



Application of methylotrophic yeast *Pichia pastoris* in the field of food industry - A review

Viviane C. Silva, Maristela F. S. Peres and Edwil A. L. Gattás *

Departament of Food and Nutrition, Faculdade de Ciências Farmacêuticas, UNESP - São Paulo State University, Rodovia Araraquara-Jaú, Km 1, Postal Code 14801-902, São Paulo, Brazil.

*e-mail: edwilg@yahoo.com.br

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Abstract

Since ancient times, the utilization of yeasts by the man has a great impact on the socio-economic development. After the advent of the technology of recombinant DNA, great advances have occurred due to the acquisition of strains of mutant yeasts in the field of applied research, and *Saccharomyces cerevisiae* has soon been outstanding as an interesting candidate for the expression of heterologous proteins of biotechnological interest. As the time goes by other alternative systems of expression have been shown because they have advantages over *Saccharomyces cerevisiae*. Among those new systems, *Pichia pastoris* is outstanding as methylotrophic yeast capable of growing in a culture medium containing methanol as the only source of carbon and energy. The induction of production of glycerol-3-phosphate dehydrogenase (GPD, NAD⁺: oxido-redutase EC 1.1.1.8) by *Pichia pastoris* was accomplished in the medium containing methanol. One of the most important key parameters in *Pichia pastoris* expression system is the methanol concentration. Bibliographic reviews on the *Pichia pastoris* production system have shown that the best culture conditions vary according to the strain used and/or kind of heterologous protein desired to be expressed. Therefore, we have sought to develop a system, involving expression of glycerol-3-phosphate dehydrogenase in the yeast *Pichia pastoris*, for generating sufficient quantities of the enzyme in order to assess its potential value for use in various food bioanalytical determination. Dehydrogenases have been widely used in the enzymatic assays of diverse composites of industrial interest, being enclosed among them glycerol and a number of important bioanalytical applications.

Key words: Yeast, *Pichia pastoris*, optimization, expression, glycerol-3-phosphate dehydrogenase, culture medium.

Introduction

The yeasts are considered as unicellular fungi that are vegetatively reproduced by sprouting or fission. The yeasts are classified according to the sexual aspects and morphological, physiological and genetic characteristics. Their identification and characterization are very important in biotechnology, mainly in industrial processes, that is to say, this procedure is indispensable for brewing, where the presence of wild yeasts can deteriorate the product, and also in the propagation of baking yeasts, where wild species, as *Candida utilis*, can grow together with *Saccharomyces cerevisiae*, due to better capacity of sugar transportation ¹.

Nowadays, the exploration of yeasts has significant impacts, in relation to the supply of renewable energy, because they are capable of fermenting carbohydrates, so producing ethanol. Biotechnological studies, including the disease control, mainly of genetic diseases and cancer ¹ are also study objects. Great advances were performed in the field of medicine, due to the production of human therapeutic proteins, by utilizing genetic engineering of yeasts. The use of yeasts in therapeutic medicine, as from the modern technology of recombinant DNA, returns to ancient times, when yeasts were utilized for the biological control of bacteriological infections ².

The yeast *Saccharomyces cerevisiae* has an outstanding role, besides being considered one of the most useful microorganisms for the man; this yeast is one of the most known eukaryotic

systems. Bread, beer and wine have represented the most expressive products in the handling process of those microorganisms along the time. Its manipulation caused a great impact on the food production and, so, it influenced the process of socio-economic development of the humanity ¹.

In relation to genetics, the yeast *S. cerevisiae* is well known and its genome was totally sequenced, such fact represented one of the greatest conquests of biology in the twentieth century. After the advent of the technology of recombinant DNA, this yeast could be used in studies of molecular genetics as from the late 70's, when it was genetically modified for the first time. Ever since, several kinds of molecular vectors were developed, including artificial chromosomes, more known as YAC (Yeast Artificial Chromosomes). With the acquisition of strains of mutant yeasts, it was possible to isolate the genes of other eukaryotic organisms. In the field of applied research, the yeast *S. cerevisiae* has soon been outstanding as an interesting candidate for the expression of heterologous genes of biotechnological interest. The intracellular environment of the yeast is appropriate for the occurrence of several reactions that normally occur in mammal cells ³.

Another microorganism of biotechnological interest is *Escherichia coli*, extensively used as a cellular host for the production of heterologous proteins. However, several

applications do not present the result desired, because this simple microorganism is defective of an intracellular system that makes it difficult to express the recombinant protein. The expression of a successful recombinant protein in *E.coli* is dependent on the primary, secondary and tertiary conformations and on the functional characteristics of the protein of interest⁴. A great variety of proteins that cannot be expressed in *E.coli*, because they need the correct post-translational maturation level, need to be produced in the methylotrophic yeast *Pichia pastoris*⁵.

Methylotrophic yeasts: Along the two last decades, other yeasts have been presented as alternative systems of expression, because they showed advantages over *S. cerevisiae*. Among those new systems, the methylotrophic yeasts are outstanding, that is to say, species of yeasts that are capable of growing in a culture medium containing methanol as the only source of carbon and energy. The lineages identified as methylotrophic belong only to four genera: *Hansenula*, *Pichia*, *Candida* and *Torulopsis*⁶, and their applications to biotechnology are outstanding in the production of recombinant proteins. The initial reactions of utilization of methanol occur in the peroxisomes, organelles that are strongly induced when this alcohol is utilized as a carbon source.

Table 1 shows studies of comparison of different species of methylotrophic yeasts, in relation to the carbon source. The glucose represses the expression of protein, while the addition of glycerol or other carbon source is dependent on the promoter and the yeast⁷.

Protein Production of the Methylotrophic Yeast *Pichia pastoris*

Pichia pastoris is a methylotrophic yeast also known as *Endomyces pastori*, *Komagataella pastori*, *Petasospora pastori*, *Saccharomyces pastori*, *Zygosaccharomyces pastori*, *Zygowillia pastori* and *Zymopichia pastori*. It is naturally found in the exudate from chestnut tree in France and in the black oak of *Quercus kelloggii* in the USA. Its taxonomy is described as being from the kingdom Fungi; from the phylum Ascomyceta; from the subphylum Saccharomycotina; from the class Saccharomycetes; from the order Saccharomycetales; from the family Saccharomycetaceae; from the genus *Pichia* and the species *P. pastoris*⁸.

Some yeasts from the genus *Pichia* are found in soil, fresh water, insects, exudate from trees, plants and fruits and are also

contaminants in a variety of food and beverages, some of those species can contribute with desirable effects on the initial stages of wine fermentation, in several kinds of brine, in different kinds of cheese, while some species have been described as human pathogens^{9,10}.

All of them utilize a specific way for the methanol, in which several unique enzymes are involved. The initial reactions occur in specialized organelles, the peroxisomes, followed by subsequent metabolic steps in the cytoplasm. The peroxisomes have an indispensable role during the growth, once they have three key enzymes for the metabolism of methanol: the alcohol oxidase, catalase and the dihydroxyacetone synthase. The subsequent reactions of assimilation and deassimilation of methanol are located in cytosol¹¹. The enzyme alcohol oxydase (AOX) catalyses the first step in the utilization pathway of methanol, the oxidation of methanol to formaldehyde and hydrogen peroxide, AOX is captured in the peroxisome together with catalase, in which it degrades the hydrogen peroxide to oxygen and water. A part of the formaldehyde generated by AOX is released from the peroxisome, and later it is oxidized to formate and carbon dioxide, through two dehydrogenases of the cytoplasm, reaction which are energy sources for the cellular growth in methanol¹². The enzyme alcohol oxydase is strongly repressed by several carbon sources such as glucose and glycerol, and it is induced by lack of carbon source. The conversion of methanol into formaldehyde is a limiting step in the utilization of methanol as a carbon source, and it is regulated by the amount of enzyme AOX which is present in the cell. There are demonstrations of this behaviour when the concentrations of methanol are suddenly increased, and the cellular growth is inhibited.

Production of heterologous proteins: The fact that the yeast *Pichia pastoris* grows up to a high cellular density, in a cheap medium, induced the Phillips Petroleum Company to propose the use of this yeast as a feeding source ("single cell protein - SCP"). After having noticed that the process of SCP production was not economically viable, because of the value of methanol due to the petroleum crisis in the 70's¹³, the company decided to transform *P. pastoris* into a system of production of recombinant proteins. The yeast *P. pastoris* has two characteristics that make it an attractive host for the production of heterologous proteins. The first is the strong promoter used to transcript heterologous genes, which is derived from the alcohol oxydase gene (AOX1) of *P. pastoris*. This provider is transcriptionally regulated by methanol,

Table 1. Repression and derepression effect of carbon sources on AOX genes of methylotrophic yeasts⁷.

Yeast	Promoter	Carbon source			
		Glucose, ethanol	Glycerol	Methanol	Glycerol+MeOH
<i>Pichia pastoris</i>	AOX1	Repression	Repression	Induction	Repression
	AOX2	Repression	Repression	Induction	Repression
<i>Hansenula polymorpha</i>	MOX	Repression	Derep. (~60-70%)	Induction	Induction (~100%)
	FMD	Repression	Derep. (~60%)	Induction	
<i>Candida boidinii</i>	AOD1	Repression	Derep. (~3-30%)	Induction	Induction (~90%)
	FDL	No activity	Derep. (~20%)	Induction	Induction (~70%)
	FDH	Repression	No activity	Induction	Induction (~30%)
	DAS	Repression	Derep. (~2%)	Induction	Induction (~70%)
<i>Pichia methanolica</i>	MOD1	Repression	Derep. (~60-70%)	Induction	Induction (~100%)
	MOD2	Repression	No activity	Induction	Induction (~100%)
	FLD	No activity	Derep. (~20%)	Induction	Induction (~70%)

a relatively cheap inducer. In cells exposed to methanol as the unique carbon source, the beginning of the transcription in the promoter AOX1 is highly effective and comparable to the promoters derived from the highly expressed genes of the glycolytic pathway. Therefore, unlike the glycolytic promoters, the promoter AOX1 is strongly regulated and repressed under conditions of growth without methanol. Once the majority of heterologous proteins is, in some way, deleterious for the cells, when expressed in high levels, the ability of maintaining the culture in a repressed or disconnected state is highly desirable. It is concerned with an important precaution, in order to minimize the selection of mutants that do not express the heterologous product during the culture growth. In order to be activated, the promoter AOX1 requires the presence of methanol and, in the absence of this inducer, it becomes repressed. Besides methanol, the system AOX1 needs the absence of glucose, in order to be totally activated. Once the promoter AOX1 is controlled by the handling of the carbon source added to a culture medium, the growth and the induction of strains of *P. pastoris*, that express heterologous proteins, are easily obtained in all scales, from bottles up to big fermentators³.

The second important characteristic of *P. pastoris* is that this yeast is not considered a fermentator source as *S. cerevisiae*. The fermentation performed by yeasts produces ethanol, which in cultures of high density, can quickly reach toxic levels (Crabtree effect). For an economically viable production of recombinant proteins, the concentration of proteins in the medium must be proportional to the amount of cells. First, it is necessary to reach the high levels of cellular density, which are not easily obtained with *S. cerevisiae*. In opposition, the strains that produce *P. pastoris* are easily grown, and cellular densities reach about of 100 g/l of dry weight, or even higher. Several genes of different sources (bacteria, fungi, invertebrates, plants and humans) have already been expressed in *P. pastoris* under the control of AOX1 promoter. The majority of the expressed genes had its products secreted to the extracellular medium and some, as the human tumor necrosis factor, were produced in the intracellular environment¹⁴. The expression systems in this microorganism allow producing proteins in the intracellular as well as in the extracellular form. However, the system of extracellular production presents the advantage of releasing the protein of interest in the supernatant of the culture medium, both models of expression are being widely studied, and they need enough cell mass, so making it possible for the proteins to be purified and utilized as a product¹². More than five hundred proteins were cloned and expressed using the system *P. pastoris*^{11,15}.

Cereghino and Cregg¹² described some of the proteins expressed in the system of *P. pastoris*, some originally found in bacteria (α -amylase originally from *Bacillus licheniformis*, D-alanine carboxypeptidase from *Bacillus stearothermophilus*), others found in fungi (glucoamylase from *Alternaria alternata* (Alt), catalase L from *Aspergillus fumigatus*), others found in protists (hexose oxidase from *Chondrus crispus* - red algae, acid α -mannosidase from *Trypanosoma cruzi*), in plants (phytochrome B from potato, acid phosphatase from nodule of soy grain), in invertebrates (bright green protein from *Aequorea victoria* (jelly fish), some in non-human vertebrates (bovine β -casein, bovine pancreatic trypsin inhibitor (aprotinine), gelatinase B from rats, intestinal carrier of peptide (PEP1 and 2 from rats) and some from

humans (seric albumin, thrombomodulin).

Fermentation media and operational conditions: According to Cos *et al.*¹³, the optimization of the expression of heterologous proteins that consists on maximizing the output and the productivity, depends on different phenotypes with promoter AOX1, from other promoters, culture media and operational strategies.

Macauley-Patrick *et al.*¹¹ report that a great part of the work in *P. pastoris* utilizes a complex medium for the growth and induction of the protein expression. The formulations of those media are found in the registers of Invitrogen Corporation (Carlsbad, CA, USA), and they are dependent on the strain used. The concentration of substrates (glucose or glycerol) is a more common limitation in the production of wide scale, because the maintenance of the initial substrate, with low concentration (non-inhibitory), is essential for the optimization of the biomass production, and consequently, in the desired protein.

Boze *et al.*¹⁶ obtained sufficiently high amounts of expression of protein in bioreactor, comparing to the growth in little bottles, when *P. pastoris* was grown using a high cellular density in fed bioreactor or in continuous fermentation process, with the controls of pH and amount of oxygen.

Bibliographic reviews on the production system of *Pichia pastoris*, demonstrated that the best conditions (culture medium, pH, temperature, etc.) vary according to the kind of used lineage and/or kind of heterologous protein that is desired to be expressed^{17,18}. Choi *et al.*¹⁹ report that, in general, for the production of heterologous protein using *P. pastoris* in a fermentator, three stages have to be obeyed in the process: the accumulation of biomass utilizing glycerol as a carbon source; a stage of transition, for adapting the cells, with strategies of controlled addition of glycerol in order to obtain limiting rates of growth, the induction by the slow addition of methanol.

According to Invitrogen Co.²⁰, the most common medium for obtaining the high cellular density in fermentations with this yeast is the BSM medium (Basalt Salt Medium). The BSM medium is supplemented with micronutrients like Fe, Mn, Cu and biotin, normally utilizing a solution of salt traits (PTM1) proposed by Invitrogen. Being considered as one of the best media, this one cannot be optimum due to some important problems (debalanced composition, precipitations, ionic force, etc.). Thus, some alternative proposals for this medium were performed^{21,22}, for example, in the variations of the concentrations of phosphate and potassium. Those culture media were formulated for obtaining high cellular densities in fed batch cultures. The influence of those elements on the development of *P. pastoris* is not well known. Some authors¹⁶ found a basal supplementation for the medium with seven vitamins and two salt traits that increase the production of the porcine follicle-stimulating hormone, with 93 mg/ml (basal) for 187 mg/ml (supplemented), showing that the vitamin and the trait elements required had an important effect on the growth of the cell and on the production of recombinant protein in *P. pastoris*.

The authors²³ utilized the BSM medium for the production of angiostatin in bioreactor, added to the growth of other carbon sources (for an improvement in the production of protein), such as glycerol, sorbitol, acetate and lactic acid, in which those were added, respectively, together with methanol in the stage of protein expression. The production of angiostatin was 108 mg/l when

glycerol was added. Having sorbitol as a carbon source the concentration of protein was 141 mg/l and only 52 mg/l with the use of acetate. The best production was obtained with 191 mg/l, utilizing the lactic acid. Initial experiments were performed in bottles with four different carbon sources, and those indicated improvements on the growth of *P. pastoris* and with different effects of protein expression. As the control of substrate and pH cannot be performed in this kind of experiment, the evidence of the results with different carbon sources was performed in bioreactors. The strategy used was to utilize a mixture of carbon sources, that is to say, glycerol was added to methanol during the stage of protein expression, but it was done being careful for the glycerol not to repress the promoter AOX and so decrease the production of the desired protein¹². Therefore, the use of carbon sources that improve the growth, but do not repress the promoter AOX, is essential for the improvement on the control of the fermentative process.

Sorbitol is also accepted as a non-repressive carbon source for *P. pastoris*, in which the level for the production can be compared to the data obtained with glycerol^{22, 24}. Mannitol, alanine and trehalose are also cited as non-repressive carbon sources in other studies^{14, 25, 26}, but regarding the use of lactic acid there are no citations.

Inan and Meagher²⁷ utilized as carbon sources alanine, sorbitol and mannitol, and each one of those sources showed an incredible production of recombinant β -galactosidase compared to cells grown with glycerol or glucose, as well as the reduction in the concentration of methanol required for the induction of the expression of proteins.

Chiruvolu *et al.*¹⁹ showed that high rates of glycerol feeding result in a high production of biomass, but some decreases in the protein activity, due to the accumulation of ethanol, as that obtained by the Mut phenotype. It is observed that long periods of feeding rate cause an unbalance in the cell, in terms of energy and amino acids.¹³

Another important factor to obtain success in the expression of heterologous protein is the nitrogen source. For Cos *et al.*¹⁹ one of the most important factors in the BSM and FM22 media is the nitrogen source that is added in the way of ammonium hydroxide, in order to control the pH. Maculey-Patrick *et al.*¹¹ emphasize that the increase in the concentrations of ammonium in the culture medium can extend the lag phase and thus inhibit the cellular growth, especially in concentrations which are equal or above 0.6 mol/l. However, Kobayashi *et al.*²⁸ determined that for the production of lipase in *Rhizopus oryzae*, the lack of nitrogen is directly related to the increase of the proteolytic activity, and consequently, to the degradation of extracellular proteins.

Choi *et al.*²⁹ investigated that the effect of different nitrogen

sources (yeast extract, peptone, casaminoacids, meat extract and two inorganic sources, ammonium sulphate and urea), for the production of α -amylase from rat pancreas utilizing *P. pastoris*, repressed the expression and the growth of *P. pastoris* G2. Those authors observed that the yeast extract and the peptone were the most appropriate sources, mainly when utilized together, so producing 300U/ml of α -amylase. They also observed that the cell utilized requires glutamic acid, alanine, valine, leucine and tyrosine, and the pH variation and the low amount of amino acids coming from inorganic nitrogen provided a low cellular growth and low expression of the enzyme. Other authors⁸ obtained best results by utilizing casaminoacids and alanine as nitrogen source for the production of the viral antigen of hepatitis B. Ohya *et al.*³¹ verified that the addition of 10% of peptone or 0.2 mol/l of arginine to the culture medium made the activity of pro-urikinase annexin V to reach 7.8 kU/ml in 168 hours of growth.³¹ According to Ohda *et al.*³², the addition of ammonium sulphate was important in the prevention of seriprotease degradation from human seric albumin secreted by *P. pastoris* during its growth.

Other media, as BMGY and BMM, were utilized by several authors^{23, 24, 33}. These media are utilized for the accumulation of biomass (BMGY) and induction of protein (BMMY). The Invitrogen²⁰ formulations are useful in studies for induction of extracellular expression of glycerol-3-phosphate dehydrogenase which composition can be observed in Table 2. Dehydrogenases have been widely used in the enzymatic assays of diverse composites of industrial interest, being enclosed among them glycerol and a number of important bioanalytical application (PI0205552-0)³⁴. This method also allowed quantifying of glycerol-3-phosphate, important intermediate metabolite of lipid biosynthesis and glycolysis.

The pH also is an important factor in the maintenance and stability of heterologous protein expressed by *Pichia pastoris*, and its control is necessary to minimize the proteolytic degradation of heterologous proteins^{35, 36}. Xavier *et al.*³⁷ observed that the pH of a culture medium has an important effect on the expression of influenza virus A (hemagglutinine) and in this case, its best output was obtained at pH 8.0. For Rosenfeld *et al.*³⁸ the recombinant hirudine, a powerful inhibitor of thrombin, was expressed at pH 5.0. According to Choi *et al.*²⁹, when the cultures were grown at pH 6.0, the activity of α -amylase remained stable and high levels of enzyme expression were obtained. Therefore, the pH of the growth medium of the yeast *Pichia pastoris* is dependent on the protein that is desired to be expressed.

The optimum temperature of growth for *Pichia pastoris* is 30°C, and above 32°C it is observed a decline in the protein expression by the cell. However, some authors use low temperatures for the production of heterologous proteins. Li *et al.*³⁹ showed that the

Table 2. Composition of media for growth and expression glycerol-3-phosphate dehydrogenase by *Pichia pastoris*²⁰.

Growth medium		Induction medium	
Constituent	Quantity (g/L)	Constituent	Quantity (g/L)
Yeast extract	10.0	Yeast extract	10.0
Peptone	20.0	Peptone	20.0
YNB	13.4	YNB	13.4
Glycerol	10.0	Methanol	5.0 mL
Biotin	4x10 ⁻³	Biotin	4x10 ⁻³
100 mM phosphate buffer, pH 6.0	1000 mL	100 mM phosphate buffer, pH 6.0	1000 mL

decrease in temperature from 30°C to 23°C, in the growth of this yeast, caused a threefold increase in the protein content. Jahie *et al.*⁴⁰ affirm that the use of low temperatures during the induction stage causes a decrease in the proteolytic activity and cellular lysis.

Conclusions

The effective expression of recombinant protein in *Pichia pastoris* needs several studies that involve knowledge about the yeast strain, the choice of the promoter, the composition of the culture medium and the kinetic behaviour of the protein to be expressed, the pH and temperature of the medium, among others. The applications of those proteins to the development of new food and bioanalytics suggest a promising future for this area.

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