World Journal of Pharmaceutical and Life Sciences
WJPLS
www.wjpls.org
SJIF Impact Factor: 6.129

FISSION YEAST *SCHIZOSACCHAROMYCES POMBE* AS A FOREIGN-GENE EXPRESSION SYSTEM

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Article Received on 24/04/2020 **Article Revised on 14/05/2020** **Article Accepted on 04/06/2020**

ABSTRACT

The expression and the production of proteins of interest are important for both basic research, and practical applications. Hence, the demand for foreign-gene expression systems is increasing rapidly. Many kinds of foreign-gene expression systems have been developed, some using *Escherichia coli* as a host, and others using *Bacillus, Streptomyces, Aspergillus*, yeast, insect cells, plant cells and mammalian cells. A number of these systems are used for industrial applications such as the production of pharmaceuticals and enzymes. Fission yeast *Schizosaccharomyces pombe*, is discussed here as host model for high-level protein production and functional analysis of eukaryotic proteins as it shares many molecular, genetic and biochemical features with higher eukaryotes. The review summarizes the use of *S. pombe* as foreign-gene expression system.

KEYWORDS: *S. pombe*, Foreign gene expression, fission yeast, gene silencing.

1. INTRODUCTION

Till date, various genes have been identified and cloned by the use of molecular biology. Their expression and the production of proteins of interest are very important for both basic research, such as the elucidation of physiological activity or structural analysis, and practical applications such as the production of pharmaceuticals. Therefore the demand for foreign-gene expression systems is increasing rapidly. The expression systems are composed of various combinations of 'host' and 'vector'. Many kinds of expression system, some using *Escherichia coli* as a host, and others using *Bacillus*, *Streptomyces*, *Aspergillus*, yeast, insect cells and mammalian cells, have been developed.[1] Some of these systems are commercially available as kits for academic research and others are used for industrial applications such as the production of pharmaceuticals and enzymes.

Each expression system has its own characteristics, so it is necessary to know the merit and demerit of each system and then make a choice based on the gene of interest, the amount to be produced, the purpose and final aim. In general, the expression of higher eukaryotic genes using prokaryotes as a host sometimes results in an inactive product without correct folding or post-translational modification, although the manipulation is easy and the production cost is low. In contrast, in the case of expression using animal cells as hosts, the problems mentioned above can be solved, but the manipulation is not easy, the production level is low and therefore the cost is high. Moreover, the mammalian system sometimes has the problem of viral infection. So, more suitable expression systems are desired, although many kinds of expression systems have already been constructed. Recently, yeast has received attention as a good host because: (i) molecular-biological research into yeast is developed; (ii) a culture method for it has been established; (iii) its manipulation is easy; (iv) it has been used in the fermentation industry for some time; and (v) its safety, such as freedom from endotoxins, is guaranteed.[2]

Schizosaccharomyces pombe is a unicellular eukaryote belonging to the Ascomycetes. It is called fission yeast because it only reproduces by means of fission, besides spores. No budding is observed, such as occurs in brewer's yeast.

It was originally isolated from East African millet beer and was sent from East Africa to Germany in 1890 and further cultivated to a pure culture. P. Lindner first described it in a German brewery journal in 1893 and named it *pombe*, which means 'beer' in Swahili.[3] He also showed that it is cylindrical and rounded at the end and that its length and diameter are around 7–15 µm and 4 µm, respectively. Unrelated is the fact that in some villages on Jawa Island in Indonesia and on Saipan Island liquor is made using *S. pombe.*

S. pombe is the most intensely studied and well characterized yeast species in terms of molecular genetics and cell biology,[4,5] *S. pombe* has been reported as one of the best experimental models for the study of cell-cycle control, chromosomal structure, signal transduction for sexual differentiation, and others as it shares many molecular, genetic, and biochemical features with higher eukaryotes such as plants and animals, and is distinguishable from other yeasts through its ability to proliferate by fission rather than budding.[6,2,7]

2. Fission Yeast *Schizosaccharomyces Pombe* As A Host

Lee and Nurse,[8] used *S. pombe* to clone eukaryotic genes by gene complementation, indicating the active state of heterologous genes expressed in *S. pombe*. Till date a number of membrane and secretory proteins, as well as cytoplasmic proteins, have been expressed in *S. pombe*, and more importantly, these expressed proteins have been shown to maintain their inherent characteristics (Table 1).

Bröker *et al.*[9] reported Expression of human antithrombin III in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe.* The signal sequence of antithrombin III was recognized by both yeast species, and antithrombin III was secreted into the medium which was biologically active. Tommasino *et al.*[10] reported Synthesis, phosphorylation, and nuclear localization of human papillomavirus E7 protein in *S. pombe.*

Sander *et al.*[19] successfully expressed human D2S dopamine receptor in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. It was first report of the heterologous expression of a G-protein coupled receptor in the fission yeast *S. pombe*. Their results indicated that the fission yeast *S. pombe* is even more suitable organism for the heterologous expression of the human D2S dopamine receptor. Compared to the expression of the same receptor protein in the budding yeast *S. cerevisiae* the amount of receptor protein per mg membrane protein produced in *S. pombe* was notably higher. Additionally, the K_d value for [^{3}H] spiperone binding and the K_i values for other specific ligands were lower, when the D2S dopamine receptor was expressed in the fission yeast.

Giga-Hama *et al,*[12] reported high level expression of human lipocortin I in the fission yeast *Schizosaccharomyces pombe* using a novel expression vector. Okada *et al.* (1998a)[29] reported efficient secretion and characterization of products of *Trichoderma reesei* cellobiohydrolase II in *Schizosaccharomyces pombe*. They also studied molecular characterization and heterologous expression of the gene encoding a low-molecular-mass endoglucanase from *Trichoderma reesei.*

Isoai *et al.*[15] reported production of Damino acid oxidase (DAO) of *Trigonopsis variabilis* in *Schizosaccharomyces pombe* and the characterization of biocatalysts prepared with recombinant cells.

Ikeda *et al.*[16] developed a fed-batch fermentation process to enable the production of large quantities of recombinant human lysosomal acid lipase (hLAL; EC 3.1.1.1.13), in *S. pombe*, for preclinical studies as a potential enzyme therapy drug. Recombinant *S. pombe* clone ASP397-21, expressed enzymatically active hLAL in the secreted form. A feedback fed-batch system was used to determine the optimal feed rate of a 50% glucose solution used as the carbon source. Expressed hLAL protein was purified in two-step process by hydrophobic interaction and anion exchange chromatographies. Then it was subjected to endoglycosidase H treatment and terminal analysis of deglycosylated hLAL showed that the protein was correctly processed in *S. pombe*.

Giga-hama *et al.*[17] constructed a protease-deficient strain set for the fission yeast *Schizosaccharomyces pombe*, useful for effective production of protease-sensitive heterologous proteins. They constructed this protease-deficient strain by respective disruption of 52 *S. pombe* protease genes. Their results indicated that some of the resultant disruptants were effective in reducing hGH degradation, as observed during the hGH expression procedure and mainly as a result of unknown serine- and/or cysteine-type proteases in the culture medium.

Six α-1,2-mannosyltransferase gene homologs, namely omh1– omh6, which are related to O-glycosylation of the fission yeast *S. pombe were* identified, and has found that deletion of omh1 blocks elongation of O-linked oligosaccharides.[30] The resultant omh1Δ strain will be useful for controlling O-glycosylation or further glycoengineering in fission yeast. They have shown that N- and O-linked oligosaccharides completely lack galactose residues in the gms1Δ-och1Δ double-mutant strains of *S. pombe*.[31] The N-linked oligosaccharides of gms1Δ-och1Δ cells consist of α-1,2-linked Man-extended core oligosaccharides (Man8–12GlcNAc2), from which the fission-yeast-specific α-linked Gal residues are completely absent. These α-1,2-linked Man-extended core structures might not cause an immune response and could therefore be better tolerated in the human body. Still, such compounds could be quickly turned over in the human body.[32]

Only a limited number of foreign secretory proteins have been produced in *S. pombe* so far. For expression of secretory proteins, they need to go through the secretory pathway consisting of cell organelles, including endoplasmic reticulum and Golgi apparatus, for correct folding, formation of disulphide linkage and glycosylation. The endoplasmic reticulum signal that is recognized by a signal peptidase is necessary for passing through the correct secretory pathway. In the case of

human antithrombin III,[9] human gastric lipase,[34] human placental alkaline phosphatase,[35] and *S. cerevisiae* invertase,[36] the secretory signals of the proteins themselves worked effectively in *S. pombe*, resulting in the secretion of the proteins.

In the case of many other secretory proteins, however, their signal peptides are not recognized by the signal peptidase of *S. pombe*, so that the precursor proteins do not enter the endoplasmic reticulum but stay in the cell cytoplasm as such. Tokunaga,[13] successfully expressed and secreted mouse a-amylase using the signal sequence derived from a killer toxin of *Kluyveromyces lactis.*

Mukaiyama,[44] found that over expression of endogenous putative protein disulfide isomerase (PDI) improved productivity of secreted human serum transferrin (hTF). Whole genome sequence analysis of *S. pombe* revealed five putative PDI genes and overexpression of two of them, SPAC17H9.14c and SPBC3D6.13c (SpPdi2p or SpPdi3p, respectively), significantly improved the productivity of secreted hTF. GFP-fused SpPdi2p and SpPdi3p were found to localize to the endoplasmic reticulum. They reported co-overexpression of SpPdi2p or SpPdi3p with hTF coupled with modifications to the growth medium reported in our previous study were able to increase the level of secreted hTF approximately 30-fold relative to conventional conditions.

Naumann *et al.*,[28] reported *Schizosaccharomyces pombe* as a new host organism for secretory production of fluorescent single-chain Fv (scFv) antibody fragments as scFv-green fluorescent protein (GFP) fusions and compared it with previously described yeast expression systems. They cloned a plasmid for the expression and secretion of the anti-p24 (human immunodeficiency virus 1) CB4-1 scFv fused to GFP. They reported after expression of the scFv–GFP fused to an N-terminal Cpy1 secretion signal sequence, fluorescence microscopy of living yeast cells indicated that the heterologous protein entered the secretory pathway. Western blot analysis of cell-free culture supernatants confirmed that the scFv–GFP was efficiently secreted with yields up to 5 mg/l. In addition, fluorescence measurements of culture supernatants demonstrated that the GFP moiety of the scFv–GFP protein to be fully functional after secretion. They claimed that *S. pombe* has the potential for being used as alternative expression host in recombinant antibody fragment production by ensuring efficient protein processing and secretion.

3. Expression systems for *s. Pombe*

Schizosaccharomyces pombe is a genetically tractable microorganism and a wide variety of fission yeast-specific plasmids have been developed to facilitate its molecular manipulation,[37] A typical *S. pombe* plasmid contains a bacterial origin of replication, an antibiotic-resistance gene that can be selected in bacteria, a yeast selectable marker, and an autonomous replication sequence (ars), which is responsible for plasmid maintenance. Numerous plasmids have been designed for regulated or constitutive expression of heterologous or endogenous genes (Fig. 3).

Some of commonly used general purpose episomes and integrating vectors are given in Table 2. A limited number of studies comparing different vectors to one another have been conducted e. g. by Forsburg,[37] Basi,[38] and Lacovoni.[39]

However, expression levels can vary significantly even for a single plasmid, depending upon the protein being expressed and the exact context of the ATG, so some caution must be used when assuming a particular level of induction (Table 3).

Table 2: General purpose episomes and integrating vectors (Forsburg *et al.*,[37]).

Plasmid	Features	Reference
pUR18 pUR19	ura4+, blue–white selection, extensive polylinker	[40]Barbet *et al.*, 1992
pAL19	LEU2, blue–white selection	[41]Craven *et al.*, 1998
pIRT2 pIRT2U	LEU2 (pIRT2), ura4+ (pIRT2U) no blue–white selection, limited polylinker	[42]Hindley *et al.*, 1987
pBG1	his3+, blue–white selection extensive polylinker	[43]Burke and Gould, 1994
pJK148 pJK210	leu1+ (pJK148), ura4+ (pJK210) blue–white selection no ars element	[45]Keeney and Boekei, 1994

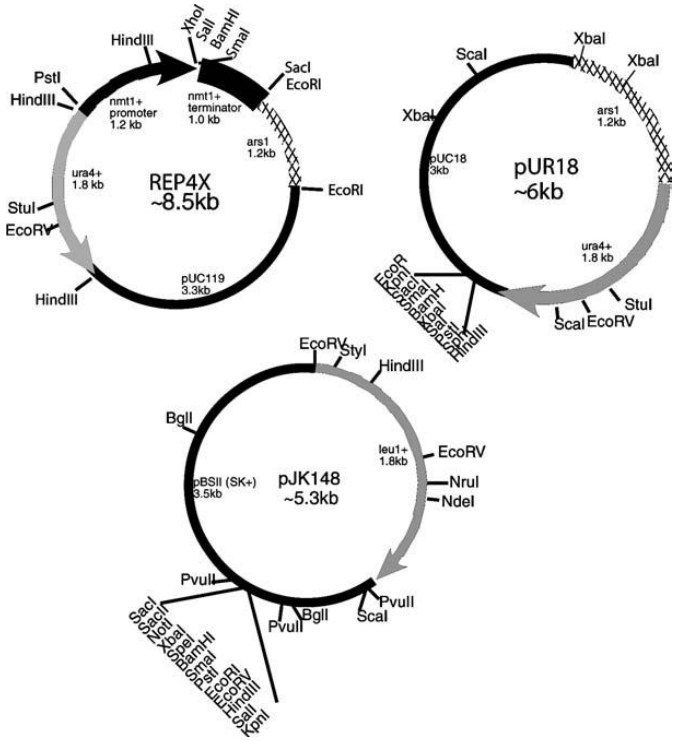

Fig. 3: Representative fission yeast plasmids. This figure shows plasmid maps for a common general use vector (pUR18), integration vector (pJK148), and expression vector (REP4X). All have multiple sites useful for cloning. pJK148 contains leu1þ, but no other sequence for stable maintenance or expression. pUR18 includes ars1 for stable maintenance and ura4þ to allow selection, but no expression or tagging elements. pREP4X contains ars1, ura4þ, and the full-strength nmt1þ promoter and terminator, to allow inducible expression of a gene cloned into its polylinker. Reproduced from Siam *et al.*[46]

Table 3: Episomal expression plasmid systems.

Promoter	Plasmid	Regulation	References
adh1þ	pART1 (LEU2)	Constitutive expression	[47] McLeod *et al.*, 1987
	pEVP11 (LEU2)		[5] Russell and Hall, 1983
SV40	pSM1/2 (LEU2)	Constitutive expression	Kaufer *et al.*, 1985
CaMV	pSLF101 (LEU2)/102 (ura4þ) in the presence of pSLF104	Tetracycline inducible	[37] Forsburg, 1993
inv1þ	pINV1-spc1þ HA6his	Glucose repressible	[39] Lacovoni *et al.*, 1999
	pINV1-spc1þ -12myc		
	pINV1-GST pREP1-spc1þ -GFP (LEU2)		
	(LEU2, ura4þ , his7þ)		
nmt1' (intermediate promoter)	REP41/42		[38] Basi, 1993
nmt1_' (weak promoter)	REP81/82		[38] Basi, 1993
nmt1þ (strong promoter)	REP3/4X	Thiamine repressible	[37] Forsburg, 1993
nmt1' (intermediate promoter)	REP41/42X		
nmt1_' (weak promoter)	REP81/82X		

3.1 ‘‘No message in thiamine’’ nmt1þ promoter

Nmt1 was the first regulatable promoter in fission yeast to be cloned and characterized and it remains the most commonly employed. The promoter is repressed by presence of thiamine (vitamin B1, Sigma #4625) in the media. Peak repression requires lM thiamine. Peak induction, which occurs when thiamine is removed, takes approximately 16–20 h, presumably reflecting depletion of pools of thiamine in the cell. Activity of the promoter can be titrated with intermediate amounts of thiamine

(0.05M). Importantly, induction requires minimal media; rich media made with yeast extract contain sufficient thiamine to repress the promoter.

Both the promoter and polyadenylation/termination sequences of nmt1 were used to construct the pREP episome and the pRIP integration plasmids.

The pREP1/2/3/4 plasmids are pUC-based plasmids containing *S. pombe* ars1 and a 1.4-kb fragment containing the nmt promoter and either LEU2 or ura4þ as the selectable marker (odd numbered vectors contain LEU2, and even numbered vectors contain ura4þ). pRIP1/2/3 plasmids lack the ars element and instead contain sup3-5, and are used for chromosomal integrations. Both pREP1/2 and pRIP1/2 have a NdeI/SalI/BamHI/SmaI polylinker, while pREP3/4 and pRIP3/4 have a BalI/ SalI/BamHI/SmaI polylinker. Both of these polylinkers contain ATG codons in the NdeI or BalI sites, respectively. The REP-X series of plasmids replaced the NdeI or BalI sites and the ATG with a XhoI linker,[37] The TATA box of the nmt1þ promoter has been mutated to modify its overall strength (Bassi *et al.*, 1993) generating intermediate-strength (nmt41; nmt*) and low-strength (nmt81; nmt**) versions. pREP41/42/X and pREP81/82/ X were constructed by using the REP1 template,[37] Bassi *et al.*, 1993).

3.2 Tetracycline inducible promoter

The tetracycline inducible promoter is a tightly repressed *S. pombe* promoter. The promoter is a derivative of the plant viral cauliflower mosaic virus (CaMV) 35S promoter, which functions as a constitutive promoter in *S. pombe.* Three Tn10-encoded Tet repressor-binding sites are located near the TATA-box of the CaMV 35S promoter. If the Tet repressor TetR is expressed in cells with this construct, transcription from CaMV 35S is blocked. Upon addition of tetracycline, up to a 400-fold induction was observed. In the absence of the Tet repressor, the CaMV promoter construct is a moderate constitutive. Derivatives of the Tet system have been generated for more general uses. The expression vectors pSLF101 and pSLF102 contain the CaMV promoter and can be used for constitutive expression. For regulated expression, they can be used with pSLF104, which contains the adh1-tetR gene and sup3-5 marker, and can be integrated randomly in the genome.[37] A copy controlled system using the same regulation, pTLM2/pAL7, has also been described.

3.3 Other regulated promoters

While other regulated promoters have been identified, they have not been used extensively for expression of cloned genes. The *S. pombe* fbp1þ gene, encoding fructose-1,6 bisphosphatase, is repressed by glucose (8%). Under derepressing carbon conditions (0.1% glucose and 3% glycerol), fbp1þ transcription is repressed in wild-type cells. One caveat to use of the fbp1þ promoter is that it can be derepressed in colonies, so experiments using fbp1þ should only be performed in liquid cultures. This promoter forms the basis for plasmid pCHY21.

Another regulatable promoter is that of invertase, the enzyme responsible for the cleavage of sucrose into glucose and fructose. In the presence of glucose, a zinc finger protein, Scr1p, binds upstream of the invertase promoter. In the absence of glucose, the repressor Scr1p is inactivated, inducing invertase expression within 1 h of the shift to sucrose-based medium. A set of inducible protein expression vectors containing the spc1þ gene (a stress-activated kinase) as a test was constructed by replacing the nmt1þ promoter of REP-based constructs with a 1.2 kb region of the inv1þ promoter to generate pINV1-spc1þ-HA6his, pINV1-spc1þ-12myc, pINV1-GST, and pINV1-GFP.[39] This system can only be used for short periods of induction (approximately 4 h at most), because the expression of invertase leads to an accumulation of glucose, which in turn causes repression of the invertase promoter.

The *S. pombe* copper transporter gene ctr4þ, which is transcriptionally regulated by copper availability, provides another regulatable promoter. When cells are grown under limiting copper conditions, the Cuf1p transcription factor stimulates transcription of ctr4þ within approximately 3 h. This expression system is a hybrid, constructed with the minimal promoter region of *S. cerevisiae* CYC1, and a fragment containing the cis-acting elements of ctr4þ and the CuSEs (necessary for copper responsive gene expression) replacing the inv1þ promoter region of pINV1-spc1þ-HA6his and pINV1-spc1þ-12myc vectors.[39] These vectors have been constructed with three different selectable markers (LEU2, ura4þ, and his7þ).

3.4 Constitutive promoters

The adh1þ+ promoter from alcohol dehydrogenase is constitutively transcribed at high levels in cells grown in glucose and glycerol.[5] This promoter was used to construct the fission yeast expression vector pART1,[47] derived from pIRT2, and also pEVP11, derived from the pBR322 vector YEp13.[5] The SV40 small-T antigen transcript is another constitutive promoter.[48] However, this promoter is weaker than the above promoters and may only be useful if limited minimal level of gene expression is desired. The pSM-1/2 expression vectors contain this promoter.

4. CONCLUSION

S. pombe has a developed Golgi apparatus and galactosyl transferase that is not found in other yeast cells. The fission yeast is therefore an ideal host for high-level production of eukaryotic proteins. However, one of the major hurdles in efficient production and purification of heterologous proteins from *S. pombe* is proteolytic degradation of the recombinant gene products by host-specific proteases. The problem becomes significant when the recombinant protein under production, is secretory and proteolytically sensitive in nature such as

recombinant mouse α-amylase,[13] In order to overcome this limitation, a number of attempts have been made, for e.g. Control of cultivation conditions e.g. culture pH, temperature and time; and changing medium contents, e.g. nitrogen and carbon sources and addition of protease inhibitors, peptone, casamino acids or specific amino acids.[49,51] But, these methods cannot ultimately prevent the proteolytic degradation of recombinant proteins or are limited by the specificities of the host species or heterologous protein molecules. For example, controlling temperature, pH and medium contents or adding protease inhibitors might also inhibit cell growth or reduce cell density, both of which are essential for high productivity of recombinant proteins during industrial production processes. Similarly, cultivation time cannot be shortened freely in some cases, e.g. in high cell density fed-batch culturing processes, where the cells must be grown for an extended period.

Alternatively, Genetic manipulation of the recombinant protein molecule itself offers protection from proteolysis, but often gives rise to serious problems for practical application. For example, a modified recombinant protein is generally regarded as a new drug and must, therefore, while keeping its original molecular structure, undergo extensive pharmacokinetic procedures prior to approval. Furthermore, such genetic modification will also cause structural or functional changes of the protein molecules.

Consequently, Genetic manipulation of the host strain is being developed as a potent method for controlling undesirable proteolytic activity of host strains. It has been done by the disruption of *S. pombe* protease genes using specialized vectors for the purpose. This method has been used to develop many protease-deficient host strains, such as *Escherichia coli*[52,53] and the yeast species *Yarrowia lipolytica*[54] and *Saccharomyces cerevisiae*[55,56,57,58] and *Schizosaccharomyces pombe*.[17]

Author aimed to develop protease deficient strain of *S. pombe* by gene silencing approach.[59] RNA silencing is an evolutionarily conserved gene regulatory mechanism with many species-specific variations by which double stranded RNA (dsRNA) causes sequence-specific degradation of mRNA sequences. A Protease silencing cassette was designed to impede the protease enzyme post trascriptionally. They succeeded to develop a custom designed protease-deficient *S. pombe* host strain.[60,61] useful for effective production of hPTH by reducing the effect of selective protease which is acting on the hPTH protein.

Future Prospects

Even being a eukaryote and sharing many molecular, genetic and biochemical features with higher eukaryotes, the fission yeast *Schizosaccharomyces pombe*, could not get much attention as a host model for high-level protein production and functional analysis of eukaryotic proteins. The major hurdle in efficient production and purification of heterologous proteins is proteolytic degradation of the recombinant gene products by host-specific proteases. Consequently, Scientists are trying genetic manipulation of the host strain as a potent method for controlling undesirable proteolytic activity of host strains. Author also has succeeded to develop a custom designed protease-deficient *S. pombe* host strain useful for effective production of hPTH by reducing the effect of selective protease which is acting on the hPTH protein. They gave a proof of our concept that this novel approach for specific silencing of protease which is acting on the heterologous protein can be applied in future to enhance protein production by *S. pombe*.

CONFLICT OF INTEREST: There is no conflict of interest in this work.

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