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Decolorization of an anthraquinone dye by the recombinant dye-decolorizing peroxidase (rDyP) immobilized on mesoporous materials

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Abstract

A recombinant dye-decolorizing peroxidase (rDyP) produced from *Aspergillus oryzae* is a novel peroxidase because of its unique tertiary structure. No conventional immobilization of rDyP has been successful. In this study, immobilization of rDyP was conducted using silica-based mesoporous materials, FSM-16 and AlSBA-15. Adsorption yields of rDyP immobilized on the two materials increased as pH decreased from 6 to 3. However, the activity yield of immobilized rDyP decreased with decreasing pH. Therefore, overall efficiency which is defined as adsorption yield × activity yield was maximum for immobilized rDyP on FSM-16 at pH 5 and on AlSBA-15 at pH 4. Leaching of rDyP from FSM-16 was much lower than that from AlSBA-15 presumably because FSM-16 has a more anionic surface, leading to stronger affinity of rDyP to FSM-16. rDyP immobilized on FSM-16 at pH 4 decolorized eight sequential batches of an anthraquinone dye, Remazol Brilliant Blue R (RBBR) in repeated-batch decolorization, but only two batches for RBBR occurred by rDyP immobilized on AlSBA-15 because of leaching rDyP from AlSBA-15.

Keywords: Recombinant dye-decolorizing peroxidase (rDyP); Mesoporous materials; Dye decolorization; Surface charge; Repeated-batch; Remazol Brilliant Blue R (RBBR)

1. Introduction

Synthetic dyes are extensively used in industry and at least 10% of the produced dye (800,000 t/year) enters the environment as waste [1]. Major synthetic dye classes include azo, anthraquinone and triarylmethane dyes which constitute more than 50% of dyes used in industrial applications. Anthraquinone-based dyes are highly resistant to microbial degradation because of their fused aromatic structure [2]. Among anthraquinone-based dyes, Remazol Brilliant Blue R (RBBR) is a toxic and recalcitrant organopollutant [3].

Considerable research during last two decades has focused on the use of enzymes to decontaminate wastewater containing dyes [4]. In our laboratory a new dye-decolorizing peroxidase (DyP), which decolorizes 20 kinds of synthetic dye, was isolated from *Thanatephorus cucumeris* Dec 1 (former name, *Geotrichum candidum* Dec 1). The *dyp* gene of *T. cucumeris* Dec 1 was transformed to *Aspergillus oryzae*, enhancing production of the recombinant dye-decolorizing peroxidase (rDyP) up to 3000 times [5,6]. For its application in industrial processes, maintenance of the stable activity, reuse of the rDyP, and reduction of inactivation by H_2O_2 are main constraints. [7,8]. Immobilization of the enzyme is a potential way to overcome these constraints and enhance catalytic efficiency of rDyP.

Techniques for enzyme immobilization include physical adsorption where weak interactions occur between the support and the enzyme, and chemical binding where covalent bonds are formed with the enzyme [9,10]. Adsorption is simple, cheap and effective but frequently reversible, and chemical covalent binding is effective and long-lasting, but is expensive and reduces the enzyme performance. Mass transfer limitation is an inherent problem in entrapment and microencapsulation of enzymes.

We tried to immobilize rDyP using different methods such as adsorption to activated carbon and glass beads, cross-linkage by glutaraldehyde and entrapping by photosensitive resins. However, no conventional techniques for immobilization of rDyP have been successful, mainly because of the completely new tertiary structures of rDyP relative to well-known peroxidases [11,12].

Ordered mesoporous materials with pore diameters in the range of 1–10 nm [13] are relatively new candidates for immobilization of enzymes because of their uniform and adjustable pore

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size, and large surface areas and pore volumes [13–17]. Additional advantages of enzyme immobilization on mesoporous materials include the mild immobilization conditions and efficient physical adsorption. Folded-sheet mesoporous materials (FSM-16) with pore sizes around 7 nm, synthesized from a layered form of sodium silicate [18], have been used for horseradish peroxidase (HRP) immobilization [15]. Furthermore, pore size limitations of these mesoporous materials led to synthesis of the larger-pore mesoporous silicates, Santa Barbara Amorphous (SBA-15) [19]. Aluminum-substituted SBA-15 (AlSBA-15) exhibited greater protein adsorption compared to their pure silica analogues [20].

In this study, we investigated immobilization of rDyP on synthesized mesoporous materials, FSM-16 and AlSBA-15 using cationic and non-ionic surfactants, respectively, and assessed the effect of pH on immobilized rDyP. Decolorization of Remazol Brilliant Blue R (RBBR) as a model anthraquinone dye by the immobilized rDyP was also investigated using repeated-batch mode decolorization.

2. Materials and methods

2.1. Chemicals

Spray-dried sodium silicate water-glass powder (kanemite) was supplied by Tokuyama Siltech, Japan. The cationic surfactant, cetyltrimethylammonium chloride (CTMA-Cl) was purchased from Kanto Chemical, Japan. The swelling agent 1,3,5-triisopropylbenzene (TIPB) was purchased from Acros Organic, Belgium. The non-ionic triblock copolymer surfactant, poly-(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) (Pluronic 123, molecular weight 5800, $EO_{20}PO_{70}EO_{20}$) and tetraethyl orthosilicate (TEOS) were purchased from Aldrich, USA. Remazol Brilliant Blue R (RBBR) and reactive blue 5 (RB5) were purchased from Nippon Kayaku, Japan.

2.2. rDyP production

rDyP, produced by cultivation of *A. oryzae* RD005 in a 10 l jar fermentor using wheat bran powder as a carbon source was purified by the previously reported method [6]. The culture supernatant (4000 ml) from a 5-day-old culture was passed through gauze to remove *A. oryzae* pellets. Then, filtered supernatant, containing 14 U/ml of rDyP, was centrifuged at 4 °C for 30 min, and concentrated to 85 ml by ultrafiltration using a 10 kDa membrane. The concentrated enzyme was precipitated in ammonium sulfate (70% saturation) at 4 °C, centrifuged at 4 °C for 30 min and dissolved in 25 mM piperazine buffer (pH 5.5). Ammonium sulfate (1.5 M) was added to 40 ml of the enzyme solution and applied to a Butyl-Toyopearl column. The gradient elution was carried out from 25 mM piperazine buffer (pH 5.5) containing 1.5 M ammonium sulfate to ammonium sulfate-free 25 mM piperazine buffer (pH 5.5). rDyP was eluted around 0.8 M ammonium sulfate.

Then, collected fractions were used for determination of activity and specific activity of rDyP. rDyP has a molecular mass of 58 kDa [6], and monomer dimensions of approximately 6.2 nm × 6.6 nm × 4.8 nm [11].

2.3. Assay of rDyP activity

The RB5 assay was conducted at its maximum absorbance of 600 nm by spectrophotometer (UV-2400PC; Shimadzu, Kyoto, Japan) to determine the activity of immobilized and free rDyP [6]. Citrate buffer (2920 μl, 25 mM pH 3.2), RB5 (15 μl, 25 mM) and 50 μl immobilized rDyP containing 0.1–0.3 mg mesoporous material or 50 μl free rDyP was added to a 3 ml cuvette. The reaction was started by the addition of 15 μl of 40 mM H_2O_2. One unit (U) of rDyP activity was defined as the amount of enzyme required to decolorize 1 μmol of RB5 in 1 min.

2.4. Synthesis of mesoporous materials

FSM-16 was synthesized in accordance with a previously reported method [15,18]. Kanemite (20 g) was dispersed in 200 ml of distilled water with stirring at ambient temperature for 70 min and filtrated through a filter paper to obtain a wet kanemite paste. The wet kanemite paste (corresponding to 10 g dry kanemite) was dispersed in 100 ml of distilled water, mixed with another 100 ml of distilled water containing 0.2 M of CTMA-Cl and 0.6 M TIPB and heated at 80 °C for 2 h with stirring. Then, the mixture pH was adjusted to pH 8.4 using 2N HCl and heated for another 2 h, then cooled to ambient temperature and filtrated. The filtered as-synthesized product was washed with distilled water and air-dried, and then calcined at 550 °C for 6 h to remove the organic fraction.

AlSBA-15 was synthesized using Pluronic 123, in accordance with a previously reported method [20]. Pluronic 123 (4 g) was dissolved in 30 ml water prior to the addition of 70 ml of 0.29 M HCl and the mixture was stirred for 5 h. Then, TEOS (9 g) and 1.32 g of alminum isopropoxide were added, and the mixture was stirred for another 24 h at 40 °C. The mixture was transferred to a Teflon-lined autoclave, heated at 100 °C for 48 h, and cooled to room temperature. The resulting white solid product was recovered by filtration, washed with distilled water and dried at 80 °C overnight. The as-synthesized AlSBA-15 was calcined at 540 °C for 6 h.

2.5. Characterization of mesoporous materials

The X-ray powder diffraction (XRD) spectra of FSM-16 and AlSBA-15 were obtained using a Rigaku RINT-2000 diffractometer equipped with Cu Kα radiation (40 kV, 40 mA). Nitrogen adsorption–desorption isotherm measurements of FSM-16 and AlSBA-15 were conducted at 77 K using a Bell Japan Belsorp 28 SA sorptometer. The specific surface areas were calculated by the Brunauer–Emmett–Teller (BET) method, using the adsorption data at relative pressure in the range of $P/P_0 = 0.05$–0.35. The pore diameter distribution curves were derived from the desorption branches by the Barrett–Joyner–Halenda (BJH) method. The aluminum and sili-

con contents of AlSBA-15 were determined using an inductively coupled plasma (ICP) source.

2.6. Immobilization of rDyP on mesoporous materials and measurement of activity

rDyP solution in a 10 mM citrate buffer (initial activity of approximately 300 U/ml) at pHs values ranging from 3 to 6 was used in immobilization experiments. FSM-16 or AlSBA-15 (30 mg) was added to 1 ml of the citrate buffered rDyP solution in a microtube (equivalent to 10,000 U rDyP per gram of each mesoporous material), and the mixture was stirred overnight at 4 °C. The mesoporous material with immobilized rDyP was recovered by centrifugation and the supernatant retained for determination of rDyP activity. The remaining solid was washed twice with 10 mM citrate buffer at the same pH as used for immobilization, and resuspended in the same buffer for determination of rDyP activity. Adsorption yield (%) was defined as

$$\frac{A_i - A_r}{A_i} \times 100$$

where A_i is the initial rDyP activity in the buffered rDyP solution before immobilization and A_r is the residual rDyP activity in the supernatant after immobilization.

The activity of immobilized rDyP is of interest in dye decolorization. Therefore, we defined activity yield as (measured rDyP activity/expected rDyP activity) × 100 (%). The expected rDyP activity was defined as $(A_i - A_r)$/concentration of the carrier. Overall efficiency (%) was defined as adsorption yield (%) × activity yield (%), which reflects overall activity performance of the immobilized rDyP.

Leaching of immobilized rDyP was assessed by storing the mesoporous material in 10 mM citrate buffer at 4 °C for 5-day periods, with sequential daily change of buffer. At each buffer exchange, the activities of immobilized rDyP and free rDyP in the supernatant were measured.

The H_2O_2 stability of immobilized rDyP and free rDyP was evaluated by placing 400 U/l of immobilized rDyP and free rDyP in 0.12 mM H_2O_2 at 30 °C and intermittently removing samples for measurement of residual rDyP activity (%) which was defined as $(A_t/A_0) \times 100$, where A_0 and A_t are the initial and time t rDyP activities, respectively. The residual free rDyP activity was plotted as a function of time, and the rate constant (k) was obtained from the exponential equation, $A_t = A_i \times \exp(-k \times t)$. The half-life of free rDyP was calculated as $\ln 2/k$.

The effect of pH on stability of rDyP activity was investigated by incubating 1000 U/l of free active rDyP in citrate buffered solution in the range of pH 3–5 at 4 °C for 48 h. The sample was taken from the buffered rDyP solution daily to measure remaining rDyP activity. The residual rDyP activity was calculated from $(A_t/A_0) \times 100$ as described above.

2.7. Repeated-batch dye decolorization by rDyP immobilized on mesoporous materials

Decolorization of RBBR was carried out in vials containing 10 ml of 10 mM citrate buffered solution (pH 3–5), 150 mg/l RBBR and 400 U/l rDyP immobilized on FSM-16 or AlSBA-15. Immobilization was carried out at the same pH as decolorization. The reaction was initiated by addition of 0.24 mM of H_2O_2 to the mixture, which was incubated at 30 °C with shaking at 100 strokes/min (spm). When decolorization ratio (%) reached more than 90%, the reaction mixture was centrifuged at 4 °C at 10,000 rpm for 10 min, the decolorized dye solution was withdrawn, and new dye solution was added for the next round of decolorization. The rDyP activity in each withdrawn solution was measured to assess leaching in rDyP from the FSM-16 or AlSBA-15. The residual activity of rDyP immobilized on FSM-16 or AlSBA-15 was assayed at the end of each round of decolorization. Controls (FSM-16 or AlSBA-15 with added RBBR but without immobilized enzyme) were included to account for non-specific dye adsorption to FSM-16 or AlSBA-15. The RBBR concentration was determined by spectrophotometry at 593 nm, and the absorbance was converted to dye concentration using a calibration curve.

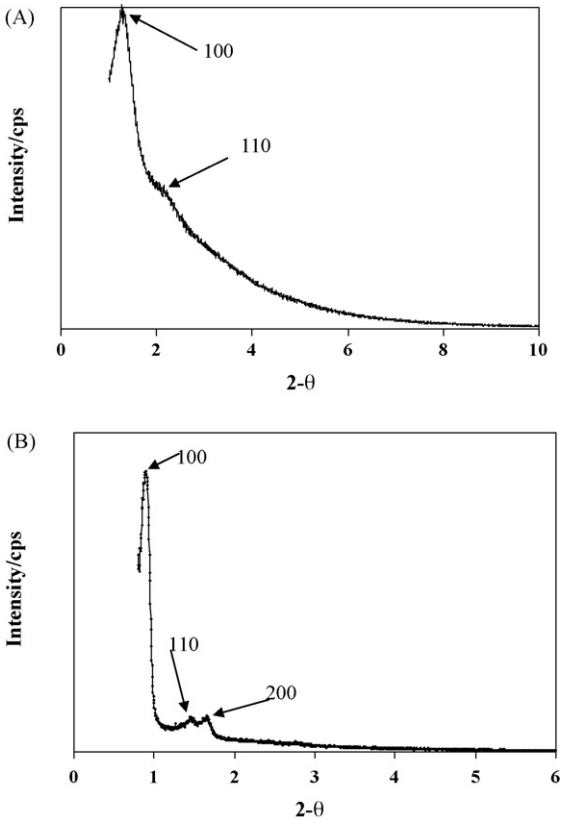

Fig. 1. X-ray powder diffraction (XRD) patterns of the calcinated mesoporous silica materials, FSM-16 (A) and AlSBA-15 (B).

3. Results

3.1. Characteristics of the mesoporous materials

Fig. 1 shows the XRD patterns of calcinated FSM-16 and AlSBA-15. The XRD spectrum of FSM-16 had an intense (1 0 0) peak and a weak (1 1 0) peak, while the spectrum of AlSBA-15 had three well-resolved peaks at (1 0 0), (1 1 0) and (2 0 0)

Table 1
Textural properties of FSM-16 and AlSBA-15

	Pore volume (cm^3/g)	Pore diameter (nm)	Specific surface area (m^2/g)	External specific surface area (m^2/g)	Si/Al
FSM-16	1.9	5.4	1108	66	–
AlSBA-15	1.29	10.6	918	22	30

–: not measured.

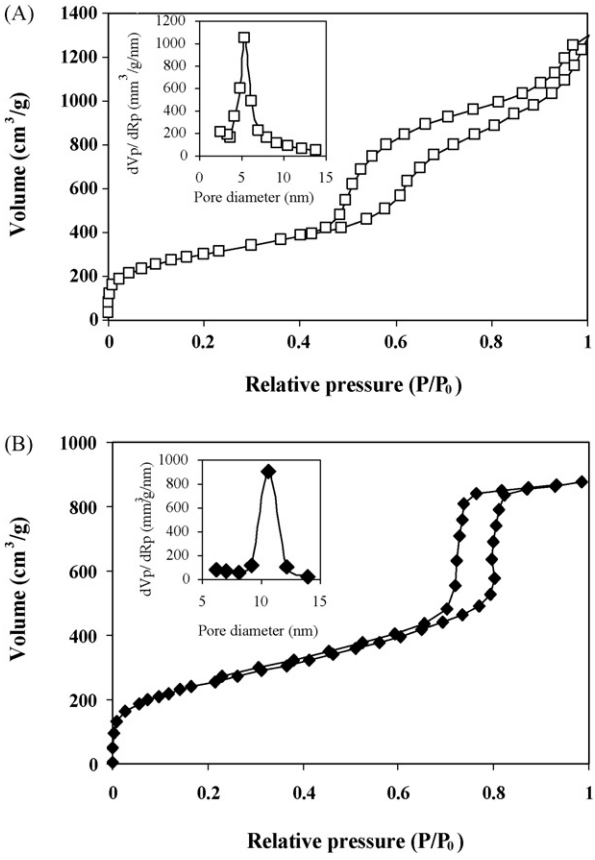

Fig. 2. Nitrogen adsorption–desorption isotherms of FSM-16 (A) and AlSBA-15 (B).

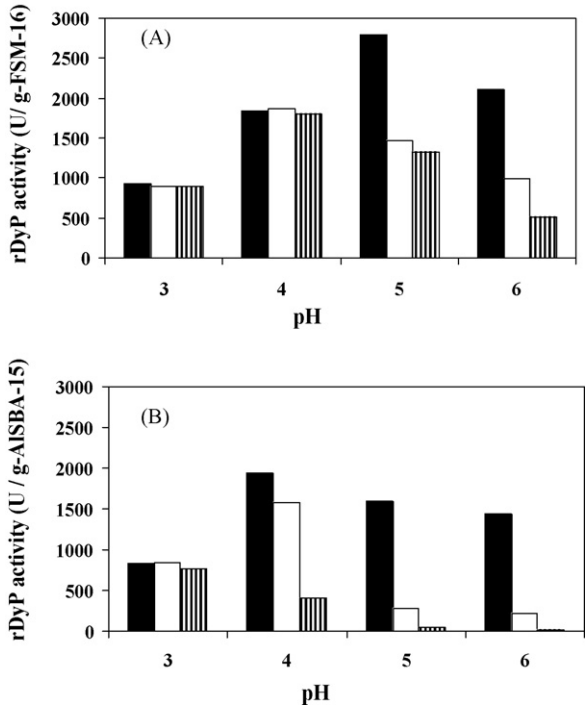

Fig. 3. Activity change of immobilized rDyP on FSM-16 (A) and AlSBA-15 (B) after daily leaching treatment under different pH conditions. Day 1 (black bars), day 5 (white bars) and day 10 (dark verticals).

reflections of the hexagonal space group *p6mm* structure. The FSM-16 and AlSBA-15 adsorption–desorption isotherms which had a typical capillary condensation step are shown in Fig. 2 with their corresponding pore diameter distributions (inserted figures). The isotherms of AlSBA-15 and FSM-16 are type IV, which is typical of mesoporous materials. The textural properties of FSM-16 and AlSBA-15 are given in Table 1.

3.2. Effect of pH on adsorption and activity yields of rDyP immobilized on mesoporous materials

Immobilization of rDyP on mesoporous materials was clearly related to pH (Tables 2 and 3). Table 2 shows that the adsorption yield of rDyP on FSM-16 increased as pH decreased from 6 to 3. Maximum adsorption yields of 98% and 97% were obtained at pH 3 and 4, respectively. Table 3 shows that in the adsorption yield of rDyP on MISBA-15, 98% of the applied rDyP was immobilized on AlSBA-15 at pH 3, but only 20% was immobilized at pH 6. The adsorption yield was significantly higher for FSM-16 than for AlSBA-15, except at pH 3. However, the activity yield of rDyP was higher for AlSBA-15. Consequently, maximum overall efficiencies were 29% at pH 5 for FSM-16 and 19% at pH 4 for AlSBA-15. The maximum measured rDyP activity at all examined pH values was 2800 U/g-FSM-16 at pH 5.

3.3. Leaching of rDyP immobilized from mesoporous materials

Leaching of rDyP from mesoporous materials was assessed by repeated daily washing of the immobilized rDyP with buffer solution. Fig. 3A shows that rDyP activity on FSM-16 dropped by 57% and 78% at pH 5 and pH 6, respectively, over 10-day period. No changes in activity of rDyP immobilized on FSM-16 were observed at pH 3 and 4, or on AlSBA-15 at pH 3. Decreased activity of rDyP immobilized on AlSBA-15 (Fig. 3B) by leaching was more significant than that on FSM-16.

3.4. Stability of immobilized and free rDyP to H_2O_2

The H_2O_2 stability of free and FSM-16-immobilized rDyP at pH 3 is shown in Fig. 4. In this experiment, immobilized and

Table 2
rDyP immobilization on 1 g FSM-16 under different pH conditions (initial rDyP activity used = 10,000 U)

pH	Adsorption yield (%)	Expected activity (U/g-FSM-16)	Measured activity (U/g-FSM-16)	Activity yield (%)	Overall efficiency (%)
3	98	9800	933	9.5	9.5
4	97	9700	1833	19	18
5	83	8330	2800	34	29
6	66	6600	2100	32	21

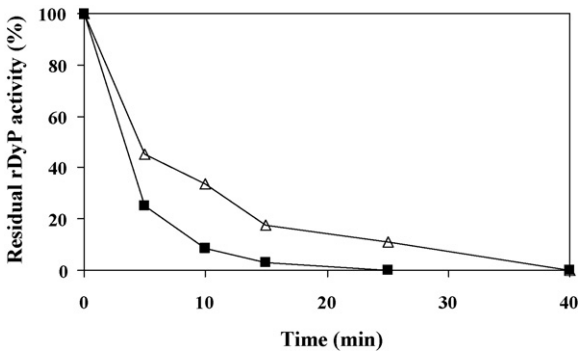

Fig. 4. Stability of free rDyP (■) and rDyP immobilized on FSM-16 (△) in 0.12 mM H_2O_2 at pH 3 at 30 °C. (initial rDyP activity 400 U/l).

free rDyPs were exposed to only H_2O_2 and no dye was added. Immobilized rDyP had higher stability to H_2O_2 compared to free rDyP. The stability of rDyP immobilized on FSM-16 at pH 4 and on AlSBA-15 at pH 3 and 4 were similar to that of free rDyP (data not shown).

3.5. Effect of pH on stability of rDyP activity

The effect of pH on stability of rDyP activity is given in Fig. 5. The decrease in rDyP activity at different pH values was negligible. More than 90% of initial rDyP activity remained active after incubation in buffer solution for 48 h. This stable rDyP in applied pHs confirms that high adsorption yield of rDyP in acidic pH in Tables 2 and 3 was a result of active rDyP adsorption on mesoporous materials. This result also suggests that the loss of rDyP activity in Fig. 4 was mainly due to rDyP inactivation by H_2O_2 and contribution of pH to rDyP inactivation was negligible.

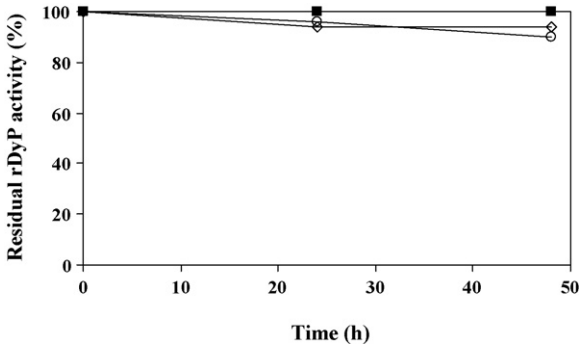

Fig. 5. Change in stability of free rDyP activity during 48 h incubation in citrate buffered solution at pH 3 (◯), pH 4 (■), and pH 5 (◊) (initial rDyP activity 1000 U/l).

3.6. Repeated-batch dye decolorization by rDyP immobilized on mesoporous materials

The results of repeated-batch decolorization of 150 mg/l RBBR by rDyP immobilized on FSM-16 under different pH conditions are given in Fig. 6. Steady decolorization of RBBR was continued for three batches of RBBR at pH 3 (Fig. 6A), but at fourth batch, decolorization rate decreased significantly. Similarly, at pH 5, after four batches, decolorization rate was decreased (Fig. 6C). At pH 4, constant decolorization occurred for seven batches (Fig. 6B).

No leaching of immobilized rDyP from FSM-16 was observed both at pH 3 or 4, as was expected from the results shown in Fig. 3. At pH 5, leaching of rDyP was observed (the data is not shown) and thus rDyP activities in decolorized dye solutions withdrawn from the first, second, third and fourth batches were 50%, 14%, 11% and 3% of the initial activity, respectively. In control samples containing only FSM-16 and RBBR approximately 10% of added RBBR was adsorbed, and this occurred independently of the application pH.

In repeated-batch decolorization by immobilized rDyP on AlSBA-15 incomplete dye decolorization occurred and no residual rDyP activity was found at the end of only one batch at pH 3 (Fig. 7A). Two batch RBBR decolorization occurred at pH 4 (Fig. 7B) and the rDyP activity in the decolorized dye solution withdrawn from the first batch was 45% of the initial activity. This means that rDyP immobilized on AlBSA-15 decreased mainly due to leaching rDyP from AlBSA-15. Dye decolorization by rDyP immobilized on AlSBA-15 was not conducted at pH 5, because of the much greater higher leaching of rDyP at pH 5. Less than 6% of the applied RBBR was adsorbed in AlSBA-15 control samples.

Table 3
rDyP immobilization on 1 g AlSBA-15 under different pH conditions (initial rDyP activity used = 10,000 U)

pH	Adsorption yield (%)	Expected activity (U/g-AlSBA-15)	Measured activity (U/g-AlSBA-15)	Activity yield (%)	Overall efficiency (%)
3	98	9830	833	8.4	8.2
4	44	4360	1933	44	19
5	17	1660	1600	96	15
6	20	1960	1433	72	14

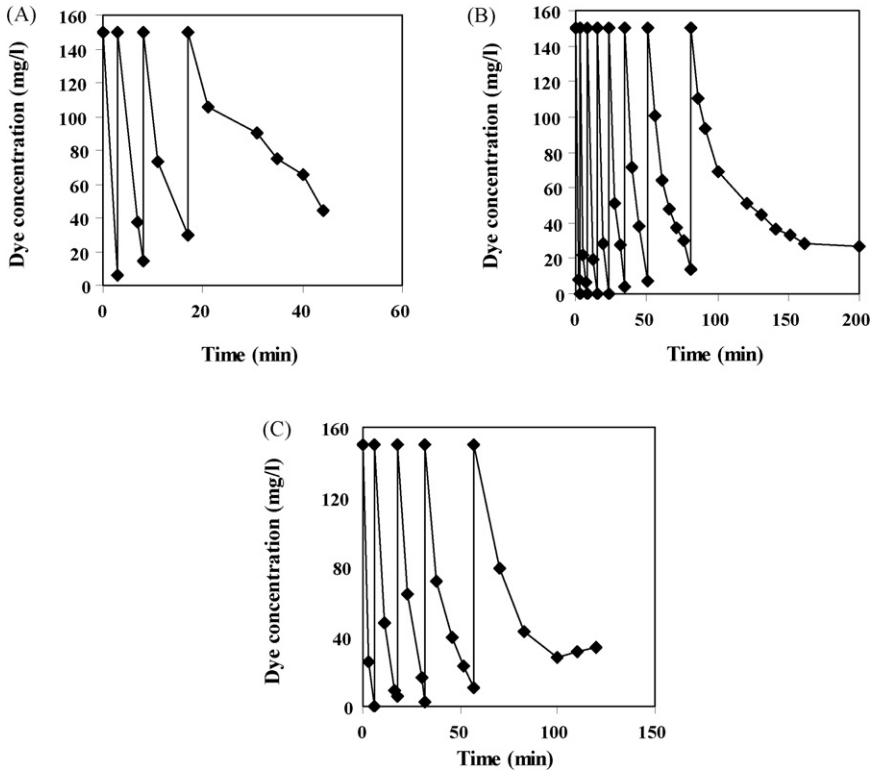

Fig. 6. Repeated-batch decolorization of RBBR by 400 U/l rDyP immobilized on FSM-16 at pH 3 (A), pH 4 (B) and pH 5 (C). Immobilization of rDyP was conducted at the decolorization pH.

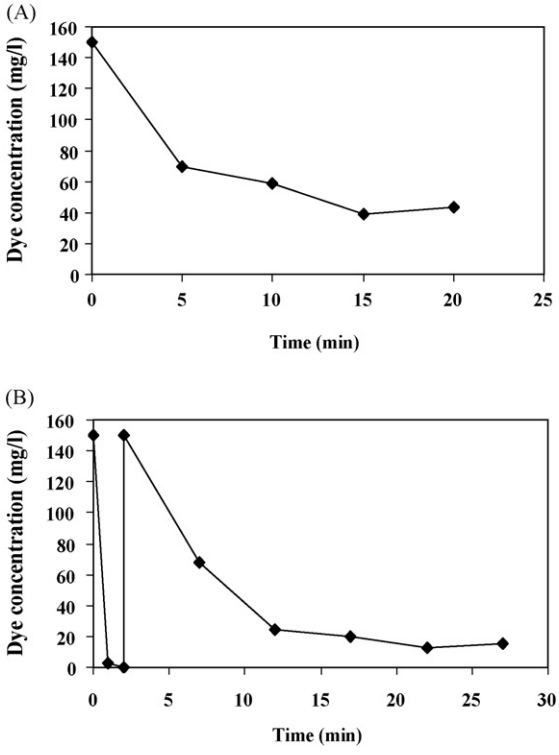

Fig. 7. Repeated-batch decolorization of RBBR by 400 U/l rDyP immobilized on AlSBA-15 at pH 3 (A) and pH 4 (B).

4. Discussion

Two factors are likely to influence enzyme immobilization on the silica-based mesoporous materials, FSM-16 and AlSBA-15. The first factor is the charges on the enzyme and the mesoporous materials [14,15]. The silica-based mesoporous materials have a net negative charge above pH 2, because they have an isoelectric point (p*I*) of 2 [20,21]. As the pI of rDyP is 4.2 [6], the enzyme is positively charged below pH 4.2 and negatively charged above it. Therefore, the electrostatic interaction strength between rDyP and the mesoporous materials is pH dependent. In the pH range 2–4.2, stronger electrostatic interactions between rDyP and mesoporous materials will have resulted in higher adsorption yields (Tables 2 and 3). Above pH 4.2, the surfaces of both rDyP and the mesoporous materials are negatively charged, and consequently the weak interaction or even repulsion between them lead to a lower rDyP adsorption yield.

In the range of pH 4–6 a higher rDyP adsorption yield was found for FSM-16 (Table 2) compared to AlSBA-15 (Table 3). This was because these materials have different surface characteristics. The cationic surfactant (CTMA-Cl) used in FSM-16 synthesis led to enhanced ionization of silanol groups (≡Si(OH)) to the ionized silanol group ($\equiv SiO^-$). However, the non-ionic surfactant (Pluronic 123) used in AlSBA-15 synthesis caused comparatively less silanol group ionization [15]. Thus, the enhanced anionic surface of FSM-16, may have facilitated adsorption to rDyP of positively charged amino acid groups (NH^+).

The second factor is the pore size of mesoporous materials relative to the molecule size of the enzyme [14,15]. The apparent pore size of FSM-16 (Fig. 2) was smaller than rDyP (6.2 nm × 6.6 nm × 4.8 nm), but the adsorption yield of rDyP on FSM-16 was high. Two possibilities could account for this finding. Firstly, the actual pore size of FSM-16 may be approximately 6.85 nm, which is large enough to accommodate rDyP, if FSM-16 is assumed to be an ideal 2-D hexagonal channel (pore diameter = $4 \times V/A$, where V and A are the specific pore volume and surface area of FSM-16, respectively). This is bigger than the pore size calculated using the BET method (5.4 nm). Secondly, rDyP may be adsorbed at the pore mouth and on the external surface of FSM-16 (approximately 60 m^2/g of FSM-16).

In spite of the higher adsorption yield, resulting from the decrease in pH and the stronger electrostatic interaction between mesoporous materials and rDyP, the activity yield of immobilized rDyP was low. It may be that the stronger electrostatic interaction of rDyP with the mesoporous material surface changed the enzyme conformation [22], and thus with decreasing pH some of the active sites of rDyP were oriented improperly in the reactions [23,24]. Transfer of rDyP immobilized on mesoporous materials from buffer solution at pH 3 to buffer solution at pH 7, resulted in almost complete release of the immobilized rDyP which was subsequently able to be recovered in a free active form. This observation supports the interpretation above. Similar changes in activity of immobilized enzymes under different pH conditions have been reported for chloroperoxidase (CPO) [22] and lipase as a result of strong electrostatic interactions of the enzyme with the surface [25,26].

Decreased activity due to leaching of rDyP from FSM-16 and AlSBA-15 was notable at pH values higher than 5 and 4, respectively (Fig. 3). Almost all of the rDyP activity losses in immobilized rDyP were found in the supernatant after each washing of the mesporous materials. This indicates that the decrease in rDyP activity on mesoporous materials at pH ≥ 5 was mainly due to leaching of the immobilized rDyP from the materials rather than enzyme denaturation or inactivation.

Another factor to affect the stability of rDyP is H_2O_2. In the presence of 0.12 mM H_2O_2 the half-life of 400 U/l free rDyP (k) was 3, 9 and 24 min at pH 3, 4 and 5, respectively, showing its pH dependence as has been found for other peroxidases such as CPO and HRP [7,27]. This indicates that an increase in pH is expected to increase the number of dye-decolorizing batches as a result of an increase in the enzyme half-life. However, rDyP leaching from mesoporous materials increased as pH was increased from 3 to 5, indicating that an increase in pH has a negative effect on the number of dye-decolorizing batches. Thus, the maximum number of batches of dye decolorization by rDyP immobilized on FSM-16 occurred at pH 4 (Fig. 6B) as a result of the effects of pH on leaching and half-life.

The number of batches of RBBR decolorized by rDyP immobilized on FMS-16 was significantly higher than for the enzyme immobilized on AlSBA-15. This suggests greater stability and less leaching of rDyP associated with the FMS-16.

Although we reported that almost no RBBR decolorization occurred by free rDyP at pH 3 [8], four batches of RBBR decolorization were possible by rDyP immobilized on FSM-16 at pH 3 (Fig. 6A). Moreover, the H_2O_2 stability of rDyP immobilized on FSM-16 at pH 3 was greater than that of free rDyP (Fig. 4). These two new properties of immobilized rDyP at pH 3 were probably a result of environmental and structural changes in the microenvironment of the immobilized rDyP [28,29]. This may have broadened the pH range applicable of rDyP immobilized on FSM-16 and increased its stability to H_2O_2.

In conclusion, rDyP produced from relatively inexpensive substrates was successfully immobilized on silica-based mesoporous materials and immobilized rDyP was stable and repeated-batch dye decolorization has been demonstrated. As a consequence, immobilized rDyP may be a promising and economical biocatalyst for treatment of colored wastewater, after optimization to minimize both leaching and inactivation of the immobilized rDyP.

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