

The fission yeast *S. pombe* is a particularly useful model for studying the function and regulation of genes from higher eukaryotes. The genome of *S. pombe* has been sequenced and DNA microarray, proteome and transcriptome analyses have been carried out. Among the well-characterized yeast species, *S. pombe* is considered as potentially the most powerful host for the production of heterologous proteins. Expression vectors for high-level expression in *S. pombe* have been developed and many foreign proteins have been successfully expressed. However, *S. pombe* has lagged behind the *P. pastoris* expression system, which is the most widely used methylotrophic yeast for production of the eukaryotic heterologous proteins. The reasons behind popularity of the *Pichia* system are availability of *AOX1* promoter, a strong methanol-inducible promoter of alcohol oxidase I and ease of growth to very high cell density on an inexpensive, non-complex and chemically defined medium. However, despite these advantages, *Pichia* expression system always has an issue related to the safety aspects of methanol, which is inflammable and combustible. Large-scale fermentation will require a significant amount of methanol and proper measures must be in place to ensure safety. Moreover, a license from Research Corporation Technologies is necessary for the use of *Pichia* vectors for commercial purpose.

In the present study, substantial progress is reported in establishing the fission yeast *S. pombe* as a possible alternative expression system over *P. pastoris* with a compendium of approaches.

One of the approaches in this study was to construct the vector with higher mitotic stability and copy number in comparison with the available vectors of *S. pombe*. These two properties of vectors are basic requirements for high and stable product yield at fermentor level. To achieve these goals, we developed a new vector (pJH4) containing *URA3m* selection marker and *mat2P-RF* as an ARS element, having higher transformation frequency and mitotic stability with low rate of plasmid loss. We have successfully increased the copy number by at least 4-fold, that is, approximately 200 copies/cell, by using *URA3m* as selectable marker as compared to vectors containing *ura4* and *ars1*. For further development, transcription terminator sequence of *nmt1* gene was isolated and inserted into the vector (pJH4) for efficient termination and polyadenylation of mRNA of the foreign gene to be expressed under a promoter. The latter features are known to enhance the stability of mRNA, thus contributing to the higher level of protein expression.

To isolate strong and regulatable promoters, we analyzed the DNA microarray data of global transcriptional responses of *S. pombe* to various environmental stresses and screened the genes whose expression level was strongly increased under the stress conditions like heat shock or oxidative stress. The promoters of three such genes were expected to drive the expression of heterologous genes in response to the said stress conditions. Approximately 1.5 Kb upstream region of the gene *SPAC1F8.02c* -named as promoter *Psp1*, ~1.2 Kb upstream region of the gene *SPBC24C6.09c* -named as promoter *Psp2* and ~1.0 Kb upstream region of the gene *SPBC16E9.16c/lsd90* -named as promoter *Psp3/Plsd90*, were PCR amplified and cloned into the newly designed high copy number vector to construct new expression vectors pJH6a, pJH6b and pJH6c, respectively.

The firefly luciferase (*Fluc*) gene was the first to be expressed as the reporter to characterize the efficacy of the newly constructed vectors. Surprisingly, all three promoters were found to be constitutive with a continuous increase in luciferase activity upto early stationary phase (48 hrs) with increase in cell density, which remained constant during later time points of growth. Out of these promoters, *Plsd90* promoter showed highest level of luciferase expression [ $2.5 \times 10^{-14}$  moles (~ 2.5 ng)] which was at least  $10^4$ -fold higher than the level achieved with promoters *Psp1* and *Psp2*, suggesting that the latter were much weaker promoters and therefore not suitable for heterologous gene expression. Further, the maximum level of luciferase under the promoter *Plsd90* was 19-fold and 39-fold higher than that achieved with promoters *adh1* and *nmt1*, respectively. Most interestingly, the level of luciferase achieved with *Plsd90* promoter after 48 hrs was ~10-fold higher and 2.5-times faster compared to that achieved with *AOX1* promoter in *P. pastoris* after 120 hrs. Thus, we have identified a strong constitutive promoter *Plsd90*, which can yield significantly higher expression than other promoters of *S. pombe*, namely *nmt1* and *adh1* and *AOX1* promoter of *P. pastoris*.

Next, we expressed another common reporter *GFP* and compared the expression level of GFP under the promoters *Plsd90*, *adh1* and *nmt1* in *S. pombe*. Similar to the results of luciferase expression, highest level of GFP fluorescence was observed under control of the *Plsd90* promoter which was almost 3-fold higher as compared to maximum level of fluorescence achieved with the promoters *adh1* and *nmt1*. Quantitative data showed that the GFP expression level constituted over 10% of total cellular protein.

Next, we expressed the therapeutic protein HBsAg as it is an injectable protein whose expression has been established in other yeasts, like *Pichia* and *S. cerevisiae* and the product is already in the market.

We compared the level of HBsAg expression under the *Plsd90* promoter with that achieved with the *adh1* and *nmt1* promoters of *S. pombe*. Expression of HBsAg achieved with the *Plsd90* promoter was estimated to be at least 10% of total cellular protein. This expression level of 100 mg/l of culture compares favorably with reports in *S. cerevisiae* or *P. pastoris*. Remarkably, expression level of HBsAg with *Plsd90* promoter was at least 20- and 60-fold higher than that achieved with *nmt1* and *adh1* promoters.

*S. pombe*-derived HBsAg protein showed good physical and immunological characteristics, resembles 22 nm particles present in the serum of HBV carriers is potentially better in immunoreactivity. The recombinant HBsAg protein expressed in *S. pombe* could provide promising, inexpensive, large scale material for diagnostic reagents and vaccine for the prevention of HBV infection.

One major hurdle in obtaining efficient export of proteins into the culture medium is the cell wall of yeast cells. To overcome this problem, mutants having a defective cell wall that could either allow proteins to be secreted and/or it could be easily broken under less harsh conditions of mechanical lysis have been generated. We isolated such seven temperature sensitive, sorbitol-dependent (*sdp*) mutants followed by characterization in terms of growth kinetics, cell morphology and mechanical lysis. Three of these mutants (*sdp1*, *sdp4* and *sdp5*) showed comparatively higher rate of growth than rest of the mutants and were characterized further. In absence of sorbitol at 37°C, these mutants showed altered morphology with cells appearing greatly enlarged either spherical or drastically distorted

Conditions were also optimized for expression of GFP and HBsAg with *Plsd90* promoter in the sorbitol-dependent mutants. Stable expression of both proteins was observed for several days of continuous growth. Presence of sorbitol in the cultures of wild-type and *sdp1* mutant caused almost 50% increases in level of GFP fluorescence, which, interestingly become detectable even under visible light. Furthermore, in the presence of sorbitol, GFP fluorescence under the promoter *Plsd90* was found to be almost 4-fold higher in comparison to the *adh1* and *nmt1* promoters of *S. pombe*. No effect of sorbitol on the level of GFP expression was observed under the promoters *adh1* and *nmt1*.

Importantly, in the cultures of wild-type and *sdp1* mutant strains, GFP level of over 30% of total cellular protein was observed in the Commassie stained gel in the presence of sorbitol while it was estimated to constitute over 10% of total cellular proteins in absence of sorbitol under the promoter *Plsd90*.

Further, sorbitol allowed to achieve at least 3-fold higher level (estimated to be at least 300 mg/l) of HBsAg under the promoter *Plsd90* in wild-type and *sdp1* mutant strains than that achieved earlier in the absence of sorbitol (~100 mg/l). Accordingly, level of HBsAg expressed under the promoter *Plsd90* in the presence of sorbitol was estimated to be 180-fold higher than that with *adh1* and at least 60-fold higher than that with *nmt1* promoter.

Thus, we report *sdp1* mutant as an expression host with advantages of growth rate equal to the wild-type and cell wall fragility that allows us maximum extraction of proteins without degradation. Furthermore, *sdp1* mutant presents the additional advantage of having a cell wall osmotically stable only in the presence of sorbitol, a factor that stimulates the expression level further by 3-fold.

Evidently, *S. pombe* offers an excellent eukaryotic expression system for inexpensive, large-scale production of functional recombinant proteins like *P. pastoris*. We report the *Plsd90* promoter as the strongest constitutive promoter in *S. pombe* till date. Other advantages of the new promoter *Plsd90* include its very simple kinetics and inexpensive culture conditions. Most interestingly, despite constitutive expression of foreign proteins, no deleterious effect on growth of cells was observed. Therefore, the promoter *plsd90* needs to be developed further at fermentor level to achieve high cell density comparable to *Pichia* for the expression of heterologous proteins of therapeutic and industrial interest at industrial scale.