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REVIEW

Fission yeast *Schizosaccharomyces pombe* as a producer and secretor of heterologous proteins

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Abstract

Microorganisms are widely used as producers of heterologous (foreign) proteins of medical or industrial interest. Bacteria are the most efficient producers, but they are not able to perform the post-translational processing of eucaryotic proteins (folding, glycosylation, phosphorylation or removal of part of their initial sequence). Eucaryotic yeasts are able to do the post-translational processing and secrete heterologous eucaryotic proteins in their native, biologically functional form. Among yeasts, the fission yeast Schizosaccharomyces pombe has the advantage of being more similar to higher eucaryotes in many respects, but its heterologus protein production is still small compared to that of other yeasts such as Saccharomyces cerevisiae, Pichia pastoris or Kluyveromyces lactis. However, the good quality of proteins obtained so far makes fission yeast an interesting producer and efforts are being made by many researchers to modify the gemome of S. pombe in order to obtain better producers of proteins. This review presents these efforts up to date and describes expression systems, signaling peptides, post-translational processing and secretion systems known in fission yeast, and the possible ways to increase their efficiency. The last paragraphs are dedicated to approaching methods and protocols of genomic modifications.

Keywords: heterologous protein, expression, traffic, secretion.

Introduction

Heterologous protein production is mainly a biotechnological topic. Unicellular microorganisms, both procaryotic and eucaryotic, are the preferred heterologous protein producers for obvious reasons: they can be cultured in big amounts, at low costs, their manipulation is simple and their genetic background is well known and accessible for manipulations. Some proteins can be secreted into the medium from where they can be easily recovered and purified. The proteins thus produced can be used as medicines, vaccines, laboratory or industrially useful enzymes or food components. A few recent or recently updated examples (restricted to yeasts as producers) are: production of insulin by *S. cerevisiae* [1], production of influenza virus proteins usable as vaccine components by the *Saccharomyces* yeast [2, 3, 4], production of hepatitis C virus proteins by *Pichia pastoris* with the aim of preparing a vaccine [5], production of industrially or laboratory usable enzymes by *Pichia pastoris* [6, 7].

As producers, a large and ever-increasing number of unicellular organisms are being used: procaryotic (bacterial) organisms such as *Escherichia coli, Bacilus (subtilis, braevis),* or simple eucaryotes such as *Saccharomyces cerevisiae, Picchia pastoris, Kluyveromyces lactis, Schizosaccharomyces pombe.* For a given protein each of the organisms may be more or less appropriate. The advantage of bacterial producers is the high yield of production and the ease

of genetic manipulation. They are most appropriate for the production of proteins of bacterial origin. Gram-negative bacteria such as *Escherichia* have the advantage of a very well known genome and the existence of a large choice of well characterized mutant strains exhibiting special qualities, but have the inconvenient of sequestering the secreted proteins in the periplasmic space; Gram-positive *Bacillus* bacteria are devoid of the outer membrane and can secrete proteins directly into the medium [8, 9]. A major inconvenient of the bacterial producers is that they lack the equipment needed for the eucaryotic proteins to be processed post-translationally and to become functional and able to be secreted. Yeasts possess that equipment and therefore they are preferred for the production of heterologous eucaryotic proteins.

From this point of view the fission yeast *Schizosaccharomyces pombe* appears to be a good choice for mammalian heterologous protein production. *S. pombe* has more similar genomic features to higher eucaryotes than other yeasts. Some human genes (like *cdk1*) were able to replace homologous genes that were deficient in fission yeast, which proves that the encoded proteins are functional in *S. pombe* [10]. Some transcription promoters from human cells work as promoters in the fission yeast as well [10]. The signal for retention of proteins in the endoplasmic reticulum (ER) - the carboxy-terminal KDEL sequence - is identical in fission yeast and in mammals, but different from that of other yeasts [11]. The Golgi system is structurally well represented in *S. pombe* but less visible in other yeasts such as *Saccharomyces cerevisiae* and *Pichia pastoris* [12, 13]. However, the fission yeast has been less used for human protein production than other yeasts so far. The reason is that the best secretion of such proteins was evaluated at 20 mg/l/24 hours [12], in *S. pombe* compared to a few grams/l/24 hours in *Bacillus* bacteria. Certainly, the quality of secreted proteins may be important, still a higher secretion is desirable and this is the target of nowadays ongoing research [13].

The steps of protein production and secretion

In order to obtain a high protein production by a microorganism, the following conditions are necessary: 1) High expression of the gene encoding that protein in the producer organism; 2) Efficient translation of the genetic information; 3) Efficient folding, post-translational processing, intracellular traffic of the protein and its secretion; 4) Avoiding the proteins' destruction both in and out of the cells by lytic enzymes. We shall present the essential of these data, stressing on fission yeast.

1. Gene expression is usually the rate limiting step of protein synthesis. It is measured as the amount of specific mRNA extracted from cells. The expression of a heterologous gene can be increased by increasing the number of copies of the respective gene in the cell. Usually a foreign gene is introduced into the producer cell through a plasmid which can multiply independently of the chromosomal DNA, this resulting in a number of copies per cell. Plasmids are not evenly distributed between the sister cells in mitosis so that, after several rounds of cell division, some cells lose plasmids altogether. This genetic instability is an inconvenient that can be circumvented by maintaining the cells in a selective medium that only allows growth of the cells possessing the plasmid (which also has to contain a selection marker gene).

Another method to obtain high expression of a heterologous gene is to place it under the control of an active promoter, i.e. a DNA sequence upstream of the gene that ensures the functioning of the transcription complex. The promoter is usually homologous (belongs to the producer organism). In the fission yeast *Schizosaccharomyces pombe* the thiamine-repressible promoter of the *nmt1* gene is largely used (see also Table 1). A gene under its control can be

expressed a few hundred folds when thiamine is absent in the growth medium than when it is present [14]. Another homologous expression system is that of the *pho1* gene. Its expression is low when adenine in the medium is abundant, but increases about 40 fold when adenine is limiting [15]. We have shown recently that expression of *pho1* is under the negative control of a system involving a 14-3-3 protein encoded by *rad24* gene. Deletion of *rad24* enhances *pho1* expression about 100 fold [16]. A heterologous promoter being used is that of the human cytomegalic virus [10].

A heterologous gene can be inserted into the chromosome downstream of the active gene promoter or, alternatively, both the promoter and the gene may be included in a plasmid which is transformed into the producer organism, thus combining the generation of multiple copies of the gene with its increased transcription.

Table 1. Expression systems designed or used for heterologuos proteins production in *Schizosaccharomyces pombe*

Category (plasmid or genomic insertion)	Promoter	Number of heterolo-gous gene copies	Observations and references
pRep	*nmt1* (homologous, thiamine-repressible)	~ 500	Constructed accordingly to [14]. Used for GFP (green fluorescent protein) over-expression by [12, 17]
pTL2MI	hCMV (heterologous, constitutive)	200	Constructed and used by [10] for the expression and secretion of human hypophyseal growth hormone .
pSL2MI	hCMV (heterologous, constitutive)	200	
Insert	*nmt1, nmt41, nmt81* (homologous, thiamine-repressible)	1	Proposed by [20] for controlled expression of several genes.
Insert	*pho1* (homologous, adenine-repressible)	1	Proposed by [15, 16] as a possible adenine-controlled expression system
Insert	hCMV	4	Constructed and used by [18, 19] for the production and secretion of human hypophyseal growth hormone.

Expression system's choice is an important step for the production and secretion of heterologous proteins. The most efficient expression system is usually chosen. However the secretion might not be as high as the expression and this fact may have some consequences. If the heterologuous protein is toxic for the producing cell, it is more convenient to choose an expression system repressible by one of the metabolites in the medium (thiamine or adenine for fission yeast, see also Table 1). Such a system will allow the expression only when this factor is consumed and the cell number is big enough to produce a high protein quantity before the cell death. Another consequence of overexpression is triggering of an inhibitory control system due to a high number of unfolded or incorrectly folded polypeptide chains, as it will be shown further in this review. In this case a lower expression system may be used.

2. When the expression of the heterologous protein encoding gene is very high, the subsequent steps may be rate-limiting for protein secretion. To our knowledge there is no data in the scientific literature to show that **translation** can be rate-limiting in the case of heterologous protein production. However, post-translational processing and the traffic mechanisms of protein exporting can.

3. Post-translational processing and protein secretion. After translation, a protein destined to be exported outside the cell may follow one of the secretion pathways existent in both procaryotes and eucaryotes. Two such pathways are known in fission yeast, followed by

its two peptide pheromones: a) pheromone P pathway or the classic secretion pathway, involving the passage through the ER (endoplasmic reticulum) and the Golgi system; b) pheromone M pathway, independent of ER and Golgi but depending on the ABC transporter, a protein system in the plasma membrane.

a) *Endoplasmic reticulum depending pathway.* The polypeptide chain synthesized in the ribosomes attached to the ER membrane (where it can be folded and subjected to the first post-translational processing) has to begin at the N terminal with the so called *leader peptide*, a sequence of about 20 amino acids. The leader peptide serves as a signal for driving the newly synthesized polypeptide (pre-pro-protein) to the ER, a signal that is recognized by the SRP (Signal Recognition Particle) protein complex. In the heterologous protein technology the leader peptide has to belong to one of the homologous proteins, i.e. the heterologous expression construct has to consist of the tandem: active promoter (homologous or heterologous) - leader peptide encoding sequence (homologous) - gene encoding the heterologous protein. Obviously, this tandem is a recombinant construct. For the production of heterologous proteins by *Schizosaccharomyces pombe* a few leader peptides have been used: the P3 leader peptide (belonging to the P pheromone precursor, encoded by *map2* gene) [19], the *pho1* leader peptide [17] and the carboxypeptidase Y leader peptide (encoded by *cpy1*gene) [12] (see also Table 2).

Table 2. Signal sequences for secretion pathways used by heterologous proteins in fission yeast

Name	Access no. in NCBI database	Amino acid sequence	Observation and references
P3	*map2* NM 001023030	MKITAVIALLFSLAAASPIPVAD	Used by [18] for hypophyseal growth hormone secretion and by [12] for GFP secretion.
Cpy 1	*cpy1* NM 001019854	MLMKQTFLYFLLTCVVSAQFNGY VPPE	Used by [12] for GFP and TNF (tumor necrosis factor) secretion.
Pho 1	*pho1* NM 001023870	MFLQNLFLGFLAVVCANAQFAE	Used by [17] for GFP and HPV (human papilloma virus) secretion.
Mfm 1	*mfm1* NM 001020207	MDSMANSVSSSSVVNAGNKPAET LNKTVK**N-Y**TPKVPYMC-VIA	Used by [37] for GFP secretion.

The leader sequences of different homologous polypeptide chains are not identical, but they keep a common general pattern with respect to the constitutive amino acid side chains: 1-5 amino acids with cationic groups (arginine-R, lysine-K, histidine-H) and a core of 6-15 hydrophobic amino acids followed by 3-7 polar amino acids that serve as signal for the peptidases that will remove the leader peptide in the ER [21]. The leader sequences are not equally efficient as secretion signals for all heterologous proteins. In fission yeast for example, the Cpy1 sequence attached to GFP (green fluorescent protein from *Aequorea victoria* jelly fish) produces a secretion that is several fold more efficient than that of the P3 sequence [12] (see also Table 2). The causes of this phenomenon remain misunderstood, especially since the leader sequence is removed from the secreted protein while this is still inside the ER. Thus in an attempt of constructing a gene encoding a heterologous protein destined to be secreted, several leader sequences have to be tested in order to choose the more convenient one.

The leader peptide is removed in the ER as one of the first post-translational processing events. At the same time, protein folding occurs. This is facilitated by some protein complexes. Two main complexes are present in the ER: *i) Lectin complex* consisting of

calnexin (an ER membrane integral protein), calreticulin (a soluble protein), a thiol oxidoreductase and a few other proteins. This complex recognizes the newly formed substrate polypeptides on their glycosyl residues after they have been N glycosylated [22, 23]; *ii) Chaperone complex* consisting of several proteins of the Hsp70 and Hsp40 classes. Chaperones contribute to correct folding of the newly synthesized polypeptides by binding them on some of the chaperone's hydrophobic residues (an ATP-dependent process) precluding in this way hydrophobic aggregation of the newly synthesized proteins among themselves and facilitating their correct folding. The absence of these proteins leads to a decrease in homologous and heterologous protein production and secretion.

Fission yeast has genes encoding both calnexin [11, 23] and BiP, an Hsp70 class homologue [24]. The roles of calnexin and of BiP protein are different: calnexin retains BiP and other proteins which are "resident" in the ER lumen due to the recognition and attachement to a carboxy-terminal tetrapeptide common to all of these proteins [11]. In *S. cerevisiae* the BiP chaperone stabilizes a protein located in the membrane of the ER tubules (Ire1p) by attaching to it. The existence of a large number of unfolded polypeptide chains competes with Ire1p for BiP, so that BiP dissociates from Ire1p and the latter dimerises and triggers an UPR (unfolded protein response) which consists of reshaping an RNA species. This RNA regulates the transcription of at least 300 genes, some of their products being able to modulate the expression of proteins such as BiP and PDI (protein disulphide isomerase), but also to decrease the expression of proteins that have triggered the UPR [25]. Calnexin and BiP probably also have common functions with respect to assisting the polypeptide chains folding, because the overexpression of one of them can compensate the lack of the other one in this process and in maintaining the cells viability [26]. These data are established for *Saccharomyces cerevisiae* and are also probably valid for *Schizosaccharomyces pombe*. Table 3 presents the proteins participating in the ER depending secretion process and the genes encoding them in *S. pombe* as well as their orthologues (homologues with the same function) in other species such as *Saccharomyces cerevisiae* and *Homo sapiens*.

Table 3. Proteins involved in the secretion of homologous or heterologous proteins in Schizosaccharomyces pombe

Name	Function	Coding gene in *S. pombe*	Orthologues in other species	Refe-rences
BiP	Chaperone from the ER. It „assists" the folding of polypeptide chains in the ER.	*Sp-BiP*	*Hs-BiP, Sc-KAR2*	[24]
Calnexin	Lectin from the ER. It retains the proteins having a „residence" signal in the ER tubules.	*Sp-cnx1*	*Sc-CNE1, Hs-CLNX*	[11, 23]
PDI – Protein disulphide isomerase	Proteins containing disulphide bonds. They „assist" S-S bonds formation in the process of protein folding in the ER lumen.	*Sp-pdi1*	*Sc-PDI1*	[27]
ERO – Endo-plasmic reticulum oxidases	Catalyzes the generation of S-S bonds in PDI enzymes.	*Sp-ero1-1*	*Sc-ERO1-1*	[8]
Ypt 1	GTPase involved in vesicular traffic.	*Sp-ypt1*	*Hs-rab1*	[31]
Ypt2	GTPase involved in vesicular traffic.	*Sp-ypt2*	*Sc-SEC4, Hs-rab8*	[32]
Ypt3	GTPase involved in vesicular traffic.	*Sp-ypt3*	*Sc-YPT3, Hs-rab11*	[33]
Ypt5	GTPase involved in vesicular traffic.	*Sp-ypt5*	*Hs-rab5*	[34]
Mam1	Component of the ABC export system.	*Sp-mam1*	*Sc-STE6*	[37]

Other proteins important for secretion and resident in the ER are those upon which disulphide bond formation depends. Protein disulphide isomerase (PDI) is one of them. This enzyme, containing itself disulphide bonds, is an isomerase (it catalyses the exchange of some disulphide bonds with others in the same molecule) and an oxidoreductase (it catalyses the formation of disulphide bonds by oxidation or their cleavage by reduction). Other oxidoreductases having disulphide bonds as substrates are ERO (oxidases from the ER). These enzymes have PDI as a substrate and are a reservoir of oxidized disulphide bonds. Proteins of both PDI and ERO type are encoded in the genome of fission yeast and of all the species that have been studied in this regard (Table 3 and included references). A series of other proteins in the chaperone and oxidoreductase category have been found in budding yeast, but not yet in fission yeast. As far as the heterologous protein production is concerned, the folding step could (when rate limiting) be improved by chaperone overexpression in *Pichia pastoris* [29] or overexpression of proteins contributing to disulphide bonds formation in *Kluyveromyces lactis* [30].

The intracellular protein traffic consists of an orderly passing of a protein through various compartments of the Golgi system where they undergo specific post-translational processing, especially glycosylations. Finally, a protein that does not have a signal sequence to direct it to other destinations (nucleus, mitochondria, plasma membrane) is exported through an exocytosis process. Both traffic and exocytosis involve forming of lipid vesicles in which proteins are included. A series of small GTP-associated proteins, named Rab in mammals and Ypt in yeasts, participate in this process (table 3 and included references). At least one of these proteins, Ypt3, has proved to be important for the export of a heterologous protein (GFP with the Pho1 leader peptide) in *S. pombe* [33]. Inositol phosphatides also have a major importance for trafficking [35]. We have reported data that suggest the requirement of inositol for intracellular traffic of the P pheromone and the M and P pheromone receptors in *Schizosaccharomyces pombe* [36].

b*) The ABC export pathway*, independent of ER and Golgi system. This pathway exists in both procaryotes and eucaryotes. The ABC transporter is located in the plasma membrane and facilitates protein translocation by using ATP. A component of the ABC system in *S. pombe* is the protein encoded by *mam1* gene [37]. Besides the membrane transporter, the other components of the system are unknown. The secretion might depend on a vesicular (vacuolar) system which is different from the Golgi apparatus. As this system represents the natural pathway of secretion for the M pheromone, it has been used as a signal for the secretion of a reporter recombinant protein (GFP) by Kjaerulff et al. [37]. They proved that the greatest secretion efficiency is obtained by placing the heterologous protein in-between the prosequence of the M pheromone precursor and the part coding for the pheromone aminoacid sequence (in Table 2 the insertion place of the heterologous sequence is in-between N-Y written in bold face). The „pro" sequence of the precursor (from the NH_2 terminal to glutamine-N 30, see Table 2) is essential for secretion, as well as the carboxy-terminal aminoacid triad. Even though apparently more simple, the export through this pathway hasn't proved to be more efficient than the one through the complex pathway involving the ER and the Golgi system [37].

4. The rate and quality of protein secretion is often dependent on both **intra** and **extracellular (secreted) proteases**. A particular case of intracellular proteases is that of pro-protein converting proteases which are involved in post-translational processing by proteolytic cleavage. Kexins represent a subclass of these proteases and have been described in many species. The fission yeast *S. pombe* has a known kexin, encoded by *krp1* gene that is involved in pro-pheromones processing. There have also been described some other genes coding for putative pro-protein converting proteases [38]. The problem of pro-protein

processing is interesting in the technology of heterologous protein production when these proteins must undergo such a processing (proinsulin, for example). Other proteases, intracellular (cytoplasmatic or lysosomal) or extracellular (secreted) are important as factors to be avoided in heterologous protein production. It has been reported that the deletion of any one of 13 genes encoding proteases in *Schizosaccharomyces pombe* improved heterologous human growth hormone (hGH) production [19]. The deletion of *ppp80* gene, encoding a cystein protease, leads to a spectacular increase of hGH secretion. Combining this deletion with the deletion of other 6 genes encoding different proteases increases the secretion 2 more times [18, 19].

5. Other factors leading to an increased efficiency of *Schizosaccharomyces pombe* yeast as a heterologous protein producer. Recently, Y. Giga-Hama's group proposed a series of genetic modifications of fission yeast which lead to a better efficiency of heterologous proteins production and secretion. The authors started from the hypothesis that some nonessential genes can be deleted without producing growth limitations, but only auxotrophies (the requirement for nutritional factors in the culture medium). These deletions would lead, on the other hand, to energy sparing and to greater productions of the proteins of interest. The deletion of a chromosomal region of 500 kb coupled with the deletion of some important genes involved in the carbohydrate and amino acid metabolism, and also of some protease encoding genes leads to a remarkable increase in human growth hormone heterologous production [13].

APPROACHING METHODS AND USEFUL PROTOCOLS

In order to introduce the genes encoding foreign proteins, sequence signals, promoters, etc. into the genome of the producing cells, there can be used **episomal modifiers** of the genome or **chromosomal insertions**.

1. Episomal factors. The recombinant plasmids must contain an autonomous replication center (the most utilised is the *ars1* sequence), a selectable marker (a gene that confers prototrophy to an auxotrophic strain or a gene conferring resistance to a particular antibiotic, etc.), and a polylinker sequence (recognized by several restriction enzymes) where the genes encoding heterologous proteins can be inserted. An expression promoter must be added at a distance of 25-39 nucleotides from the insertion point of the heterologous gene. One of the *nmt* variants (thiamine-repressible) is the most common promoter, but other promoters can be used as well (see Table 1). Such a plasmid is pRep, initially designed by Maundrell [14] and subsequently modified variants. The plasmids can be constructed or obtained from their authors. The gene coding for the heterologous protein can be PCR-amplified from the genome of the original organism using total DNA or cDNA (copy of messenger RNA) as a template. Amplification on cDNA is more advantageous as the gene contains only the coding part, without introns. The DNA fragment containing the gene of interest must be flanked by restriction sites (designed by PCR primers sequences) in order to enable the subsequent insertion in the polylinker of the plasmid vector, which has been linearized with the same restriction enzyme. After subsequent ligation, the gene encoding the heterologous protein is placed under the control of the chosen expression promoter. The transforming of plasmids into the cells can be carried out according to several protocols; one of them with good transformation efficiency is described in [20].

2. Linear modules for chromosomal insertion. In principle, such a module has to contain the gene of interest under the control of an expression promoter as well as a selectable marker gene, flanked by two fragments that are homologous to two chromosomal sequences flanking the point where the module has to be inserted (target). These homologous fragments

ensure the insertion at the target by a homologous recombination process consisting of two crossings-over. The linear modules can be made by PCR amplification of constructs containing the tandem: active promoter (homologous or heterologous) - leader peptide encoding sequence (homologous) - gene encoding the heterologous protein - selection marker, included in a plasmid. The primers of approximately 80 nucleotides are each composed of a long (approx. 60 nucleotides) sequence homologous to the target and an additional shorter sequence (about 20 nucleotides) homologous to the ends of the tandem. A collection of such modules is described in [20]. A better method (as providing longer homologous ends) seems to be the construction of linear inserts by fusing the tandem's amplicon (the insert) with amplicons of two sequences 500 bp each, homologous to the genome target sequences (flanks). The two flanking amplicons can be made by PCR amplification using genomic DNA as a template and primers that are identical partially to the flanks and partially to the insert. The insert and the flanks can then be fused together in a single construct by fusion PCR, due to their partial overlapping [39,40].

Once the strain expressing the heterologous gene has been constructed, it is grown in the most favorable conditions for producing the protein of interest. The secretion of the protein may be quantified in the growth medium by immunochemical methods (western blotting).

References

1. T. KJELDSEN, S. LUDVIGSEN, I. DIERS, P. BALSCHMITD, A.R. SORENSEN, N.C. KAARSHOLM. *Engeneering enhanced protein secretory expression in yeast with application to insulin*. *J. Biol. Chem.*, **277**, 18245-18248 (2002).
2. X. SAELENS, P. VANLANDSCHOOT, W. MARTINET, M. MARAS, S. NEIRYNCK, R. COTRERAS, W. FIERS, W.M. JOU. *Protection of mice against a lethal influenza virus challenge after immunization with yeast-derived secreted influenza virus hemagglutinin*. *Eur. J. Biochem.*, **260**(1), 166-175 (1999).
3. A. WADLE, G. HELD, F. NEUMANN, S. KLEBER, B. WUELLNER, A.M. ASEMISSEN, B. KUBUSCHOK, C. SCHEIBENBOGEN, T. BREINING, A. MEYERHANS, C. RENNER. *Cross-presentation of HLA class I epitopes from influenza matrix protein produced in Saccharomyces cerevisiae*. *Vaccine*, **11**(24), 6272-6281 (2006).
4. C.Y. WANG, Y.L. LUO, Y.T. CHEN, S.K. LI, C.H. LIN, Y.C. HSIEH, H.J. LIU. *The cleavage of the hemagglutinin protein of H5N2 avian influenza virus în yeast*. *J. Virol. Meth.*, **146**, 293-297 (2007).
5. G. MARTINEZ-DONATO, N. ACOSTA-RIVERO, J. MORALES-GRILLO, A. MUSACCHIO, A. VINA, C. ALVAREZ, N. FIGUEROA, I. GUERRA, J. GARCIA, L. VARAS, V. MUZIO, S. DUENAS-CARRERA. *Expression and processing of hepatitis C virus structural proteins in Pichia pastoris yeast*. *Biochem. Biophys. Res. Commun.*, **342**, 625-631 (2006).
6. N. JUGE, G. WILLIAMSON, A. PUIGSERVER, N.J. CUMMINGS, I.F. CONNERTON, C.B. FAULDS. *High-level production of recombinant Aspergillus niger cinnamoyl esterase (FAEA) in the methylotrophic yeast Pichia pastoris*. *FEMS Yeast Res.*, **1**, 127-132 (2001).
7. J.G. BERRIN, W.R. MCLAUCHLAN, P. NEEDS, G. WILLIAMSON, A. PUIGSERVER, P.A. KROON, N. JUGE. *Functional expression of human liver cytosolic beta glucosidase în Pichia pastoris. Insights into its role în the metabolism of dietary glucosides*. *Eur. J. Biochem.*, **269**, 249-258 (2002).
8. L. WESTERS, H. WESTERS, W.J. QUAX. *Bacillus subtilis as cell factory for pharmaceutical proteins: a biotechnological approach to optimize the host organism*. *Biochim. Biophys. Acta*, **11**, 299-310 (2004).
9. G. GEORGIU, L. SEGATORI. *Preparative expression of secreted proteins in bacteria: status report and future prospects*. *Curr. Opin. Biotechnol.*, **16**, 538-545 (2005).
10. Y. GIGA-HAMA, H. KUMAGAI. *Expression system for foreign genes using the fission yeast Schizasaccharomyces pombe. Biotechnol. Appl. Biochem.*, **30**, 235-244 (1999).
11. F. PARLATI, D. DIGNARD, J.J. BERGERON, D.Z. THOMAS. *The calnexin homologue cnx1 in Schizosaccharomyces pombe is an essential gene which can be complemented by its soluble ER domain. EMBO J.*, **14**, 3064-3072 (1995).

12. S. KJAERULFF, M.R. JENSEN. *Comparison of different signal peptides for secretion of heterologous proteins in fission yeast*. *Biochem. Biophys. Res. Commun.*, **336**, 974-982 (2005).
13. Y. GIGA-HAMA, H. TOHDA, K. TAKEGAWA, H. KUMAGAI. *Schizosaccharomyces pombe minimum genome factory*. *Biotechnol. Appl. Biochem.*, **46**, 147-155 (2007).
14. K. MAUNDRELL. Thiamine *repressible expression vectors pRep and pRip for fission yeast*. *Gene*, **123**, 127-130 (1993).
15. M.E. SCHWEINGRUBER, E. EDENHARTER, A. ZURLINDEN, K.M. STOCKMEYER. *Regulation of pho1-encoded acid phosphatase of Schizosaccharomyces pombe by adenine and phosphate*. *Curr. Genet.*, **22**, 289-292 (1992).
16. P.M. VOICU, E. PETRESCU-DĂNILĂ, M. POIȚELEA, A. WATSON, M. RUSU. *In Schizosaccharomyces pombe the 14-3-3 protein Rad24p is involved in negative control of pho1 gene expression*. *Yeast*, **24**(2), 121-127 (2007).
17. J. BRASPENNING, W. MESCHEDE, A. MARCHINI, M. MULLER, L. GISSMAN, M. TOMMASINO. *Secretion of heterologous proteins from Schizosaccharomyces pombe using the homologous leader sequence of pho1 acid phosphatase*. *Biochem. Biophys. Res. Commun.*, **245**, 166-171 (1998).
18. A. IRIDIS, K.W. BI, H. TOHDA, H. KUMAGAI, Y. GIGA-HAMA. *Construction of a protease-deficient strain set for the fission yeast Schizosaccharomyces pombe, useful for effective production of protease-sensitive heterologous proteins*. *Yeast*, **23**, 83-99 (2006).
19. A. IRIDIS, H. TOHDA, K.W BI, A. ISOAI, H. KUMAGAI, Y, GIGA-HAMA. *Enhanced productivity of protease-sensitive heterologous proteins by disruption of multiple protease genes în the fission yeast Schizosaccharomyces pombe*. *Appl. Mircrobiol. Biotechnol.*, **73**, 404-420 (2006).
20. J. BAHLER, J-Q WU, M.S. LONGTINE, N.G. SHAH, A MCKENZIE III, A. STEEVER, A. WACH, P. PHILIPSEN, J. PRINGLE. *Heterologous modules for efficient and versatile PCR-based gene targeting in Schizosaccharomyces pombe*. *Yeast*, **14**, 943-951 (1998).
21. G. VON HEINE. Signal sequences. *The limits of variation*. *J. Mol. Biol.*, **184**, 99-105 (1985).
22. V.S. STRONGE, Y. SAITO, Y. IHARA, D.B. WILLIAMS. *Relationship between calnexin and BiP in suppressing aggregation and promoting refolding of protein and glycoprotein substrates*. *J. Biol. Chem.*, **276**, 39779-39787 (2001).
23. A. MARECHAL, P.L. TANGUAY, M. CALLEJO, R. GUERRIN, G. BOILEAU, L.A. ROKEACH. *Cell viability and secretion of active proteins in Schizosaccharomyces pombe do not require the chaperone function of calnexin*. *Biochem. J.*, **380**, 441-448 (2004).
24. A.L. PIDOUX, J. ARMSTRONG. *Analysis of the BiP gene and identification of an ER retention signal in Schizosaccharomyces pombe*. *EMBO J.*, **11**, 1583-1591 (1992).
25. P. XU, D. RADEN, F.J. DOYLE III, A.S. ROBINSON. *Analysis of unfolded protein response during single-chain antibody expression în Saccharomyces cerevisiae reveals different roles for BiP and PDI in folding*. *Metab. Eng.*, **7**, 269-279 (2005).
26. H. ZHANG, J. HE, Y. JI, A. KATO, Y. SONG. *The effect of calnexin deletion on the expression level of PDI in Saccharomyces cerevisiae under heat stress conditions*. *Cell. Mol. Biol. Lett.*, **13**, 38-48 (2008).
27. S.J. KIM, Y.S. CHOI, H.G. KIM, E.H. PARK, C.J. LIM. *Cloning, characterization and regulation of a protein disulfide isomerase from the fission yeast Schizosaccharomyces pombe*. *Mol. Biol. Rep.*, **33**, 187-196 (2006).
28. K. KETTNER, A. BLOMBERG, G. ROEDEL. *Schizosaccharomyces pombe ER oxidoreductin-like proteins SpEro1a and SpEro1b*. *Yeast*, **21**, 1035-1044 (2004).
29. W. ZHANG, H.L. ZHAO, C. XUE, X.H. XIONG, X.Q. YAO, X.Y. LI, H.P. CHEN, Z.M. LIU. *Enhanced secretion of heterologous proteins in Pichia pastoris following overexpression of Saccharomyces cerevisiae chaperone proteins*. *Biotechnol. Prog.*, **22**, 1090-1095 (2006).
30. T. LODI, B. NEGLIA, C. DONNINI. *Secretion of human serum albumin by Kluyveromyces lactis overexpressing K1PDI1 and K1ERO1*. *Appl. Environ. Microbiol.*, **71**, 4359-4363 (2005).
31. S. MIYAKE, M. YAMAMOTO. *Identification of ras-related ypt family genes in S. pombe*. *EMBO J.*, **9**, 1417-1422 (1990).
32. M.W. CRAIGHEAD, S. BOWDEN, R. WATSON, J.ARMSTRONG. *Function of the ypt2 gene in the exocytic pathway of Schizosaccharomyces pombe*. *Mol. Biol. Cell*, **4**(6), 583-592 (1993).
33. T. INABA, Y. NAGANO, T. NAGASAKI, Y. SASAKI. *Distinct localization of two closely related Ypt3/Rab11 proteins on the trafficking pathway in higher plants*. *J. Biol. Chem.*, **277**, 9183-9188 (2002).
34. J. ARMSTRONG, M.W. CRAIGHEAD, R. WATSON, S. PONNAMBALAM, S. BOWDEN. *Schizosaccharomyces pombe ypt5: a homologue of the rab5 endosome fusion regulator. Mol. Biol. Cell*, **4**, 583-592 (1993).

35. P. DE CAMILI, S.D. EMR, P.S. MCPHERSON, P. NOVICK. *Phosphoinositides as regulators in membrane traffic*. *Science*, **271**, 1533-1538 (1996).
36. P.M. VOICU, M. POIȚELEA, M.E. SCHWEINGRUBER, M. RUSU. *Inositol is specifically involved in the sexual program of the fission yeast Schizosaccharomyces pombe*. *Arch. Microbiol.*, **177**, 251-258 (2002).
37. S. KJAERULFF, S. MULLER, M.R. JENSEN. *Alternative protein secretion: The mam1 ABC transporter supports secretion of M-factor linked GFP in fission yeast*. *Biochem. Biophys. Res. Commun.*, **339**, 1853-1859 (2005).
38. G. LADDS, J. DAVEY. *Identification of proteases with shared functions to the protein processing protease Krp1 in the fission yeast Schizosaccharomyces pombe*. *Mol. Microbiol.*, **38**, 839-853 (2000).
39. P.J.H. WERLER, E. HARTSUIKER, A.M. CARR. *A simple cre-lox method for chromosomal N-terminal tagging of essential and non-essential Schizosaccharomyces pombe genes*. *Gene*, **304**, 133-141 (2003).
40. E. PETRESCU-DĂNILĂ, P.M. VOICU, M. POIȚELEA, R. STĂNESCU, B. STOICA, M. RUSU. *Method for protein tagging in the fission yeast Schizosaccharomyces pombe. Rev. Med. Chir. Soc. Med. Nat. Iaşi*, **110**(2), 403-408 (2006).