Revista de la Facultad de Ciencias Universidad Nacional de Colombia, Sede Medellín
V 10 N°1 enero-junio de 2021 • ISSN-e 2357-5749 • Research Paper • Páginas 20 a 44
DOI: https://doi.org/10.15446/revfaccienc.v10n1.86805

Pichia pastoris AS BIOLOGICAL PLATFORM TO PRODUCE RECOMBINANT PROTEINS: RELEVANCE FOR DEVELOPMENT OF BIOTECHNOLOGICAL PRODUCTS IN COLOMBIA[a]

Pichia pastoris COMO PLATAFORMA BIOLÓGICA PARA PRODUCIR PROTEÍNAS RECOMBINANTES: RELEVANCIA PARA EL DESARROLLO DE PRODUCTOS BIOTECNOLÓGICOS EN COLOMBIA

JUAN DAVID ROSAS CABRERA[b], CARLOS ANDRÉS DÍAZ RODRÍGUEZ[c]

Recibido 30-04-2020, aceptado 18-08-2020, versión final 18-10-2020.
Research paper

ABSTRACT: The world market for compounds produced by biotechnological means is growing due to the search and implementation of cellular systems that allow the mass production of complex molecules with a specific biological activity. These range from drugs, to enzymes and proteins for diverse uses, such as academic research and the development of industrial processes. *Pichia pastoris* is a methylotrophic yeast that has been studied in recent decades for the expression and generation of recombinant proteins, because it has features that make it especially efficient, not only to host external DNA, but also to express it and, thus, produce a wide variety of molecules. In this study, the most important aspects related to the production of recombinant proteins are examined, by using *P. pastoris* as a model, from the most common expression strategy, to the aspects related to the cultivation at bioreactor scale and, by yielding high-value products. Some papers conducted, in Colombia, are also reviewed, as well as their approach and the current state of the expression system in the country's biotechnology and its barriers, by concluding that studies with *P. pastoris* are scarce and are mainly developed around a few academic centers.

KEYWORDS: Recombinant protein; Promoters; Methylotrophic yeast; *Pichia pastoris*; Biotechnological products.

RESUMEN: El mercado mundial de compuestos producidos por medios biotecnológicos está creciendo debido a la búsqueda e implementación de sistemas celulares que permiten la producción masiva de moléculas complejas con una actividad biológica específica. Estas van desde los medicamentos, hasta las enzimas y las proteínas para usos diversos como la investigación académica y el desarrollo de procesos industriales. *Pichia pastoris* es una levadura metilotrófica que se ha estudiado en los últimos decenios para la expresión y generación de proteínas recombinantes debido a

[a]Rosas Cabrera, J. D. & Díaz Rodríguez, C. A. (2021). *Pichia pastoris* como plataforma biológica para producir proteínas recombinantes: Relevancia para el desarrollo de productos biotecnológicos en Colombia. *Rev. Fac. Cienc.*, 10 (**1**), 20–44. DOI: https://doi.org/10.15446/revfaccienc.v10n1.86805

[b]Ingeniero Biológico. Miembro del grupo de investigación Biotecnología Industrial, Universidad Nacional de Colombia, sede Medellín email: jdrosasc@unal.edu.co

[c]Ingeniero Biológico. Miembro del grupo de investigación Microbiomas y Bioenergía - Universidad de los Andes, email: c.diazr@uniandes.edu.co

que posee características que la hacen especialmente eficiente, no sólo para albergar ADN externo sino también para expresarlo y producir así una amplia variedad de moléculas. En el presente estudio se examinan los aspectos más importantes relacionados con la producción de proteínas recombinantes utilizando *P. pastoris* como modelo, desde las estrategias de expresión más comunes, hasta los aspectos relacionados con el cultivo a escala de biorreactor y obtención de productos de alto valor agregado. Se revisan también algunos trabajos realizados en Colombia, su enfoque y el estado actual del sistema de expresión en la biotecnología del país y sus barreras, concluyendo que los estudios con *P. pastoris* son pocos y se desarrollan, principalmente, alrededor de unos pocos centros académicos.

PALABRAS CLAVE: Proteínas recombinantes; promotores; Levadura metilotrófica; *Pichia pastoris*; productos biotecnológicos.

1. INTRODUCTION

In 1969, the first report of methylotrophic yeast being able to use methanol as single carbon and energy source was published (Ogata *et al.*, 1969). Due to its ability for growing at very high cellular densities (up to 130 gDW/L, where DW refers to dry weight), *Pichia pastoris* was used for the development of the single cell protein industry, at the end of the last century (Sreekrishna & Kropp, 1996; Aw & Polizzi, 2013). The Phillips Petroleum Company tried to produce biomass for feedstock, but it resulted in a non-economically viable process. This led the company to start researching about the usage of *P. pastoris* for recombinant protein production with good results and the subsequent release of the expression system for academic research laboratories in 1993 (Sreekrishna & Kropp, 1996; Cereghino & Cregg, 2000). Currently, *P. pastoris* has been a big interest in academic and industrial purposes, mainly due to its promoter derived from Alcohol Oxidase gene (AOX1) and the similarity of genetic modification techniques with those of the well-known and well-studied yeast *Saccharomyces cerevisiae* (Cregg *et al.*, 1985; Cregg *et al.*, 2000). Other interesting features that *P. pastoris* has, are the ease for the excretion of products, its promoter strength, the simple media in which it can be cultured, the integration of multi-copies of foreign DNA, yielding in more stable transformations and the easiness of recovering proteins due to its well-studied secretion systems (Li *et al.*, 2007; Demain & Vaishnav, 2009). Unlike the bacterial systems, eukaryotes, such as *P. pastoris* can make post-translational modifications, which include protein processing, folding, and N-glycane addition. This is important because most of the biological activity of recombinant proteins (as those for medical purposes) depends on modifications carried out after the translation process (Baghban *et al.*, 2016). One important advantage of using *P. pastoris* over the traditional yeast, such as *S. cerevisiae*, is the avoidance of hyperglycosylation patterns, especially for the healthcare industry (Demain & Vaishnav, 2009). In this paper, important aspects of *P. pastoris* related to the recombinant protein production, by using it as a biological platform, are reviewed, from cell features to the culture techniques and metabolic aspects to produce yeast strains suitable for biotechnological processes. The relevance of *P. pastoris* is also highlighted for the development of the biotechnological industry, in Colombia, based on the studies conducted in the country with this expression system in the production of recombinant proteins.

2. METABOLIC ASPECTS

2.1. Methanol metabolism

Pichia pastoris results in a good expression platform under certain non-conventional conditions, such as methanol, by increasing medium concentration in which the majority of microorganisms will not be able to grow, by overcoming these milestones and, by becoming this organism a really good standpoint for industrial applications (Duan *et al.*, 2018). These features can also be found in other yeast genera, such as *Hansenula, Pichia, Candida*, and *Torulopsis* (Lee & Komagata, 1980).Thus, it would be useful to look at the genetic relationship that this microorganism has with other genera, such as Candida or Saccharomyces. Not only is determining the phylogenetic position useful for further phylogenetic description purposes, but also it can be useful for determining further genetic elements search, such as ribosomal binding sites, promoter sites, initiation, and stop codons, among others. This has been proven as shown by De Schutter *et al.* (2009) where a similarity analysis found that the codon usage for this yeast was like the one for *S. cerevisiae*, which turns out to be attractive for further genetic modifications.

The utilization of a certain number of carbon and nitrogen sources for growth by yeasts is characteristically associated with the development of unique subcellular compartments in the cell (Van Dijken*et al.*, 1975).Previously, some authors have discovered that the adaptation process that this kind of microorganisms suffer, by growing them on methanol, is associated with the proliferation of large microbodies in the cell (Avers & Federman, 1968).Thus, a key physiological feature that methylotrophic microorganisms have, is a set of particular inner structures, which are called peroxisomes. It can be defined as intracellular compartments that have certain special enzymes, such as catalases or oxidases that help in the detoxification processes within the cell (Titorenko & Rachubinski, 2004). This complex arrangement allows to carry out reactions with a specialized target without affecting the other cellular processes (Gabaldón & Carreté, 2015). It has been noted that during the exponential phase of growing methylotrophic yeasts fed with glucose, peroxisomes present within the cell, are generally very difficult to detect, as well as their physiological function (Veenhuis *et al.*, 1983). Thus, it is known that there is a considerable increase in the number and size of some cellular compartments, such as mitochondrial structures, as well as lipid droplets, under a glucose fed regime in *P. pastoris*. This result is contrary to the methanol feeding case in which mitochondrial structures are hardly visible regarding peroxisomal structures that suffer a dramatic expansion (RuBmayer *et al.*, 2015). It has been proven that peroxisomal structures can adopt different shapes, ranging from rectangular shapes to complex crystalline structures that may fulfill around 80 % of the total intracellular volume depending on the environmental conditions of the media and the kind of substrate with which the cell is fed (Veenhuis *et al.*, 1983). Thus, it is crucial to understand how different substrates enter into the cell, as well as their further transformation. Let us analyze the most important substrate for the methylotrophic yeast: methanol. Singh & Narang (2019) have assumed that methanol enters into the cell by diffusion rather than by active transport. This assumption, often made implicitly, is probably motivated by the observation that in *Saccharomyces cerevisiae*, in which ethanol enters into the cell by diffusion, which suggests that methanol, a similar and even smaller compound, also enters into *P. pastoris* cells by diffusion (Loureiro & Ferreira, 1983).

According to different authors (Cereghino & Cregg, 2000; Gao *et al.*, 2016; Krainer *et al.*, 2012; Lin *et al.*, 2000) the use of methanol as a sole carbon source by this yeast has some drawbacks during its development process. Methanol is potentially hazardous due to its toxicity and flammability capacity, which could be an issue under fermentation process conditions. Also, it is known that these cells take oxygen at a high rate so that the expression of foreign genes can be negatively affected by oxygen limitation. Finally, a considerable amount of heat is produced due to the large amounts of oxygen that is consumed through cellular growth, which causes extra difficulties in the stability of the cellular system.

About the enzymology of methanol oxidation in yeasts, it is fundamentally different from those found in bacteria (Veenhuis *et al.*, 1983).It appears most likely that *P. pastoris* and other methylotrophic yeasts, have evolved a specialized set of enzymes for sugar phosphate rearrangements that shows high transcript levels. These enzymes are located in peroxisome and are specifically induced by growth in methanol (RuBmayer *et al.*, 2015). The initial methanol assimilation step is carried out within the peroxisome where methanol is transformed into formaldehyde as a result of the alcohol oxidase (EC 1.1.3.13) enzyme action, by using oxygen as electron acceptor and, by generating hydrogen peroxide as a product (Yurimoto *et al.*, 2011; Van der Klei *et al.*, 2006).

As it is indicated in Figure 1, the formed formaldehyde is a central intermediate situated at the branch point between the assimilation and dissimilation pathways (Yurimoto *et al.*, 2005).Formaldehyde must be rapidly assimilated into biomass or dissimilated to CO_2 due to its toxicity. A portion of formaldehyde is fixed to xylulose 5-phosphate (Xu5P) by dihydroxyacetone synthase (DAS) (EC 2.2.1.3), by forming dihydroxyacetone (DHA) and glyceraldehyde 3 phosphate (GAP), which are used for the synthesis of cell constituents and the generation of Xu5P through Xylulose Monophosphate (XuMP) pathway (Yurimoto *et al.*, 2011; Zhang *et al.*, 2017).Another portion of formaldehyde is further oxidized to CO_2 by the cytosolic dissimilation pathway. At this point, it would be useful to recognize the crucial role that peroxisome has in methanol assimilation, by giving methylotrophic yeasts their main feature. However, peroxisomes not only allow alcohol degradation, but they can also assimilate nitrogen sources, such as tertiary amines like methylamine into formaldehyde through other peroxisomal enzymes called amine oxidases in order to be assimilated by the cell as it was explained earlier (Shiraishi *et al.*, 2015; Siverio, 2002; Zwart *et al.*, 1983).

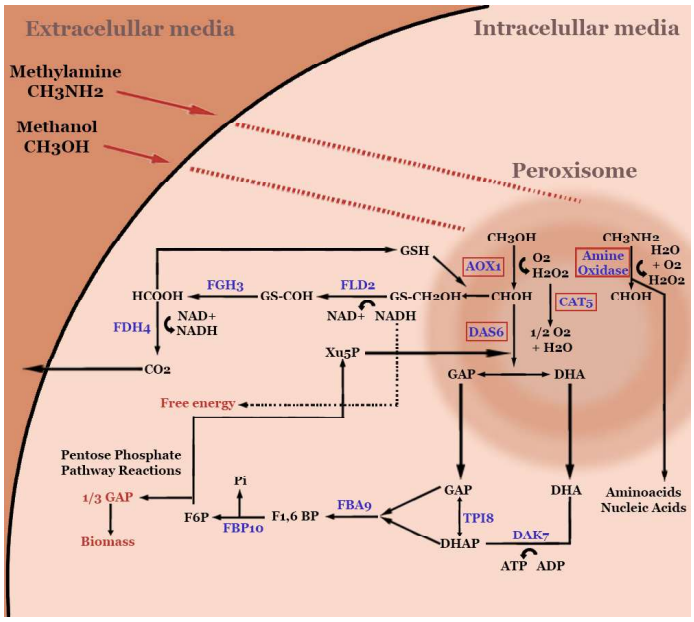

Figure 1: *Pichia pastoris* assimilation alcohol and amine pathway. Source: Own elaboration

2.2. Promoters

There are two main promoters to produce recombinant proteins: AOX1, which is induced by methanol and GAP, which is the promoter for the glyceraldehyde-3-phosphate dehydrogenase.

AOX1

The AOX1 system regulates the production of Alcohol Oxidase along with AOX2, but it is known that AOX1 is responsible for most of the enzyme activity in the cell. The promoters are so active when wild-type yeast grown in methanol as sole carbon source and energy, the enzyme can reach approximately 30 % of the total soluble cell proteins and 5 % of the mRNA. That is because, surprisingly, the enzyme has a low affinity for its substrates, methanol, and oxygen. Although AOX2 is less common and has a lower expression level than AOX1. There are studies in which truncated versions of this system are used for the production of recombinant proteins (Mochizuki *et al.*, 2001; Kuwae *et al.*, 2005; Potvin *et al.*, 2012; Vogl & Glieder, 2013).
AXO2 is activated under higher methanol concentration than AOX1 (Mayson *et al.*, 2003). The regulation of AOX1 is at transcriptional level and the promoter is repressed by glucose, glycerol, and ethanol. It is necessary to eliminate repressor carbon sources of the broth before starting the induction process with methanol, so cultures must be carried out in two stages, one of growth and the other one of induction.

The preference of AOX for small alcohols is explained by the presence of conserved bulky aromatic residues nearby the active site in which FAD cofactor is present near the active site (Vonck *et al.*, 2016).As mentioned above, methanol metabolism has undesirable consequences. To overcome the issues, mutants have been

developed: the mutant S, denoted by MutS (AOX1-) and the mutant, denoted by Mut- (AOX1- AOX2) which cannot use methanol as substrate. In general terms, the advantage of these modifications is less methanol consumption (Singh & Narang, 2019).

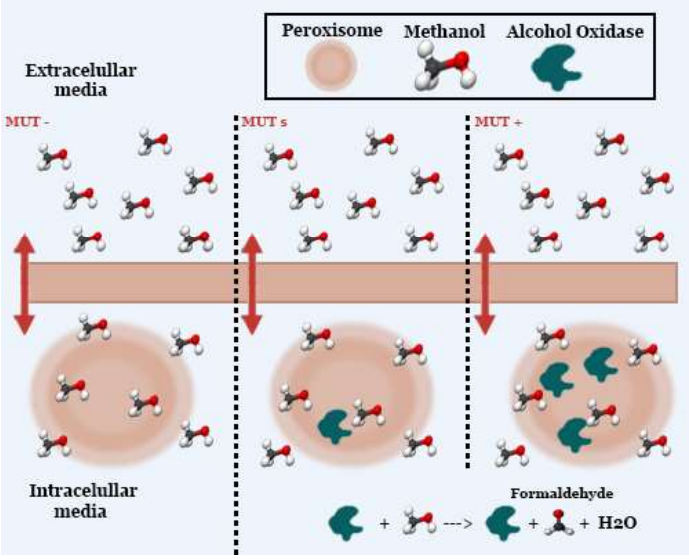

Figure 2: Methanol assimilation mechanism in *P. pastoris*. Source: adapted from Singh et al. (2019)

The previous figure clearly shows that, under the same methanol concentration condition, each mutant has a different behavior: the Mut- is not able to assimilate methanol due to alcohol oxidases absence so that methanol diffuses freely without decreasing its concentration. On the other hand, the MutS has certain methanol consumption rate thanks to the presence of some alcohol oxidase enzymes, but it is not so much. Certainly, its assimilation is slow. That is why this mutant has the suffix S. Finally, the mutant Mut+ presents the higher methanol consumption because of a considerable presence of alcohol oxidase enzymes, which allow better methanol assimilation (Lin-Cereghino *et al.*, 2006; Singh & Narang, 2019).

GAP

GAP promoter is active in a constitutively way because it regulates the production of an important enzyme involved in the glycolysis and gluconeogenesis. It was first isolated in 1993 (Waterham *et al.*, 1997).This is an advantage with respect to AOX1 because it is possible to produce foreign proteins as the cell grows. Moreover, some products are affected by what is produced in the methanol metabolism due to the increase of the oxidative stress in the broth and, of course, the danger associated with working with a flammable and toxic substance as methanol, which requires a special care (Waterham *et al.*, 1997; Shen *et al.*, 2016; Yang & Zhang, 2018). GAP promoter is optimal for continuous production processes because it allows to avoid the shift in the growth and induction phases. As Potvin *et al.* (2012) mentioned, there are some studies in which the efficiency of the AOX1 promoter is greater than GAP, but other studies claim just the opposite.

Yang & Zhang (2018) reported that the expression of an alkaline phytase, by using the GAP system, was eight-fold higher than that under the AOX1-based system. More studies are necessary to define under which culture conditions and products, the use of each one, is more suitable.

Alternative promoters

Despite GAP and AOX promoters are more common, there is an interest in developing alternative promoters, especially to replace the *AOX1* production processes because large volumes of methanol are required when bioprocess are scaled-up, besides the disadvantages mentioned above. Menéndez *et al.* (2003) reported the isolation and sequencing of the *P. pastoris* isocitrate lyase gene *(ICL1)*. They tested this promoter system for the expression of dextranase from *Penicillium minioluteum* under the control of *pICL1* in *P. pastoris.* The *ICL1* system is based on the gene used to the Isocitrate lyase (EC 4.1.3.1) production, fundamental for ethanol metabolism in order to produce Acetil-Coa, which was used before in order to generate glucose metabolic intermediates (Dunn *et al.*, 2009). In 1998 Shen *et al.* (1998) isolated and characterized the gene coding for the glutathione-dependent formaldehyde dehydrogenase *(FDL1),* (EC 1.2.1.1) of *P. pastoris,* which was an important enzyme involved in the metabolism for methanol, as an alternative for the *AOX1* expression system. They showed that *FLD1* can produce heterologous proteins at the same level as *AOX1* system, but with the difference that *FLD1* can be induced both of them for methanol as carbon source and methylamine as nitrogen source. *FDL1* expression systems needs lower oxygen supply, too (Resina *et al.*, 2005). A comparison of yields between *GAP* and *AOX* promoters is presented below.

Table 1: Expression of codon-optimized genes in *P. pastoris*, adapted from Yang *et al.*, 2018.

Gene name	Gene product	Promoter (p)	Highest protein yield	Reference
*hIFN*γ	Human interferon gamma	*pAOX1*	0.016 mg/L	(Razaghi *et al.*, 2017)
Cap	Porcine circovirus type 2 capsid protein	*pAOX1*	174 mg/L	(Tu *et al.*, 2013)
RABV-G	Rabies virus glycoprotein	pGAP	0,15 mg/L	(Ben *et al.*, 2016)
GCSF	Human granulocyte colony-stimulating factor	pGAP or pAOX1	0,66 mg/g DCW	(Maity *et al.*, 2015)
Man26A	*Aspergillus niger* mannosidase	pGAP	5069 U/mL	(Zhao *et al.*, 2011)
Pgp	P-glycoprotein	pAOX1	N.A	(Bai *et al.*, 2011)

*N.A.: Not available.

3. CULTIVATION TECHNIQUES

Due to *P. pastoris* has gained attention for the biotechnological production of different chemical compounds, companies such as Invitrogen have developed expression kits with defined culture conditions. Other conditions available in the literature are quite similar for those presented by Invitrogen but new trends go towards the development of specific culture conditions for every product and genetic construct (Looser *et al.*, 2014).

A table with culture conditions of different products is shown below (Table 2).

Table 2: Culture conditions comparison in various production processes with *P. pastoris.*

Product/ culture technique	pH	Temp. (°C)	Bioreactor volume (L)	Agitation (rpm)	Reference
β-1,3-glucanosyl-transglycosylase / FB	5,5	30	5	800	(Resina *et al.*, 2009)
Rhyzopus oryzae lipase / CC	5,5	30	1,5	700	(Berrios *et al.*, 2017)
Interferon α-2b / FB	5,4	30	2,7	N.A.	(Katla *et al.*, 2019)
N-acetylgalactosamine-6-sulfate sulfatase human lysosomal / FB	5	28	3,7	N.A.	(Rodríguez-López *et al.*, 2019)
recombinant iduronate 2-sulfatase / FB	5	28	3,7	N.A.	(Pimentel *et al.*, 2018)

*N.A.: Not available.
In the culture technique, FB: Fed batch, and CC: Continuous culture.

P. pastoris grows well in different carbon sources as glucose, glycerol, fructose, sorbitol, ethyl amine and mannitol, among others. The doubling time depends on the carbon source, and it is typically around 90 minutes in glucose, and 6 hours in methanol. The normal growth temperature is 30°C and 250 rpm when growing in liquid media. For plate cultures, it is necessary to consider the evaporation of substrate if it is methanol (Sreekrishna & Kropp, 1996).

3.1. Fed-Batch culture

Fed-Batch culture is maybe the most common production system for high-density cultures of microorganisms, such as *P. pastoris.* The process starts with culture in a carbon source efficient for the accumulation of biomass, typically glucose or glycerol. The depletion of carbon source is measured, and the flux of inducer is started to activate the promoter chosen (Yang & Zhang, 2018). The recommended process is made up of 3 phases: 1) Glycerol or glucose batch phase, 2) or glucose fed-batch phase, 3) Induction phase, typically with methanol. The objective of the two initial phases is to increase biomass concentration in order to reach high-cell densities. It is important to know that reaching high yields in dense cultures is complex because the feeding of methanol processes have to be controlled in order to avoid toxicity levels for cells and to maintain an adequate flow for its consumption as an inducer and carbon source (Liu *et al.*, 2019).
Mayson *et al.* (2003) used glucose as substrate for both batch and first fed-batch phases in a Mut+ system of *P. pastoris* for production of transferrin in a 3L bioreactor. When induction started, the culture density was 200 g/L of wet cell weight. Glucose flow did not stop during methanol induction. 450 mg of transferrin per liter after 72 h of elapsed fermentation time was obtained as a maximum. Rodrigues *et al.* (2019) used the *AOX1* Muts system for production of L-asparaginase from *S. cerevisiae* in a 3L bioreactor under substrate

limit conditions. The batch step was performed with glycerol followed by a starvation period of 1-2 hours, as well as the subsequent fed-batch step with methanol at different feeding regimes. The overall enzyme volumetric productivity obtained was 31 U/L h.

Fed-batch cultures can be performed with the *GAP* or other alternative promoter systems, too, for recombinant protein production. As *GAP* promoter is expressed during cell growth, it is desirable to maintain an exponential growth of biomass in order to keep the product generation in the same way. It is necessary to obtain the optimum specific growth rate (μ) in order to keep it constant with the adequate feeding flow rate (Yang & Zhang, 2018). Garcia-Ortega *et al.* (2013) used a *GAP* system in *P. pastoris* in order to produce a human antigen-binding fragment (Fab) in a 5L bioreactor operated in fed-batch with an initial media volume of 2L and 4L approximately at the end of the feeding. The process consisted of two phases, a batch process followed by a feeding step, both used alternatively glucose or glycerol in different fermentations and the feeding regime was programmed in order to keep a specific value of μ. The overall product substrate yield was 0.081 and 0.073 for glucose and glycerol, respectively. Resina *et al.* (2009) used the *FLD1* promoter system in *P. pastoris* to produce a *Rhizopus oryzae* lipase-encoding gene in a 5L bioreactor operated in fed-batch. The culture was carried out in three steps: a batch growth phase, by using glycerol as carbon source and ammonium sulfate as nitrogen source, a sorbitol-batch phase when glycerol was depleted, and the addition of methylamine as nitrogen source and a fed-batch induction phase with exponential feed of sorbitol and methylamine, as well. Different mutant strains were evaluated, by yielding productivity levels from 602 to 3274 AU/L h (lipolytic activity units per liter per hour).

Anane *et al.* (2016) used an oxygen tension strategy in order to program the feeding rate in 10.5 L bioreactor with 8L of working volume to compare the *AOX1* and *GAP* promoter systems in the production of b-fructofuranosidase (FFase) enzyme. In *GAP* fermentation system, glycerol was used for both batch and fed-batch steps while in *AOX1* fermentation system glycerol was used for batch phase and methanol for fed-batch phase. They found that the *AOX1* system was better for the FFase production compared with *GAP,* but it required an important external cofactor supply not needed under the *GAP* promoter system.

3.2. Continuous culture

Continuous process has the advantage of further controlling fermentation parameters, especially the specific growth rate if the process is carried out under chemostat conditions (Harmand *et al.*, 2017). It can be considered that reaching low-growth rates and high-substrate consumption can improve product yields, but decreasing feed rate can increase the possibility of cell wash if the value of μ decreases nearly to zero. Continuous culture then, is less common in *P. pastoris* due to the limited range of dilutions rates available to get a true continuous feed to the bioreactor (Rebnegger *et al.*, 2016). The retentostat system, described for the first time by Herbert *et al.* (1956) is an interesting alternative in which biomass is retained inside the bioreactor while liquid media flows through. This kind of culture, theoretically speaking, allows to main-

tain low-growth rates, but it avoids the cell damage caused by substrate depletion in long-term cultures. Rebnegger *et al.* (2016) also demonstrated a viability of 97% in retentostat cultures of *P. pastoris* when very low-growth rates ($\mu < 0,001h^{-1}$) were reached. They also demonstrated a decrease in the value of cell maintenance and a transcriptional re-programming.

Rahimi *et al.* (2019) carried out a comparison between fed-batch and continuous culture strategies in MUT+ *P. pastoris* in order to produce recombinant hepatitis B surface antigen in a 10 L bioreactor. Initially, two-stage batch cultures were carried out, the first one with glycerol and the second one with methanol. This was done to make a transition before feeding. The third step was a fed-batch process with methanol, and finally, the system was switched to a continuous process, by maintaining the same volume (5L). They observed a specific and volumetric productivity of 0,00468 mg HBsAg/g cell/h and 1,699 mg HBsAg/L/h, respectively, for chemostat fermentation and 0,00375 mg HBsAg/g cell/h and 1,13 mg HBsAg/L/h, respectively, for fed batch, by considering harvesting and next, run initiating time. HBsAg refers to Hepatitis B surface Antigen.

4. PROTEIN SECRETION

A key aspect that makes *P. pastoris* a very interesting biological platform for the production of recombinant proteins, is the possibility of introducing a group of specialized signals called secretion signals, which allow the product to be recovered from the broth media much more easily so that higher titers can be achieved due to the lower secretion levels of endogenous proteins (Chahal *et al.*, 2017). Secretion signaling can be made either by native signal peptides as that of fungal xylanase or the commercial α-mating factor (MF), which is very used in *P. pastoris* systems (Yang & Zhang, 2018).

The first step of the classical secretory pathway is the translocation of the polypeptide in the endoplasmic reticulum (ER) to make post-translational modifications (Zahrl *et al.*, 2018). This is an important step because the overload of proteins translocating to the ER can cause an intracellular accumulation, and often, also, degradation, which results in yield decrease (Garcia-Ortega *et al.*, 2016) even when cells as *P. pastoris* as other eukaryotes have the unfolded protein response (UPR) in the ER. It is a system, which detects disturbances in the ER normal functionality and augment the secretion capability with the upregulation of more than 300 genes involving protein folding, glycosylation, translocation, trafficking, and degradation (Taylor, 2016; Bankefa *et al.*, 2018). Despite that the composition of amino acids and length of signal peptides among the secreted proteins in same species are highly diverse, and it would be hard to predict whether a secretion sequence will be able to translocate a protein to secretion pathway (Karaoglan *et al.*, 2014). There are few sequences, which have been characterized and applied for secretion of proteins in addition to the *Saccharomyces cerevisiae* α-mating factor pre-propeptide (MATα) (See Table 3). The *S. cerevisiae SUC2* gene signal sequence, the bovine β-casein, and the native acid phosphatase (PHO1), (EC 3.1.3.2) signal peptide was occasionally used only (Kang *et al.*, 2016). This last secretion signal sequence

shows significant homology to repressible PHO from other yeast species, such as *Saccharomyces cerevisiae.*

Payne *et al.* (1995) demonstrated that PHO1 is translocated into the ER, glycosylated, and transported to the cell surface in about 5 minutes. Regarding AOX1 promoter, the PHO1 promoter has the advantage that it can be induced about 100-fold under conditions that do not significantly alter cell growth or morphology, so that the regulated PHO1 promoter will be a useful alternative tool for heterologous gene expression in *P. pastoris.* (Payne *et al.*, 1995). Some proteins have been produced, by using this secretion system. For example, Ben *et al.* (2016) showed that in the production of rabies virus, glycoprotein (RABV-G) in *P. pastoris,* by using PHO1 and the α-mating factor despite that the expression level of secreted RABV-G was similar, the PHO1 signal presented higher protein degradation rates in comparison to α-mating factor (Ben *et al.*, 2016). A similar result was obtained by (Tanaka *et al.*, 2004), who demonstrated that α-mating factor signal peptide resulted in two-fold higher xylanase yield than PHO1 signal sequence into growth media. Therefore, it is evident the necessity to explore the α-mating factor further because of its capacity to export different proteins as it has been previously shown.

The MATα signal leader contains a pre-region with 19 amino acids and a pro-region with 67. This is the most widely used secretion signal used in *P. pastoris,* being, even in some cases, better than the leader sequence of the native signal peptide. However, variability in the N-terminal amino acids are reported in heterologous protein secreted with this signaling system and, in some cases, the protein is retained in the ER or Golgi apparatus, by impeding transport, and triggering degradation (Macauley-Patrick *et al.*, 2005; Chahal *et al.*, 2017). Modifications have been proposed in order to enhance the secretory efficiency of the MATα, by using different approaches as codon optimization, directed evolution, in addition to spacer sequences, and site-directed mutagenesis (Duan *et al.*, 2019).

Table 3: Signal peptides used for secretion of enzymes in P.pastoris, adapted from Kang *et al.*, 2016

Signal Peptide	AA sequence	Recombinant Protein
α-Factor	MRFPSIFTAVLFAASSALAAPVNTTTEDE TAQIPAEAVIGYSDLEGDFDVAVLPFSNST NNGLLFINTTIASIAAKEEGVSLEKREAEA	Streptomyces trypsin [47,4U mL^{-1} (amidase activity)]
		Lipase (6100 $U\ mL^{-1}$)
PHO1	MFSPILSLEIILALATLQSVFA	Glucoamylase ($0,4\ g\ L^{-1}$)
		Mannosyltransferase ($0,4\ g\ L^{-1}$)
SUC2	MLLQAFLFLLAGFAAKISA	Amylase ($2,5\ g\ L^{-1}$)
		Antithrombin ($324\ mg\ L^{-1}$)
Bovine β-casein	MKVLILACLVALALA	Xylanase
E-CALB	MNLYLITLLFASLCSAEFLPSGSDPAFSQP KSVLD	Lipase ($30\ U\ ml^{-1}$)
P23	MKILSALLLLFTLAFA	Human growth hormone ($19\ mg\ ml^{-1}$)

5. RECOMBINANT PROTEINS IN COLOMBIA

According to Zapata *et al.* (2012) the Colombia's health system faces some issues that the government should solve first in the near future. Within the main topics, it was included the price of some drugs, which are included in the Mandatory Health Plan (POS for its acronym in Spanish *"Plan Obligatorio de Salud"*). Among the highest prices registered in it, there were drugs developed by biotechnological processes, which represents a 12% of the global drug sales, according to a market study published in Colombiacompra.gov.co (2019). The same market study shows that, the National Cancerology Institute in Colombia registered a capital expenditure in the 2016-2017 period up to 5,7-dollar millions for the purchase of the 10 most expensive drugs. It is estimated that, in 2013, approximately, 36% of the total pharmaceutical market, in Colombia, corresponded to drugs of biological origin, which included some for cancer treatment, such as breast cancer and other expensive diseases. As for biosimilars, it is very important to highlight that the National Institute of Cancerology of Colombia is currently conducting all the conventional tests for radiopharmaceuticals, by using nuclear medicine and Positron Emission Tomography (PET), (Vaca-González *et al.*, 2019).

For the reasons mentioned above, not only has the government of Colombia, but also all governments around the world, begun the search for affordable treatments, which allows a more integral health coverage (Blanco-García *et al.*, 2018). A very attractive solution is the use of biosimilar drugs, discussed broadly by (Uhlig & Goll, 2017; Kaida-Yip *et al.*, 2018; Nabhan *et al.*, 2018). However, still some concerns remain in the sense that these alternatives should be overcome. The topics may vary from technical issues like adverse effects (Odinet *et al.*, 2018; Colloca *et al.*, 2019) to legal process related to the specific legislation, which varies from country to country (Tesar *et al.*, 2019; Joung, 2015), as well as patents and intellectual property (Moorkens *et al.*, 2016).

Broadly speaking, a biosimilar drug should have a high degree of similarity with regard to the reference medicinal product in terms of quality features, biological activity, safety, and efficacy, based on a comprehensive comparability exercise, which needs to be established (European Medicines Agency, 2014). These requirements should be regulated by specialized institutions for drug control and distribution, such as the Food and Drugs Administration (FDA) for the United States, the European Medicines Agency for Europe, in Colombia, one remarkable institution with regard to drug control is the National Food and Drug Surveillance Institute (INVIMA, which has been recognized as a national drug regulatory authority of Reference for Drugs and Biological components (Ojeda *et al.*, 2016). This latter entity plays an important role because it is in charge of allowing or denying the circulation of all bioproducts developed or distributed throughout the Colombian territory. According to the resolution 1782 as of 2014, Article 6, published by INVIMA, there are 3 routes in which a drug can be approved, in Colombia, for new or pioneer biological drugs. The so-called full dossier route was established, which requires the submission of pre-clinical studies (*in-vivo* and/or *in-vitro*) and clinical trials. In addition to the fact that they must comply with the common requirements for their relevant authorizations (Bernal-Camargo *et al.*, 2018).

In the biotechnological drugs case, there are some expensive ones, such as transcription factors and monoclonal antibodies, which have high usage rates (Manrique-López & Jiménez-Barbosa, 2012). It has been seen that these molecules are extremely complex, and their biological activity is because of a spatial complex arrangement, which allows to carry out a reaction with a specialized target (Putz *et al.*, 2016). These molecules can be synthesized by biological platforms as is shown in Meza-Gutierrez *et al.* (2019). This will allow that recombinant proteins, which are produced throughout microorganisms can be used for biosimilar drug production (Baeshen *et al.*, 2014) as is the case of the recombinant insulin (Heinemann & Hompesch, 2014), which is likely to become widely available in the coming years, and are expected to have a major impact on diabetes care (American Diabetes Association, 2019) . However, the economic assessment of the impact that these drugs could have on the market remains unclear due to the fact that there are considerable limitations in terms of the range of included costs and reliance on assumptions carried out in the economic analysis instead of robust data for feeding the models (Simoens *et al.*, 2017).

Likewise, it has been demonstrated that *P. pastoris* can be used into the recombinant insulin production, by gaining a save in time and economic terms, by yielding an overall output from insulin precursor to human insulin of 51 % (Polez *et al.*, 2016). Rodríguez-López *et al.* (2019) and Rodríguez-López *et al.* (2019) conducted two quite similar studies in which N-acetylgalactosamine-6-sulfate sulfatase (GALNS), an enzyme used to treat the Morquio A syndrome, was produced in a 3,7L bioreactor. Also, Espejo-Mojica *et al.* (2016) showed that the enzyme can be internalized and reach the lysosome in mammalian cells while Rodríguez-López *et al.* (2019) achieved an important improvement in the enzyme activity after removing a native signal peptide and also observed that human skin fibroblasts took it up in a dose-dependent manner through a process potentially mediated by an endocytic pathway without any additional protein or host modification. Finally, Iduronate-2-sulfate sulfatases, a group of enzymes linked to the Hunter syndrome, have been expressed in a 100mL of media culture and its activity was reported as 4,213 nmol/mg h of total protein-1 at 72 h (Landázuri *et al.*, 2009). The same kind of enzyme was cultured in 3L bioreactor by Poutou-Piñales *et al.* (2005), by yielding activities between 25,4 y 29,36 nmol/mg h and by Córdoba-Ruiz *et al.* (2009) who obtained an activity between 7,3 and 29,5 nmol/mg h. Pimentel *et al.* (2018) studied this enzyme, too, in a 3,7L bioreactor, by yielding 12,45 nmol/mg h, showing a dose-dependent cell uptake.

Regarding enzymes with environmental applications, laccases, originally, produced from different organisms, have also been studied and produced, by using *P. pastoris* at different volume scales. Some authors focus on the improvement of culture conditions (Ardila-Leal *et al.*, 2019; Gouzy-Olmos *et al.*, 2018; Morales-Álvarez *et al.*, 2017); while others research different applications, such as the detoxification of pulping black liquor, and still some others, in water treatments (Rivera-Hoyos *et al.*, 2018). Other lysosomal products as β-hexosaminidases are important for Tay Sachs or Sandhoff disease treatment, and they have been produced and characterized in *P. pastoris,* by yielding important results that demonstrated the potential of this expression system (Espejo-Mojica *et al.*, 2020). Table 4 summarizes some of the Colombian research pa-

pers related to *P. pastoris* applied to recombinant proteins, most of them mainly conducted by *"Pontificia Universidad Javeriana".*

Table 4: Colombian research papers developed with *P. pastoris* as expression system

Product/ culture technique	Culture technique	Bioreactor volume (L)	Reference
N-acetylgalactosamine-6-sulfate sulfatase (GALNS)	B followed by FB	3,7	(Rodríguez-López *et al.*, 2019)
			(Rodríguez-López *et al.*, 2019)
lysosomal beta-hexosaminidases	B followed by two FB steps	3,7	(Espejo-Mojica *et al.*, 2016)
Recombinant laccase	Erlenmeyer flask	0,5	(Morales-Álvarez *et al.*, 2017)
Human Iduronate-2-sulfate sulfatase	Erlenmeyer flask	0,1	(Landázuri *et al.*, 2009)
Human Iduronate-2-sulfate sulfatase	B followed by FB	3	(Poutou-Piñales *et al.*, 2005)
rPOXA 1B laccase	Erlenmeyer flask with immobilized cells in alginate calcium beads	0,5	(Gouzy-Olmos *et al.*, 2018)
recombinant iduronate-2-sulfatase	B followed by two FB steps	3,7	(Pimentel *et al.*, 2018)
rPOXA 1B laccase	B	10	(Ardila-Leal *et al.*, 2019)

In the culture technique, B stands for batch, and FB, for fed batch

Therefore, the national panorama shows that there are few research groups actively working with *P. pastoris* and many of them are oriented towards enzyme production of whose altered function generates diseases. In Colombia, there are still many approaches and developments that can be made in order to establish solid studies on this important system of expression, which, as has been mentioned, presents great advantages, which could direct research to the industrial sector that has been little addressed in the country. It is important to emphasize that a great part of the research is concentrated on *"Pontificia Universidad Javeriana"* because it has a long-standing tradition, and several publications related to *P. pastoris* as a system of expression. At this moment, the country is putting efforts to produce biosimilar drugs inside the country to grapple with the high cost of imported health treatments. There are few papers available oriented towards the production of biosimilars in Colombia, but some of them are starting to medium and large-scale production, in which mammalian cell cultures are being used (Díaz *et al.*, 2020).

6. CONCLUSIONS

This paper reviewed the features, which make *P. pastoris* a suitable model for the development of recombinant proteins. From the advantages, which were presented by this kind of system, it was possible to see how the well-studied promoter system can be used with fed-batch (maybe the best option) or continuous culture in order to produce different heterologous proteins, by taking advantage of the secretion feature, which can contribute to easily recover proteins after culture. AOX promoters are the most used, as well as the most

studied since they represent a very good option for the development of expression systems.

Both globally and in Latin America, developments can still be considered few. Additional research is still needed in order to support the development of new products and boost national industries. To achieve this, it will be necessary to establish legislation to stimulate researchers and national companies to achieve this goal, and to stop high-cost products, which are imported in many cases, at inflated prices. Despite that, there are researchers, in Colombia, who focus their efforts on the development of interesting processes, which could be the starting point to bring solutions to the recombinant protein and enzyme industry, in the country, in the future to come.

References

American Diabetes Association (2020). 1. Improving Care and Promoting Health in Populations: Standards of Medical Care in Diabetes-2020. Diabetes care, 43(Suppl 1), S7-S13. https://doi.org/10.2337/dc20-S001

Anane, E., van Rensburg, E. & Görgens, J. F. (2016). Comparison of constitutive and inducible β-fructofuranosidase production by recombinant *Pichia pastoris* in fed-batch culture using defined and semi-defined media, *South African Journal of Chemical Engineering*, 22, 17-22. doi: 10.1016/j.sajce.2016.10.001.

Ardila-Leal, L. D., Albarracín-Pardo, D. A., Rivera-Hoyos, C. M., Morales-Álvarez, E. D., Poutou Piñales, R. A., Cardozo-Bernal, A. M., Quevedo-Hidalgo, B. E., Pedroza-Rodríguez, A. M., Díaz-Rincón, D. J., Rodríguez-López, A., Alméciga-Díaz, C. J. & Cuervo-Patiño, C. L. (2019). Media improvement for 10 L bioreactor production of rPOXA 1B laccase by *P. pastoris*. *3 Biotech*, 9(**12**), 1 -16. doi: 10.1007/s13205-019-1979-y

Avers, C. & Federman, M. (1968). The occurrence in yeast of cytoplasmic granules which resemble microbodies. *The Journal of Cell Biology*, 37(**2**), 555–559. doi: 10.1083/jcb.37.2.555

Aw, R. & Polizzi, K. M. (2013). Can too many copies spoil the broth?, *Microbial Cell Factories*, 12(**1**), 1 -9. doi: 10.1186/1475-2859-12-128.

Baghban, R., Tehran, T. E. W. & Way, T. E. (2016). Camelid-derived heavy-chain nanobody against *Clostridium botulinum* neurotoxin E in *Pichia pastoris*, *Biotechnology and Applied Biochemistry*, 98(**21**), 1–16. doi: 10.1002/bab.1226.

Bai, J., Swartz, D. J., Protasevich, I. I., Brouillette, C. G., Harrell, P. M., Hildebrandt, E., Gasser, B., Mattanovich, D., Ward, A., Chang, G., & Urbatsch, I. L. (2011). A gene optimization strategy that enhances production of fully functional P-Glycoprotein in *Pichia pastoris*. *PLoS ONE*, 6(**8**). doi:10.1371/journal.pone.0022577

Bankefa, O. E. et al. (2018). Hac1p homologues from higher eukaryotes can improve the secretion of heterologous proteins in the yeast *Pichia pastoris*, *Biotechnology Letters. Springer Netherlands*, 40(**7**), 1149-1156. doi: 10.1007/s10529-018-2571-y.

Baeshen, N., Baeshen, M., Sheikh, A., Bora, R., Ahmed, M., Ramadan, H., Saini, K. & Redwan, E. (2014). Cell factories for insulin production. *Microbial Cell Factories*, 13(**1**). doi:10.1186/s12934-014-0141-0

Ben Azoun, S., Belhaj, A. E., Göngrich, R., Gasser, B. & Kallel, H. (2016). Molecular optimization of rabies virus glycoprotein expression in *Pichia pastoris*. *Microbial Biotechnology*, 9(**3**), 355-368. doi:10.1111/1751-7915.12350

Bernal-Camargo, D., Gaitán-Bohórquez, J. & León-Robayo, É. (2018). Medicamentos biosimilares en Colombia: una revisión desde el consumo informado. *Revista Ciencias de La Salud*, 16(**2**), 311. doi:10.12804/revistas.urosario.edu.co/revsalud/a.6772

Berrios, J., Flores, M. O., Díaz-Barrera, A., Altamirano, C., Martínez, I. & Cabrera, Z. (2017). A comparative study of glycerol and sorbitol as co-substrates in methanol-induced cultures of *Pichia pastoris*: temperature effect and scale-up simulation. *Journal of Industrial Microbiology and Biotechnology*, 44(**3**), 407-411. doi:1007/s10295-016-1895-7

Blanco-García, E., Ledón-Naranjo, N. & Lage-Dávila, A. (2018). Rising Cancer Drug Prices: What Can Low-and Middle-income Countries Do?. Scielosp.org. Retrieved 29 April 2020, from https://scielosp.org/article/medicc/2018.v20n4/35-39/.

Cereghino, J. L. & Cregg, J. M. (2000). Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*, *FEMS Microbiology Reviews*, 24(**1**), 45 - 66. doi: 10.1111/j.1574-6976.2000.tb00532.x.

Chahal, S. et al. (2017). Structural characterization of the α-mating factor prepro-peptide for secretion of recombinant proteins in *Pichia pastoris*, *Gene*. Elsevier B.V., 598, 50 - 62. doi: 10.1016/j.gene.2016.10.040.

Colloca, L., Panaccione, R.& Murphy, T. (2019). The Clinical Implications of Nocebo Effects for Biosimilar Therapy. *Frontiers In Pharmacology*, 10. doi:10.3389/fphar.2019.01372

Colombiacompra.gov.co.(2019). Estudio de Mercado. Compra pública pre-comercial para la innovación. Convocatoria de ideas para la producción de bajo volumen de medicamentos biotecnológicos. Retrieved 29 April 2020, from https://www.colombiacompra.gov.co/sites/cce_public/files/cce_documentos/estudio_de_mercado.pdf.

Córdoba-Ruiz, H. A., et al. (2009). Laboratory scale production of the human recombinant iduronate 2-sulfate sulfatase-Like from *Pichia pastoris*. *African Journal of Biotechnology*, 8.9. doi:10.5897/AJB2009.000-9255

Cregg, J. M. *et al.* (1985). *Pichia pastoris* as a host system for transformations, *Molecular and Cellular Biology*, 5(**12**), 3376 - 3385. doi: 10.1128/mcb.5.12.3376.

Cregg, J. M. *et al.* (2000). Recombinant protein expression in *Pichia pastoris*, *Applied Biochemistry and Biotechnology - Part B Molecular Biotechnology*, 16(**1**), 23 - 52. doi: 10.1385/MB:16:1:23.

De Schutter, K., Lin, Y., Tiels, P., Van Hecke, A., Glinka, S. & Weber-Lehmann, J. et al. (2009). Genome sequence of the recombinant protein production host *Pichia pastoris*. *Nature Biotechnology*, 27(**6**), 561 - 566. doi: 10.1038/nbt.1544

Demain, A. L. & Vaishnav, P. (2009). Production of recombinant proteins by microbes and higher organisms, *Biotechnology Advances. Elsevier Inc.*, 27(**3**), 297 - 306. doi: 10.1016/j.biotechadv.2009.01.008.

Díaz, C., Valero, K., Zapata Loaiza, S., Buitrago, J. & Orozco Sánchez, F. (2020). Diseño conceptual de una planta de producción de un biosimilar del medicamento contra el cáncer de mama trastuzumab a partir de células CHO. *Revista Colombiana de Cancerología*. 24 (**1**). https://www.revistacancercol.org/index.php/cancer/issue/view/50/RCCAN

Duan, G. *et al.* (2019). Screening endogenous signal peptides and protein folding factors to promote the secretory expression of heterologous proteins in *Pichia pastoris*, *Journal of Biotechnology. Elsevier*, 306(October 2018), 193 - 202. doi: 10.1016/j.jbiotec.2019.06.297.

Duan, X., Gao, J. & Zhou, Y. (2018). Advances in engineering methylotrophic yeast for biosynthesis of valuable chemicals from methanol. *Chinese Chemical Letters*, 29(**5**), 681 - 686. doi:10.1016/j.cclet.2017.11.015

Dunn, M. F., Ramírez-Trujillo, J. A. & Hernández-Lucas, I. (2009). Major roles of isocitrate lyase and malate synthase in bacterial and fungal pathogenesis, *Microbiology*, 155(**10**), 3166 - 3175. doi: 10.1099/mic.0.030858-0.

Espejo-Mojica, A. J., Mosquera, A., Rodríguez-López, A., Díaz, D., Beltrán, L., Hernandez, F. L., Alméciga-Díaz, C. J. & Barrera, L. A. (2016). Characterization of recombinant human lysosomal beta-hexosaminidases produced in the methylotrophic yeast *Pichia pastoris*. *Universitas Scientiarum*, 21(**3**), 195- 217. doi:10.11144/Javeriana.SC21-3.corh

Espejo-Mojica, Angela J., et al. (2020). Human recombinant lysosomal β Hexosaminidases produced in *Pichia pastoris* efficiently reduced lipid accumulation in Tay-Sachs fibroblasts. *American Journal of Medical Genetics Part C: Seminars in Medical Genetics*. Hoboken, USA: John Wiley & Sons, Inc. doi: 10.1002/ajmg.c.31849

European Medicines Agency (2014). Biosimilar Guideline. 44(December 18), 1 - 16. doi:10.1089/blr.2011.9970

Gabaldón, T. & Carreté, L. (2015). The birth of a deadly yeast: tracing the evolutionary emergence of virulence traits in Candida glabrata. *FEMS Yeast Research*, 16(**2**), p.fov110. doi: 10.1093/femsyr/fov110

Gao, M., Dong, S., Yu, R., Wu, J., Zheng, Z., Shi, Z. & Zhan, X. (2011). Improvement of ATP regeneration efficiency and operation stability in porcine interferon-α production by *Pichia pastoris* under lower induction temperature. *Korean Journal of Chemical Engineering*, 28(**6**), 1412 - 1419. doi:10.1007/s11814-010-0527-6

Garcia-Ortega, X. et al. (2013). Fed-batch operational strategies for recombinant Fab production with *Pichia pastoris* using the constitutive GAP promoter, *Biochemical Engineering Journal*, 79, 172 - 181. doi: 10.1016/j.bej.2013.07.013

Garcia-Ortega, X. et al. (2016). A step forward to improve recombinant protein production in *Pichia pastoris*: From specific growth rate effect on protein secretion to carbon-starving conditions as advanced strategy, *Process Biochemistry*, 51(**6**), 681 - 691. doi: 10.1016/j.procbio.2016.02.018.

Gouzy-Olmos, M., Cháves-Tequia, L. M., Rojas-Fajardo, M. F., Morales-Álvarez, E. D.,Rivera-Hoyos, C. M., Poutou-Piñales, R. A., González-Neira, E. M., Reyes-Montaño, E. A., Cardozo-Bernal, Á. M., Gómez-Méndez, L. D. & Pedroza-Rodríguez, A. M. (2018). Statistical improvement of batch culture with immobilized *Pichia pastoris* cells for rPOXA1B laccase production. *American Journal of Biochemistry and Biotechnology*, 14(**2**), 88 – 107. doi:10.3844/ajbbsp.2018.88.107

Harmand, J. et al. (2017). The Chemostat. First Edit, The Chemostat. First Edit. ISTE Ltd and John Wiley & Sons, Inc. doi: 10.1002/9781119437215.

Heinemann, L. & Hompesch, M. (2014). Biosimilar Insulins. *Journal of Diabetes Science and Technology*, 8(**1**), 6-13. doi: 10.1177/1932296813516958

Herbert, D., Elsworth, R. & Telling, R. C. (1956). The Continuous Culture of Bacteria; a Theoretical and Experimental Study, *Journal of General Microbiology*, 14(**3**), 601 - 622. doi: 10.1099/00221287-14-3-601.

Joung, J. (2015). Korean regulations for biosimilars. *Generics and Biosimilars Initiative Journal*, 4(**2**), 93 - 94. doi: 10.5639/gabij.2015.0402.019

Kaida-Yip, F., Deshpande, K., Saran, T. & Vyas, D. (2018). Biosimilars: Review of current applications, obstacles, and their future in medicine. *World Journal of Clinical Cases*, 6(**8**), 161 - 166. doi:10.12998/wjcc.v6.i8.161

Kallel, H. (2016). Heterologous expression of rabies virus glycoprotein in the methylotrophic yeast *Pichia pastoris*. *New Biotechnology*, 33, S57. doi:10.1016/j.nbt.2016.06.923

Kang, Z. et al. (2016). Recent advances of molecular toolbox construction expand *Pichia pastoris* in synthetic biology applications, *World Journal of Microbiology and Biotechnology*, 33(**1**), 1 - 8. doi: 10.1007/s11274-016-2185-2.

Karaoglan, M., Yildiz, H. & Inan, M. (2014). Screening of signal sequences for extracellular production of *Aspergillus niger* xylanase in *Pichia pastoris*. *Biochemical Engineering Journal*, 92, 16 - 21. doi:10.1016/j.bej.2014.07.005

Katla, S., Mohan, N., Pavan, S. S., Pal, U. & Sivaprakasam, S. (2019). Control of specific growth rate for the enhanced production of human interferon α2b in glycoengineered *Pichia pastoris*: process analytical technology guided approach. *Journal of Chemical Technology & Biotechnology*, 94(**10**), 3111-3123. doi:10.1002/jctb.6118

Krainer, F. W., Dietzsch, C., Hajek, T., Herwig, C., Spadiut, O. & Glieder, A. (2012). Recombinant protein expression in *Pichia pastoris* strains with an engineered methanol utilization pathway. *Microbial Cell Factories*, 11(**1**), 22. doi:10.1186/1475-2859-11-22

Kuwae, S. *et al.* (2005). Production of recombinant human antithrombin by *Pichia pastoris*, *Journal of Bioscience and Bioengineering*, 99(**3**), 264 - 271. doi: 10.1263/jbb.99.264.

Landázuri, P., Poutou-Landázuri Piñales, R. A., Acero-Godoy, J., Córdoba-Ruiz, H. A., Echeverri- Peña, O. Y., Sáenz, H., Delgado, J. M. & Barrera-Avellaneda, L. A. (2009). Cloning and shake flask expression of hrIDS-Like in *Pichia pastoris*. *African Journal of Biotechnology*, 8(**12**), 2871 - 2877. doi:10.5897/AJB08.610

Lee, J. & Komagata, K. (1980). Taxonomic study of methanol-assimilating yeasts. *The Journal of General and Applied Microbiology*, 26(**2**), 133 - 158. doi:10.2323/jgam.26.133

Li, P. et al. (2007). Expression of recombinant proteins in *Pichia pastoris*, *Applied Biochemistry and Biotechnology*, 142(**2**), 105 - 124. doi: 10.1007/s12010-007-0003-x.

Lin, J., Panigraphy, D., Trinh, L., Folkman, J. & Shiloach, J. (2000). Production process for recombinant human angiostatin in *Pichia pastoris*. *Journal of Industrial Microbiology and Biotechnology*, 24(**1**), 31 - 35. doi:10.1038/sj.jim.2900766

Lin-Cereghino, G., Godfrey, L., de la Cruz, B., Johnson, S., Khuongsathiene, S. & Tolstorukov, I. et al. (2006). Mxr1p, a Key Regulator of the Methanol Utilization Pathway and Peroxisomal Genes in *Pichia pastoris*. *Molecular and Cellular Biology*, 26(**3**), 883 - 897. doi:10.1128/mcb.26.3.883-897.2006

Liu, W. C. et al. (2019). Fed-batch high-cell-density fermentation strategies for *Pichia pastoris* growth and production, *Critical Reviews in Biotechnology*, 39(**2**), 258 - 271. doi: 10.1080/07388551.2018.1554620.

Looser, V. et al. (2014). Cultivation strategies to enhance productivity of *Pichia pastoris*: A review, *Biotechnology Advances*, 33(**6**), 1177–1193. doi: 10.1016/j.biotechadv.2015.05.008.

Loureiro, V. & Ferreira, H. G. (1983). On the intracellular accumulation of ethanol in yeast. *Biotechnology and bioengineering*, 25(**9**), 2263-2269. doi:10.1002/bit.260250911

Lushchak, O. & Lushchak, V. (2008). Catalase modifies yeast *Saccharomyces cerevisiae* response towards S-nitrosoglutathione-induced stress. *Redox Report*, 13(**6**), 283 - 291. doi:10.1179/135100008x309037

Macauley-Patrick, S. et al. (2005). Heterologous protein production using the *Pichia pastoris* expression system, *Yeast*, 22(**4**), 249 - 270. doi: 10.1002/yea.1208.

Maity, N., Thawani, A., Sharma, A., Gautam, A., Mishra, S. & Sahai, V. (2015). Expression and Control of Codon-Optimized Granulocyte Colony-Stimulating Factor in *Pichia pastoris*. *Applied Biochemistry and Biotechnology*, 178(**1**), 159 - 172. doi:10.1007/s12010-015-1865-y

Manrique López, S. & Jiménez Barbosa, W. (2012). Mercado de medicamentos biotecnológicos en el Sistema General de Seguridad Social en Salud. *Ciencia & Tecnología para la Salud Visual y Ocular*, 10(**2**), 59. doi:10.19052/sv.143

Mayson, B. E. *et al.* (2003)- Effects of methanol concentration on expression levels of recombinant protein in fed-batch cultures of *Pichia methanolica*, *Biotechnology and Bioengineering*, 81(**3**), 291 - 298. doi: 10.1002/bit.10464.

Menéndez, J.; Valdes, I. & Cabrera, N. (2003). The ICLI gene of *Pichia pastoris,* transcriptional regulation and use of its promoter. *Yeast,* 20(**13**), 1097 - 1108. doi: 10.1002/yea.1028.

Meza-Gutierrez, F., Simsek, D. & Toczyski, D. (2019). A genetic approach to study polyubiquitination in *Saccharomyces cerevisiae*. *Methods in Enzymology*, 49-72. doi: 10.1016/bs.mie.2018.12.020

Mochizuki, S. *et al.* (2001). Expression and characterization of recombinant human antithrombin III in *Pichia pastoris. Protein Expression and Purification,* 23(**1**), 55 - 65. doi: 10.1006/prep.2001.1479.

Moorkens, E., Jonker-Exler, C., Huys, I., Declerck, P., Simoens, S. & Vulto, A. (2016). Overcoming Barriers to the Market Access of Biosimilars in the European Union: The Case of Biosimilar Monoclonal Antibodies. *Frontiers In Pharmacology,* 7. doi:10.3389/fphar.2016.00193

Morales-Álvarez, E. D., Rivera-Hoyos, C. M., Cardozo-Bernal, Á. M., Poutou-Piñales, R. A., Pedroza-Rodríguez, A. M., Díaz-Rincón, D. J., Rodríguez-López, A., Alméciga-Díaz, C. J. & Cuervo-Patiño, C. L. (2017). Plackett-burman design for rGILCC1 laccase activity enhancement in *Pichia pastoris* Concentrated enzyme kinetic characterization. *Enzyme Research*. doi:10.1155/2017/5947581

Nabhan, C., Parsad, S., Mato, A. & Feinberg, B. (2018). Biosimilars in Oncology in the United States. *JAMA Oncology,* 4(**2**), 241. doi:10.1001/jamaoncol.2017.2004

Odinet, J., Day, C., Cruz, J. & Heindel, G. (2018). The Biosimilar Nocebo Effect? A Systematic Review of Double-Blinded Versus Open-Label Studies. *Journal of Managed Care & Specialty Pharmacy,* 24(**10**), 952 - 959. doi:10.18553/jmcp.2018.24.10.95

Ogata, K., Nishikawa, H. & Ohsugi, M. (1969). A yeast capable of utilizing methanol, *Agricultural and Biological Chemistry,* 33(**10**), 1519 - 1520. doi: 10.1080/00021369.1969.10859497.

Ogrunc, M. (2014). Reactive oxygen species: The good, the bad, and the enigma. *Molecular & Cellular Oncology,* 1(**3**), e964033. doi:10.4161/23723548.2014.964033

Ojeda, Lisette Pérez y Cristiá & Rafael Pérez (2016). Fortalecimiento de la regulación sanitaria en las Américas: las autoridades reguladoras de referencia regional. *Revista Panamericana de Salud Pública,* 39(**5**), 294 - 298. ISSN 1680-5348.

Payne, W., Gannon, P. & Kaiser, C. (1995). An inducible acid phosphatase from the yeast *Pichia pastoris:* characterization of the gene and its product. *Gene*, 163(**1**), 19 - 26. doi:10.1016/0378-1119(95)00379-k

Perrone, G., Tan, S. & Dawes, I. (2008). Reactive oxygen species and yeast apoptosis. *Biochimica Et Biophysica Acta (BBA) - Molecular Cell Research,* 1783(**7**), 1354 - 1368. doi:10.1016/j.bbamcr.2008.01.023

Pimentel, N., Rodríguez-Lopez, A., Díaz, S., Losada, J. C., Díaz-Rincón, D. J., Cardona , C.,, Espejo-Mojica, Á. J., Ramírez, A. M., Ruiz, F., Landázuri, P., Poutou-Piñales, R. A., Cordoba-Ruiz, H. A., Alméciga-Díaz, C. J. & Barrera-Avellaneda, L. A. (2018). Production and characterization of a human lysosomal recombinant iduronate-2-sulfatase produced in *Pichia pastoris. Biotechnology and Applied Biochemistry,* 65(**5**), 655 - 664. doi:10.1002/bab.1660

Polez, S., Origi, D., Zahariev, S., Guarnaccia, C., Tisminetzky, S., Skoko, N. & Baralle, M. (2016). A Simplified and Efficient Process for Insulin Production in *Pichia pastoris*. *PLOS ONE*, 11(**12**), p.e0167207. doi.org/10.1371/journal.pone.0167207

Potvin, G., Ahmad, A. & Zhang, Z. (2012). Bioprocess engineering aspects of heterologous protein production in *Pichia pastoris*: A review. *Biochemical Engineering Journal. Elsevier B.V.,* 64, 91-105. doi: 10.1016/j.bej.2010.07.017.

Poutou-Piñales, R. A., Córdoba, H., Quevedo-Hidalgo, B. E., Landázuri, P., Echeverri-Peña, O. Y., Sáenz, H., Vanegas, A., Acero, J., González, A., Herrera, J., Algecira, N., Caicedo, L. & Barrera, L. A. (2005). Expresión de iduronato 2-sulfato sulfatasa humana recombinante (IDShr) EN *Pichia pastoris. Universitas Scientiarum,* 10(**1**), 75 - 96.

Putz, M., Duda-Seiman, C., Duda-Seiman, D., Putz, A., Alexandrescu, I., Mernea, M. & Avram, S. (2016). Chemical Structure-Biological Activity Models for Pharmacophores 3D-Interactions. *International Journal of Molecular Sciences*, 17(**7**), p.1087.

Rahimi, A. *et al.* (2019). Enhancing the efficiency of recombinant hepatitis B surface antigen production in *Pichia pastoris* by employing continuous fermentation. *Biochemical Engineering Journal,* 141, 112 - 119. doi: 10.1016/j.bej.2018.10.019.

Ray, P., Huang, B. & Tsuji, Y. (2012). Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cellular Signalling,* 24(**5**), 981 - 990. doi:10.1016/j.cellsig.2012.01.008

Razaghi, A., Tan, E., Lua, L. H. L., Owens, L., Karthikeyan, O. P. & Heimann, K. (2017). Is *Pichia pastoris* a realistic platform for industrial production of recombinant human interferon gamma? *Biologicals,* 45, 52 - 60. doi:10.1016/j.biologicals.2016.09.015

Rebnegger, C. *et al.* (2016). *Pichia Pastoris* exhibits high viability and a low maintenance energy requirement at near-zero specific growth rates. *Applied and Environmental Microbiology,* 82(**15**), 4570-4583. doi: 10.1128/AEM.00638-16.

Resina, D. *et al.* (2005). Developing high cell density fed-batch cultivation strategies for heterologous protein production in *Pichia pastoris* using the nitrogen source-regulated FLD1 promoter. *Biotechnology and Bioengineering,* 91(**6**), 760 - 767. doi: 10.1002/bit.20545.

Resina, D. *et al.* (2009). Engineering of bottlenecks in Rhizopus oryzae lipase production in *Pichia pastoris* using the nitrogen source-regulated FLD1 promoter. *New Biotechnology,* 25(**6**), 396 - 403. doi: 10.1016/j.nbt.2009.01.008.

Rivera-Hoyos, C. M., Morales-Álvarez, E. D., Abelló-Esparza, J., Buitrago-Pérez, D. F., Martínez-Aldana, N., Salcedo-Reyes, J. C., Poutou-Piñales, R. A. & Pedroza-Rodríguez,A. M. (2018). Detoxification of pulping black liquor with *Pleurotus ostreatus* or recombinant *Pichia pastoris* followed by CuO/TiO2/visible photocatalysis. *Scientific Reports,* 8(**1**), 1 - 15.doi:10.1038/s41598-018-21597-2

Rodrigues, D. *et al.* (2019). Fed-batch production of *Saccharomyces cerevisiae* L-Asparaginase II by recombinant *Pichia pastoris* MUTs strain. *Frontiers in Bioengineering and Biotechnology,* 7, doi: 10.3389/fbioe.2019.00016.

Rodríguez-López, A., Alméciga-Díaz, C. J., Sánchez, J., Moreno, J., Beltran, L., Díaz, D. & Barrera, L. A. (2016). Recombinant human N-acetylgalactosamine-6-sulfate sulfatase (GALNS) produced in the methylotrophic yeast *Pichia pastoris*. *Scientific reports*, 6, 29329. doi: 10.1038/srep29329

Rodríguez-López, A., Pimentel-Vera, L. N., Espejo-Mojica, A. J., Van Hecke, A., Tiels, P., Tomatsu, S., Callewaert, N.& Alméciga-Díaz, C. J. (2019). Characterization of Human Recombinant N-Acetylgalactosamine-6-Sulfate Sulfatase Produced in *Pichia pastoris* as Potential Enzyme for Mucopolysaccharidosis IVA Treatment. *Journal of Pharmaceutical Sciences,* 108(**8**), 2534 - 2541. doi:10.1016/j.xphs.2019.03.034

Ruβmayer, H., Buchetics, M., Gruber, C., Valli, M., Grillitsch, K. & Modarres, G. *et al.* (2015). Systems-level organization of yeast methylotrophic lifestyle. *BMC Biology,* 13(**1**). doi: 10.1186/s12915-015-0186-5

Shen, S. *et al.* (1998). A strong nitrogen source-regulated promoter for controlled expression of foreign genes in the yeast *Pichia pastoris. Gene,* 216(**1**), 93 -102. doi: 10.1016/S0378-1119(98)00315-1.

Shen, W. *et al.* (2016). A novel methanol-free *Pichia pastoris* system for recombinant protein expression, Microbial Cell Factories. *BioMed Central,* 15(**1**), 1 - 11. doi: 10.1186/s12934-016-0578-4.

Shiraishi, K., Oku, M., Uchida, D., Yurimoto, H. & Sakai, Y. (2015). Regulation of nitrate and methylamine metabolism by multiple nitrogen sources in the methylotrophic yeast *Candida boidinii. FEMS Yeast Research*, fov084. doi:10.1093/femsyr/fov084

Simoens, S., Jacobs, I., Popovian, R., Isakov, L. & Shane, L. (2017). Assessing the Value of Biosimilars: A Review of the Role of Budget Impact Analysis. *PharmacoEconomics*, 35(**10**), 1047-1062. doi: 10.1007/s40273-017-0529-x

Singh, A. & Narang, A. (2019). The Mut+ strain of *Komagataella phaffii (Pichia pastoris)* expresses PAOX1 5 and 10 times faster than Muts and Mut - strains: Evidence that formaldehyde or/and formate are true inducers of AOX. doi: 10.1101/573519

Siverio, J. (2002). Assimilation of nitrate by yeasts. *FEMS Microbiology Reviews,* 26(**3**), 277 - 284. doi:10.1111/j.1574-6976.2002.tb00615.x

Sreekrishna, K. & Kropp, K. E. (1996). *Pichia Pastoris. Encyclopedia of Food Microbiology,* 1686-1692. doi: 10.1006/rwfm.1999.1260.

Tanaka, H., Okuno, T., Moriyama, S., Muguruma, M. & Ohta, K. (2004). Acidophilic xylanase from Aureobasidium pullulans: efficient expression and secretion in *Pichia pastoris* and mutational analysis. *Journal of Bioscience and Bioengineering,* 98(**5**), 338 - 343. doi:10.1016/s1389-1723(04)00292-0

Taylor, R. C. (2016). Aging and the UPR(ER). *Brain Research,* 1648, 588-593. doi: 10.1016/j.brainres.2016.04.017.

Tesar, T., Golias, P., Inotai, A., Kawalec, P. & Wawruch, M. (2019). The impact of implemented regulations on biosimilars in Slovakia. *Health Policy And Technology,* 8(**4**), 408 - 413. doi:10.1016/j.hlpt.2019.09.004

Titorenko, V. I. & Rachubinski, R. A. (2004). The peroxisome: orchestrating important developmental decisions from inside the cell. *The Journal of cell biology,* 164(**5**), 641 - 645. doi:10.1083/jcb.200312081

Tu, Y., Wang, Y., Wang, G., Wu, J., Liu, Y., Wang, S., Jiang, C. & Cai, X. (2013). High-level expression and immunogenicity of a porcine circovirus type 2 capsid protein through codon optimization in *Pichia*

pastoris. Applied Microbiology and Biotechnology, 97(**7**), 2867-2875. doi:10.1007/s00253-012-4540-z

Uhlig, T. & Goll, G. (2017). Reviewing the evidence for biosimilars: key insights, lessons learned and future horizons. *Rheumatology,* 56(4), iv49-iv62. doi:10.1093/rheumatology/kex276

Vaca-González, C. P., Arteaga, L. & Delgado López, N. E. (2019). Magistral drug production in Colombia and other middle-income countries. *Nature Biotechnology,* 37(**3**), 216 - 217. doi:10.1038/s41587-019-0044-z

Van der Klei, I., Yurimoto, H., Sakai, Y. & Veenhuis, M. (2006). The significance of peroxisomes in methanol metabolism in methylotrophic yeast. *Biochimica Et Biophysica Acta (BBA) - Molecular Cell Research,* 1763(**12**), 1453 - 1462 doi:10.1016/j.bbamcr.2006.07.016

Van Dijken, J., Veenhuis, M., Kreger-Van Rij, N. & Harder, W. (1975). Microbodies in methanol-assimilating yeasts. *Archives Of Microbiology,* 102(**1**), 41 - 44. doi:10.1007/bf00428343

Veenhuis, M., Dijken, J. & Harder, W. (1983). The Significance of Peroxisomes in the Metabolism of One-Carbon Compounds in Yeasts. *Advances in Microbial Physiology*, 1 - 82. doi: 10.1016/s0065-2911(08)60384-7

Vogl, T. & Glieder, A. (2013). Regulation of *Pichia pastoris* promoters and its consequences for protein production, *New Biotechnology. Elsevier B.V.,* 30(**4**), 385-404. doi: 10.1016/j.nbt.2012.11.010.

Vogl, T., Fischer, J., Hyden, P., Wasmayer, R., Sturmberger, L. & Glieder, A. (2020). Orthologous promoters from related methylotrophic yeasts surpass expression of endogenous promoters of *Pichia pastoris. AMB Express,* 10(**1**). doi:10.1186/s13568-020-00972-1

Vonck, J., Parcej, D. & Mills, D. (2016). Structure of Alcohol Oxidase from *Pichia pastoris* by Cryo-Electron Microscopy. *PLOS ONE,* 11(**7**). doi:10.1371/journal.pone.0159476

Waterham, H. R. *et al.* (1997). Isolation of the *Pichia pastoris* glyceraldehyde-3-phosphate dehydrogenase gene and regulation and use of its promoter. *Gene,* 186(**1**), 37-44. doi: 10.1016/S0378-1119(96)00675-0.

Weinhandl, K., Winkler, M., Glieder, A. & Camattari, A. (2014). Carbon source dependent promoters in yeasts. *Microbial Cell Factories,* 13(**1**), 5. doi:10.1186/1475-2859-13-5

Yang, Z. & Zhang, Z. (2018). Engineering strategies for enhanced production of protein and bio-products in *Pichia pastoris:* A review. *Biotechnology Advances. Elsevier,* 36(**1**), 182 - 195. doi: 10.1016/j.biotechadv.2017.11.002.

Yurimoto, H., Kato, N. & Sakai, Y. (2005). Assimilation, dissimilation, and detoxification of formaldehyde, a central metabolic intermediate of methylotrophic metabolism. *The Chemical Record,* 5(**6**), 367- 375. doi:10.1002/tcr.20056

Yurimoto, H., Oku, M. & Sakai, Y. (2011). Yeast Methylotrophy: Metabolism, Gene Regulation and Peroxisome Homeostasis. *International Journal of Microbiology,* 2011, 1 - 8. doi:10.1155/2011/101298

Zahrl, R. J., Mattanovich, D. & Gasser, B. (2018). The impact of ERAD on recombinant protein secretion in *Pichia pastoris (Syn komagataella spp.). Microbiology* (United Kingdom), 164(**4**), 453 - 463. doi: 10.1099/mic.0.000630.

Zámocký, M. & Koller, F. (1999). Understanding the structure and function of catalases: clues from molecular evolution and in vitro mutagenesis. *Progress In Biophysics And Molecular Biology,* 72(**1**), 19 - 66. doi:10.1016/s0079-6107(98)00058-3

Zapata, J., Steiner, R., Bernal, S. & Castillo, J. (2012). Pertinencia de incentivar la competencia en el mercado de medicamentos biotecnológicos en Colombia y su impacto sobre las finanzas del sector de la salud. Retrieved 28 April 2020, from https://www.researchgate.net/publication/241754341_Pertinencia_de_incentivar_la_competencia_en_el_mercado_de_medicamentos_biotecnologicos_en_Colombia_y_su_impacto_sobre_las_finanzas_del_sector_de_la_sa.

Zhang, W., Zhang, T., Wu, S., Wu, M., Xin, F. & Dong, W. (2017). Guidance for engineering of synthetic methylotrophy based on methanol metabolism in methylotrophy. *RSC Advances,* 7(**7**), 4083 - 4091. doi:10.1039/c6ra27038g

Zhao, W., Zheng, J. & Zhou, H. bo (2011). A thermotolerant and col-active mannan endo-1,4-β-mannosidase from *Aspergillus niger* CBS 513.88: Constitutive overexpression and high-density fermentation in *Pichia pastoris*. *Bioresource Technology*, 102(**16**), 7538-7547. doi:10.1016/j.biortech.2011.04.070

Zwart, K. B., Veenhuis, M. & Harder, W. (1983). Significance of yeast peroxisomes in the metabolism of choline and ethanolamine. *Antonie van Leeuwenhoek,* 49(**4-5**), 369 - 385. doi:10.1007/BF00399317