High affinity phosphate binding protein (PBP) for phosphorous recovery: Proof of concept using recombinant *Escherichia coli*

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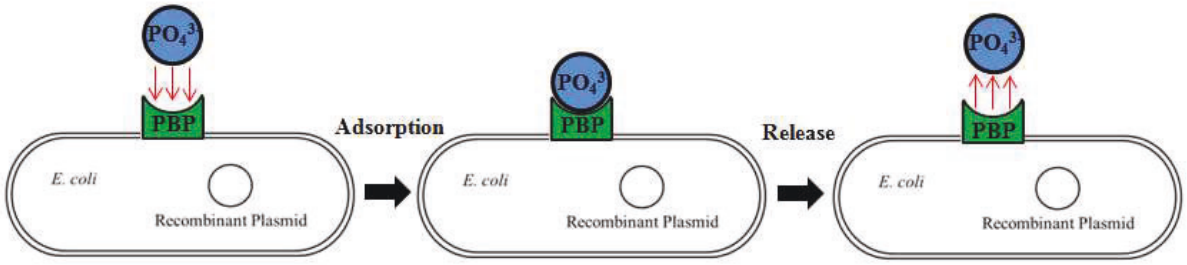
Graphical Abstract



One Sentence Summary

High-affinity phosphate binding proteins (PBP) offer an opportunity to recover phosphorus, as shown in this study, wherein *E. coli* expressing PBP adsorbed more phosphate from liquid and released more phosphate under controlled conditions in comparison to negative controls.

Abstract

Phosphorus (P) is a critical, nonrenewable nutrient; yet excess discharges can lead to eutrophication and deterioration of water quality. Thus, P removal from water must be coupled with P recovery to achieve sustainable P management. P-specific proteins provide a novel, promising approach to recover P from water. Bacterial phosphate binding proteins (PBP) are able to effectively remove phosphate, achieving extremely low levels in water (i.e., 0.015 mg-P L^{-1}). A prerequisite of using PBP for P recovery, however, is not only removal, but also controlled P release, which has not yet been reported. Phosphate release using recombinant PBP-expressing *E. coli* was explored in this study. *E. coli* was genetically modified to over-express PBP in the periplasmic space. The impacts of ionic strength, temperature, and pH on phosphate release were assessed. PBP-expressed *E. coli* demonstrated consistently superior ability to adsorb more phosphate from liquid and release more phosphate under controlled conditions relative to negative controls (unexpressed PBP *E. coli* and *E. coli* K12). Lower pH (3.8), higher temperature (35ºC), and higher ionic strength (100 mM KCl) facilitated increased phosphate release, providing a maximum of 2.1% P recovery within 3 h. This study provides proof-of-concept of the feasibility of using PBP to recover P.

Keywords: Phosphate Binding Protein (PBP), *Escherichia coli (E. coli)*, Adsorption, Water, Recovery, Phosphorus

1. Introduction

Phosphorus (P) is a biocritical element in short supply in nature, the modern terrestrial cycling of which is dominated by anthropogenic activity (Filippelli, 2008). Historically, removal of pollutant P from wastewater has been emphasized since excess concentrations can yield extraordinary phytoplankton growth, which can lead to eutrophication and subsequent development of hypoxia and acidification of surface water (Cai et al., 2011; Mayer et al., 2013; Rittmann et al., 2011). Eutrophication is a major water quality problem (Smith et al., 2014), and is the cause of at least 400 coastal dead zones worldwide (Caballero-Alfonso et al., 2015; Diaz and Rosenberg, 2008). In municipal wastewater treatment, enhanced biological phosphorus removal (EBPR) is often employed to achieve effluent concentrations as low as ~0.1 mg-P L^{-1}, which approaches the kinetic and thermodynamic limit (Blaney et al., 2007; Cooper et al., 1993; Jenkins et al., 1971; Jenkins and Hermanowicz, 1991). As P regulations and guidelines specify progressively lower concentrations for surface waters (e.g., below 0.1 mg-P L^{-1}, even as low as 0.005 mg-P L^{-1}) (Mayer et al., 2013), it is imperative to develop innovative strategies suitable for operation in water and/or wastewater that can remove P to these ultra-low levels and also facilitate P recovery. Reuse of the recovered P benefits from highly-selective separation of P (Mayer et al., 2016), making selective P adsorption an attractive treatment approach.

Removal of P from water using high-affinity phosphate-specific bacterial proteins has recently attracted research interest (Choi et al., 2013; Li et al., 2009). Bacteria import phosphate into their cells using dedicated transport systems. One of these systems, the phosphate-specific transporter (Pst) is primarily responsible for uptake when phosphate is present at low levels, which demands efficient binding and transport of phosphate to meet the cell's metabolic demands (Blank, 2012;

Botero et al., 2000; Santos-Beneit et al., 2008; Wanner, 1993). In *E. coli*, the Pst complex consists of four proteins: a dimeric ATP-binding protein (PstB), two transmembrane proteins (PstA and PstC), and a periplasmic phosphate-binding protein (PBP, also known as PstS or PhoS) (Choi et al., 2013; Santos-Beneit et al., 2008). Pursuant to the Venus flytrap model (Brune et al., 1998; Mao et al., 1982), PBP sequesters inorganic P in a deep cleft, using 12 strong hydrogen bonds to yield exceptional P specificity (Luecke and Quiocho, 1990). Previous research indicated that recombinant *E. coli* expressing PBP in the periplasmic space can remove ≥ 97% of phosphate within 6 h from water with an initial concentration of 0.2 – 0.5 mg-P L^{-1} (Choi et al., 2013) . Column tests using PBP immobilized on Sepharose beads showed removal of ^{32}P-labeled phosphate to below the detection limit of 9.5 ng-P L^{-1} using an influent concentration of 0.015 mg-P L^{-1} (Kuroda et al., 2000). Thus, PBP has considerable potential for applications requiring P removal to ultra-low concentrations. However, beyond efficient removal (Choi et al., 2013), P recovery by PBP requires controlled desorption of the sorbed phosphate, regarding which limited information exists (e.g., Brune et al., 1998; Kuroda et al., 2000).

The objective of this study was to demonstrate that PBP could increase P adsorption, and that the P could be released under controlled conditions. The focus of this work was on establishing system capabilities, rather than optimization for maximum P uptake and release. Using common methods for phosphate analysis (e.g., colorimetric or ion chromatography), large amounts of purified PBP protein would be needed to quantify P recovery during adsorption/desorption experiments. Another option is to use a small amount of protein with the ^{32}P isotope (Kuroda et al., 2000), quantification of which requires specialized analytic equipment. To avoid using P isotopes or using large quantities of purified proteins, reversible phosphate release was

demonstrated using recombinant PBP-expressing *E. coli* (PBP *E. coli*) and conditions favorable for controlled phosphate release were identified. Genetic modification of *E. coli* can be applied as a fast and easy approach to establish the feasibility of controlled, reversible phosphate sorption using PBP proteins.

2. Materials and Methods

2.1. Construct and Verify Recombinant *E. coli* Expressing PBP

We engineered PBP-expressing *E. coli* following the manufacturer's protocols (PET System manual 10th edition, Novagen, Madison,WI). The PBP gene was directly synthesized using the PBP sequence from *Pseudomonas aeruginosa* (GenScript, Piscataway, NJ), as its phosphate binding protein has demonstrated strong phosphate binding (Neznansky et al., 2014). Plasmid PET 30 a (Novagen, Madison, WI) and the target PBP gene were double enzyme digested using NcoI and XhoI (New England BioLabs, Ipswich, MA), followed by gel purification (QIAquick Gel Extraction Kit, Qiagen, Valencia, CA). Ligation was conducted using a DNA Ligation Kit (Novagen kit #69838, Madison, WI). The sequence of the inserted gene was confirmed by Sanger Sequencing. The reconstructed plasmid was introduced into *E. coli* One Shot® BL21(DE3) cells (Novagen, Madison,WI). A single colony was inoculated into Lysogeny broth (LB) containing 50 mg L^{-1} kanamycin (Sigma-Aldrich, St. Louis, MO, USA), and cultures were incubated at 37°C on a shaker at 200 rpm. After culturing for 2 h, 1 mM isopropyl-β-D-thiogalactopytanoside (IPTG, Sigma-Aldrich, St. Louis, MO) was added to induce PBP expression, and the cells were further cultured for another 12 h. Cells were harvested by centrifugation at 5,000 *g* for 10 min at 4°C, and then lysed by water bath sonication. The target PBP protein was obtained by one-step purification using a Ni-NTA agarose column (Qiagen, Valencia, CA). Fractions were pooled and dialyzed followed by 0.22 μm filter sterilization.

Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using standard protocols for molecular weight and purity measurements (Sambrook et al., 1989). The primary antibody for Western blot was Mouse-anti-His mAb (GenScript, Piscataway, NJ).

2.2. Unexpressed Controls and P Analysis

Two unexpressed controls were used for comparison against the PBP-over-expressed *E. coli*: a) *E. coli* K12 (endogenous PBP with the gene in the chromosome) and b) unexpressed recombinant PBP *E. coli* (PBP gene in both the chromosome and related plasmid). The *E. coli* were independently inoculated into LB medium at 37°C (Choi et al., 2013). The LB medium for the recombinant *E. coli* was supplemented with 50 mg L^{-1} kanamycin. After 2-h incubation, IPTG was added to one aliquot of the recombinant *E. coli* to induce PBP protein expression (hereafter called PBP *E. coli*), while the aliquot of recombinant *E. coli* without IPTG addition was used as a negative control (unexpressed PBP *E. coli*). After overnight incubation, bacteria biomass was harvested by centrifuging at 5,000 *g* for 5 minutes at 4°C. The biomass was re-suspended in 1 mM KCl solution. To minimize residual LB media associated with bacteria biomass, three consecutive centrifuge and resuspension cycles were conducted using 1 mM KCl. Prior to tests, the biomass from each of the three groups of bacteria (PBP *E. coli*, unexpressed PBP *E. coli*, and *E. coli* K12) was diluted to an optical density at a wavelength of 600 nm (OD 600) of 0.50.

Unlike previous studies directed at P removal (Choi et al., 2013), this study focused on the potential for controlled release of phosphate bound by PBP-expressing *E. coli*. To assess P sorption, initial total P content (inclusive of P integrated in cell biomass as well as extra P sorbed

by the cells) of all cultures was quantified. An aliquot of 5 mL of mixed cell suspension was collected, digested, and analyzed using a Hach Kit (Phosphorus TNT plus, Hach, CO) with a detection limit of 0.5 mg-P L^{-1}. To quantify P release, phosphate was measured for each sample by first collecting 5 mL of cell suspension, and centrifuging it at 5,000 *g* for 5 min at 4°C. The supernatant was then filtered using 0.45 μm disc filters (GF, Acrodisc®, Pall Corporation, NY) to remove the biomass. The phosphate concentration in the filtrate was measured using PhosVer® 3 Phosphate Reagent Powder Pillows (Hach, CO) with a detection limit of 0.01 mg-P L^{-1}.

2.3. Phosphate Release from Recombinant *E. coli* as a Function of Ionic Strength, Temperature, and pH

The impact of ionic strength was explored by suspending the bacteria in 1 mM, 10 mM, and 100 mM KCl solutions. The suspensions were mixed on an orbital shaker at room temperature (22°C) for 3 h. Choi et al. (2013) reported efficient removal of P using *E. coli* in 6-h batch-scale adsorption tests, indicating that cell integrity was maintained throughout the 3-h test used in this study. We also confirmed integrity of the cell using the Bradford assay, which indicated that the concentration of proteins released from the cells after 3 h was below the detection limit of 0.125 mg L^{-1}.

To facilitate comparison of the P release capabilities of PBP *E. coli* and unexpressed *E. coli*, concentrations of released P were normalized to that from the unexpressed *E. coli*. In a similar way, we explored the influences of temperature (22°C and 35°C) and pH (3.8, 6.8, and 8.4) on phosphate release. All tests were conducted in triplicate (biological replicates). Percent P recovery was calculated by dividing the concentration of phosphate released by the total P content of the cells. We also evaluated phosphate release at different pHs as a function of time:

0, 0.5, 2, 3, 6, and 9 h. Kinetic data were fit to zero and first order reaction rates for comparison using Microsoft Excel.

2.4. Statistical Analysis

Differences in released phosphate concentrations due to changes in ionic strength and temperature were assessed using one-way ANOVA conducted using SPSS 11.5 software for Windows (SPSS Inc., Chicago, IL, USA). Two-way ANOVA was used to determine the effect of the contributing factors (i.e., time and pH) on phosphate release kinetics. Tukey post hoc analysis was performed for all ANOVA analyses. A significance level of 0.05 was used for all tests.

3. Results and Discussion

3.1. Confirmation of PBP Expression by SDS-PAGE and Western Blotting Analyses

Expressed PBP isolated from the periplasmic fraction of the PBP *E. coli* was analyzed by SDS-PAGE and Western Blotting, as shown in Figure 1 (the raw image is shown in Figure S1 in the supporting information). Both approaches indicated the molecular weight of the purified PBP was approximately 35 kDa. This result indicated that PBP was successfully expressed as it agrees with previous reports of 35.6 kDa for PBP (Choi et al., 2013).

3.2. Phosphate Release from Recombinant *E. coli* at Different Ionic Strengths and Temperatures

To quantify initial sorption (including both absorption for cellular functions and additional adsorption provided by PBP), we first measured the total P content at the same biomass concentration (OD 600 = 0.50) for the three groups of *E. coli*. They were 4.54 ± 0.01, 3.59 ± 0.03, 5.63 ± 0.10 mg-P L^{-1} for *E. coli* K12, unexpressed *E. coli*, and PBP *E. coli*, respectively. Based on these measures of the total P concentrations of the three types of cells, the over-

expressed PBP *E. coli* can clearly sorb more phosphate than the unexpressed controls (one-way ANOVA, $p < 0.05$). These results provide a basis for comparatively assessing P release as a function of ionic strength, temperature, and pH.

Figure 2 depicts the percentage of released phosphate from the three different groups of *E. coli* using different ionic strength solutions, all normalized to the concentration of P released from unexpressed *E. coli* at 1 mM KCl. The unexpressed *E. coli* and PBP *E. coli* generally showed increased phosphate release as ionic strength increased. However, *E. coli* K12 released similar phosphate concentrations across the range of ionic strengths tested ($p > 0.10$). At each ionic strength evaluated, PBP *E. coli* provided greater phosphate release than the control groups. For instance, the PBP *E. coli* released nearly two times more P than the unexpressed PBP group.

Limited information on the mechanisms of P release from the PBP-P complex is currently available, but binding is known to vary as a function of ionic strength (Wang et al., 1994). Ledvina et al. (1998) observed a 20-fold increase in the dissociation constant, K_d, at 0.30 M NaCl compared to no-salt solution, which agrees with our finding that higher ionic strength promotes P release. Though the exact mechanism for increased phosphate release by higher ionic strength is not yet known, there might be two plausible reasons. First, the increase in ionic strength could also increase the hydrolysis rate of protein-phosphate complexes, as research has shown that higher conductivity may increase enzymatic hydrolysis (Butre et al., 2012). Second, the increased ionic strength might also raise the permeability of the outer membrane of the cells and facilitate phosphate transport from the periplasmic space to the outside of the membrane for phosphate release (Suzuki et al., 1999).

The effect of temperature on the release of phosphate is illustrated in Figure S2. At room temperature, PBP *E. coli* released about 3.2 times more P than the unexpressed *E. coli*, while at 35ºC, PBP *E. coli* released about 3.1 times more P than the unexpressed *E. coli*. For all three types of *E. coli* tested, the elevated temperature improved phosphate release ($p < 0.05$). Increased P release as a function of increasing temperature agrees with the expectation that rates would increase since the kinetic energy of molecules increases with temperature. Protein stability may dictate an upper bound for temperature increases, but as the denaturation temperature for most proteins is 41°C (Stoker, 2006), PBP activity is unlikely altered at 35°C. Elevated temperature can increase membrane permeability (Bischof et al., 1995; Osborne and MacKillop, 1987), and the change in permeability of the membrane could ostensibly increase phosphate release. For PBP *E. coli*, the elevated temperature may also trigger hydrolysis of the phosphate-PBP complex, although further research is needed.

3.3. Phosphate Release from Recombinant *E. coli* at Different pH Levels

Phosphate release at different pH levels is shown in Figure 3. The PBP *E. coli* and unexpressed PBP *E. coli* demonstrated similar trends. The lower pH increased the concentration of phosphate released compared to near-neutral conditions for all three *E. coli* ($p < 0.05$), while no significant difference was identified between the near-neutral condition and pH 8.4 ($p = 0.27, 0.18, 0.18$ for unexpressed PBP *E. coli*, PBP *E. coli*, and *E. coli* K12, respectively). For all three *E. coli*, lower pHs appear to facilitate phosphate release while higher pHs (i.e., pH 8.4) have negligible impact. PBP *E. coli* released more phosphate than the two negative controls at each pH level, approximately 2.3 – 3.3 fold and 1.3 – 2.2 fold greater compared to unexpressed PBP *E. coli* and *E. coli* K12, respectively, at the pH levels tested here. The interaction between P and PBP is

dominated by local dipolar interaction (Ledvina et al., 1998). Thus, pH shifts away from neutral could lead to redistribution of charge on the P-PBP complex, thereby affecting dipolar interactions. Accordingly, lower or higher pH favors the dissociation of P from the complex, as indicated by our results.

3.4. Kinetics of Phosphate Release

Before testing P release kinetics at different pHs, we measured the initial total phosphate content in each culture (after diluting each to OD 600 = 0.5), which was 4.9 ± 0.02, 3.9 ± 0.04, and 4.0 ± 0.2 mg L^{-1} for PBP *E. coli*., unexpressed PBP *E. coli*, and *E. coli* K12, respectively. This shows that the genetically modified PBP *E. coli* removed more phosphate from LB medium than the negative controls.

The results shown in Figure 3 indicated that there was negligible impact on P release using the basic solution. Therefore, the kinetics of phosphate release were evaluated at near-neutral and acidic conditions. Figure 4a depicts the kinetics of phosphate release within 9 h at near-neutral conditions (pH 6.8). In terms of P release, the PBP *E. coli* released more phosphate at each time point, yielding a final phosphate concentration of 0.07 ± 0.005 mg L^{-1} after 9 h. However, both unexpressed PBP *E. coli* and *E. coli* K12 reached the highest phosphate concentrations after 0.5 h. Two-way ANOVA between unexpressed PBP *E. coli* and *E. coli* K12 indicated no significant effects due to group ($p = 0.68$), meaning unexpressed PBP *E. coli* and *E. coli* K12 were essentially the same in terms of phosphate release. There was also no significant effect due to joint factors (group × time, $p = 0.23$); however, time did have a significant impact on phosphate release ($p < 0.05$). The change in P concentration over time was well represented using a zero order reaction for PBP *E. coli* ($R^2 = 0.85$), yielding a reaction constant of 0.006 mg L^{-1} h^{-1}. The

unexpressed *E. coli* and *E. coli* K12 produced reaction constants of 0.001 and 0.002 mg L^{-1} h^{-1}, respectively. Clearly PBP *E. coli* not only released more P than the controls, but also demonstrated a faster P release rate at pH 6.8.

All three groups of *E. coli* showed an increasing trend of phosphate release as a function of time in acidic conditions (Figure 4b). All samples released more phosphate compared to near-neutral conditions, and PBP *E. coli* consistently released more phosphate than the negative controls. To analyze the difference between unexpressed PBP *E. coli* and *E. coli* K12, two-way ANOVA analysis was conducted. The analysis showed no significant effects due to groups and time × groups (p values = 0.45, 0.10, respectively), while a significant effect was observed due to time ($p < 0.05$). Pseudo first order kinetics provided a better fit to the data than zero order, providing reaction rate constants of 1.04, 0.48, and 0.27 h^{-1} for PBP *E. coli*, *E. coli* K12, and unexpressed *E. coli* (R^2 = 0.8, 0.2, 0.4), respectively. Thus, PBP *E. coli* always released statistically greater levels of phosphate at a faster rate than the controls.

3.5. Phosphate Recovery Potential using PBP *E. coli*

Implementation of recombinant-plasmid bacteria systems in actual wastewater treatment applications introduces challenges such as expulsion of the plasmid in the absence of antibiotic pressure (Clark, 2009; Palomares et al., 2004). However, this study provides proof-of-concept for the use of PBP for P recovery by demonstrating controlled P release. The results clearly indicate the feasibility of using PBP for P recovery in that: a) bacterial expression of PBP proteins enables greater phosphate adsorption, and b) PBP-bound phosphate can be released using environmental stimuli, with lower pH, higher ionic strength, and higher temperature promoting desorption. The highest observed recovery of adsorbed P in this 3-h study was 2.1%.

Although the concentrations of P released to the water were low, optimized release of the phosphate sorbed by PBP *E. coli* into smaller volume "regenerant" solutions could facilitate subsequent use as a liquid fertilizer or solid fertilizer following precipitation of phosphate-rich solids. Successful construction of recombinant *E. coli* in this study not only demonstrated an efficient means of producing PBP, but also provides a solid preliminary basis for future work using PBP for phosphate removal. Future research is needed to address the many fundamental thermodynamic questions that remain, including what are the important cofactors for the dissociation reaction, and how do pH and ionic strength impact PBP-P complex configuration and binding? Phosphate recovery may be greatly improved through direct exposure of PBP to the water matrix, rather than expressing it in the cell's periplasmic space. Ultimately, an immobilized PBP system will be investigated to improve understanding of phosphate-PBP sorption and desorption potential.

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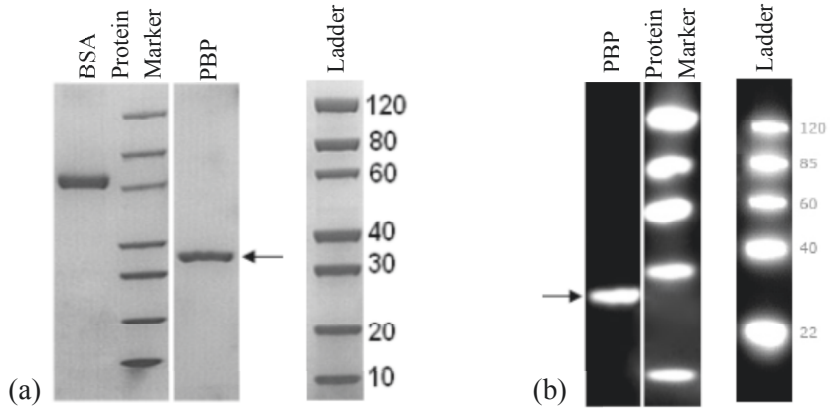

Figure 1. (a) SDS-PAGE and (b) Western blotting analyses of purified PBP protein. Bovine serum albumin (BSA) was used as a PBP-negative control for SDS-PAGE.

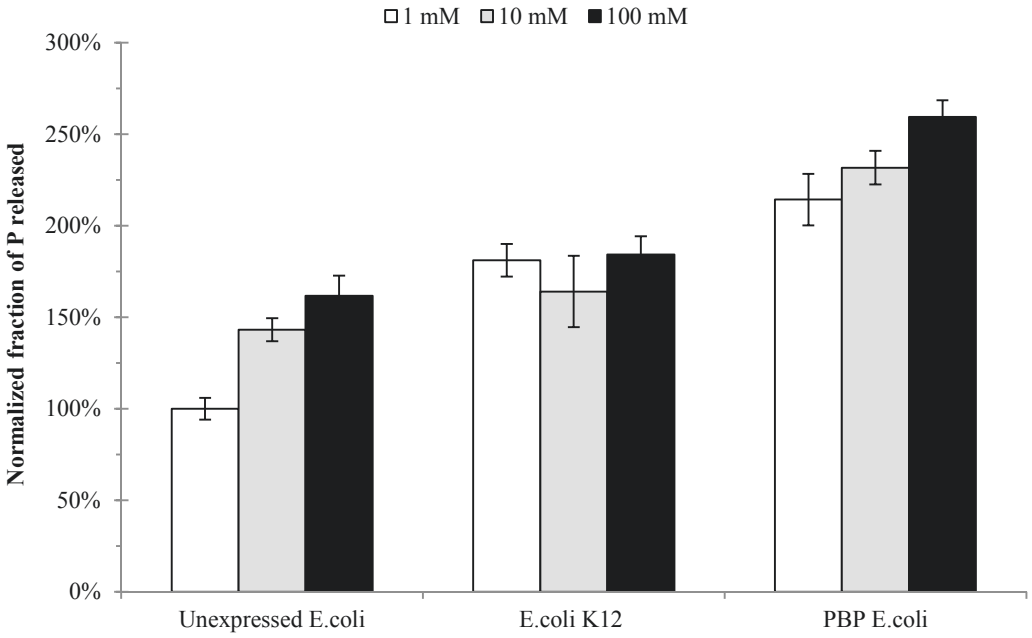

Figure 2. Phosphate release from PBP *E. coli*, unexpressed *E. coli*, and *E. coli* K12 suspension within 3 h at different ionic strengths (1 mM, 10 mM, and 100 mM KCl). All concentrations were normalized to the P concentration released from unexpressed *E. coli* at 1 mM KCl. Experiments were performed at room temperature (22°C), and the pH of all samples was initially 6.8. The initial concentration of all bacterial suspensions was OD 600 = 0.50. Bars and error bar represent mean ± one standard deviation of triplicate experiments.

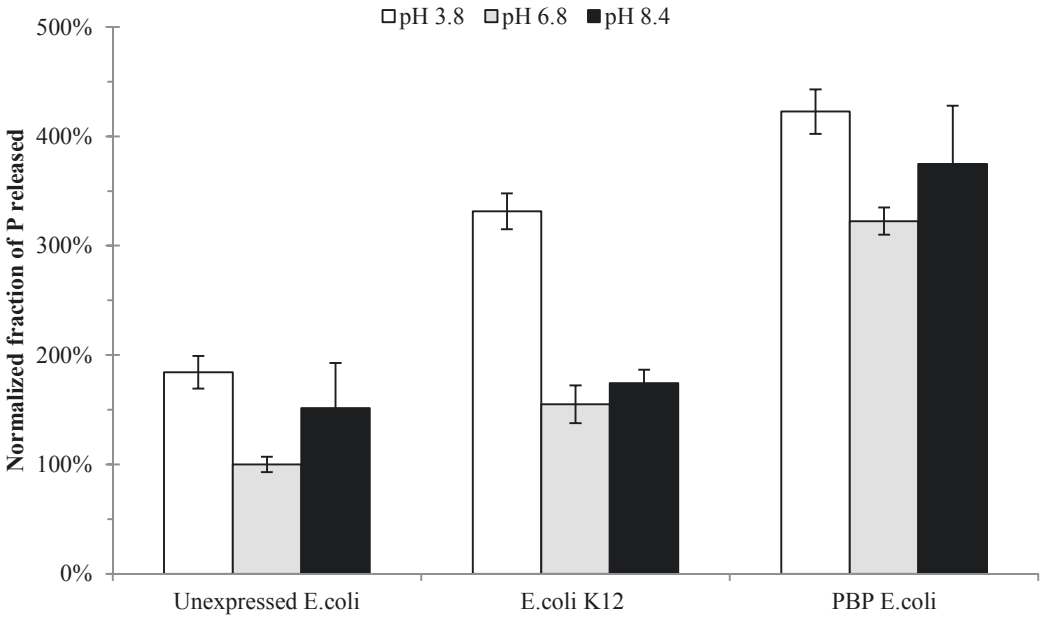

Figure 3. Phosphate concentration released from PBP *E. coli*, unexpressed *E. coli*, and *E. coli* K12 suspension within 3 h at different pHs. All concentrations were normalized to the P concentration released from unexpressed *E. coli* at pH 6.8. All tests were performed at room temperature 22°C and 1 mM KCl was used for all samples. The initial concentration of all bacterial suspensions was OD 600 = 0.50. Bars and error bar represent mean ± one standard deviation of triplicate experiments.

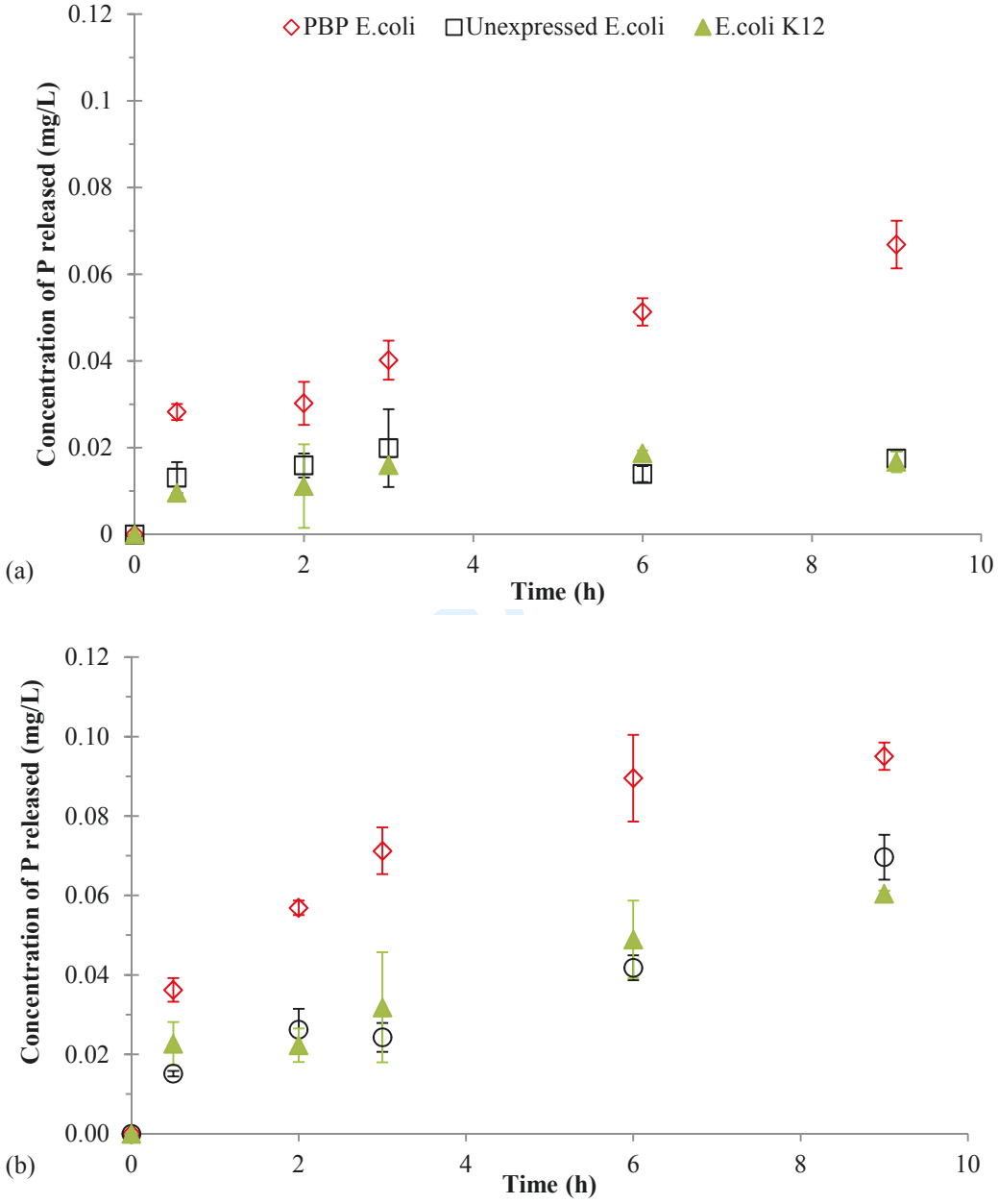

Figure 4. The change in phosphate concentration released from PBP *E. coli*, unexpressed *E. coli*, and *E. coli* K12 as a function of time at (a) pH 6.8 and (b) pH 3.8. All cell suspensions were adjusted to the same bacteria concentration of OD 600 = 0.50. Data points and error bars represent mean ± one standard deviation of triplicate experiments.

High affinity phosphate binding protein (PBP) for phosphorous recovery: Proof of concept using recombinant *Escherichia coli*

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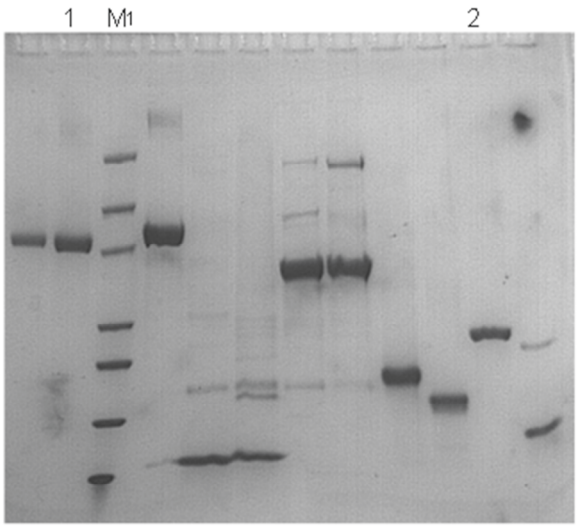
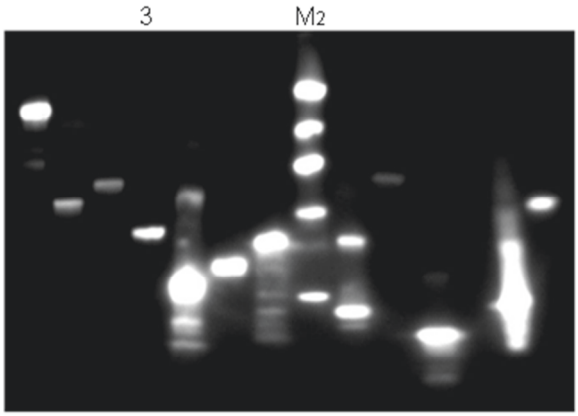


1: BSA,2 µg
M1: SDS-PAGE Marker
2: 600490-1,1.95 µg(SDS-PAGE)

(a)



3: 600490-1(Western Blot)
M2: Western Blot Marker

(b)

Figure S1. Original images for (a) SDS-PAGE analysis and (b) Western blotting using the purified PBP protein. In Figure S1a, lanes 1, M1, and 2 represent bovine serum albumin (BSA), protein marker, and purified protein PBP, respectively. In Figure 1b, lanes 3 and M2 represent purified protein PBP and protein biomarker, respectively.

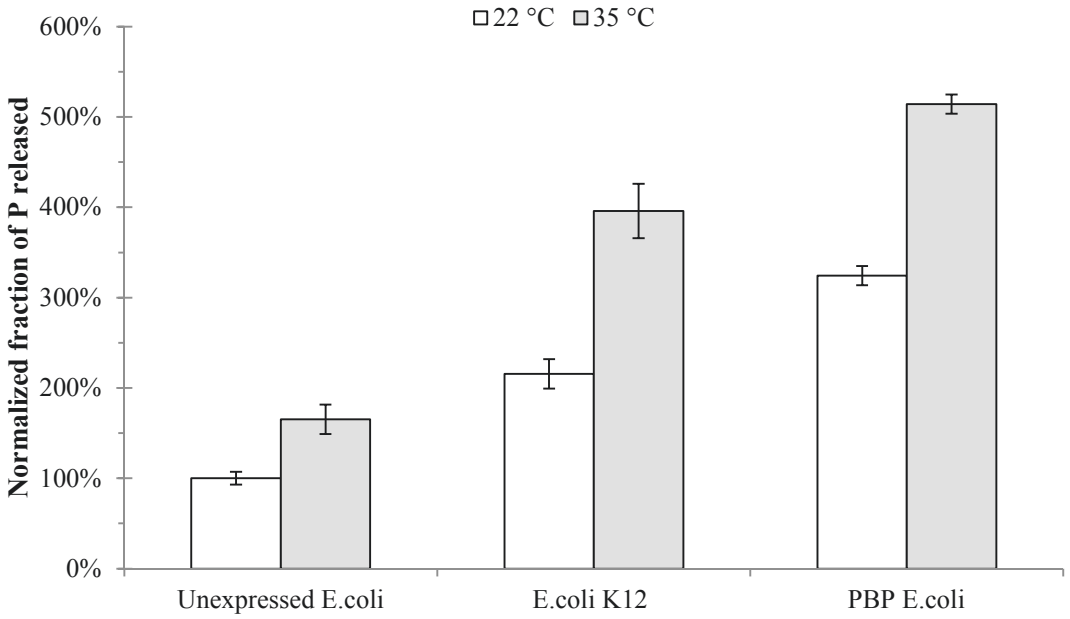


Figure S2. Phosphate concentrations released from PBP *E. coli*, unexpressed *E. coli*, and *E. coli* K12 suspensions within 3 h as a function of temperature. All concentration values were normalized to the P concentration released from unexpressed *E. coli* at 22ºC. All suspensions had a bacteria concentration of OD 600 = 0.50. Bars and error bars represent mean ± one standard deviation of triplicate experiments.

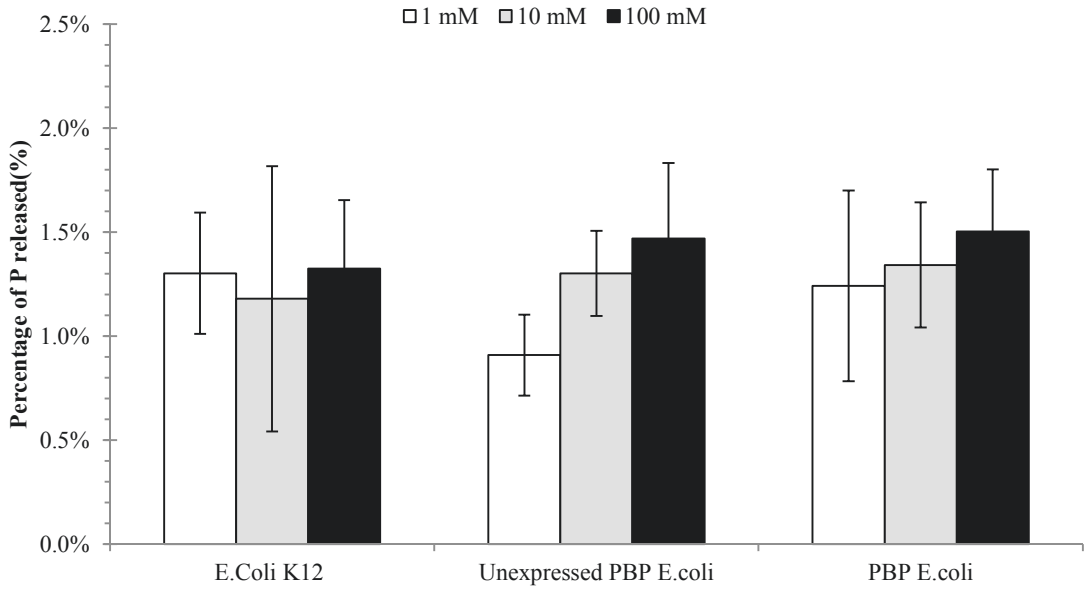


Figure S3. Percentage of phosphate released from PBP *E. coli*, unexpressed *E. coli*, and *E. coli* K12 suspension within 3 h at different ionic strengths (1 mM, 10 mM, and 100 mM KCl). All concentration values were normalized to the total P of the cell suspension. The initial pH of all samples was 6.8 and the initial concentration of all bacterial suspensions was OD 600 = 0.50. Bars and error bar represent mean ± one standard deviation of triplicate experiments.

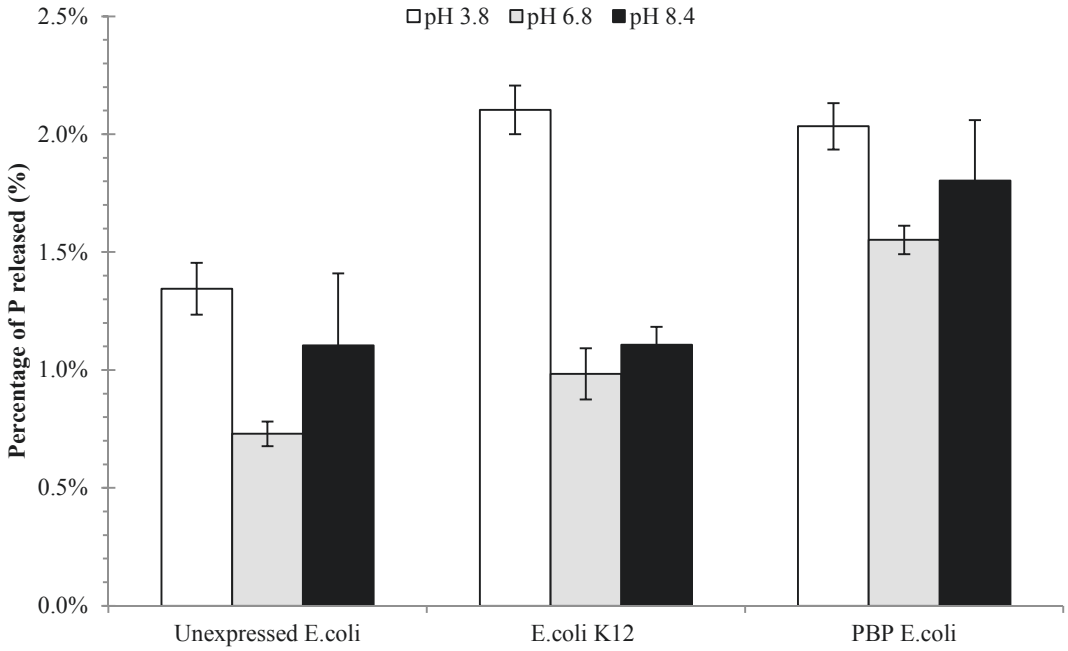


Figure S4. Percentage of phosphate released from PBP *E. coli*, unexpressed *E. coli*, and *E. coli* K12 suspension within 3 h at different pHs. All concentrations were normalized to the total P concentration of each cell suspension at pH 6.8. 1 mM KCl was used for all of tests. The initial concentration of all bacterial suspensions was OD 600 = 0.50. Bars and error bar represent mean ± one standard deviation of triplicate experiments.

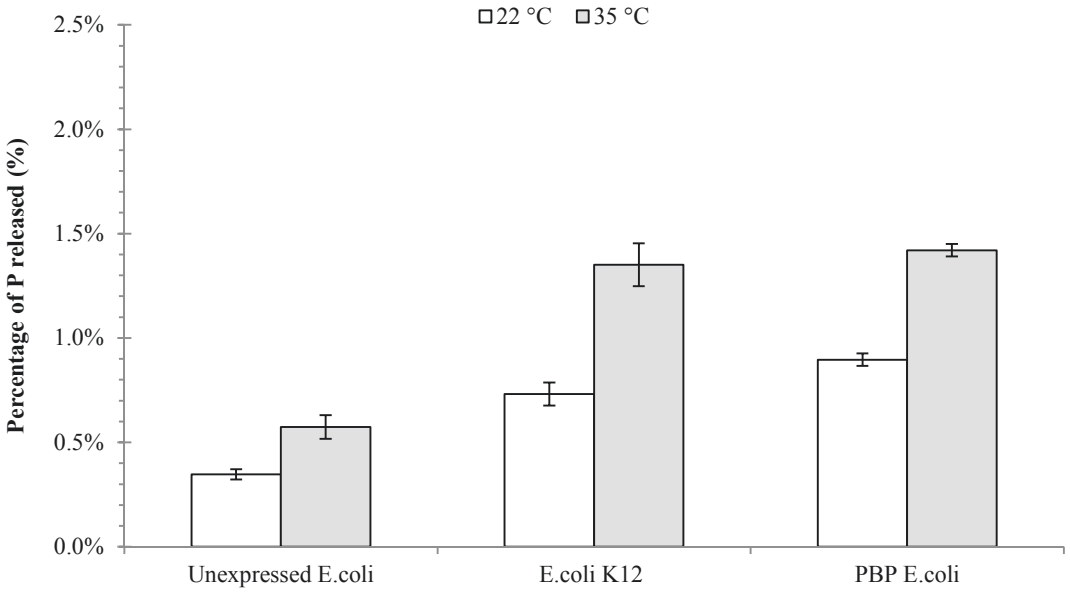


Figure S5. Percentage of phosphate released from PBP *E. coli*, unexpressed *E. coli*, and *E. coli* K12 suspensions within 3 h as a function of temperature. All concentrations were normalized to the total P of the cell suspension at 22 ºC. All suspensions had a bacteria concentration of OD 600 = 0.50. Bars and error bar represent mean ± one standard deviation of triplicate experiments.