Aerobic biodegradation of HMX by *Planomicrobium flavidum*

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Abstract

In this report, aerobic biodegradation of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine or high melting explosive (HMX), a highly explosive chemical by *Planomicrobium flavidum* strain S5-TSA-19, an isolate from an explosive-contaminated soil, was investigated. The isolate S5-TSA-19 degraded 70% of HMX in 20 days during which time nitrite ion was produced with the subsequent formation of metabolites, viz. methylenedinitramine and *N*-methyl-*N,N'*-dinitromethanediamine with molecular weights 136 Da and 149 Da, respectively. The degradation mechanism was found to follow first-order kinetics with a half-life of 11.55 days and formation of above intermediates indicate single nitrite elimination pathway. The proliferation of isolate S5-TSA-19 in the absence of nitramines indicates the cometabolic degradation of HMX. Isolate S5-TSA-19 can thus be used as futuristic microbe for degradation of HMX at explosive-contaminated site.

Keywords Aerobic biodegradation · *Planomicrobium flavidum* · High-performance liquid chromatography · Mass spectroscopy · HMX · Contaminated sites

Introduction

Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine, commonly known as Her Majesty's Explosive or High Melting Explosive (HMX) is a nitramine-based explosive and finds wide usage in high-energy propellant compositions of guns and missiles, quarrying and mining activities, implosion of radioactive nuclides and other commercial purposes (Keegan 1993; Lyman et al. 2002; Pichtel 2012; Pennington et al. 2002). HMX is stable and displays better explosive power compared to other widely used high explosives such as 2,4,6-trinitrotoluene (TNT) and royal demolition explosive (RDX) (An et al. 2010). However, the dissemination of this chemical into the environment during its production, usage at war and training ranges, improper disposal of worn-out ammunition has led to contamination of soil, surface and ground water and it adversely affects the sustainability of living beings (Pennington and Brannon 2002; Pichtel

2012). United States Environmental Protection Agency has coded HMX as class D carcinogen with 400 ppb exposure limit in drinking water and its removal from environment is extremely essential (USEPA 2004; Pennington et al. 2002). Various methods such as filtration, incineration, precipitation, alkaline hydrolysis, and adsorption have been employed for degradation of HMX (Elisangela et al. 2009). However, these methods further degrade the environment due to addition of more chemicals and involve higher cost. Bioremediation seems to be the most effective and environment-friendly method as microbes which grow at explosive-contaminated sites are used for degradation of these toxic chemicals and their by-products into non toxic molecules by utilising their metabolic activities (Cui et al. 2017; Bhatt et al. 2006). Moreover, the method is cost effective and can be applied over a vast area. Several studies on biodegradation of nitramines have been conducted under aerobic (Harkins et al. 1999; Boopathy 2001) and anaerobic (Adrian et al. 2003; McCormick et al. 1984) conditions.

Cyclic nitramines are less stable compared to aromatic nitro compound such as TNT. Therefore, any nucleophilic attack on nitramino or methylene group of nitramines causes ring cleavage leading to decomposition. Bioremediation of HMX is a bit tough due to its very low solubility (Adrian and Arnett 2007). Several studies involving different microbes have been carried out to determine their

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degradation potential for nitroaromatics (TNT) (Preuss et al. 1993; Anand et al. 2016) and nitramines (RDX and HMX) (Singh et al. 2009; Fournier et al. 2004; Coleman et al. 1998). There is, however, a lesser number of studies on aerobic and anaerobic microbial degradation of HMX. Despite being the higher homologue of RDX, HMX shows more resistance to chemical and biological degradation than RDX. Few microbes selectively degrade RDX rather than HMX (Zhao et al. 2004, 2007).

The genus *Planomicrobium* has recently been proposed by Yoon et al. (Yoon et al. 2001) to accommodate new species, viz. *Planomicrobium koreense*, *Planomicrobium okeanoites*, and *Planomicrobium mcmeekinii*. Subsequent to this *Planomicrobium flavidum* isolated from marine solar saltern was categorised under the *Planomicrobium* genus (Jung et al. 2009). So far only few studies of *P. flavidum* for degrading xenobiotic compounds have been reported. Recently Kariuki et al. reported *P. flavidum* amongst the seven metribuzin-degrading soil bacteria and assessed its growth in response to selected physicochemical conditions (Kariuki et al. 2017). The biodegradation potential of *P. flavidum* for TNT and PETN under anaerobic conditions in the presence of sewage sludge as enrichment source was also demonstrated (Amin et al. 2017). However, the degradation potential of *P. flavidum* has not yet been explored for nitramine-based explosives. In the present study HMX degradation potential of native bacterial isolate, *Planomicrobium flavidum* strain S5-TSA-19, was investigated under aerobic conditions. Our research into HMX degradation by isolate S5-TSA-19, a new strain, used for the first time for aerobic degradation will facilitate future applications of this isolate for bioremediation.

Materials and methods

Chemicals

HMX (purity > 99%) used in the study was of commercial grade and obtained from one of the HMX manufacturing facility in India. Other chemicals used were of analytical grade from Sigma-Aldrich.

Media composition

The experiments were performed in minimal salt medium (MSM) as proposed by Cook and Hutter (Cook and Hutter 1981) with few modifications. Media comprised of 40 mM potassium phosphate buffer (pH 7.2) containing 10 mM glycerol, 61.61 mgMgSO₄·7H₂O, 5 mM succinate, 5 mM glucose, trace elements and NH₄Cl. For degradation studies, 6 mg/L of HMX was added to the medium. Trace elements solution contained (per liter) 2 mg FeSO₄·7H₂O, 0.03 mg

CaCl₂·2H₂O, 0.5 mg MnCl₂·4H₂O, 0.05 mg H₃BO₃, 0.05 mg ZnCl₂, 0.03 mg CuCl₂, 0.01 mg Na₂MoO₄·2H₂O, 0.5 mg CoCl₂·6H₂O and 0.05 mg NiCl₂·6H₂O (Thompson et al. 2005). Glucose, succinate, and glycerol were added as carbon sources. MSM, excluding trace metals and glucose, was sterilized in an autoclave at 121 °C for 20 min, whereas the glucose and trace metals stock solutions were sterilized by passing through 0.22 µm pore size sterile filters (MillexGP; Millipore, Bedford, MA).

Microbial isolate characterisation and growth condition

Live culture of isolate S5-TSA-19 (Microbial type culture collection, MTCC no. 12608) was obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India where isolation and identification of native bacteria were carried out using 16S ribosomal RNA sequence technique (Krishnamurthi et al. 2009) using Eztaxon server (Yoon et al. 2017). The GenBank accession number and length of 16S rRNA gene of the isolate S5-TSA-19 are LT631687.2 and 1407 bp, respectively. The isolate S5-TSA-19 was subcultured in MSM for three generations before subculturing in MSM containing HMX. The subculturing was carried out in an Erlenmeyer flask by agitating in an orbital shaker at 120 rpm and 35 °C. The growth of isolate was monitored at regular time intervals by measuring the optical density (OD) of culture at 600 nm with UV–Visible spectrophotometer (Perkin Elmer, Model Lambda 650S). Inoculum was prepared using log-phase of cell suspension having OD of 0.8.

HMX degradation experiment

The isolate S5-TSA-19 was inoculated in MSM containing 6 mg/L HMX under aseptic condition with two controls viz. MSM containing HMX without isolate and MSM containing isolate without HMX. HMX was dissolved in acetonitrile as it has low solubility in water. Acetonitrile was evaporated under aseptic conditions before the addition of the media and bacterial isolate inoculum. All experiments were performed in triplicate under strict sterile conditions. The cultures were incubated for 20 days at 35 °C in an orbital shaker agitated at 120 rpm. At regular time intervals, aliquots were taken out to determine growth of isolate S5-TSA-19 and HMX degradation. Decrease in HMX concentration was measured using high-performance liquid chromatography (HPLC) following USEPA 8330A method (USEPA 2007). Aliquots of 2 mL were withdrawn from treated cultures at regular intervals. The cells of isolate in the aliquot were removed by centrifugation at room temperature for 10 min and supernatant were used for HPLC sample preparation. The supernatant solutions were filtered through 0.45 µm Teflon filter. HPLC measurements were performed using

Flexar model HPLC instrument of PerkinElmer Inc. consisting of a quaternary pump, a 100-vial auto sampler and a photo diode array detector. Integrations were performed using Total chrome software. Samples (10 μ L) were eluted on a 3 μ m C18 reversed-phase column (4.6 mm $\times$ 150 mm). A mobile phase consisting of acetonitrile and water (1:1, v/v) was passed through the column at a flow rate (1 mL/min). Elution of HMX was monitored at 254 nm.

Nitrite is released during the degradation of HMX by microorganism. The amount of nitrite released was quantified spectrophotometrically as described in literature (Mericmek et al. 2013). Briefly, 1 mL aliquot of incubating culture was taken out at regular intervals and centrifuged at 10,000g at room temperature for 10 min. Following centrifugation, the supernatant was analyzed for nitrite (NO_2^-) concentration. To 200 μ L of supernatant, 50 μ L of sulphanilamide solution was added. The mixture was then incubated at room temperature for 5 min. This was followed by addition of 50 μ L *N*-(1-naphthyl) ethylene diamine dihydrochloride solution and incubation of the mixture at room temperature for 20 min. The volume of reaction mixture was raised to 1 mL by adding 700 μ L of deionised water. Concentration of nitrite released was determined by measuring the OD at 540 nm with sodium nitrite as standard.

Mass spectrometric (MS) analyses were performed on Synapt G2 (Waters ACQUITY QSM) MS system using atmospheric pressure chemical ionization in the positive ion (ES^+) mode. An Acquity UPLC® BEH column (C18, 1.7 μ m) was used to separate HMX and its degraded products at a flow rate of 0.75 mL/min over 20 min. The solvent system consisted of 0.1% formic acid, methanol, and acetonitrile.

Microbial cells samples for SEM imaging were prepared using method described elsewhere (Prasad et al. 2016). The samples were then coated with Au-Pd with Jeol JEC-300 auto fine coater and viewed under scanning electron microscope Jeol JSM 6610 at 10 kV.

Results and discussion

The main objective of this study was to investigate the potential of isolate S5-TSA-19, a native bacterial species isolated from HMX-contaminated site, to aerobically biodegrade HMX. SEM image of isolate S5-TSA-19 (Fig. 1a) depicts the isolate to be rod-shaped cells and there is no variation in its shape in presence of HMX (Fig. 1b). This indicates that isolate S5-TSA-19 remains unaffected by the toxic environment of HMX. The cells of S5-TSA-19 proliferate well in both the culture conditions, i.e., MSM with and without HMX (6 mg/L) and attained the highest growth within 12 days of incubation as observed from OD at 600 nm (0.09–1.05) and started declining thereafter (Fig. 2).

Fig. 1 Scanning electron micrographs of isolate S5-TSA-19 cells in media **a** without HMX and **b** with HMX

Fig. 2 Growth curve of isolate S5-TSA-19 cells over 20 days incubation period

It was observed that isolate S5-TSA-19 degraded 70% of HMX within 20 days in MSM whereas there was insignificant variation of HMX concentration (6 mg/L) in the uninoculated control (Fig. 3). All experiments were done in triplicates and the error was within 5%. To unravel the rate of degradation of HMX in presence of isolate S5-TSA-19 first-order kinetics model was applied on the temporal concentration of HMX. The linear form of first-order rate equation is represented as Eq. (1).

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

and half-life of HMX degradation is given by Eq. (2)

$$t_{0.5} = 0.693/k, \quad (2)$$

where A_0 is initial concentration of HMX, A is concentration of HMX at incubation time t , k is the degradation rate constant and $t_{0.5}$ is the time period required for 50% degradation of HMX. Best fit of plot of logarithm of HMX concentration against incubation time (Fig. 4) results in a k value of 0.06 day^{-1} and $t_{0.5}$ of 11.55 days.

The growth and proliferation of isolate S5-TSA-19 both in absence and presence of HMX along with degradation of latter indicates that HMX acts as a non growth substrate, i.e., metabolism of HMX does not directly support cell replication of the isolate. Thus isolate degrades HMX by cometabolism (Dalton and Stirling 1982). Similar cometabolic degradation of nitroaromatics and nitramines by anaerobic consortium and phytosymbiotic *Methylobacterium* sp. strain BJ001 have been reported earlier (Aken et al. 2004; Daun et al. 1998).

The temporal disappearance of HMX was concomitant with the formation of nitrite (NO_2^-) ion in the medium. It

Fig. 4 First-order kinetics plot of HMX degradation in presence of isolate S5-TSA-19

is well established that nitrite is a by-product of HMX degradation (Bhushan et al. 2003; Balakrishnan et al. 2003). To understand the mechanism of HMX degradation, nitrite estimation was carried out during incubation period. Nitrite concentration in HMX medium inoculated with isolate S5-TSA-19 increased significantly after 48 h of incubation and continues to increase up to 15 days and reached a maximum of 0.49 mg/L on 20th day (Fig. 5). While there is no significant increase in nitrite concentration (0.04 mg/L) in control media (HMX medium incubated without isolate). The minute release of nitrite in control may be released by abiotic degradation (photodegradation) of HMX. The release of 0.5 mol of NO_2^- per mole of HMX degraded indicates that degradation of HMX by isolate S5-TSA-19 follows

Fig. 3 HMX concentration variation over 20 days in the minimal salt medium in presence and absence of isolate S5-TSA-19. Error bars represents the standard deviation of three replicate samples per time point

Fig. 5 Time-dependent accumulation of nitrite (NO_2^-) in growth medium during incubation period. Error bars represents the standard deviation of three replicate samples per time point

Fig. 6 Mass spectra of secondary metabolites formed during biodegradation of HMX with isolate S5-TSA-19

single nitrite elimination path by transfer of a single electron to HMX (Bhushan et al. 2003). The release of nitrite ions as the first metabolite in HMX degradation by isolate S5-TSA-19 indicates a mechanism same as that of alkaline hydrolysis (Hoffsommer et al. 1977).

To further elucidate the pathways of HMX degradation the ring cleavage products of HMX were analyzed using MS. Positive electron spray ionization (ESI) revealed the presence of intermediate molecular ions having $[M+H]^+$ peaks at m/z of ~137 Da and 150 Da (Fig. 6) which are formed during ring cleavage of HMX following single nitrite ion elimination. This molecular ion fragments indicate the presence of metabolites, methylenedinitramine (MEDINA), $CH_4N_4O_4$ with MW 136 Da and

N-methyl-*N,N'*-dinitromethanedi-amine, $C_2H_6N_4O_4$ with MW 149 Da (Fournier et al. 2004; Zhao et al. 2007; Eaton et al. 2013; Balakrishnan et al. 2003). The metabolite with m/z 150 is a result of multiple degradation pathways involving methylenedinitramine intermediate via ring cleavage. On basis of the above-mentioned findings the proposed degradation pathway is given in Fig. 7.

In conclusion, isolate S5-TSA-19 degrades HMX very efficiently and renders itself to be a potential microbe for remediation of effluent and soil contaminated with HMX. This study is also the first report of HMX degradation by isolate S5-TSA-19, a native soil bacterium. Further study on the determination of enzymes responsible for degradation of HMX by isolate S5-TSA-19 is underway.

Fig. 7 A scheme of biodegradation of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) by isolate S5-TSA-19 under aerobic conditions

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is 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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