J Ind Microbiol Biotechnol (2009) 36:253–260
DOI 10.1007/s10295-008-0493-8

ORIGINAL PAPER

Bioremediation of nitroexplosive wastewater by an yeast isolate *Pichia sydowiorum* MCM Y-3 in fixed film bioreactor

S. P. Kanekar · P. P. Kanekar · S. S. Sarnaik · N. P. Gujrathi · P. N. Shede · M. R. Kedargol · K. F. Reardon

Received: 29 May 2008 / Accepted: 14 October 2008 / Published online: 1 November 2008

Abstract Nitroexplosives are essential for security and defense of the nation and hence their production continues. Their residues and transformed products, released in the environment are toxic to both terrestrial and aquatic life. This necessitates remediation of wastewaters containing such hazardous chemicals to reduce threat to human health and environment. Bioremediation technologies using microorganisms become the present day choice. High Melting Explosive (HMX) is one of the nitroexplosives produced by nitration of hexamine using ammonium nitrate and acetic anhydride and hence the wastewater bears high concentration of nitrate and acetate. The present investigation describes potential of a soil isolate of yeast *Pichia sydowiorum* MCM Y-3, for remediation of HMX wastewater in fixed film bioreactor (FFBR). The flask culture studies showed appreciable growth of the organism in HMX wastewater under shake culture condition within 5–6 days of incubation at ambient temperature (28 ± 2°C). The FFBR process operated in both batch and continuous mode, with Hydraulic Retention Time (HRT) of 1 week resulted in 50–55% removal in nitrate, 70–88% in acetate, 50–66% in COD, and 28–50% in HMX content. Continuous operation of the reactor showed better removal of nitrate as compared to that in the batch operation, while removal of acetate and COD was comparable in both the modes of operation of the reactor. Insertion of baffles in the reactor increased efficiency of the reactor. Thus, FFBR developed with baffles and operated in continuous mode will be beneficial for bioremediation of high nitrate and acetate containing wastewater using the culture of *P. sydowiorum.*

Keywords Bioremediation · *Pichia sydowiorum* · Fixed film bioreactor · Nitrate · Nitroexplosive

S. P. Kanekar · P. P. Kanekar (✉) · S. S. Sarnaik · N. P. Gujrathi · P. N. Shede · M. R. Kedargol
Microbial Sciences Division, Agharkar Research Institute, G.G.Agarkar Road, Pune 411004, Maharashtra, India
e-mail: kanekarp@rediffmail.com

K. F. Reardon
College of Biological Engineering, Colorado State University, Fort Collins, CO 80523-1370, USA

Introduction

Environmental contamination by nitro compounds is associated principally with industrial chemicals such as nitroexplosives, dyes, polyurethane foams, herbicides, insecticides, solvents, etc. Nitroexplosives are mainly used for military purposes in detonators, in nuclear weapons, primers, mines, and as a solid rocket propellant in rocket boosters. Most commonly used nitroexplosives are trinitrotoluene (TNT), Royal Demolition Explosive (RDX), and High Melting Explosive (HMX). Since these chemical compounds are essential for security and defense of any nation, their production cannot be stopped. These compounds are toxic to animals including human beings. They can cause methaemoglobinaemia in human beings and are harmful to the liver and central nervous system in rats, mice, and rabbits.

One of the currently used nitroexplosives is HMX i.e. High Melting Explosive. Chemically HMX is octahydro-1,3,5,7-tetranitro-1,3,5,7 tetrazocine; a powerful and relatively insensitive polynitramine high explosive, chemically related to RDX [6]. HMX is produced by a modified Bachmann process wherein nitration of hexamine is carried out

in presence of *p*-formaldehyde and ammonium nitrate using acetic anhydride as solvent. The HMX wastewater therefore contains high concentration of nitrate and acetate. HMX is a manmade chemical, does not occur naturally in the environment, becomes resistant to biological treatment and remains in the biosphere for longer time. Since it is toxic, the wastewater containing HMX needs treatment for removal of HMX from the source.

Wastewater treatment can be categorized by the nature of the treatment process used i.e. physical, chemical, or biological. Technologies such as bioremediation and composting are being developed as methods of remediating HMX contamination in a solid matrix. Some of the bioremediation methods used for removing nitrate from nitroexplosives wastewater as well as other nitro aromatic compounds are reviewed [15, 21]. Organic mulch permeable reactive barriers were used for bioremediation of RDX and HMX contaminated groundwater [1]. Biodegradation of cyclic nitramine explosives i.e. RDX, HMX, and CL-20 with respect to their hypothetical metabolic pathways and biodegradation mechanism was reviewed [7].

Degradation of HMX can occur both aerobically and anaerobically. A reference has been made on aerobic biodegradation of HMX [15, 25]. Members belonging to the family Enterobacteriaceae namely *Morganella morganii*, *Providencia rettgeri*, and *Citrobacter freundii* were isolated from explosive contaminated soils and were studied for their ability to transform HMX [19]. Comparatively more work has been carried out on degradation of HMX under anaerobic conditions. TNB, RDX, and HMX are metabolized by anaerobic bacterial consortia wherein the bacteria used these explosives as sole sources of nitrogen [4, 5, 13, 17, 19, 23, 28, 30, 32].

A variety of the fungal species are known to secrete enzyme systems degrading xenobiotic compounds including nitroexplosives. Some bacterial and yeast strains degrade xenobiotics such as phenol and nitrophenol [10]. The thermotolerant yeast strain of *Kluyveromyces marxianus* IMB-3 degraded iminodiacetate and nitrilotriacetate [27]. Degradation of Dinitrobenzene (DNB) and wastewater generated during its production by the yeast isolate *Candida pulcherima* was demonstrated [9]. A marine yeast isolate of *Yarrowia* transformed 2, 4, 6-trinitrotoluene [14]. Other marine bacteria metabolized cyclic nitramines including HMX [31, 33]. *Phenarochaete chrysosporium* [11] and other fungi i.e. species of *Acremonium*, *Penicillium*, *Rhodotorula*, and *Bullera* could degrade HMX [2, 3, 29].

The present investigation describes potential of a soil isolate of yeast, *Pichia sydowiorum* MCM Y-3 in removing nitrate and acetate from HMX wastewater in a fixed film bioreactor developed using the yeast culture.

Materials and methods

Characterization of the HMX wastewater

Wastewater generated during production of HMX was collected from High Energy Materials Research Laboratory (HEMRL), Pune as and when required and preserved in the laboratory at ambient temperature for further studies.

The wastewater was characterized for pH, Nitrate, Acetate, and Chemical Oxygen Demand (COD) as per Standard Methods [12].

pH pH of the wastewater was recorded immediately after collection of samples.

Nitrate Nitrate estimation was carried out using spectrophotometric method by measuring absorbance at 220 and 275 nm as described in Standard Methods and its modification [12, 24]. Nitrates were also estimated using Cyberscan Ion meter (Eutech Instruments, Singapore).

Acetate Acetate estimation was carried out using Gas Chromatograph (Perkin Elmer, USA) equipped with Flame Ionization Detector (FID) and SS packed column containing 10% Free Fatty Acid Phase (FFAP) with 3% H_3PO_4 as stationary phase. Extra pure grade nitrogen at the flow rate of 32 ml/min was used as carrier gas and mixture of Hydrogen and air (1:10, v/v) was used for ignition of flame. The temperatures of oven, injector, and detector were 140, 170 and 190°C, respectively. Standard Acetic acid (Sigma, USA) was used as reference standard.

Chemical Oxygen Demand (COD) COD of the wastewater was estimated by Open Reflux Method as described in Standard Methods using potassium dichromate in presence of sulfuric acid as oxidizing agent [12].

HMX analysis Estimation of HMX was performed using High Performance Liquid Chromatography—HPLC (Agilent, Germany; equipped with autosampler). Mobile phase used was 50% acetonitrile in water with a flow rate of 1 ml/min. HMX powder was used as a reference standard.

Isolation and identification of the microorganisms capable of growing on HMX wastewater

Two garden and one compost soil sample were used for isolation of micro-organisms for degradation of high nitrate containing HMX wastewater. Moisture content and pH of the soil samples were determined as per Standard Methods [12].

Isolation by enrichment method

Micro-organisms present in soil were enriched in wastewater so as to obtain isolates capable of growing on wastewater.

Ten gram of soil sample was suspended in 90 ml 1:10 diluted and neutralized HMX wastewater (WWB) in 250 ml flask. The flasks were incubated on shaker at 120 rpm at ambient temperature (28 ± 2°C) for 7 days. Four weekly transfers were carried out using 10% inoculum. A total of 0.1 ml of enrichment culture was spread on Wastewater Agar (100 ml wastewater solidified with 2 g% agar i.e. WWA). The plates were incubated at ambient temperature (28 ± 2°C) for 5–7 days. Well isolated distinct colonies grown on WWA were transferred on nutrient agar plates for checking morphological characteristics of the isolate and maintained on WWA for further studies.

Baiting method

Neutralized wastewater was diluted 1:10 and added to 200 g soil sample in a container so as to maintain moisture content around 30%. The containers were incubated at ambient temperature (28 ± 2°C) and under stationary condition for 15 days. 10 g% suspension of the baited soil sample was diluted serially tenfold and 0.1 ml of 10^{-5} and 10^{-6} dilutions were spread on WWA. The plates were incubated for 1 week at ambient temperature (28 ± 2°C).Well isolated colonies were streaked on NA plates for further pure culture studies.

The isolated colonies from WWA were inoculated in WWB and growth was measured at 540 nm at 24 h interval till 7 days. The isolates were selected based on their growth in 4–7 days.

Testing the isolates for tolerance to high concentration of nitrate and acetate

The selected three isolates were tested for nitrate and acetate tolerance. Davis Mingioli's synthetic medium (DM) [8] was modified by replacing ammonium sulphate with ammonium nitrate and glucose/citric acid with sodium acetate (denoted as SM) for screening of the isolates.

To check tolerance of the isolates to nitrate, SM medium was used with sodium acetate (0.2 g%) and different concentrations of ammonium nitrate in the range of 0.1–5 g% (synthetic nitrate medium). Five-day-old growth in WWB was streaked on SM slants. The slants were incubated at ambient temperature (28 ± 2°C) for 5 days. The growth on SM slants was observed daily.

To check tolerance of the isolates to acetate, SM medium was used with ammonium nitrate (0.1 g %) and different concentrations of sodium acetate in the range of 0.2–5 g% (synthetic acetate medium). Five-day-old growth in WWB was streaked on SM slants. The slants were incubated at ambient temperature (28 ± 2°C) for 5 days. The growth on SM slants was observed daily for growth.

The five-day-old culture from SM slants was inoculated in SM broth containing 4 g% nitrate and 2 g% acetate and WWB and incubated at ambient temperature (28 ± 2°C) for 5 days. The growth was measured in terms of optical density (OD at 540 nm).

Based on the tolerance to nitrate and acetate in SM medium and growth in WWB, the isolate S_1III was selected for further studies.

Identification of the selected isolate

Since microscopic observation revealed the organism to be yeast, it was grown on glucose yeast extract peptone (GYP) agar medium and studied for morphological characteristics by coverslip culture technique. Biochemical tests performed to characterize and taxonomically determine the culture included sugar fermentation tests, nitrate reduction test, gelatin liquefaction test, and utilization of different carbon and nitrogen sources in yeast nitrogen base and yeast carbon base medium, respectively [20, 22].

Growth of the isolate in the wastewater

In order to find suitable culture condition for the growth of the isolate, the culture was inoculated in wastewater of pH 5, 6, 7, and 8 and incubated at ambient temperature (28 ± 2°C) under both stationary and shake culture conditions. Growth of the culture was monitored at 24 h interval up to 12 days in terms of absorbance at 540 nm using Qquant ELISA reader (Biorad, USA).

Development of fixed film bioreactor (FFBR) using the culture of *Pichia sydowiorum*

A fixed film bioreactor was developed in a 10 L capacity rectangular container of dimensions 36 × 24 × 15 cm using brick pieces as supporting medium for formation of biofilm of the culture of *P. sydowiorum*. To have better performance of the reactor in terms of increase in the residence time of fluid in the reactor, to increase the contact with microorganisms, to avoid channeling of the feed stream throughout the reactor and also to avoid short circuiting in the reactor flow, it was thought worthwhile to use baffles across the supporting media i.e. brick pieces (Fig. 1). The flow in the horizontal direction with baffle inserted in the path may result in better residence time distribution for the feed stream in the reactor.

Five kilogram brick pieces having dimensions as 2.5 × 3.5 × 3.5 cm were placed randomly in 10 L plastic container. Ninety-six hour grown culture broth (2.5 L) of *P. sydowiorum* having cell density of 1.65×10^7 cells/ml (based on direct microscopic count) was mixed with 2.5 L of neutralized 1:10 diluted wastewater. The wastewater was

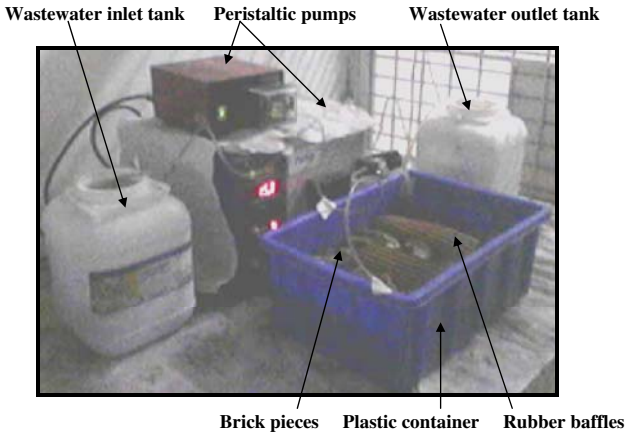

Fig. 1 Fixed Film Bioreactor developed for bioremediation of HMX wastewater using a culture of *Pichia sydowiorum*

recycled using peristaltic pump, through brick bed for 5 days at ambient temperature (28 ± 2°C) for formation of film on brick pieces used as supporting media. After development of the film of the organism as evidenced by the growth on brick pieces and microscopic observations of the culture showing typical oval yeast cells and formation of pseudomycelium, the wastewater was recirculated through brick bed using peristaltic pump.

FFBR: batch process (reactor 1)

Four litre of fresh wastewater was recycled through the reactor at the flow rate of 0.7 ml/min with residence time of 4 days and the performance of the reactor was monitored up to 168 h with respect to removal of nitrate, acetate, COD, and change in pH of the wastewater at 24 h interval by analyzing the cell free supernatant obtained after centrifugation of the samples at 14,000 rpm for 15 min at 20°C (Kubota 6930, Japan). pH was adjusted to 6 by addition of 1:10 diluted *o*-phosphoric acid at 24 h interval. The experiment was performed in duplicate and the results given are the average values of two experiments.

FFBR with baffles: batch process (reactor 2)

Two rubber baffles (rubber sheets) of length 18 cm and breadth 12 cm were introduced breadth wise in the reactor at a distance of 12 cm from each end in opposite direction. Evaluation of the reactor was carried out in duplicate as mentioned earlier.

FFBR with baffles: continuous process (reactor 3)

The FFBR with baffles was operated in continuous mode using peristaltic pumps with working volume of 4 L. Fresh wastewater was added at the flow rate at 0.7 ml/min with residence time of 4 days. The reactor was run for 168 h. pH was adjusted to 6 by addition of 1:10 diluted *o*-phosphoric acid. Performance of the reactor was evaluated by analyzing wastewater samples at 24 h interval as described earlier. The experiment was carried out in duplicate as mentioned earlier.

Statistical analysis

Performance of all the three reactors with reference to reduction in nitrate, acetate, and COD content of the waste water was evaluated statistically using student's *t* test and also by applying one-way analysis of variance (ANOVA) method to determine *F* values [18].

Results and discussions

Characterization of the HMX wastewater

Chemical characterization of HMX wastewater is detailed in Table 1. pH of the wastewater is found to be 2.38 and hence could be stored at ambient temperature till study. Since production of HMX is carried out using acetic acid and acetic anhydride as solvent, it is quite obvious that pH of the wastewater is acidic in nature. This fact also contributes to the high acetate content of the wastewater i.e. to the extent of 227,000 mg/L. There are no permissible limits specified by Maharashtra Pollution Control Board (MPCB) for acetate content but still the acetate content of the wastewater can be considered as very high. COD of the wastewater was also found to be around 300,000 mg/L and limits laid down by MPCB are 250 mg/L. The production of HMX involves nitration of hexamine using concentrated nitric acid and ammonium nitrate as raw materials and hence the nitrate content of the wastewater is very high i.e. of the magnitude of 342,350 mg/L. The permissible limit of MPCB for nitrate is only 45 mg/L. Production of HMX is a batch process, hence recovery of HMX varies from batch to batch and average amount of HMX estimated from wastewater was ~250 mg/L which again seems to be very high and far above the limits of MPCB.

Table 1 Characterization of HMX Wastewater

Parameters	Concentration	MPCB limit
pH	2.38	6.5–8.5
Nitrate	342,350 mg/L	45 mg/L
Acetate	227,000 mg/L	–
COD	300,000 mg/L	250 mg/L
HMX	254 mg/L	0.03 mg/L

Because of the acidic pH and high concentration of acetate, nitrate, and HMX in the wastewater, it was necessary to neutralize the wastewater and then dilute it suitably i.e. 1:10 (v/v) with water (designated as WWB), so as to have nitrate (34,235 mg/L), acetate (22,700 mg/L), and HMX (25 mg/L) concentration suitable for growth of microorganisms.

Isolation and identification of the microorganisms capable of growing on HMX wastewater

By enrichment method 12 isolates and from baiting method 2 isolates were found to grow on WWA in 5–7 days. Thus, in the present studies total 14 isolates were obtained.

The three isolates namely S_1III (Soil sample S_1, III week of enrichment transfer), S_1I (Soil sample S_1, baiting method) and Su (Soil sample S_2, baiting method) were selected on the basis of their ability to grow in WWB and WWA in 4–7 days at ambient temperature (28 ± 2°C). The other isolates required 7–10 days for growth.

Based on the criteria of maximum tolerance and growth in synthetic nitrate medium and WWB, the isolate S_1 III was selected for bioremediation studies. The isolate S_1 III was identified as *P. sydowiorum* based on its morphological features and biochemical tests [20, 22].

The culture is deposited in MACS Collection of Microorganisms (MCM) recognized by World Federation of Culture Collection (WFCC—Registration Number 561) with the accession number as MCM Y-3 and preserved as glycerol stocks at −20°C. The isolate was able to grow in the wastewater indicating its ability to utilize acetate as carbon source and nitrate as nitrogen source for its growth. This isolate was also able to tolerate nitrate at the concentration of 4 g% i.e. 40,000 mg/L and acetate at 2 g% i.e. 20,000 mg/L when incorporated in Davis Mingioli's synthetic medium. The culture was used for carrying out studies on bioremediation of 1:10 diluted wastewater in FFBR.

Growth of *Pichia sydowiorum* in wastewater

Growth of *P. sydowiorum* in wastewater (WWB) under stationary and shake culture condition is illustrated in Fig. 2. It was observed that the shake culture condition and pH 6 is suitable for growth of the organism as there is exponential increase in the growth during incubation period of 48–96 h. Maximum growth of the organism was seen at 96 h. Stationary culture condition was found to be unsuitable for growth in WW as there is long lag phase till 96 h. Similarly, growth at pH 5, 7, and 8 were unsuitable for growth in WW (Fig 3).

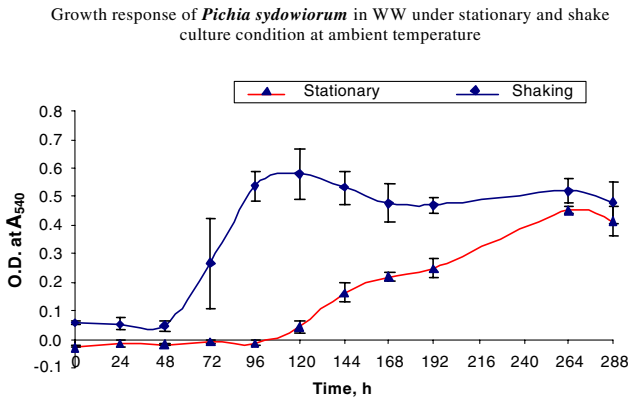

Fig. 2 Growth of *Pichia sydowiorum* in wastewater (WW) under stationary and shake culture condition

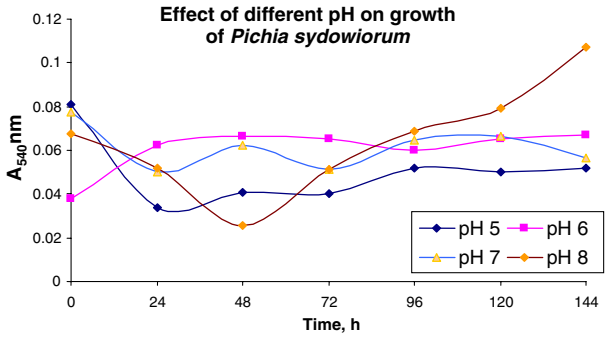

Fig. 3 Effect of different pH on growth of *Pichia sydowiorum* under shake culture condition

Performance of FFBR

FFBR: batch process (reactor 1)

The results of the change in pH of the wastewater and removal of acetate, nitrate and COD of the wastewater during batch process in FFBR (reactor 1) are presented in Fig. 4. It was seen that pH of the treated wastewater in the bioreactor increased from initial 6 to 8 during the process which may probably due to formation of ammonia qualitatively detected by turmeric paper and smell. The reduction in nitrate content was in the range of 1–50% (average 27%), acetate content from 26 to 69% (average 58%), COD content from 6 to 58% (average 32%) and HMX removal was ~29% after 168 h of incubation. All these results indicated potential of the yeast culture *P. sydowiorum* in bioremediation of high nitrate and acetate containing wastewater generated during manufacturing of HMX.

FFBR with baffles: batch process (reactor 2)

The results of the change in pH of the wastewater and removal of acetate, nitrate, and COD of the wastewater

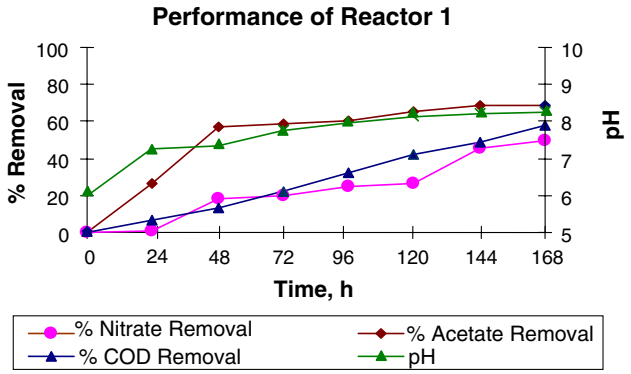

Fig. 4 Performance of reactor 1 with respect to removal of nitrate (34, 235 mg/L), acetate (22,700 mg/L), and COD (30,000 mg/L) from the wastewater and change in pH

during batch process (reactor 2) are illustrated in Fig. 5. It was observed that the pH of the wastewater increased from initial 6 to 8. The reduction in nitrate content was in the range of 7–51% (average 31%), acetate content from 63 to 90% (average 79%), COD content from 10 to 50% (average 30%) and HMX removal was ~28% after 168 h of incubation. Thus insertion of baffles in the reactor enhanced removal of acetate from the HMX wastewater.

FFBR with baffles: continuous process (reactor 3)

The results of the change in pH of the wastewater and removal of acetate, nitrate, and COD of the wastewater during continuous process in FFBR with baffles (reactor 3) are presented in Fig. 6. The reduction in nitrate content was in the range of 25–55% (average 48%), acetate content from 42 to 85% (average 68%), COD content from 6 to 67% (average 37%) and HMX removal was 50% after 168 h. Thus, the reactor when operated in continuous mode increased the removal of both nitrate and acetate to a considerable extent. However, removal of COD was not

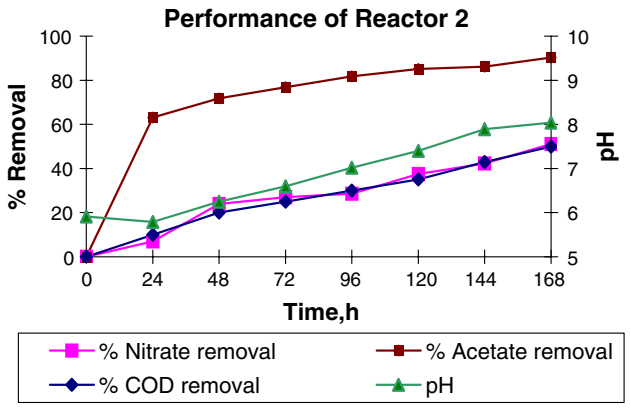

Fig. 5 Performance of reactor 2 with respect to removal of nitrate (34, 235 mg/L), acetate (22,700 mg/L), and COD (30,000 mg/L) from the wastewater and change in pH

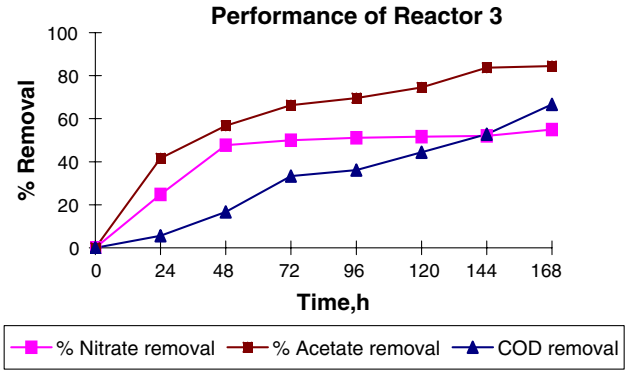

Fig. 6 Performance of reactor 3 with respect to removal of nitrate (34, 235 mg/L), acetate (22,700 mg/L), and COD (30,000 mg/L) from the wastewater

enhanced by either due to insertion of baffles or mode of operation. Since, growth of the organism was optimum at pH 6 and the process was continuous, the pH of the wastewater during operation was maintained at 6 by addition of dilute ortho-phosphoric acid. The removal of HMX was remarkably enhanced from 28% in batch process to 50% in continuous process.

The comparative performance of the three reactors in removal of nitrate, acetate, and COD of the HMX wastewater based on their average values (Table 2) was statistically analyzed. When student *t* test was applied (Table 3), it was seen that both, insertion of baffles as well as operation of the reactor in continuous mode enhanced the removal of nitrate from the HMX wastewater. In particular, this removal enhanced to a greater extent with insertion of baffles as indicated by higher calculated *t* value (15.94) than tabulated *t* value (2.179) at 5% level. In case of acetate

Table 2 Performance of reactors with respect to removal of pollutants

% Removal (average)	Reactors		
	1	2	3
Nitrate	27	31	48
Acetate	58	79	68
COD	32	30	37

Table 3 Application of Student *t* test for the comparative performance of the three bioreactors

	Reactors		
	1 and 2	1 and 3	2 and 3
Nitrate *t* value	0.55^{NS}	15.94*	4.41*
Acetate *t* value	3.47*	1.42^{NS}	1.67^{NS}
COD *t* value	0.16^{NS}	-5.84^{NS}	0.65^{NS}
Tabulated *t* value		2.179	

* Statistically significant at 95% confidence interval

^{NS} Statistically not significant

Table 4 Application of one-way ANOVA i.e. *F* test for of the efficiency of bioremediation for three bioreactors

F value	Nitrate	4.31*
F value	Acetate	4.96*
F value	COD	0.22^{NS}
F tabulated value		3.55

* Statistically significant at 95% confidence interval

^{NS} Statistically not significant

removal, the insertion of baffles has enhancing effect as indicated by higher *t* value (3.47) and mode of operation of the reactor has no effect on the acetate removal. Likewise there is hardly any effect of either insertion of baffles or continuous operation on performance of reactor in removing organic load (COD) from HMX wastewater.

These results were also subjected to one-way ANOVA test and the results are detailed in Table 4. It is seen that the efficiency of the reactor in removing nitrate and acetate depends upon the mode of operation of bioreactor, as *F* value for nitrate is 4.31 while *F* value for acetate is 4.96 both of which are higher than the tabulated 3.55 *F* value. There was no significant effect of mode of operation on the removal of organic load (COD) as indicated by low *F* value (0.22).

Overall, the experimental results indicated that the reactor with baffles operated in continuous mode will be beneficial in removing nitrate and acetate from the wastewater treated in FFBR using the yeast culture of *P. sydowiorum.*

Some yeast cultures and white rot fungi have been reported for degradation of nitro compounds and nitro explosives [2, 9, 11, 14, 27, 29]. However, these cultures have not been studied for bioremediation of wastewaters containing these nitro compounds. Some information is known on bioremediation of soils contaminated with these nitroexplosives [19, 26, 28] however, meager data are available on the bioremediation of water or wastewater containing these nitroexplosives [1, 4, 23, 25]. Kanekar and Godbole [16] have demonstrated the removal of TNT from wastewater generated during production of TNT using bacterial cultures belonging to the genera *Bacillus*, *Pseudomonas*, *Citrobacter*, *Micrococcus*, etc. Likewise Dey et al. [9] have described degradation of Dinitrobenzene (DNB) and wastewater generated during its production by the yeast isolate *Candida pulcherima*. However, all these studies were at flask level and not using a bioreactor.

This paper describes the process used for bioremediation of HMX wastewater using a yeast culture of *P. sydowiorum* and a fixed film bioreactor developed using easily available and cheap material like brick pieces for supporting the film formed by the organism.

Conclusions

The yeast culture *P. sydowiorum* capable of growing in 1:10 diluted and neutralized wastewater was able to tolerate nitrate at the concentration of 40,000 mg/L and acetate at 20,000 mg/L when incorporated in Davis Mingioli's synthetic medium. A FFBR was set up using brick pieces as solid support for formation of biofilm of the culture of *P. sydowiorum* for remediation of wastewater of pH 6. The FFBR was run in both batch and continuous mode. The reactor resulted in reduction to the extent of 50–55% in nitrate, 70–88% in acetate, 50–66% in COD and 28–50% in HMX content.

The present study showed potential of *P. sydowiorum,* a soil isolate in bioremediation of HMX wastewater and also for bioremediation of high acetate, especially high nitrate containing wastewaters generated during manufacturing of other nitroexplosives, nitroaromatic compounds, pesticides, dyes, etc.

Acknowledgments Part of the work was carried out under Indo-US project sponsored by Indo-US Science and Technology Forum (IUSSTF), Govt of India and US Govt. The authors thank authorities of HEMRL, Pune for providing HMX wastewater and related information.

References

1. Ahmad F, Schnitker SP, Newell CJ (2007) Remediation of RDX- and HMX-contaminated groundwater using organic mulch permeable reactive barriers. J Contam Hydrol 90(1–2):1–20. doi:10.1016/j.jconhyd.2006.09.005
2. Bhatt M, Zhao JS, Halasz A, Hawari Z (2006) Biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine by novel fungi isolated from unexploded ordnance contaminated marine sediment. J Ind Microbiol Biotechnol 33(10):850–858. doi:10.1007/s10295-006-0136-x
3. Bhatt M, Zhao JS, Monteil-Rivera F, Hawari Z (2005) Biodegradation of cyclic nitramines by tropical marine sediment bacteria. J Ind Microbiol Biotechnol 32(6):261–267. doi:10.1007/s10295-005-0239-9
4. Bhushan B, Halasz A, Thiboutot S, Ampleman G, Hawari J (2004) Chemotaxis-mediated biodegradation of cyclic nitramine explosives RDX, HMX, and CL-20 by *Clostridium* sp. EDB2. Biochem Biophys Res Commun 316(3):816–821. doi:10.1016/j.bbrc.2004.02.120
5. Boopathy R, Gurgas M, Ullian J, Manning JF (1998) Metabolism of explosive compounds by sulfate-reducing bacteria. Curr Microbiol 37(2):127–131. doi:10.1007/s002849900350
6. Cooper PW, Card ER, Autenrieth R (1998) Treatment of HMX and RDX contamination in explosives engineering. Wiley-VCH, New York
7. Crocker FH, Indest KJ, Fedrickson KL (2006) Biodegradation of the cyclic nitramine explosives RDX, HMX, and CL-20. Appl Microbiol Biotechnol 73(2):274–290. doi:10.1007/s00253-006-0588-y
8. Cruickshank RL (1965) Medical microbiology, 11th edn. The English Language Book Society, London
9. Dey S, Kanekar P, Godbole SH (1986) Aerobic microbial degradation of m-Dinitrobenzene. Indian J Environ Health 29(2):118–128

10. Dimkov R, Topalova Y (1993) Dependence of the microbiological degradation of aryl-containing xenobiotics on their concentration. Acta Microbiol Bulg 29:9–16
11. Fournier D, Halasz A, Thiboutot S, Ampleman G, Manno D, Hawari J (2004) Biodegradation of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) by *Phenerochaete chrysosporium*: new insight into the degradation pathway. Environ Sci Technol 38(15):4130–4133. doi:10.1021/es049671d
12. Greenberg AE, Clesceri LS, Eaton AD (1992) Standard methods for the examination of water and wastewater. American Public Health Association (APHA), American Water Works Association, Water Environment Federation, Washington DC
13. Hawari J, Halasz A, Beaudet S, Paquet L, Ampleman G, Thiboutot S (2001) Biotransformation routes of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine by municipal anaerobic sludge. Environ Sci Technol 35(1):70–75. doi:10.1021/es0013531
14. Jain MR, Zinjarde SS, Deobagkar DD, Deobagkar DN (2004) 2, 4, 6-trinitrotoluene transformation by a tropical marine yeast *Yarrowia lipolytica* NCIM3589. Mar Pollut Bull 49(9–10):783–788. doi:10.1016/j.marpolbul.2004.06.007
15. Kanekar PP, Dautpure PS, Sarnaik SS (2003) Biodegradation of nitroexplosives. Indian J Exp Biol 41:991–1001
16. Kanekar PP, Godbole SH (1984) Microbial degradation of Trinitrotoluene (TNT). Indian J Environ Health 20(2):89–101
17. Kaplan DL (1993) Biotechnology and bioremediation for organic energetic compounds. In Organic Energetic Compounds P. Marinkas, (ed). Nova Science Publishers, Inc., New York
18. Khan IA, Khanum A (eds) (1994) Fundamentals of Biostatistics. Ukaaz Publications, Hyderabad
19. Kitts CL, Cunningham DP, Unkefer PJ (1994) Isolation of three hexahydro-1,3,5-trinitro-1,3,5-triazine-degrading species of the family Enterobacteriaceae from nitramine explosive-contaminated soil. Appl Environ Microbiol 60(12):4608–4611
20. Krutzman CP, Fell JW (eds) (2000) The yeasts: a taxonomic study. Elsevier Science B. V, Netherlands
21. Kulkarni M, Chaudhari A (2007) Microbial remediation of nitroaromatic compounds: an overview. J Environ Manage 85(2):496–512. doi:10.1016/j.jenvman.2007.06.009
22. Lodder J (1970) The yeasts: a taxonomic study, 2nd edn. North-Holland Publishing Company, Netherlands
23. McCormick NG, Cornell JH, Kaplan AM (1984) The fate of hexahydro-1,3,5- trinitro-1,3,5-triazine (RDX) and related compounds in anaerobic denitrifying continuous culture systems using simulated wastewater. Tech Rep Natick TR-85(008):ADA149462. US Army Natick Research and Development Center, Natick
24. Shivran HS, Kumar D, Singh RV (2006) Improvement of water quality through biological denitrification. J Environ Sci Engin 48(1):57–60
25. Spanggord RJ, Mabey WR, Mill T, Chou TW, Smith JH, Lee S, Robert D (1983) Environmental fate studies on certain munitions wastewater constituents. Phase IV-lagoon model studies. *ADA138550*. US Army Medical Research and Development Command, Fort Detrick, Frederick
26. Strietelmeier BA, Coyne PJ, Leonard PA, Miller WL, Brian JR (2006) Initial characterization of a highly contaminated high explosives outfall in preparation for in situ bioremediation. http://www.osti.gov/energycitations/servlets/purl/752372-4ucf9X
27. Ternan NG, McMullan G (2002) Iminodiacetate and nitrilotriacetae degradation by *Kluyveromyces marxianus* IMB 3. Biochem Biophys Res Commun 290(2):802–805. doi:10.1006/bbrc.2001.6233
28. Toumi E, Coover MP, Stroo HF (1997) Bioremediation using composting or anaerobic treatment for ordnance-contaminated soils. Ann NY Acad Sci 829:160–178. doi:10.1111/j.1749-6632.1997.tb48573.x
29. Van Aken B, Agathos SN (2001) Biodegradation of nitro substituted explosives by white-rot fungi: a mechanistic approach. Adv Appl Microbiol 48:1–77. doi:10.1016/S0065-2164(01)48000-2
30. Zhao JS, Greer CW, Thiboutot S, Ampleman G, Hawari J (2004) Biodegradation of the nitramine explosives hexahydro-1,3,5-trinitro-1,3,5-triazine and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine in cold marine sediment under anaerobic and oligotrophic conditions. Can J Microbiol 50(2):91–96. doi:10.1139/w03-112
31. Zhao JS, Manno D, Hawari J (2007) Abundance and diversity of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX)-metabolizing bacteria in UXO-contaminated marine sediments. FEMS Microbiol Ecol 59(3):706–717. doi:10.1111/j.1574-6941.2006.00248.x
32. Zhao JS, Paquet L, Halasz A, Manno D, Hawari J (2004) Metabolism of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine by *Clostridium bifermentans* strain HAW-1 and several other H2-producing fermentative anaerobic bacteria. FEMS Microbiol Lett 237(1):65–72. doi:10.1111/j.1574-6968.2004.tb09679.x
33. Zhao JS, Spain J, Thiboutot S, Ampleman G, Greer C, Hawari J (2004) Phylogeny of cyclic nitramine degrading psychrophilic bacteria in marine sediment and their potential role in the natural attenuation of explosives. FEMS Microbiol Ecol 49(3):349–357. doi:10.1016/j.femsec.2004.04.008