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Peroxicretion, a Novel Tool for Engineering Membrane Trafficking

Cees M.J. Sagt

*DSM Biotechnology Center, Delft
The Netherlands*

1. Introduction

The production of proteins by recombinant micro-organisms has been possible since the late 70's. Since then enormous steps have been made to improve protein titers and to expand the range of possible proteins to be produced. The development of genetic modification tools and improved ease of use of cloning strategies have played an important role in this. The time to construct a strain and develop a cost-effective bioprocess has decreased significantly. Concurrently, sequencing data, which serve as a library for donor genes to be overexpressed, have increased exponentially. Therefore, the speed and flexibility to engineer custom-made protein factories has improved tremendously during the last decades. As in any developed technology, a certain degree of standardization is desired. In order to make this ambition more tangible the concept of cell factories is often used. This concept is based on the principle that the cell factory should be able to produce any protein to a certain desired level. Based on the concept of standardized expression vectors and expression hosts the basics of this cell factory concept were built. However, in reality this concept proved to be far too simple, as a standardized input does not result in a standardized output. This is caused by the immense complexity and dynamics of cellular systems. Even when all components are described, by sequencing the genome, the interaction, compartmentalization and dynamics of these components are largely unknown. A striking example is the difference between homologous protein expression which are produced to levels up to 50 g/l in filamentous fungi whereas heterologous proteins are produced at levels which are usually 100-1000 fold less ¹. So even when identical tools are used in a standardized approach the final result can vary over several orders of magnitude.

The general consensus is that this large difference is linked to cellular events and responses which are caused by the overexpression of the heterologous proteins ², therefore the scientific community, together with biotech industries, have been studying these effects for several decades. The cellular stress responses and intracellular events which are linked to the use of cells as protein production factories are very well described ³⁻⁹. This profound insight in the molecular mechanism of the cellular stress reactions has facilitated the increase in expression and secretion levels of some heterologous proteins. However, levels comparable to homologous proteins have not been reached ^{1, 10}. It is evident that, when even more demanding sources of biodiversity are tapped, like intracellular proteins, the engineering of a robust and versatile cell factory which is able to produce this wide range of proteins becomes a major challenge.

The production of intracellular proteins in a secreted form, to avoid complex and costly downstream processing, has hardly been touched upon. Several strategies have been developed to increase protein titers, however, these efforts have all been focused on secreted proteins. The functional diversity of intracellular proteins is very broad, while extracellular enzymes are mostly hydrolases, as shown in Figure 1. If the cell would be able to secrete these intracellular enzymes in an active form a wide range of applications could be within reach. Current strategies do not suffice but when these are combined with membrane engineering to redirect vesicle trafficking this could become a very promising approach.

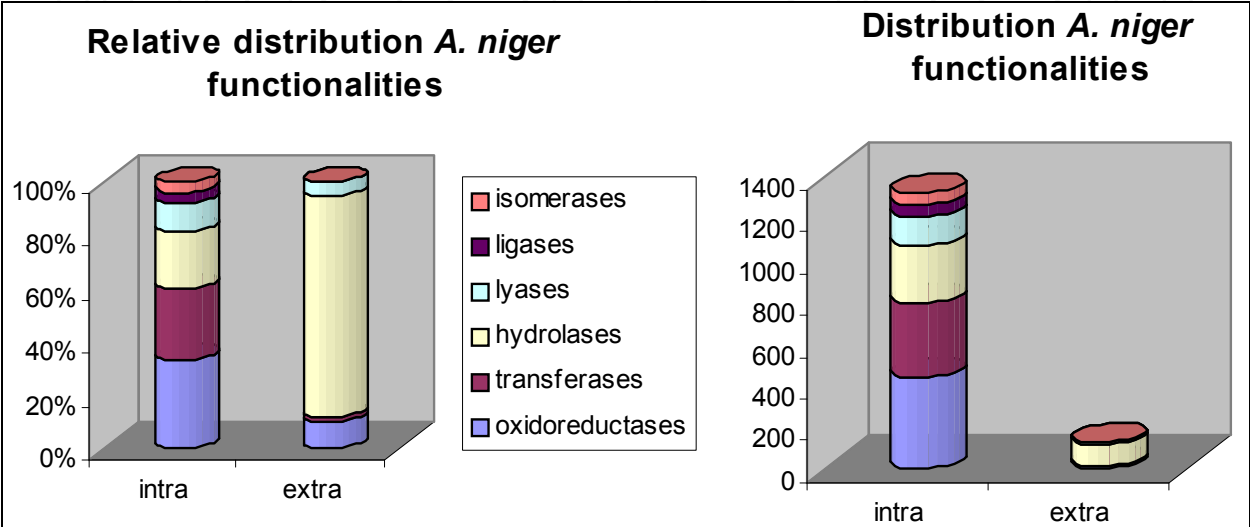


Fig. 1. Distribution of enzyme functionalities in *Aspergillus niger*. The genome sequence of *Aspergillus niger* was mined for annotated functionalities based on EC classifications. The extracellular (extra) or intracellular (intra) protein distribution is based on this annotated genome. The left panel indicates relative function distribution, the right panel shows absolute numbers of enzyme function distribution.

This chapter will discuss the different strategies to improve the productivity of the cellular protein factory. Especially the possibility of engineering membrane trafficking will be addressed. This technology is not only useful for developing a versatile and robust cell factory but can be very valuable to study intracellular membrane trafficking on an academic level as well.

2. Conventional engineering strategies for improved protein production

The conventional strategy to improve protein production is to increase the expression levels of the gene of interest by using multiple gene copies and strong promoters ^{11, 12}. As a consequence, the cell is faced with very high levels of mRNA which should rapidly be translated into active and correctly folded proteins. To handle this increased demand for folding capacity the cell often reacts with the so called Unfolded Protein Response (UPR). This UPR is a well studied mechanism ¹³⁻¹⁷ which is also applied to increase folding capacity. By upregulating the transcription factors needed, or the resulting chaperones and folding enzymes, the folding capacity of the cell is enhanced ¹⁸⁻²¹.

For heterologous proteins the fusion peptide concept has been proven to be successful ²². This concept is used for heterologous proteins which are secreted by their native host but are overexpressed in an alternative system to increase production titers. By fusing the protein of interest to a homologous protein which is naturally secreted by the host, the desired protein is also transported outside the cell, for example, *Acremonium murorum* phenol oxidase expressed in *Aspergillus awamori* ²³. This carrier protein is often an N – terminal part of a very well secreted homologous protein which is fused to the protein of interest. Often a cleavage site is engineered, like Kex2 with the aim to produce only the full length protein. In reality often a mixture of unprocessed protein, partially processed protein and fully processed protein is produced, which can have consequences for downstream processing in order to meet the required product specifications. On the other hand, there are also reports which describe secretion of heterologous proteins by using only signal sequences without any additional fusion proteins like the production of llama variable heavy-chain antibody fragments (VHHs) ²⁴ and the production of *Arthromyces ramosus* peroxidase ²⁵ both in *Aspergillus awamori*. However, the titers obtained by these systems are in the mg/l range which is generally not sufficient for an economically feasible process.

The next generation of tools to increase protein titers are based on technology developments within bio-IT combined with the possibility of designing custom-made genes. Recently, the concept of codon adaptation was demonstrated. This concept is based on the use of favorable codons so that translation of the mRNA encoding the protein of interest is not a bottleneck. An excellent *in silico* study has recently been published ²⁶ describing that translational speed and, concomitantly, ribosome density are determined by the combination of coding sequences and the tRNA pool. The first dozens of codons are translated at lower rates and create an area where ribosomes are very densely packed, especially on transcripts with a high mRNA level. This strategy could prevent ribosome jamming in later stages of the translational process and therefore be of physiological advantage of the cell. This elevation of translational speed towards the end of transcripts is often seen in fungi (which are biotechnological work-horses) and provides the cell with an effective tool against late abortions of protein synthesis which is an energy-consuming process. As suggested by the authors the fine-tuning of this ramping principle could be an additional tool to increase the efficiency of the protein production factory.

As optimizing codon usage is aimed at improving the translation of mRNA into protein, the folding capacity of the cell can become limiting, especially when high copy numbers and strong promoters are combined with codon optimization. The folding and refolding of proteins is tightly linked to membrane trafficking, a very stringent quality control system existing in the endoplasmic reticulum acts as a gatekeeper for proteins to be transported further into the secretory pathway. This quality control system has been reviewed recently ². Also the removal of proteolytic activity has been applied to improve protein production ²⁷⁻³³ this approach has shown to be very effective in specific cases. The success of the different approaches is hard to predict and highly dependent on the protein of interest being overproduced. In addition, all of the strategies above are focused to improve secretion of natural secreted proteins, most of them being hydrolases.

An overview on conventional strategies to improve the productivity of the cellular protein factory is shown in Figure 2. As shown, the modification of DNA, which includes cloning strong promoters in front of the gene of interest and constructing multiple copies of this

expression cassette is a generic strategy which can lead to improvement of protein production. Also the optimization of codon(pair) usage and mRNA stabilizing elements can be applied in a generic way. The adaptation of the folding capacity of the host cell is not a generic approach. The folding and modification of proteins is intertwined and very protein specific. Improvement of regular transport of secretory vesicles is a strategy which is less well-known, but some possible routes have been described ³⁴. In addition the overexpression of SEC4, a Rab protein associated with vesicles, doubled protein production in *Pichia pastoris* ³⁵, which is a direct proof that modification of vesicle flow can result in enhanced protein production. However all these approaches are focused on enhancing the efficiency of existing protein production and secretory processes in the host cell.

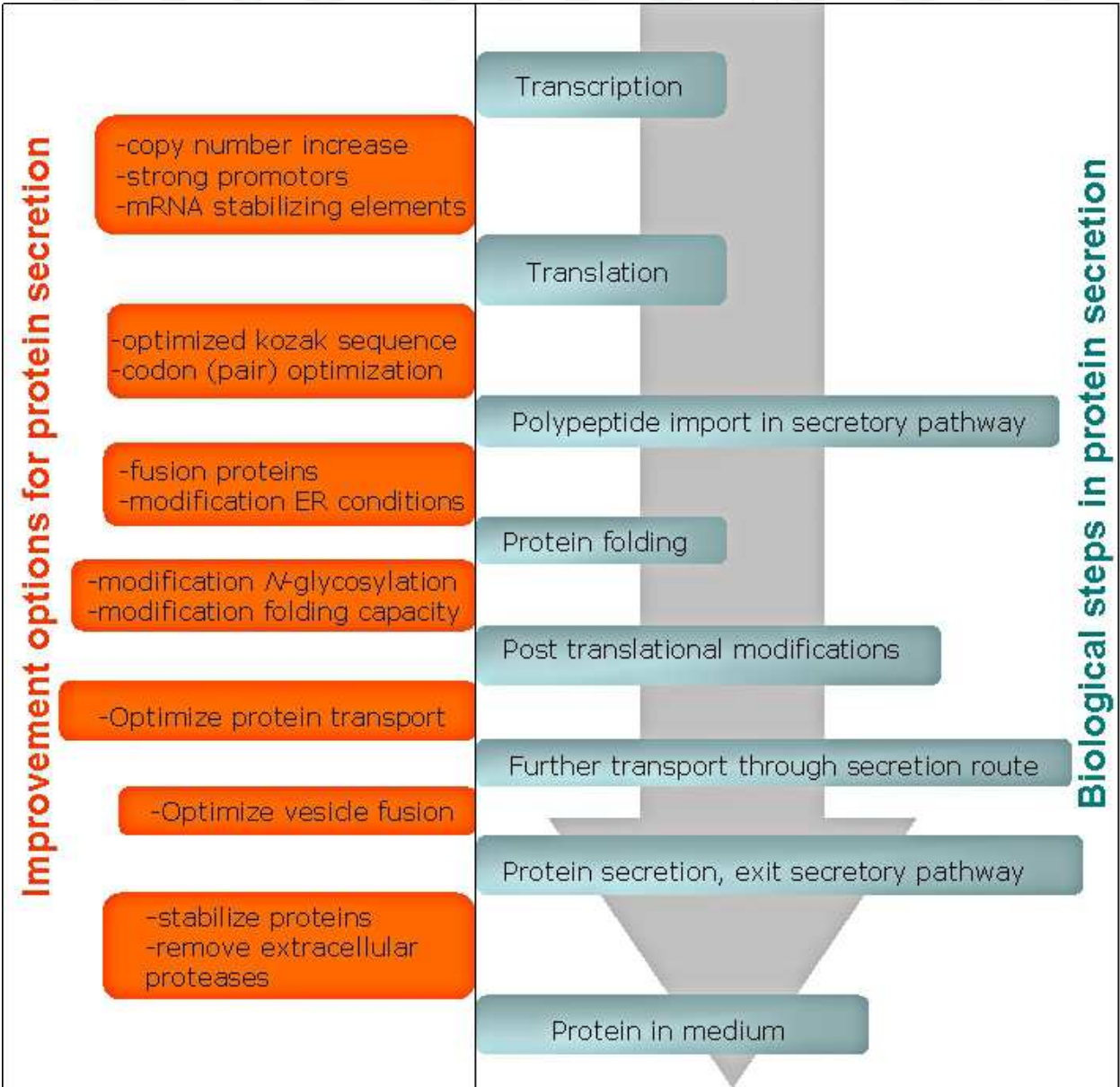


Fig. 2. Conventional improvement options for protein secretion in biological processes. Indicated in blue are the major steps in biological (eukaryotic) systems which are crucial for protein secretion, indicated in orange are improvement strategies which can be applied at the different stages of the protein secretion process.

3. Limitations of conventional strategies

The conventional strategies described above are focused on incremental improvement, overproducing proteins which are naturally secreted products. Therefore, the large diversity of intracellular proteins remains untapped. In addition, these strategies can be seen as generic, at least in part, but very large differences in secretion efficiency exist between different proteins; changes of a few amino acids can often have a detrimental effect on protein secretion^{7, 36}. This is probably linked to different interactions with chaperones and folding enzymes. In the case of cutinase secretion in yeast, the bottleneck was circumvented by engineering an N-glycosylation site at the amino terminus of cutinase⁸. The restrictions of conventional strategies are not only illustrated by the order of magnitude of differences in protein secretion but also by the limited class of proteins which are secreted. As shown in Figure 1, in *A. niger* most of the secreted proteins are hydrolases whereas the enzyme variation of intracellular enzymes is much larger. This enormous potential of enzymes is hardly being touched upon. There are strategies to overproduce intracellular proteins inside the cell but this is always followed by a complex downstream processing step to liberate the proteins and to purify them to an acceptable level^{37, 38}. In addition, toxic proteins or compounds cannot be produced by these methods at economical levels because they will very likely damage the host cell³⁹⁻⁴¹. In order to get access to the large variety of intracellular functionalities and to be able to produce toxic compounds into a cell compartment which does not impact the viability of the host cell, an additional strategy based on membrane engineering would be very valuable.

4. Engineering membrane trafficking as a novel concept

Whereas the conventional approaches are focused on reaching high levels of active proteins, the engineering of membrane trafficking is aimed at redesigning the membrane trafficking in the host cell enabling a custom made flow of vesicles which can expand the possible use of the microbial cell factory. This designed vesicle flow will give access to the large diversity of intracellular enzyme activities and will be important to produce toxic compounds without damaging the host cell.

Recently a novel strategy was described to engineer membrane trafficking, this concept is called peroxicretion⁴². The peroxicretion concept of engineering membrane trafficking is based on two features: 1) the use of cytosolic domains of SNARE proteins (soluble NSF (N-ethylmaleimide-sensitive factor) attachment receptor) which are key for specific membrane trafficking and 2) the use of transmembrane domains, which can reposition the SNARE molecules.

Every cellular compartment of the secretory route contains a specific set of SNARE molecules. They are called v-SNARE or t-SNARE molecules (vesicle or target). SNARE molecules are transmembrane molecules which direct membrane trafficking in eukaryotic cells⁴³. More recently, SNAREs are classified into Q and R SNAREs based on structural features. This nomenclature is more precise since certain SNARE molecules act as both v- and t-SNAREs depending on the direction of vesicle flow. Besides transmembrane domains which serve as membrane anchors also lipid modifications like palmitoylation or farnesylation^{44, 45} occur. Combinations of transmembrane domains and palmitoylation, preventing degradation of the SNARE, are also reported⁴⁶. SNARE molecules are

characterized by the so-called SNARE motif which is a conserved region of approximately 65 amino acids, this region also determines the specificity of the SNARE molecule ⁴⁷. This important feature enables modification of the specificity of the SNARE molecules by adapting the SNARE motifs. By fusing these SNARE motifs to transmembrane domains of other proteins it becomes possible to relocalize the SNARE motifs and modify membrane trafficking in eukaryotic systems ⁴². Hu *et al.* showed that, by flipping SNARE molecules to the outside of cell membranes, it is possible to fuse these cells, again indicating that SNARE molecules and especially the SNARE motifs are determining the specificity of fusion of membranes ⁴⁸. The N-terminal domains of SNAREs are thought to act like a zipper which acts from the N-terminus towards the transmembrane domain thereby bringing the membranes in close proximity followed by the actual fusion. This model has been reviewed in an excellent paper ⁴³. Most research on SNARE molecules has been performed in the field of neuronal cells ^{49, 50}, in relation to synaptic vesicle transport. In *Saccharomyces cerevisiae* a clear set of orthologous proteins can be found as well as in *Aspergillus niger* (see Figure 5).

The concept of SNARE-pin formation has been reviewed by Jahn ^{51, 43}. In this concept, four helices are interacting to form a so called SNARE pin. The helices are named Q- and R-helices, determined by the glutamine and arginine residues which are participating. The glutamine residues are specified further as Qa, Qb, and Qc. These SNARE complexes are very efficient in mediating membrane fusion. Recently, it was shown very elegantly, by FRET studies, that one SNARE complex is sufficient to enforce membrane fusion ⁵². Again, this indicates that modification of these SNARE complexes can have a direct effect on vesicle transport.

It has been described that N-terminal domains and SNARE motifs determine the functionality of SNARE proteins ⁵³. The functions of the transmembrane domains are not very well known, but one obvious function would be to target and anchor the SNARE in the correct membrane. By using the cytosolic domain of the SNARE and fusing this part to a transmembrane domain of a protein which is located in a different membrane it is possible to engineer membrane trafficking *in vivo* ⁴².

To reposition the SNARE molecules a transmembrane domain is needed of a protein which is located on the vesicles which need to be modified in order to engineer membrane trafficking. The protein of which the transmembrane part is used should have the N-terminus at the cytosolic side (i.e. surface) of the membrane. The concept of this approach is depicted in Figure 3.

The transmembrane domains of proteins as tools for relocalization of SNARE molecules should be selected carefully. For the proof of principle which was aimed at transforming peroxisomes into secretory vesicles in order to secrete proteins which were located in the peroxisome, the *A. niger* ortholog of a peroxisomal membrane protein described in *Arabidopsis* named PMP22 ^{54, 55} was used. The final topology of the fusion protein SNARE-membrane anchor is important. The N-terminal domains and SNARE motifs should be positioned at the cytosolic side of the vesicle in order to interact with their SNARE partner. In order to enable peroxisomal fusion with the plasma membrane, it was crucial to identify a peroxisomal membrane anchor, which could be used to decorate the peroxisome with proteins involved in membrane fusion. In order to determine whether peroxisomal membrane proteins have the correct topology we performed topology predictions at the

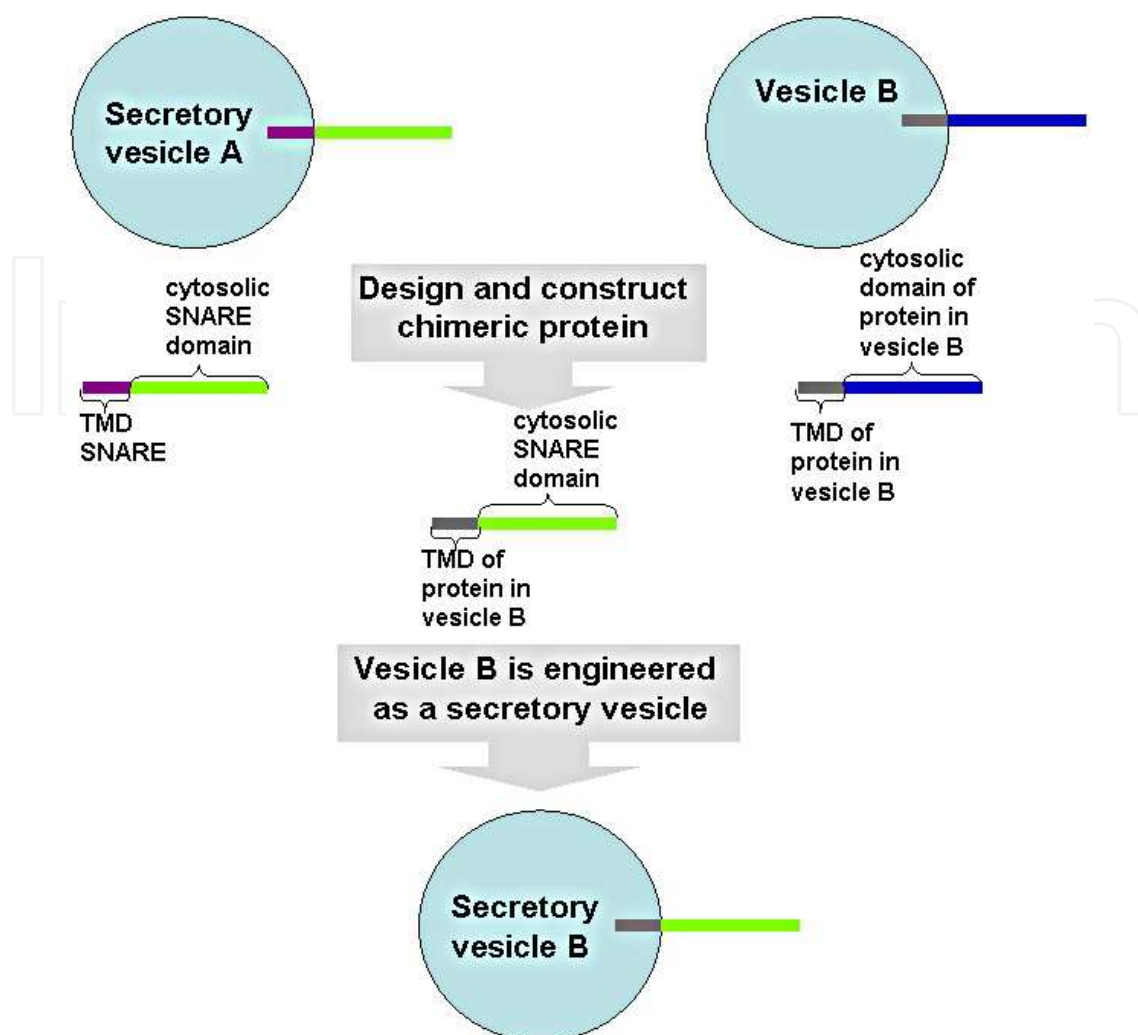
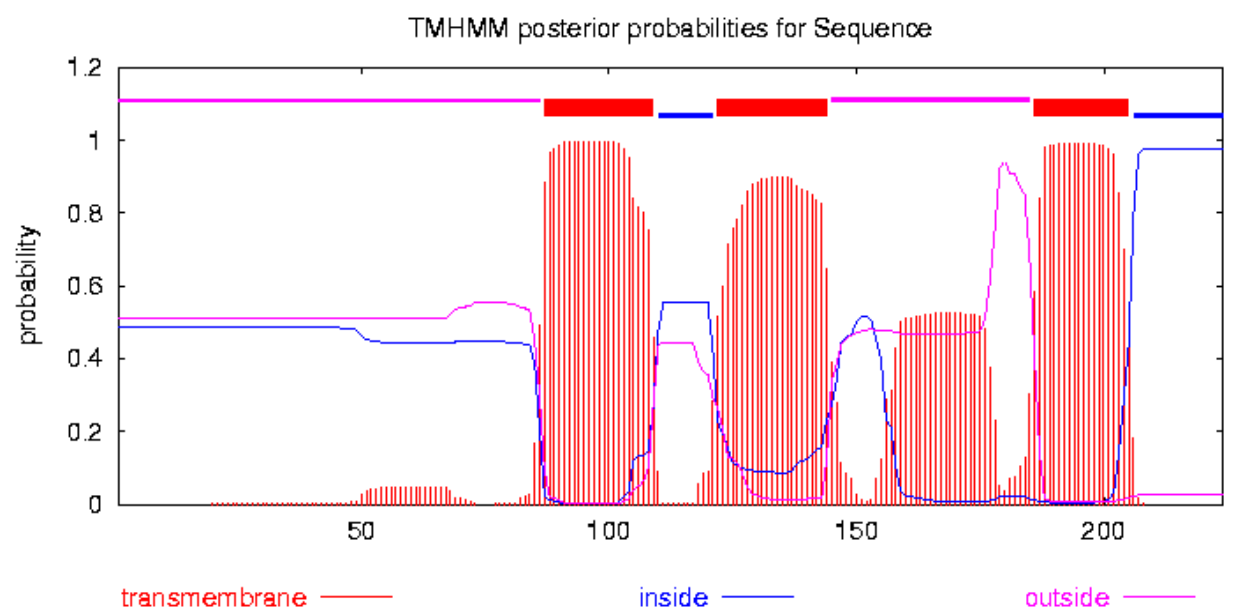


Fig. 3. The membrane trafficking engineering concept. The concept is based on using a cytosolic SNARE domain (green) which is located on a secretory vesicle A. This SNARE domain is fused to a transmembrane domain (grey) of a transmembrane protein originally located on vesicle B. The fusion protein (grey-green) should then be localized at vesicle B, and transforms this vesicle into a secretory vesicle B.

CBS prediction server, as shown in Figure 4 for the PMP22 ortholog of *A. niger* (An04g09130).

The peroxisomal membrane protein PMP22 has been studied in CHO cells⁵⁶, in *Arabidopsis*⁵⁵, and in COS cells⁵⁷. All these studies give a description of the membrane topology of PMP22, PMP22 contains 4 TMDs and has the N- and C-termini placed at the cytosolic side of the peroxisome. The precise role of PMP22 is not known. PMP22 contains two peroxisomal targeting regions with almost identical basic clusters which interact with PEX19⁵⁷. The N-terminus of PMP22 is placed towards the cytosolic side and this topology makes PMP22 useful for v-SNARE fusions at the N-terminus. To determine if PMP22 localizes to the peroxisomes a GFP-PMP22 fusion was constructed and expressed in *A. niger*⁴². Using fluorescence microscopy the localization of GFP-PMP22 was determined. The pattern of the GFP-PMP22 fusion is similar to the GFP-SKL punctuated pattern, which indicates that the PMP22 can be used as peroxisomal membrane anchor⁴².



MSAKFQDEAVTSIREDTKELVHKVGNRLTGDGYLALYLRLQSQSNPLRTKMLTSGVLSSLQEILASWIA HDVSKHGHYFSARVPKMALYGMFISAPLGHFLIGILQRFVAGRTSIKAKILQILASNLLVSPIQNAVYL CCMAVIAGARTFHQVRATVRAGFMPVMKVSWSVTSPIALAFAQKFLPEHTWVPFFNIVGFVIGTYVNT HTKKRLEALRKCGLPDMVG

Fig. 4. Predicted topology and sequence of the PMP22 ortholog in *A. niger* (An04g09130). The upper panel displays the predicted topology of the PMP22 ortholog by the CBS prediction servers (<http://www.cbs.dtu.dk/services/>). Four transmembrane domains are predicted, although the TMD between aa 150 and 175 does not exceed the threshold and therefore, 3 TMD are possible as well. It is clear that in both scenarios the N terminus of An04g09130 is positioned towards the outside (cytosolic side) of the vesicle. The lower panel displays the aminoacid sequence of the *A. niger* ortholog of PMP22 (An04g09130) used as input for the CBS prediction server TMD algorithm.

Crucial in this engineering approach is the final orientation of the novel chimeric SNARE molecule, the SNARE domain should be positioned on the outer side (i.e. surface) of the vesicle as shown in Figure 3. The resulting vesicles are decorated with a set of alternative SNARE molecules which can enable fusion of these vesicles with other cellular compartments which contain the appropriate target SNAREs.

Peroxisomes decorated with SNARE proteins have been used as alternative vesicles for transport of intracellular proteins ⁴². The use of peroxisomes as alternative vesicles for secretion of proteins which are normally located in the cytosol can be justified by several observations. Peroxisomes are organelles with a single membrane ⁵⁹ and a very elegant publication shows convincing evidence that peroxisomes originate at the ER ⁶⁰. In addition, the identification of the small GTPase Rho1p as being localized on peroxisomes by interaction of the peroxisomal membrane protein Pex25p again indicates a link of peroxisomes with the secretory machinery ⁶¹, since Rho1p is known to play a role in actin reorganization and membrane dynamics ^{62, 63}. In yeast, polarized growth is regulated by Rho1p ⁶⁴, and also in fungal systems the link between Rho1 and polarized growth has been confirmed ⁶⁵ in the filamentous fungus *A. niger*, polarized growth is linked to secretion of proteins ⁶⁶.

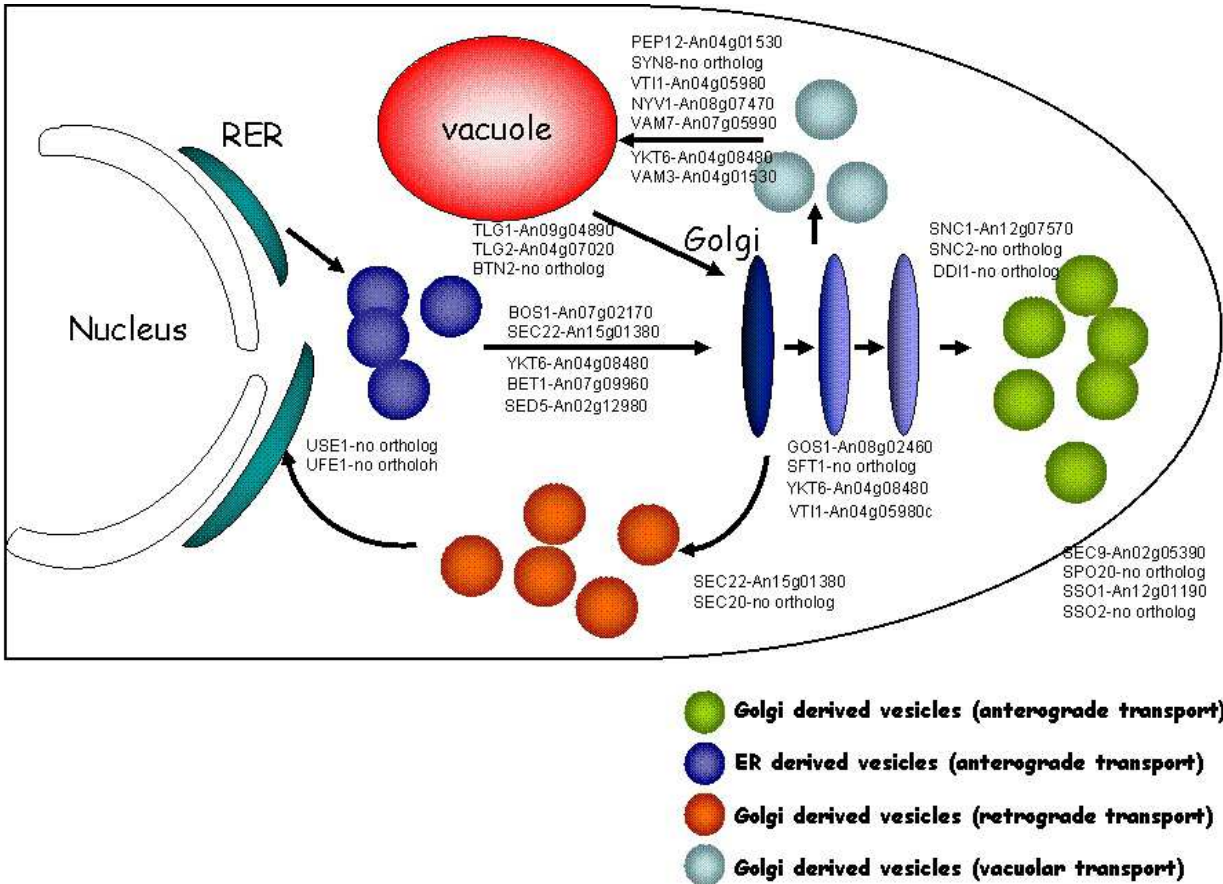


Fig. 5. Overview on SNARE molecules in *Aspergillus niger* and their orthologs in *Saccharomyces cerevisiae*. This overview is based on the *A. niger* sequencing work and the supplementary material of the resulting publication⁵⁸. In green, the vesicle flow form Golgi towards cell membrane is shown, dark blue indicates vesicle flow from ER towards Golgi and the orange vesicles are representing retrograde transport form Golgi towards ER. Finally, the light blue vesicles are representing vacuolar vesicle flow derived from the Golgi apparatus.

More recent papers also very clearly showed that peroxisomes originate from the endoplasmic reticulum^{60, 67, 68-70}. The recent finding that insertion of some peroxisomal membrane proteins occurs already at the ER⁷¹ clearly underpins that peroxisomes are linked to the secretory pathway in the eukaryotic cell. However, the same study mentioned that no conventional signal sequences can be found in a set of Peroxisomal Membrane Proteins (PMP) in *S. cerevisiae*. A possible mechanism could be that Pex19p, which is described as a chaperone for proper insertion of PMP into peroxisomes⁷²⁻⁷⁴ also acts a receptor for peroxisomal membrane proteins which are inserted at the ER membrane and become part of peroxisomes at a later stage. An alternative model could be that Pex19p is involved in de budding process of (pre)peroxisomes from the ER where PMP have been already inserted. This yields an interesting model which strongly suggest that peroxisomes are derivatives of the secretory pathway. It has been reported that the transportation of peroxisomes in *Arabidopsis* by actin filaments is dependent on MYA2 (Myosin XI isoform)⁷⁵, similar to the transportation of secretory vesicles. This common ground makes peroxisomes an attractive vehicle for transporting intracellular proteins towards the plasma membrane.

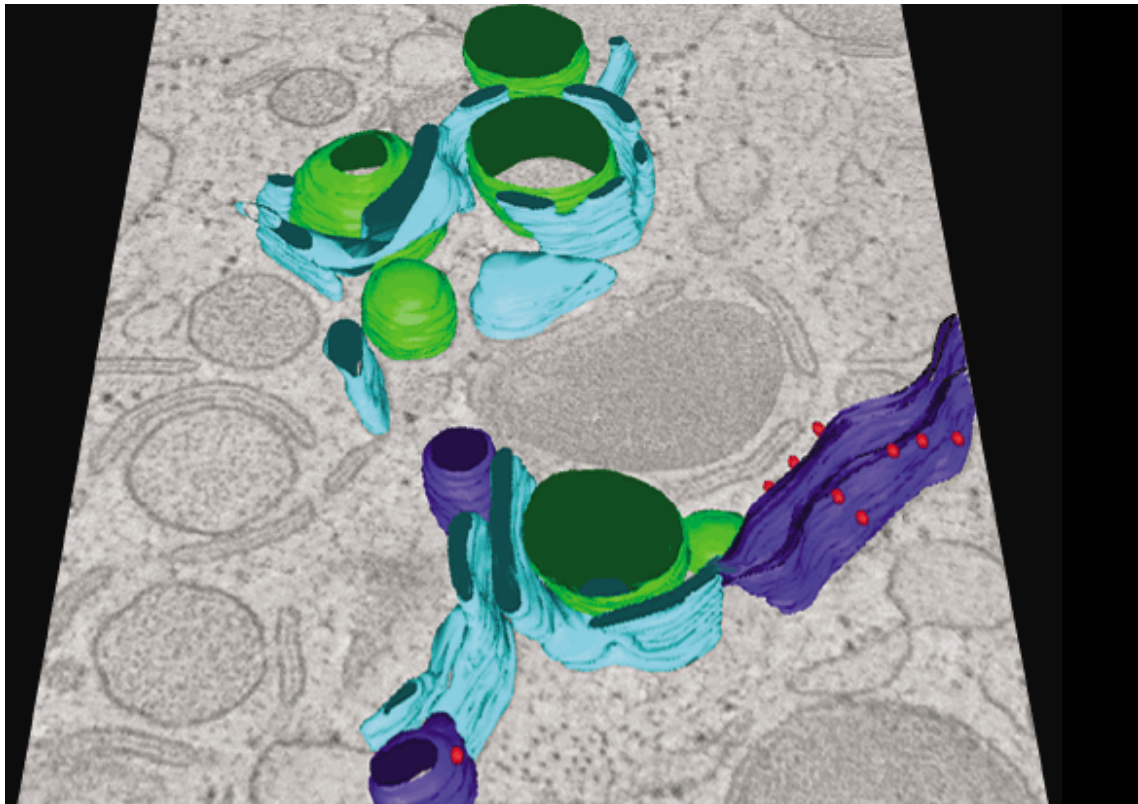


Fig. 6. Peroxisomes originate from the endoplasmic reticulum, taken from Tabak *et al.* 2003⁶⁷. In this 3D- reconstituted SEM picture⁶⁷ which was processed in silico, the peroxisomes (green) are attached to the smooth ER (light blue), as a continuous membrane structure. Other peroxisomes have already been released from the smooth ER. Rough ER is indicated by dark blue and the red dots represent ribosomes on the RER.

In addition, these organelles contain an import machinery capable of importing completely folded proteins⁷⁶⁻⁷⁹, and have a controlled way of proliferation⁸⁰⁻⁸². So, peroxisomes have the same origin as secretory vesicles and are thus candidates for alternative trafficking of proteins. The first application of such an engineered secretory pathway would be to produce intracellular proteins which show a much broader spectrum of enzymatic activities compared to secreted proteins which have predominantly hydrolase activity (as depicted in Figure 1). Normally, the intracellular proteins are folded in the cytosol, are not N-glycosylated, and disulphide bridges are not formed in this relatively reducing environment². The cytosol does not contain a major machinery for the formation of disulphide bridges or for N-glycosylation as the ER does^{83,84}. These basic differences suggest that the conventional route to secrete these intracellular proteins is not compatible with the folding environment, kinetics and modifications of these proteins.

The demonstration of engineering membrane trafficking is based on the ability of peroxisomes to import completely folded proteins. The peroxisomes containing the proteins of interest are equipped with a v-SNARE, which is normally localized at the Golgi apparatus (the study of Marelli *et al.* 2004 did not identify SNARE-like proteins on peroxisomal membranes). This ensures that peroxisomes are able to bind to t-SNAREs which are localized at the plasmamembrane. After the formation of a so called SNARE-pin the

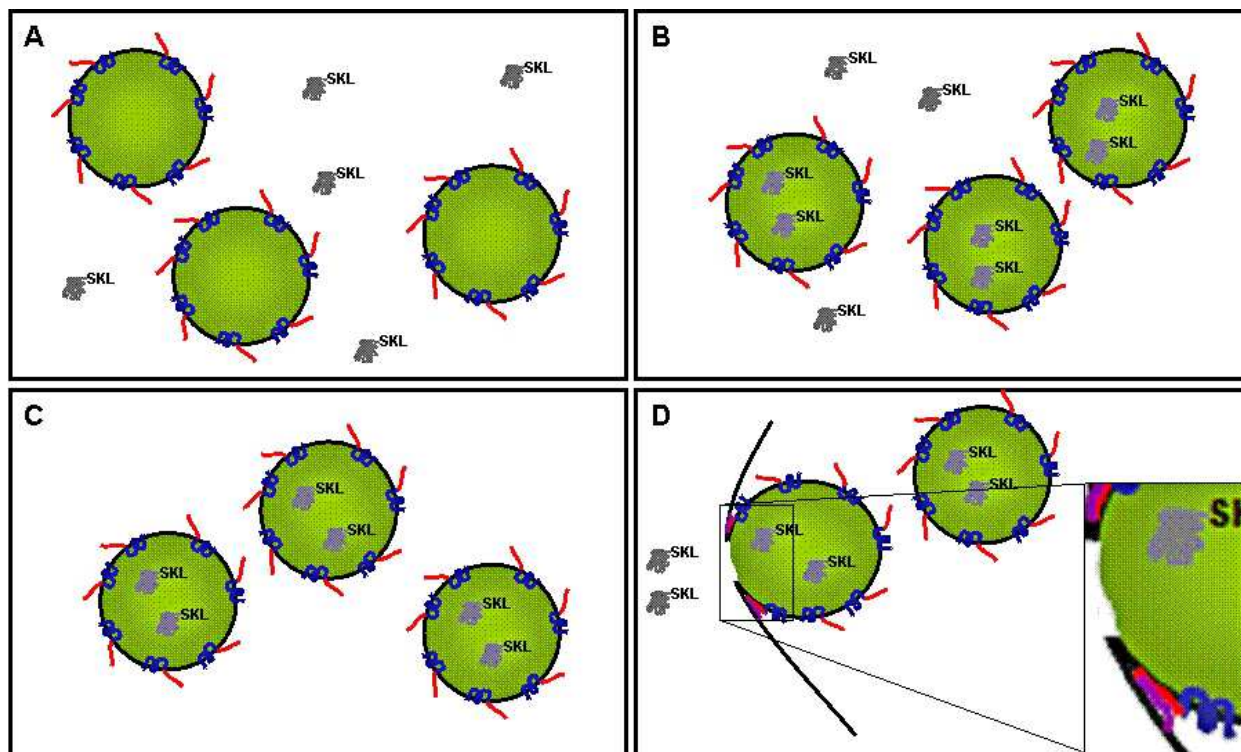


Fig. 7. Overview of the peroxicretion concept. Panel A shows the expression of SKL tagged proteins of interest together with the expression of SNC1-PMP22 fusion protein which are localized on the peroxisomal membrane. The transmembrane anchor encoded by the PMP22 is depicted in blue, the SNC1 SNARE module is encoded by red, together they ensure the decoration of peroxisomes with the *A. niger* ortholog of the v-SNARE SNC1 at the cytosolic side. Panel B shows the translocation of the SKL tagged proteins into the decorated peroxisomes. Panel C shows the complete translocation of the SKL tagged proteins in peroxisomes, the formation of peroxisomes can be increased by overexpression of PEX11 orthologs ⁸⁶. Panel D shows the fusion of the peroxisomes with the plasmamembrane, using the ortholog of the t-SNARE SSO1 (purple). The complete SNAREpin structure is more complex, and is not described here for the sake of simplicity.

peroxisomes fuse with the plasma membrane thereby releasing their luminal content. The *A. niger* ortholog of v-SNARE SNC1 (An12g07570) was placed on the peroxisome using the transmembrane domain of the PMP22 ortholog (An04g09130) as a membrane anchor. The transmembrane domain of SNC1 was omitted, the fusion protein is shown in Figure 8. It has been reported that this transmembrane domain of SNC1 is important for the function of SNC1 ⁸⁵, but the replacement of this TMD by the PMP22 TMD did not abolish the function of SNC1 (An12g07570) in *A. niger* ⁴². The replacement of the SNC1 TMD by the PMP22 protein does not diminish the potential of SNC1 to enforce membrane fusion because the peroxisomal content is released extracellularly. The paper of Grote *et al.* 2000 ⁸⁵ described inhibition of SNC1 function when the TMD was replaced by geranylgeranyl anchors. Probably membrane fusion is only possible when the TMD domains of the SNARE pairs bring the membrane bilayers in close contact so that spontaneous membrane fusion occurs.

Proteins which are produced by using the peroxicretion technology are C-terminally tagged with a PTS1 signal. The most commonly used PTS1 is SKL ⁸⁷, however variations on this

sequence have been described as well ⁸⁷. Since the Pex5p (PTS1 receptor) is highly conserved between yeasts and *A. niger*, it has been shown that SKL also functions as a PTS1 signal in *A. niger* ⁴². This raises possibilities for purifying the PTS1 tagged products on an affinity column which specifically binds PTS1 sequences. In principal nature has provided us already with a very efficient PTS1 binding protein Pex5p, which is the natural receptor for PTS1.

MSEQPYDPYIPSGANGAGAGASAAQNGDPRTREIDKKIQETVDTMRSNIFKVSEGERLD
SLQDKTDNLATSAQGFRRGANRVRKQMWKDMKMRS AKFQDEAVTSIREDTKELVHK
VGNRLTGDGYLALYLRQLQSNPLRTKMLTSGVLSSLQEILASWIAHDVSKHGHYFSARVP
KMALYGMFISAPLGHFLIGILQRVFAGRTSIKAKILQILASNLLVSPIQNAVYLCCMAVIAG
ARTFHQVRATVRAGFMPVMKVS WVTSPIALAFAQKFLPEHTWVPFFNIVGVIGTYVNT
HTKKKRLEALRKCGLPDMVG

Fig. 8. The SNC1-PMP22 fusion protein which enables peroxicretion. SNC1 ortholog sequence (no TMD) is depicted in green (based on An12g07570) the PMP22 ortholog sequence (based on An04g09130) is depicted in blue.

Mutational studies to determine whether shortening of the region between SNC1 and PMP22, thereby placing SNC1 closer to the peroxisomal membrane, could enhance peroxicretion were performed by our group. This approach was partially successful, the most efficient peroxicretion occurred when SNC1 was linked to the full length PMP22 (318 aa) or a truncated version of PMP22 (300 aa). When PMP22 was truncated further the peroxicretion decreased dramatically (< 50%). This is in contrast with a previous study ⁸⁸, which predicts an increase of vesicle fusion when the hinge region of the v-SNARE and the TMD domain is shortened. This discrepancy is most likely caused by the unnatural TMD of the SNC1-PMP22 fusion protein. The TMD of PMP22 was not evolutionarily selected for efficient membrane fusion. Moreover, the localization information of PMP22 is most likely positioned in this hinge region. GFP fusions with the different truncated PMP22 constructs showed a localization, which is not unambiguously peroxisomal. This indicated (partial) mis-localization of SNC1-PMP22 fusion products when PMP22 is truncated to less than 300 aa, explaining the dramatic decrease of peroxicretion.

Additional modifications to fine-tune the peroxicretion concept can be foreseen, like rebuilding the peroxisome in such a way that all components of the secretory vesicles (proteins and lipids) are present on the peroxisome, making them efficient cell organelles for peroxicretion. Subcellular proteomics could supply us with these leads. One of the most promising options is to use Sec4p a protein involved in SNARE-pin formation. Sec4p is a Rab-GTPase which plays an important role in polarized secretion by tethering secretory vesicles to the plasmamembrane ^{89, 90}. Sec4p is localized at Golgi-derived vesicles with a geranylgeranyl anchor. Sec4p GEF (Sec2p) and Sec4p GAP (Msb3p and Msb4p) are described and are controlling the activity of Sec4p ⁹¹. This makes Sec4p and its partners potential leads for improving the peroxicretion technology.

In the quest for alternative secretory routes the peroxicretion concept was developed ⁴², which shows that it is possible to redirect peroxisomes to the cell membrane where they are able to fuse and release their cargo in the extracellular medium, as shown in Figure 7. The peroxisomes can be loaded with intracellular enzymes by adding a C terminal peroxisomal

signal sequence like SKL. A combination of the peroxicretion approach with more conventional approaches to improve protein production like removal of proteases and increasing folding capacity of, in this case, the cytosol may further improve protein production. This proof of principle shows that by relocating the SNARE molecules to alternative compartments, it is possible to redirect vesicle trafficking in cells, which could also enable production of toxic compounds in micro organisms. This exciting possibility of constructing chimeric proteins of a specific membrane anchor with a specific SNARE functionality is an important tool to engineer membrane trafficking.

Moreover, it can also be hypothesized that due to the modified peroxisomes the complete biomass could now be used for production of enzymes, and not only the hyphal tips as is being suggested for normal secretory proteins⁹². A speculative idea would be to determine the components that allow the peroxisomes to utilize all of the biomass to release their content into the medium and adjust the conventional secretory vesicles in a similar way which would enable secretion of secretory proteins using the complete biomass instead of just the hyphal tips. Using this approach we could even increase fermentation yields of secretory proteins.

5. Designing a robust and versatile cell factory, an outlook

The cell factory of the future should be able to produce a wide range of proteins and metabolites with high productivities and yields in a robust process. It is clear that current strategies contribute in an incremental way, which is important to make the bioprocess economically feasible. In order to expand the product range and design a truly versatile cell factory more innovative approaches are needed. Until now, membrane engineering has hardly been used as an additional tool for designing cell factories. Recent developments have shown that membrane engineering can be a key for designing a truly versatile cell factory. The potential of this approach is enormous, especially in combination with conventional strategies.

In this era of synthetic biology where bio-bricks are used to engineer microbes for new functionalities the concept of membrane trafficking engineering is very well positioned. It enables a true engineering methodology for intracellular trafficking thereby unlocking a complementary approach to already applied strategies like genetic devices based on bio-bricks. In addition, the availability of -omics technologies will function as a starting platform for further fine-tuning of the membrane engineering concept. Based on this holistic approach more key components will be identified which can be used to increase the efficiency of membrane engineering and the application of engineered membranes in microbial cell factories.

This quest for further fine-tuning will be exciting and will have academic impact as well. The membrane trafficking engineering concept can serve the academic society with a new way of studying the complex vesicle flow in biological systems. By combining different components of the secretory pathway into novel chimeric proteins with new functionalities a detailed overview is within reach which can describe the molecular details of the protein secretion mechanism.

This is a major challenge and as with any technology the combined effort of industry and academia can result in significant progress when strong interaction and mutual creativity are applied and recognized.

6. Acknowledgments

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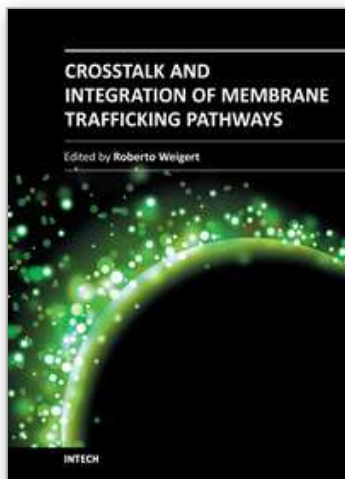
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Membrane traffic is a broad field that studies the complex exchange of membranes that occurs inside the cell. Protein, lipids and other molecules traffic among intracellular organelles, and are delivered to, or transported from the cell surface by virtue of membranous carriers generally referred as "transport intermediates". These carriers have different shapes and sizes, and their biogenesis, modality of transport, and delivery to the final destination are regulated by a multitude of very complex molecular machineries. A concept that has clearly emerged in the last decade is that each membrane pathway does not represent a close system, but is fully integrated with all the other trafficking pathways. The aim of this book is to provide a general overview of the extent of this crosstalk.

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Phone: +86-21-62489820
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