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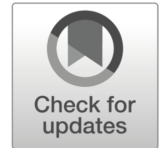
BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING



Expression and secretion of active *Moringa oleifera* coagulant protein in *Bacillus subtilis*

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Abstract

Cationic polypeptide proteins found in the seeds of the tropical plant *Moringa oleifera* have coagulation efficiencies similar to aluminum and ferric sulfates without their recalcitrant nature. Although these proteins possess great potential to augment or replace traditional coagulants in water treatment, harvesting active protein from seeds is laborious and not cost-effective. Here, we describe an alternative method to express and secrete active *M. oleifera* coagulant protein (MO) in *Bacillus subtilis*. A plasmid library containing the MO gene and 173 different types of secretory signal peptides was created and cloned into *B. subtilis* strain RIK1285. Fourteen of 440 clones screened were capable of secreting MO with yields ranging from 55 to 122 mg/L of growth medium. The coagulant activity of the highest MO secreting clone was evaluated when grown on Luria broth, and cell-free medium from the culture was shown to reduce turbidity in a buffered kaolin suspension by approximately 90% compared with controls without the MO gene. The clone was also capable of secreting active MO when grown on a defined synthetic wastewater supplemented with 0.5% tryptone. Cell-free medium from the strain harboring the MO gene demonstrated more than a 2-fold reduction in turbidity compared with controls. Additionally, no significant amount of MO was observed without the addition of the synthetic wastewater, suggesting that it served as a source of nutrients for the effective expression and translocation of MO into the medium.

Keywords *Bacillus subtilis* · Biocoagulant · Coagulant protein · *Moringa oleifera* · Water treatment · Water turbidity

Introduction

The cost to meet global clean water requirements on an annual basis could rise to several trillion dollars in the near future depending on scarcity and overall water quality (Cazcarro et al. 2016). This is especially problematic in countries with limited resources, where it is estimated nearly two-thirds of the world's population suffers from a lack of clean water (United Nations World Water Assessment Programme 2017). In order for water to become potable, it must first be treated to remove contaminants that can be particulate, chemical, and/or biological in nature. Similarly, wastewater must be treated before it is discharged into the environment, which is becoming a major concern due to rising costs associated with the removal of various classes of toxic synthetic compounds (Rajasulochana and Preethy 2016). The ever-increasing global demand for clean water is the driving force to develop new technologies to treat both water supplies and wastewater more economically (Cazcarro et al. 2016).

In conventional water treatment, the first step is to remove suspended particles using coagulation-flocculation, which is typically followed by sedimentation, filtration, and chemical treatment. Inorganic coagulants such as ferric and aluminum salts and charged organic polymers such as polyacrylamide derivatives and polyethylene imine are used extensively in typical water treatment systems for coagulation and flocculation. Although these coagulants are very effective at helping to reduce the particulate and organic loads, they are also expensive to produce, transport, and store. They can also lead to secondary water issues in the form of residual iron and aluminum species, in addition to toxic synthetic polymers (Ramavandi 2014). Moreover, sludge produced by these coagulation processes is of little secondary value due to its recalcitrant nature, which can lead to additional disposal costs.

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Biocoagulants such as proteins and polysaccharides are quickly garnering attention as potential alternatives to conventional coagulants as they are biodegradable and generally regarded as safe (GRAS). Typically, they produce significantly less sludge compared with traditional coagulants. In addition, they tend to have less influence on pH, alkalinity, and conductivity, which can affect downstream treatment processes thus lessening the need for supplemental treatment (Ndabigengesere et al. 1995; Broin et al. 2002; Narasiah et al. 2002).

Many naturally occurring coagulants capable of water clarification have been described in the literature, most of which are derived from various plant species (Yongabi 2010; Kansal and Kumari 2014). *Moringa oleifera* is one such species that has been shown to harbor proteins within the seed that have high coagulant activity (Kansal and Kumari 2014). *Moringa oleifera* is a drought resistant tree belonging to the family Moringaceae, which has been cultivated in developing countries for use as a nutritional supplement and food source, as well as for crude water purification for human consumption (Muyibi and Evison 1995, Ramavandi 2014, Ravani et al. 2017). Acting as a natural coagulant, seed extracts from *M. oleifera* have been shown to dramatically improve water quality by reducing particulate content comparable with aluminum sulfate, the most commonly used inorganic coagulant (Poumaye et al. 2012; De Souza et al., 2017). Moreover, purified *M. oleifera* seed proteins have been shown to clarify turbid water at doses < 0.5 mg/L, making them promising alternatives to traditional coagulants (Ali et al. 2010).

While coagulants found in *M. oleifera* seeds have been shown to be effective clarifiers, the growth, harvesting, and extraction process of the seeds is generally laborious and costly, making them impractical for large-scale treatment operations (Okuda et al. 2001; Ali et al. 2010). It can take years for *M. oleifera* to produce high yields of seed pods, and the species proliferates under tropical conditions making it unsuitable for growth in many climates (Ramachandran et al. 1980; Olsona 2017). To overcome these limitations, the expression of plant-based coagulant proteins has been explored in bacteria. Broin et al. (2002) successfully cloned and expressed *M. oleifera* coagulant protein in *Escherichia coli*, which resulted in a recombinant protein capable of flocculating both clays and bacteria. Suarez et al. (2002) also successfully expressed an active *M. oleifera* seed protein in *E. coli* and not only demonstrated the recombinant protein was capable of flocculating suspended mineral particles but also reported the protein had perceptible antimicrobial activity. Furthermore, large-scale recombinant protein production has been demonstrated, which resulted in yields of roughly 42 mg/L of active protein and was within the concentration required for industrial use (Pavankumar et al. 2014).

Although these studies demonstrated effective production of active *M. oleifera* coagulant protein, limitations existed in that *E. coli* was used as the host for protein expression. Recombinant protein expression within *E. coli* requires expensive inducers, and more importantly, the recombinant proteins have to be extracted from the cell cytoplasm before use. In addition, a rich growth medium is generally required to support the taxing anabolic expression process. Although effective, these methods add cost and complexity to the production process and introduce logistical burdens making them difficult to implement.

The use of *Bacillus* sp. for heterologous protein expression is becoming more common due to their designation as GRAS organisms, ease of genetic modification, low nutritional requirements, large-scale growth, and production of extremely high yields of recombinant protein (Schumann 2007). In fact, studies have demonstrated the production of active recombinant proteins in *B. subtilis* at concentrations equal to 25 g/L (Schallmey et al. 2004). In addition to high production rates, another distinct advantage of using a *Bacillus* expression system is the unique ability of the genus to translocate large amounts of protein into the surrounding growth media, making extraction unnecessary.

Secreted *B. subtilis* proteins are synthesized within the cytoplasm, translocated extracellularly, and released into the surrounding growth media as a means to digest surrounding organic matter. This process is mediated by signal peptides (SP), which are short N-terminal amino acid sequences that act as identifiers for translocation of the attached protein out of the cell (Tjalsma et al. 2000). After the protein passes through the cell wall, the SP is removed by signal peptidases, which release the protein into the surrounding medium. The most common means for this process is via the general secretory (Sec) pathway, which consists of an elaborate array of recognition factors, translocases, signal peptidases, and chaperones (Tjalsma et al. 2000; Fu et al. 2007). In this study, we take advantage of the Sec pathway within *B. subtilis* as a means to secrete active *M. oleifera* coagulant protein when grown on both rich and nutrient limited substrates.

Materials and methods

The *M. oleifera* coagulant protein gene sequence used in this study was obtained from the National Center for Biotechnology Information (NCBI) database (accession number AJ345072) and enhanced for expression in bacteria using OptimumGene codon optimization algorithm (GenScript, Piscataway, NJ). The resulting sequence and protein was abbreviated as MO in this study. The optimized gene was synthesized using de novo oligonucleotide chemistries, cloned into pUC57, and then subcloned into a pET-15b expression vector using the *Bam*HI restriction sites. Both vectors containing the codon optimized gene were propagated in chemically competent *E. coli* and purified using a commercially available plasmid mini-preparation kit. The coagulant protein gene

sequences in both vectors were confirmed by DNA sequencing using standard BigDye chemistries.

The MO protein was initially expressed using the well-characterized lac operon in a non-secreting *E. coli* strain. Chemically competent One Shot BL21(DE3) (Invitrogen, Carlsbad, CA) cells were transformed and induced according to the manufacturer's protocol, with the exception that a 0.1% inoculum was used to start cultures for coagulant gene expression. Cultures were grown to an optical density at 600 nm (OD_{600}) of roughly 0.3 and then induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM for 2 h. The cultures were harvested by centrifugation and proteins were extracted with a combination of lysis buffer (100 mM Tris pH 8, 500 mM NaCl, 10% glycerol, 25 mM imidazole, 1 mM PMSF, and 40 mM DTT) and sonication. The total lysate was then centrifuged and the supernatant was dialyzed against 50 mM Na_2HPO_4, pH 7.0, and 150 mM NaCl overnight at 4 °C. The sample was then applied to a Ni-nitrilotriacetic acid (NTA) agarose column (Qiagen, Valencia, CA) and the 6xHis-tagged fusion protein was affinity purified by eluting with 250 mM imidazole and dialyzed as previously described.

Purified recombinant protein was quantified using a Qubit Protein Assay (Invitrogen) and subsequently analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 4–20% polyacrylamide gel. A western blot was performed to confirm the presence of 6xHis-tagged protein by transferring proteins onto a 0.2-μm polyvinylidene difluoride (PVDF) membrane using a Bio-Rad Trans-Blot Turbo Transfer System per the manufacturer's recommendations (Hercules, CA). Fusion proteins were probed using a Bio-Rad Immun-Blot Assay Kit (cat. #1706461) with a 1:1000 dilution of anti-6xHis-tag IgG primary antibody (cat. #6200203) per the manufacturer's protocol. *Bacillus* lytic enzyme PlyPH tagged with N and C-terminus 6xHis-tags served as a positive control for the protein immunoblot (Yoong et al. 2006).

For MO seed extract control studies, *M. oleifera* seeds were obtained from Moringa Farms (Sherman Oaks, CA) and extracted by crushing whole seeds with a pestle and mortar and mixing 1:100 (w/v) in deionized water. The suspension was shaken vigorously for 5 min and then allowed to settle. After 5 min, the supernatant was filtered through a tissue to remove remaining debris. Inorganic coagulants were prepared to a 10 mg/mL stock solution in water, with the exception of ferric chloride which was prepared to a 10 mg/mL in 0.1 mM HCl.

Coagulation activities of both biocoagulants and traditional inorganic coagulants were determined by measuring clarification of a synthetic turbid water suspension consisting of 1 g/L kaolin in 1 mM $NaHCO_3$ and 1 mM NaCl, which exhibited a turbidity of 250 nephelometric turbidity units (NTU) and an optical density at 500 nm (OD_{500}) of approximately 0.35. Various concentrations of coagulants were added to 10-mL glass vials containing the synthetic turbid water suspension and stirred at 200 rpm for 1 min and at 15 rpm for 20 min. Stirring was then stopped and the suspension was allowed to settle for 1 h before measuring the turbidity using a LaMotte LTC3000 turbidity meter (Chestertown, MD). To conserve recombinant protein, a small-scale coagulation assay was also developed based on the method used by Ghebremichael et al. (2005). Specifically, coagulation of the buffered kaolin suspension describe above was carried out in 4-mL polyethylene cuvettes, where various concentrations of coagulants were added to a total volume of approximately 2.5 mL. Reactions were mixed end-over-end at 100 rpm in a rotating mixer for 1 min, and then speed was decreased to 10 rpm for 20 min after which the samples were allowed to settle for predetermined time points at 24 °C. After settling, OD_{500} was read using a UV-Vis spectrophotometer. Activity for both assays was measured as a function of coagulant concentration and reduction in NTU or OD_{500}. Controls without coagulant or cell-free medium were included.

After confirmation of active MO expressed in *E. coli*, the same optimized gene was cloned and expressed in *B. subtilis* strain RIK1285 (Takara Bio, Mountain View, CA). The gene was polymerase chain reaction (PCR) amplified from the pUC57 construct using primers designed with the In-Fusion Cloning Primer Design Tool (Takara Bio) to meet the downstream requirements for integration into pBE-S DNA expression vector at *Nde*I and *Xba*I restriction sites. The resulting PCR product was purified by gel electrophoresis and cloned into the multiple cloning sites (MCS) of pBE-S DNA, transformed into chemically competent *E. coli*, and purified using a commercially available plasmid mini-preparation kit. The resulting construct was named pBE-S-MO. Correct gene sequence and orientation within the plasmid were confirmed via DNA sequencing.

To create the random SP clone library, pBE-S-MO was digested with *Mlu*I and *Eag*I and gel purified. The linearized DNA was used to randomly ligate and transform 173 different *B. subtilis* SP (Takara Bio) into chemically competent *E. coli*. Roughly 2000 antibiotic resistant transformants were pooled and pBE-S-MO-harboring random SP were extracted, purified, and transformed into chemically competent *B. subtilis* strain RIK1285. Cells were plated on selective medium, and 440 random clones were propagated and screened for the presence of heterologous protein within the cell-free medium using a 96-well Cell Biolabs His-Tag Protein ELISA Kit (San Diego, CA) per the manufacturer's instructions. Absorbance at 450 nm was read using a microplate reader, and concentrations of tagged protein were quantitated using a standard curve generated with known concentrations of 6xHis-tagged Rhotekin (MW 10 kDa). Concentrations were standardized against cell-free medium from the wild type RIK1285. Signal peptides of clones that secreted 6xHis-tagged protein into the medium were identified by plasmid extraction, DNA sequencing, and alignment to known *Bacillus* SP sequences. Nomenclature for MO secreting clones was given as pBE-S-SP-MO.

After SP identification, physical traits of each SP were determined and analysis of variance (ANOVA) and linear regression analysis were performed to identify any statistically relevant correlations between the SP characteristics identified and amount of secreted protein. SignalP 4.1, with a cutoff of 0.450, was used to calculate D-scores (Petersen et al. 2011). Net charges were determined by Protein Calculator v3.4 (http://protcalc.sourceforge.net/) at neutral pH. Isoelectric point (pI) and grand average of hydropathicity (GRAVY) were calculated using ProtParam (Gasteiger et al. 2005). Peptide hydrophobicity was determined by dividing the total number of hydrophobic amino acids by the total number of residues.

Sequences of truncated SP were determined by removing N-terminus amino acids directly downstream from the translational start site until the D-scores were < 0.450. The resulting truncated SP gene sequences were then synthesized using de novo oligonucleotide chemistries and cloned into pBE-S-MO as described previously.

Cell-free medium from 100 mL cultures of pBE-S-YngK-MO (accession number MN082146) and pBE-S-AprE (no MO gene) grown with Luria broth were studied for their ability to clarify synthetic turbid water, as described earlier. After 48 h at 37 °C with shaking (180 rpm), the cells were removed from the culture by centrifugation and the resulting cell-free medium was concentrated by spin filtration to 25× and 40× of the eluent volume. Protein concentrations were determined using method of Bradford (Bradford 1976) and activity was evaluated using the small-scale coagulation assay described above. To confirm the presence of recombinant MO, concentrated cell-free media was purified by affinity chromatography and immunoblotted as previously described. Controls without coagulant were also performed.

Growth and coagulation activity of cell-free medium was also evaluated from cells grown in a mixture of synthetic black water amended with various concentrations of casein, tryptone, and milk powder (0.25—1% w/v). The defined synthetic black water was adapted from Nopens et al. (2001) and consisted of the following in diH_2O (mg/L): beef extract, 360; milk powder, 360; urea, 180; NH_4Cl, 150; yeast extract, 480; humic acid, 60; K_2HPO_4, 422; NaCl, 350; standard test dust, 10; and common top soil, 6. This formulation resulted in a solution having a target chemical oxygen demand (COD) of roughly 1200 mg/L. The synthetic black water was steam sterilized at 120 °C for 20 min prior to use. Five milliliter cultures were grown for 48 h as previously described in the defined media, cells were removed by centrifugation, and 6xHis-tagged recombinant proteins were detected in the cell-free media using ELISA as described previously. Colony forming units (CFU) were used to estimate biomass by reconstituting pellets in sterile water, serially diluting, and plating onto LB.

Results

Evaluation of prokaryotic gene optimization of MO

To evaluate activity of the codon optimized MO gene sequence, it was initially expressed in *E. coli* due to the relative ease of transformation and predictable IPTG induction. Expression and subsequent NTA purification of the *E. coli* cell lysate resulted in a band having approximately the same molecular weight as the 6xHis-tagged MO, 9.6 kDa (Fig. 1a) and the western blot confirmed the presence of the epitope tag (Fig. 1b). The protein was roughly 84% pure based on SDS-PAGE analysis and a total yield of roughly 0.6 mg/L was achieved. Coagulant activity of the *E. coli*–expressed MO was evaluated as the ability to reduce NTU in a kaolin suspension. No effect on clarification was observed with the purified MO until a dosing concentration > 20 mg/L was achieved (data not shown). At 30 mg/L, a turbidity reduction of approximately 80% was observed compared with unamended controls, and additional dosing up to 60 mg/L showed no significant increase in clarification, consistent with a charge neutralization mechanism. When compared with traditional inorganic coagulants, the recombinant protein was capable of nearly equivalent clarification (Fig. 1c). Optimal dosing concentrations within systems were defined as the concentrations that accomplished the maximum degree of clarification per milligram added. These concentrations were as found to be as follows: 200 mg/L protein from seed extract; 30 mg/L purified MO; 20 mg/L aluminum sulfate; and 50 mg/L ferric sulfate. Dosing with *M. oleifera* seed extract and purified MO resulted in reductions in turbidity of 84 ± 5% and 76 ± 1%, respectively. The addition of aluminum and ferric sulfates demonstrated a reduction in turbidity of 84 ± 11% and 82 ± 5%, respectively. Only a 1 ± 6% reduction in turbidity was observed in controls that were not amended with a coagulant.

MO expression and secretion in *B. subtilis*

After successfully demonstrating coagulation activity of the optimized MO cloned and expressed in *E. coli*, the same construct was subcloned into pBE-S DNA and expressed in *B. subtilis*. Of the 440 clones screened, 14 were capable of producing epitope-tagged proteins at concentrations ranging from 55 to 122 μg/mL (Fig. 2). The SP were identified and YngK was found to secrete 122 ± 6.0 μg/mL of tagged protein into the surrounding medium. The lowest secretor was YusW at 55 ± 5.0 μg/mL protein. No statistical differentiation was observed between YngK and the next 2 highest epitope secreting clones YxiT and PhrG, which had extracellular 6xHis-tag concentrations of 115 ± 9.1 μg/mL and 114 ± 12.5 μg/mL, respectively. AprE, which is controlled by

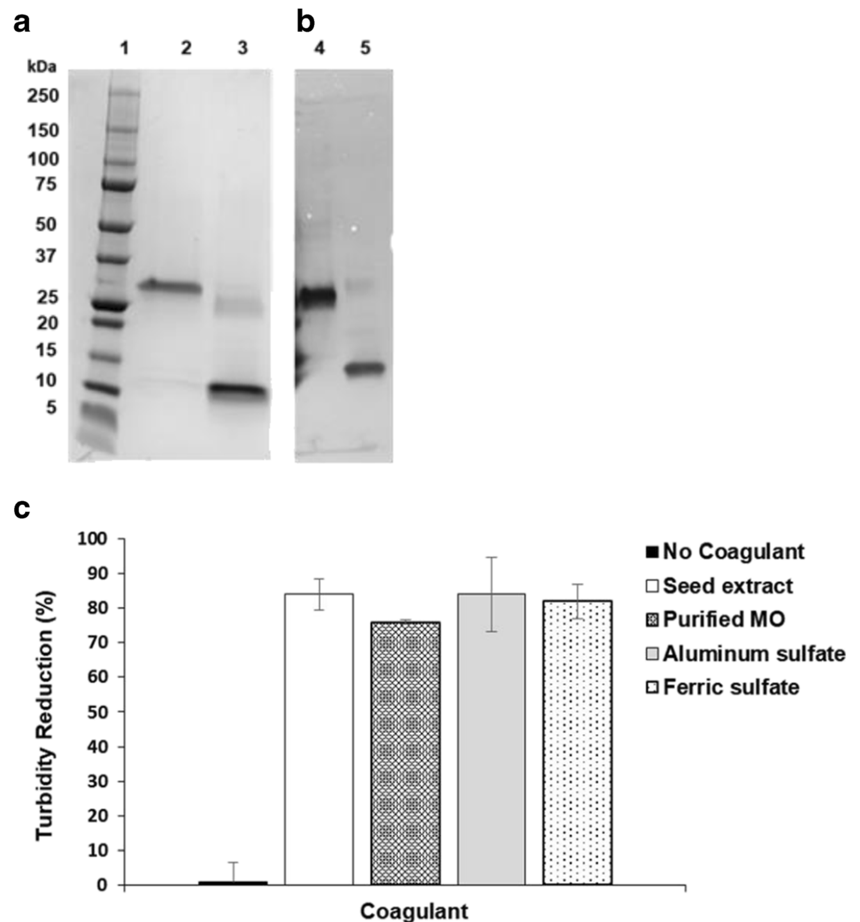


Fig. 1 Purification and coagulant activity of recombinant MO expressed in *E. coli*. **a** SDS-PAGE analysis of the NTA purification of cell-free extracts: lanes 1, molecular weight marker; 2, 6xHis-tagged positive control (PlyPH); and 3, purified MO. **b** Western blot of purified MO using 6xHis-antibody probe: lanes 4, PlyPH; and 5, purified MO. **c** Comparison of seed extract and purified MO to traditional coagulants at optimal dosing concentrations. Error bars represent ± SD of 3 experimental replicates

the native *B. subtilis*–derived subtilisin promoter (aprE promoter) secreted 74 ± 5.4 μg/mL of protein. Truncated SP YngK (tr-Yngk) and AprE (tr-AprE) were found to have significantly lower concentrations of tagged protein in the medium compared with the native SP, 8 ± 1.9 μg/mL and 11 ± 0.8 μg/mL, respectively. No tag was detected in the uninoculated controls (LB). Based on the ELISA screening, the clone containing MO fused to YngK SP (pBE-S-YngK-MO) was chosen for further study.

Correlations between the amount of protein secreted and 6 physical characteristics of the 14 SP identified were assessed (Table 1). All the SP identified had D-scores > 0.450 (0.460 to 0.893) and ranged in the number of amino acids residues from 21 to 41. Both truncated SP, as designed, had D-scores < 0.450. All native SP had a net positive charge and pI > 9.0 except YusW. The aliphatic index ranged from 94.2 to 139.5, GRAVY from 0.513 to 1.567, and hydrophobicity from 52 to 74%. Linear regression analysis of the peptide

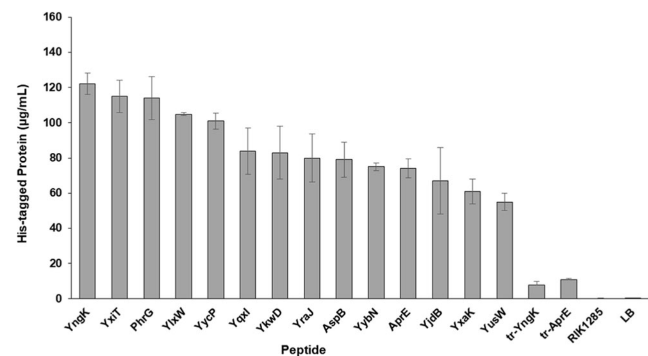


Fig. 2 Identification of SP and ELISA quantitation of 6xHis-tag protein in cell-free medium of clones grown with LB. Error bars represent ± SD of 3 replicates

Table 1 Characteristics of signal peptides identified by ELISA screening. The SP are listed in descending order from high to low concentration in the cell-free medium

SP	Amino acid sequence	D-score[a]	Charge[b]	pI[c]	Aliphatic index[c]	GRAVY[c]	Hydrophobicity (%)[d]
YngK	MKVCQKSIVRFLVSLIIGTFVISVPFMANA	0.716	+ 3	10.1	133.0	1.350	63
YxiT	MKWNNMLKAAGIAVLLFSVFAYAAPSLKAVQA	0.618	+ 3	10.0	113.1	0.878	69
PhrG	MKRFLIGAGVAAVILSGWFIA	0.464	+ 2	11.0	139.5	1.567	71
YlxW	MRGKSAVLLSLIMLIAGFLISFSFQMTKENNKSAA	0.519	+ 3	10.3	108.9	0.637	54
YycP	MKKWMITIAMLILAGIALFVFISPLKS	0.512	+ 3	10.3	151.9	1.544	74
YqxI	MFKKLLLATSALTFSLSLVLPLDGHAKA	0.668	+ 2	9.7	136.1	0.896	61
YkwD	MKKAFILSAAAAVGLFTFGGVQQASA	0.775	+ 2	10.0	94.2	0.965	62
YraJ	MTLTKLKMLSMLTVMIASLFIFSSQALA	0.741	+ 2	10.0	132.5	1.357	64
AspB	MKLAKRVSALTPSTTLAITAKA	0.603	+ 4	11.3	106.8	0.400	55
YybN	MNKFLKSNFRFLLAAALGISLLASSNFIKA	0.657	+ 4	11.3	120.7	0.787	60
AprE	MRSKKLWISLLFALTLIFTMAFSNMSVQA	0.692	+ 3	11.1	114.5	0.928	62
YjdB	MNFKKTVVSALSISALALSVSGVASA	0.893	+ 2	10.0	123.9	1.123	58
YxaK	MVKSFRMKALIAGAAVAAAVSAGAVSDVPAAKVLQPTAAYA	0.577	+ 3	10.0	105.1	0.915	68
YusW	MHLIRAAGAVCLAVVLIAGCRFNEDQHQAEG	0.460	− 1	6.5	110.3	0.513	52
tr-YngK	MIIGTFVISVPFMANA	0.431	0	5.5	121.9	1.744	69
tr-AprE	MLIFTMAFSNMSAQA	0.411	0	5.5	72.0	1.047	67

[a] Signal peptide (SP) D-scores were calculated using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/)

[b] Net charge calculated by Protein Calculator v3.4 (http://protcalc.sourceforge.net/) at neutral pH

[c] Values were calculated using ProParam (https://web.expasy.org/protparam/): isoelectric point (pI); grand average of hydropathicity (GRAVY)

[d] Hydrophobicity was calculated by dividing the total number of hydrophobic amino acids by the total number of residues

characteristics produced *p* values all in excess of 0.05 and R^2 values of < 0.3. Stepwise variable selection resulted in no variables being selected. Furthermore, ANOVA showed no positive results in the analysis.

When grown on LB, pBE-S-YngK-MO exhibited an initial increase in exogenous protein concentration when compared with untransformed RIK1285 and was seemingly capable of precipitating cells out of solution (Fig. 3). After a 24-h incubation period, the strain containing the MO gene produced 2-fold more exogenous protein than the wild type strain (Fig. 3a). However, concentrations slowly decreased over a 72-h incubation period in the culture containing pBE-S-YngK-MO, whereas in the RIK1285 containing culture, protein steadily increased. After 72 h, the cell-free medium protein concentration in the untransformed culture was nearly equal to the strain harboring the MO gene after 24-h incubation. However, the total protein in the cell-free medium in the transformed strain decreased nearly 2-fold after an additional 48-h incubation. Furthermore, when the culture was allowed to rest without shaking, a 70 to 80% reduction in turbidity in the MO gene containing culture was observed, and flocked cells could clearly be seen with the naked eye (Fig. 3b, c).

Cell-free media of pBE-S-AprE (no MO) and pBE-S-YngK-MO grown on LB were then tested for their ability to clarify water. The total concentration of protein added to the assays was 15 μg/mL. Because this dose was half of the optimal dosing identified previously (Fig. 1c), a longer settling period was required to infer activity. After a 24-h settling period, the media from pBE-S-YngK-MO was shown to reduce turbidity by roughly 90%, and pBE-S-AprE was not found to reduce turbidity relative to the control, which did not contain cell-free medium (Fig. 4a). Turbidity reduction in the culture containing the MO was seen to be significantly greater from the control after 10 h and continued to increase over the course of the experiment. Essentially no variance in turbidity was observed between the uninoculated control and pBE-S-AprE. After 24 h, the cell-free medium from pBE-S-YngK-MO showed a 50% greater reduction in turbidity compared with the control and pBE-S-AprE, which was clearly visible by the naked eye (Fig. 4b).

To confirm the presence of epitope tagged heterologous protein within the cell-free medium of pBE-S-YngK-MO, immunoblot analysis was performed on an affinity chromatography purified sample. The purified fraction was analyzed by SDS-PAGE, and while no band was observed in pBE-S-AprE inoculated culture, a band was clearly visible in pBE-S-YngK-MO inoculated culture. This band had an approximate molecular weight of 8.4 kDa, the expected weight of MO without YngK (Fig. 5a). Proteins within the gel were transferred to a PVDF membrane and probed for the presence of a 6xHis-tag

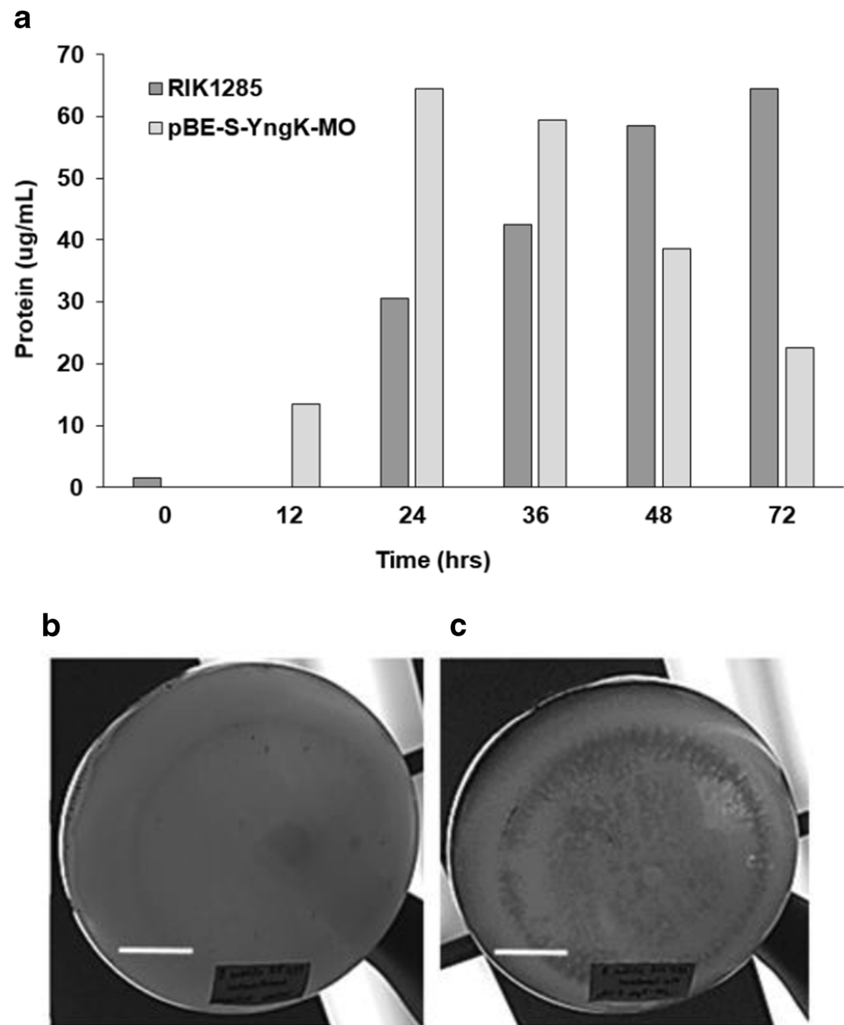


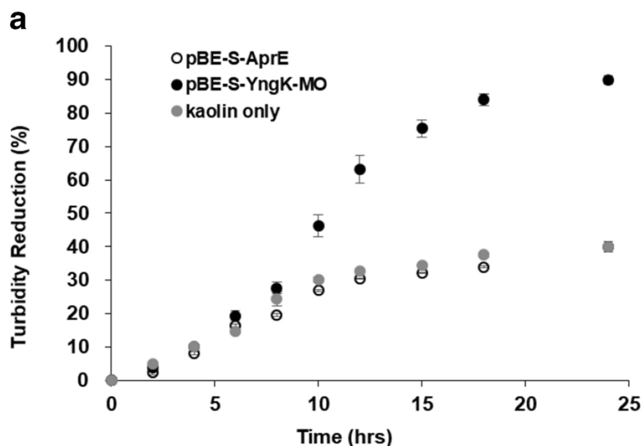
Fig. 3 Exogenous protein concentrations and visible cell flocculation in 100 mL cultures of pBE-S-YngK-MO and RIK1285 grown in LB. **a** Time course of protein concentrations in cell-free medium over a 3-day period. **b** Visual inspection of resting cultures of RIK1285 after 72-h incubation viewed from the flask bottom. **c** Visual inspection of resting cultures of pBE-S-YngK-MO after 72-h incubation viewed from the flask bottom. The scale bars represent 2 cm

via immunoblotting. Western blotting with anti-6xHis-tag IgG primary antibody confirmed the presence of the 6xHis within the purified band having the approximate calculated molecular weight as the target recombinant coagulant protein (Fig. 5b). No affinity was detected elsewhere, including the untransformed controls.

MO expression and secretion in *B. subtilis* grown in synthetic wastewater

To determine the ability of pBE-S-YngK-MO to utilize various substrates (other than LB) to secrete recombinant protein, an ELISA array was performed. Mixtures of synthetic black water with various concentrations of casein, tryptone, and milk powder were evaluated for their ability to support the secretion of 6xHis-tagged MO (Fig. 6). Synthetic black water amended with 0.5% and 1% tryptone resulted in the greatest amount of tagged protein in the cell-free media at 57.6 ± 1.7 μg/mL and 65.0 ± 2.0 μg/mL, respectively. When grown on tryptone only (no black water), 5.9 ± 0.3 μg/mL of protein was secreted. The amount of tagged protein decreased from 26.8 ± 0.4 to 9.3 ± 0.7 μg/mL as the concentration of casein increased from 0.25 to 1% in the synthetic black water. The casein only culture produced 5.3 ± 2.1 μg/mL of tagged protein in the medium. When pBE-S-YngK-MO was grown with synthetic black water and milk protein, the extracellular 6xHis-tagged protein concentration increased from 3.2 ± 1.4 to 28.0 ± 2.0 μg/mL as the milk protein increased, and no tagged protein was detected in cell-free medium with the milk protein alone without the addition of black water.

No growth was observed in either of the uninoculated controls (Fig. 6). The highest viable biomass was observed in the cultures grown in black water supplemented with 0.5% tryptone having 3.09E+08 cells/mL. The biomass decreased from 1.38E+08 to 6.79E+07 cells/mL in the 1% tryptone cultures in the absence of black water. Cell numbers ranged from 1.74E+08 to 2.12E+08 cells/mL in the black water amended with casein and only 8.70E+05 in the cultures without black water. Viable cell numbers ranged from 2.88E+06 to 1.41E+08 cells/mL in the black water amended with milk powder and only 1.00E+06 in the cultures without black water.



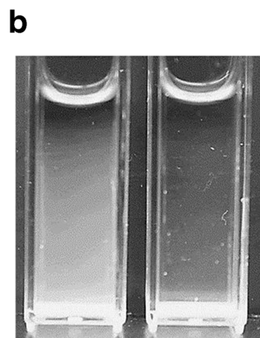


Fig. 4 Clarification of turbid water using cell-free medium from pBE-S-AprE and pBE-S-YngK-MO grown with LB. **a** Time course of turbidity reduction in a defined kaolin suspension. Error bars represent ± SD of 3 experimental replicates. **b** Clarification of kaolin suspension after 24 h: left cuvette, cell-free medium from pBE-S-AprE; and right cuvette, cell-free medium from pBE-S-YngK-MO

Cell-free medium from pBE-S-YngK-MO grown on synthetic black water amended with 0.5% tryptone was capable of reducing turbidity more than 2-fold compared with controls over a 48-h settling period (Fig. 7a). Reactions not containing cell-free medium and reactions with cell-free medium from a MO deficient strain pBE-S-AprE had reductions in turbidity of 19 ± 10% and 22 ± 8%, respectively. However, the reduction in turbidity of reactions amended with cell-free medium from pBE-S-YngK-MO was 63 ± 1%. Furthermore, water clarification mediated by cell-free medium from the strain harboring the MO gene could clearly be seen by the naked eye (Fig. 7b).

Discussion

The use of *B. subtilis* for heterologous protein expression is advantageous because of their nonpathogenic nature, ability to grow well on relatively low-nutrient feedstocks, and ease of genetic modification. More importantly, unlike *E. coli*, they do not produce endotoxin and can secrete vast amounts of recombinant protein into the surrounding media. This significantly simplifies protein recovery by eliminating the need to extract them from the cell, thus making *B. subtilis* more commercially attractive. In fact, recombinant proteins expressed in a *Bacillus* host have been routinely shown to secrete in gram-per-liter quantities into the surrounding media under commercial production (Schallmey et al. 2004). However, heterologous proteins are known to be problematic and are generally expressed in much lower concentrations than homologous proteins (Li et al. 2004; van Dijl and Hecker 2013). This is likely due to several key factors including the SP that target and guide them to the extracellular environment (Tjalsma et al. 2000; Brockmeier et al. 2006; Nijland and Kuipers 2008; Pohl and Harwood 2010). Signal peptides are short N-terminal amino acid sequences that act as targets for translocation machinery and transportation across the cytoplasmic membrane. Prokaryotic SP upstream of the protein to be secreted are on average 30 amino acids long and are composed of three regions: a positively charged N domain, a hydrophobic core region, and a hydrophilic peptidase recognition site

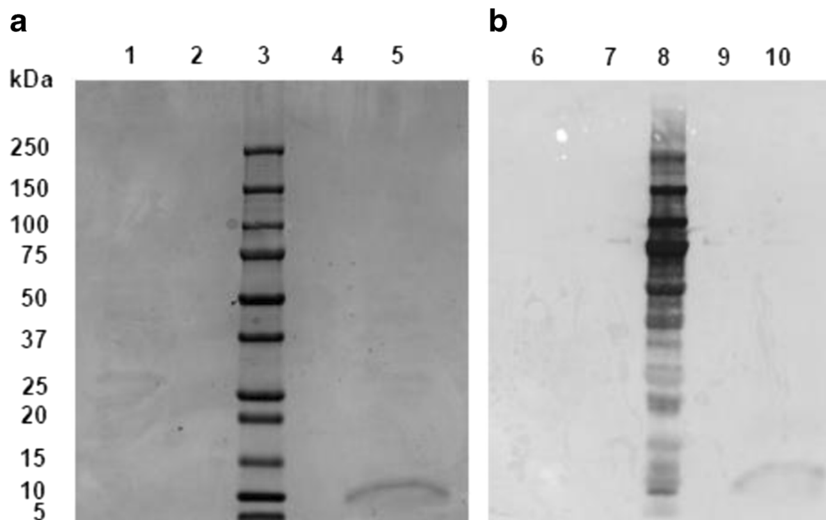


Fig. 5 Purification of recombinant MO secreted from *B. subtilis*. **a** SDS-PAGE analysis of NTA-purified cell-free medium: lanes 1, pBE-S-AprE; 2, blank; 3, molecular weight markers; 4, blank; and 5, purified MO. **b** Western blot of purified MO using 6xHis-antibody probe: lanes 6, pBE-S-AprE; 7, blank; 8, molecular weight markers; 9, blank; and 10, purified MO

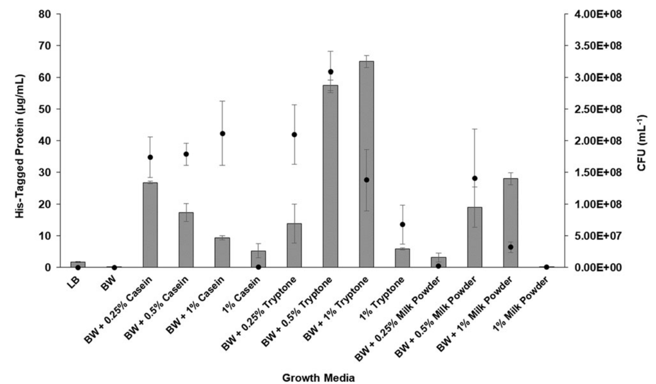


Fig. 6 Cell-free 6xHis-tag concentrations and colony forming units (CFU) of pBE-S-YngK-MO grown with synthetic black water (BW) and various nutrient sources. Bars, epitope tagged protein; circles, CFU; percentages are given as w/v and error bars represent the ± SD of 3 replicates

(Tjalsma et al. 2000; Brockmeier et al. 2006). All three regions play a critical role in the translocation process and are known to be protein specific; that is, SP action can vary significantly with different proteins. Accurate in silico predictions are currently not available; therefore, the effectiveness of SP-mediated translocation must be evaluated empirically with the specific protein of interest (Hemmerich et al. 2016).

In this study, a novel combination of SP clone libraries and ELISA was used to rapidly screen 173 different types of SP for their ability to secrete active MO from *B. subtilis*. Before cloning, the OptimumGene codon optimization algorithm was used to optimize the native MO gene sequence in an effort to promote the highest possible level of expression in the prokaryotic hosts. The algorithm revealed the native gene contained several tandem rare codons, which could reduce the efficiency of translation or even disengage the translational machinery. The codon usage bias was also changed in an effort to increase the codon adaptation index (CAI) and mRNA half-life was increased by optimizing the GC content. In addition, possible stem-loop structures, which can adversely impact ribosomal binding and stability of mRNA, were removed. In all, approximately 70% of the eukaryotic gene sequence was changed by the optimization process.

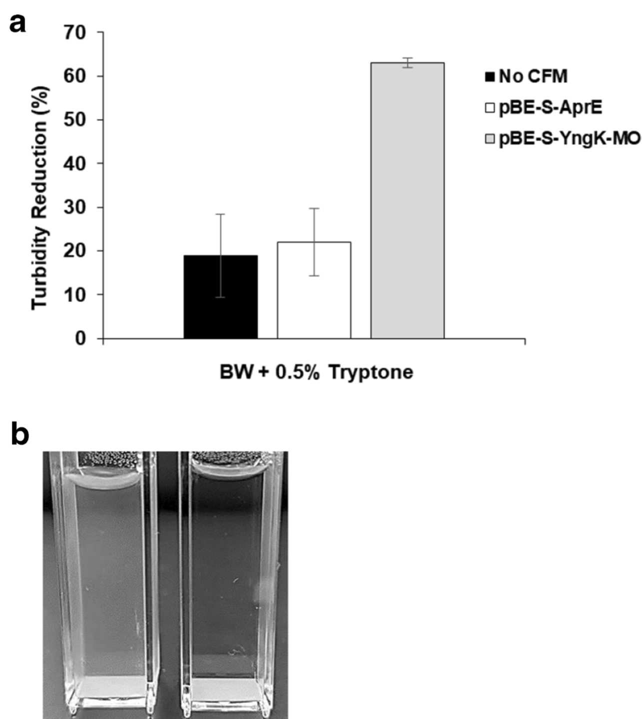


Fig. 7 Clarification of turbid water using cell-free medium (CFM) from pBE-S-YngK-MO grown with BW + 0.5% Tryptone. **a** Reduction in turbidity in a defined kaolin suspension. Error bars represent ± SD of 3 experimental replicates. **b** Clarification of kaolin suspension after 48 h: left cuvette, CFM from pBE-S-AprE; and right cuvette, cell-free medium from pBE-S-YngK-MO

After gene optimization, the resulting sequence was synthesized and cloned into pUC57 for archival purposes and then cloned into pET15b for initial expression experiments in *E. coli*. These experiments were performed to demonstrate the optimized sequence would result in active MO from a prokaryotic host and also served as a benchmark for subsequent experimentation with *B. subtilis*. Gel electrophoresis and western blotting confirmed the presence of recombinant MO and indicated a purity of roughly 84%. Additionally, the protein yield was found to be similar to other reports that used *E. coli* as a host (Pavankumar et al. 2014). The purified MO was shown to have similar water clarification capabilities as the extracted *M. oleifera* seeds, but at one-sixth the amount of protein. However, it should be noted that the protein concentration of the crude seed extract was calculated as the total amount of protein extracted from 1 g of seed into 100 mL of water. Several seed coagulant proteins have been identified in *M. oleifera*; hence, the total amount of coagulant protein in the crude extract relative to other exogenous proteins was unknown (Ali et al. 2010). Regardless, the purified MO would be advantageous in that it would reduce the organic load going into a treatment system by nearly 6-fold to accomplish equivalent clarification. Dosing rates of the inorganic coagulants were found to be similar to other published reports and served as a good benchmark to evaluate the recombinant MO (Sulistyo et al. 2012; Young, 2012; León-Luque et al. 2016). The inorganic coagulants exhibited no significant increase in clarification when compared with recombinant MO. Essentially no reduction in turbidity was observed in the control without coagulant, indicating minimal settling occurred due to the test conditions. These results clearly demonstrated

the codon optimized MO gene could be expressed in a prokaryote, and the epitope-tagged MO was capable of clarifying water at similar efficiencies as *M. oleifera* crude seed extract and traditional inorganic coagulants.

After successfully demonstrating the codon optimized MO could be expressed in *E. coli* and the resulting protein was active, it was then subcloned into the expression vector pBE-S DNA. Expression of this vector is under the control of *B. subtilis*–derived subtilisin aprE promoter, which controls production of subtilisin, a serine protease. The vector contained two origins of replication allowing it to be shuttled between *E. coli* and the target host *B. subtilis*. Construction and propagation of the SP library in *E. coli* were advantageous due to the relative ease of transformation as well as producing high plasmid copy numbers, which was necessary for creating the large plasmid library required to screen all the SP in this study. The expression vector has a MCS for the MO gene insert, in addition to *Mlu*I and *Eco52I* restriction sites upstream of the MCS that allows the insertion of random secretion SP. Restriction sites *Nde*I and *Xba*I were chosen as the cloning sites for MO to minimize extraneous sequence between SP and coagulant protein, which could negatively affect protein transport and coagulation activity. Additionally, RIK1285 is a protease deficient strain, which promotes protein persistence within the expression system (Murayama et al. 2004).

Methods to screen for secretion of active recombinant proteins typically involve activity assays. Although effective, these methodologies can be labor intensive, be time consuming, screen only limited numbers of clones at a time, and require relatively large amounts of target protein to obtain the desired reactivity and sensitivity (Tsuji et al. 2015; Ramos et al. 2016). In this study, we used ELISA in a 96-well format, which gave the ability to rapidly screen hundreds of clones simultaneously for secretion of the target recombinant protein. Of the 440 clones evaluated, 14 were found to secrete tagged proteins in sufficient concentrations to clarify turbid water based on the preliminary studies conducted with *E. coli*–derived MO. Specific SP characteristics were calculated from the 14 clones, and statistical analysis was performed to determine if any correlation existed between the amount of secreted protein and the peptide traits. All p values were found to be far > 0.05, signifying the model was not good for the data, R^2 values were all very low, and the variables explain only 31% of the variability in the model used. Stepwise variable selection resulted in no variables being selected and thus could not sufficiently explain the variance in the data to be included in a good-fit model. Furthermore, data typically has to be normal for ANOVA to be accurate, and non-normal data has higher instances of false positive results. However, there were no positive results in this analysis. Similar to linear regression analysis, this test suggests that none of the variables explain a significant amount of variance in the data and no correlation was found. Previous studies have suggested that effective SP protein combinations can only be determined empirically due to the complexity of the transport system and diversity of target proteins (Hemmerich et al. 2016). The statistical analysis supported this contention, in that no significant correlation could be found between the amount of secreted protein and any of the parameters listed in Table 1. This further illustrates the usefulness of an immunological approach for screening large libraries for efficient protein secreting clones.

Interestingly, when pBE-S-YngK-MO was grown on LB, extracellular protein concentrations peaked after 24 h incubation then gradually decreased roughly 2-fold over a 72-h period, whereas extracellular protein continued to increase in RIK1285 cultures (Fig. 3a). Cells could clearly be seen to floc and fall out of suspension in the strain harboring the MO gene, but no sedimentation was observed in the cells without MO (Fig. 3b, c). It was believed that due to high teichoic acid concentrations within *Bacillus* cell walls, it was likely the cationic MO was binding the cells and causing the aggregation (Brown et al. 2013). Others have demonstrated the ability of MO to aggregate bacterial cells (Broin et al. 2002; Ghebremichael et al. 2005). In addition, it was likely the highly positively charged MO was also coagulating and flocking endogenous proteins. Attempts were made to test this hypothesis by dissociating the protein from the organic matter by increasing both salt and pH within the cultures; however, no apparent increase in extracellular protein yield was observed. Thus, to optimize recombinant MO yields for large-scale water treatment, incubation conditions and/or modified host strains will likely need to be considered, which was beyond the scope of this study.

When pBE-S-YngK-MO was grown on LB, a significant reduction in turbidity of the model kaolin suspension was observed, which was easily seen by the naked eye (Fig. 4b). Purification and subsequent western blot probing of the protein confirmed expression and secretion of active MO. Moreover, the total amount of protein added to the coagulation reactions was roughly 15 μg/mL, which was less than the *E. coli*–derived MO for a comparable reduction in turbidity (Fig. 1). However, the coagulation process was found to be much slower. This was most likely due to the lack of purity in the samples used from the cell-free medium. As with the seed extracts, the actual MO concentration was likely < 15 μg/mL due to the presence of other endogenous proteins. Additionally, the variation in the location of 6xHis-tag and/or the linker length between MO and the tag may have affected the coagulation efficiencies. The *E. coli*–produced MO contained a C-terminus 6xHis-tag and a 15 amino acid linker between the tag and MO, whereas the *B. subtilis*–derived MO possessed only a 2 amino acid linker between MO and the N-terminus 6xHis-tag. This may also explain difficulties encountered when NTA purifying secreted MO, as linker length, position, and sequence can affect flexibility and resin binding

(Chen et al. 2013). Future experimentation with purified pBE-S-YngK-MO derived MO, as well as modified linkers or untagged MO, is warranted to accurately compare the two MO proteins and optimize coagulation efficiency. Nevertheless, the western blot and coagulation data clearly demonstrated production and secretion of active MO in *B. subtilis*.

In addition to secreting MO when grown on LB, strain pBE-S-YngK-MO was also found to grow and produce MO on a defined synthetic black water amended with various nutrients (Fig. 6). The greatest amount of protein was secreted when pBE-S-YngK-MO was grown on synthetic black water and tryptone. No growth was observed nor was MO secreted when the strain was grown on synthetic black water without supplementation, and significantly less recombinant protein was secreted when grown with casein or tryptone alone (no black water). Interestingly, a reduction in secreted protein was observed as the casein concentration increased, whereas protein secretion increased as concentrations of tryptone and milk powder increased. Casein and synthetic black water supported growth; thus, the reduction in MO may have been caused by binding to casein as was hypothesized when the strain was grown on LB and again deserves further study.

Medium consisting of synthetic black water and 0.5% tryptone supported the greatest combination of growth and MO secretion at the lowest supplemental nutrient dose and thus was selected to perform coagulation studies (Fig. 7). No statistical difference in turbidity reduction was observed between coagulation reactions without cell-free medium and cell-free medium from strain pBE-S-AprE, which did not contain the MO gene. However, more than a 2-fold decrease in turbidity was observed in reactions amended with cell-free medium from pBE-S-YngK-MO when compared with the controls. Additionally, differentiation between pBE-S-AprE and pBE-S-YngK-MO could easily be made by the naked eye. Collectively, these data confirmed active MO was expressed and secreted when grown on less than optimal medium and black water was required for effective secretion. This is beneficial in that the nutrients present in wastewater could support the in situ generation of coagulant using in line *B. subtilis* bioreactors for water treatment processes.

Although aluminum and ferric salts are very effective at removing particulates, they also form large amounts of sludge that typically contain high levels of harmful ionic iron and aluminum species (Edzwald 1993; Ramavandi 2014). Due to negative environmental and human health effects, sludge produced by these processes is strictly regulated by the US Environmental Protection Agency (EPA) (EPA 1993; Flaten 2001; Kluczka et al. 2017). Before sludge produced by inorganic coagulation processes can be released into the environment, it must first be properly treated to meet EPA requirements, which can increase operational cost up to 50% (Seiple et al. 2017). In contrast, biocoagulants are GRAS, produce significantly less sludge, have low or no toxicity, and are biodegradable (Narasiah et al. 2002). Therefore, sludge derived from biocoagulants would likely need no treatment before use or disposal, resulting in lower operational costs. Furthermore, utilizing engineered bacteria that can secrete active coagulant protein when grown on wastewater have the potential to significantly reduce operational cost by decreasing or eliminating the need for inorganic coagulants (Abdulazeez et al. 2016).

In this study, a novel methodology to produce active MO via the Sec pathway within *B. subtilis* was demonstrated. An immunological approach was taken to rapidly screen hundreds of clones for their ability to secrete MO. Strain pBE-S-YngK-MO was shown to not only secrete active MO when grown on a rich medium but also secrete active coagulant when grown on a nutrient limited substrate. Furthermore, the strain only produced significant amounts of MO when incubated with synthetic black water, suggesting it was serving as a nutrient source for efficient expression and secretion. Although further study is warranted, the strain has the potential to produce valuable biocoagulant from waste, which could be used to treat both water supplies and wastewater more economically.

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Compliance with ethical standards The authors of this article did not perform any studies with animals or human subjects.

Conflict of interest The authors declare that they have no conflicts of interest.

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