Yeast as a tool for membrane protein production and structure determination

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Editor: John Morrissey

Abstract

Membrane proteins are challenging targets to functionally and structurally characterize. An enduring bottleneck in their study is the reliable production of sufficient yields of stable protein. Here, we evaluate all eukaryotic membrane protein production experiments that have supported the deposition of a high-resolution structure. We focused on the most common yeast host systems, Saccharomyces cerevisiae and Pichia pastoris. The first high-resolution structure of a membrane protein produced in yeast was described in 1999 and today there are 186 structures of α-helical membrane proteins, representing 101 unique proteins from 37 families. Homologous and heterologous production are equally common in S. cerevisiae, while heterologous production dominates in P. pastoris, especially of human proteins, which represent about one-third of the total. Investigating protein engineering approaches (78 proteins from seven families) demonstrated that the majority contained a polyhistidine tag for purification, typically at the C-terminus of the protein. Codon optimization and truncation of hydrophilic extensions were also common approaches to improve yields. We conclude that yeast remains a useful production host for the study of α-helical membrane proteins.

Keywords: membrane protein production, Saccharomyces cerevisiae, Pichia pastoris

Introduction

From 1998 to 2002, two of us had the privilege of working in Stefan Hohmann’s laboratory as one of his PhD students (Kristina Hedfalk) and one of his postdoctoral research fellows (Roslyn Bill). Work under his guidance on the yeast aquaporin Fps1, resulted in the first publication (Bill et al. 2001) of many together over the next few years (Tamas et al. 2003, Elbing et al. 2004, Hedfalk et al. 2004, Karlgren et al. 2004, 2005, Otterstedt et al. 2004, Henriksson et al. 2005, Pettersson et al. 2006). True to his generous and supportive nature, Stefan encouraged Roslyn to be the sole corresponding author on that first article and he encouraged us both to pursue our independent research interests. One of those was the development of yeast as a host for the production of recombinant membrane proteins (Bill 2001, Oberg et al. 2009, Bill et al. 2011, Oberg and Hedfalk 2013), which is a line of investigation that we have continued to collaborate upon (Nyblom et al. 2007, Oberg et al. 2011, Bill and Hedfalk 2021).

The successful production of recombinant proteins plays a critical role in academic and industrial research. A wide range of host organisms exist for this purpose, including several yeast species that have proved to be effective and cost-efficient for the production of soluble and membrane proteins. The production of recombinant membrane proteins has facilitated the elucidation of transport mechanisms, substrate binding, and the effect of new molecules as drug leads (Gulezian et al. 2021). In common with Escherichia coli, yeasts can grow on inexpensive chemically defined media and cultivation technologies are available that produce high biomass (and hence high protein) yields. In common with mammalian cells, yeasts are eukaryotes. The production of biopharmaceuticals in yeast is also approved for human use (Gerngross 2004, Wang et al. 2017). The low fidelity of carbohydrate modifications is a known disadvantage of using yeast, a property, i.e. most pronounced for Saccharomyces cerevisiae (Veira Gomes et al. 2018). However, yeast strains with humanized glycosylation pathways have been reported (De Wachter et al. 2021). Stefan catalyzed our shared love of yeast as an experimental tool and our enduring fascination with membrane proteins. In this review, in his memory, and together with some of our students, we examine the use of S. cerevisiae and Pichia pastoris for production of membrane proteins of eukaryotic origin, for subsequent structural and functional characterization. Thus, α-helical transmembrane proteins are the focus of this mini-review.

Yeast is an attractive host for resolving new membrane protein structures

From 1999 to 2022, 190 high-resolution α-helical membrane protein structures were derived from proteins produced in yeast. Of those, 100 proteins were extracted from S. cerevisiae, 86 from P. pastoris, and 4 from Schizosaccharomyces pombe (Ago et al. 2007, Wang et al. 2019a, Deng et al. 2021). In S. cerevisiae, 57% of the 100 proteins were homologous, either extracted from the native membrane or recombinantly produced, and 43% were produced by het-

Received: July 6, 2022. Revised: September 1, 2022. Accepted: September 26, 2022.

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Figure 1. High resolution membrane protein structures derived from proteins produced in yeast from 1999 to date; unique structures derived from yeast-produced proteins (homologous and heterologous) (A) presented as a percentage of all structures (derived from proteins produced in all hosts; gray dashed line) and (B) with dotted lines showing the linear regression of the use of *P. pastoris* (green) and *S. cerevisiae* (yellow), respectively.

erologous recombinant protein production (Table S1, Supporting Information). In *P. pastoris*, only 2% of the 86 proteins were from the homologous host while almost all (98%) had an origin other than yeast (Table S2, Supporting Information).

Figure 1 shows that the use of yeast as a recombinant host has become more common with time, a development that follows the overall upward trend of membrane protein production using any host system. The first structure derived from a protein produced in yeast was reported in 1999 when we were working in Stefan’s laboratory: ATP synthase extracted from its native source *S. cerevisiae* (Stock et al. 1999). The first protein structure of a protein produced in *P. pastoris* was reported in 2005: recombinant rat Kv1.2 voltage-gated potassium channel (Long et al. 2005). From 2009 onwards, there has been an upward trajectory in the deposition of unique structures derived from *S. cerevisiae* and *P. pastoris* (Fig. 1A). Notably, production of homologous and heterologous proteins has been equally popular in *S. cerevisiae*, while there is a clear dominance of production of heterologous proteins in *P. pastoris*. The
Figure 2. From construct design to cell fractionation in yeast. In *S. cerevisiae*, the gene of interest may be produced from a constitutive (e.g. PGK) or an inducible (e.g. GAL) promoter, while the very strong and methanol inducible AOX1 promoter is commonly used in *P. pastoris*. The plasmid for expression in *S. cerevisiae* is selected for using media lacking one essential amino acid or nucleobase and the gene for its production is instead part of the expression plasmid. In *P. pastoris*, the plasmid for expression is selected by adding Zeocin to the media, a selection, i.e. applied in both *E. coli* and *P. pastoris*. Linearization and integration into the genome after transformation is the more common procedure in *S. cerevisiae* is selected for using media lacking one essential amino acid or nucleobase and the gene for its production is instead part of the expression plasmid. In *P. pastoris*, the plasmid for expression is selected by adding Zeocin to the media, a selection, i.e. applied in both *E. coli* and *P. pastoris*. Linearization and integration into the genome after transformation is the more common procedure in *S. cerevisiae*. In *expression in *P. pastoris*, the plasmid for expression is selected by adding Zeocin to the media, a selection, i.e. applied in both *E. coli* and *P. pastoris*. Linearization and integration into the genome after transformation is the more common procedure in *P. pastoris*, where high production clones are evaluated in total cell extracts using immunoblots and a suitable antibody. Independent of yeast species, it is important to verify production and purification of the protein of interest, cells are collected by centrifugation, broken, and the fraction of interest is collected by differential centrifugation.

Table 1. Protein families produced in *S. cerevisiae*.

| Protein family                                      | References                                      |
|-----------------------------------------------------|-------------------------------------------------|
| Potassium, sodium, and protein ion-selective channels | (Kintzer and Stroud, 2016), (Kintzer et al, 2018), (Dickinson et al, 2022) |
| Transient receptor potential channels               | (Huynh et al, 2016), (Hughes et al, 2018, 2019), (Dosey et al, 2019), (Pumroy et al, 2019), (Conde et al, 2021), (Ahmed et al, 2022) |
| Aquaporins and glyceroporin channels                 | (Gotfryd et al, 2018), (van den Berg et al, 2021) |
| Other ion channels                                   | (Wang et al, 2019b)                            |
| SWEET and semisweet transporters                    | (Brauer et al, 2019), (Gerondopoulous et al, 2021) |
| AAA-ATPase membrane translocators                   | (Kater et al, 2020)                            |
| Intramembrane proteases                              | (Pyró et al, 2013)                            |
| ABC-transporters                                     | (Kodan et al, 2019), (Bickers et al, 2021), (Harris et al, 2021) |
| Solute carrier transporters                          | (Coudray et al, 2017), (Parker and Newstead, 2017), (Parker et al, 2019), (Tsai et al, 2020) |
| Oligosaccharyltransferases                           | (Bai et al, 2018), (Wild et al, 2018), (Neuhaus et al, 2021) |
| Protein O-mannosyl transferases                      | (Bai et al, 2019a)                            |
| Oxidases                                             | (Ma et al, 2004), (Son et al, 2008)             |
| Antiporters                                          | (Rüprecht et al, 2014, 2019), (Winklemann et al, 2020), (Matsuoka et al, 2022) |
| P-type ATPases                                       | (Clausen et al, 2013), (Bai et al, 2019b, 2020a, 2021), (Timchenko et al, 2019, 2021), (Geurts et al, 2020), (McKenna et al, 2020), (Li et al, 2021a), (Zhao et al, 2021) |
| Permease channels                                    | (van den Berg et al, 2016)                      |
| Major facilitator superfamily transporters          | (Pedersen et al, 2013), (Parker and Newstead, 2014), (Kapoor et al, 2016), (Paulsen et al, 2019), (Qureshi et al, 2020), (Bavnhoj et al, 2021), (Custodio et al, 2021) |
| Membrane-integral pyrophosphatases                   | (Kellosalo et al, 2012), (Lin et al, 2012), (Li et al, 2016), (Tsai et al, 2019) |
| Transmembrane protein 16 family proteins            | (Brunner et al, 2014), (Falzone et al, 2019), (Kalienkova et al, 2019), (Khelashvili et al, 2019) |
| Nucleobase-cation-symport-2 family proteins          | (Alguel et al, 2016)                           |
| Sec, translocase, and insertase proteins             | (Schoebel et al, 2017), (Itskanov and Park, 2019), (Wu et al, 2019, 2020), (Bai et al, 2020b), (Matoba et al, 2020), (Miller-Vedam et al, 2020), (Itskanov et al, 2021), (Weng et al, 2021), (Zhang et al, 2021) |
| Cation antiporter family                             | (Waigt et al, 2013)                            |
| Sterol-sensing domain proteins                       | (Winkler et al, 2019)                          |
| Vacular ATPase                                       | (Oot et al, 2012, 2016), (Mazhab-Jafari et al, 2016), (Roh et al, 2018, 2020), (Vasanthakumar et al, 2019) |
| F-type ATPase                                        | (Stock et al, 1999), (Kabaleswaram et al, 2006), (Dautant et al, 2010), (Symersky et al, 2012a,b), (Robinson et al, 2013), (Guo et al, 2017), (Srivastava et al, 2018), (Luo et al, 2020) |
| Electron transport chain complexes                   | (Hunte et al, 2000), (Lange et al, 2001), (Palsdottir et al, 2003), (Lancaster et al, 2007), (Solmaz and Hunte, 2008) |
| Electron transport chain supercomplexes              | (Hunte et al, 2019, 2020), (Rathore et al, 2019), (Berndtsson et al, 2020) |
Cloning and growth in yeast

Cloning

Good construct design is key to achieving high yields of functional recombinant membrane protein (Fig. 2). Codon optimization of the gene sequence for optimal expression, the selection of tags (and cleavage sites) and the truncation of the corresponding protein sequence are all considerations in designing an effective construct (Veiera Gomes et al. 2018). Most yeast plasmids can be manipulated in E. coli to facilitate the necessary molecular biology prior to transformation, which is why they are known as shuttle vectors (Fig. 2). Three main types of shuttle vectors are used: integrative, centromeric, or episomal plasmids, which differ in several aspects including integration (or not) into the genome and plasmid copy number (Gnugge and Rudolf 2017). The polyhistidine tag is by far the most commonly used in yeast expression experiments (Table S3, Supporting Information). Sometimes, several tags are used in tandem including a GFP tag, which is enables protein yields to be monitored through the measurement of GFP fluorescence (Drew et al. 2018). Tags are most often placed at the C-terminus rather than the N-terminus of the recombinant protein, and a cleavage site is typically inserted to enable tag removal, especially with longer polyhistidine-tags or when several tags are present which might alter protein function. TEV protease is most often used for tag cleavage due to its high site specificity, but thrombin is also very common (Kapust and Waugh 2000). In some cases, the protein of interest is truncated at a terminus, which can increase yields, presumably due to stabilization of the folded protein (Bill et al. 2011). It is common practice to remove flexible termini to improve homogeneity for crystal growth (Mooij et al. 2009). Codon usage is organism dependent meaning that codons that may be abundant in the source organism of the protein target are rare in yeast. As such, optimizing the gene sequence according to the codons present in the heterologous host can facil-
tate higher recombinant protein yields (Table S3, Supporting Information). While codon optimized genes adapted for a certain host are available commercially, a higher translation efficiency can be achieved by a more complex optimization approach, which also includes the condition under which heterologous genes are being expressed (Lanza et al. 2014). Codon optimization is widely used for membrane proteins produced in P. pastoris (Table S3, Supporting Information; see also Fig. 5 below).

Growth

In optimizing growth conditions, the type of promoter used (inducible or constitutive) needs to be considered as well as the influence of media components, the presence or absence of a stabilizing ligand, the pH and oxygen levels in the culture and its temperature (Liu et al. 2013). For yeast, a common procedure is to start with smaller cultures and upscale to larger cultures once evidence of expression has been found. For cultures with constitutive expression, there is a correlation between the specific growth rate and the produced protein (Liu et al. 2013). Fed-batch cultures are typically performed in a fermenter when protein expression is under the control of an inducible promoter (Fig. 2). Normally, S. cerevisiae will first be grown in Synthetic Defined (SD) medium, which contains glucose as the main source of carbon, but lacks the amino acid or nucleobase used to maintain selection of the episome.

Figure 3. Production of α-helical membrane proteins in S. cerevisiae. (A) Membrane protein families for which high-resolution structures have been derived from protein produced in S. cerevisiae. (B) The origin of recombinant proteins for which a high-resolution structure has been derived from S. cerevisiae membranes.
Figure 4. Production of α-helical membrane proteins in *P. pastoris*. (A) Protein families successfully produced in the yeast *P. pastoris* with a resulting high-resolution structure. (B) Origin of proteins for which a high-resolution structure has been derived after extraction from the *P. pastoris* membrane.

Protein expression is commonly under the control of the GAL1 or GAL10 promoter in *S. cerevisiae*, which is repressed by the MIG1 repressor while glucose is present in the medium. This enables maximum cell growth (Kang et al. 2005) prior to the galactose addition that induces gene expression (Vieira Gomes et al. 2018). *Pichia pastoris* has a rapid growth rate and can be cultured to very high cell-densities in fermenters, which allows high protein yields. Expression in *P. pastoris* is typically under the control of strong and tightly regulated promoters (Fig. 2). The alcohol oxidase promoter (P<sub>AOX1</sub>) is induced by methanol, but repressed by glucose, glycerol, and ethanol. Constitutive promoters may also be used, such as the glyceraldehyde-3-phosphate promoter (GAP), which achieves comparable expression levels in presence of glucose as with AOX1 (Rabert et al. 2013, Ahmad et al. 2014). Strains for large-scale production are most commonly derived from *P. pastoris* CBS7435, as they are not limited by patent protection or materials ownership policies. Nevertheless, there are some modified strains that may be more useful depending on the protein of interest.
Membrane protein production in *S. cerevisiae*

**Saccharomyces cerevisiae is a useful host for a wide range of membrane proteins**

Members of 26 α-helical membrane protein families have been produced in *S. cerevisiae* (Table 1).

Overall, diverse membrane proteins have been produced in *S. cerevisiae* with Sec, Translocase, and Insertase proteins; P-type ATPases and F-type ATPase being most common (Fig. 3A).

For some protein families, like AAA-ATPase membrane translocators (Kater et al. 2020), intramembrane proteases (Pyyro et al. 2013), oligosaccharyltransferases (Bai et al. 2018, Wild et al. 2018, Neuhaus et al. 2021), protein O-mannosyl transferases (Bai et al. 2019a), cation antiporter family (Wight et al. 2013), sterol-sensing domain proteins (Winkler et al. 2019), vacuolar ATPase (Oot et al. 2012, 2016, Mazhab-Jafari et al. 2016, Roh et al. 2018, 2020, Vasanthakumar et al. 2019), F-type ATPase (Stock et al. 1999, Kabaleeswaran et al. 2006, Dautant et al. 2010, Symersky et al. 2012a, 2012b, Robinson et al. 2013, Guo et al. 2017, Srivastava et al. 2018, Luo et al. 2020), electron transport chain complexes (Hunte et al. 2000, Lange et al. 2001, Falsdottir et al. 2003, Lancaster et al. 2007, Solmaz and Hunte 2008), and electron transport chain supercomplexes (Hartley et al. 2019, 2020, Rathore et al. 2019, Berndtsson et al. 2020), only homologous production (native or recombinant) has been used with many structures reported over several years. There are also some protein families that are only produced recombinantly using *S. cerevisiae* as a heterologous host; potassium, sodium, and protein ion-selective channels (Kintzer and Stroud 2016, Kintzer et al. 2018, Dickinson et al. 2022), aquaporins and glyceroporin channels (Gotfried et al. 2018, van den Berg et al. 2021), other ion channels (Wang et al. 2019a), SWEET and semisweet transporters (Brauer et al. 2019, Gerondopoulos et al. 2021), oxidases (Ma et al. 2004, Son et al. 2008), permease channels (van den Berg et al. 2021), major facilitator superfamily transporters (Pedersen et al. 2013, Parker and Newstead 2014, Kapoor et al. 2016, Paulsen et al. 2019, Qureshi et al. 2020, Bavnhoj et al. 2021, Custodio et al. 2021), membrane-integral pyrophosphatases (Kellosoal et al. 2012, Lin et al. 2012, Li et al. 2016, Tsai et al. 2019), and transmembrane protein 16 family proteins (Brunner et al. 2014, Falzone et al. 2019, Kalienkova et al. 2019, Khelashvili et al. 2019; Table S1, Supporting Information). Overall, 58% of all α-helical membrane proteins produced in *S. cerevisiae* rely on homologous production (Fig. 3B).

**Saccharomyces cerevisiae is commonly used for homologous production of native yeast proteins**

Proteins from 11 different sources have been recombinantly produced in *S. cerevisiae* (Fig. 3B). A total of three bacterial, one parasite, and 11 plant and algal proteins have been produced. Mammalian targets together represent 19 proteins of which human proteins are represented by six targets. Fungi constitute 11 targets with the majority of targets being from yeast (58 proteins; Fig. 3B). Of these 58 proteins, 57 originate from *S. cerevisiae* and one from *S. pombe* (Matoba et al. 2020; Table S1, Supporting Information).
Table 3. Comparison of the two most widely used yeast hosts for production of α-helical membrane proteins; S. cerevisiae and P. pastoris, respectively.

| Structures | Sources | Heterologous | Homologous | Average number of proteins from each family | Protein families/total structures | Unique proteins/structures Sources/total structures |
|------------|---------|--------------|------------|------------------------------------------|-------------------------------|--------------------------------------------------|
| S. cerevisiae | 100 | 57 | 43 | 0.75 | 52 | 0.52 |
| P. pastoris | 86 | 2 | 84 | 42 | 48 | 0.56 |

Overall, this means that the majority of α-helical membrane protein structures from protein produced in S. cerevisiae are from the homologous expression (Table 3).

**Membrane protein production in P. pastoris**

*Pichia pastoris is a successful host for synthesizing channel proteins*

Proteins produced in P. pastoris represent 20 protein families (Table 2). Overall, several membrane protein families are represented, although there are slightly fewer than those produced in S. cerevisiae (Table 3). For P. pastoris, there is a clear dominance of certain targets where potassium, sodium, and protein ion-selective channels constitute one-third of all proteins (Fig. 4A). In comparison to S. cerevisiae, most structures from P. pastoris-derived protein are from recombinant production, the endogenous aquaporin Aqy1 being the only exception. Overall, P. pastoris is a suitable host system for recombinant production of α-helical membrane proteins coming from a wide range of protein families.

*Pichia pastoris is a successful host for recombinant production of human proteins*

Proteins from 13 different eukaryotic sources have been produced in P. pastoris, which is slightly less than for S. cerevisiae (Table 3). Human membrane proteins represent more than one-third of all proteins produced in P. pastoris (Fig. 4B) and are represented in more than half of all protein families (Table S2, Supporting Information). Taken together, P. pastoris is a suitable host for α-helical proteins from different sources, especially of human origin. Indeed, for the yeast P. pastoris the ratio between heterologous and homologous production is 42, as compared to 0.75 in S. cerevisiae (Table 3).

**Discussion and conclusion**

The use of yeasts as expression hosts for α-helical membrane proteins is increasing year-on-year (Fig. 1). Saccharomyces cerevisiae, but not P. pastoris, is widely used for the extraction of homologous proteins. Both systems are used to produce a wide variety of recombinant membrane proteins and the number of unique proteins produced in each host are comparable (Table 3). In comparison, slightly more protein families have been produced in S. cerevisiae compared to P. pastoris (Tables S1 and S2, Supporting Information). The number of sources for the proteins produced are also similar between the two systems.

A total of 78 proteins from seven protein families have been heterologously produced in S. cerevisiae and P. pastoris (Table S3, Supporting Information). Of these, 16 (21%) were produced in S. cerevisiae and 62 (79%) in P. pastoris (Table 3). The majority (79%) of these 78 proteins contained a polyhistidine tag for purification, but the length of the tag varied between 4 and 10 residues (Fig. 5A). The location of the histidine tag was typically at the C-terminus of the protein (Fig. 5B). In 53% of cases, a cleavage site was present between the protein sequence and the tag; the PreScission Cleavage Site (PSC) was most widely used (Fig. 5C). Other commonly applied engineering principles were truncation of hydrophilic termini as well as codon optimization, which were used in 36% and 35% of cases, respectively (Table S3, Supporting Information).

The purpose of construct engineering is to improve production yields as well as to aid subsequent purification, commonly for structural evaluation (Scott et al. 2013, Fig. 6). These approaches...
are general and not specific for a given host system (Kesidis et al. 2020). Codon optimization, however, is applied to match the codons of the host system, which improves the translation process, and it is also done to improve the stability of the mRNA template (Hanson and Coller 2018). Truncation of hydrophilic termini is commonly done to improve crystal formation (Hedfalk 2013). The start point for purification is the membrane fraction isolated by differential centrifugation (Fig. 6). A suitable detergent is necessary, i.e. both efficient in terms of solubilization and keeps the membrane protein in its functional fold (Kotov et al. 2019). The most common purification procedure is based on two steps, where the first step is generally Ni-chromatography using the polyhistidine-tag (Table S3, Supporting Information) and the second is size-exclusion chromatography. Alternatively, ion-exchange chromatography may be used instead of or in combination with Ni-chromatography. If the production level is relatively high, two purification steps are usually sufficient, but other methods and additional steps will sometimes be necessary to achieve the required purity for subsequent evaluation. To succeed with crystalllography, a monodisperse peak is desirable, being an indication of a homologous protein population (Drew et al. 2008). Also, a traditional SDS-PAGE gel is informative regarding size and stability of the purified protein product (Bill and Hedfalk 2021).

Despite the availability of more complex host systems, such as mammalian or insect cell lines, P. pastoris and S. cerevisiae are still regarded as powerful hosts (Cereghino and Gregg 2000, Rabet et al. 2013, Ahmad et al. 2014). Their genomes are fully sequenced, and they can perform complex translational and post-translational processes. The use of yeast is particularly relevant for the expression of eukaryotic proteins since post-translational modifications are important for proper folding and cannot always be achieved in prokaryotic systems such as bacteria. Furthermore, yeast is usually cheap to work with, easy to genetically modify and can result in high protein yields. Notably, S. cerevisiae is an important biotechnological host, producing 20% of all biopharmaceuticals (Nielsen 2013). In comparison, P. pastoris has been used as a protein production system for more than 25 years. Recent improvements include replacing methanol as inducer or improving protein secretion (Ahmad et al. 2014). A significant advantage of this yeast is that, there is no endotoxin, bacteriophage, or human pathogen contamination (Rabert et al. 2019). The number of new membrane protein structures is increasing daily (Fig. 1). New emerging technologies (Pandey et al. 2016), combined with ongoing improvements in host systems have allowed us to obtain many more eukaryotic membrane protein structures than were available two decades ago when we worked with Stefan. Although the majority of eukaryotic MEMBRANE PROTEIN structures are DERIVED FROM PROTEINS produced in HEK293 and insect cells (Kesidis et al. 2020), we show here the importance of yeast as a production host and its role as an essential player in the production of eukaryotic membrane proteins for structural and functional analysis.

Acknowledgments

The authors would like to acknowledge Professor Jens B Nielsen, Chalmers University of Technology, Sweden, for the invitation to write this min-review to honor Professor Stefan Hohmann.

Supplementary data

Supplementary data are available at FEMSYR online.

Conflict of interest statement. None declared.

Funding

This work was supported by the Swedish Research Council (RFI—Protein Production Sweden to K.H.), Elisabeth and Alfred Ahlqvists Foundation (A.C.) are also gratefully acknowledged for the funding. The authors have no conflict of interest to declare.

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