Journal Homepage: - www.journalijar.com
INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)
Article DOI: 10.21474/IJAR01
DOI URL: http://dx.doi.org/10.21474/IJAR01
ISSN NO. 2320-5407

RESEARCH ARTICLE

International Conference on Recent Advances in Biotechnology, Biomolecules and Pharmacy RABBP – 2020 (Organized in Virtual Mode due to COVID-19 Pandemic) during 17th to 19th December 2020 at KL University, Vijayawada, Andhra Pradesh, India.

RECOMBINANT HETEROLOGOUS PROTEIN EXPRESSION IN BACTERIAL AND YEAST SYSTEMS

Monica Adapala, Vandana Kamjula, Renuka Vemparala and Praveen Kumar Vemuri
Department of Biotechnology, Koneru Lakshmaiah Education Foundation, Guntur, Andhra Pradesh, India.

Manuscript Info

Key words:-
Escherichia Coli, Pichia Pastoris, Recombinant, Heterologous, Protein Expression, Cloning Hosts

Abstract

Recent studies have demonstrated that strain/genetic engineering is a very promising approach for evolving engineered *E. coli* strains with markedly enhanced capacities for recombinant protein production. Several unique and powerful methods have emerged recently that allow the generation of large libraries of bacterial and yeast mutants carrying different types of genetic profiles. Optimization of recombinant protein expression in prokaryotic and eukaryotic host systems has been carried out by varying simple parameters such as expression vectors, host strains, media composition, and growth temperature. The information obtained from the analysis of the genetic profiles in the isolated strains can provide invaluable and fundamental understanding about the biology of protein biogenesis, folding, stability and homeostasis in bacteria. This information can subsequently be combined and utilized to generate specialized protein expression in bacteria and yeast "cell factories" for uses in research as well as in the industrial field.

Introduction:-

Escherichia coli have been used extensively for protein overexpression due to its rapid growth rate, ease of high-cell-density fermentation, low cost and, most importantly, the availability of excellent genetic tools[1-3]**.** The extensive laboratory use of *E. coli* has resulted in technologies to target protein overexpression to various intracellular compartments (figure 1). This is advantageous because these compartments have different environments that may facilitate folding of particular proteins of interest[4]. The optimization of recombinant protein expression in *E. coli* has been carried out largely by trial and error by varying simple parameters such as expression vectors, host strains, media composition, and growth temperature[5]. During the past years, extensive studies have shown that the replacement of codons within a heterologous gene with synonymous ones used preferentially in the expression host (codon optimization), and the manipulation of the nucleotide sequence of the translational initiation region can have a profound effect on recombinant protein yields[6]. To successfully perform molecular genetic techniques, it is essential to have a full understanding of the properties of the various *Escherichia coli* host strains commonly used for the propagation and manipulation of recombinant DNA[7]. *E. coli* is an enteric rod-shaped Gram-negative bacterium with a circular genome of 4.6 Mb[8]. It was originally chosen as a model

Corresponding Author:- Monica Adapala
Address:- Department of Biotechnology, Koneru Lakshmaiah Education Foundation, Guntur, Andhra Pradesh, India.

system because of its ability to grow on chemically defined media and its rapid growth rate. In rich media, during the exponential phase of its growth, *E. coli* doubles every 20–30 min[9]; thus, during an overnight incubation period, single selected organisms will double enough times to yield a colony on an agar plate or 1–2 billion cells per milliliter of liquid media[10]. The ease of its transformability and genetic manipulation has subsequently solidified the role of *E. coli* as the host of choice for the propagation, manipulation, and characterization of recombinant DNA[11]. In the past 60 years*E. coli* has been the subject of intensive research and more is now known about these bacilli than any other organisms on earth. A wide variety of *E. coli* mutants have been isolated and characterized. Almost all strains currently used in recombinant DNA experiments are derived from a single strain: *E. coli* K-12, isolated from the feces of a diphtheria patient in 1922. Genotype indicates the genetic state of the DNA in an organism. It is associated with an observed behavior called the phenotype[12]. Genotypes of *E. coli* strains are described in accordance with a standard nomenclature and genes are given three-letter, lowercase, italicized names that are often mnemonics. The methylotrophic yeast *Pichia pastoris* is widely used as a host system for recombinant protein production[13]. It is also a commonly used model organism for basic research of peroxisome and secretory organelles biosynthesis. Furthermore, it has come into focus for the production of glycol-proteins with human-like N-glycan structures, as well as for several metabolites and recombinant proteins[14].Recently, *P. pastoris* has been reclassified into a new genus, namely *Komagataella* and divided into three species *K. pastoris*, *K. phaffii*, and *K. pseudopastoris*[15]. The strains GS115 and X-33 have been made available by Invitrogen and belong to the species *K. phaffii*. Apart from that, other strains belonging to either *K. pastoris* or *K. phaffii* are freely used by researchers. Moreover, in accordance with published literature, all strains are further named *P. pastoris*, standing for the entire genus *Komagataella*. At present, the genomes of two *P. pastoris* strains (DSMZ 70382 and GS 115) have been fully sequenced[16]. There from, two genome browsers were set up. Until then, most data on genetic and physiological background for strain X-33 and process design relied on analogies to other, well studied yeasts like *Saccharomyces cerevisiae*. Accordingly, *P. pastoris* gene names follow mainly the format established for *S. cerevisiae*[17].

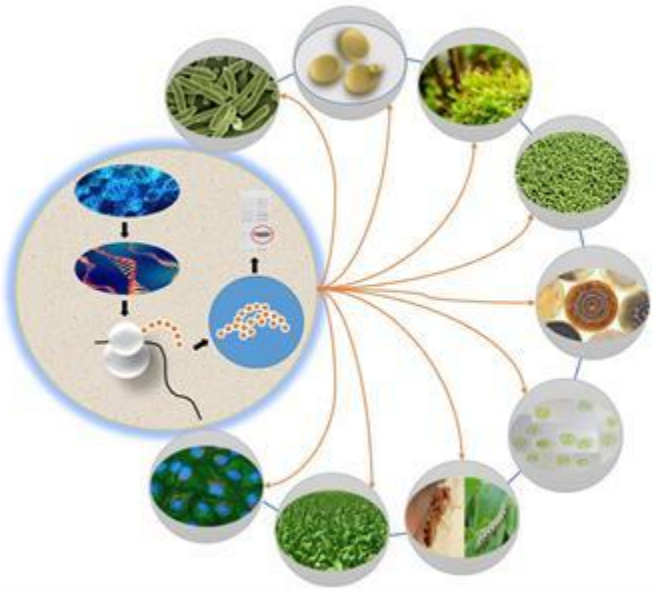

Figure 1:- Heterologous protein expression and production platforms using bacterial host systems.

Properties of Cloning Hosts

The genotypes and features of a representative selection of popular host strains used for general recombinant DNA cloning procedures are listed below. Many useful strains are available through the American Type Culture Collection (ATCC) and the *E. coli* Genetic Stock Center at Yale, as well as from commercial suppliers such as Stratagene, Fermentas, Promega, Novagen, Invitrogen and New England Biolabs. *E. coli* is a popular host for the overexpression of recombinant proteins[18]. There are a number of factors that can influence protein yields and careful strain choice can greatly improve the chance of successful expression. Recent innovations have resulted in the availability of many new host strains.

Strain Genotype

DH10B F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1

	*ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ- *rps*L *nup*G
JM109	endA1, gry96, hsR17, (Δ*lac-proAB), recA1, relA1 supE44, thi-1, traD36*
TOP10	F- *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), φ80*lac*ZΔM15 Δ*lac*X74, *rec*A1, *ara*D139, Δ(*araleu*) , 7697, *gal*U *gal*K, *rps*L(StrR), *end*A1, *nup*G
DH5α	*deoR endA1 gyrA96 hsdR17* Δ (*lac*)*U169 recA1 relA1 supE44 thi-1* (Δ 80 *lacZ* Δ M15)
INV110	*ara dam dcm dupE44 endA galK galT* (*lac-proAB*) *lacY leu* (*mcrCBhsdSMR-mrr*)*102*::Tn*10*(Tetr) *rpsL*(Strr) *thi-1 thr tonA tsx* F'[*lac*Iq *lacZ*M15 *proAB+ traD36*]
JS5	Δ (*araABC-leu*)*7697 araD139 galU galK hsdR2* Δ (*lac*)*X74 mcrA mcrBC recA1 rpsL*(Strr) *thi* F'[*lac*Iq *lacZ* Δ M15 *proAB*+ Tn*10*(Tetr)
HB101*d*	*ara-14 galK2 proA2 lacY1 hsdS20 mtl-1 recA13 rpsL20* (Strr) *supE44 xyl*-5

The methylotrophic yeast *Pichia pastoris*

Generalizing, the number of functionally annotated genes (9.4 Mb; 5.450 ORFs) is comparable to other yeasts, most metabolic enzymes are present in single copies and the number of actually secreted proteins is low, thus making secretory production of heterologous proteins attractive.*Pichia pastoris* is quite similar to *Saccharomyces cerevisiae* as far as general growth conditions and handling[19]. You should be familiar with basic microbiological and sterile techniques before attempting to grow and manipulate any microorganism. You should also be familiar with basic molecular biology and protein chemistry. X-33 is a wild-type *Pichia* strain that is useful for selection on neomycin and large-scale growth[20]. It will grow in YPD and in minimal media. The growth temperature of *Pichia pastoris* is 28–30°C for liquid cultures, plates, and slants[21]. Growth above 32°C during induction can be detrimental to protein expression and can even lead to cell death[22-24]. Growth characteristics may vary depending on the recombinant protein expressed.

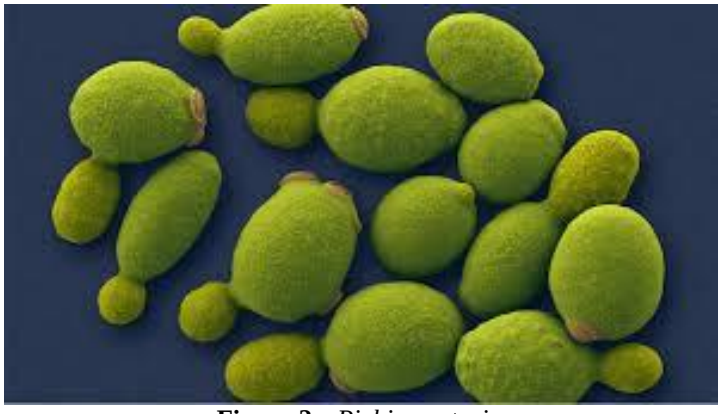

Figure 2:- *Pichia pastoris.*

Other important facts:

1. Doubling time of log phase Mut+ or MutS *Pichia* in YPD is ~2 hours
2. Mut+ and MutS strains do not differ in growth rates unless grown on methanol
3. Doubling time of log phase Mut+ *Pichia* in methanol medium (MM) is 4–6 hours
4. Doubling time of log phase MutS *Pichia* in MM is ~18 hours
5. One OD600 = ~5 × 107 cells/ml

To store cells for weeks to months, use YPD medium and YPD agar slants.

1. Streak each strain for single colonies on YPD.
2. Transfer one colony to a YPD stab and grow for 2 days at 30°C.
3. The cells can be stored on YPD for several weeks at 4°C.

To store cells for months to years, store frozen at –80°C.

1. Culture a single colony of each strain overnight in YPD.
2. Harvest the cells and suspend in YPD containing 15% glycerol at a final OD_{600} of 50–100 (approximately 2.5 × 109–5.0 × 109 cells/ml).
3. Cells are frozen in liquid nitrogen or a dry ice/ethanol bath and then stored at –80°C.

Recombinant protein production in *Pichia pastoris*

The use of *P. pastoris* as a cellular host for recombinant protein production steadily increases[25]. It is genetically easily manipulated and cultured, and can reach high cell densities (> 130 g l-1 dry cell weight) on methanol and glucose[26]. Equally important, as eukaryote, it provides the potential for producing soluble, correctly folded proteins, which have undergone post-translational modifications, such as glycosylation (O- and N-linked; less over-glycosylating than *S. cerevisiae*), disulfide bridge formation, as well as processing of signal sequences[27]. For intracellular expression, the amino-terminal methionine residue is cleaved off, unlike proteins expressed in *E. coli*, or the protein can also be acetylated and specific amino acid residues are likely to be phosphorylated, generating phospho-proteins without limitations and bottlenecks obtained by the secretory pathway.

Transformation of the haploid, homothallic *P. pastoris* host with recombinant DNA is mediated either by integrative plasmids or by autonomously replicating plasmids[28]. Directed integration or replacement requires homology of the introduced DNA with a chromosomal locus. Multiple integrations are often obtained on purpose. Integration only requires restriction at a unique site, homologous to the *P. pastoris* genome[29]. Transformation is mediated by electroporation and usually results in genetically stable transformants with high transcription rates. Most vectors are hybrids between bacterial and yeast sequences, possessing an origin of replication for *E. coli* and selection markers. The plasmids also contain a multiple cloning site (MCS). Both auxotrophic markers, such as the functional histidine dehydrogenase gene, which can be used in histidine dehydrogenase defective GS115, as well as dominant markers exist[30]. The bacterial kanamycin resistance gene also confers resistance to the eukaryotic antibiotic G418 and the bacterial *Sh ble* gene applies resistance to the antibiotic Zeocin, also appropriate for yeast.

Further benefits of the *P. pastoris* system are strong inducible and constitutive promoter systems. One important issue of recombinant protein production is the transcription efficiency; therefore, the choice of the promoter is crucial. The number of available promoters is limited and however, mainly comprises the methanol inducible alcohol oxidase 1 (AOX1) promoter and the constitutive glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter[31]. The *P. pastoris* alcohol oxidase and two other methanol regulated genes have first been isolated by Ellis and co-workers. Alcohol oxidase is the key enzyme in the methanol utilization pathway; specific for methylotrophic yeasts. It is encoded by two genes, *AOX1* and *AOX2*, and functionally and structurally characterized as well as reviewed several times. The AOX promoters are tightly regulated by a carbon source-dependent repression/induction mechanism; showing full repression during growth on glucose or glycerol excess conditions, and maximal induction during growth on methanol[32]. In contrast, the glyceraldehyde-3-phosphate dehydrogenase promoter (PGAP) is constitutively expressed, although its strength varies depending on the carbon source used for cell growth. This offers an attractive alternative to PAOX1 on glucose, especially if induction by methanol may be inappropriate or inconvenient; simultaneously increasing cell viability[33]. Furthermore, the activity of PGAP in glucose-grown shake-flask cultures is stronger than PAOX1 in methanol-grown shake-flask cultures and slightly lower on methanol[34].

Identifying alternative promoters is a promising and important tool especially for co-expressing recombinant proteins. Additionally, through regulation of transcription, promoters with varying activities increase host engineering possibilities. Auxiliary constitutive promoters include PTEF1 (translation elongation factor 1-α), PPGK1 (glycolytic enzyme 3-phosphoglycerate kinase) and PYPT1, a GTPase involved in secretion[35]. The formaldehyde dehydrogenase promoter (PFLD1), on the other hand, is inducible by both methanol and methylamine, whereas the isocitrate lyase promoter (PICL1) is inducible by glucose or ethanol. Recently, the auspicious promoter of a protein involved in synthesis of the thiamine precursor hydroxymethylpyrimidine (PTHI11) has been identified by Stadlmayr and co-workers. It appears to be the first carbon and nitrogen source independent adjustable promoter. A major focus of several researchers is therefore the identification of new applicable promoter sequences.

Selection of secretion signals in the *Pichia pastoris*

With *P. pastoris*, heterologous proteins can either be expressed intracellularly or secreted into the medium, since itsecretes only low levels of endogenous proteins, and because its culture medium contains no added proteins, a secreted heterologous protein comprises the vast majority of the total protein in the medium. Signal sequences follow a common structural motif. The preregion typically consists of a positively charged N-terminus, followed by a hydrophobic middle sequence and a polar C-terminus[36]. It targets the nascent polypeptide to the ER via SRP dependent, SRP independent or both pathways, which is regulated by the hydrophobicity of the core sequence. In this context, increasing hydrophobicity was reported to augment protein secretion of human lysozyme in *S.*

cerevisiae, however, failed to improve secretion of chicken lysozyme in *P. pastoris*. The signal peptidase cleavage site is most often followed by three hydrophobic amino acids (Ala-Phe-Val), preceded by a basic residue, determining the pro-region of varying length and amino acid composition.Galectins lack a classical signal peptide and can be localized to the cytosolic compartments where they have intracellular functions[37]. However, via one or more as yet unidentified nonclassical secretory pathways, galectins can also be secreted to function extracellularly. Individual members of the galectin family have different tissue distribution profiles and exhibit subtle differences in their carbohydrate-binding specificities. Each family member may preferentially bind to a unique subset of cell surface glycoproteins. *P. pastoris* and *S. cerevisiae* have low specificity for recognition of signal sequences and therefore, the recombinant proteins native signal may also be used successfully for protein expression[38]. A ribosome inactivating protein from *Aspergillus giganteus* (α-Sarcin) has been successfully secreted in *P. pastoris* with its native signal sequence, but not in *S. cerevisiae*. A single mutation of the leader peptide, generating a more favored Kex2p recognition site, enhanced processing and secretion of α-Sarcin. Human serum albumin (HSA) was efficiently secreted by its native leader. Additionally, human lysozyme was directed correctly processed to the supernatant in *P. pastoris* by the HSA native signal leader and the chicken lysozyme native leader[39]. The matrix metalloproteinases-1, 2, 3, 9 signal peptides are also functional in the *P. pastoris* system. Additionally, the plant lectin *Phaseolus vulgaris* agglutinin (PHA; both E- and L-forms) signal sequence was used to secrete correctly processed PHA and GFP in *P. pastoris*. Recently, a C-terminal truncated envelope protein from Dengue-2 virus has been targeted to the ER for further folding through the *S. cerevisiae* sucrose invertase 2 (Suc2) signal sequence in *P. pastoris[40]*. This signal sequence was also used for hepatitis C virus structural viral protein and *A. niger* xylanase expression in *P. pastoris*, just to name a few. Protein secretion was also obtained by using a viral secretion signal derived from the K28 prepro toxin, the precursor of the yeast K28 virus toxin in *P. pastoris* as well as by using the *P. pastoris* Pho1 (acid phosphatase 1) secretion signal, containing a Kex2p cleavage site[41]. To this end, usage of MFα1pp is mostly improving heterologous protein secretion compared to other or native secretion signals; however, fractions with N-terminal amino acids of the leader are often remaining. Different other prepro sequences were tested in *P. pastoris*, leading to various outcomes compared to the MFα1pp. Generally, the optimal secretion leader should be adjusted specifically for every recombinant protein as the leader influences folding and secretion. The establishment and characterization of novel powerful secretion signals is therefore a common and feasible goal for biotechnology applications and understanding yeast physiology.

Conclusion:-

All genes must be expressed to exhibit their biological activities. How genes are expressed and regulated is acentral question in molecular biology and our knowledge in this area has been expanding enormously in recent years.The complexity of gene regulation is compounded by the fact that gene activities reach every comer of biology.Furthermore, advances in high-throughput screening have enabled the monitoring of the overexpression phenotype at the single-cell level and the rapid isolation of the rare clones with the desired overexpression profiles.Summing up, recombinant proteins can either be secreted, thus generating a more desirable amount of protein and simultaneously mediating the first step of purification, or intracellular expressed; this choice will depend on how the protein is being expressed in its native system.

References:-

1. Tripathi NK. High yield production of heterologous proteins with Escherichia coli. Defence Science Journal. 2009;59(2):137.
2. Huang CJ, Lin H, Yang X. Industrial production of recombinant therapeutics in Escherichia coli and its recent advancements. J Ind Microbiol Biotechnol. 2012;39(3):383-99.
3. Demain AL, Vaishnav P. Production of recombinant proteins by microbes and higher organisms. Biotechnol Adv. 2009;27(3):297-306.
4. Hannig G, Makrides SC. Strategies for optimizing heterologous protein expression in Escherichia coli. Trends Biotechnol. 1998;16(2):54-60.
5. Makino T, Skretas G, Georgiou G. Strain engineering for improved expression of recombinant proteins in bacteria. Microb Cell Fact. 2011;10(1):32.
6. Gustafsson C, Govindarajan S, Minshull J. Codon bias and heterologous protein expression. Trends Biotechnol. 2004;22(7):346-53.
7. Copeland NG, Jenkins NA, Court DL. Recombineering: a powerful new tool for mouse functional genomics. Nat Rev Genet. 2001;2(10):769-79.

8. Clarke L, Carbon J. A colony bank containing synthetic CoI EI hybrid plasmids representative of the entire E. coli genome. Cell. 1976;9(1):91-9.
9. Jishage M, Iwata A, Ueda S, Ishihama A. Regulation of RNA polymerase sigma subunit synthesis in Escherichia coli: intracellular levels of four species of sigma subunit under various growth conditions. J Bacteriol. 1996;178(18):5447-51.
10. Jacobs WR, Tuckman M, Bloom BR. Introduction of foreign DNA into mycobacteria using a shuttle phasmid. Nature. 1987;327(6122):532-5.
11. Aminov RI. Horizontal gene exchange in environmental microbiota. Front Microbiol. 2011;2:158.
12. Barros SP, Offenbacher S. Epigenetics: connecting environment and genotype to phenotype and disease. J Dent Res. 2009;88(5):400-8.
13. De Schutter K, Lin YC, Tiels P, Van Hecke A, Glinka S, Weber-Lehmann J, Rouzé P, Van de Peer Y, Callewaert N. Genome sequence of the recombinant protein production host Pichia pastoris. Nat Biotechnol. 2009;27(6):561-6.
14. Kumar Vemuri P, Veeravalli S. Expression, purification and characterization of human recombinant galectin 3 in Pichia pastoris. Iran J Biotechnol. 2014 Apr 1;12(2):3-8.
15. Kurtzman CP. Description of Komagataella phaffii sp. nov. and the transfer of Pichia pseudopastoris to the methylotrophic yeast genus Komagataella. Int J Syst Evol Microbiol. 2005;55(2):973-6.
16. Mattanovich D, Callewaert N, Rouzé P, Lin YC, Graf A, Redl A, Tiels P, Gasser B, De Schutter K. Open access to sequence: browsing the Pichia pastoris genome. Microb Cell Fact. 2009;8(1):53.
17. Romanos M. Advances in the use of Pichia pastoris for high-level gene expression. Curr Opin Biotechnol. 1995;6(5):527-33.
18. Gialama D, Kostelidou K, Michou M, Delivoria DC, Kolisis FN, Skretas G. Development of Escherichia coli strains that withstand membrane protein-induced toxicity and achieve high-level recombinant membrane protein production. ACS Synth Biol. 2017;6(2):284-300.
19. Cregg JM, Vedvick TS, Raschke WC. Recent advances in the expression of foreign genes in Pichia pastoris. Biotechnol. 1993;11(8):905-10.
20. Baghban R, Farajnia S, Rajabibazl M, Ghasemi Y, Mafi A, Hoseinpoor R, Rahbarnia L, Aria M. Yeast expression systems: overview and recent advances. Mol Biotechnol. 2019;61(5):365-84.
21. Ramón A, Marín M. Advances in the production of membrane proteins in Pichia pastoris. Biotechnol J. 2011;6(6):700-6.
22. Scott RC, Juhász G, Neufeld TP. Direct induction of autophagy by Atg1 inhibits cell growth and induces apoptotic cell death. Curr Biol. 2007;17(1):1-1.
23. Varfolomeev EE, Schuchmann M, Luria V, Chiannilkulchai N, Beckmann JS, Mett IL, Rebrikov D, Brodianski VM, Kemper OC, Kollet O, Lapidot T. Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. Immunity. 1998;9(2):267-76.
24. Marciniak SJ, Yun CY, Oyadomari S, Novoa I, Zhang Y, Jungreis R, Nagata K, Harding HP, Ron D. CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. Genes Dev. 2004;18(24):3066-77.
25. Bill RM. Playing catch-up with Escherichia coli: using yeast to increase success rates in recombinant protein production experiments. Front Microbiol. 2014;5:85.
26. Ageitos JM, Vallejo JA, Veiga-Crespo P, Villa TG. Oily yeasts as oleaginous cell factories. Appl Microbiol Biotechnol. 2011;90(4):1219-27.
27. Taylor AB, Smith BS, Kitada S, Kojima K, Miyaura H, Otwinowski Z, Ito A, Deisenhofer J. Crystal structures of mitochondrial processing peptidase reveal the mode for specific cleavage of import signal sequences. Structure. 2001;9(7):615-25.
28. Gould SJ, McCollum D, Spong AP, Heyman JA, Subramani S. Development of the yeast Pichia pastoris as a model organism for a genetic and molecular analysis of peroxisome assembly. Yeast. 1992;8(8):613-28.
29. Cereghino GP, Cereghino JL, Sunga AJ, Johnson MA, Lim M, Gleeson MA, Cregg JM. New selectable marker/auxotrophic host strain combinations for molecular genetic manipulation of Pichia pastoris. Gene. 2001;263(1-2):159-69.
30. Näätsaari L, Mistlberger B, Ruth C, Hajek T, Hartner FS, Glieder A. Deletion of the Pichia pastoris KU70 homologue facilitates platform strain generation for gene expression and synthetic biology. PloS one. 2012;7(6):e39720.

31. Döring F, Klapper M, Theis S, Daniel H. Use of the Glyceraldehyde-3-phosphate Dehydrogenase Promoter for Production of Functional Mammalian Membrane Transport Proteins in the YeastPichia pastoris. Biochem Biophys Res Commun. 1998;250(2):531-5.
32. Prielhofer R, Maurer M, Klein J, Wenger J, Kiziak C, Gasser B, Mattanovich D. Induction without methanol: novel regulated promoters enable high-level expression in Pichia pastoris. Microb Cell Fact. 2013;12(1):5.
33. Weinhandl K, Winkler M, Glieder A, Camattari A. Carbon source dependent promoters in yeasts. Microb Cell Fact. 2014;13(1):5.
34. Jordà J, Suarez C, Carnicer M, ten Pierick A, Heijnen JJ, van Gulik W, Ferrer P, Albiol J, Wahl A. Glucose-methanol co-utilization in Pichia pastoris studied by metabolomics and instationary 13 C flux analysis. BMC Syst Biol. 2013;7(1):1-6.
35. Cereghino JL, Cregg JM. Heterologous protein expression in the methylotrophic yeast Pichia pastoris. FEMS Microbiol Rev. 2000;24(1):45-66.
36. Rakestraw JA, Sazinsky SL, Piatesi A, Antipov E, Wittrup KD. Directed evolution of a secretory leader for the improved expression of heterologous proteins and full-length antibodies in Saccharomyces cerevisiae. Biotechnol Bioeng. 2009;103(6):1192-201.
37. Liu FT, Patterson RJ, Wang JL. Intracellular functions of galectins. Biochim Biophys Acta Gen Subj. 2002;1572(2-3):263-73.
38. Daly R, Hearn MT. Expression of heterologous proteins in Pichia pastoris: a useful experimental tool in protein engineering and production. J Mol Recognit. 2005;18(2):119-38.
39. Cregg JM, Cereghino JL, Shi J, Higgins DR. Recombinant protein expression in Pichia pastoris. Mol Biotechnol. 2000;16(1):23-52.
40. Samsa MM, Mondotte JA, Iglesias NG, Assunção-Miranda I, Barbosa-Lima G, Da Poian AT, Bozza PT, Gamarnik AV. Dengue virus capsid protein usurps lipid droplets for viral particle formation. PLoS Pathog. 2009;5(10):e1000632.
41. Eiden-Plach A, Zagorc T, Heintel T, Carius Y, Breinig F, Schmitt MJ. Viral preprotoxin signal sequence allows efficient secretion of green fluorescent protein by Candida glabrata, Pichia pastoris, Saccharomyces cerevisiae, and Schizosaccharomyces pombe. Appl Environ Microbiol. 2004;70(2):961-6.