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Generation of continuous packed bed reactor with PVA–alginate blend immobilized *Ochrobactrum* sp. DGVK1 cells for effective removal of *N*,*N*-dimethylformamide from industrial effluents

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ABSTRACT

Effective removal of dimethylformamide (DMF), the organic solvent found in industrial effluents of textile and pharma industries, was demonstrated by using free and immobilized cells of *Ochrobactrum* sp. DGVK1, a soil isolate capable of utilizing DMF as a sole source of carbon, nitrogen. The free cells have efficiently removed DMF from culture media and effluents, only when DMF concentration was less than 1% (v/v). Entrapment of cells either in alginate or in polyvinyl alcohol (PVA) failed to increase tolerance limits. However, the cells of *Ochrobactrum* sp. DGVK1 entrapped in PVA–alginate mixed matrix tolerated higher concentration of DMF (2.5%, v/v) and effectively removed DMF from industrial effluents. As determined through batch fermentation, these immobilized cells have retained viability and degradability for more than 20 cycles. A continuous packed bed reactor, generated by using PVA–alginate beads, efficiently removed DMF from industrial effluents, even in the presence of certain organic solvents frequently found in effluents along with DMF.

1. Introduction

N,*N*-Dimethylformamide (DMF) is a widely used organic solvent in chemical, pharmaceutical and textile industries. Because of its solubility in water and high dielectric constant it is used for recovery of organic compounds. Therefore, a considerable amount of DMF is found in industrial effluents. DMF has adverse toxic effects on human and other living organisms [1–4]. It is readily absorbed through oral, dermal and inhalation exposure. The extents of toxicity induced by DMF are hepatotoxicity, embryotoxicity, teratogenicity and possible carcinogenicity. Long term exposure to DMF might also cause irreversible alterations in mitochondrial DNA [5]. DMF is the most stable organic pollutant. Once released into the environment it remains unaltered in the environment withstanding a variety of physico-chemical conditions [6]. As the rate of chemical degradation is extremely slow, biodegradation might represent a viable alternative for the DMF removal from industrial effluents and DMF-polluted sites [7].

Interestingly, certain soil microbes isolated from the industrial effluents have used DMF as sole source of carbon and nitrogen. The extraordinary potential of these soil microbes has been exploited for removal of DMF fortified into the culture flasks [1,2,6–12]. However, due to toxic effects, the free cells have failed to grow at higher concentration of DMF [11]. Bacterial cells entrapped in suitable matrix have been shown to have improved tolerance to a variety of toxic and recalcitrant compounds [13–18]. Therefore, immobilization could be a better alternative in bioremediation of toxic organic solvents. Due to obvious advantages, higher volumetric reaction rates may be obtained with immobilized cells as a result of their higher local cell concentration or altered cell permeability, the immobilized cells have been used to remove numerous toxic chemicals from industrial effluents [19–24].

We have previously reported isolation of *Ochrobactrum* sp. strain DGVK1 capable of using DMF as carbon and nitrogen source [11]. In the present study, we report immobilization of *Ochrobactrum* sp. DGVK1 cells in single and blended matrices. Studies reported in the present investigation have shown that the immobilized cells are more efficient in terms of substrate tolerance and degradation efficiency than free cells. However, among immobilized cells, the PVA–alginate immobilized cells were found to be more efficient in degradation of DMF than the cells immobilized in single matrix. PVA contributes the strength and high crosslinking capacity whereas alginate reduces the agglomeration and increases the surface properties [25]. The immobilized system was also found suitable for removal of DMF from industrial effluents containing other organic solvents.

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2. Materials and methods

2.1. Chemicals

Dimethylformamide was procured from Sigma–Aldrich, USA and PVA was procured from S.D Fine Chemicals, Mumbai, India. Alginate and culture media ingredients were purchased from Hi-Media, Mumbai, India. All other chemicals used in this study were of analytical grade.

2.2. Bacterial strain and media conditions

Ochrobactrum sp. DGVK1 was previously isolated from coalmine leftovers in our laboratory. The strain is a Gram-negative, coccoid bacterium is a member of proteobacteria capable of utilizing DMF as a sole source of carbon and nitrogen [11]. The bacterium was maintained in mineral salt medium (MM1) having following composition ($g\,l^{-1}$): K_2HPO_4 6.3; KH_2PO_4 1.8; $MgSO_4{\cdot}7H_2O$ 0.1; $MnSO_4{\cdot}4H_2O$ 0.1; $CaCl_2{\cdot}2H_2O$ 0.1; $FeSO_4{\cdot}7H_2O$ 0.1; $NaMoO_7{\cdot}7H_2O$ 0.006. The pH of the medium was adjusted to 7.0. Filter-sterilized DMF (0.8%, v/v) was supplied as the sole source of carbon and nitrogen.

2.3. Growth condition and cells harvesting

The DGVK1cells from one liter of culture grown for 48 h in the mineral salts medium (MM1) supplemented with 0.8% (v/v) DMF as carbon and nitrogen source were harvested at the early logarithmetic phase by centrifugation (8000 rpm for 15 min at 4 °C). The cell pellet was washed twice with potassium phosphate buffer (50 mM, pH 7.0) before using them for immobilization.

2.4. Immobilization of Ochrobactrum sp. DGVK1 cells

Alginate entrapment of cells was performed by following standard protocols with following slight modifications [20]. A 10 ml of cell suspension (OD A_{600} = 1.2, wet weight 2.5 g) taken in a sterile beaker was subjected to a constant mild stirring before slowly adding alginate stock solution (3%, w/v) to a final alginate concentration 2.5% (w/v). The alginate-cell mixture obtained in this manner was extruded into sterile cold calcium chloride solution (0.2 M) for cell entrapment in alginate beads. The beads formed (2–3 mm) were further hardened by stirring the beads in a fresh solution of calcium chloride (0.2 M) for two more hours. Finally, the beads were washed thrice with sterile distilled water and stored in refrigerator until further use. Immobilization of cells in PVA was essentially done by following protocols described by Sharanagouda and Karegoudar [26]. While immobilizing the cells in PVA–alginate blended matrix, the sterile solution of PVA (4.5%, w/v) and alginate (2%, w/v) was added to the 10 ml of cell suspension (OD A_{600} = 1.2, wet weight 2.5 g) under constant stirring. The blended matrix–cell mixture was extruded drop wise into cold, sterile saturated boric acid solution containing calcium chloride (0.2 M). The contents were stirred gently for 2 h and the beads formed were stored in refrigerator after thorough washing. The entrapment of cells in the matrices was later confirmed through scanning electron microscopic studies (Fig. 1). The cell content present in each gram of beads was measured by dissolving the gel beads in sodium pyrophosphate 1% (w/v) followed by serial dilution and plating on nutrient agar plates [20].

2.5. Analytical methods

Concentration of DMF was determined by using HPLC (WATERS 2489, 515 Binary pumps with C8 Sunfire column 250 mm × 4.6 mm, particle size 5 μm and ultraviolet detector 210 nm). The solvent system used was 50 mM sodium dihydrogen phosphate prepared in HPLC grade water containing 0.5% (v/v) acetonitrile. The samples from the spent medium were centrifuged and filtered through 0.2 μm filters before injection. Dimethylamine (DMA) concentration was measured according to the method of Cullis and Waddington [27]. The concentration of ammonia was determined as described by Schär et al. [9].

2.6. DMF degradation conditions

2.6.1. Degradation by free cells of Ochrobactrum sp. DGVK1

The free cells of *Ochrobactrum* sp. DGVK1 were inoculated (2 ml of exponential cells; 5.5×10^9 CFU ml^{-1}) in 250 ml Erlenmeyer flask containing 50 ml of MM1 medium with (0.6%, 0.8%, and 1%, v/v) DMF. The cultures were then incubated at 30 °C and a portion of culture media was withdrawn at fixed time intervals for estimation of DMF, DMA and NH_3 in the spent medium.

2.6.2. Degradation by immobilized cells

Ochrobactrum sp. DGVK1 cells immobilized in different matrices were independently inoculated into 100 ml of minimal medium (MM2) having composition ($g\,l^{-1}$): K_2HPO_4 0.38; $MgSO_4{\cdot}7H_2O$, 0.1; $FeSO_4{\cdot}7H_2O$, 0.1; $CaCl_2{\cdot}2H_2O$, 0.2; the pH of the medium was adjusted to 7.0 then supplemented with various amounts of DMF. These cultures were incubated at 30 °C and a portion of the culture was withdrawn for every 12 h to estimate DMF, DMA and NH_3. Polymer beads (without cells) inoculated to the minimal medium served as control to investigate the removal of DMF by adsorption to the immobilized beads. Once the supplemented DMF is completely degraded, the fermented medium was decanted and a fresh minimal medium containing DMF was added to the immobilized beads to test the reusability of beads. The process was repeated for several rounds and efficiency of DMF degradation was calculated by quantifying amount DMF degraded for each gram of beads.

2.6.3. Continuous packed bed reactor

The continuous degradation studies were carried out in a packed bed reactor. A cylindrical glass column (2.5 cm × 22 cm, volume 80 ml) with 3 outlet facility was used as a reactor. The bottom of the column was packed with circular foam pad (diameter 2 cm) followed by a porous glass-frit. The reactor was packed with 120 g of immobilized cells, to a height of 18 cm; the working volume was 25 ml. Aeration was maintained at 0.5 bar throughout the column so that culture medium was well mixed (Fig. 2). The influent MM2 medium containing DMF was fed into the reactor from bottom side arm opening by peristaltic pump (Miclins, India). The degradation process was carried out by continuous supply of influent with various initial concentrations of DMF (2.5–3%, v/v) at different flow rates (10–40 ml h^{-1}). Residual DMF, DMA and NH_3 concentration were measured in the effluent for each set of experiments. Control experiments were carried out by PVA–alginate beads lacking bacterial cells.

2.6.4. Treatment of Industrial effluent containing DMF by immobilized cells

The industrial effluent from M/s Lupin Ltd., Tarapur, Mumbai, India, a fermentation-based industry involved in the production of Rifampicin was collected and used to test the DMF degradability of *Ochrobactrum* sp. DGVK1 immobilized in PVA–alginate matrix. The typical composition of effluent discharged from this pharmaceutical industry is listed in Table 1. The initial pH of the effluent was 5.0. While performing the degradation studies, the pH of the effluent was brought to 7 by adding 1 N NaOH. Subsequently, the effluent was diluted with double strength 2× MM1 medium to adjust the concentration of DMF to 2% (v/v). The effluent treated in this manner was then inoculated with different concentrations

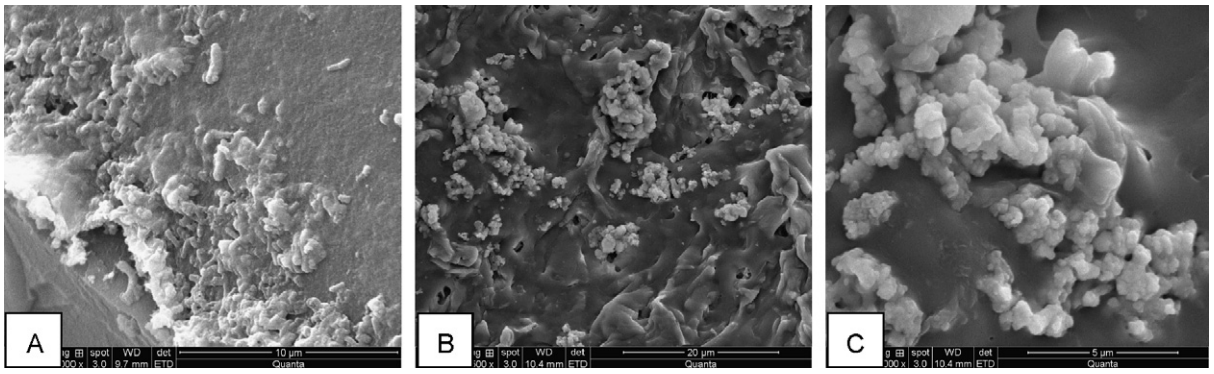

Fig. 1. Scanning electron microscopic images of (A) PVA–alginate, (B) alginate and (C) PVA.

Table 1
The composition of industrial effluent supplied by M/s. Lupin Ltd.

Effluent constituents	Concentration (%)
Dimethylformamide	4–5
Methylene chloride	0.5
Acetic acid	0.5–1
Tertiary butylamine	0.5
Dimethylamine	0.5

of immobilized cells and incubated at 30 °C on a rotary shaker. A portion of effluent was withdrawn from the flask periodically to estimate DMF, DMA and NH_3.

3. Results and discussion

3.1. Degradation by Ochrobactrum sp. DGVK1 free cells

Ochrobactrum sp. DGVK1 cells have been shown to use DMF as sole source of carbon and nitrogen. In the process of DMF metabolism NH_3 and HCOOH are generated as terminal products, indicating complete mineralization of DMF. In the present investigation an attempt is made to exploit the inherent properties of *Ochrobactrum* sp. DGVK1 for removal of DMF from industrial effluents. Initially the free cells grown in minimal medium supplemented with DMF were suspended in fresh medium to a final concentration of 5.5×10^9 CFU ml^{-1} and incubated with different initial concentrations of DMF (0.6%, 0.8%, and 1%, v/v) for various time points. The free cells have successfully degraded DMF within 96 h of incubation, only when the final concentration of DMF was less than 1% (v/v) (Fig. 3). When DMF concentration is increased beyond 1% (v/v), no degradation was seen even after incubating the culture for more than 168 h. Further the higher concentration of DMF adversely influenced the viability of the cells. As reported in our previous studies, the DMF degradation was witnessed by concomitant production of DMA and ammonia in the spent medium [11]. Further, production of ammonia increased pH of the medium from 7 to 9.2.

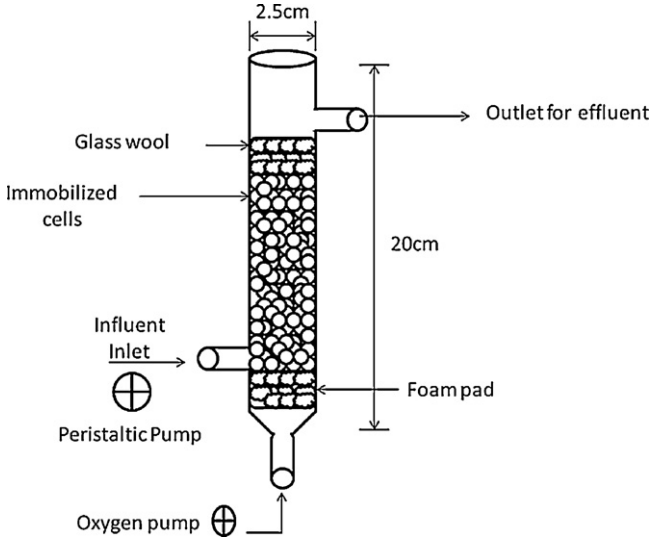

Fig. 2. Setup of continuous packed bed reactor (CPBR).

3.2. DMF degradation by alginate and PVA immobilized cells

Immobilization of *Ochrobactrum* sp. DGVK1 was very effective. About 5.65×10^{10} cells (CFU) were found entrapped per every gram of alginate. These alginate entrapped cells were then used for studying DMF degradation by incubating the alginate beads in the medium containing various concentrations of DMF. The alginate immobilized cells have degraded DMF within 60 h when the initial concentration is 1% (v/v). However, they have taken 108 h to eliminate DMF completely when the concentration was increased to 1.5% (v/v). There was no complete degradation of DMF with the increase in concentration of DMF. Immobilization of cells in alginate has clearly shown higher degradation abilities and remarkably reduced degradation time when compared to free cells. However, there is no improvement with respect to the tolerance limits. The free and alginate immobilized cells have shown almost identical tolerance limits to final DMF concentrations. A number of studies have shown influence of supporting matrices on cell viability and catalytic efficiency [19,20,22]. PVA has been shown to be an effective matrix for immobilization of bacterial cells [21,27,28]. Aiming to improve tolerance limits, the *Ochrobactrum* sp. DGVK1 cells were immobilized

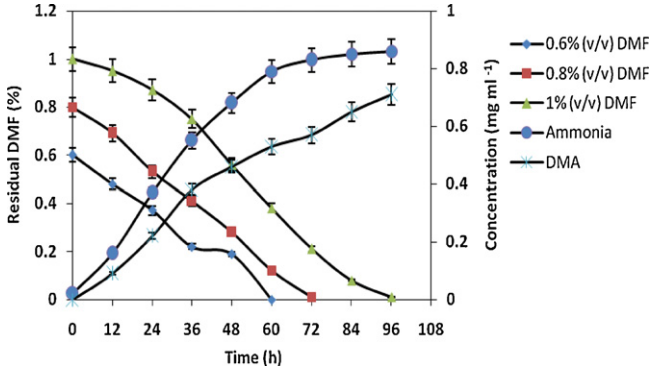

Fig. 3. Degradation of DMF by free cells of *Ochrobactrum* sp. DGVK1 with different initial concentrations (0.6%, 0.8% and 1%, v/v). Production of ammonia and DMA at 1% (v/v) DMF.

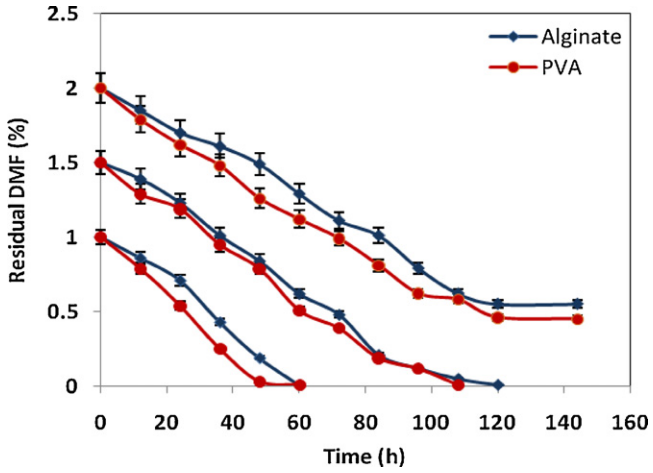

Fig. 4. Degradation of DMF by immobilized DGVK1 in alginate (blue lines) and PVA (red lines) at different initial concentrations 1%, 1.5% and 2% (v/v). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

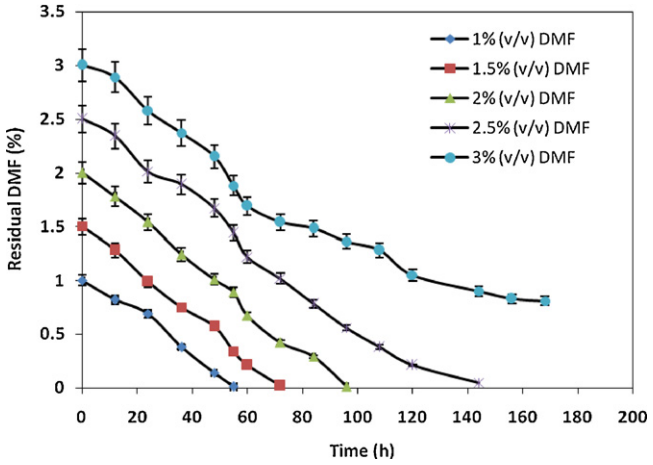

Fig. 5. Degradation of DMF by PVA–alginate blended immobilized cells with different initial concentrations 1–3% (v/v) DMF.

in PVA matrix. About 5.82×10^{10} cells (CFU) were entrapped per every gram of PVA. These immobilized cells were then used for degradation studies. As shown in Fig. 4, the PVA immobilized cells have shown better degradation capabilities when compared to the free and alginate entrapped cells. However, there was no improvement in tolerance limits to DMF, mainly due to aggregation of PVA beads with increase in concentration of DMF. The control experiments revealed that the removal of DMF by adsorption to alginate and PVA polymer beads lacking cells was very negligible. About 99% DMF remains as a residual concentration in the fermented medium.

3.3. PVA–alginate blended immobilization increased tolerance limit

As expected, blending of PVA–alginate has contributed for gaining of properties of both PVA and alginate. The PVA contributed for the stability and mechanical strength of beads whereas alginate contributed for the improvement of surface properties of the beads and reduced agglomeration. PVA–alginate composite hydrogel entrapped nearly 6.23×10^{10} cells (CFU) per gram. These PVA–alginate beads were then used for evaluating the degradation efficiency and tolerance limits. Initially these beads were incubated in minimal medium supplemented with various initial concentrations ranging from 1% to 2.5% (v/v) of DMF. Interestingly, the PVA–alginate entrapped cells mineralized DMF completely even when it is present at 2.5% (v/v) within 144 h (Fig. 5). Further increase of DMF in culture medium reduced degradation efficiency of DMF. The cells entrapped in PVA–alginate could not completely eliminate DMF when its initial concentration was increased to 3% (v/v).

After establishing degradation profile of immobilized cells they were used for testing the cell viability and degradation efficiency in a batch fermentation process. The immobilized cells incubated with the DMF containing medium were carefully collected and reused for number of cycles to assess their ability to remove DMF from the medium (Table 2). Interestingly, the PVA–alginate blended beads efficiently removed DMF for 22 cycles. However, the alginate and PVA immobilized cells have retained degradation efficiency only for 16 cycles and 12 cycles, respectively.

3.4. Treatment of industrial effluent by PVA–alginate immobilized cells

The results described in previous sections have clearly shown advantage of using PVA–alginate blended cells in removing DMF from the culture medium. In order to extend these studies to real-time situation, the composite biocatalyst was used to treat industrial effluent containing DMF as a major component. While performing these studies, the initial concentration of DMF in the industrial effluent was brought down to 2% and 2.5% (v/v) by diluting the effluent with minimal medium. The PVA–alginate immobilized cells have successfully eliminated DMF when its concentration was adjusted to 2% (v/v) with in 142 h of incubation. However, the efficiency of the biocatalyst has shown declining trend with increase in DMF concentration (Figs. 6 and 7). This may be due to presence of other organic solvents such as methylene chloride, acetic acid and tertiary butyl amine and dimethylamine in the effluents. These contaminants showed little effect on DMF degradation when present at concentrations ranging 0.5–1% (v/v) in the culture medium.

3.5. Continuous degradation of DMF in a packed bed reactor

As the composite biocatalyst has successfully eliminated DMF from effluents, a packed bed reactor was generated as described in Section 2 and the diluted effluents were continuously passed at different flow rates (10, 20, 30 and 40 ml h^{-1}). The PVA–alginate

Table 2
Repeated batch degradations of DMF by alginate, PVA and PVA–alginate immobilized cells.

Matrix	Initial DMF (%)	Percentage degradation	Incubation time (h)	No. of cycles
Alginate	1.0	100	60	18
	1.5	100	108	16
	2.0	80	156	–
	2.5	52	240	–
PVA	1.0	100	55	14
	1.5	100	106	11
	2.0	85.5	168	–
	2.5	60.8	216	–
PVA–alginate	1.0	100	60	24
	1.5	100	72	24
	2.0	100	96	23
	2.5	100	144	23
	3.0	76.6	240	–

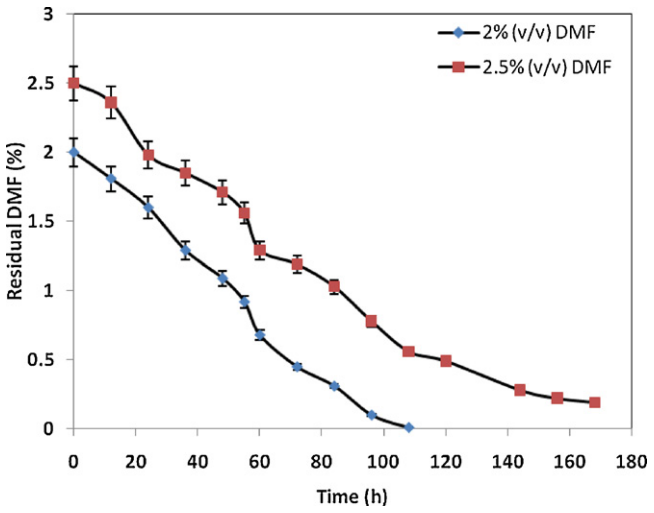

Fig. 6. Treatment of industrial effluent containing DMF by PVA–alginate immobilized cells when concentration of DMF in effluent was brought to 2% and 2.5% (v/v).

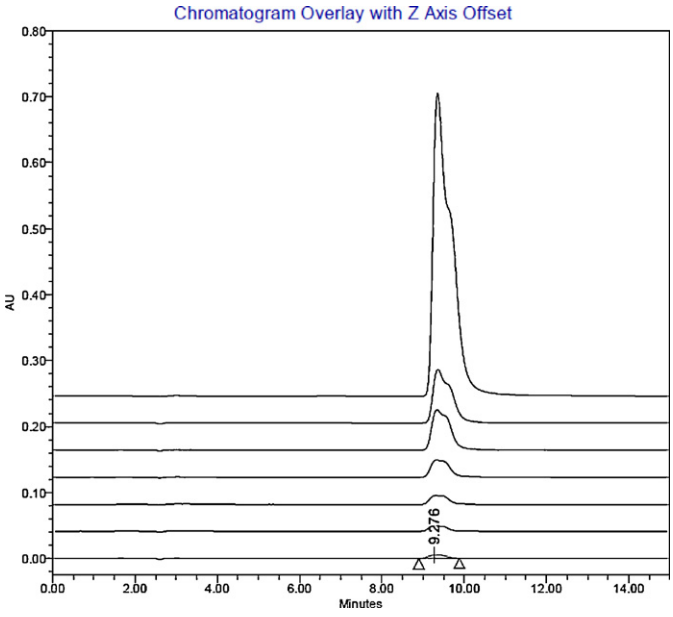

Fig. 7. Chromatograms showing decreasing in the residual DMF concentration in the effluent at different intervals of degradation.

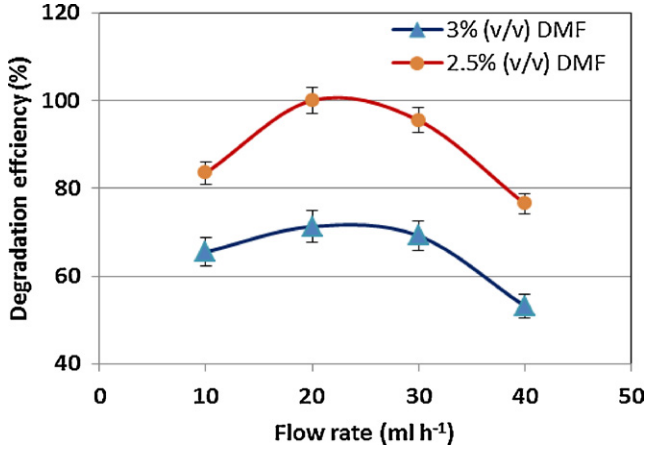

Fig. 8. Degradation effceincy of PVA–alginate immobilized cells in the continuoes packed bed reactor at different flow rates from 10 to 40 ml h^{-1} at influent concentration of 2.5% and 3% (v/v) DMF.

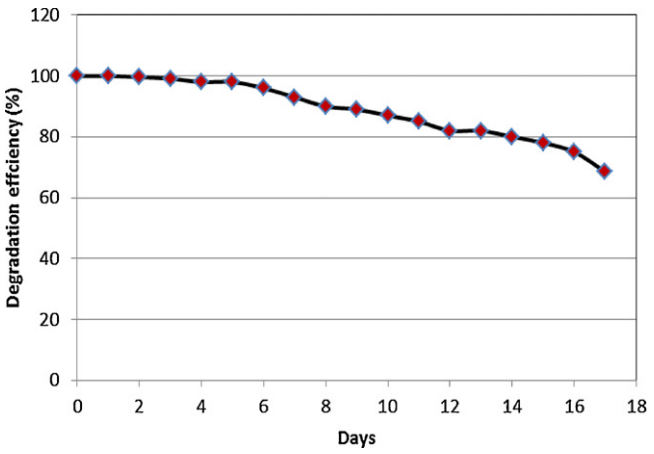

Fig. 9. The efficiency of reactor when operated continuously by PVA–alginate beads at 20 ml h^{-1} with influent concentration of 2.5% (v/v) DMF in the continuous packed bed reactor.

immobilized cells have eliminated (2.5%, v/v) DMF completely. Whereas 71.3% of 3% (v/v) DMF degradation was achieved when the flow rate was adjusted to 20 ml h^{-1} (Fig. 8). Further, the durability of packed bed reactor was tested by passing the effluents at fixed flow rate (20 ml h^{-1}) and keeping the DMF concentration at 2.5% (v/v). The flow through collected at fixed hydraulic retention time intervals were used to estimate DMF and to calculate efficiency of the packed bed reactor. The control experiments were carried out at all different flow rates with PVA–alginate polymer beads produced without cells to check the removal of DMF due to adsorption. The results revealed that 99% of the DMF which remains as residual in effluent of packed bed reactor. It clears that adsorption has very negligible effect in the removal of DMF. All the experiments were carried in triplicates and the results were expressed as average of triplicate values. The reactors efficiency was slowly gone down and reached to 68.3% after 17 days (Fig. 9).

DMF is found to be one of the most recalcitrant organic solvent extensively used in textile and pharmaceutical industries. As physico-chemical methods found to be ineffective to remove DMF from the effluents, a number of attempts have been made to find viable bioremediation strategies for effective removal of DMF involving soil bacteria capable of using DMF as sole source of carbon [1,6,8–11]. As reported in earlier studies, the free cells of *Ochrobactrum* sp. DGVK1 have shown less tolerance to DMF than their immobilized counter parts. Direct contact of free cells with DMF and its metabolites like dimethylamine and ammonia as well as the other organic solvents found in the effluents might be responsible for the reduced viability of cells. Composite catalysts generated in the study have shown clear advantage than free and immobilized cells in a single matrix. Immobilization of cells has been shown to accelerate catalytic efficiency due to increased cell density and membrane permeability to the substrate [29]. Alginate is one of the most commonly used matrices for cell immobilization. Due to increased porosity the alginate entrapped cells have better access to the substrate. However, these beads are susceptible to chemicals, especially the cation-chelating agents such as phosphate, citrate, and lactate. Chelation of cations can cause alginate bead disruption or dissolution [28]. Due to the fact that industrial effluents contain salts and organic solvents, the alginate beads have been found unsuitable to treat industrial effluents. Added to these disadvantages, due to increased porosity, the entrapped cells have maximum scope to leach out of the beads causing reduction in catalytic efficiency. Though PVA entrapped cells are stable, the increased concentration of DMF has caused beads aggregation and made them unsuitable for treating DMF containing effluents. The

PVA–alginate composite matrix resolved these problems by combing the best of these two matrices. The composite biocatalyst has tolerated more DMF concentrations and improved degradation efficiency and durability of the catalyst. The composite biocatalyst has shown to be superior than the free and PVA and alginate immobilized cells while eliminating DMF either from culture medium or from industrial effluents. The continuous packed bed reactor using the composite biocatalyst clearly demonstrates suitability of the system for effective and safe removal of DMF from the industrial effluents. Superior qualities such as, durability and increased tolerance limits, place composite biocatalyst ahead of other technologies available for removal of DMF from industrial effluents.

4. Conclusion

Ochrobactrum sp. DGVK1 cells entrapped in alginate, PVA and PVA–alginate have shown more tolerance to DMF. These entrapped cells have effectively removed DMF even in the presence of other organic solvents. These biocatalysts were used both in semi continuous and continuous mode to remove DMF from effluents collected from pharmaceutical industries. The present study clearly demonstrates development of industrially feasible and economically viable bioremediation strategy for discharging DMF free effluents into environment.

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