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Gavin C. Barnard
Dartmouth College

Jesse D. McCool
Dartmouth College

David W. Wood
Princeton University

Tillman U. Gerngross
Dartmouth College

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Integrated Recombinant Protein Expression and Purification Platform Based on *Ralstonia eutropha*

Gavin C. Barnard,[1] Jesse D. McCool,[1] David W. Wood,[2] and Tillman U. Gerngross[1]*

Thayer School of Engineering, Department of Biological Sciences, and Department of Chemistry, Dartmouth College, Hanover, New Hampshire, 03755,[1] and Department of Chemical Engineering, Princeton University, Princeton, New Jersey 08544[2]

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Protein purification of recombinant proteins constitutes a significant cost of biomanufacturing and various efforts have been directed at developing more efficient purification methods. We describe a protein purification scheme wherein *Ralstonia eutropha* is used to produce its own "affinity matrix," thereby eliminating the need for external chromatographic purification steps. This approach is based on the specific interaction of phasin proteins with granules of the intracellular polymer polyhydroxybutyrate (PHB). By creating in-frame fusions of phasins and green fluorescent protein (GFP) as a model protein, we demonstrated that GFP can be efficiently sequestered to the surface of PHB granules. In a second step, we generated a phasin-intein-GFP fusion, wherein the self-cleaving intein can be activated by the addition of thiols. This construct allowed for the controlled binding and release of essentially pure GFP in a single separation step. Finally, pure, active β-galactosidase was obtained in a single step using the above described method.

We have previously reported the development of a novel high cell density protein expression platform based on the gram-negative bacterium *Ralstonia eutropha* (22, 23). This system has been developed to overcome some of the shortcomings associated with recombinant protein expression in *Escherichia coli* (e.g., poor fermentation performance at high cell density, and inclusion body formation). Expression of organophosphohydrolase, an enzyme originally isolated from *Pseudomonas diminuta* (20) and prone to inclusion body formation in *Escherichia coli* (4, 28, 29), was demonstrated at high levels. Titers of active, soluble organophosphohydrolyase, in excess of 10 g/liter were obtained in high cell density fermentation (3), representing at least a 100-fold increase over those previously reported in *E. coli*.

While the successful expression of a recombinant protein is a necessary requirement, recovery and purification still remain a significant cost in recombinant protein production. We thus sought to integrate the existing *R. eutropha* protein expression platform with a protein purification strategy to simplify the expression and purification of recombinant proteins. This specific approach uses the natural ability of *R. eutropha* to produce a polymer known as polyhydroxybutyrate (PHB), which accumulates as insoluble granules within the cell. PHB is a member of the polyhydroxyalkanoate class of polymers, synthesized by many bacteria, as carbon storage compounds (2, 15, 16, 26, 30, 31, 32). Polyhydroxyalkanoates have received attention as biodegradable polymers and can be obtained by fermentation processes utilizing cheap, abundant renewable carbon sources (2, 24). Polyhydroxyalkanoates have been produced industrially by ZENECA Bioproducts (26) and Monsanto (10).

* Corresponding author. Mailing address: Thayer School of Engineering, Department of Biological Sciences, and Department of Chemistry, Dartmouth College, 8000 Cummings Hall, Hanover, NH 03755. Phone: (603) 646-3161. Fax: (603) 646-2277. E-mail: tillman.gerngross@dartmouth.edu.

PHB synthesis in *R. eutropha* has been the model system for studying polyhydroxyalkanoate biosynthesis in bacteria (10, 15, 16, 18, 26). The biogenesis of polyhydroxyalkanoate granules involves two distinct proteins, the polyhydroxyalkanoate synthase (PhaC) and phasins (PhaP). Phasins are low-molecular-weight proteins whose role in polyhydroxyalkanoate formation is not well understood (10, 11, 15, 16, 18, 24, 26, 30, 31, 32). Phasins accumulate during PHB synthesis, bind to PHB granules, and promote further PHB synthesis (32). It has been shown that *phaP* mutants form only one large PHB granule and that up-regulating the *phaP* gene increases the number of PHB granules while reducing their size (15, 26). Phasins accumulate at high levels in cells that are synthesizing PHB, and as much as 5% of total cellular protein can be PhaP (16). Phasins have high affinity for PHB granules and are the predominant protein present on the granule surface (24, 26).

In this study, we exploit the specific affinity between PhaP and the PHB granules for the purpose of purifying a recombinant model protein (GFP). In essence, this yields an affinity-based purification scheme wherein the cell synthesizes its own chromatography matrix and PhaP is used as the affinity tag to sequester a protein of interest to the PHB granule surface. The recombinant protein can then be recovered by cell disruption followed by a centrifugation step that separates the insoluble high-density polymer from other soluble cellular components. In an improved version, the protein of interest is linked to PhaP through an intein allowing its release by thiol induced cleavage (see Fig. 3).

Inteins are self-splicing proteins that occur as in-frame insertions in specific host proteins throughout nature, and have been adapted for use in recombinant protein expression and purification schemes (9, 27). Intein cleavage can be mediated by pH changes or the addition of thiols. The *Mxe* GyrA intein is a 198-amino-acid polypeptide, with N-terminal cleavage activity in the presence of thiols (25). By incorporating this intein into a PhaP-intein-GFP fusion we were able to show (i) the

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference or source
R. eutropha strains		
NCIMB 40124	Wild type; gentamicin resistant	National Collections of Industrial, Food & Marine Bacteria, Aberdeen, Scotland
G	pG introduced into *R. eutropha* wt	This study
PG	pPG introduced into *R. eutropha* wt	This study
PIG	pPIG introduced into *R. eutropha* wt	This study
GP	pGP introduced into *R. eutropha* wt	This study
GIP	pGIP introduced into *R. eutropha* wt	This study
PIL	pPIL introduced into *R. eutropha* wt	This study
E. coli strains		
TOP10	Host strain for plasmids derived from pCR2.1-TOPO	Invitrogen
S-17	Host strain for plasmids derived from pKNOCK-Cm	8, 17, 21
Plasmids		
pG	*phaPp*::*gfp* transcriptional fusion introduced into pKNOCK-Cm; plasmid used to create strain *R. eutropha* G	This study
pPG	*phaP* ORF::*gfp* translational fusion introduced into pKNOCK-Cm; plasmid used to create strain *R. eutropha* PG	This study
pPIG	*phaP* ORF::*Mxe* GyrA intein::*gfp* translational fusion in pKNOCK-Cm; plasmid used to create strain *R. eutropha* PIG	This study
pGP	*phaPp*::*gfp*::*phaP* transcriptional fusion in pKNOCK-Cm; plasmid used to create strain *R. eutropha* GP	This study
pGIP	*phaPp*::*gfp*::*Mxe* GyrA intein::*phaP* transcriptional fusion in pKNOCK-Cm; plasmid used to create strain *R. eutropha* GIP	This study
pPIL	*phaP* ORF::*Mxe* GyrA intein::*lacZ* translational fusion in pKNOCK-Cm; plasmid used to create strain *R. eutropha* PIL	This study
pKNOCK-Cm	Suicide vector used for introducing plasmids into *R. eutropha* chromosome	1
pGY1a+	Vector containing *phaP*p::*gfpmut2* translational fusion	30
pTWIN1	Commercially available expression vector containing *Ssp* DnaB intein and *Mxe* GyrA intein	New England Biolabs
pUCPPCm	Plasmid vector containing *phaP* promoter	22
pGB27	pKNOCK containing the *phaP* promoter	This study
pGB73	*Mxe* GyrA intein introduced into pCR2.1-TOPO	This study
pGB76	*phaP* ORF introduced into pCR2.1-TOPO	This study
pGB80	*phaPp*::*gfp*::*phaP* transcriptional fusion in pKNOCK-Cm	This study
pGB82	*phaPp*::*gfp*::NEB *Mxe* GyrA intein::*phaP* transcriptional fusion in pKNOCK-Cm	This study
pGB85	*phaP*::*gfp* transcriptional fusion in pCR4-TOPO	This study
pGB91	*phaP*::*gfp* transcriptional fusion in pKNOCK-Cm	This study
pGB93	*phaP*::NEB *Mxe* GyrA intein::*gfp* transcriptional fusion in pKNOCK-Cm	This study
pGB96	*Peptide linker*::*phaP* transcriptional fusion in pCR2.1-TOPO	This study
pGB97	*phaP*::*peptide linker* transcriptional fusion in pCR2.1-TOPO	This study
pGB470	*lacZ* ORF fragment cloned introduced into pCR4Blunt-TOPO	This study

[a] wt, wild type.

expression of a PhaP-intein-GFP fusion protein, (ii) its sequestration to PHB granules, and (iii) the subsequent release of GFP from the PHB granule by treating the cell debris with dithiothreitol.

MATERIALS AND METHODS

Strains, plasmids and oligonucleotides. Strains and plasmids that were used in this study are listed in Table 1. Oligonucleotides used in this study are listed in Table 2. Standard procedures were used for the preparation and manipulation of DNA and for PCR. All PCR products were subcloned into pCR2.1-TOPO (Invitrogen), pCR4Blunt-TOPO (Invitrogen) or pCRII-Blunt-TOPO (Invitrogen) and sequence verified at the Molecular Biology Core Facility at Dartmouth College. Methods for introducing plasmids into the *R. eutropha* chromosome have previously been described (22, 23). In brief, all pKNOCK-Cm derived plasmids are introduced into *E. coli* S17 (17, 21) before being incorporated into the *R. eutropha* chromosome by simple biparental mating.

Growth media, antibiotics and cultivation conditions. *E. coli* strains were grown in Luria-Bertani (LB) medium (13). *R. eutropha* strains were grown in one of the following media depending on the application: LB medium or Lee medium (20 g/liter glucose, 3 g/liter $Na_2HPO_4 \cdot 7H_2O$, 1 g/liter KH_2PO_4, 2 g/liter NH_4Cl, 0.2 g/liter $MgSO_4 \cdot 7H_2O$, 1 ml/liter Corn Steep Liquor [Sigma], 2.4 ml/liter trace element solution [22]). Antibiotics were added to the growth medium to the following concentrations depending on the application: chloramphenicol (50 μg/ml), kanamycin (50 μg/ml), and gentamicin (10 μg/ml). *R. eutropha* and *E. coli* strains were cultivated at 30°C and 37°C, respectively.

Fluorescence microscopy. To prepare cells for fluorescence microscopy, cells were transferred from LB agar plates into 200 μl of buffer (phosphate-buffered saline) and resuspended thoroughly; 10 μl of this cell suspension were transferred to a single well in a 15-well slide pretreated with 1% poly-L-lysine. Microscopy was carried out using a Leica epifluorescence light microscope. An ORCA-ER charge-coupled device camera (Hamamatsu) and OPENLAB software (Improvision) were used for all image acquisition and processing.

Sucrose gradient fractionation. Strains were cultivated in 50 ml of Lee medium to an approximate optical density at 600 nm of 10. The cultures were centrifuged and the cells resuspended in 2 ml of buffer B1 (20 mM Tris, 500 mM NaCl, 1 mM EDTA, pH 8.5). Cells were sonicated in a Fisher Scientific Sonic Dismembrator 550 in ten pulsed cycles (2 seconds ON, 0.5 second off, 30 second duration, 5 min cooling on ice between cycles); 1 ml of the lysate was loaded onto a sucrose density gradient. The sucrose density gradient consists of nine layered 1 ml fractions of buffer B1 containing 0 to 2 M sucrose (0.25 M increments). The

10-ml solutions were spun at 1,500 × *g* for 3 h. Ten 1 ml fractions were collected with a syringe and needle.

Fluorometry. Fluorescence was measured using the SpectraMax Gemini spectrophotometer (Molecular Devices). Excitation and emission wavelengths of 360 nm and 509 nm, respectively, were used.

PHB analysis. The concentration of PHB was quantified by the sulfuric acid-HPLC method of Karr et al. (12) with modifications (30).

Intein mediated cleavage. A published intein mediated cleavage protocol (14) has been adapted for this study; 300 μl of the lysate generated from the sonication was centrifuged, the supernatant discarded and the insoluble pellet retained. The pellet was washed three times by resuspension in 1 ml of buffer B1 followed by centrifugation. The pellet was then resuspended in 500 μl of buffer B2 (buffer B1 containing 40 mM dithiothreitol). The pellet was incubated overnight at 37°C. After incubation, the solution was centrifuged and the supernatant and pellet retained. The pellet was again washed as described above and resuspended in the original volume (500 μl). Samples were subjected to fluorometry and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on a 12% Tris-HCl polyacrylamide gel (Bio-Rad), stained with SimplyBlue SafeStain (Invitrogen).

β-Galactosidase assay. We added 70 μl of an aqueous 4 mg/ml ortho-nitrophenyl-β-D-galactopyranoside (ONPG) solution to 200 μl of 1X cleavage buffer (0.6 M $Na_2HPO_4{\cdot}7H_2O$, 0.4 M $NaH_2PO_4{\cdot}H_2O$, 0.1 M KCl, 0.01 M $MgSO_4{\cdot}7H_2O$, 2.7 ml/liter β-mercaptoethanol) and incubated in a 30°C water bath for 10 min; 5 or 10 μl of sample (diluted to an appropriate concentration in Buffer B1) was added to the cleavage buffer containing ONPG and incubated at 30°C for 30 min. The reaction was quenched by adding 500 μl Stop Buffer (1 M Na_2CO_3). The amount of ONPG hydrolyzed was determined by measuring optical density at 420 nm and using the molar extinction coefficient of ONPG (4,500 M^{-1} cm^{-1}). Total protein concentrations were measured using a Bradford protein assay kit (Bio-Rad, Hercules, CA). 1 unit of galactosidase activity was defined as the cleavage of 1 nmol of ONPG to *o*-nitrophenol and galactose in 1 min at 30°C.

Construction of plasmid pG (*phaPp*::*gfp* transcriptional fusion in pKNOCK-Cm). The *phaP* promoter from pUCPPCm was inserted into pKNOCK-Cm as a EcoRI/*Eco*RV fragment yielding pGB27. The *gfpmut2* open reading frame (ORF) was PCR amplified from plasmid pGY1a+ using oligonucleotides oGB41 and oGB42 and cloned into pCR2.1-TOPO (Invitrogen). The *gfp* ORF was cloned from the resultant TOPO derivative into pGB27 as a SacI/XhoI fragment yielding pG.

Subcloning an intein (pGB73). The *Mxe* GyrA intein was PCR amplified from plasmid pTWIN1 (New England Biolabs) with oGB176 and oGB177 and cloned into pCR2.1-TOPO to yield pGB73.

Construction of plasmids pGB80 and pGB82 (*phaPp*::*gfp*::*phaP* and *phaPp*::*gfp*::*intein*::*phaP* transcriptional fusions in pKNOCK-Cm). The *phaP* ORF was PCR amplified from *R. eutropha* genomic DNA with oligonucleotides oGB183 and oGB44 and subcloned into pCR2.1-TOPO yielding pGB76. Plasmid pGB76 was digested with *Eco*RV and KpnI and cloned into pG digested with *Mly*I and KpnI to generate plasmid pGB80. The intein from plasmid pGB73 was cloned into plasmid pGB80 as an *Asc*I/XbaI fragment, yielding vector pGB82.

Construction of plasmids pGB91 and pGB93 (*phaP*::*gfp* and *phaP*::*intein gfp* translational fusions in pKNOCK-Cm). A translational fusion of the *phaP* ORF and the *gfp* ORF was constructed by overlap PCR. The *phaP* ORF was PCR amplified from *R. eutropha* genomic DNA using oligonucleotides oGB185 and oGB186. The *gfp* ORF was PCR amplified from pGY1a+ using oligonucleotides oGB187 and oGB40. The resultant PCR products were purified and used as templates in a subsequent PCR. The resultant 1.2-kb PCR fragment was cloned into pCR4Blunt-TOPO yielding pGB85. The 1.2-kb *phaP*::*gfp* translational fusion was cloned into pKNOCK-Cm as a NotI/XhoI fragment yielding pGB91. Plasmid pGB93 was created by introducing the intein from pGB73 into pGB91 as an AscI/XbaI fragment.

Construction of plasmids pGP and pGIP (*phaPp*::*gfp*::peptide linker::*phaP* and *phaPp*::*gfp*::intein::peptide linker::*phaP* transcriptional fusions in pKNOCK-Cm). The *phaP* ORF was PCR amplified from genomic DNA using oligonucleotides oGB209 and oGB44. The resultant 0.6 kb fragment was cloned into pCR2.1-TOPO to yield pGB96. The *phaP* ORF in plasmids pGB80 and pGB82 was excised as an XbaI/KpnI fragment and replaced with the 0.6-kb XbaI/KpnI fragment of pGB96 to yield plasmids pGP and pGIP, respectively.

Construction of plasmids pPG and pPIG (*phaP*::peptide linker::*gfp* and *phaP*::peptide linker::intein::*gfp* translational fusions in pKNOCK-Cm). The *phaP* ORF was PCR amplified from genomic DNA using oligonucleotides oGB185 and oGB208. The resultant 0.6 kb fragment was cloned into pCR2.1-TOPO to yield pGB97. The *phaP* ORF in plasmids pGB91 and pGB93 was excised as a

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence[a]	Location and orientation[b]
oGB41	GAAGAAGAGCTCATGAGTAAAGGAGAAGAACTTTTC	5′ end of *gfpmut2* ORF (+)
oGB42	AAGAACTCGAGGAGTCGATTATTTGTATAGTTCATCCATGCCATG	3′ end of *gfpmut2* ORF (−)
oGB176	GGCGCGCCTATGTGCATCACGGGAGATGCACTAGTT	5′ end of *Mxe* GyrA ORF (+)
oGB177	TCTAGAAGCGTGGCTGACGAACCCGTTCGT	3′ end of *Mxe* GyrA ORF (−)
oGB183	GATATCGGCAGCATCGGCGCGCCTGCTTCTAGAGGCAGCATCATGATCCTCACCCCGGAACAA	5′ end of *phaP* ORF (+)
oGB44	GAAGAAGGTACCTCAGGCAGCCGTCGTCTTCTTTGCCGT	3′ end of *phaP* ORF (−)
oGB185	GCGGCCGCGAGCTCATGATCCTCACCCCGGAACAAGTT	5′ end of *gfp* ORF (+)
oGB186	GGCAGCCGTCGTCTTCTTTGCCGT	3′ end of *gfp* ORF (−)
oGB187	GCAAAGAAGACGGCTGCCGGCAGCATCGGCGCGCCTGCTTCTAGAGGCAGCATCATGAGTAAAGGAGAAGAACTT	5′ end of *phaP* ORF (+) including overlap region of *gfp* ORF
oGB40	GAAGAACTCGAGTTATTTGTATAGTTCATCCATGCCATG	3′ end of *phaP* ORF (−)
oGB208	GGCGCGCCGGATCCGCTGCCGCCCCCGCCCCGCTGCCGCCCCCGCCCCGGCAGCCGTCGTCTTCTTTGC	3′ end of *phaP* ORF (−) containing peptide linker
oGB209	TCTAGAGGATCCGGCGGGGGCGGGGCAGCGGCGGGGGCGGGGCAGCATGATCCTCACCCCGGAACAAGT	5′ end of *phaP* ORF (+) containing peptide linker
oGB216	GAAGAATCTAGAGGCAGCATCATGACCATGATTACGGATTCACTG	5′ end of *lacZ* ORF (+) containing peptide linker
oGB217	GAAGAACTCGAGTTATTTTTGACACCAGACCAACTGGTA	3′ end of *lacZ* ORF (−)

[a] Restriction sites engineered into the sequences are underlined.
[b] Forward (+) or reverse (−) orientation relative to ORF is indicated.

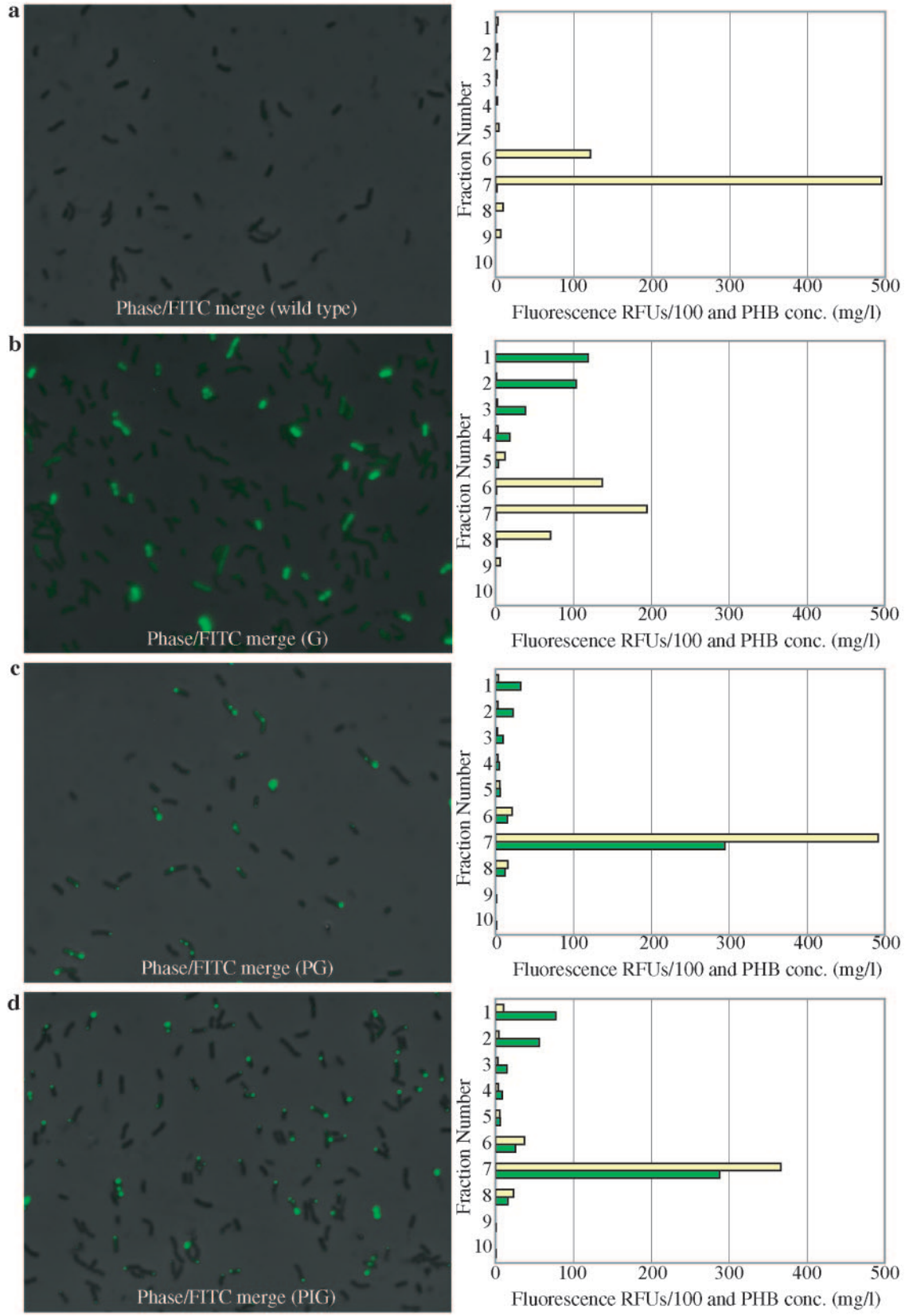

FIG. 1. Representative GFP localization. Fluorescence microscopy and sucrose density gradient fractionation of cell lysates. a) Wild-type *R. eutropha*. b) *R. eutropha* G (expressing GFP). c) *R. eutropha* PG (expressing PhaP-GFP). d) *R. eutropha* PIG (expressing PhaP-intein-GFP). Cell lysates were generated by sonication and added to the top of a sucrose density gradient. Fluorescence of individual sucrose density gradient fractions is expressed in relative fluorescence units divided by 100 and shown in green. Corresponding PHB concentrations are expressed in mg/liter and shown in tan.

SacI/AscI fragment and replaced with the 0.6-kb SacI/*Asc*I fragment of pGB97 to yield plasmids pPG and pPIG, respectively.

Construction of plasmid pPIL (*phaP*::peptide linker::*intein*::*lacZ* translational fusion in pKNOCK-Cm). The *lacZ* ORF was PCR amplified from *E. coli* S17 genomic DNA using oligonucleotides oGB216 and oGB217. The 3.0-kb fragment was subcloned into pCRII-Blunt-TOPO to generate pGB470. The *lacZ* ORF from pGB470 was cloned into pPIG as an XbaI/XhoI fragment, yielding plasmid pPIL.

***R. eutropha* strain generation.** *Ralstonia eutropha* recombinant strains were generated according to methods previously described (3, 22, 23). *R. eutropha* G, was generated using plasmid pG, which carries a transcriptional fusion between the *phaP* promoter and the *gfpmut2* ORF (6) (*phaPp*::*gfp*). Plasmid pG is a suicide plasmid and is integrated at the *phaP* promoter locus of the *R. eutropha* chromosome. Since integration occurs within the promoter region, the wild-type *phaP* gene remains intact. *R. eutropha* PG and *R. eutropha* PIG were generated using plasmids pPG and pPIG, respectively. Plasmid pPG contains an in-frame translational fusion between the *phaP* ORF and *gfpmut2* ORF (*phaP*::*gfp*). Plasmid pPIG is isogenic to pPG, with the exception of the in-frame insertion of the *Mxe* GyrA intein between the two open reading frames (*phaP*::*Mxe* GyrA intein::*gfp)*. Plasmids pPG and pPIG do not contain the *phaP* promoter and the *phaP* ORF serves as the homologous recombination locus. Therefore in *R. eutropha* PG and *R. eutropha* PIG, the wild-type *phaP* gene has been replaced by a translational fusion encoding *phaP*::*gfp* and *phaP*::*intein*::*gfp*, respectively.

RESULTS

Both fluorescence microscopy and sucrose density gradient fractionation of cell lysates were used to examine localization of GFP in *R. eutropha* strains. The fluorescence microscopy images in Fig. 1 show that the wild type exhibited no autofluorescence and that GFP is evenly distributed throughout the cell in *R. eutropha* G. However, Fig. 1 shows fluorescent foci throughout the cells in *R. eutropha* PG and *R. eutropha* PIG, presumably where GFP is localized on the surface of PHB granules.

Sucrose density gradient fractionation of cell lysates was performed to further examine GFP localization (see Materials and Methods). *R. eutropha* strains were cultivated in Lee medium, a phosphate limited growth medium that induces both PHB formation and transcription of genes under the control of the *phaP* promoter. Cells were recovered, washed, resuspended in buffer B1, and sonicated. Cell lysates were loaded onto a sucrose gradient (density from 1.02 g/ml to 1.29 g/ml) and equilibrated by centrifugation. PHB granules have a density of approximately 1.20 g/ml (19) and accumulate near the bottom of the sucrose density gradient. In contrast, soluble proteins accumulate in the low density fractions at the top of the sucrose density gradient. A fluorescence spectrophotometer was used to measure the fluorescence of each individual fraction of the sucrose gradient. *R. eutropha* G showed fluorescence predominantly in the top fractions, consistent with fluorescence micrographs that suggest that GFP is present as a soluble protein in the cytoplasm and not localized to PHB granules.

R. eutropha PG and *R. eutropha* PIG showed a strong fluorescent signal in fraction 7, which coincides with the fraction containing PHB. These results strongly suggest that in *R. eutropha* PG and *R. eutropha* PIG, the GFP is localized to the PHB granules.

A fluorescent signal also appeared in the upper fractions of the *R. eutropha* PG and *R. eutropha* PIG density gradients. We propose several possible explanations for the presence of soluble GFP in these strains: (i) excess PhaP-GFP and PhaP-intein-GFP cannot bind because the PHB granule binding capacity has been exhausted, (ii) affinity is reduced due to the C-terminal fusion of GFP to the PhaP protein, or (iii) the minor fluorescence could represent a small amount of desorption that occurs during sonication and sucrose density gradient fractionation.

From the fluorescence microscopy and sucrose density fractionation, we concluded that PhaP-GFP and PhaP-intein-GFP fusions are localized in vivo to PHB granules. A cleavage experiment was designed to demonstrate the release of pure GFP from whole cell debris (see Materials and Methods). Briefly, *R. eutropha* strains were cultivated in Lee medium, harvested, resuspended in buffer B1 and sonicated. The lysate was centrifuged and the supernatant fraction, containing the soluble protein fraction, was discarded. The pellet was washed in buffer B1. To induce intein cleavage, the pellet was resuspended in buffer B2, and incubated overnight at 37°C. The mixture was then centrifuged and the pellet and supernatant fractions both retained. The pellet was again washed with buffer B1.

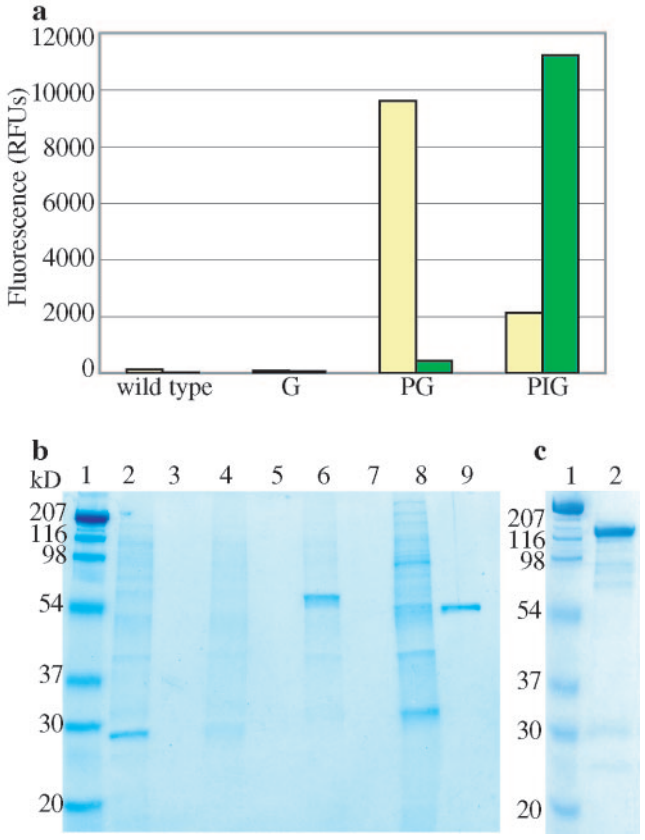

FIG. 2. Intein mediated cleavage of GFP and beta-galactosidase from whole cell debris. *R. eutropha* strains were lysed by sonication, the supernatant discarded and the insoluble pellet containing PHB granules retained. Intein mediated cleavage was activated by incubating the washed pellet overnight in buffer B2 at 37°C. After incubation, the pellet and supernatant fractions were isolated. (a) Fluorometry. Green bars show the fluorescence of the supernatant fractions. Tan colored bars denote the fluorescence of the resulting pellet fraction. (b) SDS-PAGE of fractions. Lanes 2 and 3: *R. eutropha* wild-type pellet and supernatant following dithiothreitol treatment, respectively. Lanes 4 and 5: *R. eutropha* G pellet and supernatant. Lanes 6 and 7: *R. eutropha* PG pellet and supernatant. Lanes 8 and 9: *R. eutropha* PIG pellet and supernatant. (c) SDS-PAGE. Lane 2: *R. eutropha* PIL supernatant.

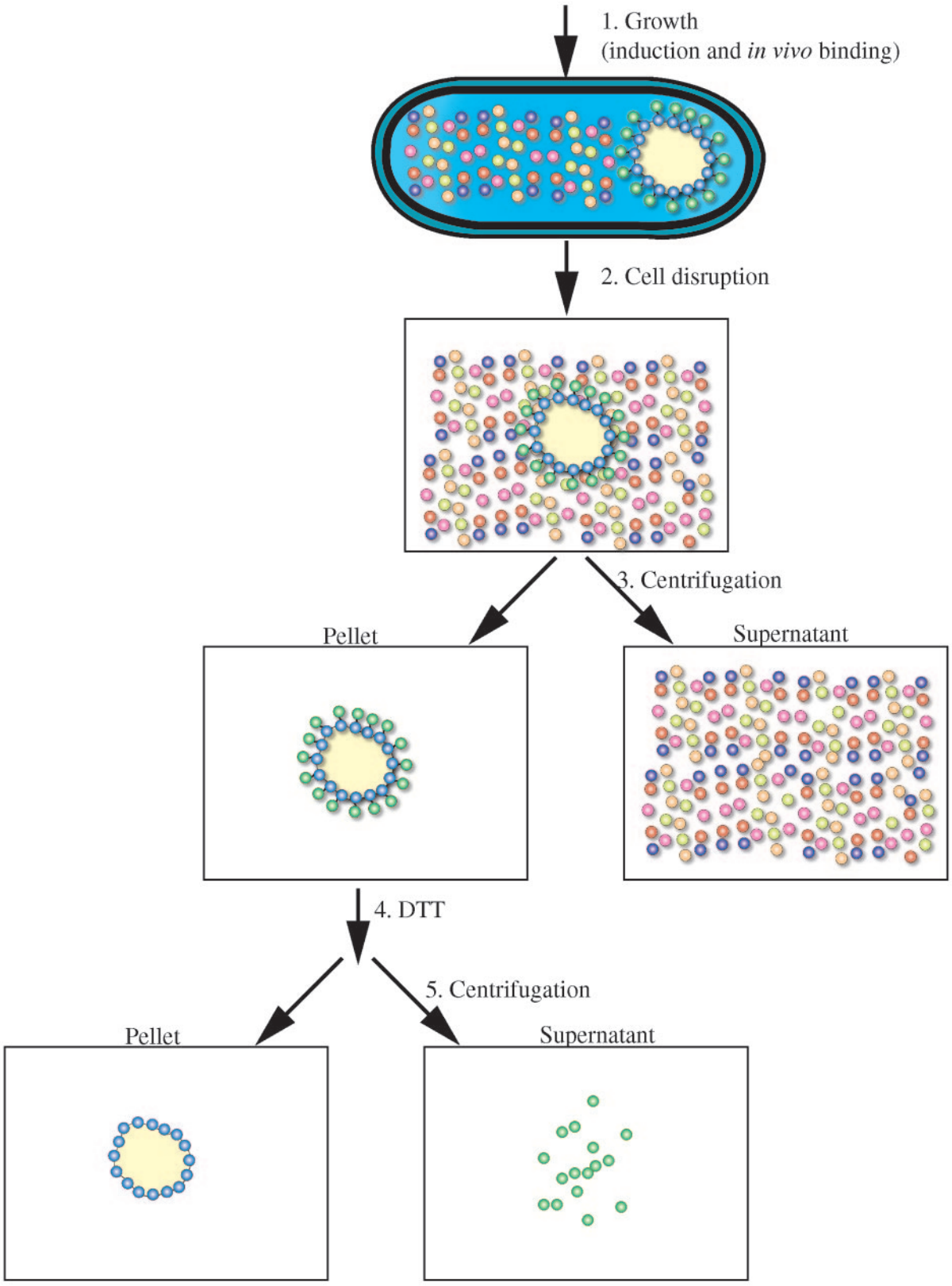

FIG. 3. Flowchart of a recombinant protein purification scheme based on PhaP mediated PHB granule sequestration. *Ralstonia eutropha* recombinants expressing phasin-intein-GFP (PIG) are cultivated in a shake flask or a bioreactor. Cells are harvested by centrifugation, washed and resuspended in buffer B1 prior to cell disruption. The cell lysate is centrifuged, the supernatant fraction discarded and the insoluble fraction retained. After washing the insoluble pellet, the fraction is resuspended in buffer B2. The intein is thiol activated and GFP is released from the whole cell debris into the supernatant. The pure protein is recovered by centrifugation, discarding the pellet and retaining the supernatant fraction.

Figure 2A shows the fluorescence of the pellet and supernatant fractions. Neither the *R. eutropha* wild-type pellet nor the corresponding supernatant showed appreciable fluorescence. Similarly, the pellet and supernatant fractions of *R. eutropha* G showed no appreciable fluorescence as expected. As expected, *R. eutropha* PG showed strong fluorescence on the pellet with no appreciable fluorescence present in the supernatant. In contrast, *R. eutropha* PIG showed very strong fluorescence in the supernatant fraction, indicating that GFP had been released from the pellet into the supernatant fraction. Although the bulk of the total fluorescence was present in the supernatant, a minor amount of fluorescence remained on the PHB granule. To confirm the dithiothreitol-mediated release of GFP and the absence of pH effects, an identical experiment was performed in which the cell debris fraction generated from *R. eutropha* PIG was resuspended in buffer B1 (i.e., buffer B2 lacking dithiothreitol). No appreciable fluorescence was found in the supernatant fraction and the fluorescence of the cell debris remained unchanged (data not shown).

Figure 2B shows an SDS-PAGE gel for the pellet and supernatant fractions with the corresponding fluorescence data depicted in Fig. 2A. As expected, the whole cell debris for each strain contains numerous proteins. No protein is visible on the gel for the supernatant fractions of *R. eutropha* wild-type, *R. eutropha* G, and *R. eutropha* PG. The PhaP-intein-GFP fusion protein is expected to be 70 kDa in size. If intein-mediated cleavage occurs, a protein of 49 kDa, corresponding to an intein-GFP (IG) fusion, should be released. Figure 2B, lane 9, shows that IG was the only protein present in the supernatant fraction. This observation confirms that intein mediated cleavage, activated by thiol addition, released GFP from the granule in the cell debris of *R. eutropha* PIG.

To further investigate the robustness of this method we attempted to purify a relatively large, multimeric, catalytically active protein (β-galactosidase, 4×116 kDa). *R. eutropha* PIL was generated using plasmid pPIL (see Table 1). Plasmid pPIL is isogenic to plasmid pPIG with the exception that the *gfpmut2* ORF in pPIG has been replaced with the *lacZ* ORF in pPIL. Therefore, in *R. eutropha* PIL, the wild-type *phaP* gene has been replaced by a *phaP*::*Mxe* GyrA intein::*lacZ* translational fusion.

Intein mediated release of pure β-galactosidase, using cell extracts of *R. eutropha* PIL was demonstrated. Craven et al. (7) reported a specific activity of 40,000 U/nmol (340,000 U/mg, 28°C, pH 7.0) for purified β-galactosidase obtained by three purification steps (ammonium sulfate precipitation, size exclusion chromatography and DEAE chromatography). Colby and Hu (5) reported 19,000 U/nmol (160,000 U/mg, 30°C, pH 7.0) for purified β-galactosidase obtained by five purification steps (ammonium sulfate precipitation, electrophoresis, DEAE chromatography, size exclusion chromatography and crystallization). Following the above described method, a specific activity of 53,000 U/nmol was measured in the supernatant following dithiothreitol treatment of whole cell debris. Thus, a single purification step, without external chromatography; results in a protein fraction of high purity consistent with previously reported data for pure β-galactosidase and SDS-PAGE (see Fig. 2C).

We have also explored the effect of orientation by using PhaP as the C-terminal fusion partner (i.e., creating plasmids pGIP and pLIP). Similar to results generated using PhaP as the N-terminal fusion partner, we have shown that soluble, active GFP and β-galactosidase can be recovered in a single purification step when PhaP was used as the C-terminal fusion partner (data not shown).

DISCUSSION

In this study, we report the development of an integrated protein expression and purification approach that obviates the need for external chromatography. By replacing the wild-type *phaP* gene with a triple translational fusion (*phaP* ORF, *Mxe* GyrA intein and *gfpmut2*), we were able to show that the fusion protein can be localized to the PHB granule and separated from the remaining cytosolic protein fraction by centrifugation. In a subsequent step, we were able to release pure GFP by resuspending whole cell debris (insoluble fraction of cell lysate, containing PHB granules) in a buffer containing dithiothreitol (see Fig. 3). We also expressed, recovered and purified β-galactosidase to demonstrate the applicability of this method to purify catalytically active proteins. We showed that the enzyme is essentially pure and that the specific activity is comparable to previously reported literature values for pure β-galactosidase.

The single step purification eliminates the need for elaborate and costly protein purification schemes. Moreover, adapting the use of inteins eliminates the need for specific endopeptidases, which are routinely used to release recombinant protein from affinity matrixes. It is contemplated that this system could be adapted for use in other hosts that either produce PHB granules naturally or hosts that have been engineered to produce PHB.

By integrating high-level recombinant protein expression with a simple, protein purification step, this system has the potential to improve upon current technologies for the large-scale production of commodity enzymes, therapeutic proteins, vaccines, and peptides.

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