Article title

Recent developments in protein extraction methods from bacteria, yeast and natural sources for various applications

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Keywords

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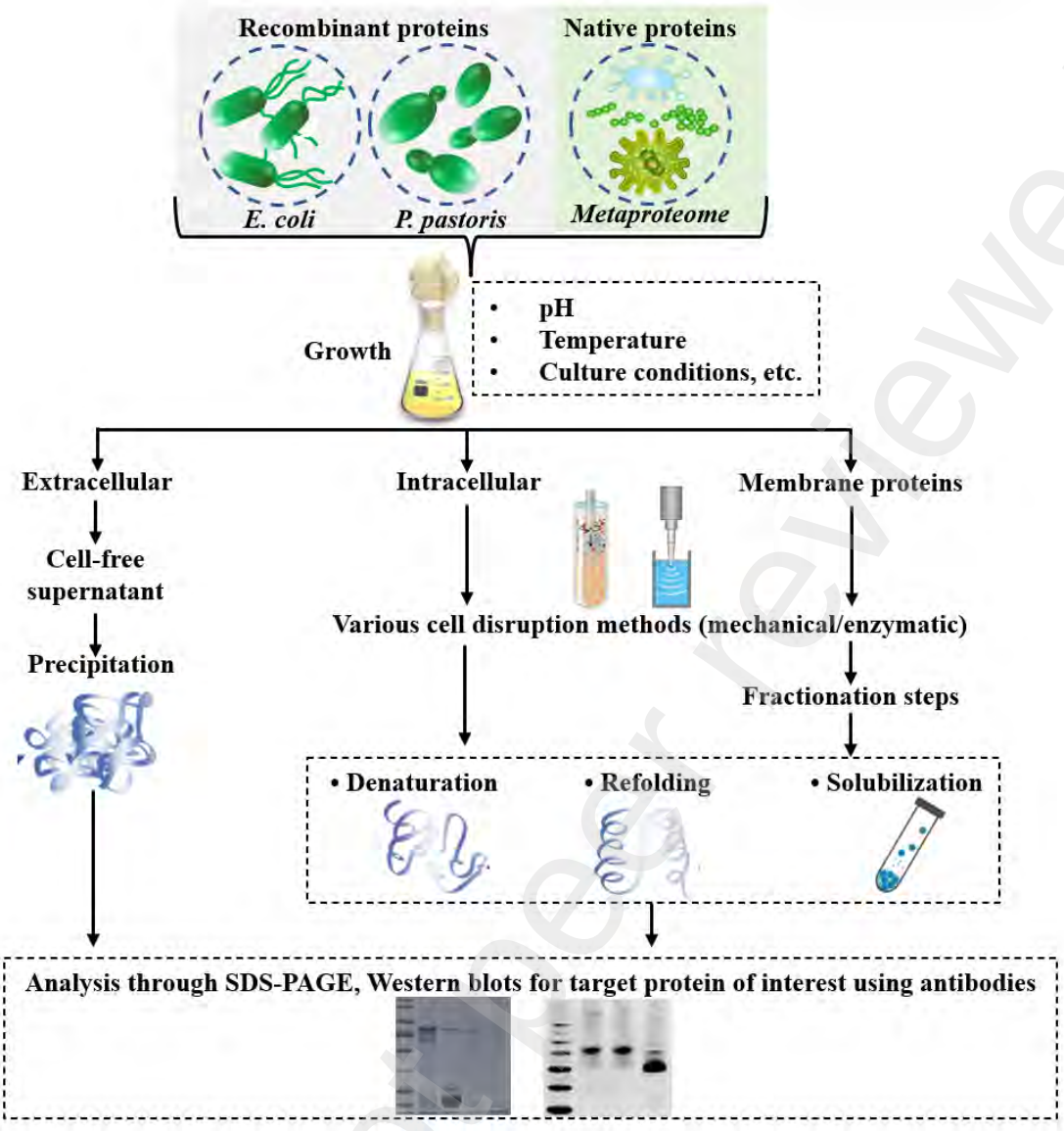
Abstract

Successful application of protein isolation and extraction method relies on the efficient characterization of target proteins. Proteins have been recovered from pure microbial cultures (recombinant and natural) for various downstream applications through strategies that minimize degradation and maximize the production of bioactive proteins from hosts. Ideally, a procedure for maximum protein recovery depends on three critical parameters: the design of suitable expression vectors, optimization of culture conditions, and the selective use of chaotropes, mild detergents, solubilization agents and/or osmolytes after the production of proteins. Following various observations, this review discusses procedures to resolve existing challenges that may pave the way for futuristic approaches in successful protein extraction and purification protocols from *E. coli*, *P. pastoris*, and natural environmental sources.

Review Highlights

- This review is intended to provide the readers with a holistic overview of the heterologous protein extraction processes from prokaryotic and eukaryotic host systems as well as challenges in methods to isolate native proteins (metaproteome) from naturally occurring microbial communities.
- Comprehensive procedures of extracellular, intracellular, membrane protein and inclusion body-based isolation methodologies of proteins from *E. coli* and *P. pastoris* discussed here allow a deeper understanding of the various challenges in the field.
- The scope of this review is to provide examples to researches for improving extraction efficiencies of difficult-to-isolate proteins from complex environments that the authors think is promising for further research.

Graphical abstract



Specifications table

Subject area	*Biochemistry, Genetics and Molecular Biology*
More specific subject area	*Protein purification for downstream applications*
Name of the reviewed methodology	*Protein extraction from prokaryotic, eukaryotic and naturally occurring microbial communities*
Keywords	Protein extraction, Cell lysis, Membrane protein, Inclusion bodies, Escherichia coli, Pichia pastoris, Metaproteome
Resource availability	*Not applicable*
Review question	What are the different techniques for extracting intracellular, extracellular, membrane-bound and inclusion body proteins from recombinant host cells? What is the impact of existing methods in the field of protein extraction and purification and how can they be developed in the future?

Method details

Factors affecting the expression of heterologous proteins in E. coli:

A number of factors control the final fate of the overexpressed protein in *E. coli*. For example, the size of the heterologous protein, codon biasing, toxicity, localization (membrane or cytoplasmic), post-translational modifications and loss of secondary structure with the ability to form insoluble aggregates during production require optimizations at several levels [1]. Expression of recombinant proteins has also been fruitful by the construction of vectors with fusion tags. For example, glutathione S-transferase (GST), maltose binding protein (MBP), hemaglutinin antigen (HA), and poly-His/Arg tags aid in successful soluble expression and/or purification [2]. Optimization is sought at two levels to obtain good quality heterologous protein from *E. coli*: ensuring overexpression within the host and stepwise extraction by cellular disruption methods. Culture conditions including concentration of inducer, induction temperature, the addition of glucose (for diauxic growth), optical density, and addition of additives, are crucial parameters ensuring overexpression and soluble expression of proteins in *E. coli* [3]. For the latter, different types of cellular disruption methods are known. The nature of buffers, ionic concentrations and detergent concentrations are crucial towards an effective isolation process of membrane proteins or proteins that form inclusion bodies. Use of nonionic (e.g., dodecyl maltoside), ionic (anionic or cationic) (e.g., SDS, LTAB) and zwitterionic (e.g., FOS-Choline 12) detergent-based protein extraction methods in bacteria has proven to be effective, but the removal of such detergents to obtain purified yields remains questionable [4]. Recently, it has been shown that the incorporation of a nuclease treatment step has been effective during the extraction process for getting structurally and functionally active yields of proteins [5].

Extraction methods using detergents:

The traditional approach both for the successful extraction of proteins from inclusion bodies and membranes in *E. coli* involves four critical levels (isolation, solubilization, refolding, and purification) that are presently used in laboratory and industry-scale productions. Membrane Proteins of Known Structure database (mpstruc/mpstruc+) (https://blanco.biomol.uci.edu/mpstruc/) and Protein Data Bank (PDB) (https://www.rcsb.org/) is useful to know more about the buffer compatibilities of individual proteins keeping in mind the variation of amino acid sequences, physico-chemical properties of their local environments and resultant isoelectric points that determines the pH of solutions for purification [6–9]. Alternatively, several modifications to the application of Triton X-100, sarkosyl or denaturants like urea in alkaline pH with presence of chelating agents like EDTA has come up in the last decade for inclusion body extractions [10]. Herein, the authors put forward a protocol using a mild solubilizing agent for efficient extraction of membrane proteins that the authors feel may substantiate further research in studying membrane proteins from *E. coli*.

Extraction of membrane proteins from *E. coli* using diglucosylated derivatives

Membrane protein extraction using diglucosylated derivatives has been described by Guillet et al., 2019.

- Briefly, *E. coli* BL21(DE3) cells are first harvested, washed with 154 mM NaCl and ultrasonication of cell pellets is performed twice for 10 min after resuspension in 100 mM Na_2CO_3 (pH 11.5).

- Cell debris are removed by centrifugation at 4 °C for 30 min at 1000g and membrane fragments are separated from soluble and peripheral proteins by centrifugation of the resultant supernatant at 4 °C for 1 h at 150000g. Membrane fraction is resuspended in 50 mM Tris, 200 mM NaCl (pH 7.4) with a protease inhibitor cocktail to a final concentration of 100 mg wet-weight pellet per 1 mL of buffer, and stock solutions of n-dodecyl-D-maltoside (DDM) or di-glucosylated (DG) compounds in buffer are mixed in a 1:1 volume ratio.

- Finally, samples are incubated for 16 h at 20 °C with continual, gentle agitation. The supernatant containing micelles is examined for the target protein after ultracentrifugation at 4 °C for 1 h at 100000g [11].

Protein production and membrane fractionation of *E. coli*:

Guillet et al., 2019 have also elucidated the use of bead beater homogenizer for BmrA membrane protein extraction of *E. coli*.

- In this method, induced cell pellets are initially treated with phosphate-buffered saline (PBS) supplemented with protease inhibitor cocktail on ice and cells are mechanically lysed using a bead

beater homogenizer with 0.1 mm glass beads giving 5 pulses of 30 sec and 2 min pauses in between each pulse.

- Sequential centrifugations of 1000g for 5 min, 15000 g for 30 min, and 100000g for 45 min are used to fractionate the internal (15000g) and plasma membrane fractions (100000g) at 4 °C which are then flash-frozen and kept at -80 °C in 20% glycerol.

- To solubilize internal and plasma membrane fractions, 2, 5, 10, and 20 mM of DDM, n-Octyl-β-d-Glucopyranoside (ODG), (N-(2-methyl-1,3-bis(O-β-D-glucose)propan-2-yl)-3-(decylthio)propanamide (DDG), or (N-(2-methyl-1,3-bis(O-β-D-glucose)propan-2-yl)-3-(dodecylthio)propanamide (DDDG), respectively, are added to PBS and protease inhibitor cocktail and incubated for 2 h at 4 °C.

- The samples are centrifuged at 100000g for 45 min at 4 °C after solubilization, and an aliquot of the total extract, the pellet, and the supernatant from each solubilization condition are analyzed for the particular protein of interest.

This procedure has been successfully applied for the extraction of BmrA protein of *E. coli* and the authors proclaim that detergents like DDM, n-Nonyl-β-d-Glucopyranoside (NDG), ODG, Lauryl maltose neopentyl glycol (LMG) and Lauryldimethylamine-N-oxide (LDAO) are often used for the initial screening test in their laboratory for assessing protein stability on small-scale [7,11]

Extraction methods using styrene: maleic acid (SMA) co-polymers:

Many of the drawbacks of traditional detergent-based methods can be overcome by the use of SMA co-polymers to extract and purify transmembrane proteins while preserving their original bilayer environment. Morrison et al. [12] have reported an efficient methodology using SMA co-polymers for membrane protein extraction and purification. Herein a summary has been described in the following sub-steps:

- SMA preparation: Firstly, 10% (w/v) commercially available SMA (1000, 2000 and 3000 variants) solution is hydrolyzed using 1 M NaOH by refluxing for 2 h and the polymers are allowed to precipitate at room temperature by addition of excess HCl. After washing, the polymers are neutralized to pH 8 using 0.6 M NaOH and freeze dried for further use.

- Protein production and membrane preparation: BmrA transformed C41 (DE3) *E. coli* cells are used to inoculate ampicillin containing Luria broth and grown at 37 °C, 200 rpm until OD_{600} reached 0.6. After induction with 0.5 mM IPTG for 18-20 h at 25 °C, cells are harvested. Resuspension of cell pellets are carried out in protease inhibitor containing lysis buffer and sonicated on ice. Membranes are obtained by ultracentrifugation (100000g, 20 min, 4 °C) after unbroken cells and debris are removed by a low-speed spin (650g, 20 min, 4 °C). At a final concentration of 60 mg/mL wet membrane weight, the membranes are resuspended in buffer, aliquoted, and kept at -80 °C.

- Solubilization trials: SMA 2000 is initially tested against the traditional detergents ODG and DDM. Membranes containing each target protein (30 mg/mL wet weight) are kept for 1 h at room temperature with gentle shaking supplemented with 2.5% (w/v) SMA 2000, 2% (w/v) ODG, or 2% (w/v) DDM. Following a centrifugation at 100000g for 20 min, the solubilized protein-containing supernatant is obtained. Further, the insoluble pellet is redissolved in buffer with 2% (w/v) SDS and the samples of soluble and insoluble fractions are analyzed by Western blot. A similar procedure is followed to screen other SMA polymer variations.

- Ni-NTA affinity purification: Solubilized proteins are purified through Ni^{2+}-NTA resin in a gravity flow column setup. The resin is rinsed with buffer supplemented with 20 mM, 40 mM and 60 mM imidazole followed by elution with 200 mM imidazole. All wash and elution buffers are additionally enhanced with 0.1% (w/v) DDM for purifications utilizing DDM. Fractions are analyzed using SDS–PAGE and elution fractions containing the target protein are pooled and stored at 4 °C.

More recently, partially esterified SMA polymers have been employed for membrane protein extraction and purification as they can solubilize both lipids and proteins effectively in complex scenarios [13].

Extraction methods from inclusion bodies:

Inclusion bodies are frequently formed when recombinant protein molecules aggregate during high-level production in *E. coli*. In the last decade, there have been established reports of purification of proteins from inclusion bodies that use mild solubilization agents instead of high concentrations of chaotropes which were often disadvantageous, as proven in earlier reports [14]. Whereas various researchers have reported yield variations at several environmental factors, with unique ideal conditions for proteins, current trends of application of design of experiment (DoE) tools and their statistical analysis can be a powerful measure for optimum recovery of proteins from inclusion bodies [15]. Herein the authors report two recent methods highlighting the significance of DoE in inclusion bodies extraction and purification.

In one such method described by Gutiérrez-González et al., 2019, the cumulative impact of post-induction temperature, post-induction time and IPTG concentration on the expression of MHC class I chain–related protein A (MICA), anti-MICA single chain variable antibody (scFv) and IL-23p19 was demonstrated using Box-Behnken method.

Thereafter, proteins were expressed in BL21(DE3) bacteria as HisTag fusion proteins. The method is outlined below:

- Cells are harvested after protein expression and then resuspended in a lysis buffer with 1% Triton X-100, lysozyme and protease inhibitors. Following sonication with 8 cycles of 20 sec and 40 resting sec, the inclusion body pellets from the centrifuged samples is collected, washed once with washing buffer containing 1% Triton X-100 and 0.1% sodium deoxycholate (DOC), and then treated with denaturation buffer containing 6 M guanidine hydrochloride.

- In case of IL-23p19, insoluble fraction obtained from centrifuged inclusion bodies is washed with the buffer without Triton X-100 and treated with the anionic detergent N-Lauroylsarcosine as denaturant. Resuspended inclusion bodies are centrifuged and taken for Ni-NTA matrix purification, which is carried out in the presence of 0.2% N-Lauroylsarcosine followed by gradual exchange of buffer until total dilution of N-Lauroylsarcosine.

- For MICA, purification is carried out in presence of 6 M guanidine hydrochloride. Denatured MICA fractions are combined and refolded by rapid dilution in renaturing buffer containing 3 mM glutathione (GSH) and 0.3 mM glutathione disulfide (GSSG). Keeping the final concentration <10 µg/mL, diluted proteins are concentrated after mixing overnight using 10 kDa centrifugal filters to a final volume <1 mL. Renaturation is confirmed by size-exclusion chromatography in FPLC.

- For scFv, in-column refolding is done by serially switching from denaturing washing buffer to washing buffer and eluting out the refolded protein.

Following elution, all three proteins were assessed by SDS-PAGE and confirmed through western blot and/or MALDI-TOF/TOF [16].

In another report, Vincenti et al., 2021 have also studied the effect of temperature, inducer concentration and induction duration to obtain optimized soluble hydroperoxide lyase (HPL) production using Box-Behnken method. Furthermore, using the optimized conditions, large-scale production of recombinant HPL was successfully performed. Soluble enzymatic activity reached 20,740 ± 537, 20,634 ± 486, 21,920 ± 356 and 20,687 ± 980 U L^{-1} of culture with various *E. coli* strain/expression vector systems such as M30, I30, B19 and A19 expression systems, respectively. Protein expression was analyzed from the soluble fractions by SDS-PAGE and Western blot [17].

Eukaryotic protein extraction methods from Pichia pastoris

Eukaryotic cells are more complicated than prokaryotic cells and have a more complex protein synthesis, folding, and modification mechanism. Eukaryotic membrane proteins, on the other hand, have unique co-translational and post-translational processing needs and membrane lipid requirements. Bacterial expression methods are insufficient in producing accurately folded eukaryotic proteins. Cell lines from insects and mammals can have several advantages in recombinant eukaryotic protein expression, but the cloning and

maintenance of these cells are exorbitant. A good middle ground is attained with yeast expression systems, which combine the benefits of ease of manipulation and inexpensive production costs with eukaryotic protein processing [18]. *Saccharomyces cerevisiae* and *Pichia pastoris* (*P. pastoris*) are the two most important yeast expression systems widely accepted by industry and academia [19]. *P. pastoris* is a methylotrophic yeast that can use methanol as its solitary carbon source. One key aspect of the *P. pastoris* expression system is its suitability for large-scale growth and culture in bioreactors [20]. Bioreactors allow the user to precisely regulate culture parameters such as OD_{600}, pH, dissolved oxygen, aeration, feed rate, and temperature in real-time [21]. The tunable culture conditions of the bioreactor permit strictly regulated culture development (>100 g/L dry cell weight), enabling ultra-high cell densities (>500 OD600 units/mL) [20]. Medium-density cultures (100 OD600 units/mL) are more commonly used for membrane proteins because they alleviate proteolysis and cellular stress with high-density cultures [22,23]. All the features mentioned above and the requirement of an inexpensive growth medium make *P. pastoris* a highly efficient and cost-effective expression system.

There are only a few vectors for *P. pastoris* expression, but the mostly employed vectors have strong, inducible alcohol oxidase I (AOX1) promoters. This promoter regulates the expression of two methanol metabolism genes, AOX1 and AOX2. The AOX1 promoter is inhibited in the presence of glycerol or glucose but substantially upregulated in the presence of methanol, indicating that the expression system is rather tightly controlled [24]. Based on the type of vector used and the nature of the gene product, the protein expression in *P. Pastoris* can be intracellular or extracellular. The sample preparation for extracting intracellular or extracellular protein from recombinant *P. pastoris* can be done using the following protocol [25]:

- Inoculate 25 mL of Buffered Glycerol Complex (BMGY) media in a 250 mL baffled flask with a single colony. Grow the culture at 28-30°C in a shaking incubator (250-300 rpm) until the OD_{600} = 2-6 (log-phase growth, 16-18 hours).
- Centrifuge the cells for 5 minutes at room temperature at 1,500-3,000 × g. Pour off the supernatant and resuspend the cell pellet to an OD600 of 1.0 in buffered minimal methanol (BMMY) medium (about 100-200 mL) to induce expression.
- Fill a 1 liter baffled flask halfway with culture. Transfer the flask to the incubator and wrap it with two layers of sterile gauze or cheesecloth to continue development.
- Add 100% methanol to a final concentration of 0.5% every 24 hours to retain induction. Monitor the amount of the culture and add methanol as required. Evaporation may decrease the volume of the culture.
- After 72-96 hours, transfer the supernatant to a new tube for secreted expression. Quickly freeze the supernatant and cell pellets in liquid N_2 or a dry ice/alcohol bath, then store at -80°C until ready for further processing.
- For intracellular protein expression, centrifuge the cells at 1,500-3,000 × g for 5 minutes and decant the supernatant. Store the cell pellets at -80°C until ready for further processing.

Different methodologies used for protein extraction from intracellular and extracellular fractions are explained below.

Protein extraction from extracellular fraction

P. pastoris can produce high titers of fully folded, post-translationally processed, and active recombinant proteins into culture media. In *P. pastoris*, the most commonly used secretion signals are derived from *S. cerevisiae* α-mating factor (α-MF). The α-MF signal sequence consists of a pre- and pro-region, which guides protein via the secretory route. The pre-region guides the nascent protein to the endoplasmic reticulum (ER) post-translationally and cleaved off by signal peptidase. The pro-region is considered to have a role in protein translocation from the ER to the Golgi compartment, which is later cleaved at the dibasic KR site by the endo-protease Kex2p [26]. Proteins released into the medium are typically more than 50% homogenous and require further purification. There are various ways to concentrate secreted *P. pastoris* proteins. One general method for extracting secreted protein from *Pichia* is as under [27]:

- Precipitate the supernatant having secreted protein using 80% saturated ammonium sulfate.
- Resuspend the precipitate in 20 mL (PBS) phosphate-buffered saline (300 mM NaCl, 20 mM potassium phosphate pH 7.4).
- Dialyze the mixture against PBS buffer to remove the ammonium salt.

- If the expressed protein is 'tagged', proceed with affinity chromatography; else, concentrate the sample using a size-specific molecular weight cutoff (MWCO) protein concentrator followed by size exclusion chromatography [28].

Protein extraction from intracellular fraction

Various methodologies for extracting intracellular protein from *P. pastoris* include mechanical and non-mechanical lysis of cells. Mechanical cell lysis of *P. pastoris* cells can be done using glass bead beating, sonication, or high-pressure homogenization. Non-mechanical methods include enzymatic or chemical breakdown of cells. Using enzymes or chemicals for cell lysis can interfere with the protein product and increase downstream costs; hence non-mechanical techniques are substantially avoided. Bio-mechanical cell lysis methods include a small amount of cell wall disrupting enzyme flow by mechanical cell lysis. The methodologies of widely used intracellular protein extraction methods for *P. pastoris* are listed below [29,30].

Mechanical intracellular protein extraction method(s):

- Thaw frozen cells on ice for 30 minutes before proceeding with cell lysis.
- To per gram of wet cell weight, add 1.5 mL of ice-cold lysis buffer (2 mM Dithiolthretol, 30 mM HEPES pH 7.4, 2 mM Magnesium acetate, 0.5 mM phenylmethylsulfonyl fluoride, 100 mM Potassium acetate).
- For sonication lysis, disrupt 10 mL of resuspended cells using a sonicator (equipped with a type of probe suitable for desired sample volume) with 45 seconds ON and 60 seconds OFF cycle at 50% of amplitude for five cycles.
- For high-pressure homogenization lysis, lyse 10 mL of resuspended cells using a homogenizer with two passes at a pressure of 1,200 bar.
- For bead-beating cell lysis, mix 10 mL of the resuspended cell with 0.5 mm glass beads in a 50 mL falcon tube. Maintain a mass ratio of 1:1 (cell: bead, g/g). Vigorously vortex the mixture using a vortex machine for 40 cycles, maintaining 1 min on vortex and 1 min on ice.
- After cell lysis using a sonicator/homogenizer/bead beating, transfer the lysate to a fresh falcon tube and centrifuge at 30,000 g and 4°C for 30 min.
- Remove the supernatant and perform buffer exchange using a dialysis bag of 3.5 kDa MWCO membrane.
- Dialyze the lysate against 50 volumes of freshly prepared dialyzing buffer (2 mM Dithiolthretol, 30 mM HEPES pH 7.4, 2 mM Magnesium acetate, 0.5 mM PMSF, 100 mM potassium acetate) for 30 minutes at 4°C. Repeat this step four times.
- If the expressed protein is 'tagged,' proceed with affinity chromatography; else, concentrate the sample using a size-specific molecular weight cutoff (MWCO) protein concentrator followed by size exclusion chromatography.

Bio-mechanical intracellular protein extraction method(s):

- Thaw frozen cells on ice for 30 minutes before proceeding with cell lysis.
- Add 10 volumes of PBS with 25 U/mL lyticase and 0.5 mM PMSF per gram of wet cell weight. Incubate the sample for 20 minutes at room temperature.
- Sonicate the sample with 45 seconds ON and 60 seconds OFF cycle at 50% of amplitude for five cycles. Perform this step at 4°C.
- Transfer the lysate to a fresh falcon tube and centrifuge at 30,000 g and 4°C for 30 min. Transfer the supernatant to another sterile falcon tube.
- If the expressed protein is 'tagged,' proceed with affinity chromatography; else, concentrate the sample using a size-specific molecular weight cutoff (MWCO) protein concentrator followed by size exclusion chromatography.

A comparative analysis of various mechanical cell disruption methods for isolating intracellular protein fraction from *P. pastoris* by Zhang et al., revealed that the highest yield could be obtained using the bead-beating approach.

Extraction of membrane proteins from P. pastoris

Several efforts have been attempted to enhance *P. pastoris* host strains and expression conditions for membrane protein synthesis. Using various tools to optimize soluble protein expression, such as modification of expression conditions, adding chemical chaperones, co-expression of chaperones or proteins activating unfolded protein response, use of protease deficient strains, and so on, has shown target-specific progress in membrane protein expression [26]. The protein extraction method for a membrane protein depends on various factors like nature, size, fold, localization, and membrane type. To date, numerous types of membrane proteins have been cloned and expressed in p pastoris. However, going through all of them would be practically difficult; hence we have listed some techniques from notable research articles for extracting various heterologous membrane proteins. However, there are many differences in each method mentioned in Table 1. The basic principle behind all of these is the same. These methods comprise cell disruption/homogenization of *Pichia* cells using mechanical cell disruptors in a buffer with components to maintain osmolarity, pH, and protease inhibitors to protect the heterologous protein from proteases. After homogenization, the protein with the membrane is subjected to solubilization using detergents. Tagged soluble fraction is then used for affinity chromatography, while untagged proteins are concentrated using an appropriate MWCO concentrator followed by gel filtration chromatography.

Table 1. Protein extraction methods for membrane protein expressed in *P. pastoris*.

Membrane Protein	Source Organism	Expression vector	*Pichia* strain used	Extra sequence (if any)	Genetic modification	Type of protein extraction method used	Reference
CmABCB1 (P-glycoprotein homologue)	*Cyanidioschyzon merolae*	pPICZ	SMD1163	C-terminal TEV protease cleavage + His tag	-	Mechanical (Homogenizer); 25,000 psi in a buffer containing 20 mM Tris HCl (pH 7.0), 150 mM NaCl, 1% (wt/vol) Nonaethylene glycol monododecyl ether	[31]
P-glycoprotein	*Caenorhabditis elegans*	pPICZ	SMD1163	C-terminal PreScission protease cleavage site followed by GFP and His tag	-	Mechanical (Cryomilling); Broken cells resuspended in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 20% glycerol, 5 mM MgCl2, 1 mM PMSF, 1 mM benzamidine, 0.1 mg ml−1 trypsin inhibitor, 3 µg ml−1 DNase, 1 µg ml−1 pepstatin A, leupeptin and aprotinin. Membrane solubilisation by 1% (w/v) n-dodecyl-β-D-maltopyranoside	[32]

SoPIP2;1 aquaporin	*Spinacia oleracea*	pPICZ	X-33	N-terminal 6His tag followed by a Thrombin protease cleavage site except	-	Mechanical (X-press-Biox AB) Peripheral membrane removed by urea/alkali treatment. Stripped membrane protein resuspended in 25ml of 50 mM NaCl, 2 mM β-mercaptoethanol, 10% glycerol, 20 mM HEPES–NaOH, pH 7.8, 50 mM. Resuspended protein was concentrated and solubilized in solubilized in 3% octyl-β-D-thioglucopyranoside.	[33]
AQP5 Aquaporin	*Homo sapiens*	pPICZ	X-33	No tag	-	Mechanical (Bead beating 0.5mm) Removal of peripheral protein by urea/alkali wash. Homogenization in buffer (2 mM β-mercaptoethanol, 20 mM Hepes-NaOH, pH 7.8, 10% (vol/vol) glycerol, 50 mM NaCl), Protein solubilisation in n-nonyl-β-D-glucopyranoside and n-octyl β-D thioglucopyranoside.	[34]
Leukotriene LTC_4 synthase	*Homo sapiens*	pPICZ	KM71H	N-terminal 6His tag	-	Mechanical (Bead beating 0.5 mm) Cell resuspension in 50 mM Tris-HCl, pH 7.8, 100 mM KCl and 10%	[35]

						glycerol; Membrane bound proteins solubilisation in sodium deoxycholate (0.5%, w/v) and Triton X-100 (1%, v/v).	
Histamine H1 receptor	*Homo sapiens*	pPIC9K	SMD1163	C-terminal GFP-8His	Codon optimised, mutated to remove N-linked glycosylation sites, N-terminal truncation, insertion of sequence encoding T4 lysozyme into the third intracellular loop	Mechanical (Bead beating 0.5 mm); Resuspension buffer (2 mM EDTA, 50 mM HEPES, pH 7.5, 5%(v/v) glycerol, 120 mM NaCl and protease inhibitor); Membrane protein solubilisation (0.2% (w/v) cholesteryl hemisuccinate and 1% (w/v) n-dodecyl-B-D-maltopyranoside)	[36]

Methods for Non-Recombinant (Native) Protein Extraction

Native protein extraction is often used to recover proteins from single pure culture organisms (proteomics), or a pool of proteins (metaproteomic) especially where there are limitations in culturing or genome sequence is unknown. It can be effective in obtaining proteins in the right folds and conformations, which recombinant protein expression frequently struggles with due to the formation of inclusion bodies caused by overexpression of non-native proteins. Nonetheless, the yield of recombinant proteins is lower than that of natural protein synthesis. Cell free extracts offers promising approach for high yield of protein [37]. As a result, the challenge is to recover as much proteins as possible from natural sources for enzyme discovery and suitable applications. Metagenomics investigations are often conducted in settings such as soil, freshwater, seawater, sediments, compost, and extreme environmental conditions likewise metaproteomics can be performed in tandem. Much like metagenomics, environment metaproteomic analysis is used to capture the true diversity of microbial proteins in the vast functional repertoire, and study microbial dynamics in the niche. It is necessitated by the fact that proteins, rather than nucleic acids, play an important part in real-time metabolic activities.

Challenges in obtaining metaproteome

Less than 1% of metaproteome protein is often recovered this is due to the limitations and biasness of extraction methods used. The intracellular proteome is frequently the focus of proteomics, however the extracellular portion has a lot of promise. The expected low protein content in external protein sample preparation is an obvious hurdle to overcome when compared to intracellular metaproteome analysis. The protein content in the sample is enhanced while evaluating intracellular proteins due to centrifugation and cell pelleting in the cell washing processes prior to cell lysis. The supernatant, on the other hand, must be collected after centrifugation for investigation of the extracellular fraction. When working with the extracellular fraction, the first straightforward concentration step of cell pelleting is no longer applicable, and alternative sample concentration procedures must be used. There is a loss of portion of extracellular fraction because

these proteins adhered to contaminants are eliminated. There might be difference in properties of both the fractions. To overcome methodological restrictions and enhance extraction of both extracellular and intracellular extracts, present approaches must be addressed [38].

Cultivation of cells and pretreatment to obtain native proteins

After determining the environmental conditions and parameters for growth, the microorganisms that are not cultured by nutrient media or standard laboratory conditions are often cultivated by supplementing the sterilised constituents from their milieu such as filter sterilized water or minimal salt media along with substrates to enrich cells that can be used as a growth medium. The growing conditions, extraction procedures, and post-processing stages differ depending on the lab, organism, and application. This is followed by cell lysis to retrieve the native protein produced. Following lysis, extracts undergo many stages, including several centrifugations, incubation, and dialysis [38,39]. In order to increase the yield of protein extracts, shake flasks have been employed in most studies in recent years due to their lower labor, skill, and equipment costs compared to fermenters. Flasks might also make it easier to investigate protocol optimizations. Shake-flask scale biomass production is feasible (1 L of cell culture in a 4 L Erlenmeyer flask). For cell cultures of 1 L, typical yields of the crude extract are between 1-2 mL. Growth-maximizing baffled flasks are widely employed since most methods are concerned with preserving rapid growth and aeration before capture at the culture mid-log phase. The majority of current techniques determine the harvest point by measuring the optical density at 600 nm during the mid-log phase, which is when translation machinery is most prevalent [39,40]. Protocols of Beloqui et al., 2010 followed for pretreatment of various samples is briefly described [41]. Pre-treatment with 20 L liquid samples such as sea water samples, where the majority of microorganisms are not cultivated, it is filtered onto a 500 kDa Nominal Molecular Weight Limit (NMWL) ultrafiltration disc such as made from polyether sulfone. After that, the filters are cut into strips (1 cm × 2 mm). For large water samples 200 L, a tangential flow filtration system (TFF), such as Pellicon TFF 0.1 µm (Millipore^{TM}) can also be used to separate solid particles and pico-eukaryotic organisms (2-4 µm). A retentate solution 10 mL, can be used to enrich cells with the desired supplement or above steps for small samples can be followed. A Nycodenz extraction method (cell disruption buffer: 0.2 M NaCl, 50 mM Tris-HCl pH 8.0) is strongly advised to prevent protein degradation or inhibition by matrix contaminants particularly for solid samples such as soil during purification from environmental materials, although it is not advised for biofilms. Biofilms require acids such as sulphuric acid wash. If the sample has high humic acid content, 0.1 M NaOH is used to elute it out followed by phenol chloroform isoamyl alcohol isolation and 0.1M amoonium acetate in methanol precipitation and subsequent washing with ammonium acetate, 80% acetone and 70% ethanol 2ml each. For 5g of soil 40 mL of the Tris Ethylenediaminetetraacetic acid (TE) buffer pH 8.0 is mixed, and inverted and low-speed centrifugation (400g for 5 min) is done to separate bulky soil particles. Then soil samples are centrifuged at 6000g for 15 to 30 min and stored at 4 °C. The Nycodenz solution is added and similar to the above step the sample is processed instead of TE buffer, the cell disruption buffer is added. After being resuspended in 0.5–2.0 mL of pH 8.0 TE buffer for pure culture, the cells pellet is prepared for lysis and protein extraction (10^5–10^{10} cells are strongly recommended for efficient extractions yields). Some samples only require a centrifugation step to isolate microorganisms, while others can be used straight for protein extraction

Methods to isolate native protein

In metaproteomic, complex protein mixtures from environmental samples are frequently separated using two-dimensional (2D) gel electrophoresis or high-performance liquid chromatography [41,42]. Two-dimensional electrophoresis can be used to separate and resolve hundreds of proteins in a microbial community with more than 100 species, and it can be used to identify proteins from various bacteria with unsequenced genomes. Recombinant protein production dwarfs that of native protein because native protein expression is constrained in bacterial species. It is challenging to stabilize proteins in the presence of denaturants like SDS, CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate), urea, or thiourea as well as other detergents, which are employed to lyse cells, according to current methods, which tend to refold inclusion bodies, denature and fragment proteins spontaneously in the nutritional medium [43,44]. Optimal conditions optimize cell lysis and the proportion of recombinant protein retrieved while avoiding protein oxidation, undesired proteolysis, and sample contamination with genomic DNA. It is possible to relieve the stability pressure by isolating native proteins. In order to acquire the desired metaproteome or proteins, a variety of cell lysis techniques are used, such as mechanical sonication, high-pressurized shear flow using French press homogenizer, bead-beating,

physical methods like freeze-thawing, and the use of enzymatic methods such lysozymes, in most circumstances it is similar. Each technique has its benefits and drawbacks.

- *Sonication*: It causes mechanical shearing and requires ice for cooling in order to avoid protein denaturation with 80W power by using a 3-mm-diameter probe for 3 min (1-s pulses) with cooling in an ice bath, in addition to the lysis buffer. The yield of protein is 0.04–0.2 mg proteins per 10^7 bacterial cells / protein 79 to 105 μg ml^{-1} of the sample. For biofilms, sonication at high power 30s pulses for 10 min in 0.1M sodium acetate pH 5.0 is required.
- *Freeze-Thaw method:* This method employs freeze-thaw cycles from liquid nitrogen baths to 30 °C incubation in lysis buffer. Similar to the above method, it is susceptible to degradations but it can depend on the type of cells. Approximately 0.01–0.19 mg proteins per 10^7 bacterial cells is obtained.
- *Combination of the Freeze-Thaw and sonication:* Average extraction yield: 0.01–0.22 mg proteins per 10^7 bacterial cells.
- *Bead beating* for sample attached to the matrix such as compost and fecal samples, glass beads ≤0.1mm is generally used. The heat generation is low than sonication and can be used for samples from cold environment. For these conditions, the extraction yield is roughly 200 μg proteins per 5 g of sample.
- *French Pressure Cell Press:* The solubilization of inclusion body aggregates is also facilitated by high hydrostatic pressure. Intermolecular connections are broken off by high pressure (2-4 kbar), which also causes inclusion bodies to disassemble.
- *Detergent methods:* detergents such as CHAPS, Triton X-100 are combined with combination with enzymatic methods such as lysozyme, 600 mg mL^{-1} and EDTA, 2mM chelating agent, these can act as denaturants such as however use of nondenaturating agents such as Triton X-100, 0.1% β-octylglucoside, can alleviate such problems. Average extraction yield in this method is 0.01–0.15 mg proteins per 10^7 bacterial cells.

For intracellular native protein extraction

Protocol of Beloqui et al., 2010 is followed throughout the extraction process with minor modifications:

- In the presence of protease inhibitors such as protease inhibitor cocktail, add two volumes of lysis buffer per volume of cells: 50 mM glucose, 10 mM ethylene diamine tetraacetic acid (EDTA), 25 mM Tris-HCl, pH 8.0, 0.6 mg mL^{-1} lysozyme, and 5 g mL^{-1} DNAse.
- Incubate the suspension at room temperature for 30-45 minutes before cooling it on ice. Sonicate the bacterial cells in lysis buffer for 6 cycles at 20% amplitude with 5 seconds on and 30 seconds off.
- Alternatively, French press homogenizer can be employed to disrupt cells resilient to plasma membrane disruption.
- Keep the tube on ice at 4°C for 2h during the entire sonication step since a lot of heat is released during sonication.
- After centrifugation (35,000 g, 35 minutes, 4°C), the supernatant is ready for protein precipitation, concentration, and further analysis.

The average extraction yield is about 260–570 μg proteins per 10^7 bacterial cells, depending on the complexity and biodiversity of the environmental samples, with lower yields for samples containing a higher number of eukaryotic cells.

For membrane proteins isolation:

Similar to above, method of Beloqui et al., 2010 is followed throughout the extraction process with minor modifications:

- The collected cells (at least 107 cells) are resuspended in 10 ml of 100 mM Triton X-114 extraction buffer (2.0% v/v Triton X-114, 300 mM NaCl, 20 mM Tris-HCl pH7.5), incubated for 2 hours at 4 °C in a shaking water bath, then sonicated on ice for 15 minutes for 5 cycles at 20 KHz with 1 minute off.
- Centrifugation at 30,000 g for 30 minutes at 4 °C separates the membrane and cytoplasmic components. Dialyzed against the same buffer, cytoplasmic proteins are kept at 20°C until usage.
- The sarkosyl method of Filip et al., 1973 is used to create the membrane fraction. To summarise, the membrane fraction was produced as previously described, then resuspended in an equal volume of

buffer, 100 mM sodium citrate (pH 3.0), containing 2% (w/v) sodium-lauryl sarcosinate, and 150 mM NaCl, and incubated at 37 °C for 1 hour to assist membrane solubilization [45].

For protein extraction from biofilms

It is critical to maintain a reducing environment for intracellular proteins. Indeed, sodium and chloride levels in the cell are extremely low, and they are almost certainly never physiologically meaningful counter-ions for intracellular proteins. In contrast to dithiothreitol (DDT), tris(2-carboxyethyl)phosphine (TCEP) is compatible with all known Immobilized Metal Affinity Chromatography (IMAC) matrices. Finally, the addition of 10% glycerol during protein purification improves the solubility and stability of many proteins. The following membrane extraction approach with acid treatment is appropriate in cases of cells that are firmly adhered to the matrix, such as acid mine drainage (AMD) biofilms. This technique is suitable for producing massive amounts of mass spectrometry data.

- Put cells back into suspension in 12 ml of either H_2SO_4 (pH 1.1) or 20 mM Tris-SO_4 (pH 8.0).
- After that, suspensions are put on ice and subjected to 10 minutes of high-intensity, 30-second pulse sonication using a microprobe.
- Supernatants are diluted with 40 ml of either H_2SO_4 (pH 1.1) or 0.1M sodium carbonate (pH 11.0) and rotated for 30 min at 4°C after centrifugation at 5,000 g for 20 min.
- The precipitates are removed by centrifuging these at 6,000 g, and the supernatants are centrifuged at 100,000 g for 1 hour.
- The ultracentrifugation process is repeated, and each membrane pellet is resuspended in 1 ml of the same solution after being washed once by resuspension in the appropriate buffer.

For cytoplasmic proteins, extraction yields ranged from 60 to 763 µg per 10^{10} cells, and for membrane proteins, they ranged from 12-51 µg per 10^{10} cells. Although the prior approaches were all equally effective, the last example is strongly advised for complex communities due to the low effectiveness of the membrane disruption processes.

Native Protein precipitation

The method of Speda et al., 2017 was followed for precipitation which yields clear spots of less abundant proteins in 2D gel electrophoresis [38].

- The samples were treated with 4 vol of 20% (w/v) Trichloroacetic acid (TCA) in acetone and 0.2% DTT (w/v) to achieve a final concentration of 16% TCA and 0.16% DTT.
- 0.2 mL of 3% sodium deoxycholate (DOC) was added to get a final concentration of 0.03% DOC.
- After 30 minutes on ice, 4 mL of 100% TCA was added to achieve a final concentration of 20% TCA.
- The samples were vortexed and incubated at 4 °C overnight.
- The precipitated proteins were pelleted by centrifugation at 15000 g for 10 minutes at 4 °C.
- The supernatant was discarded, and the pellets were resuspended in ice cold acetone for 5 minutes at room temperature before being centrifuged for 10 minutes at 15000 g qt at 4 °C.
- This washing step was repeated and the pellets were left to air-dry. The proteins can then be examined.

One can remove the stability pressure by concentrating and avoid loss by denaturation due to precipitation. By using ultracentrifugation or by Amicon concentrator, protein concentrates can be obtained.

Research needs

The use of computational methods to analyze protein structure and identification of potential product-related contaminations that could be formed during refolding can help in circumnavigating the existing challenges in inclusion body purifications by improving the overall efficacy of recovery of the active proteins. Supplementary to DoE, high-throughput process development (HTPD) techniques for exploring a wide variation of process parameters could be useful in obtaining increased protein extraction efficiencies. Also, process analytical technology (PAT) tools in real-time monitoring enhance the biologically active yield of proteins by minimizing human labour [46]. Recent developments suggest that the collective application of proteomics, metabolomics and strain engineering to execute oxidative protein folding in the cytoplasm can help researchers to isolate functionally active proteins like Fab molecules [47]. Furthermore, Royes et al, 2022 have provided futuristic insights into procedures for producing membrane proteins at high levels in *E. coli*,

using suitable construct design, shortlisting the most appropriate vector-host combinations, estimation of bacterial fitness, and selection of bacterial mutants best-suited for maximal generation of the target membrane protein. Additionally, the authors have offered a strategy for membrane protein solubilization based on their research using recent data available in the PDB [48,49]. In a nutshell, future research should focus on summarizing protocols that are effective not just for treating challenges in post-production steps, but also throw some light into the designing of tools to monitor protein production in real-time and enzyme discovery from natural sources.

Limitations

- The review provides a comprehensive insight into a couple of techniques of *E. coli* protein extraction from the last decade that has helped researchers to overcome the challenges in the isolation of membrane proteins and inclusion bodies which the authors feel might be helpful for future research.
- The authors have tried to summarize few conventional and frequently employed techniques of using *P. pastoris* as a robust host for the production of heterologous proteins and enzymes that can expedite further research in the field of eukaryotic protein production systems.
- Finally, the review creates a platform to investigate more about the challenges in metaproteome and native protein extraction from microbes naturally occurring in the environment
- Since there is no standalone protocol fully established for isolating a particular protein of interest in either prokaryotes or eukaryotes, the mechanisms highlighted here indicate towards a combinatorial approach for the successful implementation of an extraction method.

Ethics statements

All authors reviewed and agreed to MethodsX ethical guidelines.

CRediT author statement

Satyam[1], Satakshi Hazra[1], Mayur Mahindra Kedare[1]: conceptualization, methodology, data curation. **Sanjukta Patra:** Supervision. All authors contributed to manuscript writing and approved the final version. [1]These authors contributed equally to this work.

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Declaration of interests

☑The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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