10-1-2016

High-Affinity Phosphate-Binding Protein (PBP) For Phosphorous Recovery: Proof of Concept Using Recombinant *Escherichia coli*

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Accepted version. *FEMS Microbiology Letters,* Vol. 363, No. 20 (October 2016). DOI.

High-affinity Phosphate-binding Protein (PBP) for Phosphorous Recovery: Proof of Concept Using Recombinant *Escherichia coli*

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Abstract: Phosphorus (P) is a critical, non-renewable 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 (PBPs) 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 *Escherichia coli* was explored in this study. *Escherichia coli* was genetically modified to overexpress PBP in the periplasmic space. The impacts of

FEMS Microbiology Letters, Vol. 363, No. 20 (October 2016). DOI.

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

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; Rittmann *et al.*2011; Mayer *et al.*2013). Eutrophication is a major water quality problem (Smith *et al.*2014), and is the cause of at least 400 coastal dead zones worldwide (Diaz and Rosenberg 2008; Caballero-Alfonso, Carstensen and Conley 2015). In municipal wastewater treatment, enhanced biological phosphorus removal is often employed to achieve effluent concentrations as low as ~0.1 mg-P L^{-1}, which approaches the kinetic and thermodynamic limit (Jenkins, Ferguson and Menar 1971; Jenkins and Hermanowicz 1991; Cooper, Dee and Yang 1993; Blaney, Cinar and Sengupta 2007). 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 ultralow 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 (Li *et al.*2009; Choi *et al.*2013). 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 (Wanner 1993; Botero, Al-Niemi and McDermott 2000; Santos-Beneit *et al.*2008; Blank 2012). In *Escherichia 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) (Santos-Beneit *et al.*2008; Choi *et al.*2013). Pursuant to the Venus flytrap model (Mao, Pear and McCammon 1982; Brune *et al.*1998), 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 ultralow 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.

MATERIALS AND METHODS

Construct and verify recombinant *Escherichia coli* expressing PBP

We engineered PBP-expressing *E. coli* following the manufacturer's protocols (PET System manual 10th edition, Novagen, Madison, WI, USA). The PBP gene was directly synthesized using the PBP sequence from *Pseudomonas aeruginosa* (GenScript, Piscataway, NJ), as its PBP has demonstrated strong phosphate binding (Neznansky *et al.*2014). Plasmid PET 30 a (Novagen) 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, USA). Ligation was conducted using a DNA Ligation Kit (Novagen kit #69838). 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). 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) was added to induce PBP expression, and the cells were further cultured for another 12 h. Cells were harvested by centrifugation at 5000 *g* for 10 min at 4°C, and then lysed by water bath sonication. The target PBP was obtained by one-step purification using a Ni-NTA agarose column (Qiagen). 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, Fritsch and Maniatis 1989). The primary antibody for western blot was Mouse-anti-His mAb (GenScript, Piscataway, NJ, USA).

Unexpressed controls and P analysis

Two unexpressed controls were used for comparison against the PBP-overexpressed *E. coli*: (i) *E. coli* K12 (endogenous PBP with the gene in the chromosome) and (ii) 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 5000 *g* for 5 min at 4°C. The biomass was resuspended 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 5000 *g* for 5 min at 4°C. The supernatant was then filtered using 0.45 µm disk 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) with a detection limit of 0.01 mg-P L^{-1}.

Phosphate release from recombinant *Escherichia coli* as a function of ionic strength, temperature and pH

The impact of ionic strength was explored by suspending the bacteria in 1, 10 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.

Statistical analysis

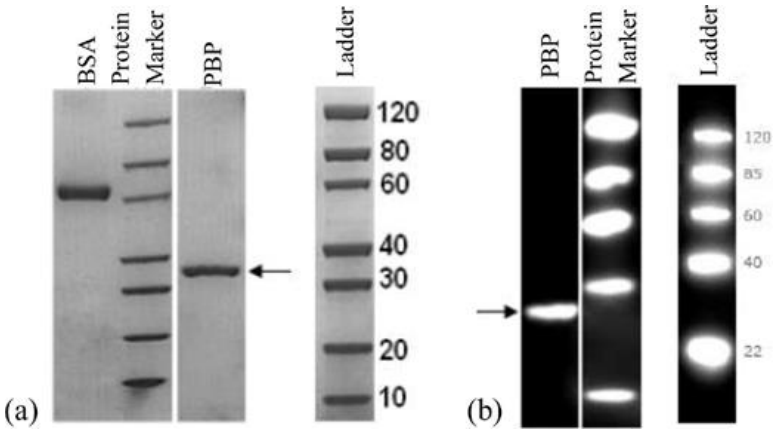
Differences in released phosphate concentrations due to changes in ionic strength and temperature were assessed using one-way ANOVA conducted using SPSS 11.5 software for Windows (SPSS Inc., Chicago, IL, USA). Two-way ANOVA was used to determine the effect of the contributing factors (i.e. time and pH) 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.

RESULTS AND DISCUSSION

Confirmation of PBP expression by SDS-PAGE and western blotting analyses

Expressed PBP isolated from the periplasmic fraction of the PBP *Escherichia coli* was analyzed by SDS-PAGE and western blotting, as shown in Fig. 1 (the raw image is shown in Fig. S1, Supporting Information). Both approaches indicated that the molecular weight of the purified PBP was ~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).

Figure 1.



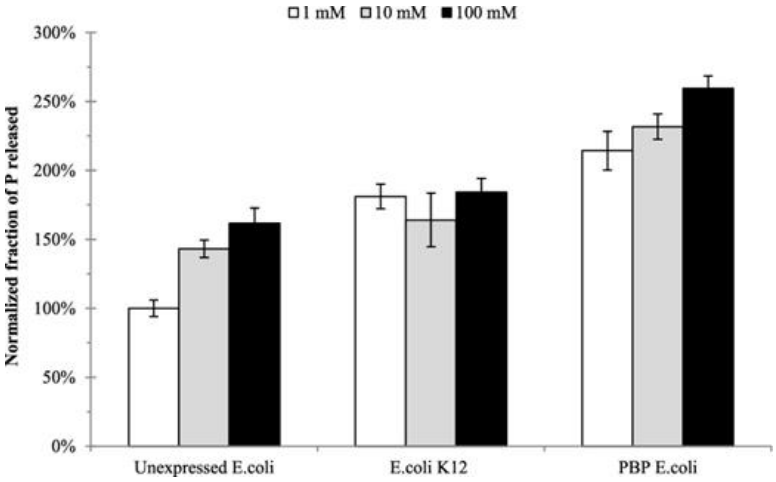
(**a**) SDS-PAGE and (**b**) western blotting analyses of purified PBP protein. Bovine serum albumin (BSA) was used as a PBP-negative control for SDS-PAGE.

Phosphate release from recombinant *Escherichia 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 $\pm$ 0.01, 3.59 $\pm$ 0.03 and 5.63 $\pm$ 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 overexpressed 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 shows 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.

Figure 2.



Phosphate release from PBP *E. coli*, unexpressed *E. coli* and *E. coli* K12 suspension within 3 h at different ionic strengths (1, 10 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.

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, Wierenga and Gruppen 2012). Second, the increased ionic strength might also raise the permeability of the outer membrane of the cells and facilitate

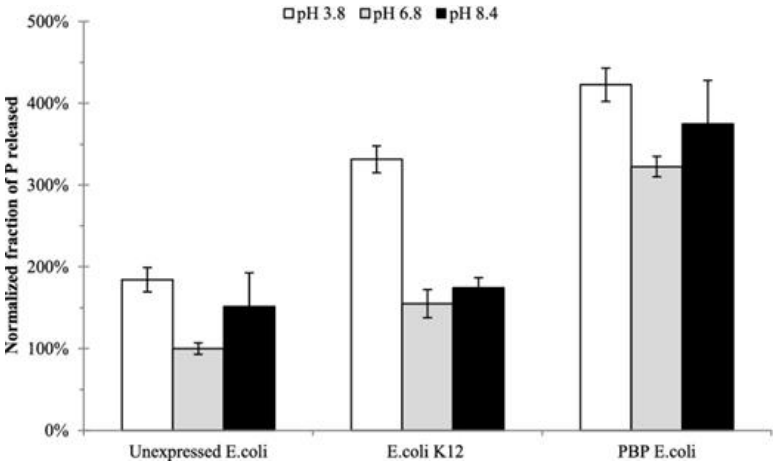
phosphate transport from the periplasmic space to the outside of the membrane for phosphate release (Suzuki *et al.*1999).

The effect of temperature on the release of phosphate is illustrated in Fig. S2 (Supporting Information). 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 (Osborne and MacKillop 1987; Bischof *et al.*1995), 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.

Phosphate release from recombinant *Escherichia coli* at different pH levels

Phosphate release at different pH levels is shown in Fig. 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 and 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, ~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.

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.

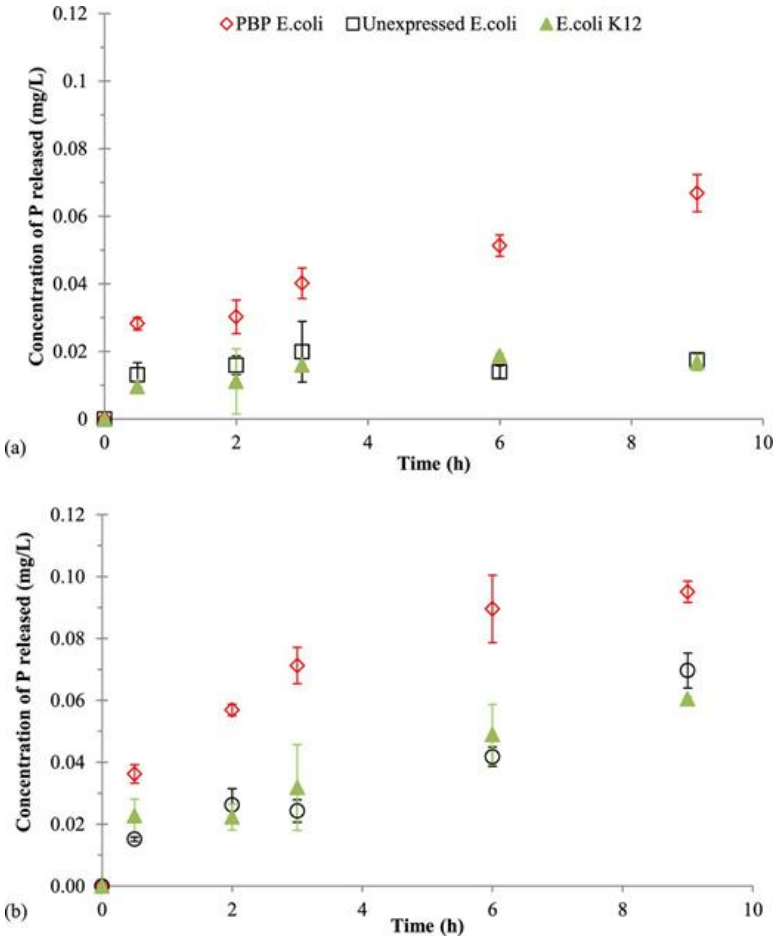
Kinetics of phosphate release

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 Fig. 3 indicated that there was negligible impact on P release using the basic solution. Therefore, the kinetics of phosphate release was evaluated at near-neutral and acidic conditions. Figure 4a shows 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.

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.

All three groups of *E. coli* showed an increasing trend of phosphate release as a function of time in acidic conditions (Fig. 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.

Phosphate recovery potential using PBP *Escherichia 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 (Palomares, Estrada-Moncada and Ramírez 2004; Clark 2009). 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 (i) bacterial expression of PBP enables greater phosphate adsorption, and (ii) 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.

FUNDING

This project is part of CAREER award [] from the . BKM acknowledges support from the for Sustainability Program [Award]. Additionally, this work was partially supported by the at . All opinions expressed in the paper are the authors' and do not necessarily reflect the views of NSF.

Conflict of interest. None declared.

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Supplementary data

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

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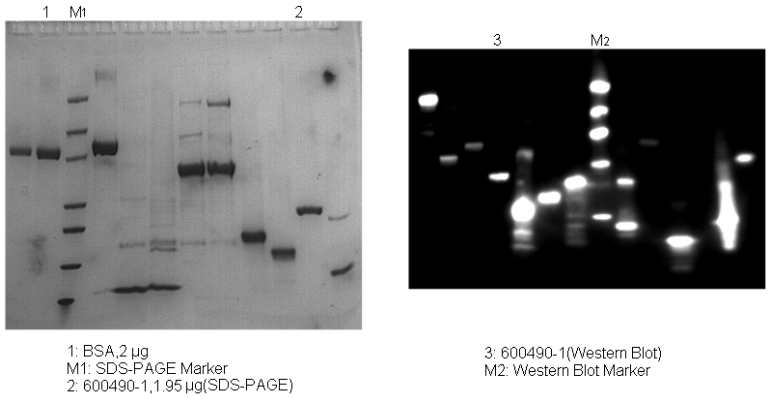

Figure S1. Original images for (a) SDS-PAGE analysis and (b) Western blotting using the purified PBP protein. In Figure S1a, lanes 1, M1, and 2 represent bovine serum albumin (BSA), protein marker, and purified protein PBP, respectively. In Figure 1b, lanes 3 and M2 represent purified protein PBP and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

(a) (b)

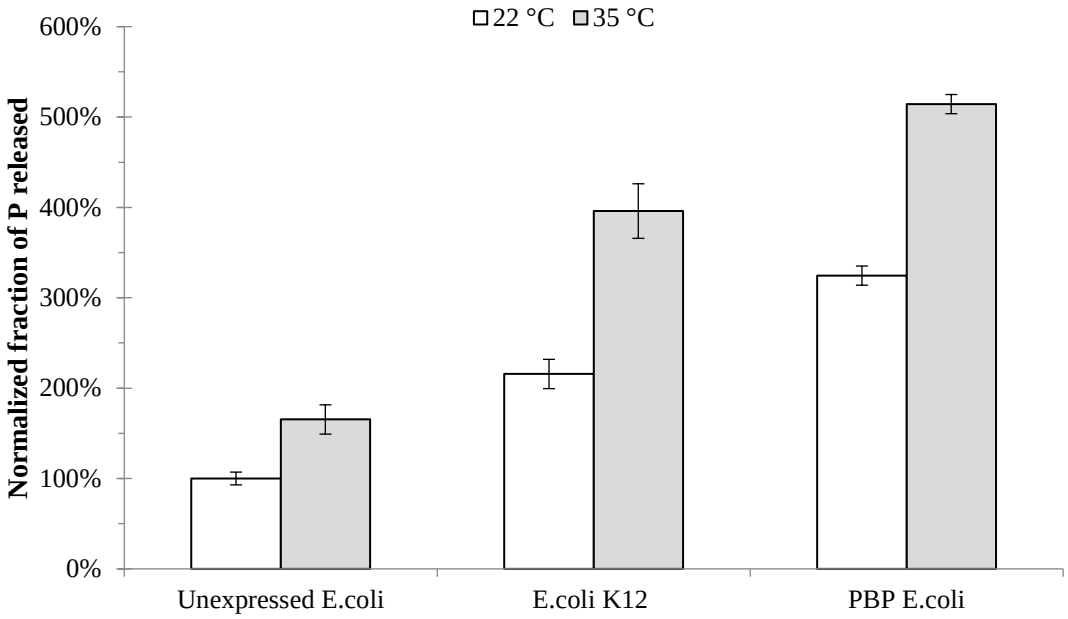

Figure S2. Phosphate concentrations released from PBP *E. coli*, unexpressed *E. coli*, and *E. coli* K12 suspensions within 3 h as a function of temperature. All concentration values were normalized to the P concentration released from unexpressed *E. coli* at 22ºC. All suspensions had a bacteria concentration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

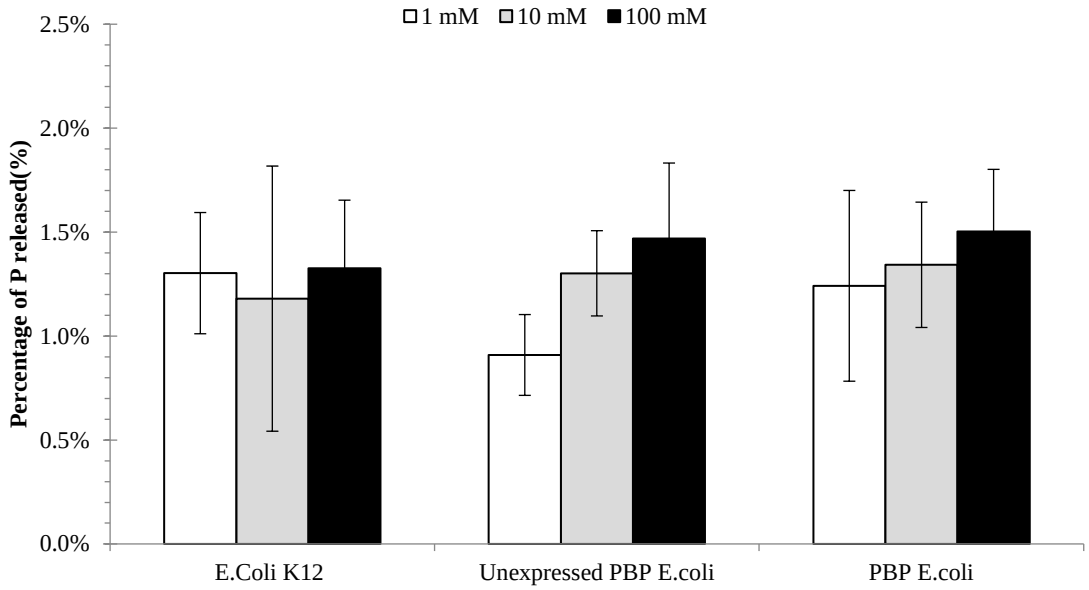

Figure S3. Percentage of phosphate released from PBP *E. coli*, unexpressed *E. coli*, and *E. coli* K12 suspension within 3 h at different ionic strengths (1 mM, 10 mM, and 100 mM KCl). All concentration values were normalized to the total P of the cell suspension. The initial pH of all samples was 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.

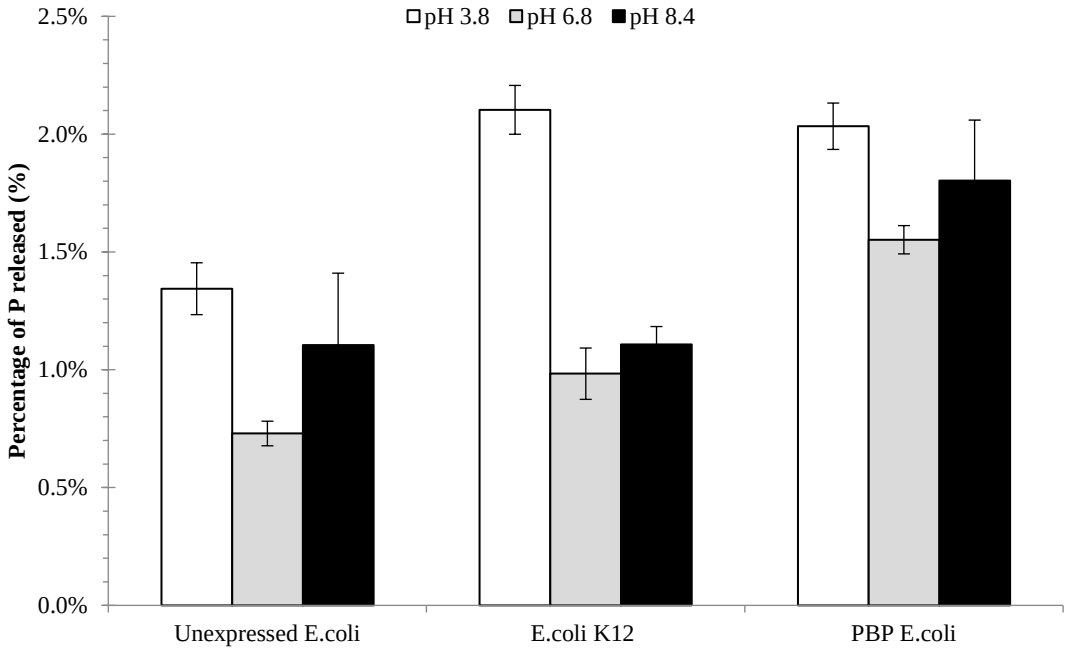

Figure S4. Percentage of phosphate released from PBP *E. coli*, unexpressed *E. coli*, and *E. coli* K12 suspension within 3 h at different pHs. All concentrations were normalized to the total P concentration of each cell suspension at pH 6.8. 1 mM KCl was used for all of tests. The initial concentration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

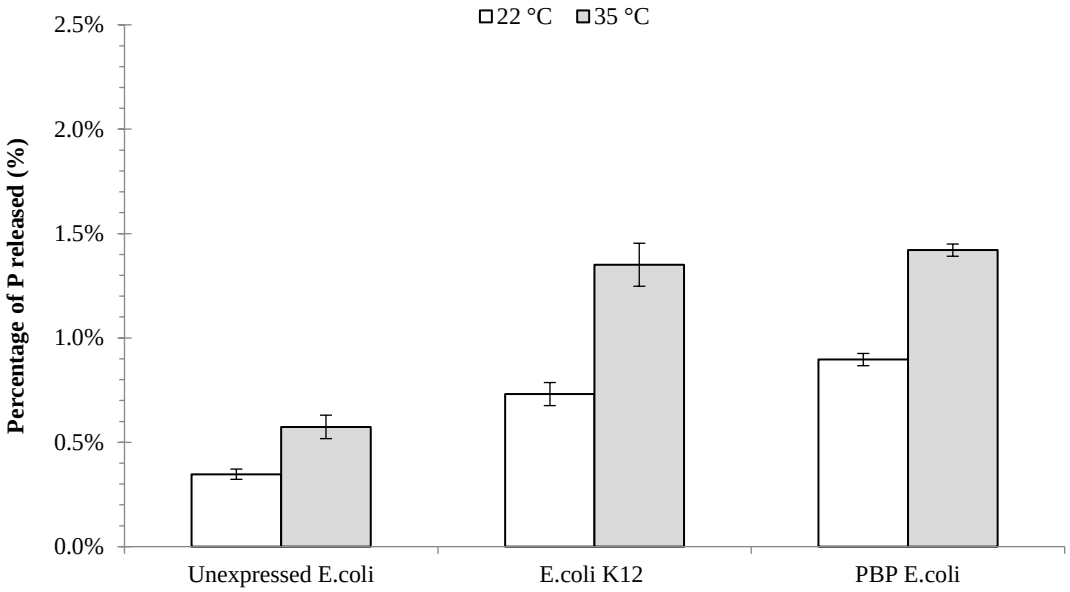

Figure S5. Percentage of phosphate released from PBP *E. coli*, unexpressed *E. coli*, and *E. coli* K12 suspensions within 3 h as a function of temperature. All concentrations were normalized to the total P of the cell suspension at 22 ºC. All suspensions had a bacteria concentration of OD 600 = 0.50.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