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Phosphate removal and recovery using immobilized phosphate binding proteins

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ABSTRACT

Progress towards a more circular phosphorus economy necessitates development of innovative water treatment systems which can reversibly remove inorganic phosphate (P_i) to ultra-low levels ($<100\ \mu g\ L^{-1}$), and subsequently recover the P_i for reuse. In this study, a novel approach using the high-affinity *E. coli* phosphate binding protein (PBP) as a reusable P_i bio-adsorbent was investigated. PBP was expressed, extracted, purified and immobilized on NHS-activated Sepharose beads. The resultant PBP beads were saturated with P_i and exposed to varying pH (pH 4.7 to 12.5) and temperatures (25–45 °C) to induce P_i release. Increase in temperature from 25 to 45 °C and pH conditions between 4.7 and 8.5 released less than 20% of adsorbed P_i. However, 62% and 86% of the adsorbed P_i was released at pH 11.4 and 12.5, respectively. Kinetic experiments showed that P_i desorption occurred nearly instantaneously (<5 min), regardless of pH conditions, which is advantageous for P_i recovery. Additionally, no loss in P_i adsorption or desorption capacity was observed when the PBP beads were exposed to 10 repeated cycles of adsorption/desorption using neutral and high pH ($\geq$12.5) washes, respectively. The highest average P_i adsorption using the PBP beads was $83 \pm 5\%$, with $89 \pm 4.1\%$ average desorption using pH 12.5 washes over 10 wash cycles at room temperature. Thermal shift assay of the PBP showed that the protein was structurally stable after 10 cycles, with statistically similar melting temperatures between pH 4 and 12.5. These results indicate that immobilized high-affinity PBP has the potential to be an effective and reversible bio-adsorbent suitable for P_i recovery from water/wastewater.

1. Introduction

The economic and ecological losses associated with eutrophication caused by excess inorganic phosphate (P_i), have inspired increasingly lower effluent phosphorus water quality guidelines for many municipal and industrial wastewater treatment facilities around the world (Amery and Schoumans, 2014; USEPA, 1995, 1986). Apart from limiting P_i loadings in wastewater effluents, there is also strong impetus to secure renewable P_i resources for use as agricultural fertilizer, i.e., via P_i recovery from wastewater. This approach stimulates the circular phosphorus economy, which is vital since in addition to being a pollutant, P_i is a geographically limited nonrenewable resource that is essential to sustain global food production (Cordell et al., 2009; Cordell and White, 2014; Mayer et al., 2016; Rittmann et al., 2011). This removal/recovery paradigm drives development of innovative water treatment systems which can effectively remove P_i to ultra-low levels (<100 μg/L) and release P_i under controlled conditions suitable for subsequent P_i reuse (Mayer et al., 2013; Rittmann et al., 2011).

A novel strategy utilizing high-affinity phosphate binding proteins (PBP) as a reusable bio-adsorbent to reversibly capture P_i was investigated in this study. The phosphate-specific transporter (Pst) system in bacteria is specifically evolved to import P_i when P_i is present at low levels, which demands efficient, selective, and high-affinity binding and transport of P_i to meet the cell's metabolic demands (Blank, 2012; Luecke and Quiocho, 1990; Santos-Beneit et al., 2008). The Pst protein complex comprises four subunits, an ATP-binding protein (pstB), two transmembrane proteins (pstA and pstC), and a periplasmic PBP (pstS) (Luecke and Quiocho, 1990; Santos-Beneit et al., 2008). The periplasmic pstS PBP has recently attracted interest as a potential high-affinity, phosphate-specific P_i

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adsorbent (Kuroda et al., 2000; Li et al., 2009; Yang et al., 2016, 2017).

Removal of P_i to ultra-low concentrations has been demonstrated using PBP expressed in bacterial cells' periplasmic space, expressed on the cells' surface, or immobilized on Sepharose beads (Choi et al., 2013; Kuroda et al., 2000; Li et al., 2009; Yang et al., 2016). For example, Choi et al. (2013) showed that recombinant *E. coli* expressing PBP in the periplasmic space can remove ≥97% P_i from water within 6 h (initial concentration of 0.2–0.5 mg-P_i/L). Kuroda et al. (2000) demonstrated P_i removal to below the detection limit (9.5 ng-P_i/L) using PBP immobilized on Sepharose beads to treat an influent concentration of 15 μg-P_i/L. To better support the circular phosphorus economy via the waste-to-resource paradigm, recovery of the captured P_i is essential. To assess P_i recovery, Yang et al. (2016) investigated the effect of varying temperature, pH, and ionic strength on P_i release from PBP over-expressed in the periplasmic space of *E. coli* cells. However, a maximum of only 1.4–2% of the adsorbed P_i was recovered after exposing the recombinant *E. coli* cells to low pH (pH 3.8), high temperature (35 °C), or high ionic strength (100 mM KCl) for 3 h (Yang et al., 2016). On the other hand, Kuroda et al. (2000) reported >90% recovery of the adsorbed P_i from PBP immobilized on Sepharose beads at pH 3. Although the data was not shown, Kuroda et al. (2000) stated that the immobilized PBP could be reused after neutralizing the pH. Thus, additional investigation of the removal and controlled release of P_i from immobilized PBP at varying pH and temperature, and the performance of PBP over multiple cycles of P_i removal and recovery is needed. Effective reusability of the PBP is a crucial aspect of the viability of this bio-adsorbent for P_i removal and recovery from water.

The objective of this study was to investigate the adsorption and desorption of P_i using PBP immobilized on an inert surface as a function of pH and temperature. In comparison to periplasmic PBP, extracellular immobilized PBP may be more effective as a reversible bio-adsorbent as it may be more conducive to both removal and controlled recovery through regulation of environmental parameters such pH and temperature. Reusability of the PBP was also investigated to assess the impact of the recovery conditions on PBP structure and P_i recovery potential over multiple cycles of P_i adsorption/desorption.

2. Materials and methods

2.1. Expression and purification of PBP

The pstS PBP used in this study was a single-cysteine mutant variant (A197C) of the mature *E. coli* PBP developed by Solscheid et al. (2015) for use as a phosphate biosensor. The pstS gene (A197C) overexpression plasmid (plasmid # 78198, Addgene, Cambridge, MA, USA) was transformed into BL21(DE3) *E. coli* competent cells, and cultured for protein expression and purification, as previously described (Solscheid et al., 2015). Briefly, a 5 mL overnight culture of the transformed BL21(DE3) cells was grown in LB media supplemented with 100 μg/mL ampicillin at 37 °C. This culture was diluted by transferring 2 mL of the overnight culture into 1 L fresh LB growth media in baffled glass flasks. The flasks were incubated at 37 °C with vigorous shaking, and the culture was allowed to grow to an OD 600 of approximately 0.8 before inducing protein expression using 500 μM isopropyl-β-D-thiogalactopytanoside (IPTG). After 4-h induction at 37 °C, cells were centrifuged for 15 min at 4000×*g* and 4 °C. All further protein purification steps were carried out at 4 °C.

To purify the proteins, the pellets were re-suspended in 100 mL of resuspension buffer (10 mM Tris-HCl, 1 mM $MgCl_2$, pH 8.0) and sonicated 4 times for 30 s at 200 W with a 5 s on/off pulse cycle. The lysate supernatant was collected following centrifugation at 6000×*g* for 45 min. The lysate was passed through a 100 mL_{BV} (settled and drained bead volume, where BV = bed volume) Q-Sepharose column (GE Healthcare Bio-Sciences, Pittsburg, PA, USA), equilibrated with resuspension buffer. The protein was eluted in a 100 mL gradient of 0–200 mM NaCl in the resuspension buffer. The presence of the protein was verified in the eluted fractions using SDS-PAGE. Fractions containing pstS were pooled and concentrated using a 10 kDa cutoff spin concentrator (Vivaspin® 20, GE Healthcare Bio-Sciences) in cases when higher protein concentrations were required. The concentration of the purified PBP was 221 ± 0.6 μM (average ± standard deviation), as quantified at 280 nm using a spectrophotometer (Agilent Technologies, Santa Clara, CA, USA), assuming an extinction coefficient of 17.8 cm^{-1} (Brune et al., 1994).

2.2. Immobilization of PBP

The purified pstS PBP was dialyzed using a Spectra/Por 2 Dialysis Membrane (MWCO 12–14 kDa, Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA). Dialysis was conducted for 16 h at 4 °C and included 6 exchanges of 0.2 M $NaHCO_3$, 0.5 M NaCl pH 8.3 buffer. The dialyzed PBP was immobilized on NHS-activated Sepharose 4 Fast Flow beads in accordance with the manufacturer's instructions (GE Healthcare Bio-Sciences). Since NHS-activated Sepharose interferes with the signal from the spectrophotometer at 280 nm, we used the Quick Start™ Bradford Protein Assay (Bio-Rad Laboratories Inc., Hercules, CA, USA) to determine the concentration of PBP for further experiments. The concentration of the dialyzed PBP was measured as 202 ± 2 μM using the Bradford assay.

Fresh NHS beads (stored in 100% isopropanol) were transferred into a 100 mL Econo-Column (Bio-Rad Laboratories Inc.) and washed with 10 bed volumes of 1 mM HCl solution at 4 °C. For the coupling reaction, 20 mL of dialyzed PBP (202 μM) was added to 20 mL of washed NHS beads (drained volume) and mixed at 30 rpm on a Roto-Torque Variable Speed end-over-end rotator (Cole Parmer, IL, USA) for 16 h at 4 °C. The supernatant was collected and the concentration of the unbound PBP was quantified using the Bradford assay. Of the initial PBP loaded onto the column (20 mL of 202 ± 2 μM), 98 ± 0.6% was immobilized onto the NHS beads, providing a coupling density of 197 ± 0.2 nmol-PBP/mL_{BV} NHS beads (mL_{BV} = drained NHS bead volume). This non-optimized coupling density was much lower than the theoretical maximum of 16–23 μmol/mL_{BV} for NHS beads reported by GE Healthcare Bio-Sciences. However, 197 ± 0.2 nmol-PBP/mL_{BV} was sufficient for the adsorption/desorption assessments in this study. Based on 1 mol of PBP adsorbing 1 mol of P_i (Brune et al., 1998, 1994; Solscheid et al., 2015), the theoretical P_i adsorption capacity of the NHS beads was 197 ± 0.2 nmol/mL_{BV}. The NHS beads conjugated with PBP (hereon referred to as PBP beads) were sequentially washed with 1_{BV} of 0.1 M Tris-HCl pH 8.5 followed by washing with 1_{BV} of buffer containing 0.1 M sodium acetate, 0.5 M NaCl pH 4.5. This cycle was repeated three times followed by five washes with 1_{BV} of buffer containing 10 mM Tris-HCl, 1 mM $MgCl_2$ pH 7.1.

To remove the legacy P_i already adsorbed on the PBP during the expression, purification, and immobilization process, the PBP beads were mopped using 0.1 unit/mL purine nucleoside phosphorylase (PNPase) and 300 μM 7-methylguanosine (7-MEG) (Brune et al., 1998, 1994). To facilitate mixing, 20 mL of 10 mM Tris-HCl, 1 mM $MgCl_2$ pH 7.1 buffer was added to 20 mL_{BV} PBP beads. Next, 0.1 unit/mL PNPase enzyme and 300 μM 7-MEG was added to the 40 mL PBP bead solution (50% suspension). The mopping reaction was performed overnight at 4 °C at 30 rpm using a rotary shaker. After 16 h, the PBP beads were washed with 5 x 1_{BV} 10 mM Tris-HCl, 1 mM $MgCl_2$ pH 7.1 buffer to remove the P_i-mop. Upon completion of this

PBP immobilization procedure, the beads were either used immediately or stored at 4 °C for up to 48 h prior to use.

A control set of beads was prepared using 20 mL_{BV} of fresh NHS beads following the same procedure used for the PBP beads, except without PBP addition.

2.3. Adsorption of P_i by immobilized PBP

Triplicate P_i adsorption experiments were conducted in batch tests in 2 mL centrifuge tubes containing 0.25 mL_{BV} PBP beads. In all tests, 1 mL reaction buffer (10 mM Tris-HCl, 1 mM $MgCl_2$) pH 7.1, 25 °C containing excess P_i (60 μM) was initially added (60 nmol P_i) versus the theoretical capacity of the PBP beads (49 nmol/0.25 mL_{BV}) to ensure P_i saturation of the PBP beads. After gentle mixing by hand and 10 min for bead settling, the supernatant was collected and analyzed for P_i. Previous studies showed that P_i binding is nearly instantaneous (Brune et al., 1998, 1994; Solscheid et al., 2015), such that 10 min was sufficient to achieve equilibrium. The pH in the centrifuge tubes was measured using a micro pH probe (Orion™ 9810BN, Thermo Scientific™, Waltham, MA, USA). No change in pH was observed over the course of the 10 min adsorption period.

2.4. Recovery of P_i from immobilized PBP as a function of temperature and pH

Following adsorption, desorption experiments were initiated by washing the beads 3 times using 1 mL reaction buffer pH 7.1 to remove unbound P_i. Each tube was then loaded with 1 mL of reaction buffer solution. In separate experiments, the influence of temperature was evaluated by adjusting the reaction buffer temperature to 25 °C, 35 °C, or 45 °C before addition to the tubes (pH 7.1). The tubes were maintained at the target temperature for the duration of the desorption experiment using an incubator (VWR 1524 digital incubator). Additional tests were performed to evaluate the influence of pH by adjusting the pH of reaction buffer to final values of 4.7, 6.5, 7.1, 8.5, 9.2, 11.4, and 12.5 using 1 M HCl or NaOH while maintaining a constant temperature of 25 °C. The pH in the microfuge tubes was measured using a micro pH probe (Orion™ 9810BN, Thermo Scientific™, Waltham, MA, USA), and did not change over the duration of the experiment. In both temperature and pH tests, gentle mixing was applied initially by inverting the microfuge tubes three times by hand. The beads were then allowed to settle for 10 min and a 1 mL aliquot of the settled supernatant was analyzed for P_i using the ascorbic acid method (APHA, 2012). For each condition tested, NHS beads with no PBP (control beads) were tested in parallel to isolate the impact of the NHS beads on P_i adsorption/desorption.

2.5. Kinetics of P_i recovery from immobilized PBP

The extent of P_i release from PBP beads was assessed as a function of time for four different pH conditions (following previous experiments showing efficient release using elevated pH). Triplicate experiments were conducted for both PBP beads and control beads, as described in Sections 2.3 and 2.4. An initial P_i adsorption cycle was performed by adding 1 mL of 60 μM P_i solution in 25 °C reaction buffer at pH 7.1 to 0.25 mL_{BV} PBP beads. After gentle mixing by hand and 10 min bead settling, the supernatant was collected and analyzed for P_i. For subsequent desorption, the PBP beads were washed 3 times with 1 mL reaction buffer at pH 7.1 to remove unattached P_i from solution. Next, the PBP beads were washed with 1.5 mL of reaction buffer, yielding final pH values in the tubes of 7.1, 10.8, 11.9 and 12.5. Aliquots of 0.1 mL were collected for P_i analysis after 5, 10, 20, 30, 40, and 50 min of reaction.

2.6. Reusability of immobilized PBP

The ability of the PBP beads to adsorb and desorb P_i over 10 cycles of sequential high and neutral pH conditions (promoting desorption and adsorption, respectively) was investigated. Tests were conducted in batch mode using 10 mL disposable Poly-Prep® reactors (Bio-Rad Laboratories Inc., Hercules, CA, USA) with 0.25 mL_{BV} beads. Based on results of previous pH tests, 3 different high pH conditions, 11.5, 12, and 12.5, were tested in independent triplicate experiments using PBP beads or control beads.

Fig. 1 illustrates the experimental approach. The first step in these experiments was to release P_i adsorbed on P_i-saturated PBP beads by washing them with 1.75 mL of reaction buffer at pH 11.5, 12, or 12.5 (25 °C) for 10 min before decanting the supernatant. Immediately following, 1.75 mL reaction buffer at pH 7.1 (25 °C) was added to adjust the beads to near-neutral pH for 10 min, before decanting the supernatant in preparation for the subsequent P_i adsorption cycle. The pH in the tubes was verified using a micro pH probe (Orion™ 9810BN, Thermo Scientific™, Waltham, MA, USA). The buffer solution decanted after each step was analyzed for P_i desorbed during the initial Cycle 0.

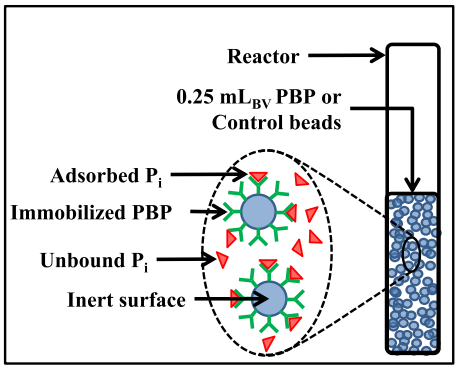
Following the initial P_i release cycle, 10 sequential cycles of P_i adsorption/desorption were performed. The first P_i adsorption step in each cycle consisted of adding 1 mL reaction buffer at pH 7.1, 25 °C containing 60 μM P_i. After gentle mixing and 10 min bead settling, the supernatant was decanted and analyzed for P_i. In the second P_i desorption step, P_i was desorbed using 1.75 mL of reaction buffer at 25 °C and pH 11.5, 12, or 12.5 for 10 min, followed by decanting the supernatant. In the third rinse step, 1.75 mL reaction buffer at pH 7.1 and 25 °C was added for 10 min to wash away any remaining high pH buffer. The buffer solutions decanted after the second and third steps were analyzed for P_i desorbed in each cycle.

2.7. Thermal shift assay

Stability of the PBP structure was analyzed in triplicate at varying pH and temperature using the thermal shift assay, as described previously (Huynh and Partch, 2015). Purified suspended PBP (non-immobilized) was diluted to 7.5 μM in 10 mM Tris, 1 mM $MgCl_2$ buffer with a final pH of 4.0, 6.6, 7.5, 9.2, 10.9, 11.9, or 12.3, similar to the pH range investigated in the P_i recovery experiment. A 20-μL aliquot of each PBP solution (7.5 μM) was mixed with 10 μL of 15X Sypro Orange (Invitrogen) for the analysis, with a resultant protein concentration of 5 μM. The mixture was dispensed into a 96-well PCR plate, sealed with an optical seal and gently shaken to remove air bubbles. A thermal scan from 25 °C to 95 °C at an incremental rate of 1 °C/min was performed on the plate using a real-time PCR instrument (Stratagene Mx3005P). The protein denaturation curve (fluorescence vs. temperature) was truncated to 2 °C past the maximum fluorescence and then fitted to a non-linear Boltzmann sigmoidal curve (R statistical package - minpack.lm::nlsLM). All raw data analysis (data truncation, non-linear curve fitting and melting temperature calculation) was conducted in RStudio (version 0.98.1091) using custom scripts.

2.8. Data analysis

All PBP bead P_i concentration data was normalized to the corresponding control bead test. The normalized data was also compared to the theoretical P_i adsorption capacity (49 nmol/0.25 mL_{BV}) of the PBP beads, to obtain percent P_i adsorbed and desorbed data. Normal data distribution was assessed using the Shapiro-Wilk test ($\alpha = 0.05$). The statistical differences in P_i concentrations between different conditions were performed using one-way ANOVA ($\alpha = 0.05$) with Tukey post hoc analysis ($\alpha = 0.05$). They were



Cycle	Step	Addition to Reactor	Analysis
0	1) P_i desorption	1.75 mL buffer at pH 11.5, 12, or 12.5	At each step, the buffer solution was removed after 10 min exposure, and subsequently analyzed for P_i.
	2) Neutral pH wash	1.75 mL buffer at pH 7.1	
n (n=1 to 10)	1) P_i adsorption	1 mL buffer with 60 μM P_i at pH 7.1	
	2) P_i desorption	1.75 mL buffer at pH 11.5, 12, or 12.5	
	3) Neutral pH wash	1.75 mL buffer to pH 7.1	

Fig. 1. Illustration of the PBP bead adsorption/desorption reusability experimental approach. Batch experiments were conducted in triplicate Poly-Prep® reactors with 0.25 mL$_{BV}$ of PBP or control beads. Cycle 0 included two steps: 1) P_i desorption wash and 2) Neutral pH wash. Cycles 1 through 10 included three steps repeated 10 times: 1) P_i adsorption, 2) P_i desorption wash, and 3) Neutral pH wash. After each step, the buffer was decanted and analyzed for P_i.

conducted using Excel 2010 (Version 14.3.2 Microsoft, USA) with an added statistical software package XLStat Pro 2014 (Addinsoft, USA).

3. Results and discussion

3.1. P_i removal from immobilized PBP

An initial adsorption test was performed to assess P_i removal by exposing the PBP beads to excess P_i. Higher levels of P_i than the theoretical capacity of the PBP beads (49 nmoles of P_i for 0.25 mL of PBP beads) were mixed with the beads for 10 min. However, the PBP beads only adsorbed 11.8 ± 4 nmoles P_i (n = 39), or 24 ± 8% of the theoretical capacity. The low degree of initial P_i adsorption suggested that not all of the PBP was available for P_i removal, possibly due to the presence of legacy P_i that bound to the PBPs' active sites during protein preparation. To address the pre-adsorption presence of P_i in the system, an initial P_i desorption wash step with a reaction buffer pH 12.5 was adopted in this study to remove legacy P_i bound to the PBP. The initial high pH wash step substantially increased the P_i adsorption capacity of the PBP beads to 41 ± 2 nmoles/mL$_{BV}$, or 83 ± 2.1% of the theoretical capacity.

3.2. Recovery of P_i as a function of temperature and pH

The results from the P_i recovery experiments using varying buffer pH and temperature conditions are summarized in Fig. 2. Increases in temperature from 25 to 45 °C and pH conditions between 4.7 and 8.5 released less than 20% of adsorbed P_i (all % P_i desorbed calculations were performed using data normalized to the theoretical P_i capacity, 49 nmoles/0.25 mL$_{BV}$). Only at higher buffer pH conditions was a substantial proportion of the bound P_i released from the PBP beads. At pH 11.4 and 12.5, the total P_i recovered from the PBP beads was 30.2 ± 3.9 nmoles P_i (62%) and 42.2 ± 5.5 nmoles P_i (86%), respectively. These results demonstrated that pH ≥ 11.4 (25 °C, 10 min reaction time) provided the best conditions for recovery.

Higher P_i recovery was achieved using the immobilized PBP in this study (86% P_i recovery at pH 12.5) in comparison to that observed using PBP over-expressed in the periplasmic space of recombinant *E. coli* (2.1% P_i recovery at pH 3.8) (Yang et al., 2016). Alternately, the recovery achieved in this study was similar to Kuroda et al.'s (2000) report using immobilized PBP from *P. aeruginosa* (>90% P_i recovery at pH 3). Accordingly, the immobilized PBP with direct exposure to the water matrix demonstrated greater pH dependency compared to intracellular proteins. Of note,

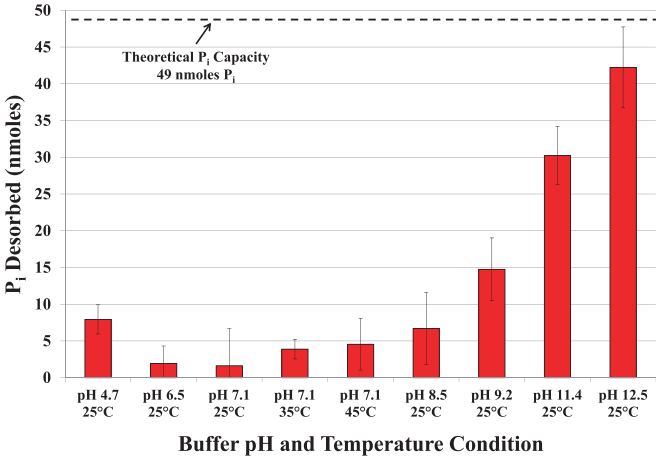


Fig. 2. P_i recovery from PBP beads at varying pH and temperature showing that elevated pH (≥11.4) facilitated desorption. The bars represent means, while error bars show ±1 standard deviation of triplicate experiments.

however, Kuroda et al. (2000) observed no P_i desorption above pH 5, but did not investigate pH ≥ 10, whereas the lowest pH investigated in this study was pH 4.7, which provided the lowest extent of P_i desorption. Thus, greater understanding of the response to a wide range of pH conditions is needed in future studies of immobilized PBP.

3.3. Kinetics of P_i recovery from immobilized PBP

Desorption of P_i occurred nearly instantaneously (<5 min), regardless of pH conditions, which is advantageous for operation of a phosphorus recovery process. This is illustrated in Fig. 3, which shows that the amount of P_i released did not change between 5 and 50 min (p value < 0.05, n = 6). However, as described in Section 3.1, pH significantly influenced P_i desorption. Average P_i desorption within 5 min of reaction time was 2 ± 9.6% (pH 7.1), 35 ± 4.4% (pH 10.8), 79 ± 13% (pH 11.9), and 97 ± 9.4% (pH 12.5). This indicates that high pH condition, not exposure time, was the critical factor determining P_i release.

3.4. Reusability of immobilized PBP

The viability of the immobilized PBP system for P_i removal and recovery hinges on PBP's adsorption/desorption efficiency over repeated cycles. In this study, performance over 10 cycles (plus an

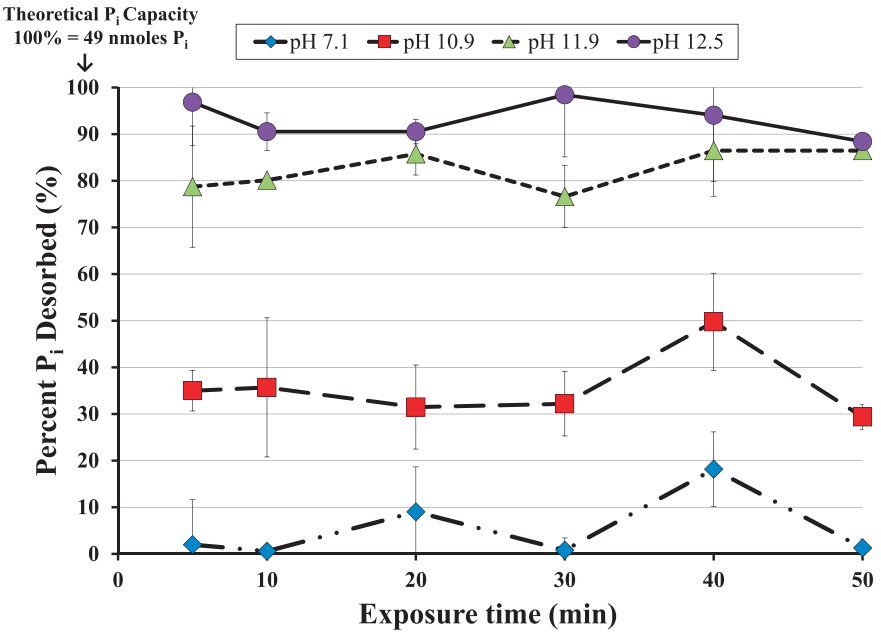


Fig. 3. Kinetics of P_i recovery from PBP beads at varying pH showing near instantaneous desorption (<5 min). The percent P_i desorbed was calculated based on the theoretical P_i capacity (49 nmoles/0.25 mL_{BV}). All tests were conducted in 25 °C reaction buffer (10 mM Tris-HCl, 1 mM $MgCl_2$). The data points represent means, while error bars show ±1 standard deviation of triplicate experiments.

initial desorption wash to remove legacy P_i) was evaluated. In the initial P_i desorption wash (Cycle 0), the average percent P_i desorbed at pH 11.5, 12, and 12.5 was 46 ± 0.6%, 59 ± 1.2%, and 77 ± 1.2%, respectively (Fig. 4). This agrees with our earlier observations, wherein P_i desorption increases with increased pH. Poor initial P_i desorption can also explain the lower 10 cycle-average P_i adsorption observed compared to desorption for pH 11.5 and 12 (63 ± 8.8% and 64.6 ± 6.2%, respectively) compared to pH 12.5 (83 ± 5%). Incomplete removal of P_i negatively affects subsequent P_i adsorption due to fewer available PBP-P_i binding sites in subsequent adsorption cycles.

As pH 12.5 buffer yielded the most complete desorption, it also demonstrated the most consistent and effective function during reuse of the immobilized PBP over 10 cycles. Specifically, pH 12.5 provided the highest 10 cycle-average desorption, 89 ± 4.1%, compared to 71 ± 6.8% using pH 11.5 and 70.7 ± 5.0% using pH 12. Additionally, this level of desorption was achieved consistently over all 10 cycles (p value > 0.05, n = 60), whereas when desorption was conducted at 11.5 or 12, lower sorption was observed in the initial cycles, followed by improvements in successive cycles. For example, percent P_i adsorption and desorption during Cycles 1 through 4 remained relatively low when desorbing with pH 11.5 (average of 58.7 ± 10.3% and 66.5 ± 8.1%, respectively) and 12 (average of 60.3 ± 1.3% and 66.7 ± 3.4%, respectively) (Fig. 4). As shown, desorption at pH 11.5 was significantly greater than adsorption ($p < 0.05$) in Cycles 1–4, whereas adsorption was generally equivalent to desorption in Cycles 5–10, indicating that incomplete desorption initially hampered adsorption. Improvements in sorption owing to incrementally more complete desorption are evident in the consistently higher (p value > 0.05, n = 18) average percent P_i adsorption and desorption observed in Cycles 5–10 for both pH 11.5 (66.1 ± 6.2% adsorption and 72.6 ± 5.1% desorption) and 12 (67.5 ± 6.5% adsorption and 73.4 ± 3.9% desorption).

These results demonstrate that pH 12.5 buffer provided the highest average P_i adsorption and desorption using PBP beads at room temperature. Additionally, the extent of adsorption and desorption observed was consistent over 10 cycles, which is crucial for the PBP bead's viability as a reusable adsorbent. As the average P_i desorbed was either statistically higher or similar to P_i adsorbed, it is reasonable to conclude that all adsorbed P_i (including some P_i not desorbed during Cycle 0) was recovered. Accordingly, these results demonstrate that extracellular immobilized PBP can successfully remove and recover P_i for at least 10 sequential cycles.

3.5. *Influence of pH and temperature on PBP structural stability and PBP-P_i interaction*

To further assess the system's potential, the mechanisms of P_i adsorption and desorption were explored. The release of adsorbed P_i triggered by elevated pH may derive from changes in overall conformation of PBP, local changes in the coordination of P_i in the active site, or a combination of these phenomena. Since the activity of PBP was retained over multiple cycles, we probed potential changes in PBP structure under the varying conditions using a fluorescent thermal shift assay. In this experiment, PBP is unfolded as a function of temperature in the presence of a non-specific hydrophobic protein-binding SYPRO orange fluorophore. Upon protein unfolding, the dye binds and generates a change in fluorescence, which was quantified by the Q-PCR instrument. Structural changes under a particular condition (e.g., changes in pH) would result in a shift in the thermal stability (i.e., melting temperature). As shown in Fig. 5, the thermal shift analysis showed that buffer pH conditions did not influence the melting temperature (T_M) of the PBP (p value > 0.05, n = 21). This indicates that there were no conformational changes to the structure of PBP. Thus, desorption of P_i from PBP at high pH conditions (>10) was not caused by changes in the structure of the protein.

Since there were no observable global changes in the structure of PBP as a function of the pH range tested, changes in the coordination of P_i in the active site likely accounted for P_i dissociation. To test this hypothesis, we analyzed the coordination of P_i in the high resolution crystal structure of PBP (PDB ID: 1IXh, www.rcsb.org) on the molecular visualization software PyMOL (Version 2.0

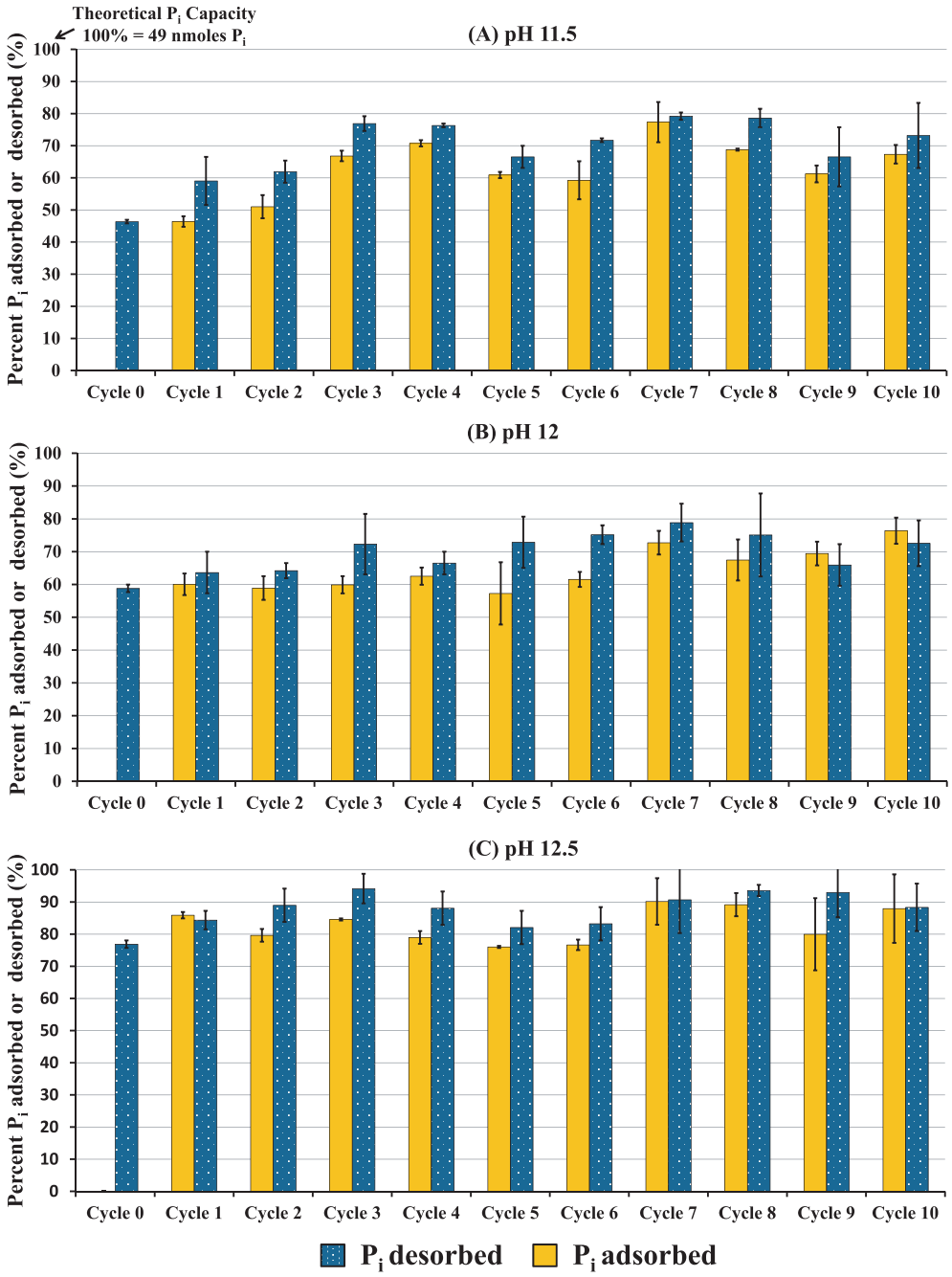


Fig. 4. Summary of PBP bead reusability experiment showing 10 sequential cycles of P_i adsorption at pH 7.1 and P_i desorption at (A) pH 11.5, (B) pH 12, and (C) pH 12.5. The percent P_i adsorbed and desorbed was calculated with respect to the theoretical adsorption capacity (49 nmoles/0.25 mL_{BV} = 100%). Cycle 0 represents the initial P_i desorption wash, while Cycles 1–10 consisted of 10 subsequent P_i adsorption and desorption cycles. The bars represent means, while error bars show ±1 standard deviation of triplicate experiments.

Schrödinger, LLC, USA). The structure of PBP bound to P_i reveals that the 7 different amino acid residues form 12 strong hydrogen bonds with P_i (Fig. 6). The P_i interacts with the peptide backbone of Thr10, Phe11, Ser38, Thr141, and Gly140. Side chain interactions with the amino group of Arg135 and the hydroxyl groups of Ser38, Ser139, Thr10, and Thr141 were observed. Finally, the carboxylate group of the Asp56 side chain also stabilizes P_i (Fig. 6) (Luecke and Quiocho, 1990). The pKa values of the 7 different amino acid acid residues that interact with P_i in the active site range from pH 9.04 to 9.6. At pH > 10, these amino acid residues are primarily deprotonated, thereby inhibiting the formation of hydrogen bonds between P_i and the PBP active site. We propose that the deprotonation at elevated pH explains P_i desorption at high pH, and subsequent P_i re-adsorption as the residues are protonated during the wash with the neutral pH buffer.

In summary, the results from the thermal shift analysis and the PBP bead reusability experiment indicated that the P_i adsorption/desorption capacity of the extracellular immobilized PBP will not be impacted by repetitive cycles of neutral and high pH to promote adsorption and desorption, respectively. Thus, reuse of the immobilized PBP is possible, lending credence to its application in water/wastewater treatment settings.

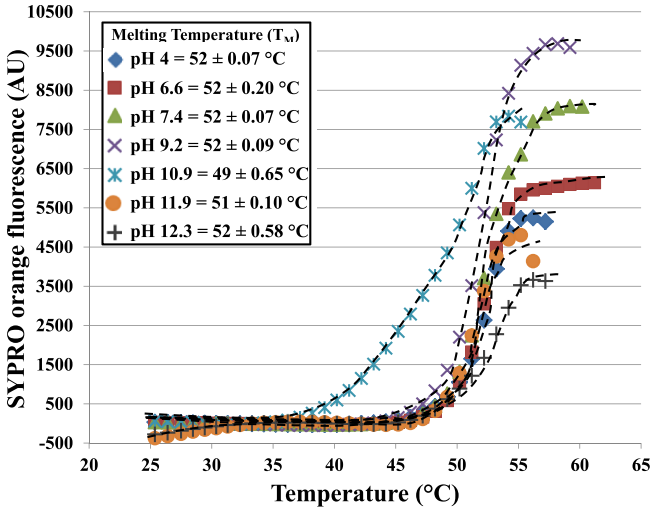


Fig. 5. Thermal shift analysis of PBP stability showing the truncated denaturation curve data from a typical 96-well screen of a range of buffer pH conditions used to test P_i desorption efficacy. The PBP melting temperatures (T_M) associated with different pH values are shown on the figure. Changes in buffer pH conditions did not significantly influence the PBP T_M values (p value > 0.05, n = 3).

3.6. *P_i removal and recovery: evaluation of PBP in comparison to other adsorbents*

The global importance of P_i removal and recovery has encouraged the development and implementation of a variety of different adsorbent materials characterized by a range of capacities, selectivities, reversibility, and affinities (some of which are summarized in reviews such as De-Bashan and Bashan (2004), Mayer et al. (2013), Mehta et al. (2015), and Rittmann et al. (2011)). Accordingly, evaluation of PBP's potential for P_i removal and recovery applications relative to other systems is prudent.

One existing P_i recovery approach that has attracted considerable interest is ion exchange using synthetic iron-based resins (Mayer et al., 2013). Commercially-available hybrid anion ion exchange (HAIX) resins with hydrated ferric oxides (e.g., ArsenX, SolmeteX, PhosX, etc.) have demonstrated effectiveness as a reversible P_i adsorbents that can remove P_i to ultra-low levels (<100 μg/L) (Blaney et al., 2007; Mayer et al., 2013; Pan et al., 2009; Sarkar et al., 2007; SenGupta and Cumbal, 2007). Approximately 95% of the P_i adsorbed by HAIX can be released using a NaOH + NaCl regenerant solution (Blaney et al., 2007). In this study, we observed similar levels of controlled P_i desorption from immobilized PBP (approximately 86–97%) using a basic solution. With similar desorption performance, PBP may offer a viable alternative for P_i recovery. However, the advantages and disadvantages of alternative adsorbents must be weighed in each application, and future optimization and evaluation of the PBP system are needed to facilitate direct comparisons.

The P_i adsorption selectivity of immobilized PBP may match, or even surpass, existing iron-based adsorbents, e.g., HAIX. The mutant variant of *E. coli* PBP (A197C) used in this study was originally developed as a P_i sensor and was extensively characterized for its P_i affinity and selectivity (Brune et al., 1998, 1994; Solscheid et al., 2015). Brune et al. (1994) demonstrated rapid P_i adsorption using *E. coli* PBP (A197C) ($k_{on} = 1.36 \times 10^8\ M^{-1}\ s^{-1}$), with adsorption limited only by diffusion (Zhou et al., 1983). Moreover, *E. coli* PBP can adsorb P_i to ultra-low levels (<100 μg/L) (Brune et al., 1994). A similar result was reported by Kuroda et al. (2000) using immobilized PBP from *P. aeruginosa*.

With respect to competition, *E. coli* PBP does not adsorb anions such as sulfate, vanadate, chloride, and fluoride (Brune et al., 1994; Luecke and Quiocho, 1990). Similarly, HAIX resins are unaffected by the presence of competing ions such as sulfate, carbonate, fluoride, and chloride (Acelas et al., 2015; Blaney et al., 2007; Martin et al., 2009; Pan et al., 2009; Sarkar et al., 2007; You et al., 2016). The only significant competitor for P_i adsorption using HAIX or other iron-based adsorbents is arsenate. Although PBP can adsorb arsenate, *E. coli* PBP (A197C) offers 50 to 100 times higher P_i binding affinity than arsenate (dissociation constant, k_d, = 0.03–0.07 μM for P_i and 3 μM for arsenate) (Brune et al., 1994). The PBPs from other microorganisms such as *P. fluorescens*, *Halmonas* sp. *GFAJ-1*, and *K. variicola* are also able to discriminate P_i from arsenate, even when arsenate is present at concentrations in excess of 3000–4000-fold higher than P_i (Elias et al., 2012). Therefore, PBP has the potential to provide a distinct P_i adsorption advantage over existing iron-based ion exchange resins with respect to selectivity of P_i over arsenate.

Although previous studies show promising results for PBP's selectivity, affinity, and kinetics of adsorption, PBP systems are still in the very early stages of development, and future advances are needed to overcome significant limitations in order for PBP to be a cost-effective alternative to existing adsorbents such as HAIX. Improving the adsorption capacity and reusability of immobilized PBP is critical for improving the technical and economic feasibility of P_i removal and recovery. Commercially available HAIX resins are robust (high reusability) with high adsorption capacities ranging from 20 to 40 mg-P_i/g resin in real wastewater conditions (Acelas et al., 2015; Blaney et al., 2007; Pan et al., 2009; You et al., 2016). In comparison, the NHS activated Sepharose beads used in this

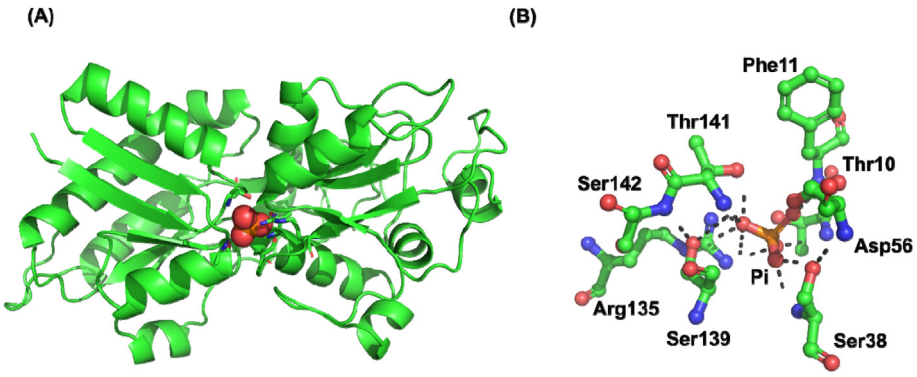


Fig. 6. Crystal structure of PBP showing (A) P_i bound to the active site and (B) the network of hydrogen bonds coordinating P_i in the protein's active site. The coordination file of PBP (PDB ID: 1IXh) was acquired from www.rcsb.org, and the crystal structure was analyzed using PyMOL (Molecular visualization software, Version 2.0, Schrödinger LLC, USA).

study have the potential to immobilize 16-23 μM-PBP/mL of bead (GE Healthcare Bio-Sciences), providing a potential P_i adsorption capacity of approximately 1.5–2 mg-P_i/mL bead, which is roughly an order of magnitude lower than HAIX resins. Additionally, the effects of real water/wastewater constituents on PBP-P_i adsorption/desorption and long-term reusability of the immobilized PBP system is yet to be investigated. In comparison to inorganic HAIX resins, PBP is biodegradable and may be negatively affected by the presence of natural organic matter, microorganisms, and enzymes (e.g., protease), which can severely hamper adsorption capacity and reusability in real water/wastewater conditions. Therefore, in order to further develop PBP as a highly selective alternative to current iron-based P_i adsorbents, future studies should focus on optimizing P_i adsorption capacity (e.g., optimized protein coupling density) and investigating the reusability of immobilized PBP in actual water/wastewater matrices.

4. Conclusions

Harnessing the selective, sensitive P_i adsorption abilities of the high-affinity PBP offers an opportunity to engineer an innovative water treatment system which can effectively remove P_i to ultra-low levels (<100 μg/L) and release P_i under controlled conditions suitable for subsequent P_i reuse. Previous studies have established PBP's capability as an effective P_i adsorbent; however, conditions for controlled P_i release have not yet been conclusively established.

This study investigated the ideal pH and temperature conditions for P_i release from extracellular PBP immobilized on an inert surface. The results showed that P_i adsorbed using immobilized PBP can be recovered nearly instantaneously using a high pH (≥12.5) wash. The immobilized PBP maintained consistent P_i adsorption capacity after 10 high pH wash cycles, supporting its reusability. As PBP structure is highly stable from pH 4 to 12.5, the P_i release at high pH is most likely due to reversible deprotonation of amino acid residues at the active binding site.

The structural stability of PBP and consistent P_i adsorption capacity after 10 high pH wash cycles also suggest that extracellular immobilized PBP could endure more than 10 repetitive cycles of neutral and high pH to promote adsorption and desorption, respectively. Accordingly, immobilized PBP appears to provide a strong foundation for an effective and reusable P_i removal/recovery adsorption system providing high-affinity, ultra-low, and ultra-fast P_i binding. Future investigations targeting optimization of the immobilized PBP system, e.g., improvements in capacity linked to higher protein/bead coupling efficiency and protein stability in actual water matrices, are needed to further improve reusability and capacity and establish PBP as a viable P_i recovery alternative. Additionally, the application of immobilized PBP for P_i recovery from real water/wastewater (and the specific influence of constituents such as natural organic matter, microorganisms, and enzymes) must be directly assessed.

Conflict of interest

The authors report no conflict of interest.

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