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Phosphate removal and recovery using immobilized phosphatebinding proteins

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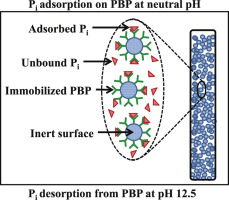
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# Abstract

Progress towards a more circular phosphorus economy necessitates development of innovative water treatment systems which can reversibly remove inorganic phosphate (Pi) to ultra-low levels (<100 μg L−1), and subsequently recover the Pi for reuse. In this study, a novel approach using the high-affinity E. coli phosphate binding protein (PBP) as a reusable Pi bio-adsorbent was investigated. PBP was expressed, extracted, purified and immobilized on NHS-activated Sepharose beads. The resultant PBP beads were saturated with Pi and exposed to varying pH (pH 4.7 to 12.5) and temperatures (25–45 °C) to induce Pi release. Increase in temperature from 25 to 45 °C and pH conditions between 4.7 and 8.5 released less than 20% of adsorbed Pi. However, 62% and 86% of the adsorbed Pi was released at pH 11.4 and 12.5, respectively. Kinetic experiments showed that Pi desorption occurred nearly instantaneously (<5 min), regardless of pH conditions, which is advantageous for Pi recovery. Additionally, no loss in Pi adsorption or desorption capacity was observed when the PBP beads were exposed to 10 repeated cycles of adsorption/desorption using neutral and high pH (≥12.5) washes, respectively. The highest average Pi adsorption using the PBP beads was 83 ± 5%, with 89 ± 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 Pi recovery from water/wastewater.

## Graphical abstract



# Keywords

Adsorbent. Phosphorus, Nutrient reuse, pstS, Water, Wastewater

# 1. Introduction

The economic and ecological losses associated with eutrophication caused by excess inorganic phosphate (Pi), have inspired increasingly lower effluent phosphorus water quality guidelines for many municipal and industrial wastewater treatment facilities around the world ([Amery and Schoumans, 2014](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "bib2); [USEPA, 1995](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "bib26), [1986](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "bib27)). Apart from limiting Pi loadings in wastewater effluents, there is also strong impetus to secure renewable Pi resources for use as agricultural fertilizer, i.e., via Pi recovery from wastewater. This approach stimulates the circular phosphorus economy, which is vital since in addition to being a pollutant, Pi is a geographically limited nonrenewable resource that is essential to sustain global food production ([Cordell et al., 2009](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "bib8); [Cordell and White, 2014](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "bib9); [Mayer et al., 2016](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "bib17); [Rittmann et al., 2011](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "bib21)). This removal/recovery paradigm drives development of innovative water treatment systems which can effectively remove Pi to ultra-low levels (<100 μg/L) and release Pi under controlled conditions suitable for subsequent Pi reuse ([Mayer et al., 2013](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "bib18); [Rittmann et al., 2011](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib21)).

A novel strategy utilizing high-affinity phosphate binding proteins (PBP) as a reusable bio-adsorbent to reversibly capture Pi was investigated in this study. The phosphate-specific transporter (Pst) system in bacteria is specifically evolved to import Pi when Pi is present at low levels, which demands efficient, selective, and high-affinity binding and transport of Pi to meet the cell's metabolic demands ([Blank, 2012](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "bib4); [Luecke and Quiocho, 1990](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "bib15); [Santos-Beneit et al., 2008](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "bib22)). 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](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib15); [Santos-Beneit et al., 2008](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib22)). The periplasmic pstS PBP has recently attracted interest as a potential high-affinity, phosphate-specific Pi adsorbent ([Kuroda et al., 2000](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "bib13); [Li et al., 2009](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "bib14); [Yang et al., 2016](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "bib28), [2017](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "bib29)).

Removal of Pi 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](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "bib7); [Kuroda et al., 2000](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib13); [Li et al., 2009](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib14); [Yang et al., 2016](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib28)). For example, [Choi et al. (2013)](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib7) showed that recombinant E. coli expressing PBP in the periplasmic space can remove ≥97% Pi from water within 6 h (initial concentration of 0.2–0.5 mg-Pi/L). [Kuroda et al. (2000)](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib13) demonstrated Pi removal to below the detection limit (9.5 ng-Pi/L) using PBP immobilized on Sepharose beads to treat an influent concentration of 15 μg-Pi/L. To better support the circular phosphorus economy via the waste-to-resource paradigm, recovery of the captured Pi is essential. To assess Pi recovery, [Yang et al. (2016)](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib28) investigated the effect of varying temperature, pH, and ionic strength on Pi release from PBP over-expressed in the periplasmic space of E. coli cells. However, a maximum of only 1.4–2% of the adsorbed Pi 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](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib28)). On the other hand, [Kuroda et al. (2000)](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib13) reported >90% recovery of the adsorbed Pi from PBP immobilized on Sepharose beads at pH 3. Although the data was not shown, [Kuroda et al. (2000)](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib13) stated that the immobilized PBP could be reused after neutralizing the pH. Thus, additional investigation of the removal and controlled release of Pi from immobilized PBP at varying pH and temperature, and the performance of PBP over multiple cycles of Pi removal and recovery is needed. Effective reusability of the PBP is a crucial aspect of the viability of this bio-adsorbent for Pi removal and recovery from water.

The objective of this study was to investigate the adsorption and desorption of Pi 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 Pi recovery potential over multiple cycles of Pi 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)](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "bib25) 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](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib25)). 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 MgCl2, 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 mLBV (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](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "bib5)).

## 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 NaHCO3, 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/mLBV NHS beads (mLBV = drained NHS bead volume). This non-optimized coupling density was much lower than the theoretical maximum of 16–23 μmol/mLBV for NHS beads reported by GE Healthcare Bio-Sciences. However, 197 ± 0.2 nmol-PBP/mLBV was sufficient for the adsorption/desorption assessments in this study. Based on 1 mol of PBP adsorbing 1 mol of Pi ([Brune et al., 1998](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "bib6), [1994](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib5); [Solscheid et al., 2015](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib25)), the theoretical Pi adsorption capacity of the NHS beads was 197 ± 0.2 nmol/mLBV. The NHS beads conjugated with PBP (hereon referred to as PBP beads) were sequentially washed with 1BV of 0.1 M Tris-HCl pH 8.5 followed by washing with 1BV 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 1BV of buffer containing 10 mM Tris-HCl, 1 mM MgCl2 pH 7.1.

To remove the legacy Pi 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](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib6), [1994](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib5)). To facilitate mixing, 20 mL of 10 mM Tris-HCl, 1 mM MgCl2 pH 7.1 buffer was added to 20 mLBV 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 1BV 10 mM Tris-HCl, 1 mM MgCl2 pH 7.1 buffer to remove the Pi-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 mLBV of fresh NHS beads following the same procedure used for the PBP beads, except without PBP addition.

## 2.3. Adsorption of Pi by immobilized PBP

Triplicate Pi adsorption experiments were conducted in batch tests in 2 mL centrifuge tubes containing 0.25 mLBV PBP beads. In all tests, 1 mL reaction buffer (10 mM Tris-HCl, 1 mM MgCl2) pH 7.1, 25 °C containing excess Pi (60 μM) was initially added (60 nmol Pi) versus the theoretical capacity of the PBP beads (49 nmol/0.25 mLBV) to ensure Pi saturation of the PBP beads. After gentle mixing by hand and 10 min for bead settling, the supernatant was collected and analyzed for Pi. Previous studies showed that Pi binding is nearly instantaneous ([Brune et al., 1998](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib6), [1994](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib5); [Solscheid et al., 2015](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib25)), 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 Pi 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 Pi. 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 Pi 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 Pi adsorption/desorption.

## 2.5. Kinetics of Pi recovery from immobilized PBP

The extent of Pi 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 Adsorption of P](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "sec2.3), [2.4 Recovery of P](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "sec2.4). An initial Pi adsorption cycle was performed by adding 1 mL of 60 μM Pi solution in 25 °C reaction buffer at pH 7.1 to 0.25 mLBV PBP beads. After gentle mixing by hand and 10 min bead settling, the supernatant was collected and analyzed for Pi. For subsequent desorption, the PBP beads were washed 3 times with 1 mL reaction buffer at pH 7.1 to remove unattached Pi 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 Pi 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 Pi 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 mLBV 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](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "fig1) illustrates the experimental approach. The first step in these experiments was to release Pi adsorbed on Pi-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 Pi 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 Pi desorbed during the initial Cycle 0.

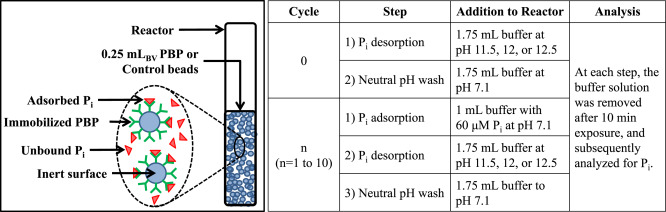


Fig. 1. Illustration of the PBP bead adsorption/desorption reusability experimental approach. Batch experiments were conducted in triplicate Poly-Prep® reactors with 0.25 mLBV of PBP or control beads. Cycle 0 included two steps: 1) Pi desorption wash and 2) Neutral pH wash. Cycles 1 through 10 included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

Following the initial Pi release cycle, 10 sequential cycles of Pi adsorption/desorption were performed. The first Pi adsorption step in each cycle consisted of adding 1 mL reaction buffer at pH 7.1, 25 °C containing 60 μM Pi. After gentle mixing and 10 min bead settling, the supernatant was decanted and analyzed for Pi. In the second Pi desorption step, Pi 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 Pi 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](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "bib12)). Purified suspended PBP (non-immobilized) was diluted to 7.5 μM in 10 mM Tris, 1 mM MgCl2 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 Pi 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 Pi concentration data was normalized to the corresponding control bead test. The normalized data was also compared to the theoretical Pi adsorption capacity (49 nmol/0.25 mLBV) of the PBP beads, to obtain percent Pi adsorbed and desorbed data. Normal data distribution was assessed using the Shapiro-Wilk test (α = 0.05). The statistical differences in Pi concentrations between different conditions were performed using one-way ANOVA (α = 0.05) with Tukey post hoc analysis (α = 0.05). They were 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. Pi removal from immobilized PBP

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

## 3.2. Recovery of Pi as a function of temperature and pH

The results from the Pi recovery experiments using varying buffer pH and temperature conditions are summarized in [Fig. 2](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "fig2). Increases in temperature from 25 to 45 °C and pH conditions between 4.7 and 8.5 released less than 20% of adsorbed Pi (all % Pi desorbed calculations were performed using data normalized to the theoretical Pi capacity, 49 nmoles/0.25 mLBV). Only at higher buffer pH conditions was a substantial proportion of the bound Pi released from the PBP beads. At pH 11.4 and 12.5, the total Pi recovered from the PBP beads was 30.2 ± 3.9 nmoles Pi (62%) and 42.2 ± 5.5 nmoles Pi (86%), respectively. These results demonstrated that pH ≥ 11.4 (25 °C, 10 min reaction time) provided the best conditions for recovery.

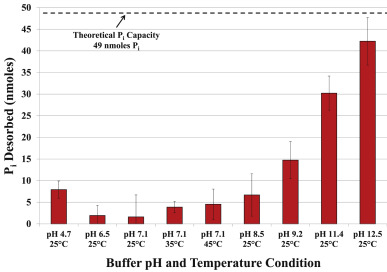


Fig. 2. Pi recovery from PBP beads at varying pH and temperature showing that elevated pH (≥11.4) facilitated desorption. The bars represent means, while error bars show ±1 standard deviation of triplicate experiments.

Higher Pi recovery was achieved using the immobilized PBP in this study (86% Pi recovery at pH 12.5) in comparison to that observed using PBP over-expressed in the periplasmic space of recombinant E. coli (2.1% Pi recovery at pH 3.8) ([Yang et al., 2016](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib28)). Alternately, the recovery achieved in this study was similar to Kuroda et al.'s (2000) report using immobilized PBP from P. aeruginosa (>90% Pi 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, however, [Kuroda et al. (2000)](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib13) observed no Pi 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 Pi 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 Pi recovery from immobilized PBP

Desorption of Pi 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](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "fig3), which shows that the amount of Pi released did not change between 5 and 50 min (p value < 0.05, n = 6). However, as described in Section [3.1](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "sec3.1), pH significantly influenced Pi desorption. Average Pi 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 Pi release.

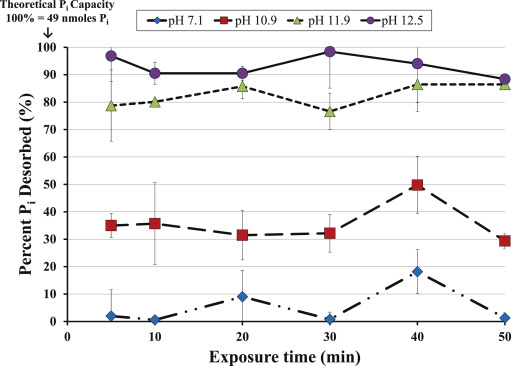


Fig. 3. Kinetics of Pi recovery from PBP beads at varying pH showing near instantaneous desorption (<5 min). The percent Pi desorbed was calculated based on the theoretical Pi capacity (49 nmoles/0.25 mLBV). All tests were conducted in 25 °C reaction buffer (10 mM Tris-HCl, 1 mM MgCl2). The data points represent means, while error bars show ±1 standard deviation of triplicate experiments.

## 3.4. Reusability of immobilized PBP

The viability of the immobilized PBP system for Pi removal and recovery hinges on PBP's adsorption/desorption efficiency over repeated cycles. In this study, performance over 10 cycles (plus an initial desorption wash to remove legacy Pi) was evaluated. In the initial Pi desorption wash (Cycle 0), the average percent Pi desorbed at pH 11.5, 12, and 12.5 was 46 ± 0.6%, 59 ± 1.2%, and 77 ± 1.2%, respectively ([Fig. 4](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "fig4)). This agrees with our earlier observations, wherein Pi desorption increases with increased pH. Poor initial Pi desorption can also explain the lower 10 cycle-average Pi 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 Pi negatively affects subsequent Pi adsorption due to fewer available PBP-Pi binding sites in subsequent adsorption cycles.

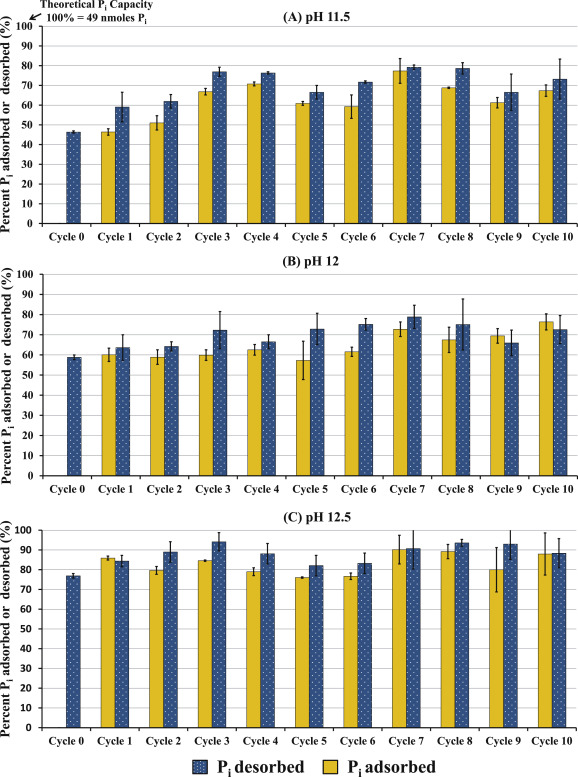


Fig. 4. Summary of PBP bead reusability experiment showing 10 sequential cycles of Pi adsorption at pH 7.1 and Pi desorption at (A) pH 11.5, (B) pH 12, and (C) pH 12.5. The percent Pi adsorbed and desorbed was calculated with respect to the theoretical adsorption capacity (49 nmoles/0.25 mLBV = 100%).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

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 Pi 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](https://www.sciencedirect.com/science/article/pii/S2589914718300033#fig4)). 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 Pi 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 Pi 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 Pi desorbed was either statistically higher or similar to Pi adsorbed, it is reasonable to conclude that all adsorbed Pi (including some Pi not desorbed during Cycle 0) was recovered. Accordingly, these results demonstrate that extracellular immobilized PBP can successfully remove and recover Pi for at least 10 sequential cycles.

## 3.5. Influence of pH and temperature on PBP structural stability and PBP-Pi interaction

To further assess the system's potential, the mechanisms of Pi adsorption and desorption were explored. The release of adsorbed Pi triggered by elevated pH may derive from changes in overall conformation of PBP, local changes in the coordination of Pi 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](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "fig5), the thermal shift analysis showed that buffer pH conditions did not influence the melting temperature (TM) 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 Pi from PBP at high pH conditions (>10) was not caused by changes in the structure of the protein.

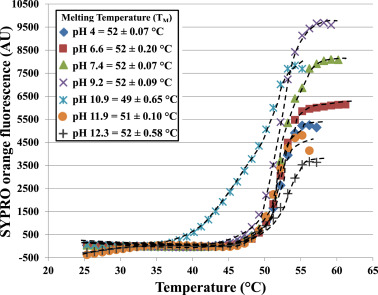


Fig. 5. Thermal shift analysis of PBP stability showing the truncated denaturation curve data from a typical 96-well screen of a range of buffer pH conditions used to test Pi desorption efficacy. The PBP melting temperatures (TM) associated with different pH values are shown on the figure. Changes in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

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 Pi in the active site likely accounted for Pi dissociation. To test this hypothesis, we analyzed the coordination of Pi in the high resolution crystal structure of PBP (PDB ID: 1IXh, [www.rcsb.org](http://www.rcsb.org/)) on the molecular visualization software PyMOL (Version 2.0 Schrödinger, LLC, USA). The structure of PBP bound to Pi reveals that the 7 different amino acid residues form 12 strong hydrogen bonds with Pi ([Fig. 6](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "fig6)). The Pi 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 Pi ([Fig. 6](https://www.sciencedirect.com/science/article/pii/S2589914718300033#fig6)) ([Luecke and Quiocho, 1990](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib15)). The pKa values of the 7 different amino acid acid residues that interact with Pi 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 Pi and the PBP active site. We propose that the deprotonation at elevated pH explains Pi desorption at high pH, and subsequent Pi re-adsorption as the residues are protonated during the wash with the neutral pH buffer.

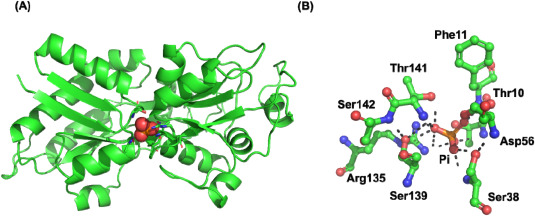


Fig. 6. Crystal structure of PBP showing (A) Pi bound to the active site and (B) the network of hydrogen bonds coordinating Pi in the protein's active site. The coordination file of PBP (PDB ID: 1IXh) was acquired from [www.rcsb.org](http://www.rcsb.org/), and the crystal structure was analyzed using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

In summary, the results from the thermal shift analysis and the PBP bead reusability experiment indicated that the Pi 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.

## 3.6. Pi removal and recovery: evaluation of PBP in comparison to other adsorbents

The global importance of Pi 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)](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "bib10), [Mayer et al. (2013)](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib18), [Mehta et al. (2015)](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "bib19), and [Rittmann et al. (2011)](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib21)). Accordingly, evaluation of PBP's potential for Pi removal and recovery applications relative to other systems is prudent.

One existing Pi recovery approach that has attracted considerable interest is ion exchange using synthetic iron-based resins ([Mayer et al., 2013](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib18)). Commercially-available hybrid anion ion exchange (HAIX) resins with hydrated ferric oxides (e.g., ArsenX, SolmeteX, PhosX, etc.) have demonstrated effectiveness as a reversible Pi adsorbents that can remove Pi to ultra-low levels (<100 μg/L) ([Blaney et al., 2007](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "bib3); [Mayer et al., 2013](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib18); [Pan et al., 2009](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "bib20); [Sarkar et al., 2007](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "bib23); [SenGupta and Cumbal, 2007](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "bib24)). Approximately 95% of the Pi adsorbed by HAIX can be released using a NaOH + NaCl regenerant solution ([Blaney et al., 2007](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib3)). In this study, we observed similar levels of controlled Pi desorption from immobilized PBP (approximately 86–97%) using a basic solution. With similar desorption performance, PBP may offer a viable alternative for Pi 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 Pi 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 Pi sensor and was extensively characterized for its Pi affinity and selectivity ([Brune et al., 1998](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib6), [1994](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib5); [Solscheid et al., 2015](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib25)). [Brune et al. (1994)](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib5) demonstrated rapid Pi adsorption using E. coli PBP (A197C) (kon = 1.36 × 108 M−1 s−1), with adsorption limited only by diffusion ([Zhou et al., 1983](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "bib31)). Moreover, E. coli PBP can adsorb Pi to ultra-low levels (<100 μg/L) ([Brune et al., 1994](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib5)). A similar result was reported by [Kuroda et al. (2000)](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib13) 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](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib5); [Luecke and Quiocho, 1990](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib15)). Similarly, HAIX resins are unaffected by the presence of competing ions such as sulfate, carbonate, fluoride, and chloride ([Acelas et al., 2015](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "bib1); [Blaney et al., 2007](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib3); [Martin et al., 2009](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "bib16); [Pan et al., 2009](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib20); [Sarkar et al., 2007](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib23); [You et al., 2016](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "bib30)). The only significant competitor for Pi 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 Pi binding affinity than arsenate (dissociation constant, kd, = 0.03–0.07 μM for Pi and 3 μM for arsenate) ([Brune et al., 1994](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib5)). The PBPs from other microorganisms such as P. fluorescens, Halmonas sp. GFAJ-1, and K. variicola are also able to discriminate Pi from arsenate, even when arsenate is present at concentrations in excess of 3000–4000-fold higher than Pi ([Elias et al., 2012](https://www.sciencedirect.com/science/article/pii/S2589914718300033" \l "bib11)). Therefore, PBP has the potential to provide a distinct Pi adsorption advantage over existing iron-based ion exchange resins with respect to selectivity of Pi 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 Pi removal and recovery. Commercially available HAIX resins are robust (high reusability) with high adsorption capacities ranging from 20 to 40 mg-Pi/g resin in real wastewater conditions ([Acelas et al., 2015](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib1); [Blaney et al., 2007](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib3); [Pan et al., 2009](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib20); [You et al., 2016](https://www.sciencedirect.com/science/article/pii/S2589914718300033#bib30)). In comparison, the NHS activated Sepharose beads used in this study have the potential to immobilize 16-23 μM-PBP/mL of bead (GE Healthcare Bio-Sciences), providing a potential Pi adsorption capacity of approximately 1.5–2 mg-Pi/mL bead, which is roughly an order of magnitude lower than HAIX resins. Additionally, the effects of real water/wastewater constituents on PBP-Pi 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 Pi adsorbents, future studies should focus on optimizing Pi 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 Pi adsorption abilities of the high-affinity PBP offers an opportunity to engineer an innovative water treatment system which can effectively remove Pi to ultra-low levels (<100 μg/L) and release Pi under controlled conditions suitable for subsequent Pi reuse. Previous studies have established PBP's capability as an effective Pi adsorbent; however, conditions for controlled Pi release have not yet been conclusively established.

This study investigated the ideal pH and temperature conditions for Pi release from extracellular PBP immobilized on an inert surface. The results showed that Pi adsorbed using immobilized PBP can be recovered nearly instantaneously using a high pH (≥12.5) wash. The immobilized PBP maintained consistent Pi adsorption capacity after 10 high pH wash cycles, supporting its reusability. As PBP structure is highly stable from pH 4 to 12.5, the Pi 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 Pi 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 Pi removal/recovery adsorption system providing high-affinity, ultra-low, and ultra-fast Pi 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 Pi recovery alternative. Additionally, the application of immobilized PBP for Pi 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.

## Acknowledgements

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