PICHIA PASTORIS: A PLATFORM ORGANISM TO PRODUCE PROTEINS

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Contents

Summary

Pichia pastoris has become one of the most extensively used expression systems. Together with *E. coli*, *B. subtilis*, *S. cerevisiae*, *H. polymorpha*, and species of *Aspergillus* and *Trichoderma*, *P. pastoris* is now an established industrial platform for production of proteins. Versatile host strains of *P. pastoris* in combination with appropriate vectors are easily available and expressing genes, coding for protein of interest, is routinely performed. In simple mineral salt medium and controlled environment of bioreactors, *P. pastoris* easily grow to high cell densities. An efficient and tightly regulated promoter (*AOX1*) allow for desired control and separation of growth and production. Furthermore, while *P. pastoris* secrete only a few endogenous

proteins, the secretion of recombinant proteins is very efficient. Separation of the recombinant protein from the simple salt medium is a rather simple task. Thus, *P.pastoris* offers very efficient recombinant protein formation and purification and is therefore ideal for production.

1. Introduction

Proteins benefit major sectors of biopharmaceutical, enzyme, and agriculture industries. Products of these industries in turn augment the field of medicine, diagnostics, food, nutrition, detergents, textiles, leather, paper, pulp, polymers, and plastics. Starting in 1970s, scope of prospective for all these fields had been dramatically changed by recombinant DNA technology.

Recombinant proteins are produced in systems like bacteria, yeast, filamentous fungi, insect cells, mammalian cells, transgenic animals, and transgenic plants. Overall, 39% of recombinant proteins are made by E. coli, 35% by CHO cells, 15% by yeasts, 10% by other mammalian systems and 1% by other bacteria and other systems. Over half of the industrial enzymes are made by yeasts and molds, with bacteria producing about 30%, animals provide 8% and plants 4%.

Generally, proteins that are larger than 100 kD are expressed in a eukaryotic system while those smaller than 30 kD are expressed in a prokaryotic systems. Bacterial cells offer simplicity, short generation time, and large yields of product with low costs. Yeasts as single-celled microorganisms that are easy to manipulate and cultivate as bacteria also offer eukaryotic environment, which is often required for production of large and complex proteins.

The initial application of the methylotrophic yeast *Pichia pastoris* for production of single cell protein occurred in the early 1970's. For this purpose, a cheap medium and a high cell density fermentation process were developed by Phillips Petroleum Company. In the 1980's, researchers at the Salk Institute Biotechnology/Industrial Associates, Inc. (SIBIA, La Jolla, CA) isolated the *AOX1* gene, coding for alcohol oxidase, and its promoter and developed vectors, strains, and methods for molecular genetic manipulation of *P. pastoris*. Today, *P. pastoris* expression system is very attractive for recombinant protein production for many reasons: *(i)* molecular genetic manipulations are simple and protocols are available, *(ii)* a tightly regulated and efficient promoters may be used for controlled expression of heterologous genes, *(iii) P. pastoris* has a strong tendency for respiratory growth, *(iv)* can be grown to high cell density in a bioreactor, *(v)* has a good secretion capacity for proteins, *(vi)* can be grown in a simple mineral salts medium with secretion of few endogenous proteins, which simplifies the product recovery and purification, and *(vii)* compared to *S. cerevisiae* has less extensive glycosylation.

The *P. pastoris* expression system can be useful especially in the cases when *E. coli* protein synthesis machinery fails to deliver correctly folded functional protein and *S. cerevisiae* glycosylation pattern results in hyperglycosylated inactive protein. Compared to other systems *P. pastoris* productivity is usually higher (Table 1). Alternative bioreactor cultivation and downstream process techniques, adapted for *P. pastoris*

specificity, have contributed to increased efficiency of this expression system. Moreover, success has been achieved in genetically engineering the *P. pastoris* secretory for production of human type N-glycosylated proteins.

In this review, components of *P. pastoris* expression system are described in connection with available fermentations techniques.

Recombinant hosts	**mg/L**
BHK cells	0.05
Insect cells	0.40
Streptomyces lividans	0.25–0.5
Escherichia coli	200–300
Saccharomyces cerevisiae	40–500
Hansenula polymorpha	1500
Pichia pastoris	1500

Table 1. Comparison of productivities of hirudin by recombinant hosts

2. The *P. Pastoris* Expression System

Linear vectors are integrated in the *P. pastoris* genome via homologous recombination and can generate stable transformants. Although some recombinant proteins are produced intracellularlly, the ability of *P. pastoris* to secrete large amounts of recombinant proteins to the growth medium is its greatest attribute. Expression of heterologous genes by *P. pastoris* is driven by the efficient and tightly regulated *AOX1* promoter. This promoter is highly repressed in cells grown on glucose, glycerol and most other carbon sources, but is strongly induced by methanol. This tight regulation is used in *P. pastoris* cultures to grow cells on glycerol with subsequent induction with methanol. The cultivation process is scalable to industrial levels of production. Current applications in pharmaceutical industry include, among others, insulin-like growth factor, hepatitis B vaccines and human serum albumin.

2.1. The *AOX1* Promoter and Alternative Promoters

2.1.1. *AOX1* Promoter.

The use of methanol as sole carbon source for the growth of *P. pastoris* stimulates the expression of a family of genes. The *AOX1* gene was first isolated by Ellis in 1985 AOX is a homo-octomer with each subunits containing one noncovalently bound flavin adenine dinucleotide (FAD) cofactor. The genome of *P. pastoris* contains two alcohol oxidase genes, *AOX1* and *AOX2*. The protein-coding regions of the genes are 92% and 97% homologous at the nucleotide and predicted amino acid sequence levels, respectively. However, the *AOX1* gene is responsible for the vast majority of AOX activity in the cells. Expression of *AOX1* is tightly regulated at the transcriptional level and appears to be controlled by both repression/derepression and induction mechanisms. AOX has a low affinity for oxygen and the cell compensates this by producing large amounts of the enzyme. In methanol limited fed-batch cultures, the AOX concentration in cells can be more than 30% of total soluble protein.

Thus, the methanol-regulated *AOX1* gene is highly expressed and tightly regulated and the *AOX1* promoter is suitable for foreign gene expression. A major advantage of this tight regulation is that foreign genes whose products are toxic to the cells can be readily maintained in cells by culturing under repressing growth condition to prevent the selection of nonexpressing strains. This promoter has been successfully used to produce numerous recombinant proteins in milligrams to grams per liter levels.

2.1.2. Strongly Expressed Alternative Promoters.

In some circumstances in which use of methanol is not suitable, alternative promoters are used. The glyceraldehyde-3-phosphate dehydrogenase (GAP) gene promoter of *P. pastoris* provides strong constitutive expression on glucose at a level comparable to that seen with the *AOX1* promoter. However, the GAP promoter is constitutively expressed and not suitable for production of proteins that are toxic to the yeast.

In methylotrophic yeasts, glutathione-dependent formaldehyde dehydrogenase (FLD) is a key enzyme required for the metabolism of methanol as a carbon source and certain alkylated amines such as methylamine as nitrogen source. Using ß-lactamase as a reporter, it was shown that the FLD1 gene promoter is strongly and independently induced by either methanol as sole carbon or methylamine as sole nitrogen source. With methanol or methylamine induction, levels of ß-lactamase are comparable to those obtained with the *AOX1* promoter. Thus, the FLD1 promoter provides a choice of carbon (methanol) or nitrogen source (methylamine) regulation with the same expression strain.

2.1.3. Moderately Expressed Alternative Promoters

For certain foreign genes, expression from strong promoters may overwhelm the post-translational machinery of the cell, causing a significant portion of the protein to be misfolded, unprocessed, or mislocalized. The *P. pastoris* YPT1 gene encodes GTPase involved in secretion, and its promoter is constitutive and moderately expressed. When the ß-glucuronidase (GUS) was used as reporter, expression levels from YTP1 promoter were about 10 to 100-fold lower than those from the GAP promoter. An alternative way to moderate expression is to use *AOX1* promoter and induction with mannitol. When *P. pastoris* cells were grown on mannitol, GUS expression from the *AOX1* promoter was 30-fold lower than in methanol-grown cells. Thus, mannitol appears to be a useful carbon source for intermediate level of expression from *AOX1* promoter.

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Biographical Sketch

Mehmedalija Jahic did graduate studies **f**rom 1998 to 2003 at Royal Institute of Technology (KTH) in Stockholm, Sweden. During this time he was conducting studies on bioprocess scale up strategies based on integration of microbial physiology and fluid dynamics and process techniques for production of recombinant proteins with *Pichia pastoris.* From 2004 to 2007 he was a project leader for set up a core facility at KTH for rapid development and pilot plant scale protein production. Year 2007 he started at DuPont Company in Wilmington, DE, USA. At DuPont he has been working in field of biofuels production.