

TITLE

Process for producing a membrane protein

5 TECHNICAL FIELD

The present invention relates to a process for producing a membrane protein. The process according to the invention is suitable for industrial application in which a large amount of membrane proteins is to be produced. Thus, the process
10 aims at applying unit operations suitable for at least a pilot plant production. Notably, the present invention aims at avoiding an ultracentrifugation apparatus.

BACKGROUND

Approximately one-third of the genes in the human genome
15 encode membrane proteins. Membrane proteins play a role in many important cellular activities including energy conversion, cell signaling, cell-cell interactions, cell adhesion, cell migration, protein trafficking, viral fusion, neural synaptic activities and ion and metabolite transport.
20 Membrane proteins are embedded in the lipid bilayer of the cell membrane and are comprised of both hydrophobic and hydrophilic moieties.

Recently, membrane proteins have been successfully integrated
25 into a thin film composite layer grafted onto a porous structural support layer. Furthermore, membrane proteins represent important pharmaceutical targets and interesting subjects of study with respect to cellular biology and protein

biochemistry. A need therefore exists for pilot plant or large-scale production of membrane proteins.

WO2017137361 (A1) discloses self-assembled nanostructures
5 formed between transmembrane proteins, such as aquaporin water channels (AQPs), and polyalkyleneimines (PAI). The self-assembled nanostructures are subsequently incorporated into thin film composite (TFC) membranes grafted onto a porous support membrane. The porous support membrane may be a hollow
10 fiber, a flat sheet, a spiral wound membrane etc. for reverse osmosis or forward osmosis.

WO2013/043118 discloses thin film composite (TFC) membranes in which aquaporin water channels (AQPs) are incorporated in
15 the active layer of the membrane. In addition, it discloses a method of producing thin film composite membranes and their uses in filtration processes, such as nanofiltration and osmotic filtration processes. The TFC membranes comprise lipid- AQP/copolymer-AQP vesicles that are incorporated in
20 the TFC active layer. WO2010/146365 describes preparation of TFC-aquaporin-Z (AqpZ) filtration membranes that use an amphiphile triblock copolymer as a vesicle forming substance for incorporating immobilised AQPs.

25 WO2014/108827 discloses a hollow fiber (HF) module having fibers modified with a thin film composite (TFC) layer comprising aquaporin water channels in which the aquaporin water channels are incorporated in vesicles before
incorporation into the TFC layer.

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Industrial methods for isolating high amounts of membrane proteins from cells are generally not available for the person skilled in the art. In the prior art focus has been on the production of relatively small amounts of membrane proteins for research purposes. Therefore, a relatively cumbersome and labor-intensive production method has been tolerated. However, with the recent higher demands for membrane proteins in industrial membranes for reverse osmosis and forward osmosis a need for a more efficient production method has become clear. The object of the present invention is to provide a process for production of membrane proteins, where the process can produce relatively large amounts of membrane proteins in an efficient way without compromising the quality of the end product.

SUMMARY OF THE INVENTION

The present invention relates to a process for producing a membrane protein, comprising the steps of:

- a. Expressing a membrane protein in a host organism present in an aqueous medium,
- b. Liberating the membrane protein from the host organism,
- c. Adding a detergent solution to solubilize the membrane protein,
- d. Recovering a liquid fraction of the solubilized membrane protein as the supernatant by centrifugation,
- e. Subjecting the liquid fraction to chromatography to bind or retain the membrane protein on a stationary phase, and
- f. Eluting the stationary phase with an elution buffer to produce the membrane protein

wherein the centrifugation in step d is performed at 500 g to 30,000 g.

The present process has the advantage of being able to handle
5 a large amount of aqueous medium containing the host organism.
Thus, the process is capable of handling amounts of 50L or
more, such as 100 L or more applying unit operations suitable
for pilot plant or full-scale production. Furthermore, the
process is scalable and may easily be adapted to high amounts
10 of aqueous medium containing the host organism.

It has surprisingly been found by the inventors that the
membrane proteins expressed by the host organism become
solubilized to a higher extent than other proteins, when a
15 detergent solution is added. It is estimated that at least
around 60 % of the proteins in the vesicles formed by the
detergent solution are the membrane protein of interest.
Furthermore, the vesicles formed of the detergent has been
observed of a size which is larger than usual, such as around
20 0.5 μm , suggesting that the membrane protein forms a more
stable super form with the detergent than the phosphor lipids
used by nature.

The aqueous medium containing the host organism may be used
25 directly in the process. However, to obtain a cleaner product
and to avoid handling of excess amounts of aqueous medium,
generally the aqueous medium comprising the host organism of
step a is filtered prior to the liberation of the membrane
protein according to step b.

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Generally, the aqueous medium comprising the host organism is up concentrated by filtering through a filter having pores small enough to allow cell debris and medium to pass, whereas the cells are retained. In a suitable embodiment, the aqueous medium comprising the host organism of step a is filtered through a microfiltration membrane having a pore diameter of 0.5 micrometer or less. Preferably, the pore diameter is 0.1 μm or less.

After the filtration step, suitably performed by microfiltration, the host organism may be isolated from the remainder of the aqueous medium. While several possible separation processes are possible it is generally preferred that the host organism is isolated after the filtration by centrifugation of the aqueous medium comprising the host organism. The centrifugation process is usually performed at a g-force which within a suitable time period forms a pellet. Therefore, the centrifugation is usually performed at 500 g or more, such as 1000 g or more, and preferably 2000 g and more. The density of the pellet should not be too high to prevent difficulties in the later steps. Therefore, the centrifugation is usually not performed above 30,000 g, such as not above 20,000 g, suitably not above 15,000 g and preferably not above 8,000 g.

The cells are harvested as the pellet and the supernatant may be discarded. It is preferred that the isolated host organism is washed with an isotonic saline solution to dissolve contaminating salts and subsequently centrifuged as described above to isolate the washed host organism. The spent washing solution appearing in the supernatant may be discarded.

The pellet containing the washed cells may be stored by freezing at -20°C or used directly in the next step. Suitably, a dilution buffer is added prior to step b. The dilution
5 buffer may contain protease inhibitors to prevent degradation of the membrane protein, pH regulating substances like TRIS and phosphate for maintaining a pH value within a desired range, and/or ion scavengers, such as EDTA.

10 The membrane protein may be liberated from the host organism in a number of ways, preferably by chemical or mechanical lysis of the cells. When chemical lysis of the cells is performed suitably an aqueous lysis solution is added to liberate the membrane protein from the host organism. In a
15 preferred aspect of the invention, the aqueous lysis buffer is a detergent solution, which simultaneously solubilizes the membrane protein.

For the lysis of the cells and the solubilization of the
20 membrane protein to take place, generally, the host cells are allowed to be subjected to the action of the detergent during agitation. Generally, the host cells are allowed to react with the detergent for at least one hour, such as 2 hours, and preferably at least 6 hours.

25 When mechanical lysis of the cells is used generally the liberation of the membrane protein from the host cells is performed by a homogenizer. It is suitable to use a homogenizer of the same type used in dairy industry for
30 homogenization of milk. A suitable example includes the

Stansted 7575 homogenizer. After treatment in the homogenizer the cells break open, liberating the membrane protein.

After the liberation of the membrane protein a cationic
5 flocculant may be added. The cationic flocculant is believed to interact with inter alia negatively charged cell debris, thereby forming flocs. In a preferred aspect of the invention, the cationic flocculant is a polyamine compound, such as Superfloc C581.

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The formation of flocs usually does not occur instantly. It is therefore preferred that the cationic flocculant is allowed to react with the cell parts of the resuspension during gentle agitation to form flocs. Usually the cationic flocculant and
15 the cell debris are allowed to interact between 10 minutes and 2 hours while stirring at room temperature.

When chemical lysis of the cells is used, the liquid fraction of step e is recovered as the supernatant from a
20 centrifugation of the suspension containing the flocs. The membrane protein appears in the supernatant as the membrane protein is solubilized by the detergent solution.

When mechanical lysis of the cells is used, the liquid
25 fraction of step e is recovered from a resuspension of a solid fraction, wherein the solid fraction is resuspended in a detergent solution for solubilizing the membrane protein. In an embodiment of the invention, the solid fraction is formed due to the fact that the cell fragments containing the
30 membrane protein interacts with the flocculant and form flocs, which may be separated from the liquid by centrifugation. In

another embodiment of the invention, it was surprisingly found that it was possible to harvest the membrane protein in the pellet when a mechanical lysis of the cells was used, without the use of a flocculant. The pellet obtained by the
5 centrifugation may be resuspended in a detergent solution for solubilizing the membrane protein. In this step, the liquid fraction of step e is harvested as the supernatant by centrifugation.

10 The centrifugation process is usually performed at a g-force which within a suitable time forms a pellet. Therefore, the centrifugation is usually performed at 500 g or more, such as 1000 g or more, and preferably 2000 g or more. The density of the pellet should not be too high to prevent difficulties in
15 the later steps. Therefore, the centrifugation is usually not performed above 30,000 g, such as not above 20,000 g, suitably not above 10,000 g, and preferably not above 8,000 g. The application of a g-force below 30,000 g makes it possible to use a clarifier commonly used in dairies, such as spore-
20 removing centrifuges from GEA, Tetra Pak, Alfa Laval, and SPX Flow Seital Separation Technology. The clarifiers useful in the present invention may also be referred to a Bactofuges by some manufactures. Thus, the present invention may omit the application of ultracentrifuges, which are only capable of
25 batch centrifugation and treatment of small amounts.

Detergents used in the present invention include alkyl maltopyranosides, such as n-dodecyl- β -D-maltopyranoside (DDM), n-decyl- β -D-maltopyranoside (DM), or 5-
30 cyclohexylpentyl β -D-maltoside (Cymal-5); alkyl glucopyranosides, such as n-octyl- β -D-glucopyranoside (OG);

amine oxides, such as n-lauryl dimethylamine N-oxide (LDAO); phosphocholines such as n-Dodecylphosphocholine (FC-12), n-tetradecylphosphocholine (FC-14), or n-Hexadecylphosphocholine (FC-16); or polyoxyethylene glycols.

5 In suitable embodiment of the invention, the detergent is selected among from the group consisting of lauryl dimethylamine N-oxide (LDAO), octyl glucoside (OG), dodecyl maltoside (DDM) or combinations thereof. LDAO is a preferred detergent because large and stable vesicles are produced,
10 suggesting that the detergent is capable of displacing the naturally occurring phospholipids.

The liquid fraction is subjected to a chromatography process in which the membrane protein is bonded to or retained by a
15 stationary phase. The type of chromatography may be selected as affinity chromatography, ion exchange chromatography, size exclusion chromatography, displacement chromatography, liquid chromatography, high performance liquid chromatography, reversed-phase chromatography, hydrophobic interaction
20 chromatography, etc. Usually, the chromatography process is preparatory chromatography, opposed to analytical chromatography, to form a purification of the membrane protein.

25 In a currently preferred embodiment, the chromatography method is selected as affinity chromatography, according to which a first part of an affinity pair is associated with the membrane protein and the second part of the affinity pair is associated with the stationary phase. Examples of a stationary
30 phase include beads and column material. In a preferred embodiment of the invention, the stationary phase associated

with a second part of the affinity pair is present in a column. Usually, the parts of the affinity pairs are associated with covalent bindings to the membrane protein or the stationary phase. However, other types of association are
5 also possible such as hybridization, affinity binding through antibody-antigen interaction, etc.

A number of affinity pairs applicable in the current invention is known to the person skilled in the art and include a
10 biotin-streptavidin pair, an antibody-antigen pair, an antibody-hapten pair, an aptamer affinity pair, a capture protein pair, an Fc receptor-IgG pair, a metal-chelating lipid pair, a metal-chelating lipid-histidine (HIS)-tagged protein pair, or a combination thereof. In a currently preferred
15 embodiment the affinity pair is a metal-chelating lipid-histidine (HIS)-tagged protein pair.

The metal is usually immobilized on the column material in a technology referred to as immobilized metal affinity
20 chromatography (IMAC). The metal is usually selected as Cu(II) or Ni(II), preferably Ni(II). Using a Ni-NTA resin, His-tagged proteins can be automatically or manually be purified. A suitable stationary phase is "Capto Chelating" from GE healthcare or alternatively HisTrap Gel filtration material
25 (Ni Sepharose 6 Fast Flow) from GE Healthcare.

The first part of the affinity pair being a histidine tag is usually attached to the C-terminal of the membrane protein. Whereas the histidine tag normally comprises 6 consecutive
30 histidine amino acids it is preferred in the present invention that, the histidine tag comprises 8 or more histidine

molecules. The high number of histidine amino acids in the histidine tag makes it possible to effectively separate the specific and the nonspecific binding proteins.

5 After the addition of the liquid fraction containing the membrane protein to the column the liquid is allowed by gravity or pressure to penetrate the resin. Suitably, the eluting buffer comprises imidazole. The imidazole has the ability to interact with the binding of the His tag to the
10 metal ion immobilized on the resin. At a certain concentration of imidazole, the His tagged membrane protein will be liberated.

To separate the nonspecific binding membrane protein from the
15 membrane protein of interest, generally, the column prior to the elution with the elution buffer, is washed with a washing buffer comprising 40% or less of the concentration of imidazole in the elution buffer. The concentration of imidazole in the elution buffer is generally in the range of
20 200mM to 2000mM imidazole. In preferred embodiments of the invention, the concentration of imidazole in the elution buffer is 400mM or more. To obtain a more effective elution of the His tagged membrane protein the concentration of imidazole in the buffers usually 600mM or 800mM or more.

25 For most applications, the His-tag generally has a negligible influence on protein structure, function, and immunogenicity. However, it may be desirable for certain applications to remove the His tag after it has served its function of binding
30 the membrane molecule to the column. The His tag may be removed by introducing a cleavable link between the membrane

protein and the His tag. Suitable cleavable links include pH sensitive linkers, disulfide linkers, protease sensitive linkers, and beta-glucuronide linkers.

5 Membrane proteins span in their natural environment the entire bilipid membrane, i.e. from the interior of the cell to the extracellular space. Many of the transmembrane proteins functions as gateways for specific substances, thereby allowing exchange of these substances between the interior of
10 the cell and the extracellular liquid. A characteristic feature of transmembrane proteins is the presence of a hydrophobic area, which will ensure integration of the transmembrane protein into the membrane. The transmembrane protein furthermore has hydrophilic segments on both sides of
15 the hollow fiber area, said hydrophilic segments being directed to the interior of the cell and the extracellular fluid, respectively.

While it is believed that any membrane protein can be produced
20 in accordance with the present invention, it is generally desired to use the process to produce membrane proteins that transport ions (ion channels) and water (aquaporin water channels). Ion channels include chloride channels and metal ion transporters. Certain chloride channels in addition to
25 the chloride ion also conducts HCO_3^- , I^- , SCN^- , and NO_3^- . The metal ion transporters include magnesium transporters, potassium ion channels, sodium ion channels, calcium channels, proton channels etc. In a certain embodiment of the invention, the membrane protein is outer membrane protein A
30 (OmpA).

In a preferred embodiment of the invention, the membrane protein is an aquaporin water channel. Aquaporin water channels facilitate the transport of water in or out of a cell. In an industrial membrane, the aquaporin water channels ensure the flow of water by osmosis, while other components in the solution are rejected. The membrane protein, such as an aquaporin water channel, may emanate from various sources including prokaryotic and eukaryotic organisms. A prokaryotic source for aquaporin includes *E. coli*, *Kyrpidia spormannii*, *Methanothermobacter* sp., *Novibacillus thermophilus*, *Saccharomyces cerevisiae*, and *Halomonas* sp. An eukaryotic source for aquaporin includes *Oryza sativa Japonica* (Japanese Rice), *Eucalyptus grandis*, *Solanum tuberosum* (Danish potato), and *Milnesium tardigradum* (water bear).

The nucleic sequence of the membrane protein from the source organism is generally codon optimized using Geneart's (Subsidiary of Thermo Fischer Scientific) service for improving expression in the host organism. The resulting gene is suitably synthesized with the addition of histidine encoding codons C-terminally, along with flanking restriction sites N-terminally and C-terminally. The synthetic gene fragment may be digested with the restriction enzyme and ligated into a vector fragment. The resulting ligation mixture is preferably transformed into an organism, such as *Escherichia coli* DH10B. The antibiotic resistant transformants are suitably selected on a medium with the antibiotic. Transformants were confirmed by sequencing of the genetic construct. Isolated vector DNA was subsequently transferred to the production host. The production host may

be selected among a number of suitable prokaryotic or eukaryotic organisms, such as *Escherichia coli* and *Saccharomyces cerevisiae*.

5 The host organism is generally manipulated to express a native membrane protein in a higher amount than usual or to express non-native membrane protein. One way of expressing the membrane protein may be by transforming the host organism with a vector comprising DNA encoding the membrane protein,
10 as discussed above. Another possibility for obtaining over-expression of the membrane protein would be by up-regulating the expression of a native membrane protein, e.g. by attenuating a repressor or insertion of a suitable promotor region. A further possibility for obtaining expression of a
15 non-native protein would be to transfect a host organism with a virus or a bacteriophage containing a nucleic acid encoding the membrane protein.

20 EXAMPLES

Example 1

An *E. coli* BL21 strain is prepared comprising a vector producing aquaporin proteins linked to a His tag at the C-
25 terminal. The His tag contains ten consecutive histidine molecules attached to the primary sequence of the aquaporin membrane protein.

The *E. coli* strain is nurtured in a standard medium to obtain
30 a total fermentation broth of 150 L. The *E. coli* cells was harvested by filtering the fermentation broth through a microfiltration membrane having a pore diameter of 0.05 µm.

The filtrate containing the *E. coli* cells is reduced to about 50 L and subsequently subjected to centrifugation at 5300g for 20 minutes. Thus, the *E. coli* cells are up-concentrated by microfiltration and the remaining medium is subsequently removed as the supernatant by centrifugation.

The pellets obtained by the centrifugation are collected and added 1:1 volume 0.9% sodium chloride to wash the cells and dissolve contaminating salts. Subsequently, the washing solution is removed in a centrifuge running at 5300g for 20 minutes. The supernatant was discarded and the washed cells collected as pellets. The pellets may be stored by freezing at -20°C or used directly in the next step.

The pellets comprising the *E. coli* cells were solubilized in about 47 L TRIS buffer used as a binding buffer. After stirring for about one hour 6.4 L detergent (5% LDAO) was added for solubilization to a final concentration of 0.6%. The mixture was incubated overnight at room temperature with gentle stirring. By the resuspension of the cells in a buffer containing a detergent a cell lysis occurred. By the cell lysis membrane proteins are liberated from the internal membranes of the cells and solubilized by the detergent.

To remove negatively charged cell material a polyamine (Superfloc C581) was added in a volume of 427 mL. The mixture was incubated for 30 minutes with stirring at room temperature for coagulating the negatively charged molecules such as cell debris, DNA, RNA and to a certain extent of proteins. The membrane protein solubilized by the detergent remains in the aqueous phase. The mixture is centrifuged at a maximum speed

of 5300 g for 15 minutes. The pellet containing the cell debris, DNA, RNA and solubilized proteins are discarded and the supernatant collected. The supernatant is transferred to a container containing 107 L dilution buffer to obtain a final
5 volume of 160 L with a final concentration of 0.2% LDAO.

A column containing the affinity resin "Capto Chelating" from GE healthcare is provided. The resin is charged with Ni²⁺ which binds to the His10 tag. The column was loaded with the
10 diluted supernatant and subsequently washed with 10 column volumes wash buffer containing 200mM imidazole to wash out the nonspecific binding components. Subsequently, 2.5 column volumes elution buffer of aqueous 1000mM imidazole was used to release the membrane protein from the column. The protein
15 eluted from the column was tested by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which primarily showed a single band indicating a purity of above 80%.

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Example 2

An *E. coli* strain BL21 is prepared comprising a vector producing aquaporin proteins linked to a His tag at the C-
25 terminal. The His tag contains ten consecutive histidine molecules attached to the primary sequence of the aquaporin membrane protein.

The *E. coli* strain is nurtured in a standard medium to obtain
30 a total fermentation broth of 250 L. The *E. coli* cells were harvested by filtering the fermentation broth through a PES

flat sheet membrane having a pore diameter of 0.05 μm . The filtrate containing the *E. coli* cells is reduced to about 50 L and subsequently subjected to centrifugation at 5300g in a Sorvall 16 L centrifuge for 20 minutes. Thus, the *E. coli* cells are up-concentrated by microfiltration and the remaining medium is subsequently removed as the supernatant by centrifugation. The pellets may be stored by freezing at -20°C or used directly in the next step.

The pellets comprising the *E. coli* cells were resuspended in about 50 L buffer (aqueous solution of the protease inhibitor PMSF and EDTA) and homogenized at 1000 bar in a Stansted nm-GEN 7575 homogenizer. To isolate the cell material of interest a polyamine (Superfloc C581) is added in a concentration of 12 ml/L. The temperature was maintained around 10-15°C. The mixture was incubated for 30 minutes with stirring at room temperature. The mixture was centrifuged at a maximum speed of 5300 g for 30 minutes. The pellet contains the membrane protein and the supernatant is discarded.

The pellet was resuspended in a 0.9% sodium chloride solution to obtain a total protein concentration of approximately 50 mg/ml. Solubilization of the membrane protein was performed by adding 28 L TRIS binding buffer and 4.5 liter 5% LDAO to 5 L of the resuspended pellet material. At room temperature and gentle stirring the mixture was allowed to incubate for 2 to 24 hours.

After the solubilization process the mixture was centrifuged in 2 L containers at 5300g for 90 minutes. The supernatant

was recovered and the LDAO concentration was adjusted to 0.2% by addition of dilution buffer.

A column containing the affinity resin "Capto Chelating" from GE healthcare is provided. The resin is charged with Ni²⁺ which binds to the His10 tag. The column was equilibrated by loading binding buffer having 0.2% LDAO. Subsequently, the column was loaded with the diluted supernatant and washed with 10 column volumes wash buffer containing 200mM imidazole to wash out the nonspecific binding components. Subsequently, 2.5 column volumes elution buffer of aqueous 1000mM imidazole is used to release the membrane protein from the column. The protein eluted from the column was tested by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) chromatography, which showed only a single band indicating a purity of above 95%.

Example 3

An *E. coli* strain BL21 is prepared comprising a vector producing aquaporin proteins linked to a His tag at the C-terminal. The His tag contains ten consecutive histidine molecules attached to the primary sequence of the aquaporin membrane protein.

The *E. coli* strain is nurtured in a standard medium to obtain a total fermentation broth of 250 L. The fermentation batch had an OD600 13 when harvested, and it was induced for 42.5 hours. The material was homogenized twice at 100 MPa in a Stansted nm-GEN 7575 homogenizer. 10 mL was taken from the lysed material and added to a 15 mL falcon tube.

Centrifugation was then carried out for 1 hour with 5300 g and the pellet was separated from the supernatant. The pellet was then resuspended in 10 mL 0.9% NaCl. BCA assay was used to determine the concentration (mg/mL total protein) of the resuspended pellets and the separated supernatants after the first spin.

The resuspended pellets and the separated supernatants were finally solubilized (1 mL) for 2 hours in 0.6 % LDAO (120 uL of 5 % LDAO stock from Carbosynth was used to 1 mL material) and were centrifuged again at 5300 G for 15 minutes. Once again, the supernatants were separated from the pellets. The pellets were resuspended (up to 1 mL again) in binding buffer w/o LDAO.

All samples were analyzed on SDS-gel, which showed that around 60% of the proteins in the supernatant was the aquaporin membrane protein.

Example 4

Following the purification of the Aquaporin Z in example 2, the size distribution of the solubilized protein in LDAO was measured.

A comparison was made between the same elution buffer, with and without protein, to evaluate whether the particle size distribution was influenced by the buffer components (including detergent) or the membrane protein.

The Aquaporin Z protein was eluted from the IMAC column using Elution buffer with 1000 mM imidazole and 0.2% w/v LDAO. The purified protein was measured for its protein concentration by amino acid analysis. 6.44 mg/mL of clear, solubilized, protein in Elution buffer was loaded into 3 separate cuvettes (Sarstedt, 4 mL, PMMA, ca. no. 67.755, Nümbrecht, Germany) and analyzed for the size distribution by the Malvern Zetasizer Nano-ZS (Malvern Instruments Ltd., Malvern UK) using the Malvern Zetasizer software v.7.02. Results presented in table 1 are the average of the samples run in triplicate.

Table 1:

Sample	Concentration of AqpZ (mg/mL)	Size, diameter (d.nm) Population intensity (%)		
		Pop. 1	Pop. 2	Pop. 3
Purified AqpZ in Elution buffer (avg.)	6.44	315.6 ± 116.8 nm 45.9%	108 ± 32.3 nm 35.5%	17.1 ± 2.5 nm 14.9%
Elution buffer (avg.)	0	0.85 ± 0.16 nm 56.8%	8.6 ± 1.2 nm 41.1%	103 ± 5.5 nm 2.1%

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The Elution buffer sample (no protein) contains a population distribution with 97.9% of the particles with a size of 8.6 nm or smaller, indicating there are no large detergent micelles in solution that would be retained by microfiltration. 81.4% of the particles in the sample with protein show a size of 108 nm or larger, thereby indicating that the presence of the protein and detergent together form large soluble particles that are retained during microfiltration. The experiment surprisingly shows that

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large, stable, particles composed of LDAO micelles and membrane protein are produced.

Example 5

5 Expression of histidine tagged aquaporin from *Oryza sativa Japonica* (Japanese Rice) in *Escherichia coli* and its purification using IMAC

The gene encoding aquaporin from *Oryza sativa Japonica*
10 (UNIPROT: A3C132) was codon optimized using Geneart's (Subsidiary of Thermo Fischer Scientific) service for improving expression in *E. coli*. The resulting gene was synthesized with the addition of ten histidine encoding codons C-terminally, along with flanking NdeI/XhoI restriction sites
15 N-terminally and C-terminally, respectively (Gene ID: aquaporin_*Oryza sativa Japonica*). The synthetic gene fragment was digested with NdeI/XhoI restriction enzymes and ligated to NdeI/XhoI - digested and purified vector pUP1909 fragment. The resulting ligation mixture was transformed into
20 *Escherichia coli* DH10B and kanamycin resistant transformants were selected on LB agar plates with kanamycin. Transformants were confirmed by sequencing of the genetic construct. Isolated vector DNA was subsequently transferred to the production host, *Escherichia coli* BL21.

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In order to heterologously express aquaporin in *E. coli*, the production host was grown in minimal medium consisting of 30 g/L Glycerol, 6 g/L (NH₄)₂HPO₄, 3 g/L KH₂PO₄, 5 g/L NaCl, 0.25 g/L MgSO₄·7H₂O, 0.4 g/L Fe(III)citrate and 1 mL/L sterile
30 filtered trace metal solution. The trace metal solution consisted of 1 g/L EDTA, 0.8 g/L CoCl₂·6H₂O, 1.5 MnCl₂·4H₂O,

0.4 g/L $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.4 g/L H_3BO_3 , 0.8 g/L $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 1.3 g/L $\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$. After inoculation and overnight growth, additional 0.25 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ was added.

5 *E. coli* was cultivated in 3L Applikon Bioreactors with ez-Control in a batch fermentation process. Protein production was induced by addition of IPTG to a final concentration of 0.5 mM at an optical density (OD 600 nm) of approximately 30. The culture was induced for approximately 24 hours and the
10 bacterial cells were harvested with centrifugation at 5300 g for 20 min.

The pellets comprising the *E. coli* cells were resuspended in buffer (aqueous solution of the protease inhibitor PMSF and
15 EDTA) and homogenized at 1000 bar in a Stansted nm-GEN 7575 homogenizer. The temperature was maintained around 10-15°C. The mixture was centrifuged at a maximum speed of 5300 g for 30 minutes. The pellet contains the membrane protein and the supernatant is discarded.

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The pellet was resuspended in a 0.9% sodium chloride solution to obtain a total protein concentration of approximately 50 mg/ml. Solubilization of the membrane protein was performed by adding 28 L TRIS binding buffer and 4.5 liter 5% LDAO to
25 5 L of the resuspended pellet material. At room temperature and gentle stirring the mixture was allowed to incubate for 2 to 24 hours.

After the solubilization process the mixture was centrifuged
30 in 2 L containers at 5300g for 90 minutes. The supernatant

was recovered and the LDAO concentration was adjusted to 0.2% by addition of dilution buffer.

After solubilization and clarification, the protein was
5 captured using IMAC and eluted in Elution buffer containing
1000 mM imidazole and 0.2% w/v LDAO. The elution fractions
were analyzed by SDS Page and only revealed a single major
band which migrated at 27 kDa which corresponds to the size
of aquaporin from Japanese rice. Furthermore, the result was
10 confirmed by comparison to a negative control purification
from *E. coli* transformed with an empty vector. The negative
control resulted in no purified protein. Western blot analysis
with antibodies (TaKaRa Bio) specific for the histidine-tag
resulted as expected in a clear signal from the purified
15 protein and no signal from the negative control confirming
the origin of the purified protein as the histidine tagged
membrane protein.

Example 6

20 Expression of histidine tagged aquaporin from *Eucalyptus grandis* in *Escherichia coli* and its purification using IMAC

The gene encoding aquaporin from *Eucalyptus grandis* (UNIPROT: A0A059C9Z4) was codon optimized using Geneart's (Subsidiary
25 of Thermo Fischer Scientific) service for improving
expression in *E. coli*. The resulting gene was synthesized
with the addition of ten histidine encoding codons C-terminally,
along with flanking NdeI/XhoI restriction sites N-terminally and C-terminally, respectively (Gene ID:
30 aquaporin_Eucalyptus_grandis). The gene was cloned and
expressed as described in Example 5. The protein was

successfully purified and confirmed as described in Example 5.

Example 7

5 Expression of histidine tagged aquaporin from *Solanum tuberosum* (Danish potato) in *Escherichia coli* and its purification using IMAC

The gene encoding aquaporin from *Solanum tuberosum* (UNIPROT: Q38HT6) was codon optimized using Geneart's (Subsidiary of Thermo Fischer Scientific) service for improving expression in *E. coli*. The resulting gene was synthesized with the addition of ten histidine encoding codons C-terminally, along with flanking NdeI/XhoI restriction sites N-terminally and C-terminally, respectively (Gene ID: aquaporin_Solanum_tuberosum). The gene was cloned and expressed as described in Example 5. The protein was successfully purified and confirmed as described in Example 5.

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Example 8

Expression of histidine tagged aquaporin from *Milnesium tardigradum* (water bear) in *Escherichia coli* and purifying using IMAC

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The gene encoding aquaporin from *Milnesium tardigradum* (UNIPROT: G5CTG2) was codon optimized using Geneart's (Subsidiary of Thermo Fischer Scientific) service for improving expression in *E. coli*. The resulting gene was synthesized with the addition of ten histidine encoding codons C-terminally, along with flanking NdeI/XhoI restriction sites

N-terminally and C-terminally, respectively (Gene ID: aquaporin_Milnesium_tardigradum). The gene was cloned and expressed as described in Example 5. The protein was successfully purified and confirmed as described in Example 5.

Example 9

Expression of histidine tagged aquaporin from *Halomonas* sp. in *Escherichia coli* and its purification using IMAC

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The gene encoding aquaporin from *Halomonas* sp. (UNIPROT: A0A2N0G6U6) was codon optimized using Geneart's (Subsidiary of Thermo Fischer Scientific) service for improving expression in *E. coli*. The resulting gene was synthesized with the addition of ten histidine encoding codons C-terminally, along with flanking NdeI/XhoI restriction sites N-terminally and C-terminally, respectively (Gene ID: aquaporin_Halomonas_sp). The gene was cloned and expressed as described in Example 5. The protein was successfully purified and confirmed as described in Example 5.

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Example 10

Expression of histidine tagged aquaporin from *Kyrpidia spormannii* in *Escherichia coli* and its purification using IMAC

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The gene encoding aquaporin from *Kyrpidia* sp. (UNIPROT: A0A2K8N5Z5) was codon optimized using Geneart's (Subsidiary of Thermo Fischer Scientific) service for improving expression in *E. coli*. The resulting gene was synthesized with the addition of ten histidine encoding codons C-

30

terminally, along with flanking NdeI/XhoI restriction sites N-terminally and C-terminally, respectively (Gene ID: aquaporin_Kyrpidia_spormannii). The gene was cloned and expressed as described in Example 5. The protein was successfully purified and confirmed as described in Example 5.

Example 11

Expression of histidine tagged aquaporin from *Methanothermobacter* sp. in *Escherichia coli* and its purification using IMAC

The gene encoding aquaporin from *Methanothermobacter* sp. (UNIPROT: A0A223ZCQ2) was codon optimized using Geneart's (Subsidiary of Thermo Fischer Scientific) service for improving expression in *E. coli*. The resulting gene was synthesized with the addition of ten histidine encoding codons C-terminally, along with flanking NdeI/XhoI restriction sites N-terminally and C-terminally, respectively (Gene ID: aquaporin_ *Methanothermobacter*_sp). The gene was cloned and expressed as described in Example 5. The protein was successfully purified and confirmed as described in Example 5.

Example 12

Expression of histidine tagged aquaporin from *Novibacillus thermophilus* in *Escherichia coli* and its purification using IMAC

The gene encoding aquaporin from *Novibacillus thermophilus* (UNIPROT: A0A1U9K5R2) was codon optimized using Geneart's

(Subsidiary of Thermo Fischer Scientific) service for improving expression in *E. coli*. The resulting gene was synthesized with the addition of ten histidine encoding codons C-terminally, along with flanking NdeI/XhoI restriction sites N-terminally and C-terminally, respectively (Gene ID: aquaporin_ Novibacillus_thermophilus). The gene was cloned and expressed as described in Example 5. The protein was successfully purified and confirmed as described in Example 5.

Example 13

Expression of outer membrane protein A (OmpA) from *Escherichia coli* in *E. coli*

Expression and purification of OmpA (UNIPROT: POA910) in *E. coli* was carried out essentially as described in Example 5, except that *E. coli* was transformed with an empty vector and solubilization was prolonged to 24 hours. The resulting solubilized protein was estimated to a purity of approximately 30-50% by SDS-PAGE, thus clearly stating that OmpA was successfully isolated in the membrane fraction following the standard purification procedure. An additional size exclusion chromatography (SEC) step could have further purified OmpA.

Example 14

Constructing a *Saccharomyces cerevisiae* strain expressing aquaporin-Z (AqpZ) from *Escherichia coli* fused to an N-terminal histidine-tagged yEGFP and separated by a Tobacco Etch Virus (TEV) protease cleavage site.

AqpZ (UNIPROT ID: P60844) from *E. coli* was codon optimized for expression in *S. cerevisiae* using Geneart's service for improved expression in *S. cerevisiae*. For expression in *S. cerevisiae*, AqpZ was fused with yeast enhanced green fluorescent protein (yEGFP) N-terminally (Brendan P. Cormack et al, Microbiology (1997), 143, 303-311) to enable visual detection and quantification of membrane protein expression. Furthermore, eight histidines (His8) was added N-terminally to yEGFP as an IMAC purification tag. The His8-yEGFP and AqpZ was genetically separated by a TEV protease cleavage site incorporated by PCR primers during construction.

Rapid and efficient construction of the plasmid encoding His8-yEGFP-TEV-AqpZ fusion was carried out by in vivo homologous recombination of overlapping regions incorporated by the primers in *S. cerevisiae* between a His8-yEGFP-TEV PCR fragment, a TEV-AqpZ PCR fragment, and the linearized expression plasmid derived from SalI, HindIII and BamHI digestion of the pEMBLyex4 plasmid, as described in (Scientific Reports 7: 16899). The TEV cleavage site enables subsequent removal of the His8-yEGFP protein by the TEV protease.

Selection of *S. cerevisiae* transformants was carried out on minimal medium plates deficient in uracil but supplemented with leucine and lysine to ensure survival of bacterial cells with correctly recombined DNA fragments. Medium composition and amino acid concentrations used in this example were identical to the ones listed in Example 15.

Example 15

Expression of His8-yEGFP-TEV-AqpZ with *Saccharomyces cerevisiae*.

5 A single colony of transformed yeast cells was selectively propagated until saturation in 5 ml of glucose minimal medium supplemented with 60 mg/L leucine and 30 mg/L lysine. 200 µl of this culture was subsequently propagated in 5 ml of glucose minimal medium supplemented with 30 mg/L lysine for selection
10 for high plasmid copy numbers. Freeze stocks of the high plasmid copy number cells were prepared.

200 µl of a thawed freeze-stock was added to 10 ml of minimal medium supplemented with lysine and grown until saturation.
15 1 ml of the culture was transferred to 100 ml of the same medium. After overnight growth, an aliquot corresponding to a final OD600 of 0.05 was transferred to 1.5 liters of minimal medium with an initial concentration of 20 g/L glucose as carbon source and 30 g/L glycerol supplemented with extra
20 amino acids. The culture was grown in a 3L Applikon® bioreactor equipped with ez-Control connected to a PC running Lucillus® software (Applikon, Holland, and SecureCell, Switzerland).

25 The initial part of the fermentation was performed at 20°C in minimal medium. The bioreactor was fed with glucose to a final concentration of 3% w/v when the initial amount of glucose had been metabolized. The pH of the growth medium was kept at 6.0 by computer-controlled addition of 1 M NH₄OH. When the
30 CO₂-exhaust gas leveled off, due to limited access of glucose, the bioreactor was cooled to 15°C prior to induction of

recombinant AQP production. Addition of 50 mL/L expression medium consisting of 400 mL/L ASD-10, 400 mL/L extra amino acids, 200 g/L glycerol and 20 g/L galactose started the expression of the recombinant protein. Yeast cells were
5 harvested after ~96 h.

The minimal medium consisted of 20 g/L glucose, 100 mL/L ASD-10, 5 mL/L V-200, 30 g/L glycerol, 0,1 g/L Ca₂Cl. The ASD-10 consisted of 50 g/L (NH₄)₂SO₄, 8.75 g/L KH₂PO₄, 1.25 g/L K₂HPO₄,
10 5 g/L MgSO₄·7H₂O, 1 g/L NaCl, 5 mg/L H₃BO₃, 1 mg/L KI, 4 mg/L MnSO₄·1H₂O, 4.2 mg/L ZnSO₄·7H₂O, 0.4 mg/L CuSO₄·5H₂O, 2 mg/L FeCl₃ and 2 mg/L Na₂MoO₄·2H₂O. The V-200 consisted of 4 mg/L Biotin, 400 mg/L D-pantothenic acid, 0.4 mg/L Folic acid, 2000 mg/L Myo-inositol, 80 mg/L Niacin, 40 mg/L p-
15 Aminobenzoate, 80 mg/L Pyridoxine, 40 mg/L Riboflavin and 80 mg/L Thiamine. When stated, the medium contained additional amino acids which consisted of 600 mg/L Alanine, 600 mg/L Arginine, 600 mg/L Cysteine, 3000 mg/L Glutamic acid, 2000 mg/L Lysine, 600 mg/L Methionine, 1500 mg/L Phenylalanine,
20 600 mg/L Proline, 10000 mg/L Serine, 900 mg/L Tyrosine, 4500 mg/L Valine, 2000 mg/L Aspartic acid, 4000 mg/L Threonine, 600 mg/L Histidine and 600 mg/L Tryptophan.

Example 16

25 Cloning of His8-yEGFP-TEV-AQP5 from *Milnesium tardigradum* in a Bioreactor with *Saccharomyces cerevisiae*

A His8-yEGFP-TEV-AQP5 construct was prepared following the procedure outlined in Example 14. The AQP5 protein from *M.*
30 *tardigradum* (UNIPROT: G5CTG2) was codon optimized for

expression in *E. coli* despite the need for expression in yeast.

Example 17

- 5 Purification of His8-yEGFP-TEV-AqpZ and His8-yEGFP-TEV-AQP5
from *S. cerevisiae*

The purification of heterologously expressed membrane protein
from *S. cerevisiae* was carried out as described in Example 2,
10 except that applied buffer volumes were scaled down to match
the reduced culture volumes and that lysis was carried out at
1800 bar. Protein production and purity was successfully
confirmed for both fusion proteins with SDS page and Western
Blot and no contaminating proteins could be detected.

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