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Mesoporous molecular sieve (MCM-41) as support material for microbial cell immobilization and transformation of 2,4,6-trinitrotoluene (TNT): a novel system for whole cell immobilization☆

Avinash M. Tope [a], N. Srinivas [b], S.J. Kulkarni [b,*], Kaiser Jamil [a]

[a] *Environmental Biotechnology Division, Indian Institute of Chemical Technology, Hyderabad 500007, India*
[b] *Catalysis Division, Indian Institute of Chemical Technology, Hyderabad 500007, India*

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Abstract

The physical immobilization of whole microbial cells of *Arthrobacter* sp., *Bacillus subtilis* and *Micrococcus luteus* on the mesoporous molecular sieve of MCM-41 was studied. Cells of *Arthrobacter* sp. immobilized on the matrix of MCM-41 could be successfully employed in the treatment of 2,4,6-trinitrotoluene (TNT) and its subsequent transformation to amino products. Some significant advantages observed in this novel method of immobilization were the proliferation of whole cells while on the matrix of MCM-41, increase in tolerance (from 60 to 400 mg l^{-1}) to TNT, repeated use of the immobilized cells for 20–23 cycles and total regeneration of support material.

Keywords: Immobilization; MCM-41; Molecular sieve; *Arthrobacter* sp.

1. Introduction

Immobilized cell systems are of great significance as they find applications in biomedicals, biosensors and processes for commodity and specialty chemical products. In wastewater treatments, encapsulated cell systems with well-defined microbial cultures are specially employed either for production of methane, removal of organic materials, radioactive wastes and heavy metals [1]. The principal advantage in all the immobilized techniques devised so far is that, they provide a means for retention of microorganisms within a reactor at concentrations well above those that would normally exist due to suspended growth alone. Hence, captured cell systems allow biological reactors to operate at much higher loading rates than suspended culture reactors. However, artificially captured cells enzymatically mediate conversion processes that are usually associated with loss in activity with time.

Many immobilization techniques have been reported, using inorganic support materials like activated carbon, ground glass, aluminosilicates, etc. These materials have been shown to be excellent carriers for immobilized enzymes and whole cells [2–5]. But many of these methods suffer from innate limitations of (a) leaching of the biocatalyst with time, (b) denaturation of biomolecules and blockage in in situ growth of microbial cells, and (c) resistance shown by the

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* Corresponding author. Tel.: 91-40-7173874; fax: +91-40-7173757.
E-mail address: sjkulkarni@iict.ap.nic.in (S.J. Kulkarni).

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matrix material towards inflow of nutrients/reactants and outflow of products. The support should allow the immobilization of large quantities of enzyme/viable whole cells with well induced catalytic activity, as well as provide a robust physical and chemical environment and reusability of the support. However, most of these support materials, such as activated charcoal, cannot be regenerated.

Zeolites are aluminosilicates having uniform pores and crystalline structures with molecular sieve properties. These are often used in many acid catalyzed reactions over a wide temperature range (60–550°C). These substances usually find applications in adsorption and separation processes, environmental pollution control [6,7], petrochemical processes [8,9], fine chemical synthesis [10,11] and in biochemistry for synthesis of nanocomposite materials [12], etc. Mesoporous molecular sieve (MCM-41), a uniformly porous material that has regular hexagonal mesoporous structure with molecular sieve properties was first reported by Mobil researchers in 1992. The unique physical properties of MCM-41 have made this material highly desirable for catalytic applications. The extremely high specific areas ($>1000\,m^2\,g^{-1}$) are conducive for high catalytic activity. The large pore size range (20–100 Å) [13,14] allows fixation of large active complexes, to act as 'host' for photo-induced electron transfer reactions with bulky substrates [15], as conducting polymers and for accommodating small enzymes, metal complexes within the channels [16–18], etc. This material was investigated as a support matrix for immobilization of microbial whole cells since it has an optimum balance of more than one parameters like the pore diameters and surface area, hydrophilicity, hydrophobicity, mechanical and chemical resistance and also offers scope for development of electrostatic interactions.

2,4,6-Trinitrotoluene (TNT) is a mutagen and believed to be a recalcitrant compound [19,20]. On long exposure, it is known to cause severe diseases such as aplastic anemia, toxic jaundice, less serious being skin rashes, mucosal hemorrhages in humans. Its mutagenic nature decreases with reduction of the nitrogroups to amino [21]. TNT and other nitrotoluenes released into the environment from munitions manufacturing plants in the form of effluents as well as the retention of obsolete (unused) munitions pose a great difficulty in their safe environmental management. A number of studies have been carried out using the matrix of activated carbon for selective removal of TNT from the wastewaters [22–25]. But these methods suffer from some severe limitations in that, once used, the carbon containing the high-energy materials poses danger even in the process of incineration. Furthermore, incineration is the only chemical option in remedying TNT without biological intervention. However, the process is neither economically viable nor environmentally safe. So far, only one fungus, viz. *Phanerochaete chrysosporium* has been reported to mineralize TNT [26]. But many microbes, including *P. chrysosporium* metabolize TNT by reductive pathways, yielding to reduction products like 4-amino dinitrotoluene (4-ADNT), 2-amino dinitrotoluene (2-ADNT), 2,4-diaminonitrotoluene (2,4-DANT), etc. [27,28].

In this paper we report for the first time, physical immobilization of bacterial cells of *Bacillus subtilis* ((0.3–2.2) μm × (1.2–7) μm), *Arthrobacter* sp. ((0.4–0.81) μm × 1.6 μm), *Micrococcus luteus* (0.5 μm × 3.5 μm) being selected keeping the differences in their cell dimensions in view, on the support MCM-41. The catalytic activity of the immobilized cells of *Arthrobacter* sp. was studied in biotransformation of TNT to its reduction products 4-ADNT, 2-ADNT and 2,4-DANT, compared with and without immobilization on MCM-41.

2. Experimental

2.1. General

TNT (99%) was procured from Government sources, while 4-ADNT, 2-ADNT were generous gifts from Thomas Lewis, University of Idaho, Moscow. 2,4-DANT was kindly made available by Ron Spanggord, SRI International Palo Alto, CA, US.

2.2. Synthesis of MCM-41

MCM-41 was synthesized as reported in [13], 0.3 g of NaOH, 0.68 g of aluminum isopropoxide were taken in 20 ml water and stirred while heating till a clear solution was formed. Then 9.4 ml of tetraethylammonium hydroxide (TEAOH) was added dropwise while cooling. This was added to

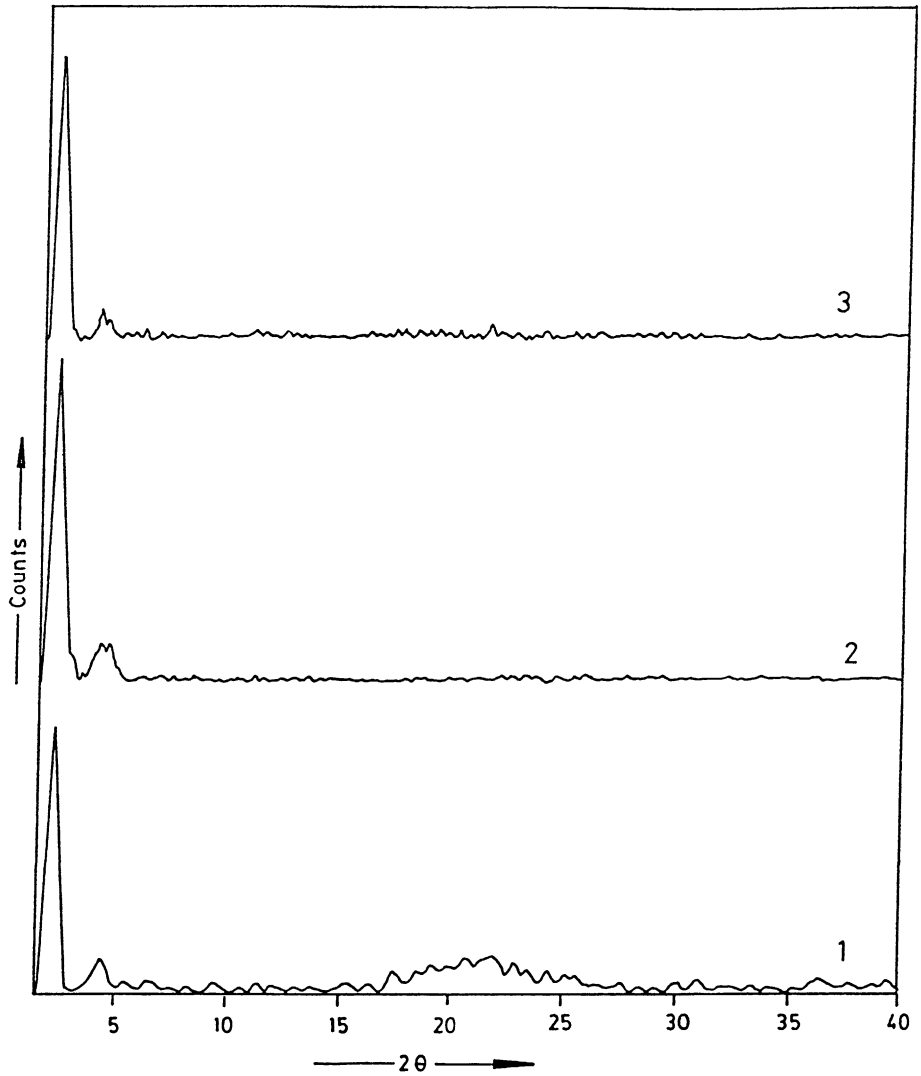

Fig. 1. XRD pattern of MCM-41. (1) Uncalcined; (2) calcined; (3) regenerated.

ludox silica (11.5 ml) in 50 ml water with vigorous stirring. This solution was stirred for another 1 h, then 10.5 g of hexadecyltrimethyl ammoniumbromide (HDTMABr) was added. The final pH was adjusted to 11.5. This gel was transferred into a Teflon coated autoclave and heated at 120°C for 24 h and calcined at 500°C for overnight. The XRD patterns of the as synthesized and calcined MCM-41 were compared and matched with those reported in the literature (Fig. 1).

2.3. Growth of the microorganisms

Cultures of *B. subtilis* (MTCC 121) and *M. luteus* (MTCC 1538) were procured from microbial type culture collection (MTCC), Chandigarh, India and *Arthrobacter* sp. (NCIM 2989) from national collection of industrial microorganisms (NCIM), Pune, India. Cells of all cultures were grown in a medium of the following composition: yeast extract 2%, peptone 5%, Na_2HPO_4 0.1%, NaH_2PO_4 0.2%, NaCl

0.5%, $MgSO_4 \cdot 7H_2O$ 0.02%, and 60 mg l^{-1} TNT (pre-dissolved in 3 ml acetone). Growth was monitored by absorbance (A_{600} nm) and viability. The values of A_{600} nm were found to be 1.78 (+0.05), 2.0 (+0.2) and 1.6 (+0.02) for *B. subtilis*, *Arthrobacter* sp. and *M. luteus*, respectively.

2.4. Immobilization set-up

The immobilization process was carried out in a down flow, fixed bed, pyrex reactor of 20 mm internal diameter, with a provision for continuous supply of disinfected air and nutrients. Pre-calcined MCM-41 (5 g) was placed into the reactor on the support of glass wool. The cells, contained in the liquid medium were collected in a syringe (50 ml) and mounted on an infusion pump (B Braun). Liquid culture containing the cells was trickled over the fixed bed of MCM-41 with a flow rate of 2 ml h^{-1}. Efficiency of immobilization was estimated through the difference in the A_{600} nm values (using Shimadzu UV–VIS 210A spectrophotometer) before and after trickling on the support material. The cells once immobilized were maintained in a supply of disinfected air (Fig. 2).

2.5. Transformation of TNT by Arthrobacter sp.

Immobilization of cells on MCM-41: to the immobilized cells, the minimal salt medium (50 ml) containing 20 mg TNT was used throughout the experiment. This was trickled over the packed bed with cells with a flow rate of 2 ml h^{-1}. Conversion of TNT was monitored every 24 h.

2.6. Analysis of products

After every 24 h, the eluted liquid (~48 ml) was extracted with ethyl acetate (100 × 2 ml), separated from the aqueous phase and was dried using anhydrous sodium sulfate. The dried ethyl acetate was evaporated in vacuum and the remaining mixture was reconstituted in acetone (2 ml) for further analytical work.

The products were separated and confirmed by TLC, GC–MS, respectively. For TLC, a solvent system of toluene and acetic acid (8:2 v/v), respectively, was used. Compounds were matched with the standards. GC–MS analysis was performed on an HP-5890 II model, using an HP-5 glass capillary column (30 m × 0.25 mm i.d.), where the column was

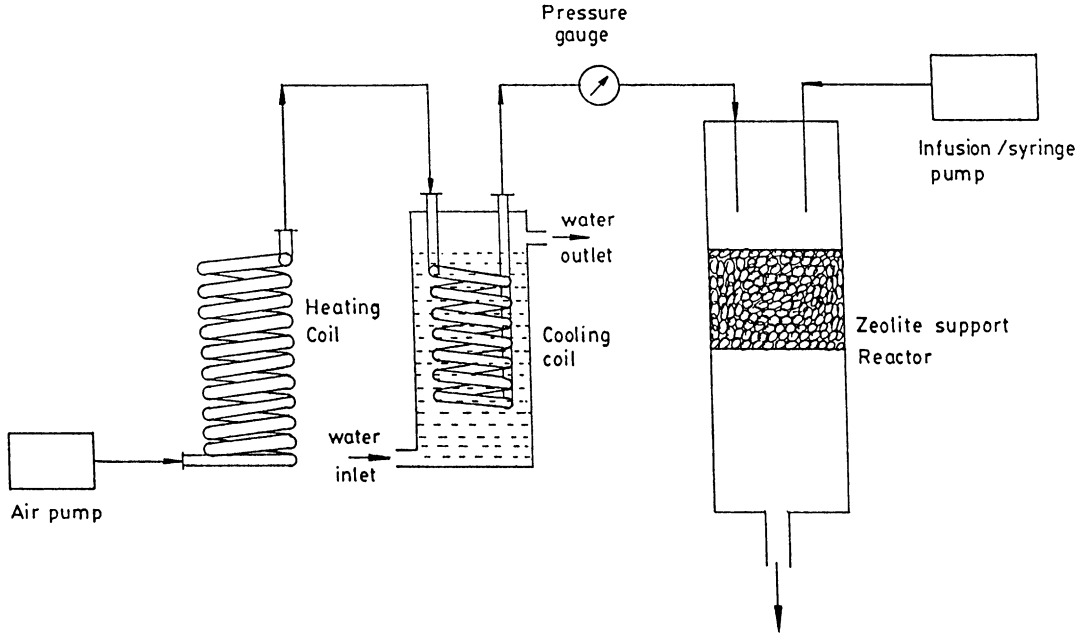

Fig. 2. Experimental set-up for immobilization of cells on MCM-41.

maintained initially at 60°C and the temperature was programmed with a rise of 10°C min^{-1} to a final of 200°C. Metabolites were confirmed with parallel running of standards of each.

2.7. Viability of immobilized cells

In order to verify the viability of cells, after every 24 h, 50 mg of the support material with the cells was withdrawn from the column, suspended in sterile physiological saline and its viability was assayed by inoculating on nutrient broth and agar. Growth in liquid medium was monitored by absorbance at 600 nm, while the same was monitored by colony counts on the solid medium and after a 24 h incubation at 30°C.

2.8. Regeneration of MCM-41

On an average, after every 23–25 cycles of TNT transformations, the column was unloaded and the contents were maintained at 600°C for 12 h for total regeneration of the starting material. Retention of structural features of MCM-41 were confirmed after every calcination by XRD and no changes were noticed.

3. Results and discussion

Microbial cells of various cellular dimensions could be successfully entrapped onto the matrix of MCM-41. In addition to the changes in the turbidity of each culture before and after immobilization (Table 1), entrapment of cells on the fixed bed was also confirmed by EDX analysis. For EDX confirmation, pre-grown cells of *Arthrobacter* sp. were harvested by centrifugation (15,000 rpm for 15 min) and the pelletized cells were resuspended in 50 ml potassium phosphate buffer (pH 7.2). The suspension was allowed to settle overnight to minimize carbon and nitrogen containing traces of nutrients, which could get carried over with the cells. After immobilizing the cells on MCM-41, the column was washed continuously with sterilized potassium phosphate buffer for 1 week and dried for 3 days. After ensuring that the column was dry, EDX analysis was performed, which indicated the presence of C and N. Coupled with the EDX analysis, presence of cells on the matrix of MCM-41 was also confirmed by SEM (Fig. 3). TG-DTA further confirmed the presence of cellular material as weight loss in the range of 150–400°C (Fig. 4), which was not observed in the calcined MCM-41.

Table 1
A_{600} nm values for the cultures immobilized on MCM-41

	B. subtilis	*Arthrobacter* sp.	*Micrococcus luteus*
Initial (before)	1.78	2.0	1.6
Final (after)	0.01	0.02	0.01

In order to study the effect of flow rate on the overall efficiency of the immobilized process, exclusive experiments were conducted on separate columns of the standard dimensions. Fig. 5 shows the different turbidity values for each flow rate before and after trickling over the packed bed of MCM-41. Flow rates higher than 2 ml h^{-1} indicated ineffective entrapment, significant leaching of cells was observed.

Disappearance of TNT with subsequent transformation to the monoaminodinitrotoluenes was confirmed to be due to the microbial activity of the immobilized cells. This was evaluated by keeping the conditions of flow rate constant to 2 ml h^{-1}, TNT solution was trickled over the fixed bed of cell free MCM-41. The eluent analysis revealed absence of transformation products. Furthermore, in another experiment, the immobilized cells were killed by maintaining the column with live cells under steam at 120°C and 15 psi for 15 min. Again TNT was found to remain unchanged.

In the suspended cell conditions, the cells of *Arthrobacter* sp. could completely transform 60 mg l^{-1} TNT in 36 h. Increase in the concentration above this could not be tolerated as a sudden drop in its viability was observed. However, a drastic tolerance as high as 400 mg l^{-1} was observed in case of cell of *Arthrobacter* sp. immobilized on MCM-41.

Complete transformation of TNT was observed per batch of 50 ml for first 8 days with the immobilized cell system. A decline in the transformational efficiency as well as viability was observed. For total loss of viability and biocatalytic efficiency, the cells required 16.5 days (average of four runs). To extend the phase of viability and biotransformation efficiency, instead of circulation of TNT, nutrient broth (50 ml) was trickled over the fixed bed after 8 days. Increase in viability was noted again (Fig. 6) and the reactions

(a)

(b)

Fig. 3. (a) SEM of MCM-41 revealing its porous structure; (b) SEM of cells of *Arthrobacter* sp. (arrows) immobilized on the support of MCM-41.

could be continued. Total transformation of TNT could be achieved for 10 more cycles (Fig. 7, Table 2).

Cells of *Arthrobacter* sp. entrapped in alginate beads also showed improvement in its biocatalytic efficiency, i.e. the cells could retain their viability to transform 60 mg l^{-1} TNT for almost 7 days. In contrast, these cells in suspended culture condition could not tolerate this concentration of TNT for more than 48 h, after which they lost their biocatalytic activity. But no tolerance to increased concentration of TNT was noted in the alginate immobilized cells [29].

Overall, this process of whole cell immobilization on the support of MCM-41 offers the scope for retention of the basic biocatalytic activity of the cells and

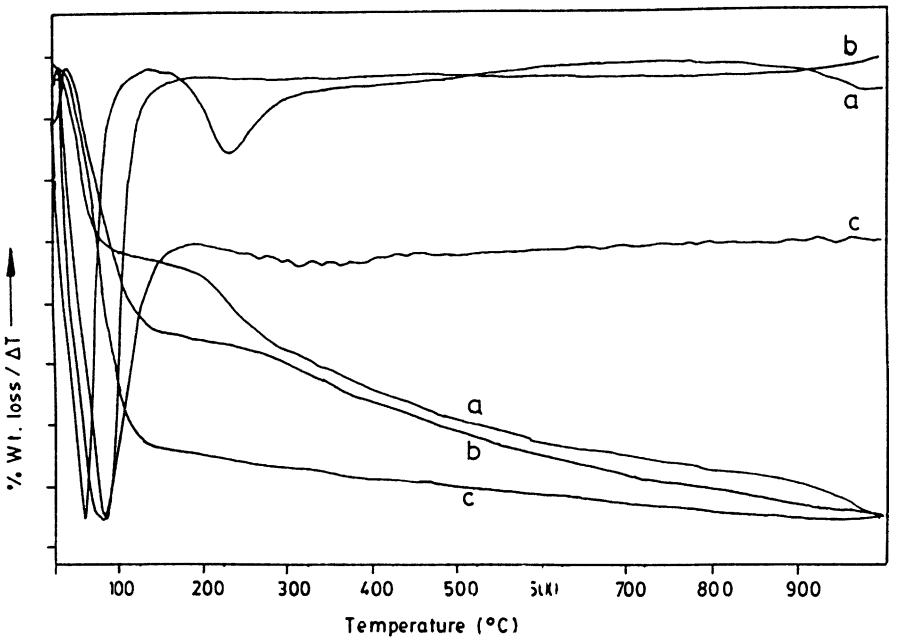

Fig. 4. TG-DTA curves of MCM-41. (a) Uncalcined; (b) calcined (i.e. before immobilization); (c) after immobilization.

consequently adds many operational advantages over the existing methods in degradation of TNT.

Though *P. chrysosporium* has been suggested to be the organism of choice, it is reported to be sensitive to concentration of TNT and is inhibited when TNT is found in concentration greater than 24 mg l^{-1}. However, no toxicity related inhibition is reported with reduction compounds of TNT, the monoaminodinitrotoluenes, 4-ADNT, 2-ADNT, etc. Hence, development of a system wherein TNT is initially converted to reduction products, which would help in facilitating quicker (faster) and complete degradation by *P. chrysosporium*, is envisaged [30].

Attempts were made to entrap the cells of *M. luteus* on the matrix of HY and HZSM-5(30) under the same experimental conditions. The cells of *M. luteus* were deliberately selected, keeping their small size as well as small pore sizes of the zeolites in focus. However,

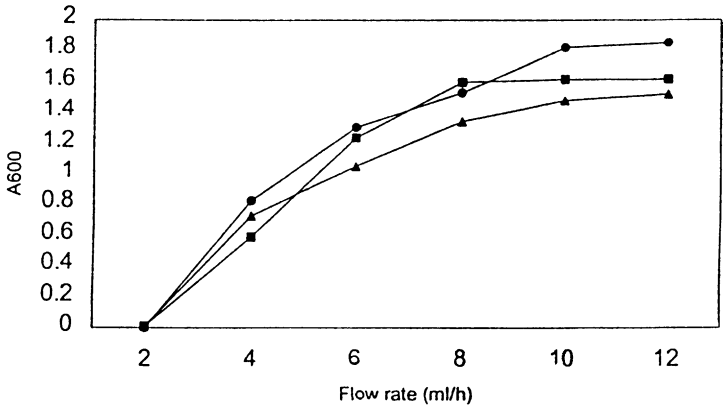

Fig. 5. Effect of flow rate on the immobilization of MCM-41 (A_{600} nm values after immobilization).

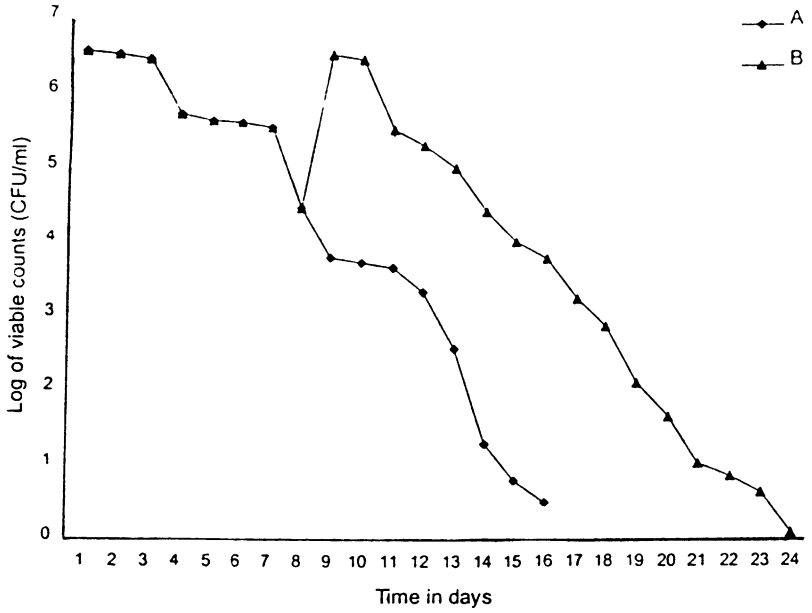

Fig. 6. Viability of *Arthrobacter* sp. entrapped on MCM-41.

no significant changes in the turbidity of the cultures could be seen before and after the cells were fed on the fixed bed (Table 3).

The exact mechanism of immobilization of cells on MCM-41 is not yet clear. The pore size of a molecular sieve plays a relatively significant role in whole cell immobilization. The average cell size of all microbes in this study are in the range of 70–100 Å, which indicates why MCM-41 can entrap these cells while HZSM-5(30) and HY zeolites fail, as they lack the required pore size. Being a surface attachment phenomenon, the presence of silanol groups of MCM-41 may be involved through hydrogen bonding with the hydrophilic centers of the microbial cells.

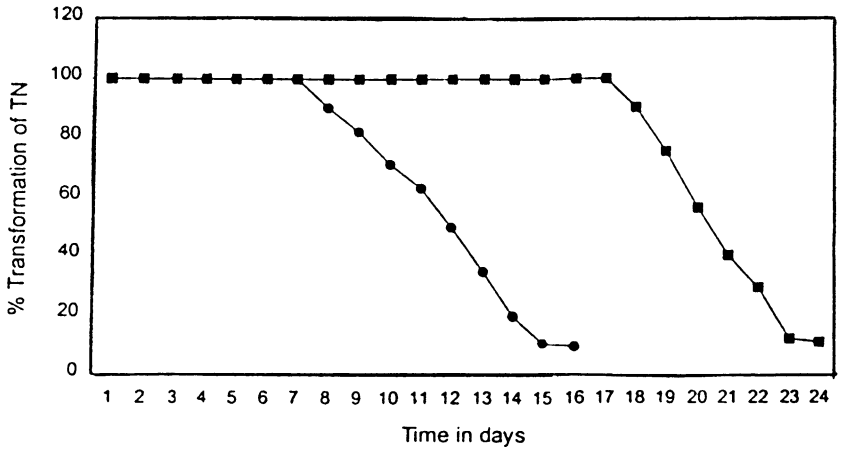

Fig. 7. Efficiency of immobilized cells of *Arthrobacter* sp. on MCM-41 in transforming TNT.

Table 2
Transformation of TNT over immobilized cells of *Arthrobacter* sp.

Time (days)	Conversion of trinitrotoluene	Yield of products (wt.%)		
		Aminodinitrotoluene	Diaminonitrotoluene	Others
2	100	83.85	16.15	–
4	100	97.8	2.2	–
6	99.75	99.75	–	–
8[a]	100	85.0	15.0	–
10	100	100	–	–
14	100	95.0	3.3	1.7
16	100	85.0	15.0	–
20	49.1	45.1	–	4.0
22	19.4	12.3	–	6.9

[a] After the supply of nutrient broth.

Table 3
Immobilization of *Micrococcus luteus* on various zeolites (A_{600} nm values)

	HZSM-5(30)	HY	MCM-41
Initial (before)	1.630	1.628	1.6
Final (after)	1.57	1.56	0.01

Being a thermostable material MCM-41 withstands high temperatures, it is inert towards treatments with organic solvent and is resistant to changes in the pH range of 4–11. The aluminosilicate structure of MCM-41 simulates natural soil conditions, thereby augmenting their physiological stability and biocatalytic activity.

Cells immobilized on silica materials and zeolites have been designed and applied to effluent treatment [31], detoxification of specific organic compounds, synthesis of specialty chemicals, biotransformation of compounds, for sensors and diagnostics, etc. Since the support permits in situ proliferation of immobilized cells, this technique can be best exploited in processes that need cells to be continued in the growth phase. Moreover, the supporting matrix can be regenerated after every cycle.

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