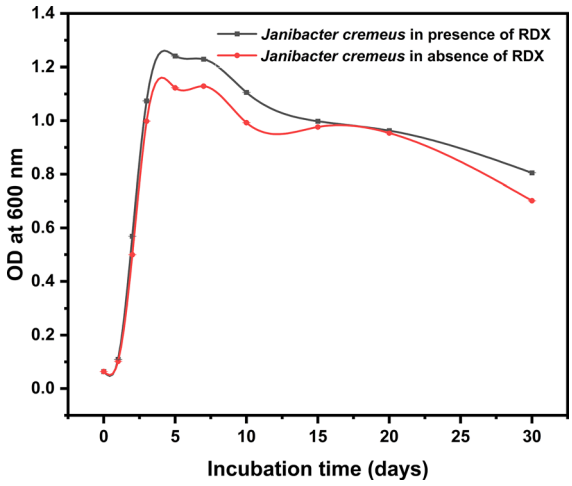


Fig. 2 Comparison of growth of *J. cremeus* in presence and absence of RDX. Error bars denote standard deviation of mean (n = 3) at a given time

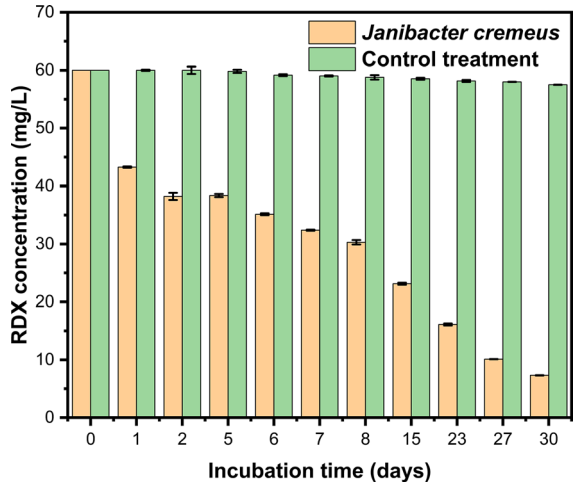


Fig. 3 RDX degradation in treatments with and without *J. cremeus*. Error bars denote standard deviation of mean (n = 3) at a given time

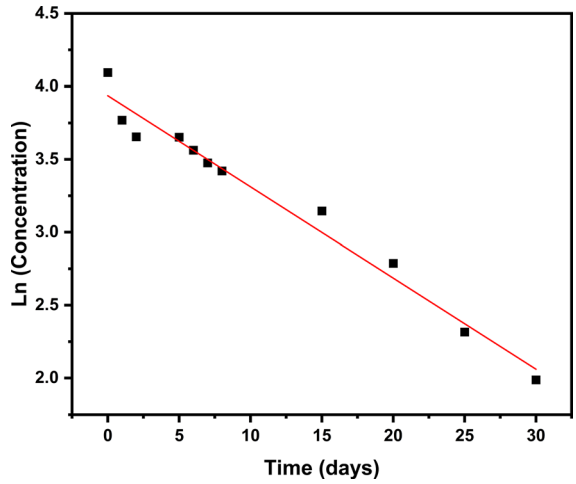


Fig. 4 First- order rate kinetics of RDX degradation by *J. cremeus*

growth of 1.241 and 1.123 for with and without RDX (respectively) on fifth day. The proliferation of *J. cremeus* in presence of RDX proves that, the explosive compound doesn't negatively affect its growth. Growth of the isolate in presence and absence of RDX as well as degradation of the explosive compound later shows that *J. cremeus* utilizes RDX as a non-growth substrate. This proves that, the bacterium degrades RDX via cometabolism (Van Aken et al. 2004; Solyanikova et al. 2011; Nagar et al. 2018).

Degradation of RDX in aqueous phase

RDX degradation efficacy of the bacteria was monitored using HPLC. It was observed that *J. cremeus* could degrade 88% of 60 mg/L of RDX in MSM in 30 days. The controls without any bacterial treatment reported negligible degradation (Fig. 3).

The reaction kinetics for the biodegradation was also calculated to determine the rate of reaction. First order kinetic model as given by Eq. (1), was applied to the residual RDX concentrations (in treatment sets).

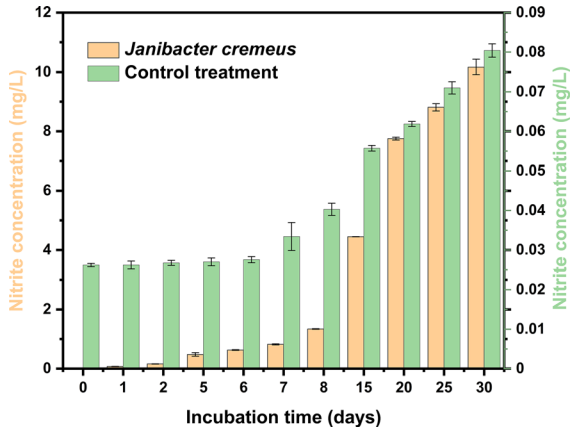


Fig. 5 Nitrite release in treatments with and without *J. cremeus* during RDX degradation. Error bars denote standard deviation of mean (n = 3) at a given time

$$\ln A = -kt + \ln A_0 \quad (1)$$

Equation (1) represents the linear form of the first-order kinetic model, wherein,

A_0 represents initial RDX concentration (mg/L), A is the RDX concentration (mg/L) at time t (days), k is the RDX degradation rate constant. The rate constant for degradation of RDX by *J. cremeus* was obtained from the graph shown in Fig. 4 as 0.0625 day^{-1}. The half-life for RDX degradation was then calculated using the following Eq. (2)

$$t_{\frac{1}{2}} = \frac{0.693}{k} \quad (2)$$

where, $t_{1/2}$ is the half-life of RDX, that is the time required for degradation of RDX by half.

The half-life of RDX on treatment with *J. cremeus* was calculated to be 11.088 days.

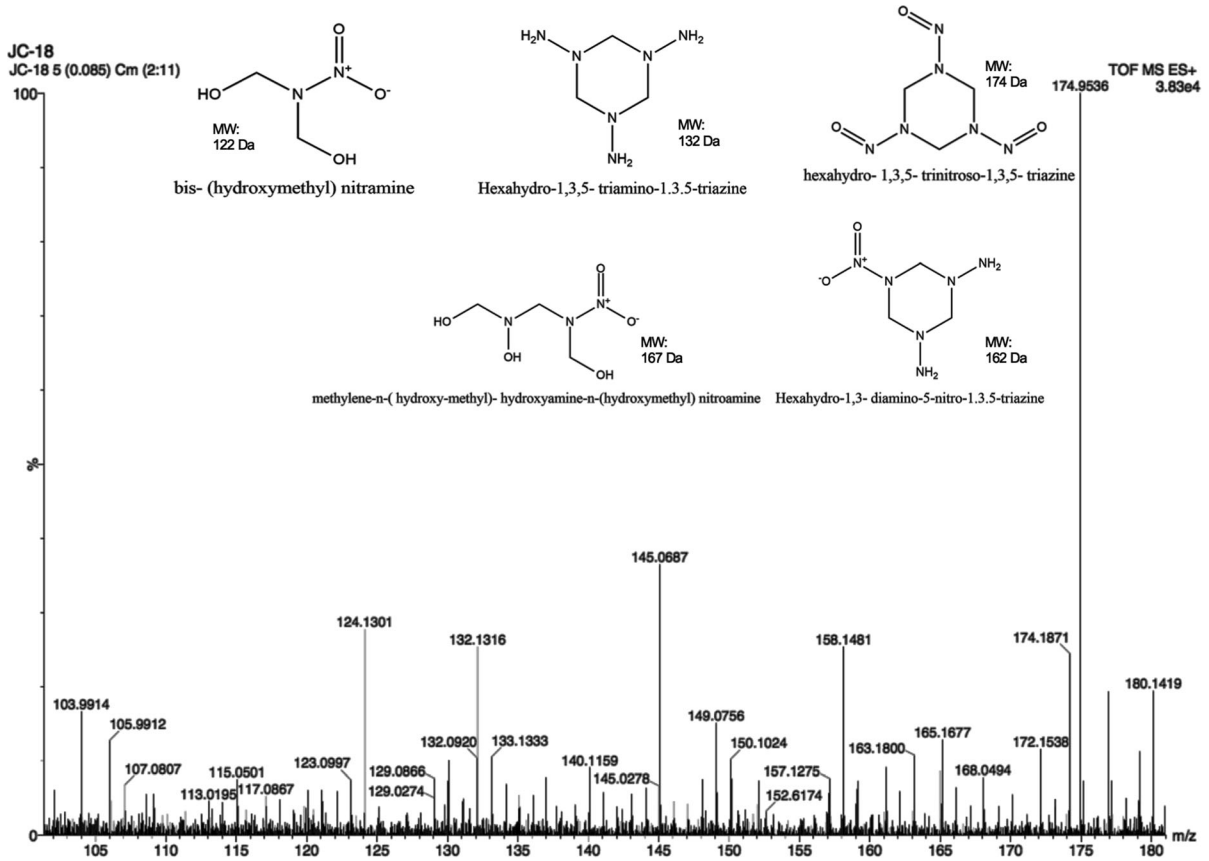


Fig. 6 Mass spectrum showing the intermediates obtained during RDX degradation by *J. cremeus*

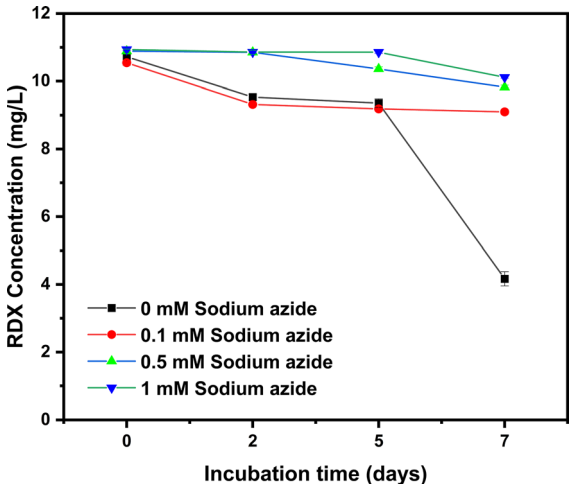


Fig. 7 Effect of increasing concentrations of sodium azide, a specific inhibitor of nitroreductase enzyme on RDX degradation. Error bars denote standard deviation of mean (n = 3) at a given time

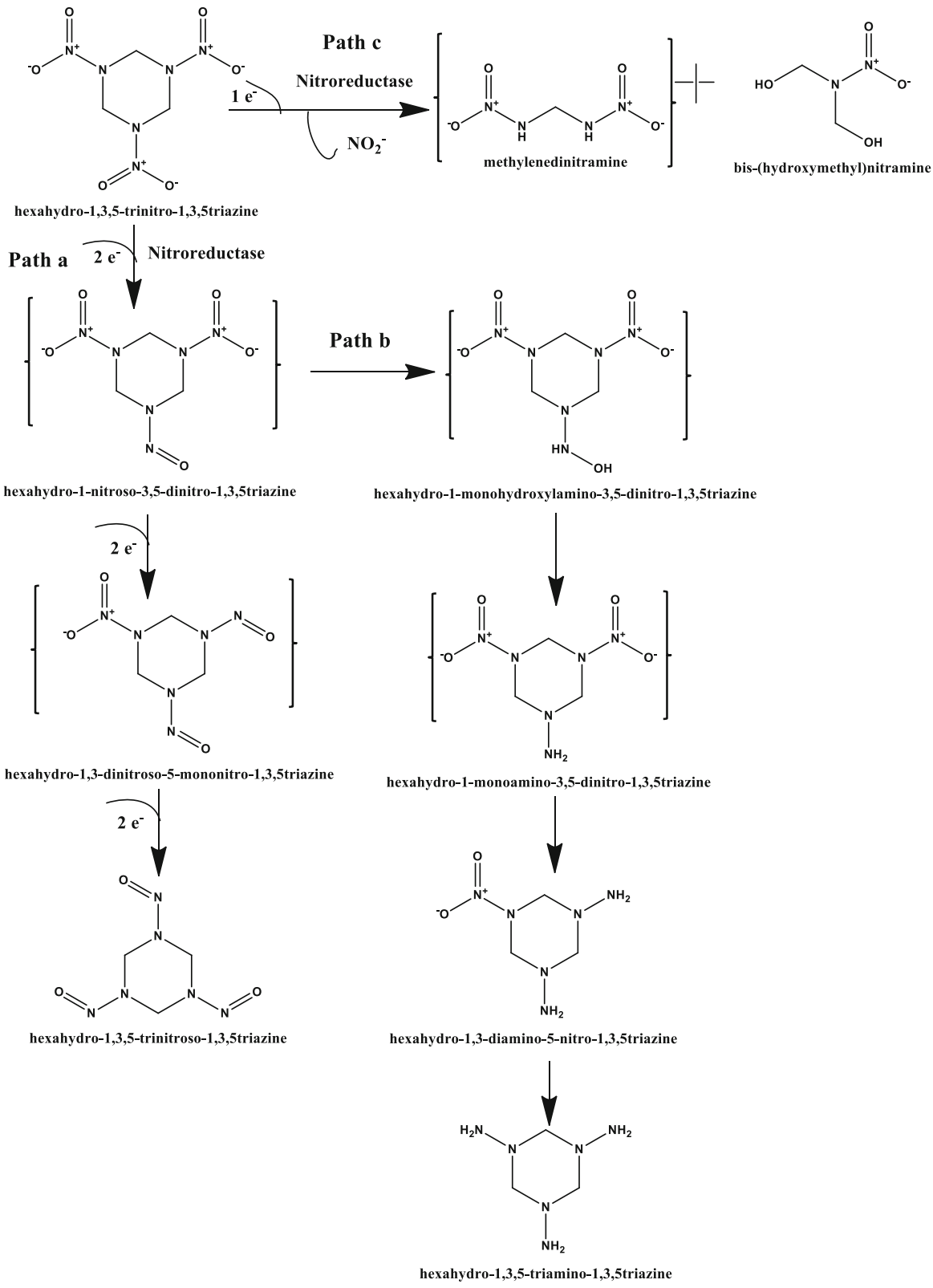
Analysis of metabolites

Nitrite is an important metabolite released during the biodegradation of RDX. It is an important initial step, which involves the removal of nitro groups present in the RDX molecule (Coleman et al. 1998). Figure 5 shows the nitrite released on incubation of RDX with *Janibacter cremeus* and control experiments. As is evident, the control experiments show negligible release of nitrite. The negligible nitrite release could be due to the photodegradation of RDX. Whereas, incubations with *J. cremeus* led to release of 0.78 mol of nitrite for a mole of RDX at the end of 30 days of incubation. The release of nitrite indicates that, RDX degradation in *J. cremeus* follows the single electron transfer/ denitration pathway (Crocker et al. 2006).

To further elucidate the pathway and identify the ring cleavage products of RDX, the samples were subjected to MS, using positive electron spray ionization (ESI). The chromatogram reveals the presence of intermediate with peak at *m/z* of 175 Da (Fig. 6). This fragment indicates the presence of Trinitroso RDX (TNX), which is formed during the double electron reductive pathway as explained by McCormick et al. Though no peaks for Mononitroso and Dinitroso-RDX (MNX and DNX) were observed which could be due to the instability of these metabolites in water (Crocker et al. 2006). But presence of TNX showed that the RDX was transformed to TNX via the mono and dinitroso- intermediates. Bhatt et al. (2006) reported the formation of nitroso derivatives by *Acremonium sp.* HAW-OCF3 under aerobic conditions. Recently, Yadav et al. (2020) also reported the formation of nitroso derivatives during aerobic degradation of RDX using, *Microbacterium esteraromaticum*. Another intermediate metabolite ion peak was obtained at *m/z* of 123 Da, which refers to the presence of bis-(hydroxymethyl) nitramine (BHNA) with a molecular weight of 122 Da. MDNA (methylenedinitramine) derivative, methylene- *N*- (hydroxy- methyl)- hydroxylamine- *N*-(hydroxymethyl) nitroamine, with an empirical formula $C_3H_9N_3O_5$ (molecular weight of 167 Da) was also observed, showing that, MDNA could have been formed by single electron reduction or denitration of RDX, as observed by Binks et al. (1995) in *Stenotrophomonas maltophilia* PB1.The presence of nitrite also proves the denitration step occurring in RDX degradation. Presence of MDNA and BHNA shows that the bacteria also follows the direct enzymatic cleavage of RDX (Hawari et al. 2000). Also, observed were peaks at *m/z* of 163 and 133 Da. These intermediate peaks correspond to presence of Diamino-RDX (162 Da) and Triamino-RDX (132 Da) respectively. A similar RDX degradation pathway was observed by Zhang and Hughes (2003) in *Clostridium acetobutylicum*. The formation of Triamino- RDX leads to no ring cleavage products. Thus the study shows that RDX is degraded by *Janibacter cremeus* by multiple degradation routes.

To observe the role of nitroreductase in RDX degradation, its activity was inhibited using specific inhibitor, sodium azide (Villanueva 1959; Roldán et al. 2008). Sodium azide at different concentrations, viz, 0.1 mM, 0.5 mM, 1 mM, was used to study the effect on RDX degradation in resting cell incubations. *J. cremeus* grown in presence of RDX as sole nitrogen source was harvested in its late log phase and resuspended in RDX at 10 mg/L concentration. The RDX degradation was monitored using HPLC. Figure 7 shows the comparative RDX degradation at different inhibitor concentrations. With no inhibitor, RDX degradation was found to be 61.13% in 7 days. It

Fig. 8 The proposed mechanism for RDX degradation by *J. cremeus*. The intermediates shown in brackets {} were not detected in this study. Path a: McCormick et al. (1981); Path b: Zhang and Hughes (2003); Path c: Hawari et al. (2000) ▶


Path c
Nitroreductase
1 e-
NO2-
hexahydro-1,3,5-trinitro-1,3,5triazine
methylenedinitramine
bis-(hydroxymethyl)nitramine
Path a
2 e-
Nitroreductase
Path b
hexahydro-1-nitroso-3,5-dinitro-1,3,5triazine
hexahydro-1-monohydroxylamino-3,5-dinitro-1,3,5triazine
2 e-
hexahydro-1,3-dinitroso-5-mononitro-1,3,5triazine
hexahydro-1-monoamino-3,5-dinitro-1,3,5triazine
2 e-
hexahydro-1,3,5-trinitroso-1,3,5triazine
hexahydro-1,3-diamino-5-nitro-1,3,5triazine
hexahydro-1,3,5-triamino-1,3,5triazine

was observed that, with increasing concentration of sodium azide, RDX degradation decreased, showing that, nitroreductase was a key player in RDX degradation pathway in *J. cremeus*. Type I nitroreductases (oxygen insensitive) may be responsible for the two electron transfer process leading to the formation of MNX, DNX and TNX, denitration and ring cleavage leading to the formation of BHNA and MDNA (Kitts et al. 1994, 2000; Young et al. 1997a, 1997b).

On basis of the above results, the proposed degradation pathway has been given in Fig. 8. Due to the substantial release of nitrite, we propose the denitration pathway (leading to the formation of BHNA and MDNA) could be a major degradation pathway for RDX transformation.

Conclusion

RDX is a very commonly used military explosive. It is found to be toxic to human and environmental health. Due to its recalcitrant nature, RDX biodegradation is a major hurdle in eco-friendly treatment of the munition compound. *Janibacter cremeus*, a bacterial isolate from an explosive contaminated soil was evaluated for its ability to degrade RDX aerobically in aqueous medium. This study showed that, *J. cremeus* was a potential degrader, which exhibited 88% degradation in 30 days. The degradation followed the first order with a half-life of 11.088 days. The pathway for degradation was also elucidated. The bacterium followed three different pathways for RDX degradation. It was also observed that, inhibition of Nitroreductase led to the inhibition of degradation, thereby proving that nitroreductase was the major enzyme involved in RDX degradation. The results of the study prove that *J. cremeus* can find further application in site remediation as it could effectively reduce RDX aerobically.

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Supporting information Online Resource 1—Media composition.

Online Resource 2—Analysis of nitrite released.

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Compliance with ethical standards

Conflict of interest All the authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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A novel egg shell-based bio formulation for remediation of RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) contaminated soil

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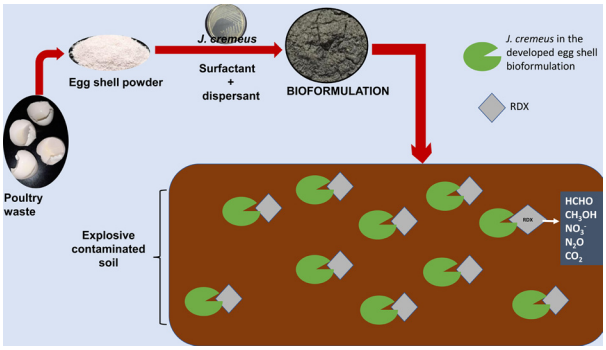
GRAPHICAL ABSTRACT



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ABSTRACT

Environmental contamination by secondary explosive has been posing threat to human health and the ecosystem. We investigated the potential of a novel bioformulation developed from poultry waste for the bioremediation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) contaminated soils. Eggshells and additives immobilized with an indigenous explosive degrading microbe *Janibacter cremeus* were utilized for the development of the wettable powder bioformulation. Treatments carried out under unsaturated and saturated soil conditions resulted in 62 and 73 % removal of RDX respectively in 35 days meeting the soil clean up goals. The saturated treatment sets exhibited better microbial growth during the study in terms of live cell count and total enzyme activity. The bacteria, *J. cremeus* was observed to exhibit significant release of nitrite under both unsaturated as well as saturated conditions. Mass spectrometric studies showed that, both the conditions lead to the formation of nitroso-derivatives of RDX. But under saturated condition, an intermediate, 5-hydroxy-4-nitro-2,4-diazapentanal was observed which is a precursor to 4-nitro-2,4-diazabuatnal ultimately leading to mineralization. An accessible bio resource from poultry waste when used as a carrier for explosive degrading microbe has proven effective for *in situ* remediation of explosive contaminated soils.

1. Introduction

Explosives are high energetic compounds that are characterized by release of large amounts of gases and energy on detonation. Of all the classes, secondary explosives are most commonly used in defence application. Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), a cyclic

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nitramine is one of the most widely used high explosive. It has been classified by the United State Environment Protection Agency (USEPA) as a potential carcinogen. Exposure to RDX has been associated with convulsions, neurological and liver damages (Singh et al., 2012). There are various ways through which RDX finds its way into the environment, viz, improper handling and disposal of waste from manufacturing, loading, assembly and packaging (LAP) activities, live firing ranges, open burning/ detonation sites and washout lagoon soils of former manufacturing plants (Thorn et al., 2004). RDX is characterized by a low sorption coefficient leading to easy migration from the surface soil to the ground water table (Agency for Toxic Substances and Disease Registry (ATSDR), 2012; EPA, 2005). This contributes to its persistence in the environment. EPA in 2017, has put forth soil screening limit (SSL) for RDX as 28 mg/Kg at industrial sites. SSL's are risk-based concentrations which are derived from combining the exposure and toxicity data and can be used as preliminary remediation goals. Concentrations below SSL warrant no further action or clean up. Hence remediation of an explosive contaminated site, requires to meet the SSL, so as to be declared safe (EPA, 1996).

Many technologies have been known for the remediation of explosive contaminated soil, viz, physical, chemical and biological. Physical and chemical technologies, are associated with use of high temperature or chemicals for achievement of treatment goal. These technologies are not successful as they majorly lead to phase transfer of explosive pollutant, rather than mineralization, thereby leading to secondary pollution. To combat these drawbacks, focus has been laid on bioremediation, as an eco-friendly tool to manage explosive contaminated soils (Kalderis et al., 2011). Bioremediation techniques have been in place for some time now, and can be broadly classified as microbial or plant mediated remediation. Microbial remediation utilizes the ubiquitous nature of microbes. These methods are sustainable and inexpensive alternative to physical and chemical methods. But, the process is associated with few drawbacks, viz, longer treatment durations, lower efficiencies and dependence on environmental factors (Xin et al., 2013). Hence, research needs to be focused on increasing the efficiency of the process.

Recently, bioaugmentation and/ or biostimulation for remediation of soils has gained much importance. But the major drawback lies in the limited survivability of the augmented microbe. Hence, use of carriers for delivery of microbes into the soil has been proposed for remediation of hazardous compounds (Wang et al., 2019; Zhang et al., 2019; Kalsi et al., 2020). Carriers that aid in increasing the bioavailability as well as provide nutrients to microbe help in better degradation. Therefore, selection of carrier materials based on these criteria help in better remediation of the explosive compound.

Explosives are characterized by low bioavailabilty due to their low sorption in soil (Monteil-Rivera et al., 2003). Use of carriers that can aid in increasing the bioavailability by increasing the sorption of explosive to soil can aid in better and rapid degradation by the immobilized microbe. Also, the carrier can help provide nutrients for survival of the bacteria, as well as provide conditions to help its growth in adverse conditions present in the contaminated soil. Many types of natural and synthetic materials have found application in remediation of various hazardous contaminants. With a shift in focus towards cost effectiveness, environmental and social sustainability, use of natural materials as a source of biochar or catalyst has already been studied in detail and many natural sources have been experimented with, for degradation of various contaminants (Ye et al., 2019 a, 2019b; Ye et al., 2020). With the present scenario, exploring use of otherwise waste materials as carriers is also gaining acceptance (Simons et al., 2012). Examples include, egg shells and cocopeat derived from coconut husks. Egg shells are very easy to source and inexpensive source of calcium carbonate. Calcium carbonate is a known amendment used in reclamation and remediation of soils contaminated with hazardous compounds (EPA 542-R-07-013, 2007). It has been reported as an amendment in treatment of military range soils contaminated with heavy metals (Siebielec and Chaney, 2012). Egg shells are also known to contain other essential nutrients that may contribute to survival of microbes (Antwi-Akomeah et al., 2018). Cocopeat is rich in organic matter and a very good adsorbent. It is known that increased organic carbon may increase the sorption of organic contaminants (Tucker et al., 2002) and make it bioavailable to microbe. Cocopeat has already been reported in sorption of dyes like, crystal violet, methylene blue, malachite green (Vijayaraghavan et al., 2015; Premkumar and Vijayaraghavan, 2014). It can also provide nutrients for microbial survival and growth (Nunal et al., 2014). Use of these egg shells as carriers for explosive degrading microbes hasn't been explored yet. The current study aims at development of a sustainable waste management approach wherein a bioformulation consisting of egg shells immobilized with a native explosive degrading microorganism was evaluated for *in situ* remediation of high explosive contaminated soil.

2. Materials and methods

2.1. Chemicals

RDX in pure (> 99.9 %) form was obtained from a manufacturing facility in India. All other chemicals, microbial growth media and reagents were of analytical grade. High Performance Liquid Chromatography (HPLC) grade solvents were used.

2.2. Carrier materials

Waste egg shells obtained from a local egg vendor were washed thoroughly with water, air dried and finely powdered before use. The cocopeat obtained from a local farm was sieved through 600-micron sieve for uniform size distribution. Tween, a very common and environmentally safe surfactant, sodium bi carbonate (dispersing agent) was also procured from standard manufacturer (Sigma Aldrich). Powdered egg shell, cocopeat, tween and sodium bi carbonate were mixed in the ratio 8:1:0.6:0.4 (w/w) respectively. The carrier mixture was sterilized by autoclaving twice with an intermittent incubation of 24 h. The explosive degrading indigenous bacterium *Janibacter cremeus* was then inoculated in the carrier mix.

2.3. Microbial culture

Janibacter cremeus (MTCC No. 12869), an indigenous explosive degrading bacterium was isolated from the soil of an explosive manufacturing facility in India and was obtained from IMTECH, Chandigarh, India. The bacterial culture was subjected to enrichment in increasing concentrations of RDX in liquid minimal salt medium (Supplementary section, S1). The bacteria were enriched to a concentration of 60 mg/L of RDX. The enriched bacterium was then maintained in nutrient broth (Supplementary section, S1) and sub-cultured twice a month.

2.4. Preparation of bioformulation

J. cremeus was grown in submerged aerated culture in Luria Bertani broth (Supplementary section, S1) for 48 h. The cells were harvested at 10,000 rpm for 10 min at 4 °C. The resultant cell suspension was observed to have a cell count of 10^{14} cells/ mL of the broth. This was diluted in sterile water (40 mL) and added to the sterile carrier mix (100 g) under aseptic conditions. The developed wettable powder formulation was then air dried in laminar air flow chamber overnight and then sealed in autoclaved bags with 75 % head space (for aeration) at 4 °C until further use (Pandey and Maheshwari, 2007). The viability of the prepared bioformulation was also monitored for 6 months using plate count method. Scanning electron microscopy (SEM) of the developed bioformulation was performed to confirm immobilization. Fourier Transform Infrared spectroscopy (FTIR, Perkin Elmer Spectrum 2) was performed on the egg shell powder to determine its composition.

Table 1
Physico- chemical characterization of the soil used in study.

Sl. No.	Physico- chemical property	Value
1	Texture	Sandy loam
2	pH	6.91
3	Electrical conductivity (mS m^{-1})	0.9
4	Bulk density (g cm^{-3})	1.15
5	Moisture content (%)	1.007
6	Water holding capacity (%)	46.8
7	Total organic carbon (%)	0.0611

Also, FTIR for egg shells immobilized with *J. cremeus* and RDX adsorbed to egg shells were also performed to observe their respective interactions.

2.5. RDX degradation experiments

Uncontaminated background soil (0 − 20 cm depth) was collected from an RDX manufacturing facility in India. The soil was air-dried, ground with a pestle to break the aggregates, passed through 2 mm sieve. The soil was characterized for its texture, pH, electrical conductivity (E.C), bulk density, moisture content, water holding capacity and total organic matter (TOM) (Table 1). The texture of the soil was determined by United States Department of Agriculture (USDA) texture analysis method (Thien, 1979). pH and E.C were determined using a bench scale pH and E.C probe with 1:1 soil to water ratio. Bulk density was measured by sand replacement method. The moisture content and water holding capacity of the soil was determined using standard USDA methods. The total organic matter (TOM) was evaluated gravimetrically by burning the sample at 550 °C for 4 − 5 hours in a muffle furnace (Drozd et al., 1997). RDX degradation experiments were carried out in 25 mL Erlenmeyer flasks as bioreactors. 5 g of soil were weighed in each. These were autoclaved at 121 °C and 15 psi for 15 min. They were autoclaved twice with intermittent incubation of 24 h. Soils were spiked with RDX at a concentration of 65 mg/ Kg (based on concentration present at site). The experiments were carried out under two moisture conditions: unsaturated, wherein, moisture was maintained at 50–60 % of the water holding capacity (soil: liquid ratio of 1:0.5) and saturated, wherein, the treatment sets were maintained in flooded condition (soil: liquid ratio of 1:2). Control treatments consisted of sterile spiked soil only and spiked soil amended with uninoculated bioformulation only. Treatment sets consisted of spiked soils inoculated with the developed bioformulation at 10 % (w/w) (IS: 8268, 1976). The experiments were carried out at 35 °C for 5 weeks. Sacrificial sampling was performed weekly and analyzed for microbial growth in terms of live cell count, total enzyme activity using fluorescein diacetate (FDA) hydrolysis. Removal of RDX, nitrite released during the microbial reduction and the intermediates formed were investigated. Experiments were carried out in triplicates and mean values are reported.

2.6. Analytical techniques

Live cell count was monitored using the plate count method. Briefly, 1 *g* of soil sample was suspended in 9 mL sterile distilled water. The suspension was serially diluted. Different dilutions were plated on nutrient agar plates and incubated at 30 °C for 24–48 hrs. The colonies were counted using a colony counter to determine the colony forming units (CFU). The total enzyme activity was monitored using fluorescein diacetate (FDA) hydrolysis. 0.5 g of soil was taken in a conical flask, followed by addition of 20 mL 0.06 M sodium phosphate buffer at pH 7.6 and 0.2 mL FDA solution. The reaction was allowed to proceed for 20 min and was then stopped using acetone. Samples were analyzed spectrophotometrically at 500 nm (Chen et al., 1988; Adam and Duncan, 2001). The values were then determined using standard curve prepared using the soil used in study (Fig. S1, Supplementary section S2). Enzyme activity was expressed as FDA hydrolyzed in µg per gram of soil in 20 min reaction time. The soil nitrite was extracted using 2 M potassium chloride solution as described by Keeney and Nelson (1982). The extract was centrifuged at 10,000 rpm for 5 min to remove any soil particles. To 600 µL of the supernatant, 150 µL of sulphanilamide solution was added. The reaction was allowed to proceed for 5 min at room temperature. To this, 150 µL of *N*-(1-naphthyl) ethylene diamine dihydrochloride solution was added. The reaction was allowed to proceed at room temperature for 20 min. The volume was then raised to 3 mL. The nitrite present in the reaction mix was monitored spectrophotometrically at 540 nm. The concentrations were quantified against sodium nitrite standard curve (Mercimek et al., 2013).

2.7. Chromatographic techniques

The explosive, RDX was extracted from the soil systems using the USEPA 8330 B method. 2 g of soil samples were mixed with 10 mL acetonitrile and was placed on a platform shaker for 18 h. The samples were allowed to rest for 30 min and filtered through 0.22 µm polytetrafluorethylene (PTFE) Millex filters.

RDX was analyzed using High Performance Liquid Chromatograph (HPLC) equipped with a diode array detector (Dionex Ultimate 3000 ThermoFisher, USA). Reversed phase C-18 column with 5-µm particle size was used as the stationary phase. Separation was obtained using acetonitrile: water (50:50) as mobile phase, with a flow rate of 1 mL min^{-1}. RDX was detected at 254 nm wavelength (USEPA Method 8330 B). The quantification was done against a standard curve with known RDX concentrations (Fig. S2, Supplementary section S2).

Mass spectrometric analysis (Synapt G2 Waters ACQUITY QSM, USA) of the acetonitrile extracted sample was performed in positive ion mode. RDX and its degradation products were separated on a C18 column (130 A, 1.7 µm, 2.1mm* 100 mm) at 0.75 mL/ min flow rate over a period of 20 min.

3. Results and discussion

3.1. Immobilization of J. cremeus

A novel wettable powder bioformulation was prepared using eggshells, cocopeat, tween and sodium bi carbonate. The immobilized bioformulation was viewed under Scanning electron microscope (Fig. 1a).

The viability of *J. cremeus* cells immobilized on the carrier mix under storage at 4 °C was observed for six months (Fig. 1b). The viability of the developed bioformulation was found to be stable with a loss of only 2 log units at the end of 180 days. The cell counts in terms of CFU (Colony forming unit) at the time of preparation was observed to be in the order of 10^{12} per gram of formulation, which is much higher than the recommended cell count as prescribed by the Indian standards (IS: 8268, 1976; Pandey and Maheshwari, 2007). The presence of live cells at the end of six months, prove that the carrier material is successfully able to provide nutrition and maintain the cells under storage.

The main carrier, egg shell was selected due to the following criteria: 1) a sustainable bio resource obtained from poultry waste 2) economically viable 3) derived from waste material that has no application elsewhere. Egg shells are mainly composed of the inorganic calcium carbonate (94 %), magnesium carbonate and calcium phosphate (1 % each). It also contains organic matter (4 %) and other insoluble proteins (Abdulrahman et al., 2014). They are also a rich source of essential nutrients like, N, P, K, Na and C, which are necessary for microbial growth and survival (Antwi-Akomeah et al., 2018). The FTIR spectrum of egg shell powder shows strong peak at 1423 cm^{-1}, 875 cm^{-1} and 712 cm^{-1} (Fig. 2). The presence of these peaks prove that calcium carbonate is the major inorganic component of egg shell powder (Iram et al., 2019). Calcium carbonate has been reported as a common soil amendment in reclamation and remediation of soils

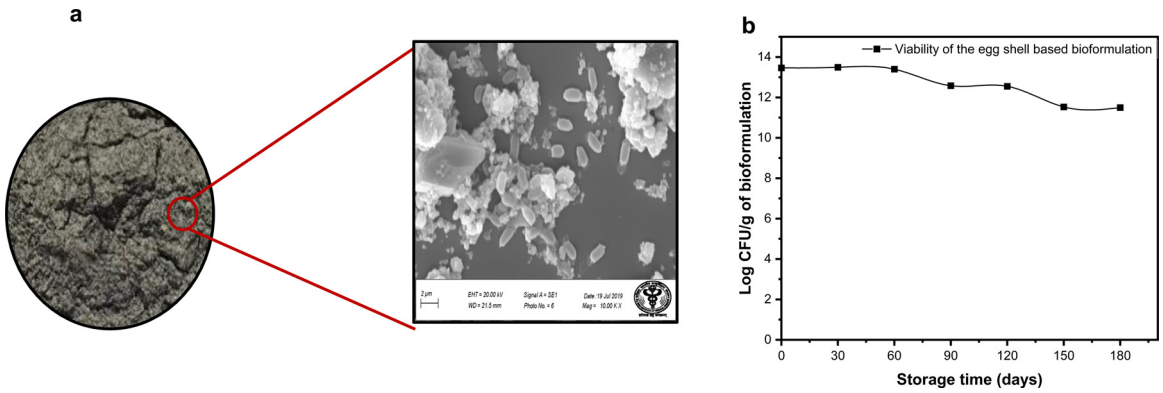

Fig. 1. (**a**) Electron micrograph image of the egg shell based bioformulation (**b**) Viability of the developed bioformulation under storage at 4 °C.

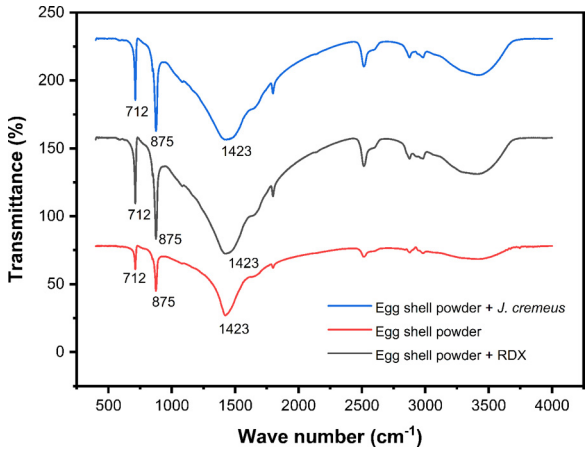

Fig. 2. FTIR spectrum of the egg shell powder, egg shell power immobilized with *Janibacter cremeus* and egg shell powder with RDX.

contaminated with hazardous compounds (EPA 542-R-07-013, 2007). The presence of organic content in the egg shells can also contribute in the maintenance of bacterial cells immobilized. The bacterium immobilize to egg shells by adsorption. The microbe could be trapped in the abundant pores in the egg shells (Li et al. 2019). Broadening of peak at 1423 cm^{-1} on FTIR for egg shell powder immobilized with *J. cremeus* and RDX (separately) suggests interaction due to hydrogen bonding (Coates, 2000). This confirms that, egg shell powder can adsorb both the bacterial culture as well as explosive chemical, RDX, increasing the bioavailability of explosive for bacteria.

Cocopeat, a plant based bioresource derived from coconut husk is a rich source of organic matter, is economical and easy to source. It helps in increasing the adsorption of explosives, the moisture holding capacity of soil and is also a rich source of carbon, phosphorous and other nutrients, thereby providing favourable environment for microbial growth (Nunal et al., 2014). The surfactant, tween is a nature friendly detergent that functions as a wetting agent. It enhances the emulsifying, dispersing, spreading and wetting properties of the developed bioformulation. Sodium bi carbonate acts as a dispersing agent as well as a buffer for pH adjustment. It improves the dispersion of the developed bioformulation (Brar et al., 2006).

3.2. RDX degradation studies

The developed novel bioformulation was tested for its efficacy in the remediation of RDX contaminated soil. The soil spiked with 65 mg/Kg of RDX was subjected to remediation under unsaturated as well as saturated conditions. Unsaturated conditions corresponded to the aerobic transformation of RDX, whereas, the saturated conditions led to anoxic conditions. The soil saturation lead to slower diffusion of oxygen thereby leading to the anoxic conditions (Sims and Kanissery, 2019). Since *J. cremeus* is a facultative anaerobe, it was found to function in both conditions.

HPLC was used to determine the residual RDX concentration at different sampling times. The control treatment sets under unsaturated as well as saturated conditions exhibited negligible degradation (Fig. S3 and S4, Supplementary section S4). The treatments with bio formulation, under unsaturated conditions, exhibited 62 % degradation whereas, under saturated conditions, the degradation obtained was 73 % (Fig. 3a & b). Though bioaugmentation hasn't been yet studied with different soil water content, bioremediation (of explosives) by natural attenuation has been studied previously. Under different moisture contents, Sagi-Ben Moshe et al. (2012), found that increase in soil water content led to better remediation of explosives RDX, HMX and TNT. They postulated that, higher soil water content led to reduction in redox potential thereby enhancing the remediation. Ringelberg et al., 2003 had also found a similar pattern. They also observed higher RDX degradation with higher soil moisture content. The goal of any treatment set up is to attain the soil screening level (SSL) as put forth by EPA. EPA (2017), stated the SSL for RDX to be 28 mg/Kg for industrial soils. Our results of both the treatments indicate that, the SSL as prescribed was achieved in 35 days.

3.3. Bacterial growth during RDX remediation

The growth of *J. cremeus* immobilized in the developed bioformulation was monitored for its growth during RDX degradation study under unsaturated and saturated conditions (Fig. 4a). *J. cremeus* showed growth in presence of RDX under unsaturated as well as saturated conditions. The highest CFU was observed on day 21 (for both unsaturated as well as saturated conditions), followed by decline in growth. The loss of bacterial cells was not lower than 10^7 g^{-1} of the soil sample, which is a requisite cell count for bioremediation (Pandey and Maheshwari, 2007). The saturated treatment sets showed better growth than unsaturated sets. The rapid growth of the bacteria shows that *J. cremeus* was not adversely affected by the presence of RDX.

Another parameter monitored to assess the growth and function of immobilized *J. cremeus* was estimation of the total enzyme activity in terms of FDA hydrolysed per gram of soil in 20 min. Day 21 showed the highest enzyme activity in both unsaturated and saturated treatment sets inoculated with the developed bioformulation (Fig. 4b). The unsaturated sets exhibited a maximum enzyme activity of 23 µg FDA hydrolysed/g of soil, whereas, saturated sets exhibited a maximum of 45 µg FDA hydrolysed/g of soil. The results obtained correlates with the bacterial live cell count monitored. The total enzyme activity of the bacterium in unsaturated condition though increasing with time, is far less than the activity expressed in saturated conditions. Liang et al., 2003, had also observed a similar pattern. They observed that increasing moisture content led to a significant increase the microbial

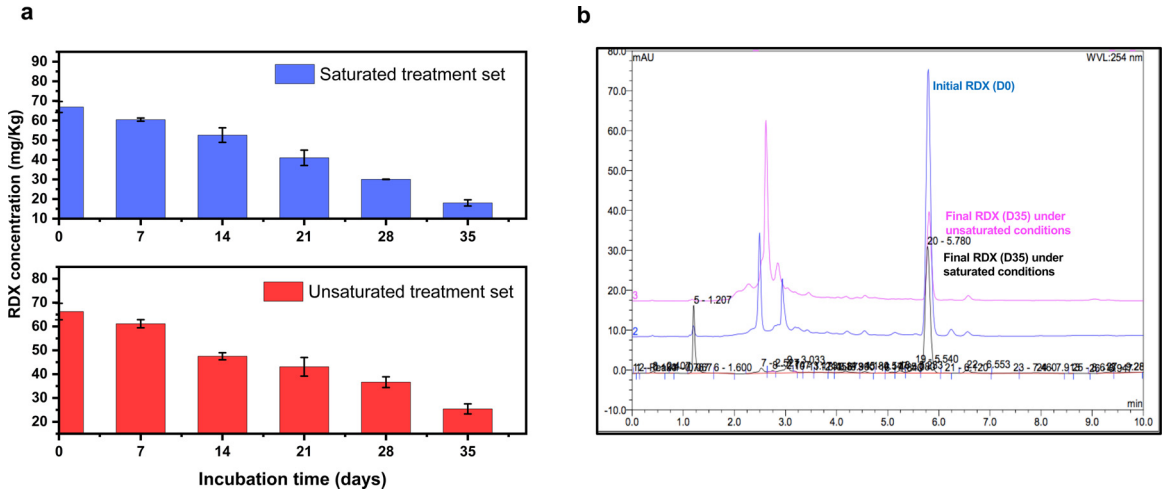

Fig. 3. RDX degradation potential of the developed bioformulation under unsaturated and saturated conditions **(a)** Residual RDX concentration in the treatment sets **(b)** Overlaid HPLC chromatogram showing initial and final RDX concentrations after treatment with egg shell based bioformulation under unsaturated and saturated conditions.

activity. Moreno-Espíndola et al., 2018, had studied the effect of water content in soil on the enzyme activity of the bacterial community. They had also observed that, higher water content promoted enzyme activity.

3.4. Elucidation of RDX degradation pathway by immobilized J.cremeus in soils

The RDX degradation pathway followed by *J. cremeus*, the active ingredient in the bioformulation was elucidated by monitoring the intermediates produced. Nitrite being an important metabolite, is generally the first released intermediate during degradation (Crocker et al., 2006). Hence, monitoring of the nitrite release is an important step. The nitrite release was measured spectrophotometrically (Mercimeck et al. 2013).Fournier et al., 2002, studied the key metabolite in the biodegradation of RDX by *Rhodococcus* sp. strain DN22. They found that nitrite was the first metabolite released which they suggested to be produced by denitration prior to ring cleavage. Zhao et al. (2002 and 2003), Bhushan et al., 2003, have also postulated pathways with the concomitant release of nitrite. Our results (Fig. 5) showed that the nitrite released in unsaturated RDX microcosms inoculated with the developed bioformulation, increased up to 21 days with a peak at concentration of 12.47 mg/ Kg with a further decrease in concentration later. This decrease in nitrite concentrations in the later stages can be attributed to the uptake of nitrite ions by nitrite reductase enzymes as reported by Lenke et al., 1992. Also, Coleman et al., 1998, reported a similar pattern of nitrite release and uptake during the aerobic

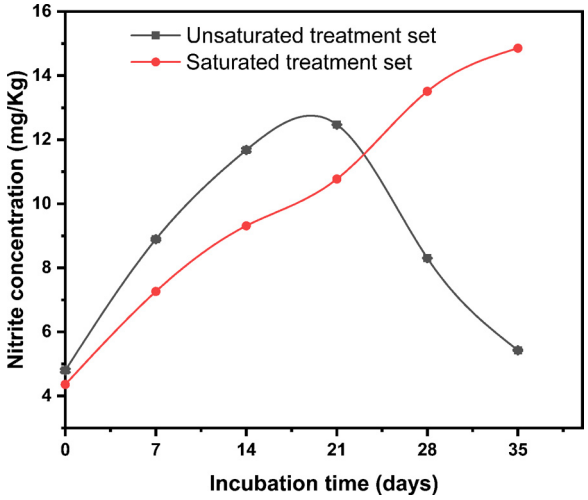

Fig. 5. Nitrite released during the bioremediation of RDX using the developed bioformulation under unsaturated and saturated conditions.

degradation of RDX by *Rhodococcus* sp. strain DN22. Sadani et al., 2017 also monitored nitrite changes during bioremediation of Pentaerythritol Tetranitrate (PETN) contaminated soils. They observed a similar pattern with an increase in nitrite concentration in the initial stages

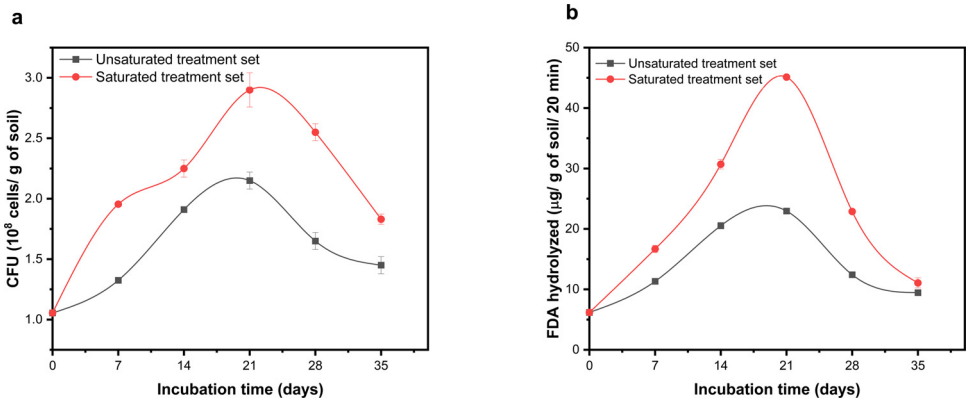

Fig. 4. Growth of immobilized *J. cremeus* in RDX contaminated soil under unsaturated and saturated conditions in terms of **(a)** Colony Forming Units **(b)** Total enzyme activity.

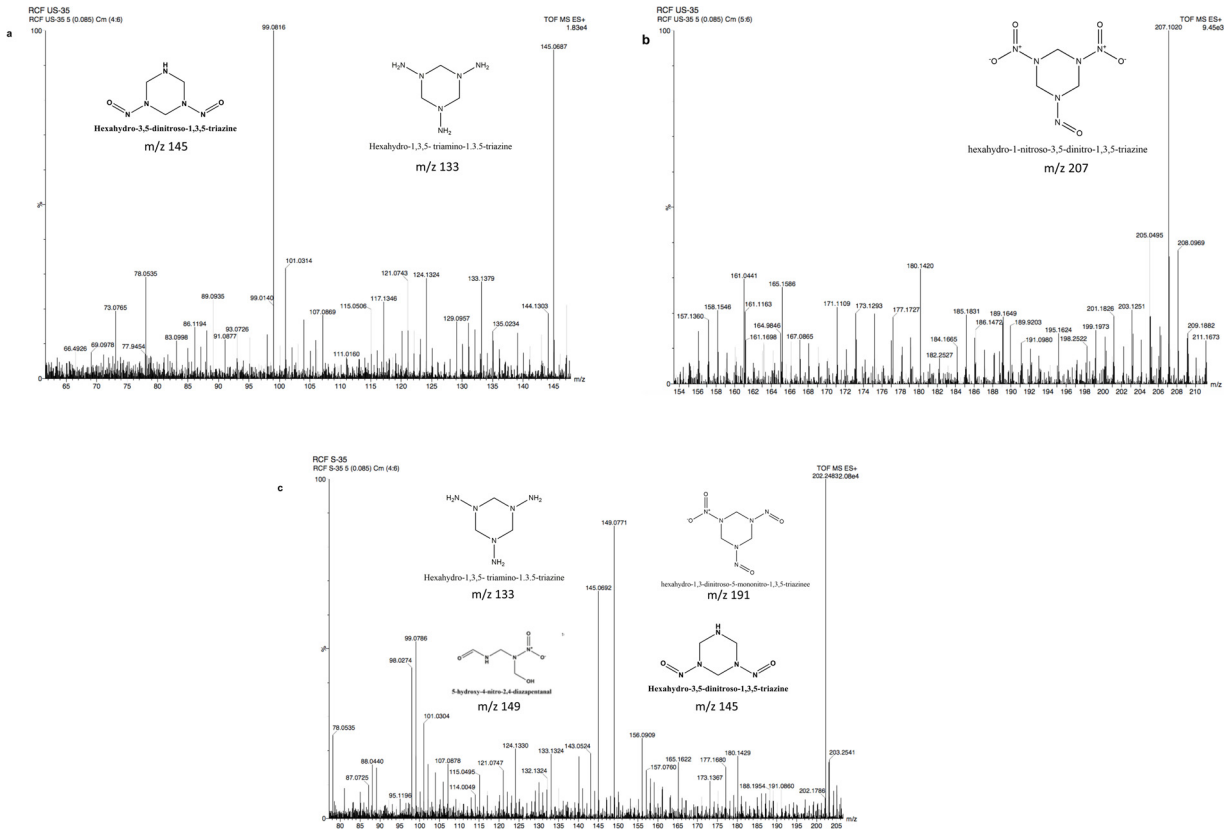

Fig. 6. MS chromatogram for RDX degradation under **(a &b)** unsaturated and **(c)** saturated conditions.

followed by its decrease later. The saturated treatment sets showed continuous increase in the nitrite release, with a concentration of 14.85 mg/Kg at day 35.

Mass spectrometry (MS) using positive electron spray ionization (ESI) was used to determine the intermediates formed during the degradation of RDX. The intermediates were then used to deduce the proposed pathway. The ring cleavage products of RDX degradation under unsaturated and saturated conditions were monitored. The mass spectra (Figs. 6a & b) reveal the presence of peak at *m/z* 207 in the unsaturated treatment sets, indicating the presence of mono nitroso RDX (MNX). Another major peak was observed at *m/z* 145 (M-NO), which signifies the presence of trinitroso derivative of RDX (TNX) (Florián et al., 2007; Naja et al., 2008). This shows that, *J. cremeus* exhibited the two-electron reduction pathway as proposed by McCormick et al. (1981). Another intermediate peak was observed at *m/z* 133. This corresponds to the triamino RDX derivative as observed by Zhang and Hughes (2003) in *Clostridium acetobutylicum* under anaerobic conditions. Though the unsaturated soil conditions are thought to be aerobic, it is very important to remember that in soil, different sites and pockets may exhibit different redox potential. Therefore, some sites may exhibit a local anoxic environment leading to the formation of an anaerobic pathway intermediate (Sims and Kanissery, 2019). In this pathway, MNX is further converted to a hydroxylamino derivative which is further converted to an amino derivative followed by a triamino derivative. Though no other denitration intermediates were observed, the nitrite released under unsaturated conditions could be attributed to the denitration of MNX as observed by Zhao et al. in 2002 and 2003. *Clostridium bifermentans* HAW1 and *Klebsiella pneumoniae* SZC1 exhibited similar pathway of denitration of RDX *via* the MNX route, which led to ring cleavage and mineralization of RDX to formaldehyde, methanol and nitrous oxide.

Mass spectrum for RDX biodegradation under saturated treatment (Fig. 6c) reveal the presence of a peak at m/Z 191, which corresponds to dinitroso RDX (DNX). Similar to the unsaturated system, a peak at *m/z* 145 correspond to TNX (M-NO). This shows that, under saturated conditions, the bacteria, *J. cremeus* breaks RDX by two electron reductive pathway to MNX, which on further reduction is converted to DNX and then to TNX. Such a pathway is typical in case of anaerobic RDX degradation as observed by Kwon and Finneran in 2008 in the bacterium *Geobacter metallireducens* strain GS-15. A similar path was observed by Perreault et al., 2012 in *Shewanella oneidensis* MR1 and by Kwon and Finneran (2006) in *Geobacter sulfurreducens* strain PCA. Another intermediate peak was observed at *m/z* 133 (triamino RDX) as observed by Zhang and Hughes in 2003 during anaerobic degradation of RDX. The nitrite release during RDX degradation could be attributed to the presence of an intermediate at *m/z* 149 which corresponds to 5-hydroxy-4-nitro-2,4-diazapentanal. This intermediate was observed by Balakrishnan et al. in 2003. This pathway led to the release of 4NDAB (4-nitro-2,4-diazabuatnal) ultimately leading to mineralized product, formaldehyde. Fig. 7(a & b) shows the proposed pathway under unsaturated and saturated conditions respectively.

4. Conclusion

With focus on sustainable remediation methods, upcycling of waste for use in various applications is gaining impetus. In this study, an egg shell based wettable bioformulation was developed. Egg shells are very common easily available waste, rich in calcium carbonate. and can help in increasing the adsorptive capacity of soil for explosives, thereby slowing down its transport to the water table. It also acts as a nutrient source, thereby supporting the immobilized microbe. The developed novel bioformulation was found to be a successful eco-friendly tool for *in situ* bioremediation of explosive contaminated industrial sites, as the formulation was able to achieve the soil screening level in 35 days.

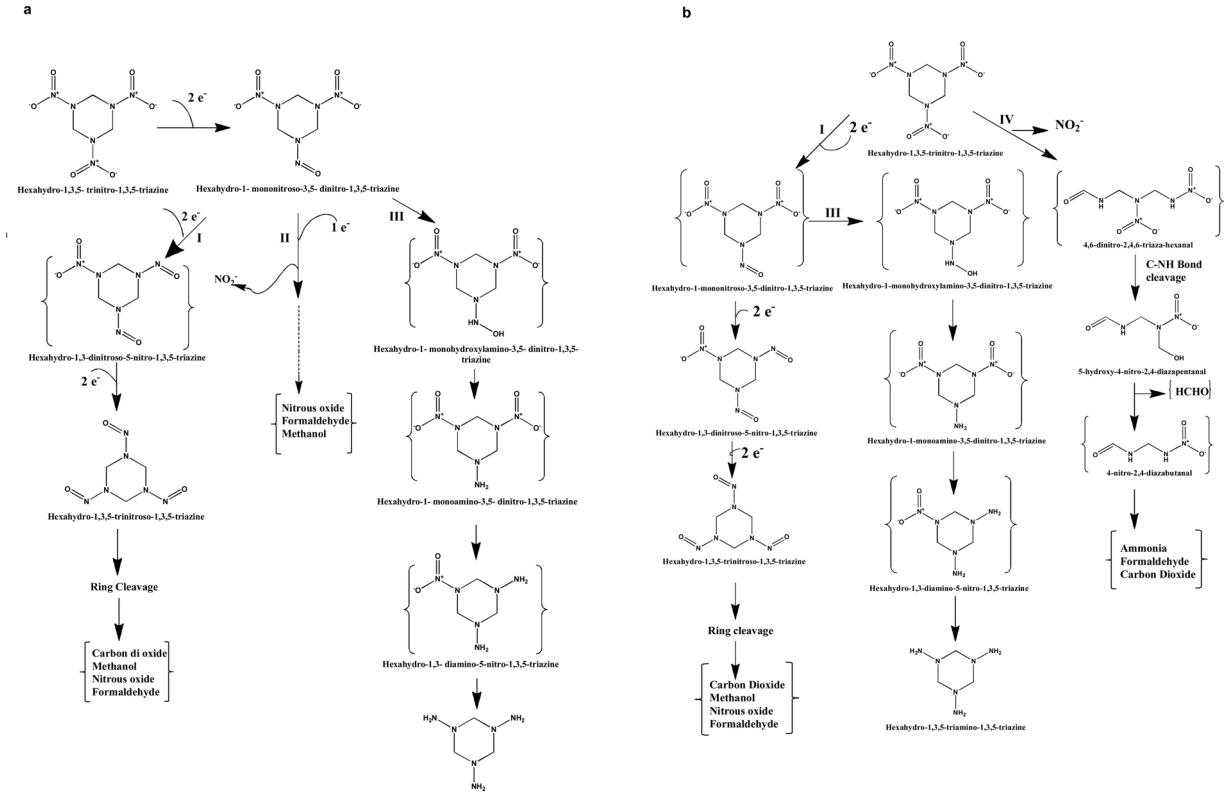

Fig. 7. Proposed degradation pathway for RDX in soil by *Janibacter cremeus* under **(a)** unsaturated conditions **(b)** saturated conditions.
Path I: McCormick et al., 1981; Kwon and Finneran, 2008, Path II: Zhao et al., 2003; Path III: Zhang and Hughes, 2003. Path IV: Balakrishnan et al., 2003.
The compounds shown in brackets {} weren't observed/ analyzed in this study.

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CRediT authorship contribution statement

Anchita Kalsi: Investigation, Methodology, Writing - original draft. **S. Mary Celin:** Conceptualization, Methodology, Writing - review & editing, Supervision, Funding acquisition. **Pallvi Bhanot:** Data curation. **Sandeep Sahai:** Resources. **Jai Gopal Sharma:** Supervision, Resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2020.123346.

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