An aqueous two-phase system to pre-purify a heterologously produced siderophore

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Natural products span broad activities and applications; however, their access and production are often limited by native cellular sources. As a result, the heterologous production of a siderophore termed yersiniabactin (Ybt) was completed using the surrogate host Escherichia coli. Post-production and purification steps are complicated by the complex nature of most media used for cell growth, prompting the development in this work of an aqueous two-phase pre-purification system capable of rapidly and simply enhancing the concentration of the target Ybt compound.

Keywords: Siderophore; Yersiniabactin; E. coli; Aqueous Two-Phase System; Heterologous Biosynthesis.

INNOVATION
The biological sources of natural products result in production processes that are complex in media composition and complicated compound purification. Such is the case for the heterologous production of yersiniabactin (Ybt) using E. coli. Production through E. coli establishes an engineering-friendly platform for compound access and eliminates the need to handle the native producer Yersinia pestis, a priority pathogen. In addition, the final compound has several potential applications in metal sequestration. However, these and other options will benefit from an economical (rapid and requiring simple reagents), green (solvent-free) and simple (non-chromatographic) pre-purification step. Toward this end, we employed an aqueous two-phase system (ATPS) for the enhanced separation of Ybt from a crude cellular extract. The separation approach is unique in its application to Ybt and enables a straightforward means of simplifying purification strategies for future applications in metal removal, retrieval and reuse. The approach also offers broader potential as an option for other natural products produced in heterologous formats.

NARRATIVE
Ybt is a mixed non-ribosomal peptide–polyketide siderophore natively produced by the pathogen Yersinia pestis1. Siderophore compounds enable iron-scavenging capabilities upon host infection, and Ybt is used in this capacity during Y. pestis virulence transition. The metal-binding properties of siderophores offer interesting alternative uses that range from corrosion resistance to heavy metal remediation2, and recently, our group has been exploring such options using Ybt. However, in undertaking these studies, the production of the Ybt compound must be accomplished safely and efficiently, which places limits on using the native Y. pestis host.

As an alternative, the production of Ybt has been established using E. coli as a host3. This step eliminates the need to handle a priority pathogen and offers all of the innate and engineering capabilities provided by E. coli. However, while the heterologous system provides new opportunities for the upstream biosynthesis of Ybt, all biological production efforts will require downstream purification steps that are complicated by the media formulations that accompany cell growth. The case for heterologous Ybt production is no different.

The heterologous experimental process for Ybt production is outlined in Fig. 1a. As a first step in purification, the resulting culture is extracted with an organic solvent (ethyl acetate) that concentrates organic-soluble culture components, including Ybt. However, the resulting extract retains numerous contaminants, prompting us to utilize an ATPS strategy as another simple, non-chromatographic approach toward Ybt purification.

The ATPS approach is outlined in Fig. 1b and involves mixing the dried extract from a Ybt-producing E. coli culture with a poly(ethylene glycol) (PEG)/salt solution, resulting in distinct phases influenced by pH, temperature and salt concentrations that affect polymer distribution. The process has been used to separate various biomolecules4,5, often proteins or peptides, but has rarely been applied to natural products and has not been used previously in heterologous biosynthetic efforts. As such, we saw an opportunity to utilize the approach for an early separation step that would simplify final purification.

Figure 2 presents ATPS efforts across several parameters designed to assess the partitioning of Ybt (as defined in the ‘Methods’ section) into the upper phase of the extract/PEG/salt mixture. To configure optimal separation conditions, we first studied the effect of PEG molecular weight and solution pH on Ybt partitioning (Fig. 2a). PEG molecular weight was varied across 300, 600, 1,000 and 2,000 Da. Using this configuration, pH was adjusted at 6, 7 or 8 using phosphate buffer (100 mM). PEG-1000 showed the highest Ybt partitioning across all pH values.

The molecular weight of the phase-forming polymer will affect the partitioning in two aspects: first by changing the equilibrium phase diagram and second by altering the number of polymer–biomolecule
Increasing PEG molecular weight will increase interfacial tension between the resulting two phases which provides a stronger barrier to the transport of solute molecules from the upper PEG phase to the lower salt phase and, thus, prompting higher \( K_d \) values. However, PEG chains with a higher molecular weight will also increase repulsive interactions with solute and potentially decrease \( K_d \) values. Positive partitioning trends are reflected in Fig. 2a as PEG molecular weight increases from 300 to 1,000, followed by a drop in \( K_d \) as PEG molecular weight is increased to 2,000. For all cases, increasing the pH increases Ybt partitioning, resulting in PEG-1000 and pH \( \text{pH}=8 \) as the best condition.

The pH influences solute surface charge by affecting ionizable groups of Ybt, resulting in partitioning preferences. Furthermore, according to the Flory–Huggins theory on ATPS, increasing pH will heighten separation of the PEG and salt phases which can influence solute partitioning. Increasing temperature increases PEG hydrophobicity which drives water into the salt-rich lower phase. These changes are reflected in the slope and length of equilibrium tie-lines. Figure 2b indicates that raising the temperature to 30°C increases Ybt accumulation in the upper phase. However, a further increase in temperature causes a slight drop in \( K_d \) which may be explained due to increased PEG concentrations resulting in solute repulsion.

The effect of PEG concentration on protein partitioning has been studied previously. In a similar manner to PEG molecular weight variation, increasing PEG concentration will affect viscosity and interfacial tension between aqueous phases with the potential of reduced \( K_d \) values as concentration values continue to rise, a trend observed in Fig. 2c. Therefore, moderate PEG concentrations are recommended for better purification and separation. Salt type will influence the hydrophobic interaction between the two aqueous phases, and salt selection is highly dependent on the solute and polymer being used. For example, when using a PEG-400/salt ATPS, the distribution coefficient for cephalosporin varied in the order \( \text{Na}_2\text{SO}_4 > \text{KPO}_4 > (\text{NH}_4)_2\text{SO}_4 > \text{MgSO}_4 \). In this study, three sulfate salts were studied (Fig. 2d), and the partitioning order was \( (\text{NH}_4)_2\text{SO}_4 > \text{MgSO}_4 > \text{Na}_2\text{SO}_4 \). As a result, PEG-1000 (20% wt/wt), 30°C, \( \text{pH}=8 \) and \( (\text{NH}_4)_2\text{SO}_4 (20\% \text{ wt/wt}) \) were chosen as the optimal ATPS parameters for Ybt pre-purification, and the resulting HPLC analysis of the upper aqueous phase confirmed separation effectiveness (Fig. 2e). It should be noted, however, that future design of experiment studies would...
allow a more thorough assessment of the interrelated effects of the parameters tested here and the potential for an even greater impact on product purity.

**METHODS**

**Materials**

Salts, PEG and solvents were purchased from Sigma-Aldrich (St. Louis, MO) and were used without further purification. Millipore water was the only source of water in experiments.

**Yersiniabactin heterologous biosynthesis and extraction**

To heterologously produce Ybt, plasmids pBP198 and pBP205 were transformed into E. coli strain BAP1 using electroporation. The strain was then used to start a 5-mL lysogeny broth (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) culture containing plasmid selection antibiotics that was used to inoculate (1% v/v) a 500-mL culture of M9 minimal medium (12.8 g/L Na2HPO4 ∙ 7H2O; 6 g/L Na2HPO4; 3 g/L KH2PO4; 0.5 g/L NaCl; pH adjusted to 7.4 with NaOH) supplemented with glycerol (0.5 wt%), casamino acids (1 wt%) and plasmid selection antibiotics. Post-inoculation, cultures were incubated at 37°C with shaking to an OD600nm of 0.4–0.6 before salicylate was added to a final concentration of 1 mM and induction initiated using 100 μM of isopropyl β-D-1-thiogalactopyranoside (IPTG). Cultures were then continued with shaking at 22°C.

After five days, FeCl3 was added (final concentration of 5 mM) to supernatants separated by centrifugation from the 500-mL cultures. After incubating for 30 min at 22°C, supernatant was extracted twice with an equal volume of ethyl acetate each time. Extracts were evaporated to dryness under vacuum, and the resulting residue was resuspended in methanol (with the final volume concentrated 100× relative to the original culture volume).

**Aqueous two-phase system**

Experiments were conducted in either 1.4-mL or 15-mL centrifuge tubes to which crude extract containing Ybt-Fe3+ was added and evaporated such that 0.15 mg/mL Ybt-Fe3+ was introduced to each ATPS. Phosphate buffer (100 mM; adjusted to different pH values by altering the ratio of mono- and dipotassium phosphate) was used to dissolve salt (20 wt%) and PEG content to maintain a total mass of either 1 or 10 g, and the resulting solution was mixed thoroughly and incubated at varying temperature values. After 4 h, the Ybt-Fe3+ contents of upper and lower phases were measured using either LC-MS or HPLC by comparing against a calibration curve. The partitioning (or distribution) coefficient (Kd) is defined as the ratio of Ybt-Fe3+ concentrations in the upper (C_u) and lower (C_l) phases:

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K_d = \frac{C_u}{C_l}
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HPLC and LC-MS analysis

Ybt-Fe$^{3+}$ concentrations were measured using a ZORBAX Eclipse XDB-C18 column connected to an Agilent 1120 HPLC system equipped with a diode array detector. Solvent A was pure water and solvent B was acetonitrile, and samples (25 μL) were analyzed at a flow rate of 1 mL/min using a linear gradient of solvent B over 20 min. The Ybt-Fe$^{3+}$ product was detected at 385 nm and peak area quantification was conducted compared to a standard calibration curve of purified product and the known extinction coefficient for Ybt-Fe$^{3+}$ (ε = 2,884)$^{15}$. Percent purity values in Fig. 2c were calculated by dividing the Ybt-Fe$^{3+}$ peak area by total peak area assessed at 385 nm. Purified product was achieved using an Agilent 1200 preparatory HPLC system equipped with a Waters C18, 5 μM, 300 Å, 150 × 3.9 mm ID column. In this setting, a flow rate of 1 mL/min was used together with a 10–100% acetonitrile (balance water) gradient over 15 min. LC-MS analysis was performed using an API 3000 Triple Quad MS with a Turbo Ion Spray source (PE Sciex) coupled with a Shimadzu Prominence LC system. All MS analyses were conducted in positive ion mode, and chromatography was performed on a Waters X Terra C18 column (5 μm, 250 × 2.1 mm ID). After an injection of 4 μL of sample, conditions for LC-MS were 10–100% acetonitrile (balance water) gradient over 15 min at a flow rate of 0.2 mL/min. LC-MS-based compound quantification was completed using a standard curve generated from purified Ybt-Fe$^{3+}$.

Statistical analysis

Error bars represent standard deviation values generated from three independent experiments.

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REFERENCES