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ORIGINAL PAPER

A comparison of sodium sulfate, sodium phosphate, and boric acid for preparation of immobilized *Pseudomonas putida* F1 in poly(vinyl alcohol) beads

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Abstract Poly(vinyl alcohol) (PVA) gel beads crosslinked with sodium phosphate or boric acid have been widely utilized for microorganism immobilization. We previously utilized sodium sulfate to induce crosslinking of PVA for preparing immobilized yeast cells in PVA beads. In this study, we compared the toxicities of sodium sulfate and conventional crosslinkers (sodium phosphate and boric acid) toward *Pseudomonas putida* F1 (PpF1) and the performance of the corresponding immobilized PpF1 in PVA beads. The toxicity of sodium sulfate to PpF1 was lower than those of the conventional crosslinkers. PVA–sodium sulfate beads showed a higher gel fraction of PVA and a lower swelling ratio in water than PVA–sodium phosphate beads, which indicates that the former had higher stability. PpF1 immobilized in the PVA–sodium sulfate beads completely degraded the pollutant trichloroethylene (TCE) with an initial concentration (0.42 mg/l) within the most common range of TCE concentration found in contaminated field sites.

Keywords Poly(vinyl alcohol) · Sodium sulfate · Sodium phosphate · Boric acid · Microorganism immobilization · Wastewater treatment

Introduction

Extensive environmental contamination with toxic chemical pollutants such as trichloroethylene (TCE) and polychlorinated biphenyls (PCBs) originating from

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industrial wastewaters is both widespread and challenging to remediate [1]. Bioremediation is an attractive technology that utilizes the metabolic potential of microorganisms to clean up the environment [2–4].

Immobilization of microorganisms in spherical polymeric matrices has been shown to be promising for improving the efficiency of bioremediation. Advantages of immobilized cells over non-immobilized cells include: (i) protection from harsh environmental conditions such as pH, temperature, and toxic compounds, (ii) relative ease of cell separation from bioreactors or targeted sites, (iii) reusability, and (iv) reduced susceptibility to contamination by foreign microorganisms [5–13]. Several natural (agar, alginate, and chitosan) and synthetic [polyacrylamide, polyurethane, and poly(vinyl alcohol) (PVA)] polymers have been used as matrix materials for cell immobilization [14, 15]. PVA offers advantages over the other polymers, including lower production costs, higher robustness, higher chemical stability, and non-toxicity toward viable cells, all of which are very important for the successful application of immobilized cells to bioremediation [16]. The simplest and most economical technique for preparing PVA gel beads is the PVA–boric acid method developed by Hashimoto and Furukawa [17]. This method, however, has two drawbacks [18]. One disadvantage is agglomeration of the PVA beads during the bead preparation process due to the low crosslinking rate of PVA with boric acid, which hinders diffusion of substrates into the beads. The other is a drastic decrease in the viability of immobilized cells caused by the high acidity (pH <4) of boric acid solution. The first of these drawbacks was improved by adding a small amount of calcium-alginate gel to PVA beads [18]. The latter problem was overcome by use of lower cytotoxic sodium orthophosphate in place of boric acid for crosslinking of PVA [15]. At present, PVA–sodium phosphate beads have been widely used for immobilization of microorganisms [14, 19]. However, further reduction of cell damage resulting from the presence of phosphate and improvement of stability of the PVA–sodium phosphate beads are essential for successful application of the beads to bioremediation of contaminated wastewaters [20, 21].

Idris et al. and Nunes et al. are pioneers in the preparation of PVA beads using sodium sulfate for enzyme immobilization [16, 22, 23]. We previously applied PVA–sodium sulfate beads to microorganism immobilization and demonstrated that the beads immobilizing *Saccharomyces cerevisiae* showed higher stability and induced higher ethanol production activity with immobilized yeast cells than PVA–boric acid beads [13]. The next step is to compare characteristics of PVA–sodium sulfate and PVA–sodium phosphate beads. Further, a comparison of the toxicities of sodium sulfate and conventional crosslinkers (sodium phosphate and boric acid) toward other microorganisms is essential for evaluation of the versatility of PVA–sodium sulfate beads because every microorganism has a unique susceptibility to chemical damage.

This study first compared the toxicities of three chemicals (sodium sulfate, sodium phosphate, and boric acid) toward *Pseudomonas putida* F1 (PpF1). PpF1 degrades TCE by aerobic co-metabolism in the presence of primary substrates such as toluene [24, 25]. TCE is a common contaminant (a suspected carcinogen) found in soil and ground water. In view of the results, we modified the previously described process for immobilization of microorganisms in PVA–sodium sulfate

beads. In addition, we compared the stability of PVA–sodium sulfate and PVA–sodium phosphate beads, and evaluated degradation rates of TCE by immobilized PpF1.

Experimental

Materials and microorganism

PVA (degree of saponification: 98 %, degree of polymerization: 2,000) and sodium alginate (Na-Alg) (viscosity of 1.0 % (w/v) aqueous solution at 20 °C: 80–120 mPa s) were purchased from Wako Pure Chemical Co. (Osaka, Japan). PpF1 DSM 6899 was obtained from The German Collection of Microorganisms and Cell Cultures (DSMZ, Brunswick, Germany). PpF1 was cultured in 220 medium (pH 7.3, 15 g of peptone from casein, 5 g of peptone from soymeal, and 5 g of NaCl in 1 L distilled water) at 30 °C and harvested by centrifugation in the late exponential phase of growth. The collected cells were washed with 0.9 % NaCl solution in triplicate and then re-suspended in 0.9 % NaCl solution. The cell suspension was used in subsequent experiments.

Toxicities of sodium sulfate, sodium phosphate, and boric acid toward PpF1

PpF1 was suspended in 30 ml of sodium sulfate (0.5–1.5 M), sodium orthophosphate (0.5–1.5 M), saturated boric acid (0.8 M), and aqueous solutions or distilled water and gently shaken at 30 °C. After 1 and 6 h of incubation, PpF1 was collected by centrifugation and washed with distilled water in triplicate to remove the chemicals from the cell suspension. The cells were incubated in 5 ml of 220 medium (1×10^7 cells/ml) with gentle stirring at 30 °C. The cell density in the solutions was determined using a hemacytometer. The turbidity (600 nm) of the suspensions was determined at various times using a spectrometer (UV-2500PC, Shimadzu, Tokyo, Japan) to assess cell proliferation.

Preparation of PVA beads immobilizing PpF1

PVA aqueous solution, Na-Alg aqueous solution, and PpF1 suspension were mixed at room temperature. The resultant suspension contained 10 % (w/v) PVA and 1.0 % (w/v) Na-Alg. The suspension was extruded from a needle (inner diameter 270 μm, outer diameter 450 μm) as droplets into 100 mM calcium chloride aqueous solution and immersed for 25 min with gentle stirring for crosslinking of Na-Alg. The beads were then transferred to sodium sulfate (0.5–1.5 M) or sodium phosphate aqueous solutions (0.5–1.5 M), and immersed for 1–6 h with gentle stirring for crosslinking of PVA. The resultant beads were washed with a large amount of distilled water. The experimental conditions are shown in Table 1. An identical protocol except for adding PpF1 to polymer solution was applied for preparing PVA beads without the microorganism. Diameters of the beads were determined using an optical microscope.

Table 1 PVA bead preparation conditions

Abbreviation	Na_2SO_4 (M)	NaH_2PO_4 (M)	Incubation time (h)	Bead diameter (mm) ($n = 80$)
S-0.5/1 h	0.5	–	1	N.D.
S-0.5/3 h	0.5	–	3	N.D.
S-0.5/6 h	0.5	–	6	N.D.
S-1.0/1 h	1.0	–	1	N.D.
S-1.0/3 h	1.0	–	3	3.3 ± 0.3
S-1.0/6 h	1.0	–	6	2.9 ± 0.2
S-1.5/1 h	1.5	–	1	3.2 ± 0.2
S-1.5/3 h	1.5	–	3	2.9 ± 0.1
S-1.5/6 h	1.5	–	6	2.8 ± 0.2
P-0.5/1 h	–	0.5	1	N.D.
P-0.5/3 h	–	0.5	3	N.D.
P-0.5/6 h	–	0.5	6	3.6 ± 0.2
P-1.0/1 h	–	1.0	1	N.D.
P-1.0/3 h	–	1.0	3	3.3 ± 0.3
P-1.0/6 h	–	1.0	6	3.0 ± 0.1
P-1.5/1 h	–	1.5	1	3.2 ± 0.1
P-1.5/3 h	–	1.5	3	3.1 ± 0.1
P-1.5/6 h	–	1.5	6	3.0 ± 0.3

N.D. means that gel beads did not form during incubation in sodium sulfate or sodium phosphate solutions

Stability test of PVA beads

The gel fraction of PVA in beads was determined as follows [13]. In brief, 10 g (wet weight) of PVA beads without PpF1 just after bead preparation was immersed in a large amount of 55 mM trisodium citrate aqueous solution containing 10 mM HEPES (pH 7.4), and gently stirred for 12 h at 37 °C to liquefy the calcium-alginate gel in the beads. The beads were then immersed in a large volume of distilled water for 24 h at room temperature with gentle stirring to remove the alginate from the beads, then dried under vacuum. The gel fraction of PVA was determined by the following equation:

$$\text{Gelfraction of PVA} = W_t \times 100/W_i,$$

where W_t and W_i are the dry weight of the beads and the weight of PVA powder used for bead preparation, respectively.

The swelling ratio of the beads was also determined. PVA beads without PpF1 (1 g, wet weight) were added to 25 ml distilled water and gently stirred at 30 °C for 12 days. The diameters of the beads were measured after 12 days of incubation using an optical microscope and the volumes of the beads calculated from their diameters. The swelling ratio was evaluated by the following equation:

$$\text{Swelling ratio } (\%) = V_{12d} \times 100/V_{0d},$$

where V_{12d} and V_{0d} are volumes of the beads at 12 and 0 days of incubation, respectively.

Biological activity of immobilized PpF1 in PVA beads

Immobilized PpF1 in PVA beads (2 g, wet weight) or non-immobilized cells were added into 10 ml of 457 medium (pH 6.9, 2.44 g of Na_2HPO_4, 1.52 g of KH_2PO_4, 0.5 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4{\cdot}7H_2O$, 0.05 g of $CaCl_2{\cdot}2H_2O$, 5 mg of ethylenediaminetetraacetic acid, 2 mg of $FeSO_4{\cdot}7H_2O$, 0.1 mg of $ZnSO_4{\cdot}7H_2O$, 0.03 mg of $MnCl_2{\cdot}4H_2O$, 0.2 mg of $CoCl_2{\cdot}6H_2O$, 0.01 mg of $CuCl_2{\cdot}2H_2O$, and 0.03 mg of $Na_2MoO_4{\cdot}2H_2O$ in 1 L distilled water) in glass vials (155 ml total volume) crimp-sealed with inert rubber septa. The number of PpF1 cells added to the vial was 1×10^{12} cells. A 2.1 µl aliquot of TCE solution diluted with *N*,*N*-dimethylformamide (0.03 mg TCE) and toluene (2 mg) were injected into the vials through the septa [26]. The initial theoretical concentration of TCE in the liquid phase in the vials was 0.42 mg/l, which was calculated according to Henry's law [27]. The vials were then incubated with gentle stirring at 30 °C. The degradation of TCE and toluene was determined by measuring their concentrations in the gas phase using the headspace method [28]. In brief, at appropriate intervals, gas samples (200 µl) were taken from the headspace of the vials. The concentrations of TCE and toluene in the sample were determined using a gas chromatograph (GC-17A, Shimadzu Corp., Kyoto, Japan) equipped with a DB-624 column (Agilent Technologies, California).

Results and discussion

Toxicities of sodium sulfate, sodium phosphate, and boric acid toward PpF1

We previously showed that sodium sulfate has a lower toxicity toward *S. cerevisiae* than boric acid. In this experiment, we compared the cytotoxicity of sodium sulfate, sodium phosphate, and boric acid toward PpF1 by examining proliferation profiles of the cells preincubated for 1 and 6 h in aqueous solutions of each chemical.

Figure 1 shows the proliferation profiles of PpF1 pretreated for 1 h with each chemical. PpF1 pretreated with distilled water began to proliferate within 1 h of incubation. The cells pretreated with 0.5 M sodium sulfate solution also had a short lag phase, while an increased concentration caused a longer lag phase. This trend was similar for sodium phosphate. A noteworthy finding is that the lag phase for the sulfate was shorter than that for the phosphate at an identical concentration. In all conditions, there was little difference between the lengths of lag phase for 1 and 6 h preincubation in the salt solutions (data not shown). A longer lag phase indicates more severe damage to microorganisms [29]. These results show that sulfate is less toxic toward PpF1 than phosphate. We note that proliferation of PpF1 pretreated with saturated boric acid solution for 1 h was not observed even after 72 h of

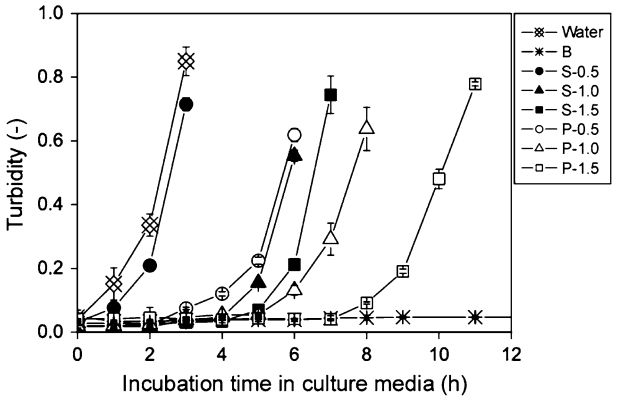

Fig. 1 Proliferation profiles of PpF1 preincubated in sodium sulfate solution (S), sodium phosphate solution (P), saturated boric acid solution (B), or distilled water for 1 h. *Bar* Mean ± SD ($n = 3$). Each value following "S" and "P" gives the concentration (molarity) of the reagents

incubation in a medium. A shortened incubation time (30 min) in the boric acid solution was ineffective in improving the outcome (data not shown). This result indicates that all cells were killed by the acidity of the boric acid solution (pH < 4) [15] so we used only PVA–sodium sulfate and PVA–sodium phosphate beads in the following experiments.

Preparation of PVA beads

The simplest procedure for preparation of PVA–sodium sulfate beads involved dropwise addition of a cell suspension with dissolved PVA to a sodium sulfate solution. However, spherical beads could not be obtained using this approach, due to the low crosslinking rate of PVA by the sulfate [13]. This suggested that addition of a small amount of calcium-alginate gel to the PVA beads was necessary to prepare spherical beads, similar to the conventional PVA–sodium phosphate and PVA–boric acid beads, because crosslinking rate of Na-Alg with calcium ions is rapid (<1 s). Therefore, we planned to deliver a cell suspension with dissolved PVA and Na-Alg to a mixed solution of sodium sulfate and calcium chloride for simultaneous crosslinking of Na-Alg and PVA. However, the procedure was not feasible because mixture of the two reagent solutions caused precipitation of calcium sulfate, which has low solubility in water. As a result, we previously delivered a cell suspension with dissolved PVA and Na-Alg into a mixed solution of saturated boric acid and calcium chloride, and incubated this for a short time (30 min) to allow simultaneous crosslinking of Na-Alg and PVA, then transferred it to a sodium sulfate solution for further crosslinking of PVA [13]. However, this procedure could not be applied to PpF1 because the cells were killed during the incubation in boric acid solution, as mentioned above. Consequently, we decided first to deliver a cell suspension with dissolved PVA and Na-Alg into calcium chloride solution for crosslinking of Na-Alg, then transfer the resulting beads to sodium sulfate solution for crosslinking of PVA. We confirmed that spherical beads could be obtained using this procedure.

Diameters of PVA beads prepared in each condition are shown in Table 1. A low salt concentration and short incubation time, especially 0.5 M and 1 h, respectively, caused failure of bead formation. Diameters of the resultant beads decreased with increasing salt concentration and incubation time, due to an increased degree of crosslinking of PVA.

Stability of PVA beads

In our previous report, we clarified that the physical crosslinking mechanism for PVA by sodium sulfate involves PVA salting-out by sulfate due to its extremely high hydration force, followed by crystallite formation (crosslinking sites) of PVA molecules [13]. A possible crosslinking mechanism for PVA by sodium phosphate is based on esterification of PVA by the salt [15] and crystalline formation of PVA via salting-out of PVA, similar to sodium sulfate [30]. In this experiment, we evaluated the characteristics of the beads, including the gel fraction of PVA and the swelling ratio, which are closely related to bead stability.

Figure 2 shows the gel fraction of PVA just after bead preparation. The gel fraction increased with increasing salt concentration and incubation time in the salt solution. The fraction for the sulfate was higher than that for the phosphate at an identical concentration and incubation time. In particular, S-1.5/6 h beads showed the highest gel fraction. Leakage of PVA from the beads would occur mainly during incubation in the calcium chloride solution used for crosslinking of Na-Alg.

Subsequently, we investigated the swelling behavior of PVA beads incubated in water (Fig. 3) because polymer matrices for bioremediation of contaminated wastewater are required to have high stability in water [13]. All PVA–sodium phosphate beads, except for P-1.5/6 h beads, completely dissolved during incubation in water for 12 days, as shown in Fig. 3c, g, k. On the other hand, all PVA–sodium sulfate beads maintained their spherical shapes for 12 days, although a

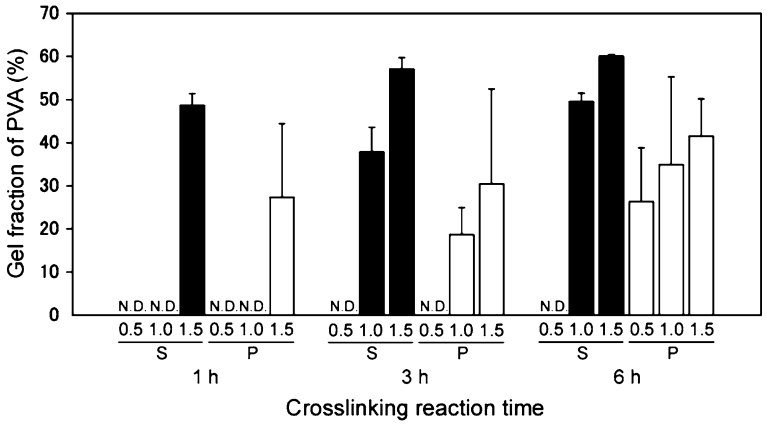

Fig. 2 Gel fraction of PVA in the beads prepared using sodium sulfate (S) and sodium phosphate (P). *Bar* Mean ± SD ($n = 3$). Each value following "S" and "P" gives the concentration (molarity) of the reagents. N.D. means that beads did not form during incubation in the salt solutions

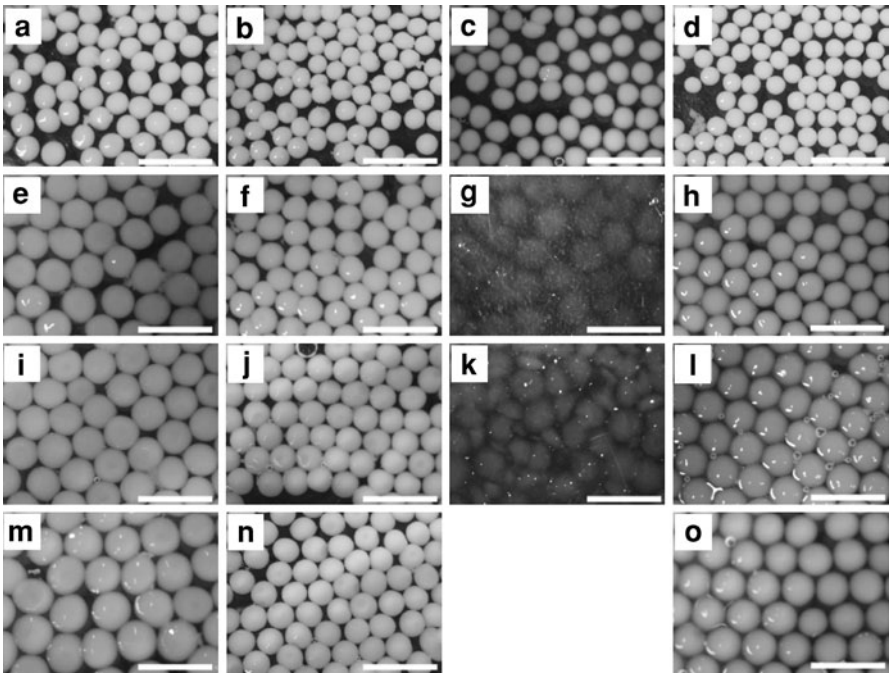

Fig. 3 Appearances of S-1.5/1 h (**a**, **e**, **i**, and **m**), S-1.5/6 h (**b**, **f**, **j** and **n**), P-1.5/1 h (**c**, **g**, and **k**), and P-1.5/6 h beads (**d**, **h**, **l**, and **o**) incubated in distilled water for 0 days (**a**–**d**), 3 days (**e**–**h**), 6 days (**i**–**l**), and 12 days (**m**–**o**). *Scale bars* 1 cm

certain degree of swelling was observed. Figure 4 shows the swelling ratio of the beads after 12 days of incubation. A noteworthy finding is that the swelling ratio of S-1.5/6 h beads was much lower than for P-1.5/6 h beads, showing that sulfate induces a higher degree of crosslinking of PVA due to its higher hydration force compared with phosphate [31]. From the results of both cytotoxicity and stability tests, we conclude that sodium sulfate is more useful than sodium phosphate and boric acid for preparation of robust PVA beads immobilizing PpF1 cells for biological treatment of contaminated wastewater.

Biological activity of immobilized PpF1 in PVA beads

Finally, we examined the ability of PpF1 immobilized in PVA–sodium sulfate beads (S-1.5/1 h and S-1.5/6 h) to degrade TCE. The initial theoretical concentration of TCE in 457 medium (0.42 mg/l) was within the most common range (0.05–5 mg/l) of TCE concentration found in contaminated field sites [28, 32]. Both types of beads completely degraded toluene within 2 h (data not shown). S-1.5/1 h beads degraded 66.1 ± 17.8 % of TCE within 2 h, while S-1.5/6 h beads degraded 30.0 ± 11.8 % (Fig. 5). The lower TCE degradation rate obtained by S-1.5/6 h beads would be due to the higher degree of cell damage associated with the longer incubation time in the sulfate solution. Both beads completely degraded TCE within 10 h. Leakage of

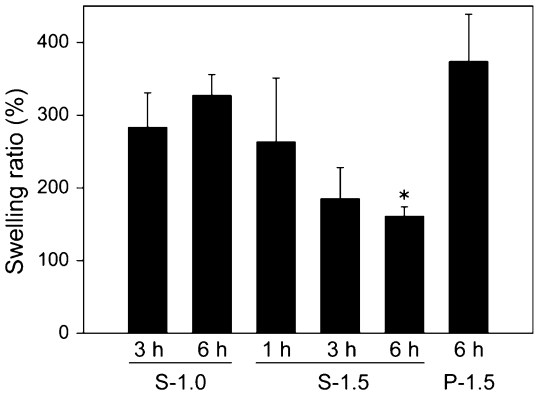

Fig. 4 Swelling ratio of PVA–sodium sulfate and PVA–sodium phosphate beads after 12 days of incubation in distilled water. *Bar* Mean ± SD ($n = 3$). *$p < 0.01$ versus P-1.5/6 h beads

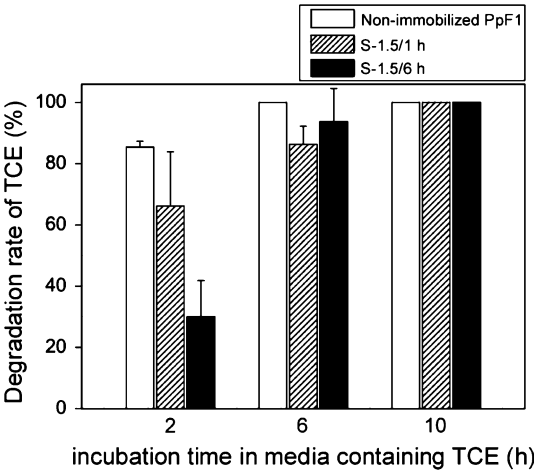

Fig. 5 TCE degradation rate by PpF1 immobilized in S-1.5/1 h and S-1.5/6 h beads as a function of incubation time in 457 medium containing TCE. *Bar* Mean ± SD ($n = 3$)

PpF1 from PVA beads was not observed during the experiment. Non-immobilized PpF1 completely degraded toluene within 2 h. The cells degraded 85.4 ± 1.9 % of TCE within 2 h and completely degraded TCE within 6 h. The lower TCE degradation rate for S-1.5/1 h and S-1.5/6 h beads than that for non-immobilized cells would be because of both resistance to the transport of TCE by PVA matrix and deactivation of the cells by sodium sulfate. Reduction of bead size for increasing specific surface area of the beads would improve the degradation rate. In preliminary study, we confirmed reusability of the beads by repeated TCE degradation tests. These results indicate that PpF1 in PVA–sodium sulfate beads retained the inherent degradation activity toward TCE and toluene.

Conclusion

Sodium sulfate has a lower toxicity toward PpF1 than sodium phosphate and boric acid. PVA–sodium sulfate gel beads showed a higher stability in water than

PVA–sodium phosphate beads. PpF1 was successfully immobilized in PVA–sodium sulfate beads without losing their inherent biological activity.

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