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A less laborious approach to the high-throughput production of recombinant proteins in *Escherichia coli* using 2-liter plastic bottles

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Abstract

Contemporary approaches to biology often call for the high-throughput production of large amounts of numerous proteins for structural or functional studies. Even with the highly efficient protein expression systems developed in *Escherichia coli,* production of these proteins is laborious and time-consuming. We have simplified established protocols by the use of disposable culture vessels: common 2-liter polyethylene terephthalate beverage bottles. The bottles are inexpensive, fit conveniently in commonly available flask holders, and, because they are notched, provide sufficient aeration to support the growth of high-density cultures. The use of antibiotics and freshly prepared media alleviates the need for sterilization of media and significantly reduces the labor involved. Uninoculated controls exhibited no growth during the time required for protein expression in experimental cultures. The yield, solubility, activity, and pattern of crystallization of proteins expressed in bottles were comparable to those obtained under conventional culture conditions. After use, the bottles are discarded, reducing the risk of cross-contamination of subsequent cultures. The approach appears to be suitable for high-throughput production of proteins for structural or functional studies.

Keywords: Structural genomics; Functional genomics; High-throughput; Heterologous expression

The emerging fields of structural and functional genomics embrace many different objectives, but both depend in part on the production of numerous proteins at a much faster rate than it occurs currently [1–3]. Structural genomics research typically requires simultaneous production of distinct, novel proteins, whereas functional analyses may call for production of numerous variants of a specific protein. To achieve these objectives, every step involved in the production of proteins must be accelerated, including cloning, expression, and purification. Automated methods using robotic liquid handlers and newly developed chromatography stations help to simplify the cloning and purification of proteins [4,5], but the process of producing target proteins in *Escherichia coli* remains laborious. New strategies of protein production have been developed, such as cell-free translation systems and miniaturized fermentation batteries [6–9], but these processes are highly specialized, requiring considerable investment in training or equipment. Inexpensive, convenient methods that reduce the effort required for production of proteins in *E. coli* but use commonly available equipment and simple protocols would be of value.

Improved volumetric yields of proteins can be obtained by expressing proteins in high-density cultures of *E. coli* grown in a highly enriched medium such as terrific broth (TB) [10,11]. This commonly used approach reduces the effort by using a smaller volume of culture medium, simplifying media preparation, centrifugation, and waste disposal. Even with this improvement, however, the process is time-consuming and cumbersome. In addition to the time required for the essential biological stages of cell growth and protein expression, conventional approaches spill over into the days preceding and following these steps, in the preparation and sterilization of media and in the cleanup of the vessels used for the cultures. The method described here—expression of proteins in non-

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sterilized media in disposable flasks—greatly reduces the time required for media preparation and eliminates much of the cleanup that follows harvesting of the cells.

The ubiquitous 2-liter polyethylene terephthalate (PET) bottles, normally used for soft drinks, fortuitously are well suited for use as disposable culture vessels. The bottles fit in standard shaker baskets designed for 1-liter flasks, and the notches in the base of the bottles provide effective aeration of cultures. They do not need to be sterilized because the antibiotics typically required for heterologous protein expression prevent the growth of contaminants. Disposal of the bottles afterward reduces the risk of cross-contamination of subsequent cultures. Expression of proteins in the bottles gave yields of soluble protein comparable to flasks, and proteins purified from both culture conditions exhibited similar properties.

Materials and methods

Cloning and expression of target proteins

Target proteins were cloned into the vector pMCSG7 [12] by automated, robotic polymerase chain reaction (PCR) and ligation independent cloning (LIC) using previously described protocols [5]. Expression was performed in the *E. coli* host strain BL21-Gold(DE3) (Stratagene, La Jolla, CA) that contains an extra plasmid encoding three rare tRNAs (complementary to codons AGG and AGA for arginine and AUA for isoleucine). All media were supplemented with 100 μg/ml ampicillin and 30 μg/ml kanamycin to maintain the two plasmids. Cultures were grown in either terrific broth (TB), Luria broth (LB), or M9 minimal medium [11]. TB was prepared from EZMix preformulated solids (Sigma Chemical, St. Louis, MO), LB was from premixed LB broth (Fisher Scientific, Fair Lawn, NJ), and M9 was from premixed mineral salts (Sigma). EZMix TB contains tryptone (pancreatic digest of casein), 12 g/liter; yeast extract, 24 g/liter; K_2HPO_4, 9.4 g/liter; KH_2PO_4, 2.2 g/liter; and inert binder, 0.6 g/liter. The medium was supplemented with 8 g/liter glycerol or 4 g/liter glycerol plus 4 g/liter glucose. Premixed LB contains tryptone, 10 g/liter; yeast extract, 5 g/liter; and NaCl, 10 g/liter. Premixed M9 Mineral Salts (Sigma M-6030) contains Na_2HPO_4 6.78 g/liter; KH_2PO_4, 3.0 g/liter; NaCl, 0.5 g/liter; and NH_4Cl, 1.0 g/liter. The medium was supplemented with NaCl, 0.5 g/liter; $MgSO_4$, 0.24 g/liter; $CaCl_2$, 0.01 g/liter; thiamine hydrochloride, 1 μg/ml; and glucose, 4 g/liter.

Production of target proteins in high-density cultures in 2-liter PET bottles

Inocula were prepared by streaking glycerol stocks on LB plates containing ampicillin and kanamycin at 100 and 30 μg/ml, respectively, and incubating overnight at 37 °C. A consistent-sized sample of the cells was then scraped from the plate using a disposable 1/4-in. plastic inoculation loop, resuspended in 2 ml sterile TB medium, and added to non-sterile TB medium prepared immediately prior to inoculation in 2-liter PET bottles (below). Bottles were obtained as a generous gift from Plastipak Packaging (Plymouth, MI) or purchased through Continental Glass and Plastic (Chicago, IL).

For expression in high-density cultures in TB medium, two protocols were employed: expression at 37 °C in medium supplemented with 4 g/liter glucose plus 4 g/liter glycerol, and expression at 20 °C in medium containing 8 g/liter glycerol. Specifically, the medium was prepared immediately prior to use by adding 12 g of preformulated EZMix medium solids through a powder transfer funnel, followed by addition of 1 cup (236 ml) of high-purity water (not sterilized) and 4 ml of a sterile solution containing concentrated ampicillin, kanamycin, and carbon sources. The freshly prepared medium was used immediately, without sterilization, by inoculation with the resuspended cells described above. In both cases, cultures were incubated at 37 °C in a C25 Incubator Shaker (New Brunswick Scientific, Edison, NJ) rotary shaker (radius of gyration: one inch) with agitation at 300 rpm. The OD_{600} of the cultures was monitored periodically. For expression at 37 °C, cultures were induced at OD_{600} 4–8 by addition of 1 mM isopropyl-(3-D-thiogalactopyranoside) (IPTG) and harvested after 3–4 h of continued growth at 37 °C. For expression at 20 °C, cultures were induced at $OD_{600} \approx 1$, then moved to an Innova 4230 Refrigerated Incubator Shaker (New Brunswick Scientific, Edison, NJ) rotary shaker (radius of gyration: three-quarter inch) at 20 °C, and agitated at 220 rpm overnight (approximately 16 h). In both cases, cells were harvested by centrifugation, resuspended in lysis buffer (50 mM Hepes, pH 7.8, containing 500 mM NaCl, 10 mM imidazole, 10 mM β-mercaptoethanol, and 5% glycerol), and frozen at −80 °C.

Production of proteins in low-density cultures and in minimal medium

Proteins were also produced in less rich media that supported lower cell densities, both in sterile 2-L baffled Erlenmeyer flasks and in 2-L PET bottles. For both types of vessels, 1 liter of medium was used. LB medium was prepared from 25 g of preformulated solids (Sigma) and M9 medium was prepared from 11.3 g of preformulated M9 salts (Sigma) supplemented with the appropriate sterile stock solutions (above) to give complete M9 medium [11]. For production in LB medium, inocula were grown on plates, as described above, but for M9 medium, inocula were grown overnight at 37 °C at 180 rpm in 15 ml sterile M9 medium in 125 ml baffled Erlenmeyer flasks. The production cultures were inoculated to give an initial $OD_{600} = 0.02$ to 0.05, grown at

37 °C with agitation at 200 rpm until $OD_{600} = 0.5$, induced with 1 mM IPTG, and then incubated overnight (approximately 16 h) at 20 and 30 °C, respectively, for the LB and M9 cultures.

Purification and crystallization of proteins

Expression of target protein from the vector pMCSG7 attaches a polyhistidine affinity tag, followed by a tobacco etch virus (TEV) protease recognition site to the N-terminus of the target protein. Details of the purification will be published elsewhere, but briefly, cells were lysed by sonication, and the target proteins were purified by two steps of immobilized metal affinity chromatography (IMAC). First, target proteins were partially purified by IMAC, followed by gel filtration on an Akta-3D purification station (Amersham Biosciences, Piscataway, NJ). The polyhistidine-tag leader was removed by treatment with his-tagged TEV protease (a generous gift of D. Waugh [13]), and a second IMAC step removed the TEV protease, uncleaved target, and contaminants from *E. coli* that had interacted with the first IMAC column. These two steps typically generated homogeneous or nearly homogeneous protein based on analytical sodium dodecyl sulfate (SDS) gel electrophoresis [14]. Glycerophosphodiester phosphodiesterase was purified further by ion-exchange chromatography on a Mono Q column (Amersham). Purified proteins were screened for crystallization using the Hampton screen kits (Hampton Industries, Laguna Niguel, CA) or stored for later crystallization or enzymatic assay by flash freezing by dripping into liquid nitrogen. For diffraction analysis, crystals were treated with an appropriate cryoprotectant, flash-frozen in liquid nitrogen, and analyzed at the 19-ID or 19-BM beamlines of the Advanced Photon Source, Argonne National Laboratory (www.sbc.anl.gov).

Analytical methods

Expression of target proteins was determined by SDS gel electrophoresis of cell lysates. A volume of cells equivalent to 1 ml of a culture at $OD_{600} = 3$ was centrifuged, resuspended in 50 µl of 0.1 M Tris/HCl, pH 8.0, and then solubilized by addition of 50 µl of solubilization buffer (6% SDS, 1 M dithiothreitol, 0.06% bromphenol blue, and 20% sucrose), followed by heating for 5 min at 90 °C. The sample was cooled and diluted with 200 µl more 0.1 M Tris buffer, and 25 µl of the solution was analyzed on a prepoured 4–20% gradient polyacrylamide gel (Gradipore, Hawthorne, NY) and stained with Coomassie Brilliant Blue R. To evaluate the solubility of the expressed proteins, samples of induced cells (1.5 ml of high-density cultures and 5 ml of low-density cultures) were centrifuged, resuspended in 600 µl of 0.1 M Tris/HCl, pH 8.0, and treated with 2.4 µl "Ready-Lyse" lysozyme (Epicentre Technologies, Madison, WI) and 1 µl Benzonase DNAase (Novagen, Madison, WI) for 15–30 min at room temperature. The sample was then transferred into a 20 ml scintillation vial containing 3 ml of 0.1 M Tris/HCl, pH 8.0, and frozen. The cells were thawed and sonicated in an ice bath with a Branson Sonicator 450 (Branson Ultrasonics, Danbury, CT) fitted with a 1/2-inch tip and using 30 pulses at power setting 4 and duty cycle 50%. Following centrifugation of 250 µl of the ruptured cells, 25 µl of the supernatant was treated with 5 µl of solubilization buffer and heated for 5 min at 90 °C. The pellet from the centrifugation step above was washed gently with buffer and then suspended in 40 µl of 0.1 M Tris, pH 8.0, plus 40 µl of solubilization buffer, treated for 5 min at 90 °C, and diluted with 160 µl Tris buffer. Protein was analyzed as described above by SDS–polyacrylamide gel electrophoresis of 25 µl of each preparation.

The kinetic parameters of glycerophosphodiester phosphodiesterase (APC172, Table 1) were determined by measuring the conversion of glycerophosphorylcholine to glycerol-3-phosphate, which was detected by its NAD^+-dependent oxidation by the coupling enzyme glycerophosphate dehydrogenase [15,16]. The assay mixture (1 ml) contained 0.9 M hydrazine/0.18 M glycine buffer, pH 9.0, 0.5 mM NAD^+, 10 mM $CaCl_2$, 20 U glycerol-3-phosphate dehydrogenase (Sigma), and 62–500 µM L-glycerophosphorylcholine as substrate. The

Table 1
Target proteins expressed in PET bottles

Target[a]	Accession Nos.	Identity
APC172	P09394	*E. coli* glycerophosphodiester phosphodiesterase[b]
APC1303	O34682	*Bacillus subtilis* hypothetical cytosolic protein
APC1313	P71020	*B. subtilis* hypothetical cytosolic protein
APC1317	O34632	*B. subtilis* hypothetical cytosolic protein
APC1324	P37104	*B. subtilis* hypothetical cytosolic protein
APC1341	P32729	*B. subtilis* ribosomal protein L7A
APC1343	Q04809	*B. subtilis* dipicolinate synthase
APC1468	RBS2257[c]	*B. subtilis* prephenate dehydrogenase

[a] Full information available at www.mcsg.anl.gov.
[b] Mature form of protein lacking leader sequence (residues 1–25).
[c] Annotation available online at WIT database, wit.mcs.anl.gov.

glycerophosphorylcholine was prepared from the $CdCl_2$ adduct (Sigma) by addition of Na_2CO_3, followed by incubation on ice for 10 min and removal of the precipitated $CdCO_3$ by centrifugation [17]. Assays, performed in duplicate, were initiated by addition of 0.4 μg purified glycerophosphodiester phosphodiesterase. Data were fitted to the hyperbola with the program Prism (GraphPad Software, San Diego, CA) assuming simple Michaelis–Menten kinetics.

Results

Culture of E. coli in 2-liter PET bottles

The common 2-liter PET bottles normally used for soft drinks can also serve as disposable culture vessels for the expression of proteins in *E. coli* (Fig. 1). The notches in the base of the bottles, which are designed to provide mechanical stability to the bottom and allow them to stand upright, serve in this context to disrupt vortices that would form during rotary agitation, thereby providing efficient aeration of cultures, just as do the notches in conventional baffled Erlenmeyer flasks. Photographs of PET bottles containing 250 ml water and agitated at 250 rpm (taken in the dark with flash lighting to freeze the motion) reveal the effectiveness of the notches in dispersing the liquid (Fig. 1B). Comparison of the growth of *E. coli* in PET bottles and baffled Erlenmeyer flasks gave indistinguishable results (Fig. 2). Strain BL21-Gold(DE3) containing a derivative of the expression plasmid pMCSG7 [12] was cultured in 250 ml TB medium in either a 2-liter PET bottle or in a sterile 1-liter baffled Erlenmeyer flask. Growth, as measured by OD_{600}, was identical in each culture until the very end of the experiment when, in this case, a slightly higher final cell density was attained in the PET bottle. In this experiment, induction of the protein encoded by the plasmid was initiated at $OD_{600} = 5$, and analysis of total cell lysates by gel electrophoresis revealed comparable expression under the two conditions (Fig. 3).

A major difference between the protocol using conventional flasks and that using PET bottles is that in the latter case the media and the bottles are not sterilized (see Materials and methods). The inclusion of two antibiotics in the culture medium, ampicillin for maintenance of the pMCSG7 derivative and kanamycin for the tRNA-encoding plasmid, effectively prevents the growth of contaminating bacteria; no measurable growth occurred during the time course of expression in an uninoculated control containing non-sterilized medium in a non-sterile PET bottle (Fig. 2, diamond symbols). Critical aspects of the protocol allow this approach to work: the inocula are scrupulously handled to maintain their sterility and prevent cross-contamination, and the medium is prepared immediately prior to use. This control experiment has been repeated many times, and not once have we observed measurable growth of contaminants during the time required for growth, induction, and harvesting of the experimental cultures.

Fig. 1. Two-liter PET bottles as culture vessels. (A) A culture of *E. coli* expressing a heterologous protein in a 2-liter PET bottle. The medium, 250 ml TB, was not sterilized, but the presence of ampicillin and kanamycin prevented the growth of other microbes during the time required for expression of the target protein. The culture was agitated at 250 rpm. (B) Close-up view of a bottle containing 250 ml water, agitated at 250 rpm, to illustrate the effective aeration provided by the notches in the base of the bottle.

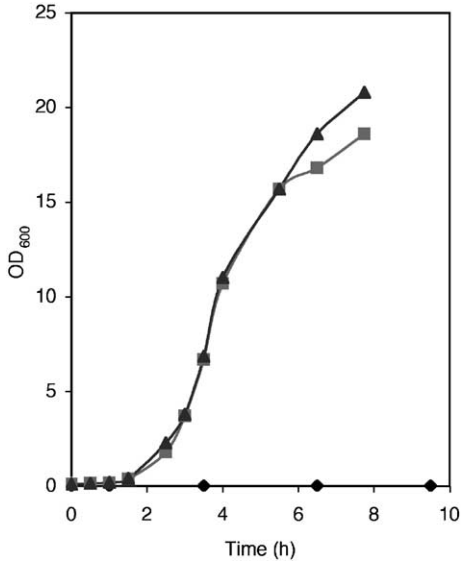

Fig. 2. Growth of cells in PET bottles and in baffled Erlenmeyer flasks. Cultures of *E. coli* containing the gene encoding APC1468 in the expression vector pMCSG7 were grown in 250 ml TB medium supplemented with 4 g/liter glucose and 4 g/liter glycerol either in a PET bottle (triangles) or in a 1-liter baffled Erlenmeyer flask (squares) at 37 °C with agitation at 250 rpm. The Erlenmeyer flask and its medium were sterilized, but the bottle and its medium were not. The cultures were induced by addition of 1 mM IPTG at $OD_{600} = 5$. Growth was measured by determination of the OD_{600} of appropriate dilutions of the cultures. No growth was observed for an uninoculated control of non-sterilized TB medium in a PET bottle (diamonds).

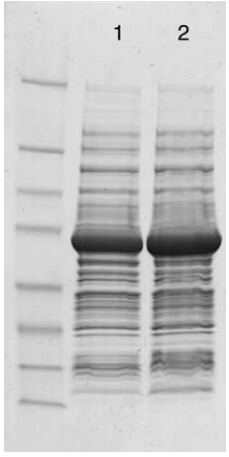

Fig. 3. Expression of ACP1468 in cultures grown in PET bottles and in baffled Erlenmeyer flasks. Polyacrylamide gel electrophoresis of total protein extracts of cells expressing APC1468 grown: (1) in non-sterile medium in a PET bottle or (2) in sterile medium in a baffled Erlenmeyer flask. Proteins were stained with Coomassie Brilliant Blue R. Molecular weight markers were the Board Range Prestained Markers of New England Biolabs (Beverly, MA), which run with apparent molecular weights of 175, 83, 62, 47.5, 32.5, 25, 16.5, and 6.4 kDa.

High-throughput production of diverse proteins for structural studies

Structural genomics studies often call for the simultaneous production of different proteins of unknown function and properties. Attaining both a high yield and high solubility of the expressed targets is critical to maintaining high-throughput production of crystals. Expression of a random set of six target proteins (Table 1), previously scored as producing soluble products by a small-scale preliminary screen, was compared under two conditions: 'classical' expression conditions (low-density cultures in sterile LB performed in 1 liter of medium in 2-liter, baffled Erlenmeyer flasks) and the reduced-labor conditions described here (high-density cultures in 250 ml non-sterile TB in 2-liter PET bottles). The yield and solubility of the proteins, as estimated by SDS gel electrophoresis, were essentially identical (Fig. 4). Both approaches gave high yields of the target proteins, with virtually identical distributions between the soluble and insoluble fractions. We also observed that inoculation of expression cultures with cells resuspended from plates gave more consistent growth, with less variability in the lag before exponential growth, than did inoculation with cells grown overnight in liquid medium, provided the experimenter resuspended a roughly equivalent amount of each strain. This result may be a consequence of the heterogeneity of growth conditions that cells experience on plates, reducing the chance that the entire inoculum has entered stationary phase.

Three of the proteins illustrated in Fig. 4 were purified to homogeneity from cultures grown under both conditions. The yields of purified protein obtained from high-density cultures grown in PET bottles were equal to or greater than those obtained from the lower-density cultures grown in Erlenmeyer flasks (Table 2). The yields, which reflect the efficiency of purification as well as of expression, vary greatly, but the relative yields of the different proteins were the same for the two culture methods (Table 2). The higher overall yield from the high-density cultures resulted primarily from the higher amounts of cells obtained in those cultures; yields of pure protein per gram of cells were comparable for the two methods (Table 2). In crystallization trials, proteins purified from either culture gave the same patterns of crystallization. Of the three proteins, one yielded crystals in a preliminary screen using the Hampton kits: APC1341 expressed under both growth conditions crystallized under the same conditions, producing crystals of the same morphology and same quality. In this particular case, unfortunately, both crystals diffracted poorly. However, other proteins not included in these comparisons that were expressed only under the high-density conditions in the PET bottles have given high-quality crystals that have led to full structural characterization. Three of these structures have already been deposited in the Protein Data Bank (PDB); APC1167, PDB identification code 1NNI; APC1490, PDB identification code 1MK4; and APC1644, PDB identification code 1NC5 (www.mscg.anl.gov, www.rcsb.org/pdb/).

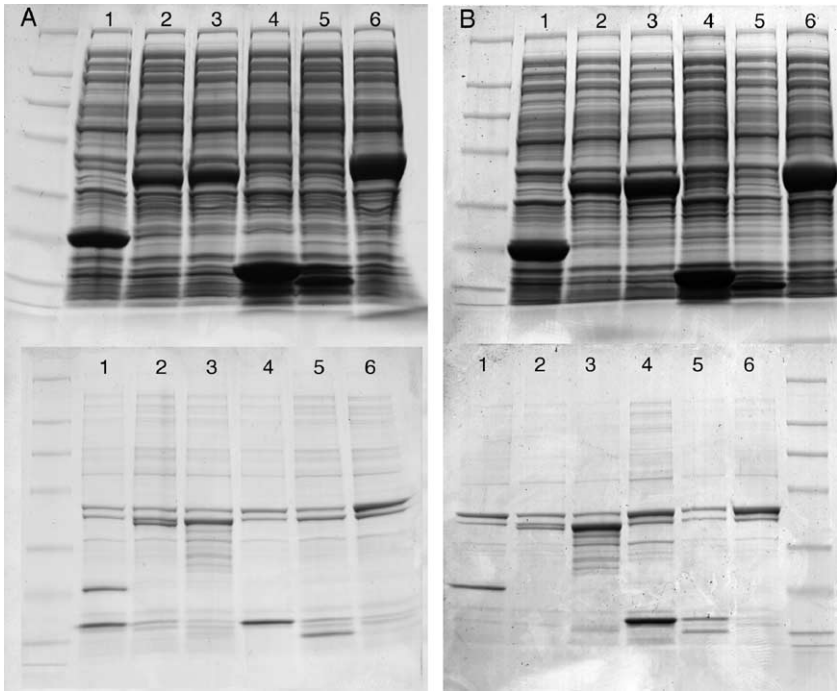

Fig. 4. Solubility of proteins expressed in PET bottles and in baffled Erlenmeyer flasks. Polyacrylamide gel electrophoresis of the soluble and particulate protein fractions of proteins expressed (A) in 250 ml non-sterile TB medium containing 8 g/liter glycerol in PET bottles or (B) in 1 liter sterile LB medium in 2-liter baffled Erlenmeyer flasks. In both cases, the cultures were grown at 37 °C until induction with 1 mM IPTG (at $OD_{600} = 1$ for the TB cultures and 0.5 for the LB cultures) and then shifted to 20 °C for overnight expression (approximately 16 h). The upper panel represents the soluble fraction; the lower, the particulate fraction. The target proteins expressed (Table 1) were: 1, APC1303; 2, APC1313; 3, APC1317; 4, APC1324; 5, APC1341; and 6, APC1343. Molecular weight markers (unlabeled lanes) are described in the legend to Fig. 3.

Table 2
Yields of cells and purified target proteins from cultures grown in PET bottles or baffled Erlenmeyer flasks

Protein[a]	Growth condition[b]	Cell yield (g wet weight)	Protein yield[c] (mg)	Pure protein/Cell mass (mg/g wet weight)
APC1317	PET bottle	8.8	120	13.6
	Erlenmeyer flask	4.6	50	10.9
APC1324	PET bottle	10.6	183	17.3
	Erlenmeyer flask	5.2	103	19.8
APC1341	PET bottle	9.1	29	3.2
	Erlenmeyer flask	6.0	33	5.5

[a] Details of target proteins are available at http://www.mcsg.anl.gov/.

[b] Growth conditions for PET bottles were: 250 ml of TB (containing glycerol at 8 ml/liter), 37 °C at 250 rpm until $OD_{600} = 1$, induction with 1 mM IPTG followed by shift to 20 °C overnight. Growth conditions for baffled Erlenmeyer flasks were: 1 liter of LB medium, 37 °C at 250 rpm until $OD_{600} = 0.5$, induction with 1 mM IPTG followed by shift to 20 °C overnight. Both cultures contained ampicillin at 100 μg/ml and kanamycin at 30 μg/ml.

[c] Yield of purified protein after hydrolysis of his-tag and second IMAC column (see Materials and methods).

Expression of heterologous proteins at lower temperature has long been recognized to improve the solubility of some proteins that give insoluble products when expressed in *E. coli,* and this was found to be true for expression in PET bottles in TB medium as well (Fig. 5). When the same six proteins were expressed in cultures grown at 37 °C (that is, when they were not shifted to 20 °C upon induction) some of the proteins gave a significant fraction of insoluble product (Fig. 5).

Expression of proteins for functional characterization

For proteins of known function that produce soluble, active products efficiently in *E. coli,* expression may also be performed at 37 °C, allowing production of cells containing high levels of the desired protein in a single day. Of the six proteins analyzed above, for example, APC1324 gave equivalent yields of soluble protein when expressed at either 20 or 37 °C, with no significant formation of insoluble material at 37 °C (Figs. 4 and 5).

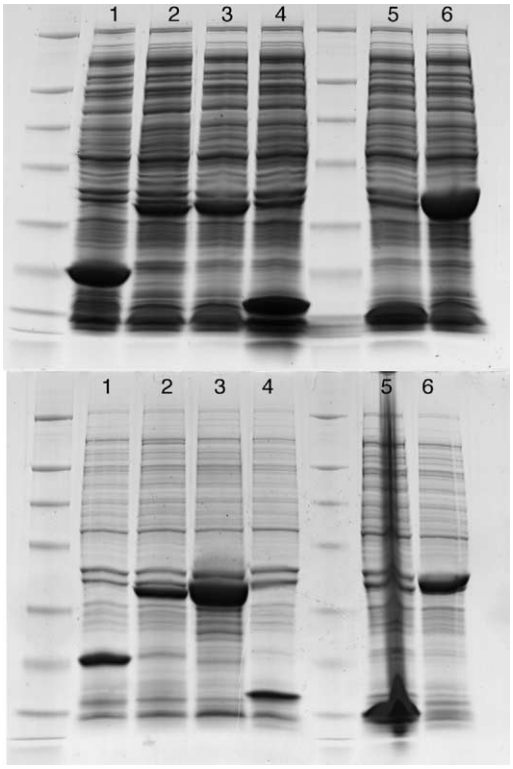

Fig. 5. Solubility of proteins expressed in PET bottles at 37 °C. Polyacrylamide gel electrophoresis of the soluble and particulate protein fractions of proteins expressed at 37 °C in 250 ml non-sterile TB medium supplemented with 4 g/liter glucose and 4 g/liter glycerol in PET bottles. Expression was induced by addition of 1 mM IPTG at $OD_{600} \approx 5$. Cells were harvested after 4 h induction at 37 °C. The upper panel represents the soluble fraction; the lower, the particulate fraction. The proteins expressed were: 1, APC1303; 2, APC1313; 3, APC1317; 4, APC1324; 5, APC1341; and 6, APC1343. Molecular weight markers (unlabeled lanes) are described in the legend to Fig. 3.

ACP172, the glycerophosphodiester phosphodiesterase of *E. coli*, is another such protein. The enzyme is normally excreted into the periplasm by signal peptide mediated transport [16]. When the mature protein, lacking the signal peptide, was cloned into pMCSG7, it was expressed in high yield in the cytoplasm (data not shown). The phosphodiesterase, expressed in cultures grown either at high density in 250 ml non-sterile TB medium in a PET bottle or at low density in 1 liter of sterile LB medium in a 2-liter flask, was purified to homogeneity from both cultures and its kinetic parameters were evaluated. The yields of pure protein and the kinetic properties of the two preparations were similar (Table 3). The V_m determined for the enzyme purified from the high-density culture was 29% lower than that of the enzyme purified from the LB culture, but its K_m was also lower, by 22%. The calculated V_m/K_m values were 0.965 and 1.02 for the enzyme obtained from the TB and LB cultures, respectively. These results indicate that both culture methods give material suitable for functional characterization. These kinetic values did, however, differ significantly from those reported for the native, mature enzyme purified from the *E. coli* periplasm (values for hydrolysis of glycerophosphorylcholine were: $V_m = 180\,\mu mol/min/mg$, $K_m = 280\,\mu M$ [17]). These differences could be due to slight differences in the proteins (expression from pMCSG7 results in addition of the amino acids serine and asparagine to the N-terminus of the protein after removal of the polyhistidine tag), but could equally well be attributed to variation between laboratories, or differences in the quality of the substrate and coupling enzyme used in the assays.

Use of PET bottles for lower-density cultures

Under appropriate conditions, PET bottles can also be used as culture vessels for the production of proteins in low-density cultures grown in less rich medium than TB. When ACP172 was grown at 37 °C to $OD_{600} = 0.5$ in either 1 liter non-sterile LB medium in a 2-liter PET bottle or in 1 liter sterile LB in a 2-liter, baffled Erlenmeyer flask, then induced and incubated overnight at 20 °C, the final cell density of the culture was only 15% lower in the PET bottle (Table 4). Similarly, growth of induced cells in 1 liter of M9 medium in PET bottles was less than 10% lower than that in a 2-liter flask. The slight reduction in cell yield is most likely due to the lower surface to volume ratio of the medium in the PET bottle cultures. In both LB and M9 media, uninoculated, non-sterile controls incubated in bottles under identical conditions showed no growth of contaminants during

Table 3
Yield and kinetic parameters of purified APC172 obtained from cells grown under different conditions[a]

Culture vessel	Medium	Yield[b] (mg)	V_m(SE)[c] (μmol/min/mg)	K_m(SE)[c] (μM)
PET bottle	TB	85	109[d] (5.3)	113[d] (16)
Erlenmeyer flask	LB	76	141[d] (12)	138[d] (33)

[a] For expression in the PET bottle, cells were cultured at 37 °C with agitation at 300 rpm in 250 ml TB containing glycerol at 8 ml/liter until $OD_{600} = 1$, then induced with 1 mM IPTG, and shifted to 20 °C and 220 rpm for overnight induction (approximately 16 h). For expression in the baffled Erlenmeyer flask, cells were cultured in 1 liter LB at 37 °C with agitation at 250 rpm until $OD_{600} = 0.5$, induced with 1 mM IPTG, and shifted to 20 °C and 220 rpm for overnight induction. Both cultures contained ampicillin at 100 μg/ml and kanamycin at 30 μg/ml.

[b] Yield of purified protein after MonoQ column (see Materials and methods).

[c] SE, standard error.

[d] Literature values reported for these parameters, with glycerophosphorylcholine as substrate, were: $V_m = 180\,\mu mol/min/mg$; $K_m = 280\,\mu M$ [17].

Table 4
Yields of cells obtained in low-density cultures in PET bottles

Medium	Condition[a]	Cell yield[b] (SE)[c] (OD_{600})
LB	Erlenmeyer flask	4.88 (0.40)
	PET bottle	4.15 (0.29)
	PET bottle, uninoculated	0.03 (0.02)
M9	Erlenmeyer flask	3.59 (0.32)
	PET bottle	3.27 (0.11)
	PET bottle, uninoculated	0.001 (0.001)

[a] Both types of culture vessels (baffled, 2-liter Erlenmeyer flasks and 2-liter PET bottles) contained 1 liter of medium. Cultures were incubated at 37 °C with agitation at 200 rpm until $OD_{600} = 0.5$, induced with 1 mM IPTG, and incubated overnight at 20 °C or 30 °C, respectively, for the LB and M9 cultures. Shaking rates were maintained at 200 rpm in both cases. All cultures contained ampicillin at 100 μg/ml and kanamycin at 30 μg/ml.

[b] Yield of cells was estimated by OD_{600} taken at the time of harvesting cells (approximately 16 h after induction).

[c] SE, standard error of the mean.

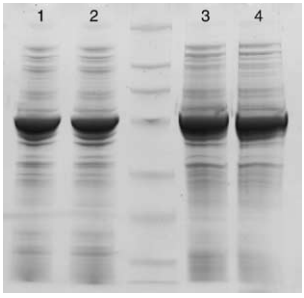

Fig. 6. Expression of target proteins in low-density cultures grown in PET bottles. Polyacrylamide gel electrophoresis of APC172 expressed in (1) 1 liter sterile LB medium in a 2-liter baffled Erlenmeyer flask, (2) in 1 liter of non-sterile LB medium in a 2-liter PET bottle, (3) 1 liter sterile M9 medium in a 2-liter baffled Erlenmeyer flask, and (4) in 1 liter non-sterile M9 medium in a 2-liter PET bottle. In all cases, the cultures were incubated at 37 °C to $OD_{600} = 0.5$ and induced by addition of 1 mM IPTG. Induction occurred overnight at 20 °C and 30 °C, respectively, for the LB and M9 medium cultures. Total cell lysates were compared. Molecular weight markers (unlabeled lane) are described in the legend to Fig. 3.

the time course of the experiment (Table 4). Comparison of protein expression showed that equivalent high amounts of the target protein were produced under all culture conditions (Fig. 6).

Discussion

The critical difference between the protocols described here and conventional approaches to protein production is the use of non-sterile media and disposable flasks; these are the adaptations that save time and reduce labor. With the elimination of sterilization and cleaning of flasks, virtually all non-productive handling of culture vessels is avoided, and production experiments occupy little time more than that required for microbial growth and expression. Sterilization can be bypassed because of the presence of antibiotics in the production medium. Structural and functional genomics experiments almost always produce proteins using expression systems that require addition of an antibiotic to maintain an expression vector, and frequently a second antibiotic is needed to maintain a helper plasmid that provides rare tRNAs. Our results show that these antibiotics also effectively prevent the growth of contaminants in non-sterile media for a longer period of time than is needed for the expression of the target proteins (Fig. 2, Table 4). This condition is clearly essential to the successful implementation of the approach, and is insured by preparation of the medium immediately prior to inoculation and by the use of a healthy inoculum. In repeated control experiments, uninoculated cultures always had final OD_{600} values less than 0.05 at the time when the experimental cultures, with OD_{600} values between 3.5 and 20, were harvested. The absence of growth in uninoculated controls also indicates that the approach described here successfully avoids cross-contamination by other ampicillin and kanamycin resistant strains present in the laboratory, a major concern in high-throughput protein production facilities. The use of a disposable vessel avoids potential contamination through the reuse of culture vessels; after harvesting the cells, the bottles are simply discarded.

Because of their notches, PET bottles provide efficient aeration of cultures and support the production of high-cell densities with strong overexpression of soluble proteins as well as baffled Erlenmeyer flasks (Figs. 2–4). Very high yields of pure protein were obtained from a single bottle containing 250 ml of a high-density culture (Table 2), and proteins isolated from these cultures had properties equivalent to those isolated from low-density, conventional cultures. Three proteins were purified from cultures grown under both conditions and screened for crystal formation. Of the three, one crystallized, yielding similar crystals under similar conditions from both preparations. Likewise, the kinetic parameters measured for a target protein of known function, the *E. coli* periplasmic glycerophosphodiester phosphodiesterase, were similar for the enzyme purified from cells cultured under both conditions (Table 3). These results indicate that expression of target proteins in high-density cultures in the PET bottles provides a satisfactory source of material for structural and functional characterization. In support of this conclusion, three structures have recently been deposited in the PDB for proteins expressed in non-sterile TB in PET bottles: PDB identification codes 1MK4, 1NC5, and 1NNI (www.mcsg.anl.gov, www.rcsb.org/pdb).

The bottles were also able to support growth and expression of heterologous proteins in 1-liter volumes of

LB or M9 medium, in spite of their small cross-section. When 1-liter cultures were induced at low cell density and shifted to 20 or 30 °C, cell yield was only slightly lower in the bottles compared to baffled Erlenmeyer flasks. The final cell densities obtained in bottles were approximately 85–90% of those in the flasks (Table 4), and expression of a representative target protein was equally strong in both cases (Fig. 6). Sterilization of these media was not required either, as no growth occurred in uninoculated, non-sterile controls incubated in parallel to the experimental bottles. These results indicate that the approach should be applicable to expression experiments conducted in less rich media, such as LB, that generate lower cell densities, or in minimal media such as M9, which allow the incorporation of selenomethionine or isotopically labeled amino acids for crystallography or nuclear magnetic resonance experiments [18–20], saving time and reducing the effort required for these experiments as well.

The methods described here require only commonly available laboratory equipment and employ standard techniques used in all laboratories. Unlike in vitro expression systems or miniature fermentation banks [7,21] no special expertise or equipment is required, making the approach suitable for diverse research situations, from small laboratories to high-volume, high-throughput production facilities. One drawback of the method is that there currently is no simple way to purchase the bottles. Almost all 2-liter PET bottles are produced for captive, local markets of soft drink bottlers. The bottles are not normally sold in small volumes. Nonetheless, we were able to consign a shipment of over 2200 bottles with little difficulty through contacts made in the packaging and bottling industries. The 2-liter PET bottles may, in the end, merely be convenient surrogates for a future, optimized disposable culture vessel designed specifically for high-throughput production of proteins for genomic studies. Bottles of different sizes or shapes would be desirable, as would bottles constructed of a more easily degraded polymer to reduce waste generation. If the general approach—production of proteins in non-sterile media in disposable vessels—is widely adopted, manufacturers may develop new bottles specifically tailored for microbiological applications, but they will most likely cost more. In the interim, at least, 2-liter PET bottles provide a readily available source of disposable vessels that can reduce labor costs and free staff for other experiments.

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