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A step forward in recombinant protein production regulated by the constitutive GAP promoter in Pichia pastoris through bioprocess engineering approaches

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A step forward in recombinant protein production regulated by the constitutive *GAP* promoter in *Pichia pastoris* through bioprocess engineering approaches

Memòria per obtenir el Grau de Doctor
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dins del Programa de Doctorat en Biotecnologia
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Francisco Valero i José Luis Montesinos

Xavier Garcia Ortega

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Francisco Valero Barranco i **José Luis Montesinos Seguí**, Professor Catedràtic i Professor Titular membres del Grup d'Enginyeria de Bioprocessos i Biocatàlisi Aplicada del Departament d'Enginyeria Química, Biològica i Ambiental de la Universitat Autònoma de Barcelona,

CERTIFIQUEM:

Que el Biotecnòleg Xavier Garcia Ortega ha dut a terme sota la nostra direcció al Departament d'Enginyeria Química, Biològica i Ambiental de la Universitat Autònoma de Barcelona, el treball que, amb el títol "**A step forward in recombinant protein production regulated by the constitutive *GAP* promoter in *Pichia pastoris* through bioprocess engineering approaches**" es presenta en aquesta memòria, la qual constitueix la seva Tesi per optar al grau de Doctor per la Universitat Autònoma de Barcelona dins del Programa de Doctorat en Biotecnologia.

I per tal que se'n prengui coneixement i consti als efectes oportuns, signem la present a Bellaterra, Novembre 2015.

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Abstract

Recombinant protein, including biopharmaceuticals proteins and industrial enzymes, is a multi-billion dollar market. Among all the suitable host organisms commonly used for its production, the yeast *Pichia pastoris* is currently one of the most effective and versatile expression platform. From an operational and industrial point of view, alternatively to the widely extended methanol-based processes, the use of the constitutive *GAP* promoter offers very important advantages. However, the development of cultivation strategies for efficient production processes regulated by the constitutive *GAP* promoter can be considered that still remains in its infancy.

In the course of this thesis various crucial factors towards increasing the yield and productivity of the process for the production of recombinant proteins with the mentioned expression system were addressed through bioprocess engineering approaches. The development and implementation of the new cultivations strategies was performed for a *P. pastoris* strain expressing constitutively the human 2F5 antigen-binding fragment (Fab) as a model protein. Nevertheless, it is expected that the production process developments achieved are also applicable for the expression of other recombinant proteins of interest.

Initially, in order to evaluate the effect caused by the reported limiting secretory capacity of the host cell system, a cell disruption and a protein extraction procedure were developed and optimized. These methods allowed achieving an important increase in the accuracy, and therefore reliability, of intracellular product quantification.

The use of glucose and glycerol as a carbon sources was compared for both phases of a fed-batch cultivation. Important positive effects were observed selecting different substrate for each phase. Furthermore, the effect of the specific growth rate on the specific production rate was studied showing a plain correlation between product formation and biomass growth as a result of the strong transcriptional effect of the specific growth rate in the central carbon metabolism, and in turn, protein of interest expression. In contrast, no clear effect of the specific growth rate could be observed in the secretory capacity of the yeast. Accordingly, fed-batch cultivation at constant and high specific growth rates were carried out reaching

production rates significantly higher than cultures based on others carbon source feeding strategies.

The impact of environmental stress conditions on the host cell physiology has been characterized and used in order to enhance significantly the yields and production rates of the recombinant protein. Important production increases have been achieved by carrying out *P. pastoris* cultivations implementing either carbon-starving or oxygen-limiting conditions. Once more, the increments were hypothesised to be caused by the regulation of the central carbon metabolism induced by the host adaptation response to the new environment.

Overall, the outcome of this study is a successful attempt to apply the host cell system physiology understanding on the rational design of cultivation strategies towards maximizing the yields and productivity of recombinant protein production processes by the exploitation of key factors that enhances the protein expression.

Resum

La producció de proteïnes recombinants, tant per aplicacions terapèutiques com industrials, dóna lloc a un mercat que anualment genera milers de milions de dòlars. Entre els organismes hostes destacats que solen ser emprats per a la seva producció, actualment el llevat *Pichia pastoris* és una de les plataformes d'expressió més efectives i versàtils. Des d'un punt de vista operacional i industrial, i com a alternativa als àmpliament estesos processos basats la utilització de metanol, l'ús del promotor constitutiu *GAP* ofereix avantatges molt importants. Malgrat aquestes avantatges, però, el desenvolupament d'estratègies de cultiu que doni lloc a processos de producció eficients regulats pel promotor *GAP* està considerat, encara avui en dia, en un estadi molt primerenc.

Al llarg del curs d'aquesta tesi, diversos paràmetres claus per a assolir millores en termes de rendiment i productivitat en els processos de producció de proteïnes recombinants amb l'esmentat sistema d'expressió van ser abordats a través de les eines de l'enginyeria de bioprocessos. El desenvolupament i implementació de noves estratègies de cultiu va ser dut a terme per a una soca de *P. pastoris* que expressa constitutivament el fragment d'unió a antigen (Fab) humà 2F5 com a proteïna model. No obstant, s'espera que les millores en els processos de producció assolits siguin també aplicables a l'expressió d'altres proteïnes recombinants d'interès.

Inicialment, amb l'objectiu de valorar l'efecte causat per la capacitat de secreció limitada àmpliament estudiada en aquest hoste d'expressió, un protocol de disrupció cel·lular i d'extracció de proteïnes va ser desenvolupat i optimitzat. Aquests mètodes van permetre assolir un increment molt important en la precisió, i per tant en la veracitat, de la quantificació de producte retingut intracel·lularment.

L'ús de glucosa i de glicerol com a fonts de carboni va ser comparat per les dues fases del cultiu en fed-batch. La selecció de diferents substrats per a cadascuna de les fases va donar lloc a efecte positius importants en el cultiu. Addicionalment, l'efecte de la taxa específica de creixement a la taxa de específica producció va ser estudiat mostrant una clara relació entre la formació de producte i el creixement de biomassa com a resultat d'un important efecte

transcripcional de la taxa específica de creixement en el metabolisme central del carboni, i a la vegada, en l'expressió de la proteïna d'interès. Per contra, no va poder ser observat un efecte evident de la taxa específica de creixement a la capacitat de secreció del llevat. Per tant, es van dur a terme cultius en fed-batch a una taxa de creixement alta i constant assolint taxes de producció significativament més elevades respecte d'altres cultiu basats en altres estratègies d'alimentació de font de carboni.

L'impacte de condicions ambientals d'estrès a la fisiologia cel·lular de l'hoste va ser caracteritzat i estudiat amb l'objectiu de millorar significativament els rendiments i les taxes de producció de la proteïna recombinant. Concretament es van obtenir increments importants de producció implementant tant condicions limitants d'oxigen com de deprivació de font de carboni en cultius de *P. pastoris.* De nou, s'hipotitza que aquests increments estan causats per la regulació del metabolisme central del carboni induïda per la resposta adaptativa de l'hoste a les noves condicions ambientals.

Globalment, el resultat d'aquest estudi és una temptativa amb èxit d'aplicar el coneixement de la fisiologia de l'hoste d'expressió en el disseny racional d'estratègies de cultiu amb l'objectiu de maximitzar el rendiment i la productivitat de processos de producció de proteïnes recombinants explotant paràmetres claus del sistema que augmenten l'expressió de la proteïna.

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Introduction

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1.1 Biotech century

Each century has been coined by the scientific and technological progress. The 19th century was the age of Chemistry, when the atomic theory and the periodic law were developed. Along the 20th century, the age of Physics, the atom was split and silicon was turned into computing power. Nowadays, the 21st century has been denominated as the "The Biotech Century". It is considered that its primary seed was spawned in 1953 when James Watson and Francis Crick elucidated the structure of DNA. Afterwards, in the early 1970s, the development of the first tools for the manipulation of genetic material also supposed an important turning point in the history of Biotechnology. Now, in the 21st century, it is expected that Biotechnology faces global challenges such to combat diseases, reduce our environmental footprint, feed the hungry, use less and cleaner energy, and have safer, cleaner and more efficient industrial manufacturing processes.

The Organisation for Economic Co-operation and Development (OECD) defined Biotechnology as the application of science and technology to living organisms as well as parts, products and models thereof, to alter living or non-living materials for the production of knowledge, goods and services. Examples of biotechnological use of microorganisms, such as alcoholic fermentation and bread making, have been registered since ancient times.

Currently, the growing and expansion of its applications have in turn brought about the need to classify biotechnology in different areas based on common features or final purposes, which have been identified by a colour system. Different authors have proposed different classifications that divide the biotechnological applications in a range between 3 and 10 colours. Below, a classification in 5 branches is described:

- *Red Biotechnology* includes all those applications related to Medicine, and thus the research and development of new drugs, vaccines and antibiotics, as well as molecular diagnostic techniques and regenerative therapies to cure diseases.
- *White Biotechnology* is focused on industrial processes. The goods that can be produced by bioprocesses have a very wide range of applications, from biopharmaceuticals to enzymes, bulk and fine chemicals, and biofuels. In this area is very important to consume fewer resources and be more energy efficient and less polluting than the traditional processes.
- *Green Biotechnology* brings together all those applications on agriculture such developing new plant varieties resistant to pests and diseases as well as producing of biofertilizers and biopesticides reducing the soil working, improving the nutritional properties of common cultures, and finally, also using plants as biofactories to produce substances and molecules of interest.

- *Grey Biotechnology* involves the areas directly related to the environment. The main applications are focused on the biodiversity maintenance and the efficient removal of contaminants, also known as bioremediation, from which valuable by-products can also be obtained.

- *Blue Biotechnology* deals with the exploitation greatest diversity of sea resources in order to obtain products of interest for the health, industrial and alimentation sectors.

1.2 Production and application of recombinant proteins

Proteins, the building blocks of life, are synthesized by all living organisms as part of their natural metabolism and have a role in virtually every cellular process. Some, such as enzymes, serve as biocatalysts by increasing the rate of metabolic reactions, while others form cellular scaffolds and are central to signalling, transport, and regulatory functions. Nowadays, proteins are used in a wide range of industries such medicine, diagnostics, food, detergents, biofuels, textiles, polymers, paper and pulp [1,2].

While some proteins can be isolated from native sources including fungi, algae, animal tissues or plants, many cannot because they are either intrinsically unstable or are present in impractically low quantities [3]. Proteins are complex molecules in terms of their structure and function and, unlike many pharmaceuticals, cannot be synthesised chemically. Genes encoding many recombinant proteins of interest have been cloned into different cell factories obtaining levels of expressions hundreds of times higher than those naturally produced [4]. Therefore, in order to be able to obtain big quantities, proteins are produced in biological systems; usually inside host cells (although a growing number of cell-free expression technologies are available). These proteins, synthesised in a host cell frequently of a different species to their origin, are termed 'recombinant proteins' [5]. This biotechnological process has been allowed by the recombinant DNA technologies (rDNA) which was developed in the early 70's, when *Cohen et al*. firstly described the first *in vitro* construction of functional bacterial plasmids in *Escherichia coli* [6]. It was soon followed by the recombinant expression of human proteins, which rapidly lead to the commercialization of the first recombinant biopharmaceutical, human insulin. Initially, in 1982, it was expressed in *E.coli* and launched by Eli Lilly and Company of Indianapolis; later, in 1986, Novo Nordisk started the industrial production using the yeast *Saccharomyces cerevisiae* as a host [7,8]. Nowadays, recombinant proteins has become into a reliable source of biopharmaceuticals that can be used as a therapeutics, prophylactics, and diagnostics for both human and veterinary medicine, as well as has also transformed industrial processes such food processing, paper, detergents, biofuels and textile production [9,10].

The wide range of applications and the increasing demand of recombinant proteins are leading to a very important growth of its market, which has been fairly immune to the recent turmoil in the global economy. The biopharmaceuticals market in 2014 was valued at US$ 162 billions (Bn), and is expected to keep growing at a compound annual growth rate (CAGR) of around 10% until 2020, reaching an estimated value of US$ 278 Bn. During the last years the increase in this market has been even bigger than the forecast published in early 2000s, which estimated market values significantly lower. The number of approved products currently on the market in the US and UE has also rapidly increased up to more than 200 protein drugs, which represents around the 25% of products and the 40% of sales of the total pharmaceutical industry. It is also important to mention that the dominance of biopharmaceuticals products in the market has changed from the beginning in the 1980s, when mainly hormones and cytokines were being produced. Nowadays, cancer targeted therapeutic antibodies dominates the market (Figure 1.1). The description of the current state of biopharmaceuticals market has been summarised by merging the data found in the following references (Figure 1.2) [8,9,11–16].

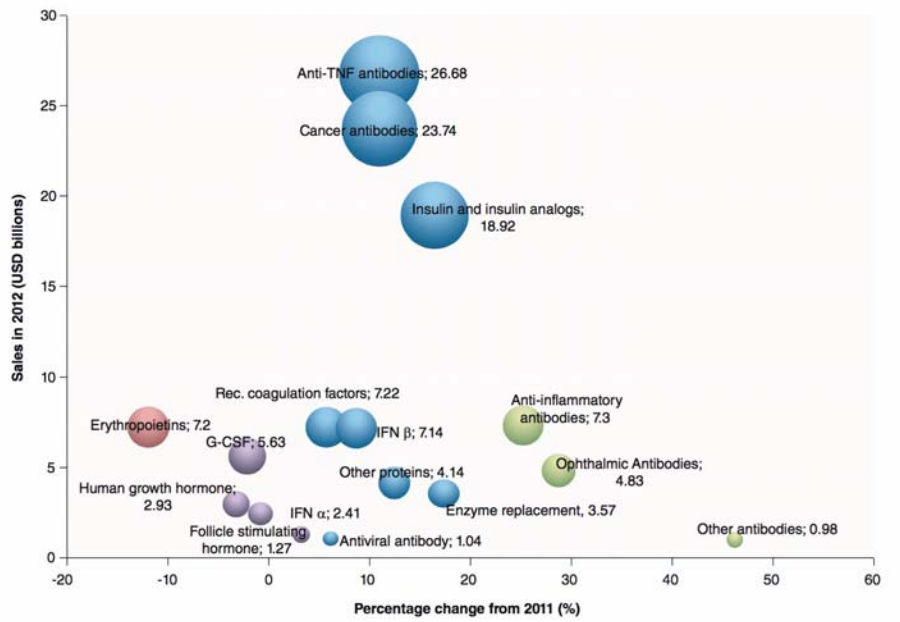

Figure 1.1: Sales distribution of biopharmaceuticals in 2012. The size of the circles represents sales; the colour of the circles represents the percentage change from 2011, red: <-5%; purple: -5% to 5%; blue: 5% to 20%; green: >20%. Figure taken from [14].

Accordingly, but in a lower order of magnitude, during the last years the market for industrial enzymes is also growing fast. This market was valued in 2014 at US$ 4.2 Bn and is expected to keep growing at a CAGR of around 7% until 2020, reaching an estimated value of US$ 6.2 Bn. Currently, over 500 industrial products are being manufactured by enzyme-based bioprocesses. Proteases and laundry detergents constitute respectively over 60% and 25% of the worldwide sales of enzymes. The description of the current state of the industrial enzymes market has been summarised by merging the data found in the following references (Figure 1.2) [1,10,17,18].

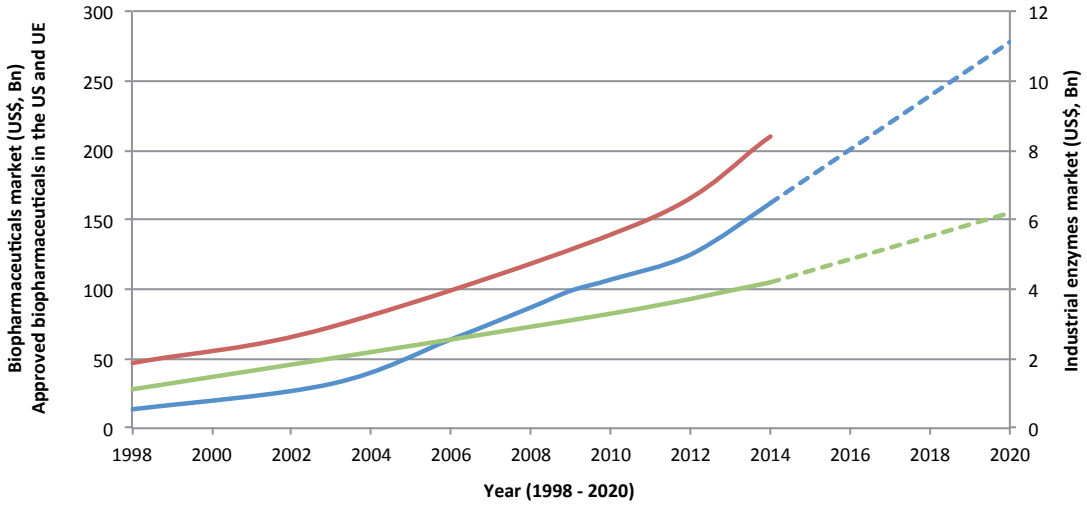

Figure 1.2: Time evolution of the sales of biopharmaceuticals (blue), sale of industrial enzymes (green) and approved biopharmaceuticals approved in the US and UE (red) between the 1998-2020. Data taken from [1,8-17].

The vast majority of recombinant proteins of first-generation products were un-engineered, thus their amino acid sequence was identical to the native protein. Unfortunately, many naturally occurring proteins are not suitable for its desired applications because of their low stability, poor selectivity, slow reaction rates, and substrate or product inhibition. Therefore, over the last 20 years, besides the increasing demand of novel recombinant proteins for different and new applications, the demand of key proteins that exhibit improved or new properties is also growing [17]. The developing of novel engineered products is possible by the application of the protein engineering. There are two major ways in which proteins can be modified to adapt their functions to applied ends: rational redesign of existing products and directed evolution, both are not mutually exclusive. Rational design involves site-specific alterations of selected residues in a protein to cause predicted changes in function, whereas directed evolution mimics the natural evolution process in the laboratory and involves repeated cycles of generating a library of protein variants and selecting the variants with desired properties [19].

As an example, a reported case of directed evolution for a microbial acetyltransferase for modification of glyphosate has described an enzyme activity increase by 7.000-fold as well as a 5-fold increment in its thermostability [20].

Nowadays for biopharmaceutical proteins, since in the 1990s was launched the first wave of engineered products being a very small fraction of the total approvals, the proportion of new products with enhanced properties approved per year has increased up to 75% in the years 2010 and 2011 [9].

1.3 Cell factories for recombinant protein production

The high demand of recombinant proteins for different applications ant its current increase with a high annual growth rate gives ground for improving the protein expression platforms, both to enable the production of novel proteins and to reduce the production costs of the existing processes. Nowadays, while high yields of active proteins obtained in many cases, there are still important limitations for the production of complex proteins in large quantities and as functional entities conserving native structure and biological activity [21].

The choice of a suitable host as an expression system for the production of the recombinant protein is therefore very important and probably one of the most challenging and critical step that should be decided at the very beginning of the bioprocess design [5]. In a perfect scenario, one universal expression system would enable expression of all possible recombinant genes in a fast, cheap, and proper manner with respect to yield, folding and biological activity [22]. However, the important limitations of the current existing systems make necessary an empirical determination of the most suitable system among the qualified candidates.

The selection of the expression system must be based on production parameters such costs, yield, production timescale, scale up capacity and downstream processes as well as on the properties and use of the product [14]. Different applications have different requirements for both quantity and quality. The characteristics of the target protein must also be taken into account in terms of structure (mass of the polypeptide, soluble, membrane bound, multi-domain, …) and the requirement to incorporate post-translational modifications (PTM's; disulphide bonds, the desired glycosylation pattern, …) [22]. Glycosylation represents the most complex and the most widespread PTM, being associated with 40% of all approved products [13]. Selecting a wrong expression host can result in the protein being misfolded or poorly expressed, lacking the necessary PTM's or containing inappropriate modifications.

In general, bacteria are an excellent expression system for proteins that are not required to be synthesised in a glycosylated or extensively post-translationally modified form, allowing fast and inexpensive production processes. This fact is mainly due to its simplicity both biological (in terms of biochemistry and physiology) and from a process perspective [5]. Among them, *E. coli* has been considered the working horse of the recombinant protein production since always has stood out as the pre-eminent host cell for producing recombinant proteins both in commercial and research (Table 1.1) [2]. It is a reflection of the quantity and quality of information available about its genetics, molecular biology, biochemical, physiological, and fermentation technologies. Using *E. coli*, recombinant protein fraction can reach up to 50% of total biomass and protocols for high cell density cultivation are established, reaching up to 100 g biomass per litre [23]. However, producing eukaryotic

proteins, the production often results in inclusion body formation and/or specific low yields. This fact can be explained by the rates of protein synthesis and folding, which are an order of magnitude faster in prokaryotes [24].

Table 1.1: Summary of the recombinant gene expression in the most commonly used host cells between the years 1980-2013. Table taken from [2].

Year	All host cells	*E. coli*	*S. cerevisiae*	*P. pastoris*	Insect cells	Mammalian cell-lines
1980	0	0	0	0	0	0
1985	0	0	0	0	0	0
1990	12	75% (9; 4E)	8% (1)	0	17% (2)	0
1995	37	70% (26; 17E)	5% (2)	5% (2)	5% (2)	8% (3)
2000	50	70% (35; 17E)	0	4% (2)	12% (6)	12% (6)
2005	121	85% (103; 53E)	0	5% (6)	6% (7)	2% (2)
2010	172	76% (131; 67E)	0	9% (15)	5% (6)	5% (9)
2013	128	73% (94; 54E)	2% (2)	11% (14)	4% (5)	4% (5)

Other bacterial systems commonly used for recombinant protein production are the *Bacillus* species, which in contrast to *E. coli*, has an enormous secretion potential and is considered a GRAS (Generally Recognized As Save) organism. The important advantage in terms of downstream processing due to secretion of the desired protein to the fermentation medium is balanced out by the production of many proteases, which sometimes destroys the recombinant protein [1]. Furthermore, other bacterial organisms are efficiently used as cell factories for recombinant protein production such *Pseudomonas fluorescens, Staphylococcus carnosus or Streptomyces lividans* [17]. It also important to mention that the currently development of bacteria as expression system made become obsolete old dogma for recombinant protein production in bacteria. The advances in the strain host engineering nowadays allows the incorporation of eukaryotic-like PTM's, the extracellular protein production for *E. coli*, or the proteases defective strains for *Bacillus* species [25].

In contrast to bacteria, the use of mammalian cell-lines should be able to overcome the most of limitations of producing recombinant eukaryotic proteins. Consequently, due to the capacity to perform human PTM's as well as to fold and assembly correctly human proteins, mammalian cells has become the dominant recombinant production system for medical applications [26]. Specially, the authenticity of the glycosylation patterns performed is an important advantage over all the other expressions hosts, thus allowing correct function, presenting good pharmacology-kinetics *in vivo* and eliminating the risk of an immunogenic response in patients due to incompatible N-glycans on the protein [27].

In the last years have been developed important improvements in terms of media composition and process control that has allowed more than a 100-fold yield improvement

over titres for similar processes in the mid-1980s [28]. CHO (Chinese hamster ovary) cells are the preferred cell type for producing monoclonal antibodies and some other recombinant proteins. However, other types are also commonly used such various mouse myelomas (NSO murine myeloma cells), baby hamster kidney (BHK) cells for production of cattle foot-and-mouth disease vaccine, green monkey kidney cells for polio vaccine and human cell lines such as human embryonic kidney (HEK) cells [4]. Nowadays this expression system still present important drawbacks such very low growth rates and product yield, expensive costs derived from the use of complex medium and serums, big potential for viral product contamination [29]. In addition, in terms of bioprocessing, the operational culture mode, process control and scaling-up steps are also very challenging [30]. Consequently, the specific yields obtained with this bioprocesses based on mammalian cell-lines are often low [29], which is leading into a declining trend in their use (Table 1.1) [2].

The yeasts, single-celled eukaryotic fungal organisms, offer a halfway house between bacterial and mammalian cells as expression system presenting important advantages for protein production processes [5,14]. Yeast are simple, genetically and physiologically well characterized, able to grow fast and up-to very high cell densities in cheap chemically defined mediums generating high yields of recombinant proteins at low cost, which incorporate PTMs such as disulphide bonds and glycosylation [8]. Since this thesis is mainly focused on the recombinant protein production in yeast, the characteristics as well as the advantages and drawbacks of this expression system will be widely discussed in the following section.

Besides the mentioned expression system there are some others that have less impact on the recombinant protein production both in research and industry, which will be shortly commented below.

- Filamentous fungi are also attractive as a host for recombinant expression of proteins of interest because of their ability to secrete high levels of bioactive proteins with PTMs processing [1]. However, these organisms are slow-growing and difficult to handle in fermenters, which are important drawbacks from an industrial point of view. Recombinant moulds as Aspergillus niger, Aspergillus oryzae, Aspergillus awamori or Chrysosporium lucknowense are sources of enzymes for industrial applications [4].
- Insect cells are also used for recombinant protein production by means of baculovirus infections. Although the PTMs that can be incorporate to the desired protein can be more complex than using yeast or fungi, the production of a recombinant viral vector for gene expression is very time-consuming, the cell growth is slow when compared with other expression systems and the cost of growth mediums are very high [12].

– Transgenic animals such as goats, mice, cows, pigs, rabbits and sheeps are being developed as a recombinant protein production system in milk, egg white, blood, urine or seminal plasma. Animals offer very high quality of the proteins and production yields at low cost. On the contrary, the production times are very long and present very important safety concerns such as transmission of infectious diseases, adverse allergenic, immunogenic and autoimmune responses [31]. Currently, the development of this system has been slower to the above systems [1].

– Transgenic plants also offer an interesting possibility to produce recombinant proteins. Plant cells are able to fold proteins, associate subunits and to perform PTMs as efficiently as animal cells, but also presents important advantages compared to animals in terms of production costs, timescale, safety risks, and in storage and distribution of the recombinant proteins [32]. The main disadvantage is that the PTMs introduced can generate adverse immune responses. Moreover, the possibility to spread the proteins in open fields and the negative public perception of the transgenic plants precludes the use of plants as an attractive expression system of therapeutic proteins [12]. The development of this expression system is also slower in comparison with other systems.

Table 1.2: Capacities comparison among different expression hosts to produce recombinant pharmaceutical proteins. Table taken from [31].

Points to consider	Host cell					
	Bacteria	***Yeast***	**Insect cells**	**Mammalian cell-lines**	**Transgenic animals**	**Transgenic plants**
Theoretical production level	+++++	+++++	++++	+	+++++	+++++
Practical production level	++(+)	++(+)	+	+	++++	++
Investment cost	+++++	+++++	++	+	+++	++++
Production cost	+++++	+++++	++	++	++++	+++++
Flexibility	+++++	+++++	++	+	++++	+++++
Line conservation	+++++	+++++	+++	+++	+++++	+++++
Line stability	+++++	+++++	++++	+++	+++++	+++++
Delay for the first production	+++++	+++++	+++	+++++	+++(+)	++++
Scaling up	+++++	+++++	++	+	++++	+++++
Collection	+++++	+++++	+++++	+++++	++++	+++++
Effect on organism	+++(+)	+++(+)	+++(+)	+++(+)	+++	+++(+)
Post-translational modifications	+	++	+++	++++	++++	+++
Glycosylation	+	++	+++	++++	++++	++
Stability of product	+++++	+++++	+++	+++	++++	++++
Purification	+++	+++	+++	++++	+++	+++
Contaminant pathogens	+++++	+++++	+++++	++++	++++	+++++
Intellectual property	++++	++++	+++	++	+++	+++
Products on the market	++++	++++	+++	+++++	+++	+

In Table 1.2 were compared the different capacities that present the different host expression systems. Besides the characteristics of the different recombinant expression systems commented above, it is also very interesting to compare the use of the different expression systems in the market for the two main applications of recombinant proteins, biopharmaceuticals and enzymes.

In the biopharmaceutical market, since the apparition of the proteins produced in mammalian cells its progression in the market has been very important and regular, thus being the most used system since 1998. This system is currently used for producing around 43% of biopharmaceutical proteins. *E. coli* (30%) and yeasts (15%) are also widely used for producing an important number of biopharmaceuticals but in lower percentages. In contrast insect cells and transgenic animals has a residual use with only one approved product for each system (Figure 1.3) [12,14].

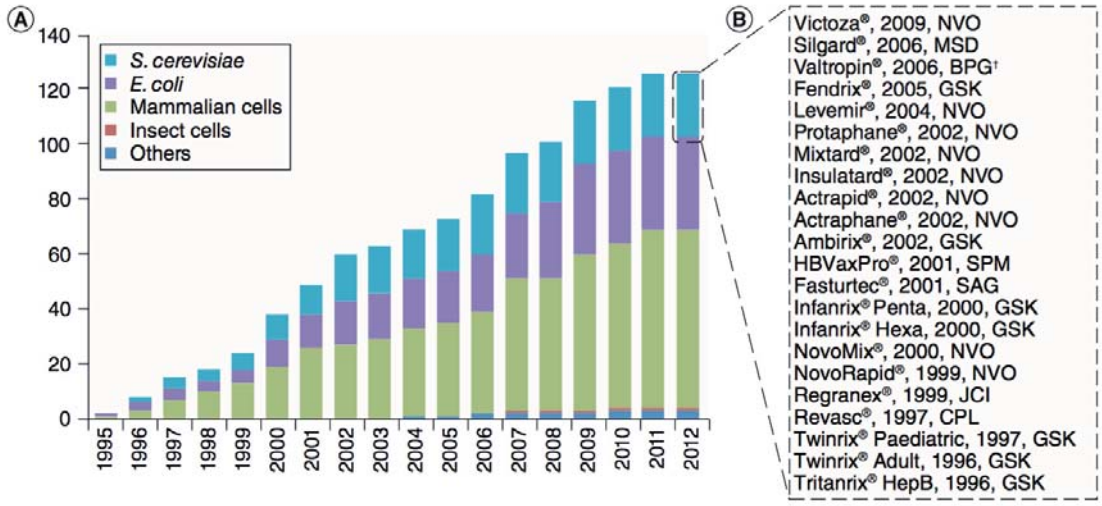

Figure 1.3: (A) Cumulative number of recombinant biopharmaceuticals proteins approved by the European Medicines Agency and the distribution of the protein production platforms used. (B) Biopharmaceutical proteins produced in *S. cerevisie* approved by the European Medicines Agency, authorized year, company. Figure taken from [14].

For the industrial enzyme market, in which the 90% of proteins are expressed recombinantly [17], *Bacillus* species is considered the workhorse of industrial enzymes producers [33]. About 60% of the commercially available enzymes are produced by *Bacillus* sp, which most of them are homologous proteins naturally secreted in the growth medium [34]. Bacteria, yeast and filamentous fungi are also commonly used for the production of industrial recombinant enzymes. In addition, both and transgenic animals and plants are currently used for producing a small fraction of the total enzymes in the market [4]

1.4 Yeasts as a powerful recombinant production platform and *Pichia pastoris* as emerging host cell system

Yeasts are widely used in traditional and modern biotechnology for the production of food, wine, beer, a broad range of biochemical products and, of course, production of recombinant proteins. The first example is dated back to around the year 6000 BC, when there is evidence for the production of the first fermented beverages in today's Iran [35].

As was briefly introduced in the previous section, yeasts can be a very versatile and efficient expression system for recombinant protein production processes. As eukaryotic microbes they combine the eukaryotic ability for protein processing such folding, assembling and introducing PTMs, with important bacterial advantages such ease gene genetic manipulation and capacity to grow fast up to very high cell densities in chemically defined media. In addition, yeasts are able to secrete recombinant proteins to the extracellular medium, which is very appreciated in downstream processing [36]. Since yeasts were implemented as recombinant protein platform in 1981 using *Saccharomyces cerevisiae* [37], lots of eukaryotic proteins have been expressed and commercialized obtaining higher product yields respect to other more complex expression system such fungi or mammalian cells. This fact is mainly due to the better scalability of yeast-based bioprocesses [25].

S. cerevisiae is a yeast well known as a beer and bread producer. Besides to be the first yeast host for recombinant protein expression, is also the most common yeast system used for producing therapeutic proteins [12]. Since Novo Nordisk could approve their recombinant insulin as the first biopharmaceutical protein produced in yeast in 1986, dozens of other therapeutic proteins such hormones (insulin and human growth hormone), vaccines (hepatitis B, human papilloma viruses) and therapeutic adjuncts (human serum albumin) produced by *S. cerevisiae* entered the market [38]. Still nowadays Novo Nordisk is the leading producer of insulin and insulin analogues using *S. cerevisiae* with a 44% of market share [14] (Figure 1.3). One of the major drawbacks of this yeast producing biopharmaceuticals is the glycosylation pattern, which may differ substantially from the performed by mammalian cells, a difference that could limit the therapeutic use. N-glycosylation in yeast, for example, is of the high mannose type whereas human N-glycans are mainly of the complex or hybrid type. In addition to N-glycosylation, yeast O-glycosylation characterized by shorter glycan structures, also differs from the human type [39].

Besides *S. cerevisiae*, in the last years other yeasts have been developed into alternative recombinant protein expression systems presenting some clear advantages respect to their predecessor, from which stands out *Pichia pastoris* [40], the recombinant protein production platform that has been studied in the present thesis. It is important to mention that the

number of articles published about recombinant gene expression in *P. pastoris* is significantly higher than in *S. cerevisiae* (Table 1.1) [2,22].

P. pastoris is currently one of the most effective and versatile systems for the expression of heterologous proteins [41]. This methylotrophic organisms was developed as a host system in 1985 by Cregg et al. [42]. Shortly after, in the 1900s, was established the first large-scale industrial production processes of plant-derived enzyme (hydroxynitrile lyase from the tropical rubber tree *Hevea brasiliensis*) obtaining more than 20 g or recombinant protein per litre of culture volume [43]. As *S. cerevisiae*, is considered a GRAS organism since it does not generate detectable levels of endotoxins. *P. pastoris* presents all the previous mentioned advantages of yeasts producing recombinant proteins such ability for protein processing (folding, assembling and introducing PTMs), ease genetic manipulation, capacity to grow fast up to very high cell densities in chemically defined media and ability to secrete recombinant proteins to the extracellular medium combined with low levels of secretion of native proteins. If it was necessary, recombinant protein production could be also driven intracellularly.

In addition, *P. pastoris* as a host also offer substantial advantages respect to *S. cerevisiae*, which are mostly listed below:

- Availability of strong promoters that regulates the expression of the desired protein allowing high production yields. The different alternatives of strong promoters on *P. pastoris* will be compared in following paragraphs.
- *S. cerevisiae* is a highly fermentative Crabtree-positive yeast, and thus able to produce ethanol aerobically in the presence of high concentrations of glucose, which reduces the production of biomass. In contrast, *P. pastoris* is Crabtree-negative; it is more sensitive to the availability of oxygen than to variations in substrate concentration. Consequently its growth on glucose is considered non fermentative due to that the uptake under aerobic conditions is limited, thus avoiding the metabolic overflow that generates ethanol [44].
- Whereas in *S. cerevisiae* is still dominant the use or vectors that are episomally replicated, in *P. pastoris* the recombinant gene of interest is integrated in the genome's host, which is a method significantly more stable [45]. In addition, an increasing collection of molecular tools are available, and many more are being developed in order to obtain high level expression of recombinant proteins [46].
- *P. pastoris* secretion pathway is more similar to higher eukaryotes rather than *S. cerevisiae*. Organelle structure and proliferation are different [47]. Also the regulation pattern of Unfolded Protein Response (UPR), a very important mechanism

that controls the protein folding, presents significant differences between these two yeast species [48]. Moreover, by cell engineering, successful developments has been recently achieved by overexpression or deletion of assisting proteins in order the increase the success in protein folding and secretion [49,50].

– The processing and secretion pathways of *P. pastoris* introduces into recombinant proteins less elaborate hyperglycosylation than *S. cerevisiae*, and therefore more similar to mammalian cells [51]. In addition, only *P. pastoris* has been successfully glyco-engineered, the humanized N-glycosylation pathway has replaced the native pathway in order to generate a set of strains producing proteins with human glycosylation patterns. However, engineering of the O-glycosylation pathway is not as advanced [39].

This expression host also presents some disadvantages such the incapacity for processing correctly complex proteins of high eukaryotes. Particularly, lower yields are obtained when the complex proteins expressed are hetero-oligomers, membrane-attached or prone to proteolytic degradation [52]. Moreover, *P. pastoris* present also two other important drawbacks, the native glycosylation patterns is different to mammalian cells and the use of methanol as a carbon source for some cultivation strategies supposes an important safety risk from an industrial point of view [53]. An overview of the advantages of *P. pastoris* as a host expression system is presented in Table 1.3.

Table 1.3: Summary of the advantage of the use of *P. pastoris* as a recombinant protein expression system.

Genetic Engineering	Protein processing	Bioprocess engineering
Simple and stable genetic manipulation	Eukaryotic capacity for folding, assembling and performing PTMs	GRAS organism, lacks detectable endotoxins
Numerous tools available for genetic manipulation	Protein processing and secreting pathway similar to high eukaryotes	Non-fermentative growth on glucose
Different strong and efficient promoters	Ability to secrete efficiently target proteins combined with low levels of secretion for native proteins	Growth in chemically defined medium up to 120 gDCW L-1
Reported high yield and stable production strains	Reduced hypergliscosilation and reported human-like glyco-engineered strains	Well established large scale production and downstream processing

As was mentioned above, *P. pastoris* is a methylotrophic yeast able to grow on methanol as sole carbon source. This is enabled by the activity of two alcohol oxidase (*AOX*) genes (*AOX1*,

AOX2), both having a strong methanol-inducible promoter (P_{AOX1}, P_{AOX2}) [54]. Therefore, in order to exploit the regulation advantages of these genes, in most of the recombinant protein expression studies using *P. pastoris,* the gene encoding the recombinant protein is placed under the tight control of the promoter of the *AOX1*, which accounts for the majority of alcohol oxidase activity (90%) [55].

Although this protein production strategy allows obtaining very high product titres in a cheap strategy, presents very important disadvantages for industrial scale bioprocesses associated with the storage and delivery large quantities methanol, which is a fire hazard [53]. In addition, as a consequence of the important impact that supposes to the cells the consume of methanol as a carbon source, very important increases up to 10-fold of heat production and oxygen requirements of the processes have been reported [56,57], which is represents a very drawback from an industrial point of view. Also other effects have been reported on *P. pastoris* cultures growing on methanol, such a significant increment of cell lysis, and subsequently release of native protein and proteolysis of the secreted product of interest [44] and the accumulation of by-products of methanol metabolism, such as formaldehyde and hydrogen peroxide [58]. All the mentioned negative consequences of using methanol as an inducer and carbon source of the cultivations has given ground to the need to develop a new strategy that exploits the advances of *P. pastoris* as an expression system, while at the same, avoids the very important drawbacks associated to this flammable substance.

As an alternative methanol-based expression system, in 1997 Waterham et al. proposed the used of the glyceraldehyde-3-phosphate dehydrogenase promoter (*GAP*, P_{GAP}) for constitutive expression of recombinant proteins in *P. pastoris* [59]. The *GAP* gene is involved in the glycolysis, in which the enzyme synthetized is an essential key factor of the metabolic pathway [60,61]. Consequently P_{GAP} regulates the levels of protein production leading to strong and constitutive gene expression when the yeast grows on glucose, glycerol or other carbon sources as a substrate (including also methanol) [59]. Several studies have reported that P_{GAP} is more efficient than P_{AOX1} [62–64], whereas others showed opposite results [65,66]. Thus, it appears that expression levels achieved for a given protein using different promoters vary significantly based on properties of the expressed protein. Additional research would be required to determine which factors impact the efficiency of both promoters [41]. Actually, an eventually low yield of protein production for some specific proteins obtained working with P_{GAP} in comparison with P_{AOX1} is the only drawback described for this protein expression system so far [61]. Therefore, the advantages in large scale production derived from avoiding the use of methanol, which were mentioned above and discussed in other works [56,57], makes the P_{GAP}-based platform a very promising alternative for industrial recombinant protein production using *P. pastoris*.

Invitrogen commercializes plasmids able to integrate in the genome of *P. pastoris* gene sequences under the regulation of P_{AOX1} or P_{GAP}, such as the pPICZα and pGAPZα vector series (www.invitrogen.com). Some of these commercial vectors also include the α-factor secretion signal for *S. cerevisiae*, polyhistidine tag for purification, Zeocin resistance gene as a selection marker and other important features essential for the genetic engineering of *P. pastoris*. Also, different strains with phenotype are commercialized in order to allow the generation of cell factories with different characteristics (Figure 1.4). The commercial use of Invitrogen products is subjected to the pay of royalties, however other sources for vectors and strain are also available [52]. In addition, currently other genetic engineering tools and strains royalties-free are being developed and obtained by research groups and companies.

Table 1.4: Different strains offered by Invitrogen. Table taken from [67].

***Pichia* strain**	**Genotype**	**Purpose**
X-33	wild type	Expression of recombinant proteins from vectors with Zeocin resistance as the only selectable marker.
GS115	his4	Expression of recombinant proteins from vectors containing the HIS4 or Zeocin resistance gene as the selectable marker(s).
KM71	his4, aox1:ARG4	Expression of recombinant proteins from vectors containing the HIS4 or Zeocin resistance gene as the selectable marker(s) in a MutS background when using methanol induction for expression.
SMD1168	his4, pepA	Protease deficient strain for the expression of recombinant proteins from vectors containing the HIS4 or Zeocin resistance gene as the selectable marker(s).

As was described in Table 1.1 [2], the use of *P. pastoris* as a protein recombinant expression system is growing during the last years. Currently over 500 recombinant proteins have been expressed in *P. pastoris* systems with expression levels as high as 80% total secreted protein or up to 30% total cell protein [41]. The range of application fields is very wide, from industrial enzymes (recombinant nitrate reductase), to animal feed additives (recombinant phytase) and biopharmaceutical proteins (www.pichia.com). In the last area, several products from *P. pastoris*, such as human serum albumin, insulin, IFN-a and hepatitis B vaccine are in the market in India and/or Japan. In 2009, the US FDA approved the first recombinant protein produced with *P. pastoris* (Kalbitor®, Dyax, MA, USA) [68]. Recently another biopharmaceutical produced in *P. pastoris,* Jetrea®, has been recently approved by the FDA and the European Commission [52]. Several more therapeutic product candidates that are produced with this yeast are still in the clinical pipeline [25,69].

In the last years, in addition to its use as a powerful recombinant protein production host, *P. pastoris* has emerged an efficient cell factory for the production of biorenewable fuels and chemicals [70,71]. The use of microbial fermentation platforms from cellulosic biomass as a renewable feedstock has become a desirable alternative to petrochemical processes for the

production of fuels and chemicals towards a circular sustainable economy [72]. These bioprocesses require the engineering of biocatalysts that can quickly and efficiently convert sugars to target products at a cost that can be competitive with the existing petrochemical production processes [73]. A wide range of products can be obtained using yeast fermentation technology including a variety of metabolites such as amino acids, nucleotides, organic acids, carbohydrates, lipids, vitamins, solvents, antibiotics, etc. [8]. Different successful microbial production processes has been reviewed in recent articles [74,75].

The genetic engineering tools and bioprocess engineering know-how developed in *P. pastoris* can be exploited also in this application field. As an example, it has been already reported the engineering of a metabolic pathway in order to make the cells be able to convert glycerol, considered a byproduct of biodiesel production processes, to propylene glycols such 1,2-propanediol, 1,2-dihydroxypropane, which are valuable fuel additives and commodity compounds [76].

1.5 Current state and innovative developments for improving the recombinant protein production processes applied to *Pichia pastoris*

A bioprocess is a specific process that uses complete organisms or their components to manufacture new products and/or destroy harmful wastes [77] . The wide range of applications fields and the increasing demand of recombinant proteins of both therapeutic and industrial interest has become their industrial production into one of the most successful and profitable bioprocesses [36]. As any other production process, to be implemented on an industrial sale, a recombinant production process must be highly productive, efficient, economic and sustainable [8]. However, despite their increasing popularity, these bioprocess have not reached yet the same maturity as traditional chemical processes [78]. Improvements in both, the overall yields and quality of the products, are needed to reduce the costs and therefore increment their accessibility in low and middle resources countries [79]. Nowadays important advances have been achieved in both host cell engineering as well as in fermentation engineering, in order to increase the productivity and efficiency of the recombinant production processes [61]. For the recombinant protein processes, fermentation engineering is commonly generally considered as bioprocess engineering, which deals with the design and study of the fermentation as a production process. Other technological advances such process monitoring and control of critical process parameters and quality attributes of the bioprocess by using process analytical technology (PAT) and procedures of downstream purification are also very important to increase the yield and the quality of the product in a real bioprocess [80,81]. However, this thesis will be only focused in the bioprocess engineering advances applied to the constitutive expression of recombinant proteins in the yeast *P. pastoris* regulated by the *GAP* promoter.

During the development of a bioprocess design must be taken into account that an efficient production of a recombinant protein requires the integration of different elements that influence each other crosswise and cannot be considered alone.

1. Genetic engineering tools that allow the protein of interest expression in the ensuring efficient transcription, translation and desired localization of the product.
2. Understanding of the host cell physiology towards reaching the highest recombinant protein production yield and quality.
3. Fast, cheap, efficient and reproducible fermentation processes that allow obtaining enough product quantity to supply the market demand in a competitive price.

1.5.1 Genetic engineering tools and advances towards a more efficient expression

Many factors related with the gene expression have been studied in *P. pastoris* to elucidate their influence in the production of the recombinant proteins efficiency. Some examples are listed below: selection of the promoter to achieve high expression levels and its genomic integration [46,52,82], use of different signal peptides for efficient protein processing and secretion to the extracellular space [83], gen dosage [84], codon usage [85] and many others. The combination of all the mentioned factors for the design of the genetic manipulation of *P. pastoris* strains probably will lead to important increases in the expression levels of the protein of interest (Figure 1.4).

Also related with the genetic engineering in *P. pastoris* processes, very important clonal variability has been widely reported for the generation recombinant strains. This fact is inherent in the transformation protocols, in which the expression cassettes are integrated in the genome of the host by means of recombinant events that cannot be directed controlled [86]. Therefore, the screening of the "best producer clone" has become a critical aspect during the development of a bioprocess. The most important advances for the clone screening procedures have been extensively reviewed recently [87].

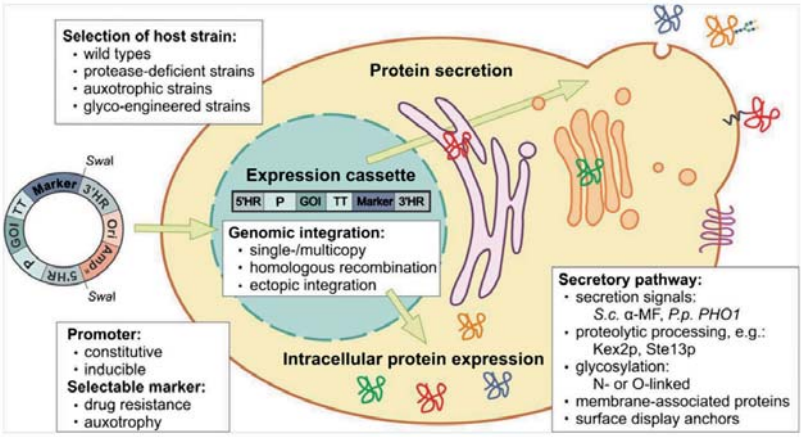

Figure 1.4: General considerations for heterologous gene expression in *P. pastoris*. Figure taken from [52].

1.5.2 Understanding the host cell physiology and its engineering development

The knowledge of physiology of the host cell is also very important and complementary to the genetic engineering for increasing the specific production rates of a desired product. At the same time, it must be also very closely related to the next bioprocess design step, the bioprocess engineering, which requires a deep understand of the host cell physiology in order to that the fermentation provides the best environment to the cells for producing the protein of interest. This aim is often hampered by the lack of knowledge of the production pathways and its dynamic profile in the producing cells. Therefore, deeply physiological studies are required for achieving a successful recombinant protein production process [23]. Actually, improved cultivation strategies are usually rationally designed from the physiological characterisation of producer strains [87]. In this respect, it is very important to identify the limits in productivity of the host cells, which can not only be attributed to just an stoichiometric drain of energy from biomass formation towards product formation [88].

For *P. pastoris* as a host have been reported several physiological limitations that are often considered bottlenecks in the heterologous protein production. Generally, the major limiting factors considered by most of authors are those related with protein processing such folding, assembling, performance of PTM's and secretion (Figure 1.5). Recently have been published excellent reviews about this important bottleneck [49,89,90]. The over expression of recombinant proteins often results into an overload of the endoplasmic reticulum (ER) folding and secretion capacity, thus leading into an accumulation of unfolded or misfolded protein in the ER that triggers the unfolded protein response (UPR). This pathway induced the synthesis of genes involved in protein folding (chaperones) [50], but also ER-associated protein degradation, which have an important impact lowering the yield of recombinant protein of interest [91,92]. Metabolic limitations are also considered a reason for suboptimal productivity due to metabolic burden caused by recombinant protein production [56]. In this sense, for cells producing recombinant proteins recombinants, have been described important effects on the metabolism regulation such increase of tricarboxylic acid cycle (TCA) flux and ATP production [93]. In addition, it has been also reported a reduction of the recombinant protein expression caused by the limited availability of certain amino acids, which its *de novo* synthesis is energetically costly [94].

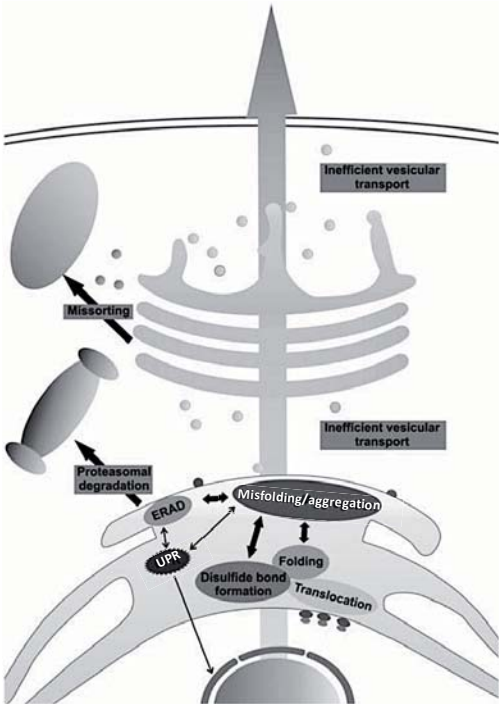

Figure 1.5: Bottlenecks encountered by recombinant proteins on their way through the secretory pathway in *P. pastortis*. Figure taken from [50].

In the last years, the –omics technologies, including genomics, transcriptomics, proteomic and metabolomics, together with the information obtained from the genome sequencing [44], has allowed to increase importantly the physiological knowledge of the *P. pastoris* [95]. Currently systems biology is able to integrate all the available information into models that offer the opportunity to address ideally all metabolic processes of a cell, including, for example, the interplay among protein production, energy demand and biomass formation [96]. This information is considered highly valuable in order to predict targets for host engineering to enhance the productivity of the bioprocesses (Figure 1.6) [97].

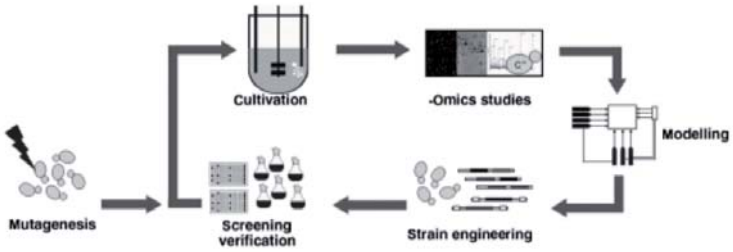

Figure 1.6: The systems biology circle for host engineering development. Figure taken from [95].

Successful examples of *P. pastoris* cell engineering to overcome the bottleneck production related to protein processing and secretion and others can be found in the literature. As an example, Gasser et al. reported very important production increases (up to 2.5-fold) by overexpressing several secretion factors that were previously identified by transcriptomic analysis [98]. As other example for other bottlenecks, different authors have reported several disruptions of different protease genes. However, so far, the influence of the different process is difficult to predict [90].

1.5.3 Fermentation technology and bioprocess engineering developments

In Biotechnology, during the last years, several authors have claimed the need of strategies and workflows for systematic bioprocess development from a bioprocess engineering point of view [99]. This is could be due to the scientific community, in most of cases, have been focused on the investigation of the proteins and organisms of interest rather than continuing developing the production processes [100].

Fermentation processes have been used for production and conservation of food for thousands of years [78]. However, the role of microorganisms was not elucidate until late 1800's Louis Pasteur carried out extensive physiological studies of fermentation by intact living yeast cells and later, in 1897, Eduard Buchner, achieved fermentation by cell-free extracts [101]. Shortly after, in the early 1900s, the microbial fermentation industry was developed leading to the first large-scale anaerobic fermentations to manufacture chemicals such as acetone and butanol, as well as aerobic fermentations for citric acid production [1]. Nowadays, in addition to the food applications, fermentation processes are increasingly used for the industrial production of bulk chemicals, fine chemicals and pharmaceuticals [78].

Although the current increasing interest of *P. pastoris* as a cell factory for different production processes [2], the fermentation technology developed for this yeast is still far from achieving the maturity of other common organisms used in microbial fermentation industry such *E. coli* or *S. Cerevisiae* [87]. Consequently, it is currently required the development of methods that allows increasing the yield and productivity of the *P. pastoris*-based bioprocesses. Especially, for the constitutive expression systems, since most of production improvement strategies have been developed so far for the methanol-regulated system based on *AOX1* promoter [61,87]. Thus, it is often considered that the development of cultivation strategies for production processes regulated by the constitutive *GAP* promoter is still in its infancy. As others production processes, a fermentation aims to produce the maximum amount of product in the minimum process time (space-time-yield, STY; volumetric productivity, Q_P), which requires to reach the maximum amount of biomass (X) growing with a high specific production rate (q_P) in the minimum process time to harvest a product with the desired quality specifications [87].

Generally two operational modes for fermentation processes are commonly used for manufacturing the wide range of products generated nowadays by different cell factories. In order to be commercially viable, any cultivation method has to meet crucial criteria. These include a high volumetric productivity, a high final product concentration, highly soluble substrates, applicability at low-cost as well as high stability and reproducibility of the process [102].

– Fed-batch cultivation are used to extend the cultivation time by feeding the selected substrate(s) [102]. Usually these cultures are divided into two phases, batch phase and fed-batch phase. Initially, in the batch phase the producer strain is cultivated in an initial batch medium to generate a moderate amount of biomass. Usually during this first step the cells grows at their maximum specific growth rate until the carbon source depletion (or any other limitation), which often can be identified with a sudden increase of dissolved oxygen concentration (pO_2) [103]. Subsequently starts the fed-batch phase, which its main concern is the cellular proliferation. This can be achieved by the feeding of the substrates necessary for the yeast growth, carbon source, and a nitrogen source, together with the necessary minerals and trace elements (Figure 1.7) [61]. The cultivation volume often increases significantly. Different feeding profiles can be used for the addition of substrate, which will determine the biomass growth and the behaviour of the cultivation [104].

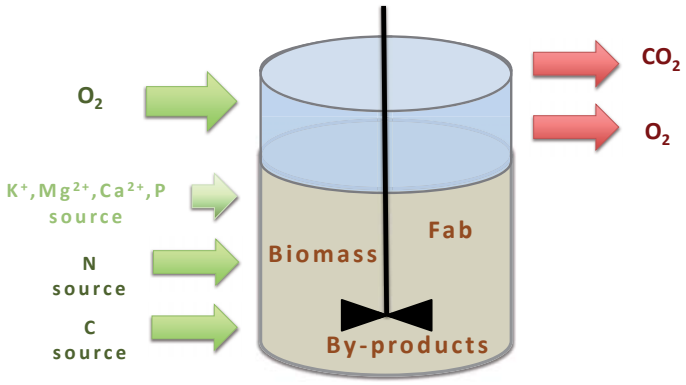

Figure 1.7: Fed-batch cultivation scheme. The cultivation volume increases due to the feeding of nutrients.

– Continuous cultivations, also called chemostat, is a culture in which fresh medium including the needed substrates is continuously added, while culture liquid containing left over nutrients, metabolic end products and microorganisms is continuously removed at the same rate to keep the culture volume constant (Figure 1.8) [105]. This operation mode also starts with a batch phase equivalent to the described above for fed-batch cultivations. Therefore the feeding and extracting bombs start the continuous operation. It can be considered that a chemostat operates in steady state conditions when the specific growth rate of the cells equals the dilution rate. Such equilibrium is further assumed to be established when the properties of the culture are constant at any point of the reactor, and when the macroscopic growth parameters and metabolic activities can be kept constant [106]. Consequently, since cells grow in a strictly controlled environment, beside the potential product manufacturing applications, continuous cultivations are considered a highly valuable tool for the physiology and systems biology studies. By means of this cultivation strategy, the cellular responses to a particular perturbation (oxygenation, temperature or osmolarity effects) can be analysed while keeping the

other parameters constant (pH, agitation, nutrients, etc.) avoiding possible fluctuations of environmental and culture parameters [107].

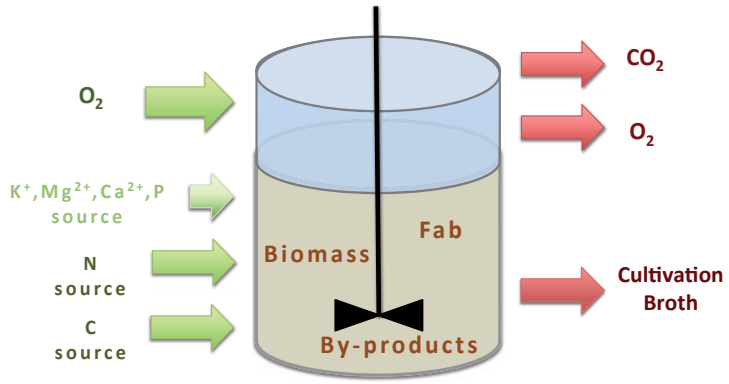

Figure 1.8: Continuous cultivation scheme. The cultivation volume is constant.

P. pastoris cultivations in industrial processes are often carried in large size stirred tank bioreactor (up tp $10m^3$) achieving very high cell densities in order to obtain the maximum amount of product [23]. Besides de commercialization of *P. pastoris* strains and vectors, Invitrogen have overviewed some basic guidelines as a starting point for the development of lab-scale fed-batch fermentations [103]. Other fermentation protocols based in the mentioned Invitrogen guidelines have been also published by other authors [108,109].

Due to the important consumption rates of oxygen during the growth, the aeration requirements are high, and in turn, also electricity consumption. The yeast metabolism generates large amounts of heat; therefore efficient cooling systems are also needed. Actually, oxygen transfer and heat removing capacity are most often considered the limiting factors of high-cell density [110]. Both factors are dependent on the specific growth rate and it is especially dramatic in the production processes regulated by *AOX1* promoter, in which the utilization of methanol as a carbon source and inducer increase, strongly, the oxygen requirements and heat production [56]. Another important technical limitation is considered the maximal biomass concentration, which as was also observed in the present work, should not exceed 100-120 g L^{-1} of dry cell weight (DCW). However, some authors reported biomass concentrations up to 150 or 200 g L^{-1} of DCW both in glucose and methanol-based fermentations [56]. For DCW values higher than 100 g L^{-1} important physical limitations (including heat and mass transfer, especially O_2) as well significant lack of homogeneity in the cultivation broth have been observed during the cultivations performed in this work. In addition, biomass separation from high cell density cultures is a challenging task in downstream processing [81]. Therefore, it is very important that the bioprocess design takes into account the technical limitations of the equipment. Usually, the main expenses of yeast fermentations are related to the costs of substrate and energy. However, taking into account the overall bioprocess, the purification costs represent a very high percentage of the total cost of bioproducts, especially those high added value [23].

In general, the efficiency of the fermentation processes is importantly influenced by operational parameters including temperature (T), pH, osmolality, dissolved oxygen concentration (pO_2) and medium composition. However, excluding pO_2, the selection of the optimal set-point as well as the control of these parameters along a cultivation often considered straight forward. The best ranges of use are widely known, extensively reviewed and used by most of authors [61,111]. In the present thesis, since they were not pretended to be object of study.

Nowadays, the published conventional fermentation procedures mentioned above [103,108,109] should be considered obsolete from a modern bioprocess engineering point of view. These protocols only aim the generation of a moderate amount in the batch phase and the implementation of the simplest feeding strategy during the fed-batch phase, constant feed rate, which is not designed in order to cover the physiological requirements of the cells. In contrast, the current trends in bioprocess engineering are being directed towards a conceptual approach that aims the design of processes based on the properties of both, the product and the physiology of the host cell system as well as to the characteristics of the bioreactor equipment available [112,113]. In order to reach a deeper understanding that allows the rational design of bioprocesses it is very important that the information obtained in specific study cases can be suitable to be applied in other similar bioprocesses. Therefore, the use of parameters independent of process-specific settings such as μ, q_S, q_P or yields should be selected for implementing innovative bioprocess strategies instead of others non comparable between different systems such biomass concentration, reactor volume, process time or addition feed rates, which have been often published in works towards the development and optimization of the fermentation technology [87].

As said before, for enhancing the efficiency of a bioprocess the physiology of the cell factories must be taken into account as determining factor for both yield and productivity optimization. Microbial product formation kinetics is dependent on many factors, which can affect importantly the rate of product formation. These include the environmental growth conditions that are usually controlled (temperature, pH, osmolorarity, dissolved oxygen concentration or medium composition, etc.), but also the metabolic state of the cell (the energy charge, the redox status, the presence of competing metabolic pathways, etc.). The specific growth rate of an organism reflects to a certain extent the metabolic state of the cell. Therefore, it is not surprising that direct correlations between the specific rate of product formation and the specific growth rate can be found [105]. The relationship between specific production rate (q_P) and specific growth rate (μ), also termed “production kinetics”, reflects the equilibrium between all processes that take in the place in the cell during the product formation. It includes induction of gene expression, translation, protein folding and degradation in the ER, flux of folded protein out of the ER, and secretory trafficking through

the secretory machinery [87]. Therefore, the interdepence between q_P and μ for the host strain must be crucial in the design of the bioprocess [114].

Several production kinetics have been reported for the production of recombinant proteins expressed in different host cell systems and specifically also in *P. pastoris.* The trends can be grouped in different groups: increasingly or decreasingly linear, monotonic increasing (hyperbolic) or non-monotonic (bell-shaped) [115–120]. Since the experience with *P. pastoris* as an expression host system is still limited, it is important to take into account that the production kinetics of a product is not predictable *a priori* and has to be determined empirically [118]. In this sense, important effects of the host physiology have been reported due to genetic burdens introduced by strain engineering and/or metabolic burdens of recombinant protein production [56,121].

Once the production kinetics have been elucidated, high-performance process strategies regarding the optimal biomass growth rate trajectory can be implemented in the different cultivation strategies [102]. For chemostat cultivations, since it works in steady stationary conditions, the application of the desired specific growth rate can be considered straight forward. In contrast, in fed-batch, in which the process conditions are dynamic, the determination of the optimal feeding profile is much more challenging. Reaching the desired specific growth rate of the biomass can be relatively easily achieved by the implementation of the widely described exponential fed-batch feed-profile (Figure 1.9) [112]. However, the technical limitations observed in fermentations at very high cell densities related oxygen, heat transfer and maximum biomass concentration is leading to the develop of customised feeding profiles towards to maximise the product titre and space-time-yield (STY) [115,117,122]. Therefore, the development on innovative fermentation strategies often has to deal with a competition between the formation of the product and the biomass growth, which limits the process time [102]. Actually, in many of the well-established large scale industrial fermentation processes, the product formation is the goal, and biomass is considered as an inevitable waste product [105].

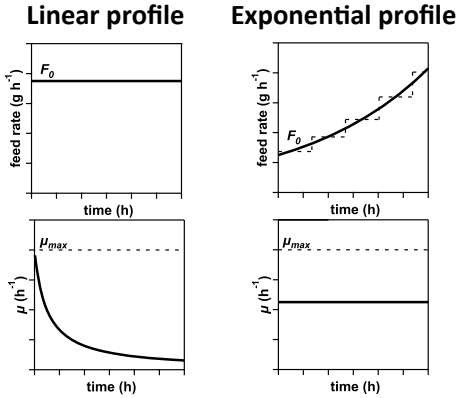

Figure 1.9: Example of different feeding profiles and its effect on the specific growth rate. Figure taken from [87].

Also, as a bioprocess engineering development based on the host cell physiology, the activation of cell stress responses is considered a very interesting way towards a more efficient and cost-effective production processes [14]. The development of these strategies is due to several cases reported in which the increasing cell stress may couple with overexpression of protein [69].

Detailed transcriptomic and proteomic studies of cellular reactions to environmental stress factors have been performed with different microorganisms, among them *P. pastoris* and *S. cerevisiae* [123,124]. In *P. pastoris* cultivations the effects of different media osmolarity and oxygen supply were compared at both transcriptome and proteome levels, revealing strong transcriptional and expression regulation of core metabolic genes, and in turn, also the recombinant protein of interest [123–125]. Similar positive effects of the oxygen limitation have been recently published for *S. cerevisiae* [126,127]. Similarly, for *P. pastoris and Hansenula polymorpha* have been reported that periods of carbon starvation along cultivation induce a considerable stress to host cell. This environmental stress may be couple with an important increase in protein production upon glucose depletion short times [128,129]. Therefore, a systematic study of different environmental stress effect on the cellular machinery of cell factories can lead to the development of innovative cultivation strategies that aims to exploit the positive effects of the cellular stress response on the production of recombinant proteins of interest.

On the other hand, other stress factors non-directly related to environment can be exploited in order to enhance the efficiency of recombinant protein production processes. As was described in the section 1.5.2, the expression of recombinant proteins often results into an overload of the ER folding and secretion capacity. Consequently, the accumulation of misfolded proteins in the ER results into a stress situation that triggers the UPR and in turn, the expression of chaperones that help to process correctly the misfolded proteins [48]. Therefore, the use of engineered strains that overexpresses certain chaperones upregulated by the UPR in order to increase the recombinant protein production can be also considered a bioprocess development based on the cell response to stress.

In order to achieve process improvements in the industrial production of recombinant protein production, the scaling up of fermentations is currently considered one of the most important bioprocess engineering challenges [78]. Along a bioprocess development, large-scale performance losses respect to the initial lab-scale results is not unusual. For industrial microbial fermentations, publications in the field have reported significant performance losses (10-30%) in terms of biomass formations, product substrate yield and productivity [130].

Scaling up a fermentation process aims to transforming the optimal operating conditions found in lab-scale or pilot plant-scale bioreactors to the industrial-scale bioreactors in order to reach the maximal volumetric productivity of the production-scale process [78]. Most of authors in the literature focused on scaling up fermentations often consider the mixing differences between scales as a very important cause of the commonly observed performance differences [130–132]. Thus, while lab-scale bioreactors are considered perfectly mixed, the lack of homogeneity is considered an important problem in large-scale cultivations. As a result, spatial gradients of important parameters such dissolved gases, pH, temperature, concentration of substrates and other are all likely to emerge leading to a potential conditions of oxygen or other essential nutrients starvation [133]. The spatial heterogeneity, in turn, lead to important difficulties for monitoring and control large-scale cultivation, due to the process sensors only can display an average of the whole system [78]. As mentioned in previous section, these environment conditions may suppose stress conditions for the cells, which can often affects importantly the biomass growth industrial-scale bioreactors. In addition, the difference of mixing often also leads to significant differences in mass and heat transfer in the processes, which are also considered limiting factors in large-scale [134].

The development of scaling up processes should aim the improvement of mixing quality in larger scales to ensure homogenous reaction conditions and to reduce both, the size of stress zones and the zonal residence times [131]. The advances in the field have been usually based on empirical approaches rather than mathematical process model nor design of experiments [78]. The conservation of some specific parameters across scales has been used as guidelines to achieve success. The most commonly selected to maintain constant are: geometry (stirrer and reactor dimensions), maximum stirrer tip speed, volumetric power input, Reynolds numbers for the stirrer, oxygen uptake rates, k_La or minimum dissolved oxygen concentrations, aeration numbers or superficial velocities, shear stress and/or mixing times [132]. However, it is not possible to conserve all the parameters across scales, so the final criteria for the scaling up should be formulated as a trade-off between them [135].

In addition, currently different bioprocess engineering tools are being used to improve the scaling up processes from which stand out computational fluid dynamics (CFD) and scaling down. CFW can provide a detailed characterization of the hydrodynamic conditions for investigation and description of gradients and oscillations of substrate concentrations (Figure 1.10) [131]. The scale down consists in being able to study in lab-scale reactors the effect of relevant process parameters that have an effect in the large-scale performance. Consequently, once suitable down-scaled versions of the full-scale reactors are available, the interest can be focused only in maximizing the volumetric productivity of the process [78]. Scaling up a fermentation process, searching a compromise between the different critical parameters discussed, the chances to reach success are maximized. Therefore, by avoiding

the usual performance losses of large-scale cultivation, important productivity increments can be achieved in industrial recombinant protein production processes.

Novel bioprocesses are initially developed at lab-scale, from which results is evaluated its economic viability. Consequently, impairments of product amounts, qualities and yields experienced in large scale immediately affect the anticipated economic benefits [132]. Therefore, the development of scaling up bioprocesses in order to prevent efficiency and productivity loses is not only and academic goal, but also an economic necessity [136].

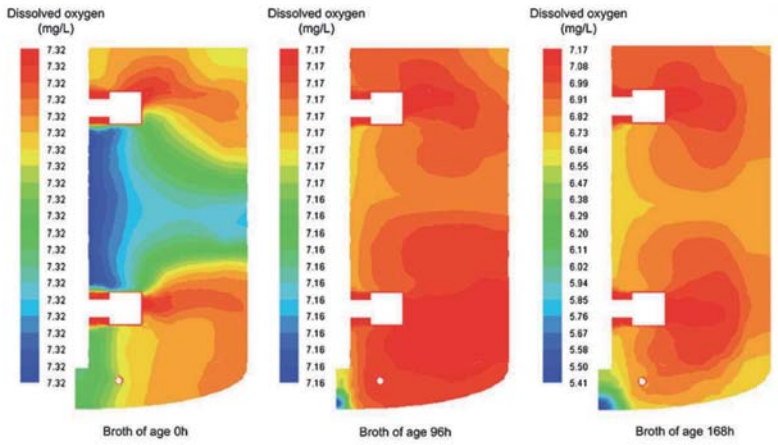

Figure 1.10: CFD-predicted pO_2 profile at 100 rpm simulated for 10 min after the introduction of oxygen gas phase at different fermentation times of a filamentous fungi. Figure taken from [137].

Another interesting way for improving the efficiency of bioprocess could be the selection of a different operational mode, which is usually decided at the very beginning of the production process development [99]. Nowadays, most of protein production processes using yeast as cell factories are still carried out with fed-batch fermentations, which allow reaching very high cell concentrations, thus also obtaining very large amount of product [23]. However, such in other industries, the current trend is leading to the transition from batch to continuous manufacturing in order to exploit the benefits of the continuous production such flexible operations, higher productivity and quality, decreased cost, smaller facilities as well as the integration and the simplification of the processes (Figure 1.11). However, the use of this cultivation mode is still its infancy and present important drawbacks including losses of productivity due to genetic instabilities of the cells, higher risk of contamination, lack of homogeneity in the reactors and poor short-term flexibility to handle multiple products, due to long run times [138–140].

Continuous cultivations are currently being used for industrial recombinant human insulin production processes, which are carried out using *S. cerevisiae* as a host expression system [141]. For *P. pastoris*, also several recombinant proteins have been successfully produced in chemostat cultures for both constitutive (P_{GAP}) and methanol-inducible (P_{AOX1}) expression [117,120,142]. The change of operational mode should be considered a very effective

strategy for improving the efficiency of a bioprocess. Actually, the US FDA has encouraged the development of continuous processing for biopharmaceuticals manufacturing [138,143].

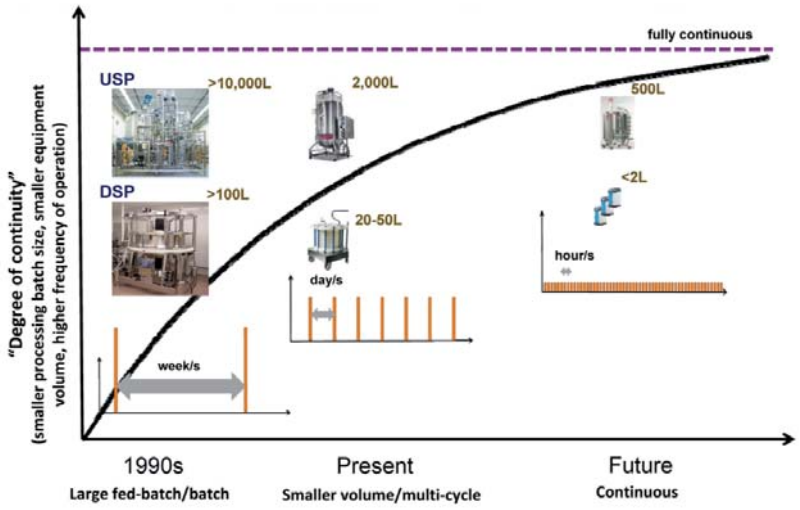

Figure 1.11: Example of the evolution a monoclonal Antibody production platform over the years. Figure taken from [139].

The different alternatives discussed for improving the efficiency of recombinant protein production using the yeast *P. pastoris* as a host expression system under the control of the GAP promoter are summarised in Table 1.4.

Table 1.4: Summary of the bioprocess engineering improvements proposed.

Bioprocess engineering improvements			
Host physiology		**Production-scale operation**	
Production kinetics	**Cell stress response**	**Scale up**	**Operational mode**
To design and of high performance process strategies regarding production kinetics (μ vs qP)	To exploit environmental stress conditions coupled with increases in the protein production rates	Optimization of key operational parameters to increase the large-scale performance of bioprocesses	Transition from batch to continuous production bioprocesses

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2

State of the art, general aims and objectives

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2.1 State of the art

It is currently considered that the fermentation processes have not reached yet the same maturity degree as traditional chemical processes [1]. Furthermore, among the wide range of host expression systems commonly used for the recombinant protein production with different applications, the use of the yeast *P. pastoris* as a production platform is still considered relatively novel respect to the workhorse systems mainly used to the date such as *E. coli, Bacillus* sp, *S. cerevisiae* and mammalian cell-lines [2]. Consequently, the operational strategies developed in order to achieve a high-performance bioprocess with *P. pastoris* are still far from achieving the maturity of the mentioned workhorses [3]. Most of the cultivation strategies to improve yields and productivities of recombinant protein have been developed so far for the methanol-regulated system based on *AOX1* promoter, which is the most widely used for *P. pastoris* due to good results obtained [4]. In contrast, although the methanol-free processes offer very important advantages from an industrial point of view, the development of cultivation strategies for production processes regulated by the constitutive *GAP* promoter is still in its infancy [5].

At the initial point of the thesis, relatively little was known and published about the developing of cultivation strategies to enhance the recombinant protein production based on the P_{GAP}-regulated expression in *P. pastoris.* Only the first studies comparing linear feeding profile and a primary design of optimized feeding profiles were published [6,7]. In these works, some essential parameters of the cultivations, such the effect of the carbon source used in both phases of a fed-batch cultivation or the effect of the specific growth rate on the productivity of fed-batch cultures, were not even properly described. This lack of knowledge gave ground to the systematic study of some crucial factors affecting the production of recombinant with this expression system.

Initially, although the research group in which has been developed the present thesis has a vast experience in *P. pastoris* fermentation, performing fed-batch cultivations at very high cell densities became quite challenging. This fact was due to the problems associated to the sample analysis of very high cell densities reached, often above 100 g L^{-1} DCW, as well as the higher specific growth rates used. In fed-batch cultivations, the specific growth rates set points used are importantly higher respect the methanol-based fermentation usually carried out. In function of the methanol-utilisation capacities of the strain used, up to 15-fold higher for Mut^S and up to 4-fold for Mut^+. It supposes very high process requirements in terms of oxygen supply and heat removal to deal with. The combination of the mentioned factors made necessary to the fermentation and analytical methods previously used.

During the development of this thesis important milestones have been reached towards increasing the physiological knowledge of *P. pastoris* including the publication of the complete genome sequence [8]. Systems biology and the "-omics" technologies have become into a new a very valuable source of data about its physiology [9]. By exploiting this techniques have been observed and reported important impacts of environmental factors on *P. pastoris* producing recombinant proteins. One of the highlights was the very important increment of the production rates observed under oxygen-limiting conditions reaching up to a 2.5-fold increase in terms of specific production rate [10,11]. It was used as a starting point in order to develop innovative cultivation strategies that exploit the potential positive impact coupled between environmental stress effects on the cells and important increases in the production of recombinant proteins.

Alternative and complementary approaches to directed strain improvement in order to increase the host cell system productivity have also experienced new developments, some of them successfully applied to yeasts, including *P. pastoris*. Such approaches are usually based on the valuable information provided by systems biology studies that predicts targets for host engineering to enhance the productivity of the bioprocesses [12]. Most of the efforts in host engineering have been focused on overcoming on of the main bottleneck of the recombinant protein processes, which is considered related with the protein folding and secretion processes [13].

The framework for the present thesis is the global study of *P. pastoris* as a host cell system for expressing recombinant proteins performed by the research group "Bioprocess engineering and applied biocatalysis", in which have been developed this project. The group has been studying the yeast *P. pastoris* for producing recombinant proteins for more than 15 years. Currently important advances are being reached at all levels, including systems biology, host engineering and bioprocess engineering. The present thesis has been the first one of the group focused in the development of operational strategies for *P. pastoris* cultivations in which the constitutive *GAP* promoter drives the expression of the protein of interest. For a high comparability of results between the different lines of the group focused on P_{GAP} processes, the same model protein, a human antigen-binding fragments (Fab) was produced. The structure of Fab fragments requires the formation of disulphide bonds and depends substantially on the availability of their multiple subunits and on the stoichiometric relation they bind to each other. Its structure becomes the Fab into very complex proteins, and thus a very important challenge for *P. pastoris* in terms of protein expression, folding and secretion.

2.2 General aims and objectives

The aim of this work was to significantly contribute on the development of high-performance recombinant protein production processes using the yeast *Pichia pastoris* under the regulation of the constitutive *GAP* promoter. Based on the host physiology but always from a bioprocess engineering point of view, several crucial factors for the protein production have been studied. Among them, highlights the impact of the production kinetics and the environmental stress effects on the cells yield and productivity of the bioprocess. Therefore, different innovative operational improvements have been designed, developed and implemented into *P. pastoris* cultivations driven by P_{GAP}.

Also, due to the important bottleneck that causes the protein folding and secretion processes, the effect of the cultivation strategy on the protein secretion capacity and the amount of protein of interest retained intracellularly have been evaluated. To carry out these studies is essential to have procedures available that allow a reliable intracellular protein of interest quantification.

In order to reach these aims, different specific objectives have been defined throughout the progress of the present thesis:

- Development of a reliable method towards an accurate quantification of the recombinant protein retained intracellularly. Specifically, will be aimed to recover the protein retained along the secretory pathway, and thus inside organelles such ER or Golgi.

- Evaluation of the carbon-source effect fermentation performance of fed-batch cultivations. Specifically, glycerol and glucose will be compared as alternative carbon sources for both phases of the cultivation.

- Study of the relationship between specific growth rate and specific production rate in both and fed-batch cultivations. Furthermore, determine a potential effect of specific growth rate in the protein secretion capacity of the host.

- Determine the impact of environmental stress factors on the host cell physiology and the protein production. Therefore, design, develop and implement innovative cultivation strategies that allow take advantage of the cellular response to stress towards enhancing the production of recombinant protein processes.

The following chapters have been written in terms of research articles that have been published or are currently submitted to scientific journal. Consequently, its structure may not have to exactly match one to one with the list of objectives defined above. However, in a next section, overview (chapter 7), the results obtained will be presented and discussed following strictly the way the objectives have been presented above.

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3

Overall performance indicator to optimizing operation of high-pressure homogenizers for a reliable quantification of intracellular components in *Pichia pastoris*

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3.1 Abstract

The most commonly used cell disruption procedures may present lack of reproducibility, which introduces significant errors in the quantification of intracellular components. In this work, an approach consisting in the definition of an overall key performance indicator (KPI) was implemented for a lab scale high-pressure homogenizer (HPH) in order to determine the disruption settings that allow the reliable quantification of a wide sort of intracellular components. This innovative KPI was based on the combination of three independent reporting indicators: decrease of absorbance, release of total protein and release of alkaline phosphatase activity. The yeast *Pichia pastoris* growing on methanol was selected as model microorganism due to it presents an important widening of the cell wall needing more severe methods and operating conditions than *Escherichia coli* and S*accharomyces cerivisae.* From the outcome of the reporting indicators, the cell disruption efficiency achieved using HPH was about 4-fold higher than other lab standard cell disruption methodologies, such bead milling cell permeabilization. This approach was also applied to a pilot plant scale high-pressure homogenizer validating the methodology in a scale-up of the disruption process. This innovative non-complex approach developed to evaluate the efficacy of a disruption procedure or equipment can be easily applied to optimize the most common disruption processes, in order to reach not only reliable quantification, but also recovery of intracellular components from cell factories of interest.

3.2 Introduction

Pichia pastoris has been widely used as cell factory in the last years [1]. The cytoplasm of yeast cells is a rich source of bio-products such proteins, cytoplasmic enzymes, or polysaccharides valuable in biotechnology, pharmacology and food industry [2]. The quantitative recovery of the intracellular compounds is determined by the disruption processes, which may affect the stability and the biological activity of the desired product. Thus, the selection of a suitable cell disruption method to recover these compounds and its reliable quantification is very important [3]. Disruption can be considered a general term that describes different processes related to cellular disintegration that range from slight release of internal metabolites to full cell breakage [4]. The efficiency of cell disruption implies selective and complete release of the product to achieve a high recovery of the target products, reduced contaminants and minimal micronization of cell debris [5–7].

The existence of cell wall in the yeast cells requires that the disruption and release of intracellular components destructs the strength-provide components of the wall, in the case of yeasts, namely glucans [2]. The basic structural components of the yeast cell wall were identified by Smith *et al.* [8] In the case of *P. pastoris* disruption procedures, the use of methanol in the cultivation has a relevant impact on the cell wall in comparison with other

carbon sources such glycerol or glucose. An important widening of cell wall thickness of *P. pastoris* cells growing on methanol was described by Canales *et al.* [9], which rather increased twice. Furthermore, after the observation of the difficulties to obtain reproducible and reliable results for disruption methods for *P. pastoris* cells grown on methanol, one can consider that more severe methods and operating conditions than the standard reported for *Escherichia coli* or *Saccharomyces cerevisiae* are needed [7].

Several methods for disruption of microbial cells are described in the literature, the most commonly used are summarized in some reviews [5,6,10]. Most of them have been applied in *Pichia*: sonication [11], bead milling [12,13], enzymatic and chemical lysis [14,15], cell permeabilization [16,17] and high-pressure homogenization (*HPH*) [18–20]. The last one is described as the most used for large scale cell disruption processes in the biopharmaceutical manufacturing industry [11,21].

Despite cell disruption is a field widely studied, among the works published in the literature there is not agreement about the reporting indicators that should be chosen for its study. The selection of reliable and simple indicators to measure the degree of cellular disruption is a key point to assess the efficiency of the disruption methods. These indicators must not be degraded in the rupture processes and released from the cell consistently through different cycles. Usually, the measure of the target protein released is the best method to quantify the efficiency of the cell rupture [6]. However, in some cases the release of other intracellular components can be used as an alternative to determine the extent of cell disruption. Direct and indirect measurements indicating the cellular disruption degree, using *S. cerevisiae* as model, have been recently reviewed concluding that different indicators provide different information to monitor the level of disruption [4]. Thus, the combination of different reporting indicators in a single parameter of the disruption efficiency is useful to integrate the information given by each indicator, which can facilitate the efficacy study of the process.

Accurate quantification of intracellular proteins, enzymatic activities and metabolites is basic to carry out research in biochemistry and biotechnology, from determining cellular components to metabolomics and systems biology studies. The reliability of the target component quantification relies on whether the cell disruption process is efficient and reproducible due that the use of non-optimized procedures may not allow to achieve the complete release of the elements that are being studied, which could lead to important errors in the determination of this cellular components. Thus is essential that the cell disruption procedures used always assures efficacy and reproducibility. Furthermore, since *P. pastoris* is commonly used as a recombinant production cell factory, the reliable recovery and quantification of the intracellular product is of capital interest to completely evaluate the efficiency of the bioprocess [22,23].

In this sense, the aim of this work is to present a methodology to determine the disruption settings that allows the reliable quantification of a wide sort of intracellular components. This approach can be applied to the most used cell disruption processes. Specifically, the work aims to characterize and optimize the working conditions of a lab scale *HPH* using *P. pastoris* suspensions. This study was performed through the definition of an overall key performance indicator (*KPI*) based on the combination of the following reporting indicators: decrease of absorbance, release of total protein, and release of alkaline phosphatase activity. The reporting indicactors have been selected among the main parameters used in other references from the literature, those being preferred which are simple, rapid, and do not require expensive equipment [6]. Since this *KPI* aims to be applicable to study different disruption processes, it is important that the reporting indicators selected are not specific for particular organisms, as could be some intracellular small molecules or metabolites. The usefulness of the methodology has been confirmed for a bigger process scale using a pilot plant *HPH*. Finally the optimal results for *HPH* were compared with other commonly used disruption methodologies.

3.3 Materials and methods

3.3.1 Microorganism

Suspensions of a wild-type X-33 *P. pastoris* strain growing on mixed feeds of glucose and methanol were obtained from steady state chemostat cultures. The cultivations were set at a D of 0.09 h^{-1} by feeding a defined growth medium containing 50 g L^{-1} of glucose/methanol mixture (80% glucose / 20% methanol, w/w) as a carbon source, dissolved oxygen levels were kept at a minimum of 15% of air saturation, pH was controlled at 5 and temperature at 25ºC. More details about the cultivation conditions can be found elsewhere [24].

3.3.2 Cell disruption methodologies

Prior the disruption processes the cell suspensions were always cleaned three times by centrifugation and resuspension in fresh *PBS*. The clean suspensions were vortexed vigorously for homogenizing the samples and dispersing any cellular aggregates. All the samples were kept on ice within the disruption steps in order to avoid the activity of the endogenous proteases. To discard the cell debris after the cell rupture procedures, the disrupted samples were always clarified by centrifugation (4200 x g, 4ºC, 15 min).

3.3.2.1 High-pressure homogenization

HPH is the most employed method for the disruption of microbial cells in large scale bioprocesses. The cell suspension is released at high pressure through a specially designed valve assembly, where the cells are disrupted as a consequence of the different forces produced by the interaction between the fluid and the solid walls of the valve [25].

The One-Shot Cell Disrupter (Constant Systems Ltd., Warwickshire, UK) was used at lab scale, being 8 mL the volume of the disruption samples. In previous studies different pressures were compared in order to optimize the method. 2 kbar and up to 3 passes were selected as the best working pressure and maximal number of passes (N) for P. pastoris as a compromise between the efficacy of disruption and the amount of foams produced during the disruption passes, which introduces lack of reproducibility and uncertainty in the process [26].

Additionally, at pilot plant scale the homogenizer used was the TS Series Cabinet Disruption System (Constant Systems Ltd., Warwickshire, UK), being 250 mL the volume of the disruption samples. The working pressure was 2.7 kbar, the highest of the equipment, because better disruption results were observed without a substantial increase in foaming production. It is accordingly to its exponential dependence previously referred for HPH [6].

3.3.2.2 Bead milling

It is a standard cell disruption in which the intracellular cell components are released after the cell cracking caused by the collisions between beads and cells [26]. The performed procedure using glass beads (Sigma-Aldrich G-9268, 425-600 μm) was adapted from the literature [27]. The disruption mixture composed by equal volumes of cell suspension with OD_0 = 25 and glass beads were vortexed for 1 minute 10 times, each followed by 1 minute on ice.

3.3.2.3 Cell permeabilization

It is an alternative method for the recovery of intracellular proteins from yeast and other microbial cells and organisms, which aims avoiding the common disadvantages of high-pressure homogenization such the own complex background of the host producer and mechanical stresses that may affect the recovery and biological activity of the target protein [28]. The used protocol was adapted from a previous published work [16]. This is based on suspending and incubating the cells in an aqueous solution containing *N,N*-dimethyl-tetradecylamine. The working conditions were: 5 g L^{-1} of *N,N*-dimethyltetradecylamine, equivalent initial OD (OD_0) of 9, and incubation time of 15h.

3.3.3 Analytical methods

3.3.3.1 Optical density (OD)

OD at 600 nm is commonly used to determine cellular concentration. *OD* measures, in absorbance units (*AU*). It can be easily converted to dry cell weight values (*DCW*, g L^{-1}) using the following conversion factor: OD (AU) x 0.2 = DCW (g L^{-1}) [29]. Additionally, in the presented work, *OD* has been used as a direct measure of the cell integrity of the samples; hence, a relative decrease of *OD* was associated to the proportion of cells disrupted [4]. All spectrophotometric analyses were taken in triplicate.

3.3.3.2 Total protein released (TPR)

TPR was considered a suitable indirect performance indicator to be correlated to disruption efficiency [6]. It was determined by Bradford assay, which was performed with Coomassie Plus™ Protein Assay Reagent (Pierce, Rockford, IL, USA) using a bovine albumin as standard. *TPR* assays were taken in triplicate and the relative standard deviation (*RSD*) was about 5%.

3.3.3.3 Alkaline phosphatase released (APAR)

As an intracellular enzyme, the *APAR* gives not only an indication of the protein released but also the preservation of enzymatic activity, which was considered as a reliable indirect performance index [30]. The protocol was adapted from the literature [31]. Alkaline phosphatase was assayed at pH 10.0 using p-nitrophenyl phosphate as substrate, incubation time at 37ºC was 20 min, after which time, absorbance was measured at 410 nm. *APAR* assays were taken in triplicate and the relative standard deviation (*RSD*) was about 6%.

3.3.3.4 Data analysis

The *OD* decrease (*ODD*) was determined as a normalized quotient between the pre-homogenized (OD_0), and the post-homogenized (OD_H) values (Equation 1):

$$ODD = 1 - \frac{OD_H}{OD_0} \quad (1)$$

In the evaluation of the disruption efficacy, the effect of the initial *OD* was one of the variables studied. Since this parameter is directly related to the total amount of biomass that will be disrupted, *TPR* and *APAR* will be affected by this variable. Thus, in order to be able to compare disruption results between samples with different biomass content, these performance indicators were always normalized with the pre-homogenized *OD* of the samples, hence using the specific form; specific *TPR* (mg OD^{-1} L^{-1}); specific *APAR* (AU OD^{-1} L^{-1}).

In the parity plot depicted in Figure 1, all the performance indicators values (Y_k) were normalized with the corresponding maximal value observed at the best process conditions ($Y_{k,max}$). In this way, the disruption indicators were scaled and shown together in the same plot.

$$\overline{Y}_k = \frac{Y_k}{Y_{k,\max}} \quad (2)$$

3.3.4 Experimental set-up and statistical analysis for the Design of Experiments (*DoE*)

The effect of OD_0 and *N* on *ODD*, *TPR* and *APAR* was studied by means of a Box-Wilson Central Composite Design (*CCD*) and response surface methodology (*RSM*). The *CCD* performed was a face-centred design (*CCF*), which was composed by 13 experiment based in two variables having 3 levels each and five central points for replication. The OD_0 and *N* range were 20-100 and 1-3 respectively. These ranges were selected from results obtained in preliminary disruption experiments. The empirical response surfaces were built from the values of *ODD*, specific *TPR* and specific *APAR*. The data results were fit to the empirical model expressed at Equation 3:

$$Y_k = \beta_{0,k} + \sum \beta_{i,k} \cdot X_i + \sum \beta_{ii,k} \cdot X_i^2 + \sum \beta_{ij,k} \cdot X_i \cdot X_j \qquad (3)$$

where, X_1 = OD_0, X_2 = N; k = 1 for *ODD*, k = 2 for *TPR*, and k = 3 for *APAR*.

The Sigma Plot statistical package (*SigmaPlot 11.0*; *Systat Software*, *Inc*., Chicago, *IL*, *USA)* was used in order to perform the statistical analysis and fit the response surfaces. The quality of the fit is expressed by the coefficient of determination R^2 obtained by regression analysis. Additionally, a lack of fit test was performed in order to compare the experimental error to the prediction error. The overall significance of the model was determined by analysis of variance (*ANOVA*) F-test, whereas the significance of each coefficient was determined by the corresponding t-test. Standard error of the estimate (*SEE, %*) was also calculated for all three models to test their estimation capabilities.

3.4 Results and discussion

3.4.1 Characterization of the HPH by means of DoE

DoE and the *RSM* were used to describe the effects of OD_0 and *N* in the cell disruption of *P. pastoris* using a *HPH*. *ODD*, *TPR*, and *APAR* measures were used as quantitative indicators of the disruption degree. This work seeks to take into account more than one reporting parameter for the disruption efficiency evaluation. The experimental results presented were used to estimate the coefficients of the quadratic polynomial equation described in the Equation 3 (Table 3.1).

A parity plot including all the experimental and model-predicted data is used to present graphically the estimation capabilities of the models (Figure 3.1). All the experimental points are within the range 5 % of error of the fitted model. This also confirms the robustness of the models estimating disruption efficacy in terms of the performance indexes studied.

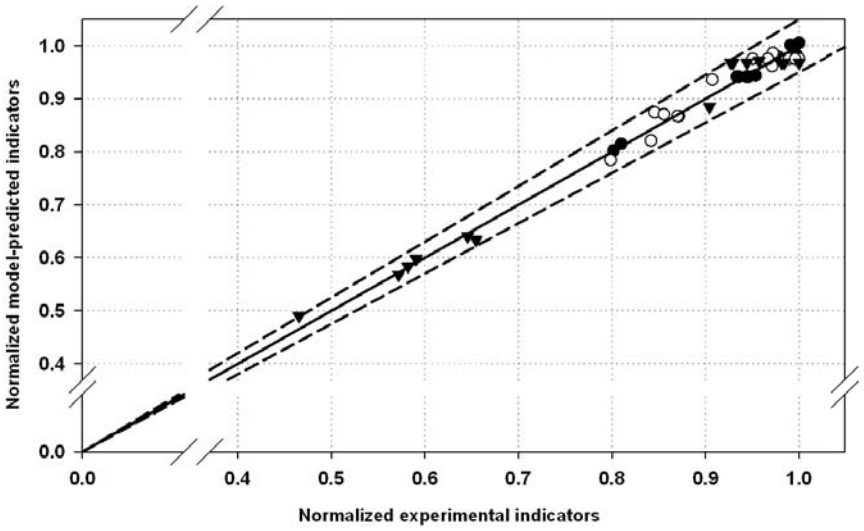

Figure 3.1: Normalized parity plot for the selected performance indicators with 5 % of error: (●), optical density decrease (*ODD*); (▼), total protein released (*TPR*); (O), alkaline phosphatase activity released (*APAR*). Maximal values for *ODD*, *TPR* and *APAR* were 88.3 %, 85 mg ·OD^{-1}·L^{-1} and 0.27 AU·OD^{-1}·L^{-1} respectively.

Table 3.1: Experimental setup for a *CCF* design for two factors, matrix design and response.

Experiment	Initial *OD*	Number of passes	*OD* decrease (%)	Total protein released (mg OD^{-1} L^{-1})	*AP* activity released (AU OD^{-1} L^{-1})
1	20 (-1)	1 (-1)	71.0	49.1	0.218
2	20 (-1)	2 (0)	84.5	55.5	0.231
3	20 (-1)	3 (+1)	87.8	56.2	0.230
4	100 (+1)	1 (-1)	77.2	40.0	0.238
5	100 (+1)	2 (0)	86.6	50.0	0.265
6	100 (+1)	3 (+1)	88.6	50.7	0.265
7	60 (0)	1 (-1)	71.7	77.7	0.234
8	60 (0)	2 (0)	83.8	79.8	0.264
9	60 (0)	2 (0)	82.8	85.9	0.260
10	60 (0)	2 (0)	83.6	79.6	0.273
11	60 (0)	2 (0)	82.9	84.3	0.270
12	60 (0)	2 (0)	82.9	84.6	0.272
13	60 (0)	3 (+1)	88.3	82.2	0.248

Table 3.2 outlines the estimated coefficients determined for the models. The *ANOVA F*-test associated *p*-value can be used as indicator of the statistical significance of the coefficients on the response. Coefficients without significance are those with *p*-value > 0.05. The high values of R^2 and low values of *SEE,* always below 4 %, point out a proper goodness of the fit for all the models.

Table 3.2: Estimated coefficients of the models and ANOVA analysis for the three disruption models in which the experimental results were fitted to the equation 3, where, i = 1 for OD_0, i = 2 for N; k = 1 for *ODD*, k = 2 for *TPR*, and k = 3 for *APAR*.

	OD decrease *ODD*-model			Total protein released *TPR*-model			*AP* activity released *APAR*-model		
	Coefficient	*t*-Value	*p*-Value	Coefficient	*t*-Value	*p*-Value	Coefficient	*t*-Value	*p*-Value
β_0	51.05	22.97	<0.0001	-0.32	-0.05	0.9594	0.132	6.771	$3.0\ 10^{-4}$
β_1	-0.03	-0.61	0.5618	2.19	18.43	<0.0001	1.10E-03	2.906	$2.2\ 10^{-2}$
β_2	24.51	11.72	<0.0001	16.01	2.80	0.0266	0.081	4.453	$3.0\ 10^{-3}$
β_{11}	0.00	3.54	0.0094	-0.02	-22.72	<0.0001	-7.63E-06	-2.846	$2.5\ 10^{-2}$
β_{22}	-3.76	-7.62	0.0001	-3.41	-2.53	0.039	-0.020	-4.558	$2.6\ 10^{-3}$
β_{12}	-0.03	-3.29	0.0133	0.02	0.82	0.4408	9.88E-05	1.103	0.3051
R^2		0.9882			0.9900			0.9210	
SEE (%)		0.99			3.32			2.86	

3-D graphs shows the effects of the key selected variables in the responses (Figure 3.2). For the *ODD*-model, OD_0 does not have a clear influence in the response, so the cell concentration of the samples does not influence the loss of cell integrity within the range studied. In contrast, *N* seems to affect the outcome. The higher number of passes, the better result is. However, the difference between 2 and 3 passes is slight, what could be related to a plateau

effect on the cell rupture phenomenon with increasing *N*. In the case of the *TPR*-model, the OD_0 causes a strong effect in the outcome resulting to a maximal response for intermediate values of initial *OD*. The cell breaking processes that occurs with intermediate cell concentrations seems to be optimal to recover the maximal amount of total protein. *N* does not affect significantly, hence one single pass through the high-pressure homogenizer is enough to let it out most of the total protein that can be released. For the *APAR*-model, the differences in the response using different conditions are clearly slighter. However, a double plateau effect in both studied variables can be observed, so medium and high OD_0 and *N* leads to high responses of the reporting parameters.

Since *TPR* and *APAR* are indirect measures, the results obtained are not only due to cell rupture, but also to other processes that can degrade the proteins and may have an important influence on the results. Physical and chemical effects, as well as the action of the proteases of the host cell are considered as the main causes of this degradation. Similarly is important to bear in mind that these parameters are also conditioned by the foaming formation during homogenization, what could lead to an inaccurate quantification of the parameters [19,32]. Normally, these effects occur on processes aiming to recover the maximal amount of protein as a target product, so it is important that models also take it into account. This fact is corroborated by the slightly higher *SEE* obtained for *TPR* and *APAR* models in comparison with the *ODD*-model.

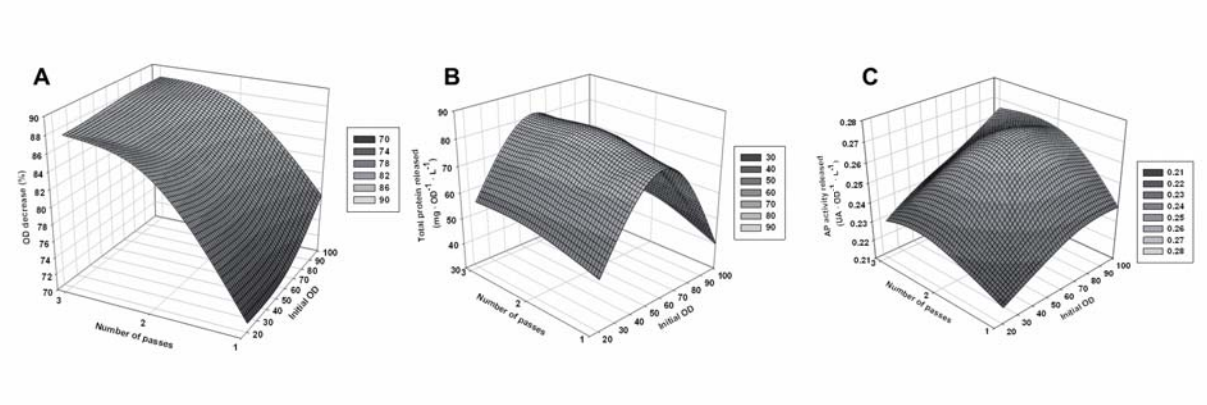

Figure 3.2: Response surface graphs based on the results of the *DoE* performed. (A) Optical density decrease (*ODD*); (B) total protein released (*TPR*); (C) *AP* activity released (*APAR*).

3.4.2 Identifying the optimal conditions for HPH

According to the results shown and discussed in the previous section, different reporting parameters must be taken into account for analyzing accurately the efficiency of the disruption procedures. Using a lab scale *HPH*, three models have been defined in order to maximize the *ODD*, *TPR* and *APAR* with two operational variables, OD_0 and *N*. Nevertheless, since different operational conditions must be used to achieve optimal results of the diverse performance indicators, it is of great interest to define an overall performance indicator that can be stated

as a global key performance indicator (*KPI*). Thus, this *KPI* is used as a global quantification parameter of disruption efficiency and it is calculated as follows:

$$KPI = \alpha_1 \cdot \overline{Y}_1 + \alpha_2 \cdot \overline{Y}_2 + \alpha_3 \cdot \overline{Y}_3 \qquad (4)$$

where $\overline{Y}_k$ is the normalized Y_k calculated dividing by its maximal value obtained $Y_{k,max}$; α_k is a weighting factor, being $\sum \alpha_k = 1.0$; k = 1 for *ODD*, k = 2 for *TPR*, and k = 3 for *APAR*.

Table 3.3: Maximal overall performance indicator (KPI_{max}) obtained with a different set of weighting factors and their corresponding number of passes and initial *OD*.

	α_1	α_2	α_3	Number of passes	Initial *OD*	KPI_{max}
1	0.3	0.6	0.1	2	62	0.975
2	0	0.5	0.5	2	61	0.989
3	0.5	0	0.5	2	80	0.971
4	0.5	0.5	0	2	65	0.964
5	1/3	1/3	1/3	2	65	0.970

From the results obtained after evaluating the effect of the different variables with the *KPI* (Table 3.3), it is shown that the *N* value that maximizes the *KPI* is 2 in any of the analyzed cases. Nevertheless, the OD_0 optimal values vary between 60 and 80.

Among the different weighting criteria, the one that does not take into account the *TPR* is the option that results into a higher difference in the optimal OD_0. This fact leads to conclude that the *TPR* is the key parameter that causes the major differences in the disruption efficiency. However, since this work seeks to take into account more than one reporting parameter, the final selected criterion was the one that took into consideration all the indicators but giving to the *TPR* weighting factor a higher value.

Consequently, the optimal working conditions of the studied *HPH* have been defined as: working pressure, 2 kbar; OD_0, 60; *N*, 2 passes. These settings are close to the operational conditions proposed for different commercial *HPH* disrupting bacteria and yeast [4,33]. Nevertheless, as a significant novelty, the recommended conditions given in the presented work has been determined as optimal through the use of *DoE* and an overall key performance indicator *KPI* based on the combination of simple cell disruption indicators. These process conditions are clearly more advantageous than those suggested for *P. pastoris* disruption by Tam et al., [19] in which the *N* value proposed is 20, resulting into lower overall disruption efficiencies.

Although other authors that published previous works in the field concluded that cell concentration does not have an important effect on the disruption efficiency [6,19,32,34,35], revising accurately their results on figures, slight differences were observed. The mentioned

differences in the results due to the cell concentration are in the same order of magnitude that the described in the present work. Since the aim of this work is to achieve and assure a very accurate, reliable and reproducible cell disruption procedure, consequently it has been concluded that the effect of the cell concentration is a significant factor that must be taken into account for optimizing the performance of a HPH.

3.4.3 Comparison among the alternative disruption methodologies

In order to compare the performance of the *HPH* with some common alternative disruption procedures, bead milling and cell permeabilization were also carried out with the same samples of *P. pastoris*. The previously used quantitative indicators; *ODD*, *TPR* and *APAR* were also considered as reporting parameters of the disruption efficiency. Results obtained for each procedure are summarized in Figure 3.3. The operating conditions for the *HPH* were the optimal determined in the previous section.

For the other disruption methods; incubation time, number of passes (*N*) and cell and reagent concentrations (OD_0; *R*) were selected following a heuristic procedure and adapted protocols described in the Materials and Methods section (data not shown).
As can be stated from all three single performance indexes, results obtained with the *HPH* were clearly better than using the other alternative procedures. *TPR* and *APAR* results using *HPH* were about 4-fold higher than using other methodologies. *ODD* results were also significantly higher, at least 50% better. Thus, one can conclude that studying intracellular components, the results obtained with *HPH* will be significantly more consistent and reliable than using other common methods.

These results are in accordance with the literature comparing different methods for cell disruption. The use of *HPH* is preferred due to the higher disruption efficiencies obtained and the possibility to scale-up the processes However, the higher cost of the HPH equipment and maintenance are important drawbacks to be taken into account in comparison with other procedures [7,10].

From the presented results, it is also important to point out that the profile of the different performance indicators studied is certainly different among the different disruption methodologies. This fact reinforces the need to consider more than one indicator in order to analyse accurately the efficiency of any disruption processes.

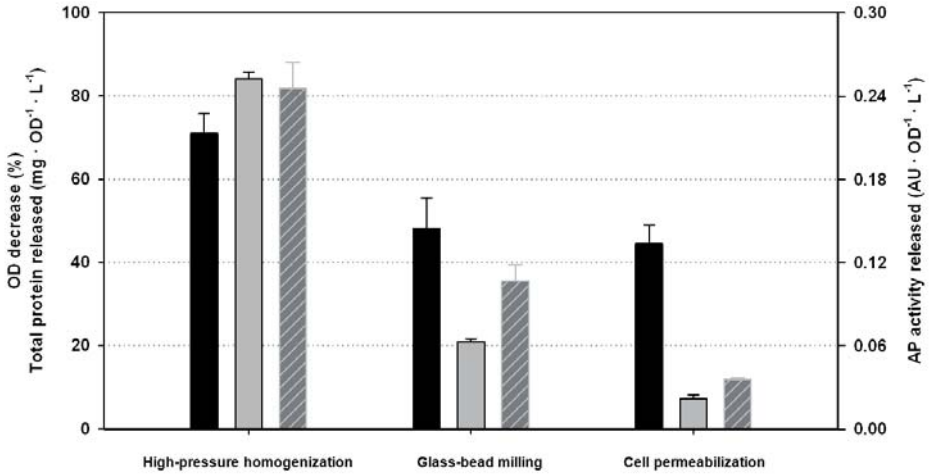

Figure 3.3: Comparison of the performance indicators among the alternative disruption methods studied: Black, optical density decrease (ODD); grey, total protein released (TPR); with stripes, AP activity released (APAR).

3.4.4 Working conditions comparison between HPH at lab and pilot plant scale

In order to compare the performance parameters at a bigger scale, similar working conditions were evaluated for an equivalent *HPH* at pilot plant scale but with operating pressure 2.7 kbar, as previously justified in materials and methods section. Cell disruption procedures for two different OD_0, 60 and 100, were performed. The *N* range studied was between 1 and 3 passes. The disruption efficiency obtained for OD_0 = 60 were significantly higher than those for OD_0 = 100 (data not shown). Consequently OD_0 = 60 was selected for the comparison between lab and pilot plant scale *HPH*. The results are presented in Table 3.4.

Table 3.4: Comparison table of the performance indicators for the *HPH* at different scales using samples at OD_0 = 60. SD indicates standard deviation.

High-pressure homogenizer	*N*	*OD* decrease (%)	SD	Total protein released (mg OD^{-1} L^{-1})	SD	*AP* activity released (AU OD^{-1} L^{-1})	SD
Lab scale	1	71.7	2.8	77.7	1.1	0.234	0.013
Pilot plant scale	1	79.8	1.2	55.7	0.7	0.181	0.010
Lab scale	2	83.2	2.3	83.1	2.1	0.271	0.005
Pilot plant scale	2	88.2	1.5	60.2	2.2	0.154	0.003
Lab scale	3	88.3	1.7	82.2	1.5	0.248	0.009
Pilot plant scale	3	91.4	1.8	62.1	1.9	0.129	0.003

In terms of *ODD*, the efficacy of the pilot plant scale is slightly better, especially in the first passes. However, the efficiency decreases significantly for both *TPR* and *APAR*. Longer disruption (residence) times for the pilot plant *HPH*, as well as different geometry of the equipment could be feasible reasons for this fact. The decrease of *APAR* in pilot plant scale

could be related with proteolysis activity of the endogenous proteases during the longer disruption times.

Afterwards, using the criteria based on the *KPI* and selecting the same weighting factors that in the lab scale (detailed in a previous section), the optimal working conditions at pilot plant scale were determined. These were: working pressure, 2.7 kbar; OD_0, 60; *N*, 3 passes. Since a substantial difference was observed in the disruption performance parameters either using 2 or 3 passes, it has been conclude that for this pilot plant *HPH* working with 3 passes is more effective.

3.5 Conclusions

A *DoE* was conducted to study the effect on the disruption of OD_0 and *N* in a lab scale *HPH*. Three different performance indicators were selected for evaluating the cell disruption degree: *ODD*, *TPR* and *APAR*. The optimal working conditions of the *HPH* at lab scale were determined by means of the definition of an overall *KPI*, because of the need to consider different indicators for analysing accurately the efficiency of the disruption processes. Thus, results obtained led to the following optimal operational conditions: 2 kbar; OD_0, 60; *N*, 2 passes. This disruption method was compared with other commonly used procedures, bead milling and cell permeabilization, showing a disruption efficiency significantly higher for all the reporting parameters studied. These differences were up to 4-fold higher in *HPH* for *TPR* and *APAR*.

Finally, the developed approach was also applied to a pilot plant scale *HPH* obtaining similar results for the *ODD.* Nevertheless, an important decrease were observed in the *TPR* and *APAR* indicators, what could be caused by the effect of the endogenous proteases, accompanied by longer residence times and different geometry of the equipment. In this case, optimal working conditions were: 2.7 kbar; OD_0, 60; *N*, 3 passes.

ODD, *TPR* and *APAR* can be stated as general disruption indicators since similar release pattern is expected for other intracellular components of interest. The methodology described to evaluate the efficacy of a disruption procedure or equipment can be applied to optimize these processes, which aim reliable quantification of intracellular cell components. From the results presented in this work, one can conclude that using non-optimized cell disruption procedures can introduce important error in the assays and processes derived from it. Therefore, the quantification of intracellular components, such proteins, metabolites and other cellular elements of interest, may not be accurate. In addition, the important decrease in recovery yields due to use of non-optimized cell disruption procedures may affect dramatically the efficiency of a bioprocess.

This article demonstrates the importance of the efficiency in cell disruption procedures for research studies derived from the quantification of intracellular components. Furthermore, the contribution is expected to have a big interest in bioprocesses for the recovery of the intracellular components of different cell factories, such recombinant or homologous proteins and enzymes, metabolites, and others.

3.6 Nomenclature

APAR Alkaline phosphatase activity released, AU DO^{-1} L^{-1}

DoE Design of experiments

HPH High-pressure homogenization

KPI Key performance indicator

N Number of passes

OD Optical density

OD_0 Initial OD

ODD OD decrease, %

RSD Relative standard deviation, %

RSM Response surface methodology

SEE Standard error of the estimate, %

TPR Total protein released, mg DO^{-1} L^{-1}

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4

Fed-batch operational strategies for recombinant Fab production with *Pichia pastoris* using the constitutive *GAP* promoter

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4.1 Abstract

Carbon source and growth rate are two major parameters affecting recombinant protein production in *Pichia pastoris*. The effect of the most commonly used carbon sources (glycerol or glucose) and the specific growth rate (μ) has been studied on the production of a human antigen-binding fragment (Fab) in this cell factory under the constitutive *GAP* promoter in fed-batch cultures.

Glycerol for batch phase and glucose for fed-batch phase was the most successful carbon source combination. During batch phase, by-products were detected when glucose was used, despite maintaining DO at values higher than 35%. Also, the presence of cell aggregates was detected affecting the reproducibility and operability of the bioprocess.

Conversely, glucose was the best substrate for fed-batch phase. When working at C-limiting conditions, neither by-products nor aggregates were detected and Fab production levels were comparable to those obtained with glycerol. In addition, the lower heat yield ($Y_{Q/X}$) and oxygen to biomass yield ($Y_{O_2/X}$) for glucose-supported cultures made this substrate the best alternative from an industrial operational point of view.

In addition, the effect of specific growth rate on fed-batch Fab production was studied. Medium and high μ (0.10 and 0.15 h^{-1}) set-points showed similar Fab production yield. However, in terms of total and volumetric productivity, higher μ was the best process conditions.

4.2 Introduction

Recombinant proteins, including biopharmaceuticals proteins and industrial enzymes, is a multi-billion dollar market [1,2]. *Pichia pastoris* is currently one of the most effective and versatile systems for the expression of heterologous proteins [3]. This fact is due to the combination of traits of this yeast, such as its accessibility for genetic manipulation including well characterized genetic elements, the ability to grow on defined media at very high-cell densities, its capacity to perform post-translational modifications including glycosylation and disulphide bond formation and the possibility of driving the protein production intracellularly or secreting it to the extracellular medium [4–6].

Different promoters have been successfully employed for recombinant protein production in *P. pastoris* [7]. The alcohol oxidase promoter (P_{AOX1}), which is strongly induced in the presence of methanol, has been extensively used, obtaining very high expression levels [8–10]. Nevertheless, during the last years, the glyceraldehyde-3-phosphate dehydrogenase constitutive promoter (P_{GAP}) has become an increasingly used alternative [11–13]. *GAP* promoter allows the protein expression using glucose, glycerol and other carbon sources as a substrate [14]. Several studies have reported that P_{GAP} is more efficient than P_{AOX1} [15–17], whereas others showed opposite results [18,19]. Thus, it appears that expression levels

achieved for a given protein using different promoters vary significantly based on properties of the expressed protein. Additional research would be required to determine which factors impact the efficiency of both promoters [3]. The use of the constitutive *GAP* promoter avoids the use of methanol in the fermentations, which reduces the cell lysis of the cultures, and subsequently, the proteolysis of secreted proteins [20]. From a large-scale processing perspective, the P_{GAP} expression system may be advantageous because it eliminates the hazard and cost associated with the storage and delivery of large volumes of methanol [21] and significantly decreases heat production and oxygen requirements of the processes [22].

Full-size monoclonal antibodies (mAbs) and their fragments are an increasingly class of therapeutic agents. Main characteristics and applicability have been widely described previously [23,24]. Antigen-binding fragments (Fab) are complex proteins composed of the antigen binding regions of an antibody molecule, containing both the heavy chain domains vH-cH and the entire light chain vL-cL chains, which are connected via disulfide bonds [25]. Microbial expressions systems have been investigated for their potential to produce mAbs and different mAbs fragments. The major advantages of these systems lies in their shorter process times and lower production costs as compared to mammalian cell culture [26]. In *P. pastoris,* high expression levels of mAbs and their fragments have been already achieved [27,28].

In this study, the human Fab 2F5 was used as a model protein complex in order to evaluate the effect of the carbon source and the specific growth rate for the production of heterologous protein under the control of the glycolytic *GAP* promoter. Glucose and glycerol were selected and compared as alternative substrates in both phases of the process, batch and fed-batch. Once the best combination of substrates was selected, three different specific growth rates were evaluated and compared. Thorough studies concerning specific rates including cell growth, substrate consumption and production formation have been carried out.

4.3 Materials and methods

4.3.1 Strains

The *P. pastoris* strain X-33 pGAPZαA Fab2F5 expressing the human 2F5 Fab under the control of *P. pastoris* constitutive *GAP* promoter was used throughout this study. Briefly, the expression cassettes for the light and heavy chain of the human Fab 2F5 antibody fragment were separately introduced under the control of the *P. pastoris GAP* promoter and combined on one plasmid [25]. This strain expresses the human Fab using the *Saccharomyces cerevisiae* α-mating signal sequence for secretion of the heterologous protein to the extracellular medium.

4.3.2 Cultivation methods

4.3.2.1 Inoculum preparation

The inoculum for bioreactor cultures were grown for 24 h in 1 L baffled shake flasks at 25 ºC, 130 rpm, in YPG medium (2% peptone, 1% yeast extract, 2% glycerol; pH=7) adding zeocin (100 $\mu g\ mL^{-1}$). The shake flasks containing 150 mL of culture medium were inoculated from cryostocks of the recombinant strain, grown, harvested by centrifugation and re-suspended in batch medium to be inoculated into the bioreactor.

4.3.2.2 Fed-batch cultivation

Fed-batch cultivations were carried out in a 5 L Biostat B Bioreactor (Braun Biotech, Melsungen, Germany) working at initial volume of 2 L and finishing the process at approximately 4 L. The culture conditions were monitored and controlled at the following values: Temperature, 25 ºC; pH, 5.0 with addition of 30% (v/v) ammonium hydroxide; pO_2, above 20% saturation by controlling the stirring speed between 600 and 1200 rpm and using mixtures of air and O_2 at aeration within 1.0 and 1.25 vvm. Water evaporation losses were minimized during the processes using an exhaust gas condenser with cooling water at 8 ºC.
The standard fermentation strategy consisted of two phases. First, the batch phase starts with a standard carbon source concentration (40 g L^{-1}). It was performed using the batch medium described elsewhere [11]. Glycerol and glucose were alternatively used as sole carbon sources in different fermentations. During this step, yeast grows at maximum specific growth rate until the depletion of the C-substrate achieving a moderate concentration of biomass (≈ 20 g L^{-1}). Just after that, begins the fed-batch phase, the most important of the process, where the culture reaches high concentrations of biomass and product.

Second, the fed-batch phase was carried out by adding feeding medium, which have similar composition to that described by Maurer et al.[11] with minor modifications detailed below. Glycerol and glucose were alternatively used as carbon source (400 g L^{-1}). Biotin 0.02% (6 mL), PTM_1 (15 mL) trace salts stock solution [29] and antifoam Struktol J650 (0.2 mL) were added per litre of feeding medium.

The fermentation strategy during the fed-batch phase aimed to achieve pseudo-steady-state conditions for specific rates during carbon-limiting growth. A pre-programmed exponential feeding rate profile for substrate addition derived from mass balance equations to maintain a constant specific growth rate (μ) was implemented [30]. This open-loop control structure allows maintaining a constant μ, which enhances process reproducibility and facilitates the systematic study of growth rate-related effects on heterologous protein production [3].
The equations derived from the fed-batch substrate balance used to apply this strategy were described elsewhere [31,32]. A constant biomass to substrate yield ($Y_{X/S}$ = 0.5 $g_X\ g_S^{-1}$) was considered to determine the initial feed rate. The feeding medium was added by automatic Crison micro-burettes MicoBU-2031 (Alella, Barcelona, Spain).

4.3.3 Analytical methods

4.3.3.1 Biomass determination by Dry Cell Weight (DCW)

Biomass concentration of the samples was determined as DCW using the method previously described [31]. Determinations were performed by triplicate and the relative standard deviation (RSD) was about 4%.

4.3.3.2 Product quantification

The 2F5 human Fab was quantified by ELISA as previously described [33]. Determinations were performed by triplicate and the RSD was about 4%.

4.3.3.3 Protein quantification

Extracellular protein concentration was determined with the bicinchoninic acid protein assay kit (Pierce BCA Protein Assay, Prod. No. 23225, Rockford, IL, USA), according to the manufacturer's instructions. Bovine serum albumin (BSA) was used as the protein standard for the calibration curve.

4.3.3.4 Carbon source and by-products quantification

Glucose, arabitol, glycerol and ethanol were determined by HPLC with a HP 1050 liquid chromatograph (Dionex Corporation, Sunnyvale, CA, USA) using an ICSep ICE COREGEL 87H3 column (Transgenomic Inc., Omaha, NE, USA). The mobile phase was 8 mM sulphuric acid. Injection volume was 20 μL. Data was quantified by Chromeleon 6.80 Software (Dionex Corporation, Sunnyvale, CA, USA). The estimated RSD was below 1% for all the analytes.

Acetate was determined by GC with an Agilent 7820-A gas chromatograph (Agilent Technologies, Inc, Santa Clara, CA, USA) equipped with a flame ionization detector (FID) and a SGE Analytical Science BP21 column (30 m x 0.25 mm x 0.25 μm) (SGE Analytical Science Pty Ltd, Victoria, Australia). Helium was used as carrier gas. Samples were mixed (1:1, v/v) with a 0.2% hexanoic acid solution in acid as an internal standard. Injection volume was 1 μL. Data was quantified by EZChrom Elite Compact Software (Agilent Technologies, Inc, Santa Clara, CA, USA). RSD was about 6 %.

4.4 Theory and calculations

4.4.1 Dynamic estimation of the working volume during a fed-batches cultivations

In fed-batch fermentation the determination of culture volume is a key parameter for the precise determination of specific rates and yields of the bioprocess. Applying mass balance equations for the total mass of broth in the reactor, a procedure has been developed to estimate the culture volume throughout the bioprocess. This approximation is composed of different elements: feeding rate of substrate, addition of ammonium hydroxide used in the pH control loop, volume of the samples withdrawn from the bioreactor, water evaporation losses and gaseous exchange term as stated in equation (1).

$$\frac{d(V_{broth}\rho_{broth})}{dt} = F_{feed}\,\rho_{feed} + F_{NH_3}\,\rho_{NH_3} - V_{sample}\,\rho_{broth} - F_{evap}\,\rho_{H_2O} + V_{broth}(32\,OUR - 44\,CPR) \quad (1)$$

where, V_{broth} is the volume of broth in the bioreactor (L); ρ_{broth}, broth density (g L^{-1}); F_{feed}, volumetric addition rate of feeding medium (L h^{-1}); ρ_{feed}, feeding medium density (g L^{-1}); F_{NH_3}, base feeding rate (L h^{-1}); ρ_{NH_3}, 30% (v/v) ammonium hydroxide density; $V_{sample,}$ volume of the samples taken out from the bioreactor (L); F_{evap}, water evaporation rate (L h^{-1}); ρ_{H_20}, water density (g L^{-1}); OUR, oxygen uptake rate (mol O_2 L^{-1} h^{-1}); CPR, carbon dioxide production rate (mol CO_2 L^{-1} h^{-1}). Densities, flow rates and V_{broth} were determined gravimetrically at the end of the process. OUR and CPR were estimated according to previously determined observed yields $Y_{O_2/X}$ (mol O_2 g_X^{-1}) and $Y_{CO_2/X}$ (mol CO_2 g_X^{-1}) [34,35].

At the end of all the fermentations, slight deviations between estimated and measured volumes were observed (3% - 5%). Consequently, specific correction factors were applied to all the fermentations.

4.4.2 Correction of substrate and product concentration for total volume

Biomass, substrates and product concentrations were determined as described above, in section 2.3. Although biomass concentration (X) was related to the total volume (V_{broth}), the substrates (S_{Liq}) and Fab titer (P_{Liq}) were measured on the supernatant, therefore, not considering the volume of biomass. Consequently, substrates (S) and Fab titer (P) were recalculated on the total volume applying equations (2) and (3), as described previously in the literature [36].

$$S_{(t)} = S_{Liq,(t)}\left(1 - \frac{X_{(t)}}{\sigma\,\rho}\right) \quad (2)$$

$$P_{(t)} = P_{Liq,(t)}\left(1 - \frac{X_{(t)}}{\sigma\,\rho}\right) \quad (3)$$

where ρ is the yeast density and σ is the fraction of dry matter in the biomass. Both were determined experimentally, ρ = 1068 g L^{-1} and σ = 0.304 g g^{-1}.

4.4.3 Calculation of specific rates during the fed-batch phase of a process

The estimation procedure for the determination of discrete specific rates: $\mu_{(t)}$, $q_{S(t)}$, and $q_{P(t)}$ was adapted from Cos et al. [37]. Global state variables (XV, SV and PV) were estimated through all the fed-batch phase by applying the smoothing tool (Matlab R2009a Curvefit Toolbox, The Mathworks Inc., Natik, USA) from the off-line data. The first derivatives of the smoothed curves were also obtained. Finally, $\mu_{(t)}$, $q_{S(t)}$, and $q_{P(t)}$ were calculated by using their corresponding mass balances in fed-batch mode. Uncertainties for discrete specific rates were estimated by error propagation from mass balance equations.

Additionally, applying the mass balances mentioned above, mean specific rates of fed-batch cultivations can be calculated by fitting the experimental data to a linear function. Some authors [27] determined q_s and q_p considering the μ value previously estimated, which may lead to a significant error accumulation. The procedure applied in this work determines q_s and q_p independently from the μ estimation. This is an advantage in processes where the relationship between μ and q_s or q_p is not always constant. In this sense, the method used has been based on linear regression as follows:

$$\int_{(XV)_0}^{(XV)} d(XV) = \mu_{mean} \int_{t_0}^{t} XV\ dt \qquad (4)$$

$$S_{feed} \int_{t_0}^{t} F_{feed}\ dt - \int_{(SV)_0}^{(SV)} d(SV) = q_{S_{mean}} \int_{t_0}^{t} XV\ dt \qquad (5)$$

$$\int_{(PV)_0}^{(PV)} d(PV) = q_{P_{mean}} \int_{t_0}^{t} XV\ dt \qquad (6)$$

The standard error of each mean specific rate is estimated as the standard error of the slope from the linear regression data.

4.4.4 Estimation of process heat production

Heat production has direct consequences on the cooling requirements of the bioprocess for temperature control. Usually, in large-scale cultivations that reach very high-cell densities, this parameter can become an important process bottleneck. The heat generation from microbial cultures (Q) can be estimated with the following equation:

$$Q = \mu \cdot X \cdot V_{broth} \cdot\ Y_{Q/X} \qquad (7)$$

where μ is the specific growth rate (h^{-1}); X, biomass of the process (g L^{-1}); V_{broth}, volume of broth in the bioreactor (L); $Y_{Q/X}$, Heat yield (kJ g_X^{-1}). This last parameter describes the amount of heat produced per amount of biomass formed, this being independent of biomass concentration as well as growth rate. This yield can be estimated using equation (8):

$$Y_{Q/X} = \frac{\Delta H_S - Y_{X/S}\,\Delta H_X}{Y_{X/S}} \qquad (8)$$

where ΔH_S is the combustion enthalpy of substrate (kJ g_S^{-1}); $Y_{X/S}$, biomass to substrate yield (g_X g_S^{-1}); ΔH_X, combustion enthalpy of biomass (kJ g_X^{-1}). The combustion enthalpies of substrates and biomass were estimated from bibliographic data. ΔH_S (glucose): -15.58 kJ g_S^{-1} [38]; ΔH_S (glycerol): -17.98 kJ g_S^{-1} [38]; ΔH_X (yeasts): -21.21 kJ g_X^{-1} [39]. Otherwise, the $Y_{X/S}$ values used were obtained from experimental data.

4.5 Results and discussion

4.5.1 Comparison of carbon sources during the batch phase

In P_{GAP}-based recombinant production processes in *P. pastoris*, either glucose or glycerol is conventionally used as the sole carbon source. However, no definitive conclusions have been drawn as to which substrate is the best for recombinant protein production [3]. Several studies have been carried out comparing both substrates showing that glucose is more efficient than glycerol [14,40], whereas others reported opposite results [17,41]. In this work, both alternative substrates were compared at each bioprocess phase, i.e the batch and fed-batch phases.

The first part of this study was focused on the batch phase. For this step, most of the reported studies use glycerol. Nevertheless, there is a lack of systematic studies of this phase comparing different carbon sources. In the present work, glucose and glycerol were compared as two possible alternatives. As observed in Table 4.1, the main difference among the substrates was the final biomass reached, and consequently, biomass to substrate yield. Both parameters were 55% higher when cells were grown on glycerol. Nonetheless, no significant difference was observed for the maximum specific growth rate. Total Fab production also presented differences below 4%, although $Y_{P/X}$ was higher for glucose due to its lower $Y_{X/S}$ value. Relevant differences observed in the final biomass obtained should be related to the production of fermentative by-products.

Time profiles are shown in Figures 4.1A and 4.1B. Ethanol, arabitol and acetate were detected in glucose-supported cultivations (maximum concentrations: ethanol ≈ 4 g L^{-1}, arabitol ≈ 1 g L^{-1}, acetate ≈ 0.5 g L^{-1}). Conversely, concentrations of these by-products were always below 0.1 g L^{-1} when cells were grown on glycerol. Notably, pO_2 and OUR time profiles were significantly different depending on the C-source, reflecting different oxygen consumption rate profiles of the cultures (Figure 4.1A). Whereas in cultures using glycerol the oxygen consumption rose exponentially until the depletion of the substrate, an unexpected behaviour of oxygen consumption was observed when using glucose. Specifically, during the first hours, the oxygen consumption also rose exponentially, causing a decrease on pO_2. Afterwards, when pO_2 attained values around 35%, no clear trend was observed. In this case, the appearance of significant amounts of fermentative by-products in the broth was detected, despite the DO level was kept always above 30% for both cases. From this point, the exponential expected behaviour of the OUR time evolution changed, presenting a lower slope. Besides, estimated OUR values showed higher oxygen consumption for the glycerol-supported cultures.

These observations suggest that, although cultivation conditions of both batches were fully aerobic throughout the whole experiment, when growing in excess of glucose at high growth rate, *P. pastoris* appears to show a limited Crabtree effect, that is showing a

respirofermentative metabolism. Also, one is tempted to speculate that this yeast could sense the decrease of available oxygen in the broth and switch its metabolism from fully respirative to respirofermentative, even under fully aerobic conditions. Specifically, this metabolic change could be triggered when, in excess of glucose in the broth, the DO level of the cultivation reaches values around 35%. In these cultivation conditions, the metabolic state of cells could be similar to a culture with a lack of oxygen, which could lead to an accumulation of intracellular reduced NADH because of a limitation of the oxidative capacity of the cells and hence to an imbalance in the redox state of the cells [42,43]. This metabolic phenomenon have been already suggested in other works for cultures with oxygen limitation [42]. All these metabolic changes could explain the alteration observed in the oxygen consumption profiles, the important reduction of biomass yield and the production of fermentative by-products mentioned above. The results are in concordance with others published where ethanol [13,43,44] and acetate [13,43] were produced under similar conditions.

The flocculation is a common phenomenon widely described for yeast cell, specially for brewer yeasts [45]. In glucose-supported batches it is important to note that when the substrate was rather depleted the appearance of cell flocs was followed by microscopic observation. The presence of aggregates affected the reproducibility and operability of the bioprocess.

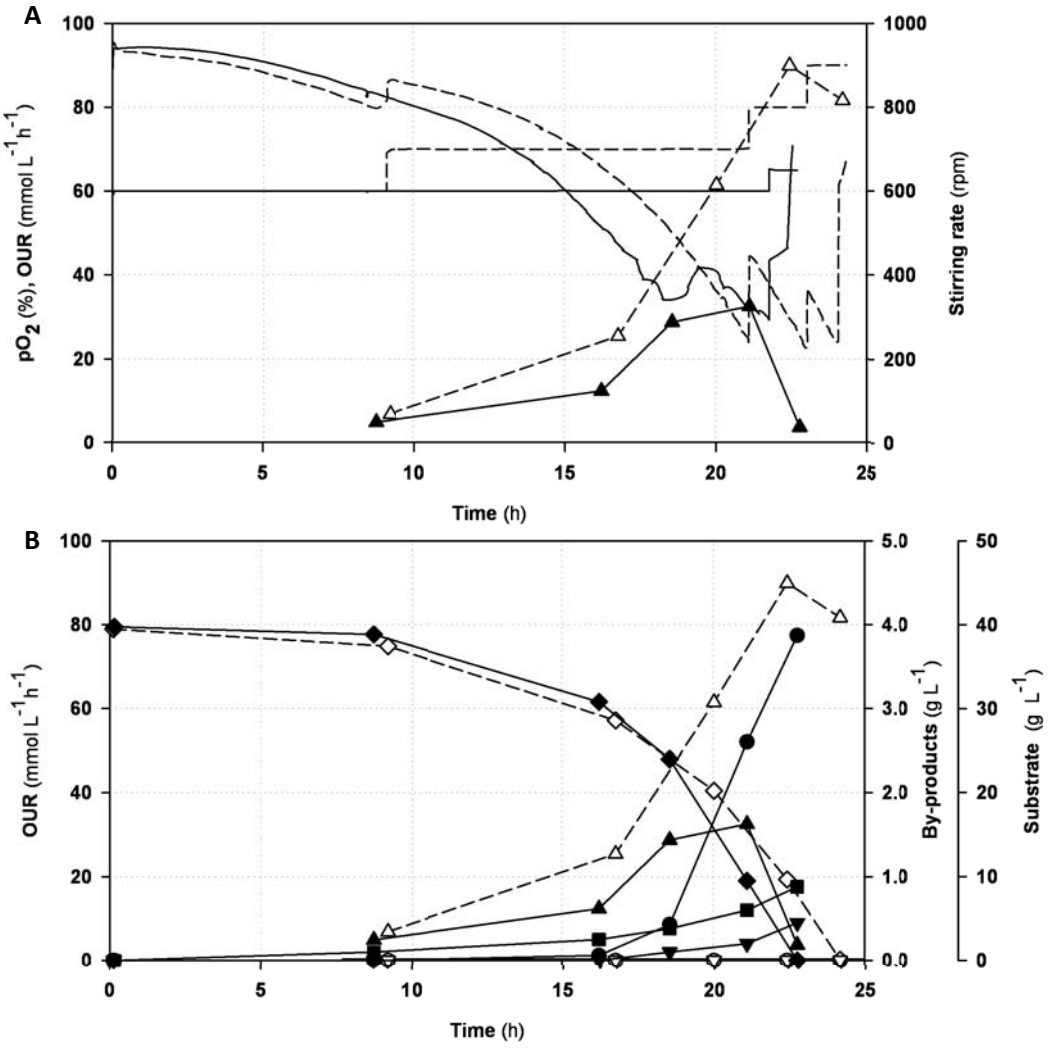

Figure 4.1: Comparison of time profiles among the use of glycerol (– – – –) and glucose (————) as a substrate in the batch phase. (A) Glycerol: OUR (△), pO_2 and stirring rate; Glucose: OUR(▲), pO_2 and stirring rate. (B) Glycerol: OUR(△), glycerol (◇), ethanol (○), arabitol (□), acetate (▽); Glucose: OUR (▲), glucose (◆), ethanol (●), arabitol (■), acetate (▼).

Similar changes observed for the biomass yields were also reported in previous studies comparing glucose and glycerol as a sole carbon sources in batch cultures [41], and comparing among batch and fed-batch cultivations growing on glucose [22]. Some of the observed by-products were also reported with chemostat cultures growing under glucose and oxygen limitation [46].

Based on the presented results, glycerol should be selected as the most suitable carbon source for batch cultivations

Table 4.1: Summary of batch fermentation results using different carbon sources as substrate.

Substrates	Carbon Source ($g\ L^{-1}$)	Max. Biomass ($g\ L^{-1}$)	Max. Fab Concentration ($mg\ L^{-1}$)	Max. specific growth rate (h^{-1})	Overall $Y_{X/S}$ ($g_X\ g_S^{-1}$)	Overall $Y_{P/X}$ ($mg_{Fab}\ g_X^{-1}$)	Overall $Y_{P/S}$ ($mg_{Fab}\ g_S^{-1}$)
Glycerol	39.5 ± 0.2	19.3 ± 0.2	3.2 ± 0.1	0.197 ± 0.002	0.49 ± 0.01	0.17 ± 0.01	0.081 ± 0.001
Glucose	39.7 ± 0.2	12.3 ± 0.2	2.9 ± 0.1	0.190 ± 0.002	0.31 ± 0.01	0.24 ± 0.01	0.073 ± 0.001

4.5.2 Comparison of carbon sources for both phase of fed-batch cultivations

Although glycerol seems to be the most promising carbon source for the batch phase, the combination of both carbon sources was tested for the two phases of fed-batch cultivation. In order to avoid the phenomenon's described above, using glucose at the batch phase, DO was kept above 50% throughout the whole experiments. As a result, the production of fermentative by-products was significantly reduced, and consequently, higher biomass yield was reached (0.43 $g_X\ g_S^{-1}$ instead of 0.31 $g_X\ g_S^{-1}$).

Therefore, the differences observed among using both substrates in the batch phase were reduced making easier the comparison. Four different fed-batch cultivation strategies were performed using all the possible combinations of carbon source, glycerol or glucose, within batch and fed-batch phase. All the cultures had the same termination criterion: similar values of DCW of about 100 g L^{-1}. Up to this value mass transfer problems are not relevant. All the cultivations were aimed to keep the same set-point of specific growth rate (μ), 0.10 h^{-1}. The set-point was achieved without significant deviations. Physiological and production parameters obtained from the cultivations are shown in Table 4.2. As example, the time evolution profile of the fermentation for a combination glycerol-glucose is shown in Figure 4.2A. Discrete and mean specific rates were calculated as described in section 4.3.3 and are presented in Figure 4.2B and 4.2C.

Slight differences were observed in biomass to substrate yields, these were among the range 0.45 - 0.50 $g_X\ g_S^{-1}$, being faintly higher when glycerol was the carbon source of the fed-batch phase. When comparing these values with those calculated in the batch cultures, significant differences were observed in the biomass yield of glucose-supported cultivations depending on whether the substrate was limiting or in excess, being higher at limiting conditions. Similar differences were also reported and discussed in previous studies [13]. The main differences were found in Fab production among the cultivations growing with glucose or glycerol throughout the batch phase. When using glycerol, final product titers were around 30% higher than in glucose-grown cultures. Consequently, similar increases were observed in other parameters directly related to it such as q_p, Q_p (volumetric productivity), total productivity and product yields. In contrast, when comparing cultivations that used the same carbon source in the batch phase but different substrates in the fed-batch-phase, minor differences were found in Fab production and related parameters mentioned above. These differences were quantified around 5 – 6 %. The only exception to this trend was observed in the total Fab production in fed-batch cultivations grown on glycerol during the batch phase. Total Fab production was 13% higher in the glucose-supported fed-batch phase.

The results obtained in fed-batch cultures combining different carbon sources indicate that the batch phase has a strong influence on the overall process yield and productivity, being

significant higher when the substrate used for this first phase was glycerol. These differences could be related to the metabolic changes described in the previous section. Specifically, during the last hours of the glucose batch and the first hours of the fed-batch phase, when the metabolic changes are taking place in the cells, the productivity of heterologous proteins lowers until the metabolic quasi-steady state is reached. Conversely, the results of production parameters (q_P and Q_P) and biomass yields did not differ significantly among cultures using glycerol or glucose as alternative carbon sources throughout the fed-batch phase. Since glucose and glycerol are both limiting substrates during the fed-batch phases, one can hypothesize that both metabolic pathways are fully respirative, opposite to the batch phase using glucose, where the respirofermentative metabolism can be suggested.

Thus, the selection criterion between both alternatives must be based on other parameters than the mentioned above. From an industrial point of view, heat production and oxygen consumption could become bottlenecks in large-scale processes. Therefore, both parameters have been used to compare different carbon sources in order to identify the best alternative for industrial *P. pastoris* fed-batch cultivations. The comparison of these parameters among glucose-based processes and methanol-based processes have been already reported in previous works, showing prominent advantages for glucose-supported cultivations [22].

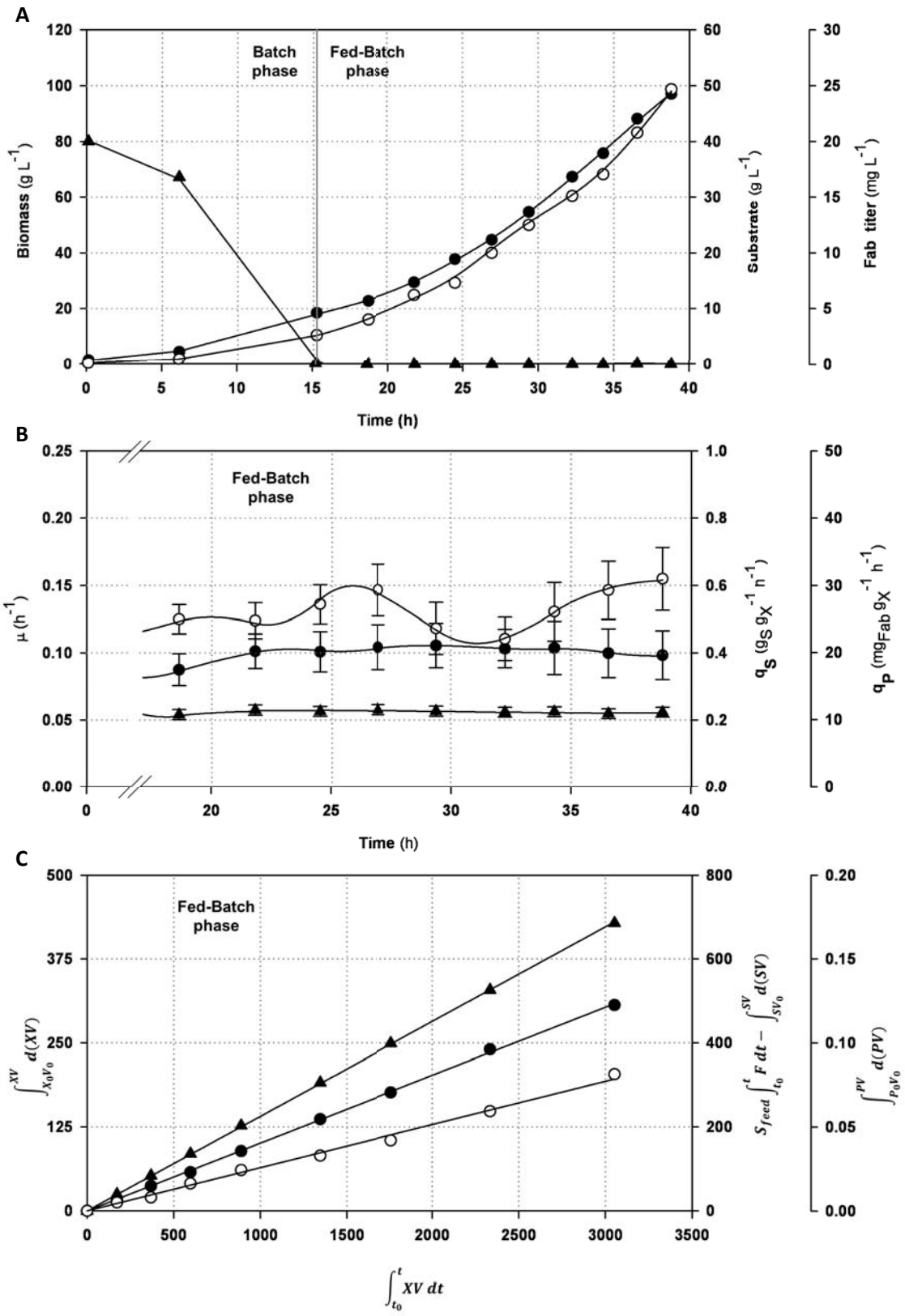

Figure 4.2: Standard fed-batch culture with a combination of glycerol-glucose at nominal μ=0.10 h^{-1}: (A) Biomass (●), substrates (▲), and Fab titration (O) time profiles; (B) Specific growth rate (μ) (●), specific substrate uptake rate (q_s) (▲) and specific Fab production rate (q_p) (O) time evolution, error bars indicate the uncertainty of the discrete estimated specific rates; (C) Determination of the mean specific rates: specific growth rate (μ) (●), specific substrate uptake rate (q_s) (▲) and specific Fab production rate (q_p) (O).

Table 4.2: Physiological and production parameters of *P. pastoris* during fed-batch cultivations at nominal specific growth rate μ = 0.10 (h^{-1}), using different combinations of carbon sources as a substrate.

Substrates	Max. Biomass ($g\ L^{-1}$)	Max. Fab Concentration ($mg\ L^{-1}$)	Fed phase μ mean (h^{-1})	Fed phase q_S mean ($g_S\ g_X^{-1}\ h^{-1}$)	Fed phase q_P mean ($mg_{Fab}\ g_X^{-1}\ h^{-1}$)
Glycerol - Glucose	97.0 ± 3.9	24.7 ± 0.9	0.101 ± 0.001	0.225 ± 0.001	25.7 ± 0.4
Glycerol - Glycerol	101.7 ± 4.1	23.2 ± 0.8	0.098 ± 0.001	0.185 ± 0.002	23.2 ± 0.3
Glucose - Glucose	95.3 ± 3.8	17.7 ± 0.6	0.099 ± 0.003	0.203 ± 0.001	17.4 ± 0.3
Glucose - Glycerol	99.6 ± 4.0	18.6 ± 0.7	0.098 ± 0.001	0.206 ± 0.001	19.6 ± 0.3

Substrates	Overall $Y_{X/S}$ ($g_X\ g_S^{-1}$)	Overall $Y_{P/X}$ ($mg_{Fab}\ g_X^{-1}$)	Overall $Y_{P/S}$ ($mg_{Fab}\ g_S^{-1}$)	Total production (mg_{Fab})	Total productivity ($mg_{Fab}\ h^{-1}$)	Q_p,Volumetric productivity ($mg_{Fab}\ L^{-1}\ h^{-1}$)
Glycerol - Glucose	0.45 ± 0.02	0.26 ± 0.01	0.113 ± 0.004	89.6 ± 3.1	2.31 ± 0.08	0.64 ± 0.02
Glycerol - Glycerol	0.50 ± 0.02	0.23 ± 0.01	0.114 ± 0.004	79.6 ± 2.8	2.13 ± 0.07	0.62 ± 0.02
Glucose - Glucose	0.45 ± 0.02	0.19 ± 0.01	0.088 ± 0.003	70.4 ± 2.5	1.73 ± 0.06	0.44 ± 0.01
Glucose - Glycerol	0.47 ± 0.02	0.19 ± 0.01	0.087 ± 0.003	72.3 ± 2.5	1.80 ± 0.06	0.47 ± 0.02

In the present study a comparison of estimated heat production and oxygen requirements for either glucose or glycerol supported cultivations of *P. pastoris* were carried out. In order to identify which is the most suitable carbon source in terms of heat production, equivalent bioprocesses using glucose or glycerol were compared applying the estimation methodology described in section 4.3.4. All the terms of the heat production equation (7) are identical except $Y_{Q/X}$. Therefore, this is the key factor that was used as a selection criterion. Applying the equation (8) with the bibliographic data shown in section 3.4, the estimated value of $Y_{Q/X}$ for glycerol-supplied cultivations was 10% higher than growing on glucose. It must be remarked that this heat yield is highly sensitive to $Y_{X/S}$ changes.

On the other hand, in aerobic systems, oxygen is a key substrate employed for growth, maintenance and in other metabolic routes including product synthesis. Due to its low solubility in cultivation broths and the important requirements of it in fed-batch cultivations at very high cell densities, the oxygen transfer to the cells is crucial, especially in large-scale aerobic bioprocesses [47].

In *P. pastoris* fed-batch cultivations, the maximum oxygen transfer capacity of the reactor can be the limiting factor that determines the maximum amount of biomass that can be reached. Air supplementation with pure oxygen is possible at small scale but it presents important drawbacks at large-scale production in terms of cost and safety risk [48]. Assuming as equivalent the rest of system parameters that affects to the oxygen consumption, oxygen yield ($Y_{O_2/X}$) must be taken into account as a key factor for the comparison between both substrates.

Literature values of this parameter for *P. pastoris* growing in different carbon sources have been compared: glucose, 0.022 mol O_2 g_X^{-1} [35]; glycerol, 0.043 mol O_2 g_X^{-1} [34]; methanol (P_{AOX1} system, Mut^+ strain), 0.185 mol O_2 g_X^{-1} [49]. The biggest difference of these estimations is observed among methanol-based and glucose or glycerol-based cultivations. Comparing glucose and glycerol a clear difference is also observed, that is, using glycerol oxygen requirements are 2-fold higher.

In summary, glucose was the carbon source selected for fed-batch phase. The lower $Y_{Q/X}$ and $Y_{O_2/X}$ have important advantages from an industrial application point of view.

4.5.3 Analysis of the effect of the specific growth rate in fed-batch cultivations

Specific growth rate is a key parameter for the production of heterologous protein. It affects directly to the productivity of the bioprocess. As a result, the impact of this parameter have been comprehensively studied for different yeast hosts using chemostats [11,50–52]. However, to our knowledge, there are no equivalent studies using fed-batch cultivations, due the higher complexity of the experimental set up.

In this work, once glycerol was selected for batch phase and glucose for fed-batch phase as best substrates, a study of the influence of specific growth rate on Fab production, using this substrate combination, was made. A pre-programmed exponential feeding rate profile for substrate addition to maintain constant μ was the operational strategy selected. The selected set-points of specific growth rate in fed-batch phase growing on glucose were 0.05, 0.10 and 0.15 h^{-1}. The maximum selected set-point of μ was lower than the μ_{MAX} obtained in batch experiments (0.19 h^{-1}) in order to avoid a possible glucose accumulation. Again, fermentations were stopped when DCW values reached around 100 g L^{-1}. All fermentation parameters and shown in Table 4.3, and the evolution of biomass and specific growth rate during the fermentation are presented in Figures 4.3A and 4.3B. As can be seen in Figure 4.3B and the calculated values of μ mean, one can conclude than the pre-programmed exponential feeding rate was accurately implemented. As observed in Figure 4.3C, the evolution of specific glucose consumption rates in each growth rate condition is consistent with the corresponding specific growth rates selected.

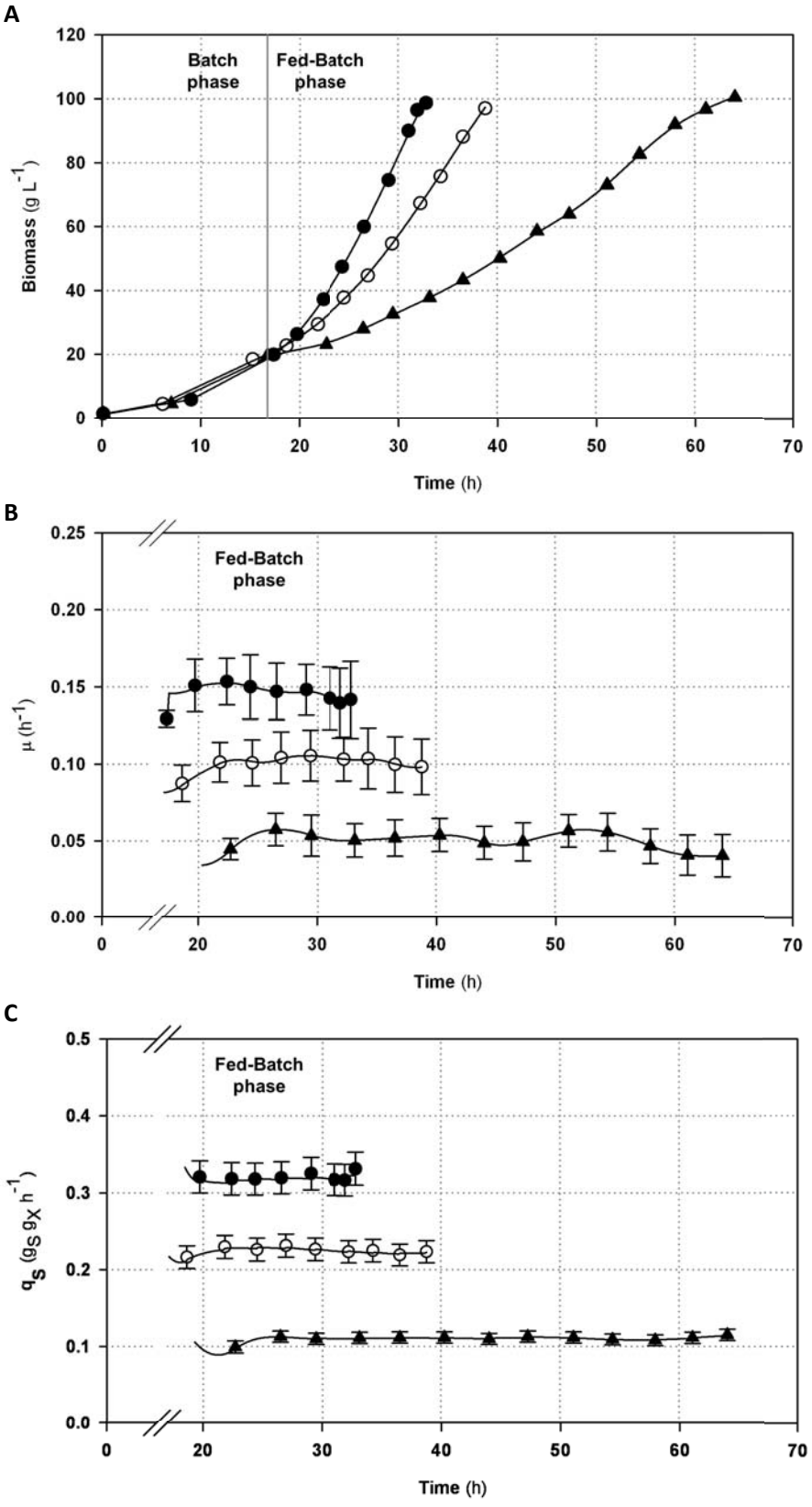

Figure 4.3: Biomass, μ and qs time evolution in fed-batch cultures at different nominal specific growth rates, (▲) μ = 0.05 h-1 , (O) μ = 0.10 h-1, (●) μ = 0.15 h-1; (A) Biomass concentration profiles; (B) Discrete specific growth rate (m); (C) Discrete specific substrate uptake rate (qs). Error bars indicate the uncertainty of the discrete estimated specific rates.

Table 4.3: Physiological and production parameters of *P. pastoris* during fed-batch cultivations with glycerol (batch phase) and glucose (fed-batch phase) as substrates at different nominal specific growth rates.

Nominal μ (h^{-1})	Max. Biomass ($g\ L^{-1}$)	Max. Fab Concentration ($mg\ L^{-1}$)	Fed phase μ mean (h^{-1})	Fed phase q_S mean ($g_S\ g_X^{-1}\ h^{-1}$)	Fed phase q_P mean ($mg_{Fab}\ g_X^{-1}\ h^{-1}$)
0.05	100.5 ± 4.0	15.1 ± 0.5	0.050 ± 0.001	0.111 ± 0.001	7.7 ± 0.4
0.10	97.0 ± 3.9	24.7 ± 0.9	0.101 ± 0.001	0.225 ± 0.001	25.7 ± 0.4
0.15	98.7 ± 3.9	24.1 ± 0.8	0.146 ± 0.001	0.312 ± 0.002	35.2 ± 0.7

Nominal μ (h^{-1})	Overall $Y_{X/S}$ ($g_X\ g_S^{-1}$)	Overall $Y_{P/X}$ ($mg_{Fab}\ g_X^{-1}$)	Overall $Y_{P/S}$ ($mg_{Fab}\ g_S^{-1}$)	Total production (mg_{Fab})	Total productivity ($mg_{Fab}\ h^{-1}$)	Q_p, Volumetric productivity ($mg_{Fab}\ L^{-1}\ h^{-1}$)
0.05	0.46 ± 0.02	0.15 ± 0.01	0.069 ± 0.002	55.0 ± 1.9	0.86 ± 0.03	0.24 ± 0.01
0.10	0.45 ± 0.02	0.25 ± 0.01	0.113 ± 0.004	89.6 ± 3.1	2.3 ± 0.08	0.64 ± 0.02
0.15	0.45 ± 0.02	0.24 ± 0.01	0.110 ± 0.004	90.41 ± 3.2	2.75 ± 0.10	0.73 ± 0.02

Although the total Fab produced for 0.10 and 0.15 μ set-points (h^{-1}) is similar, the different fermentation time employed made the highest specific growth rate selected as the strategy to maximize the productivity (Figure 4.4).

For all the μ tested the amount of extracellular Fab titered represents about 10 – 15 % of the total extracellular protein produced.

Relationships between specific rates are presented in Figure 4.5. From the slope of the q_S variation against the μ, one can conclude that the overall substrate to biomass yield is independent from the μ applied. Also, a typical specific growth rate monotonic increasing pattern for the specific production rate is depicted. The higher the μ, the higher the q_P is. However, $Y_{P/X}$ is dependent on the specific growth rate attained.

These results are consistent with previous works that studied the impact of the specific growth rate in the production of Fab in *P. pastoris* under the control of glycolytic promoter P_{GAP} [11,52]. Specifically, the production of the human Fabs 3H6 and 2F5 were studied in chemostat under glucose limitation using the same expression system, presenting also the best production rates at the highest specific growth rate.

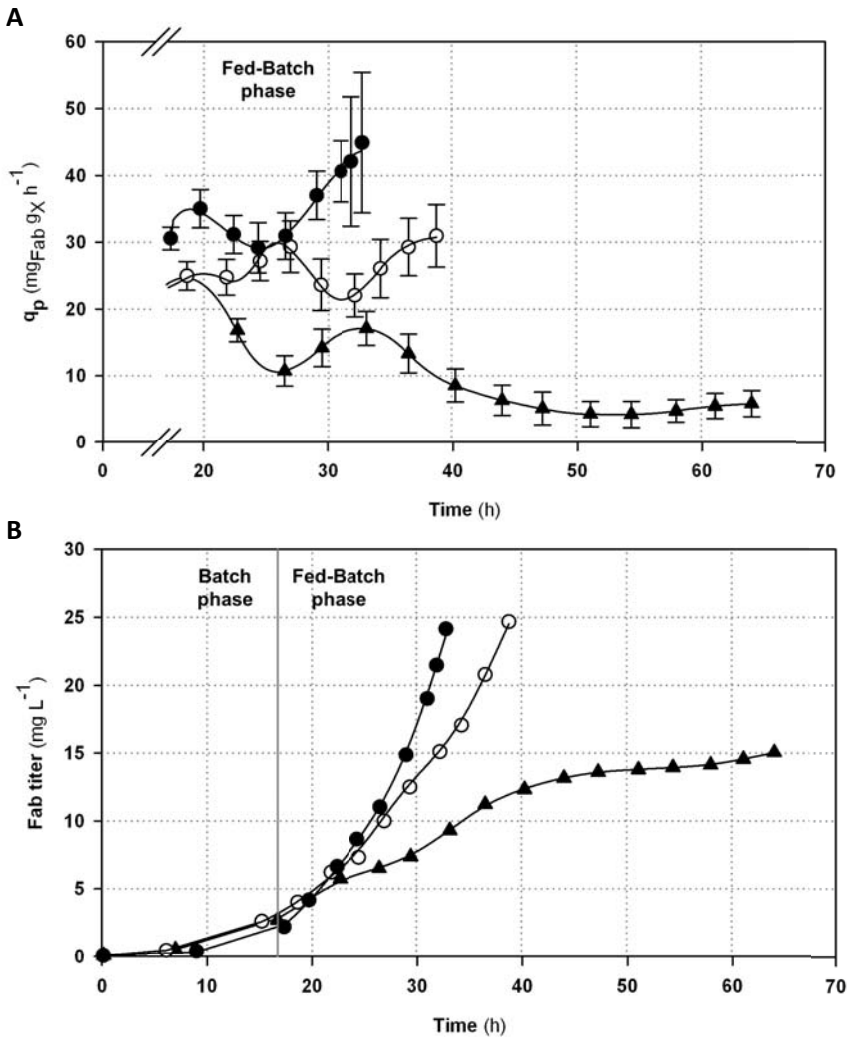

Figure 4.4. Product time profiles in fed-batch cultures at different nominal specific growth rates: (▲) μ = 0.05 h^{-1}, (O) μ = 0.10 h^{-1}, (●) μ = 0.15 h^{-1}: (A) 2F5 Fab concentration; (B) Discrete specific Fab production rates (q_p), error bars indicate the uncertainty of the discrete estimated specific rates.

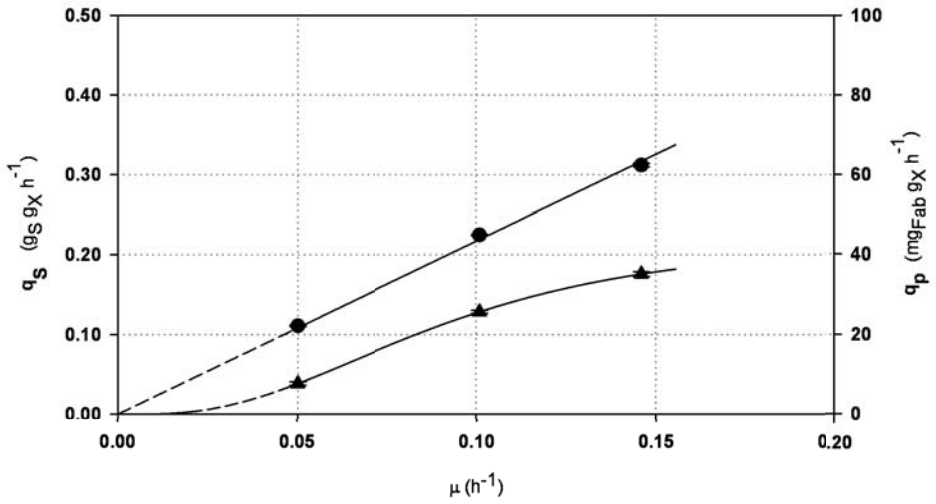

Figure 4.5. Relationship between specific rates (q_S, q_P) and specific growth rate (μ). Specific substrate uptake rate (●) and specific Fab production rate (▲).

The best results of Fab production in fed-batch cultivations obtained in this study can be also compared with previous works where the same Fab was produced in fed-batch process using the same strain. In these studies, different feeding strategies were applied in the fed-batch phase, namely, a constant feed rate [25] and an optimized feeding profile that aimed to maximize the volumetric productivity [11]. In order to compare the results obtained with different operational strategies mentioned above, mean specific production rates (q_P) were estimated as time-averaged values from discrete available literature data.

Thus, the q_P value reached in this work, 35.2 $\mu g_{Fab}\, g_X^{-1}\, h^{-1}$, was about 4-fold higher than using a constant feed rate, 8.2 $\mu g_{Fab}\, g_X^{-1}\, h^{-1}$, and 25% higher than applying an optimized profile, 28.0 $\mu g_{Fab}\, g_X^{-1}\, h^{-1}$. To maximize the volumetric productivity, Q_P, can be also a criterion to evaluate different operational strategies, but always considering biomass produced within the processes. Focusing on this performance parameter, the results presented in this work are slightly higher than the reported applying an optimized profile, 0.73 vs. 0.67 ($mg_{Fab}\, L^{-1}\, h^{-1}$). Nevertheless, both strategies increased about 3.5-fold the values obtained using a constant feed rate, 0.21 ($mg_{Fab}\, L^{-1}\, h^{-1}$), in which it is also important to note that the biomass reached was 50% higher than in the other fed-batch cultivations.

The major advantage of the strategy described in this work is the simplicity of the whole system based on an exponential feed compared to the application of optimal trajectories, somewhat more complex.

4.6 Conclusions

Glucose and glycerol have been used as alternative carbon sources in *P. pastoris* fed-batch bioprocesses producing recombinant proteins under the constitutive *GAP* promoter. In this work, both options have been compared producing the human Fab 2F5 as a protein model.

For the first step of the bioprocess, the batch phase, maximum specific growth rate and total yield attained was quite similar for both substrates. Nevertheless, when glucose was used as C-source the production of fermentative by-products and the observed cell aggregates affected the reproducibility and operability of the batch phase.

In fed-batch cultivations using both substrates, an increase of DO set-point until 50% for glucose-supported cultures in the batch phase reduced the fermentative by-products, but the overall Fab production was still negatively affected. Thus, glycerol was selected as the best substrate at the batch culture phase.

When glycerol and glucose were compared in the fed-batch phase, yields and productivities did not differ significantly. Nonetheless, the carbon source selected for the fed-batch phase was glucose, due to provide lower heat yield ($Y_{Q/X}$) and oxygen to biomass yield ($Y_{O_2/X}$)

comparing with glycerol. Hence, minor cooling and oxygen requirements would be great advantages from an operational point of view. Other authors also recommended the use of glucose only under substrate limited growth conditions [13].

Finally, the effect of the specific growth rate on Fab production was also evaluated for the constitutive Fab 2F5 production. The lower the μ implemented, the lower $Y_{P/X}$, $Y_{P/S}$ and final production were. The maximum values of these parameters were obtained from medium to high μ set-point. This fact seems to be a common characteristic for the constitutive Fab production and general for this constitutive P_{GAP}-based expression system of *P. pastoris* [11,52]. Exponential feeding at high specific growth rates would be the most suitable strategy due to its simplicity and to provide similar productivities than those previously reported for the production of the same Fab in fed-batch cultivations [11] .

4.7 Nomenclature

List of symbols

CPR	Carbon dioxide production rate (mol $_{CO_2}$ L^{-1} h^{-1}).
F_i	Volumetric feeding rates of the components (L h^{-1})
OUR	Oxygen uptake rate (mol $_{O_2}$ L^{-1} h^{-1}).
P	Product concentration (mg $_{Fab}$ L^{-1})
Q_P	Volumetric productivity (mg $_{Fab}$ L^{-1} h^{-1})
q_P	Specific product formation rate (mg $_{Fab}$ g_X^{-1} h^{-1})
q_S	Specific substrate uptake rate (g_S g_X^{-1} h^{-1})
S	Substrate concentration (g L^{-1})
V_i	Volume (L)
X	Dry biomass concentration (g L^{-1})
$Y_{CO_2/X}$	Carbon dioxide to biomass yield coefficient (mol $_{CO_2}$ g_X^{-1})
$Y_{O_2/X}$	Oxygen to biomass yield coefficient (mol $_{O_2}$ g_X^{-1})
$Y_{P/S}$	Product to substrate yield coefficient (mg $_{Fab}$ g_S^{-1})
$Y_{P/X}$	Product to biomass yield coefficient (mg $_{Fab}$ g_X^{-1})
$Y_{Q/X}$	Heat yield coefficient (kJ g_X^{-1})
$Y_{X/S}$	Biomass to substrate yield coefficient (g_X g_S^{-1})

Greek symbols

μ	Specific growth rate (h^{-1})
ρ_i	Density (g L^{-1})
σ	Fraction of dry matter in the biomass (g g^{-1})
ΔH_i	Combustion enthalpy of the components (kJ g^{-1})

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5

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

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5.1 Abstract

The recombinant protein production platform based on the *GAP* promoter and *Pichia pastoris* as a host has become a very promising system from an industrial point of view. The need for highly productive bioprocesses gives grounds for the optimization of fermentation strategies maximizing yields and/or productivities, which are often associated with cell growth. Coherent with previous studies, a positive effect of high specific growth rate (μ) on the productivity was observed in carbon-limited chemostat cultivations secreting an antibody fragment. Notably, no significant impact of this factor could be observed in the balance intra- and extracellular of the product. Accordingly, fed-batch cultures operating at a constant high μ were established. Furthermore, short carbon-starving periods were introduced along the exponential substrate feeding phase. Strikingly was observed an important increase of specific production rate (q_P) during such short carbon-starving periods in relation, to the exponential substrate feeding intervals. Therefore, we propose the application of carbon-starving periods as an innovative operational strategy resulting into increments up to 50% of both yields and total production. The implementation of the proposed substrate feeding profiles should be complementary to cell engineering strategies to improve the relation q_P vs μ, thereby enhancing the overall bioprocess efficiency.

5.2 Introduction

Currently, a wide range of products such biopharmaceuticals, organic acids, antibiotics, enzymes or amino acids are industrially produced in biological systems using bioprocess technology [1]. Over the last two decades, bioengineering has made a significant progress on the production of heterologous proteins of both therapeutic and industrial interest, being one of the most successful and profitable bioprocesses [2].

Among all the suitable host organisms commonly used for its production, the methylotrophic yeast *Pichia pastoris* is considered one of the most effective and versatile expression system [3,4]. The combination of traits that makes *P. pastoris* a very interesting cell factory for recombinant protein production has been extensively described in several reviews [5,6]. Although this yeast is mainly known for its strong methanol-inducible *AOX1* promoter (P_{AOX1}) [7,8], during the last years, the constitutive production driven by the *GAP* promoter (P_{GAP}) has been perceived as an efficient alternative production strategy to avoid the use of methanol in the bioprocess [9,10]. A comparison between the advantages and drawbacks of both production alternatives can be found in the literature, in which P_{GAP}-based processes offer important advantages from an industrial point of view, such important decreases on heat production and oxygen requirements of the processes [11,12]. Hence, several alternative fermentation strategies have been extensively studied for this expression system [13,14].

A key advantage of *P. pastoris* as a host in front of other alternatives, especially the prokaryotic systems, is its ability to secrete the product to the cultivation broth, which facilitates importantly the downstream processes [15]. In addition, the passage of proteins through the secretory pathways permits posttranslational events that usually are essential for the biological activity of the proteins [16]. Nevertheless, high levels of heterologous protein expression can lead to saturation or overloading of the secretory pathways, where the product is accumulated intracellularly and often also degraded, resulting into an important decrease of the production yield. This fact is often a major bottleneck of great importance for this biotechnological process development [17–19].

To study the effect of the secretory pathway saturation on the bioprocess efficiency, it is of capital interest the reliable quantification and recovery of the total amount of product accumulated intracellularly along a cultivation [20,21]. Some previous studies focused on the efficacy of high-pressure homogenization disruption procedures on methanol-based cultivations, as it is known that cells growing on this substrate present a significant widening of the cell wall thickness [22,23]. In addition, since an important amount of the protein of interest is expected to be retained through the secretory pathway, besides the soluble part of the cell lysates, the insoluble fraction must be taken into account in order to avoid a underestimation of the target product, as it contains the cell membranes, endoplasmic reticulum (ER), Golgi and other organelles where the protein of interest may be retained [24]. A reliable quantification of the product present in the insoluble fraction requires an extraction procedure that involves the use of detergents, which its efficiency is protein-dependent.

Previous studies concerning the P_{GAP}-based expression system described an important effect of the specific growth rate (μ) on the bioprocess productivity in both chemostat and fed-batch cultivations. These studies conclude that a high μ positively affects the production rates of protein [25,26]. The most commonly used cultivation strategies for this system are relatively simple, these are basically based on the implementation of feeding rate profiles for the substrate addition that maintain the desired specific growth rate; constant feeding rate for chemostat operations, and pre-programmed exponential feeding profiles for fed-batch cultivations [11,13]. On the other hand, Kern et al. [27] described an important productivity increase of proteins driven by P_{GAP} upon short-time depletion of glucose. This effect was observed in shake-flask cultures, but it has not been reported for high-density fed-batch cultures.

The aim of the present work is to systematically elucidate the effect of the specific growth rate on protein secretion by studying the balance intra- and extracellular product in carbon-limited chemostat cultures of *P. pastoris* growing in a wide range of dilution rates. Based on these studies, high-cell density fed-batch cultures at high specific growth rate were conducted to

both study the effect of carbon source starvation periods on the secretory efficiency of the recombinant protein and in the overall process productivity and yields, all together as an innovative operational strategy.

A strain expressing the human 2F5 antigen-binding fragment (Fab), has been used as model protein in this work. Fabs have a wide range of applicability as therapeutic agents [28] and are complex proteins composed by different domains connected via disulfide bonds [29], which makes them a suitable model protein for studying the efficiency of recombinant protein production processes.

5.3 Materials and methods

5.3.1 Strain

A *P. pastoris* strain X-33 expressing both light and heavy chain genes of the human Fab 2F5 under control of the constitutive *GAP* promoter was used in this study. This yeast strain is able to secrete the Fab to the medium by means of the *Saccharomyces cerevisiae* α–mating factor signal sequence. The details of the strain construction were described previously [29].

5.3.2 Fermentation

The preparation of the inoculum cultures for the cultivations in bioreactors were performed as described by Garcia-Ortega et al. [11].

5.3.2.1 Chemostat cultivation

Chemostat cultivations were performed in a 2 L Biostat B Bioreactor (Braun Biotech, Melsungen, Germany) at a working volume of 1 L. Cells were grown under carbon-limiting conditions at wide range of dilution rates (D) from 0.025 to 0.15 h^{-1}. The cultivation were carried out using the batch and chemostat medium compositions detailed elsewhere [13]. Minor differences were applied to the cited compositions, which are detailed below. Glucose concentration was 50 g L^{-1}, Biotin 0.02% (1 mL), PTM_1 (1.6 mL) trace salts stock solution (also described by Maurer et al. 2006) and antifoam Glanapon 2000kz (0.2 mL; Bussetti & Co GmbH, Vienna, Austria) were added per liter of chemostat medium.

Culture conditions were monitored and controlled at set points: temperature, 25 °C; pH, 5.0 with addition of 15% (v/v) ammonium hydroxide; culture vessel pressure, 1.2 bars; pO_2, above 20% saturation by controlling the stirring rate between 600 and 900 rpm during the batch phase, in the continuous phase it was kept constant at 700 rpm; air gas flow, 0.8 vvm by means of thermal mass-flow controllers (TMFC; Bronkhorst Hi-Tech, Ruurlo, The Netherlands). An exhaust gas condenser with cooling water at 4ºC minimizes mass loses by water evaporation and other volatile compounds. In all the experiments, the continuous cultivations were carried out for at least five residence times (τ) before taking samples and shifting the inlet feeding rate to attain other specific growth rate set points.

5.3.2.2 Fed-batch cultivation

Fed-batch cultivations were performed aiming to achieve pseudo-steady-state conditions for specific rates during carbon-limiting growth as was previously described [11]. In brief, cells were grown at 25 ºC, pH=5 by adding ammonium hydroxide (30%, v/v) and pO_2 above 20% saturation by controlling the stirring speed between 600 and 1200 rpm and using mixtures of air and O_2 at total aeration within 1.0 and 1.25 vvm. All the fed-batch cultivations were carried out at the same specific growth rate, 0.15 h^{-1}, by means of the implementation of a pre-programmed exponential feeding rate profile for substrate addition derived from mass balance equations. In addition, determined stops in the feeding profiles were scheduled in order to study the effect of controlled carbon-starving conditions.

5.3.3 Cell disruption and protein extraction

5.3.3.1 High-pressure homogenisation

Fermentation broth samples corresponding to an initial OD_{600} ≈ 125 were harvested by centrifugation (4500 *g*, 3 min, 4 ºC) and pellets were washed twice in cold PBS (pH 7.0) in order to remove all media components and other contaminants. Cells were then resuspended in 8 mL of cold breaking buffer (PBS, pH 7.0, 1mM PMSF) and disrupted by high-pressure homogenization using a *One-Shot Cell Disrupter* (Constant Systems Ltd, Deventry, UK). Once disrupted, homogenates were clarified by centrifugation (15000 *g*, 30 min, 4 ºC). Supernatants were collected and stored as soluble cytosolic fraction (SCF) while pellets were kept as the insoluble membrane fraction (IMF). The whole disruption process was carried out at low temperature in order to preserve protein properties as well as to avoid possible protease activity. In addition, the PMSF added in the breaking buffer was used as a protease inhibitor.

5.3.3.2 Evaluation of cell disruption efficacy

Cell number was determined after each disruption pass by means of flow cytometry assays (Guava EasyCyte™ Mini cytometer, Millipore, Hayward, CA, USA). The extent of disruption was expressed as:

$$X\ (\%) = 100 \cdot \frac{n-n_i}{n}\ (\%) \qquad (1)$$

Where *X* (%) is the degree of disruption; n, the initial number of cells before disruption; n_i, number of non-disrupted cells after each pass. Determinations were performed by triplicate and the relative standard deviation (RSD) was about 2%.

5.3.3.3 Protein extraction from insoluble membrane fraction (IMF)

Protein extraction was carried out in chilly conditions. Fraction pellets were resuspended by pipetting in 1 mL extraction buffer supplemented with detergent and then vortexed. Extracts were incubated in gently shaking at 4 ºC, clarified by centrifugation (2300 *g,* 5 min, 4 ºC) and supernatants were stored as insoluble membrane fraction (IMF) extracts.

For the initial study focused on a screening for the detergent and buffer optimization, three different buffers were compared for the target protein extraction. Buffer A: 50 mM Tris-HCl pH

7.4, 300 mM NaCl, 5 mM EDTA, 1 mM PMSF; Buffer B: 10% glycerol, 20 mM HEPES pH 7.0, 100 mM NaCl, 1 mM PMSF; Buffer C: 8% glycerol, 10 mM sodium phosphate pH 8.0, 5 mM EDTA, 500 mM NaCl, 1 mM PMSF. In addition, all the extraction buffers were also supplemented with 1% of three different detergents, Tween 20, Triton X-100 and CHAPS (Sigma-Aldrich, St. Louis, MO, US) for the detergent-buffer screening.

Once the optimal combination of buffer and detergent was selected, different concentrations of CHAPS were tested for an improved Fab extraction. They were also screened different incubation times. In this sense, extractions combining 1 – 2.5% CHAPS and 0 h, 2 h and 16 h (overnight) incubation times were performed and compared.

5.3.4 Analytical methods

5.3.4.1 Biomass determination by dry cell weight (DCW)

P. pastoris biomass concentration of the cultivations samples was determined as DCW using the method described elsewhere [30]. Determinations were performed by triplicate and the relative standard deviation (RSD) was about 3%.

5.3.4.2 Quantification of the Fab 2F5 antibody

Fab 2F5 was quantified in secreted fractions, as well as in the SCF and the IMF. Fab 2F5 concentration was measured by sandwich ELISA as previously described [31]. Determinations were performed by triplicate and the relative standard deviation (RSD) was about 4%.

5.3.4.3 Total protein quantification

Total protein was determined with the bicinchoninic acid protein assay kit (Pierce BCA Protein Assay, Prod. No. 23225, Rockford, IL, USA), according to the manufacturer's instructions. Bovine serum albumin (BSA) was used as the protein standard for the calibration curve.

5.3.4.4 Carbon source and by-products quantification

Glucose, arabitol, glycerol and ethanol concentrations were determined by HPLC with a HP 1050 liquid chromatograph (Dionex Corporation, Sunnyvale, CA, USA) using an ICSep ICE COREGEL 87H3 column (Transgenomic Inc., Omaha, NE, USA). The mobile phase was 8 mM sulphuric acid. Injection volume was 20 μL. Data was quantified by Chromeleon 6.80 Software (Dionex Corporation, Sunnyvale, CA, USA).

5.3.4.5 Off-gas analyses

A quadrupole mass spectrometer (Balzers Quadstar 422, Pfeiffer-Vacuum, Asslar, Germany) was used for on-line exhaust gas analysis in chemostat cultivations. Exhaust gas humidity was reduced by using a condenser (water at 4 ºC) and two silica gel columns. The Faraday cup detector was used for its simplicity, stability, and reliability, determining responses of *m/z* corresponding to the major gas peaks (N_2: 28, O_2: 32, CO_2: 44, Ar: 40).

5.3.5 Process parameters determination, consistency check and data reconciliation

5.3.5.1 Mass balance and stoichiometric equations

The oxidative and oxidoreductive growth can be described on a C-molar basis by a single overall reaction, a so-called Black Box model, which is a simplification of all the biochemical reactions involved:

$$S + Y^*_{O_2/S}\, O_2 \rightarrow Y^*_{x/s}\, X + Y^*_{CO_2/S}\, CO_2 + Y^*_{p/s}\, P \qquad (2)$$

where *S* denotes one single limiting substrate as the carbon and energy source; O_2, oxygen; *X*, biomass; CO_2, carbon dioxide; *P,* products. $Y^*_{i/s}$ are stoichiometric coefficients that can also be called overall "i" component-substrate yields.

Specific rates (q_i) typically conversions rates related to the biomass concentration (equation 3). Yields are defined as ratios between rates (equation 4) and positive.

$$q_i = \frac{r_i}{X} \qquad (3)$$

$$Y_{i/j} = \frac{r_i}{r_j} = \frac{q_i}{q_j} \qquad (4)$$

From off-gas results obtained with the mass spectrometer O_2 and CO_2 balances were carried out in order to estimate accurately the oxygen uptake rate (OUR), carbon dioxide production rate (CPR), and respiratory quotient (RQ), as well as their corresponding specific rates for O_2 and CO_2.

For an ideal stirred tank-reactor, considering conversion rates of biomass formation, substrate uptake and product formation the following mass balance equations for the continuous operation at steady state can be formulated:

$$\begin{bmatrix} \mu \\ q_S \\ q_P \\ q_{O_2} \\ q_{CO_2} \end{bmatrix} XV = \begin{bmatrix} F_{out}\, X \\ -F\, S_0 + F_{out}\, S \\ F_{out}\, P \\ OUR\, V \\ CPR\, V \end{bmatrix} \qquad (4)$$

where μ is the specific growth rate (h^{-1}); q_S, specific substrate uptake rate (g g^{-1} h^{-1}); q_P, specific production rate (U g^{-1} h^{-1}); q_{O2}, specific oxygen uptake rate (mol g^{-1} h^{-1}); q_{CO2}, specific carbon dioxide production rate (mol g^{-1} h^{-1}); *F*, substrate feeding rate (L h^{-1}); F_{out}, outlet flow rate (L h^{-1}); *V*, volume of broth in the reactor (L); S_0, substrate feeding concentration (g L^{-1}); *OUR*, oxygen uptake rate (mol L^{-1} h^{-1}); *CPR,* carbon dioxide production rate (mol L^{-1} h^{-1}). F_{out}, the outlet flow rate can be obtained by the total mass balance for an ideal stirred tank reactor in continuous operation at steady state, as follows:

$$F_{out} = \frac{\rho_{Feed}F - \rho_{H2O}F_{Evap} + \rho_{Base}F_{Base} - \rho_{Broth}F_O + M_{GAS}}{\rho_{Broth}} \qquad (5)$$

where F_{Evap} is the water evaporation rate (L h^{-1}); F_{Base}, base feeding rate (L h^{-1}); F_O, withdrawal rate (L h^{-1}); M_{GAS}, net mass gas flow rate (g h^{-1}); ρ_{Feed}, substrate feed density (g L^{-1}); ρ_{H2O}, water density (g L^{-1}); ρ_{Base}, base density (g L^{-1}); ρ_{Broth}, mean broth density (g L^{-1}). The net mass gas flow rate is calculated with the equation (6):

$$M_{GAS} = -(W_{O2} OUR\ V + W_{CO2} CPR\ V) \qquad (6)$$

where W_{O2} is the oxygen molar mass (g mol^{-1}); W_{CO2}, carbon dioxide molar mass (g mol^{-1}). In case of product stripping for ethanol or any other compound, an additional term is included in equation (4) in order to not underestimate its corresponding specific rate. Substrate and product concentrations were referred to the whole medium, including biomass volume [32]. Corresponding equations to fed-batch culture were previously described [11].

5.3.5.2 Consistency check and data reconciliation

The consistency of the measurements was checked by standard statistical tests considering elemental balances as constraints [33]. Five key specific rates in the black-box process model: biomass generation (μ), glucose uptake (q_s), product formation (q_p), oxygen uptake (q_{O2}), and carbon dioxide production (q_{CO2}) were measured. Carbon and redox balance were used as constraints and protein production considered negligible within these balances.

The χ^2-test performed (α=0.95) for all the experimental data obtained from chemostat cultivations showed the measurements satisfied mostly the stoichiometric model and hence, both C-balance and e-balance. Data reconciliation procedures were used to obtain the best estimates of reaction rates to fit constraints imposed [34].

5.4 Results and discussion

Previous studies on recombinant protein production in *P. pastoris* under the control of the constitutive *GAP* promoter have reported that high specific growth rate (μ) positively affects the production rates of secreted proteins [11,13,35]. This important productivity increase has been related to the transcriptional regulation of certain genes [26,36], as well as to the capacity of the secretory pathways to release the recombinant protein to the cultivation broth [25,37]. In fact, although the biological mechanisms that drive the correlation between protein secretion and growth are complex and by far not fully understood, protein secretion has been considered an important bottleneck in the recombinant protein production processes, at least at high μ [18,25,38].

In order to evaluate the impact of growth conditions on the secretion efficiency of the target protein, initially was performed a comparative study of the extracellular/intracellular product ratio among cells growing at different specific growth rates. For this purpose, cell disruption and protein extraction procedures were specifically optimized in terms of cell disruption settings, extraction buffers and detergent choice for our protein of interest (Fab 2F5), host strain and growth conditions.

5.4.1 Cell disruption and protein extraction procedures

Disruption of *P. pastoris* by using *One-Shot Cell Disrupter* was optimized for the recovery of an antibody fragment (Fab 2F5) produced by *P. pastoris* cells growing on glucose, which its expression was regulated by the constitutive *GAP* promoter.

Initially, cell number of the fermentation samples were counted by flow cytometry, and then disrupted at 2 kbar or 2.5 kbar for 1 to 8 passes. Cell cytometry was used as direct indicator of cell disruption, since cell counting has been described as the most accurate and reproducible measure of cell rupture [39]. Whole cells and cell debris could be easily differentiated in gates, being a fast and reliable method to determine accurately the extent of cell disruption (Figure 5.1A). From the cell number results of each gate, percentage of disruption was calculated using Eq. 1. Percentage of cell disruption and Fab releasing levels in front of pressure disruption and number of passes is showed in Figure 5.1B.

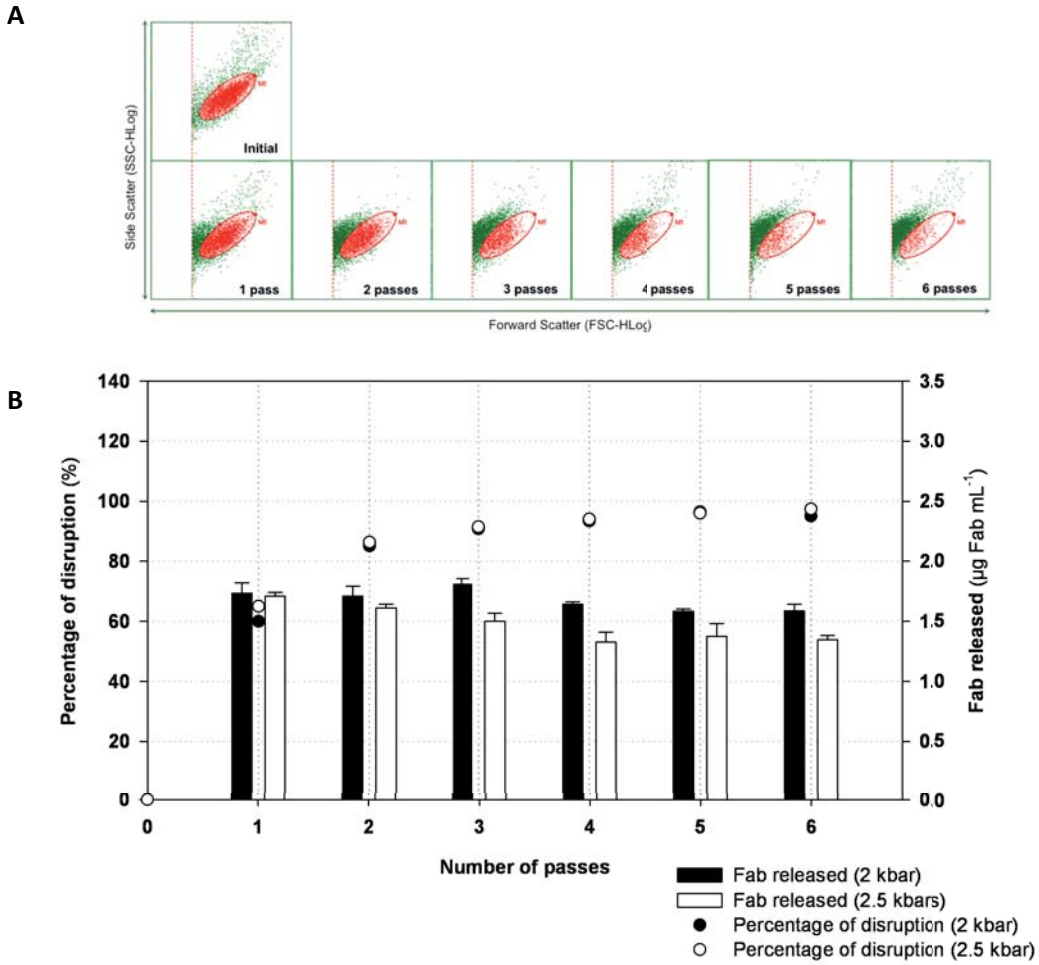

Figure 5.1: Cell disruption of *P. pastoris* cells by high-pressure homogenisation. A: Cell counting by flow cytometry used to determine the extent of cell disruption after different passes; ellipse gate differentiates the whole undamaged cells from damaged and broken cells. B: Comparison between extent of cell disruption and amount of 2F5 Fab released after different passes. Error bars indicate standard deviation.

No major differences were observed in terms of cell disruption between using 2 kbar or 2.5 kbar. Otherwise, an important increase in the extent of cell disruption from one disruption pass to two has been observed, while further passes only increased it slightly. In relation to

Fab 2F5 amounts, levels of recovery were similar when using 2 kbar regardless the disruption passes, while a decrease on Fab recovered amounts was observed when using 2.5 kbar as working pressure. Thus, 2 passes at 2 kbar were selected as the optimal working conditions for Fab 2F5 recovery.

In order to achieve a reliable quantification of intracellular proteins and its possible recovery, a protein extraction step is necessary to determine the proteins associated to membrane fraction and cell organelles present in the IMF, which makes possible to take into account the total amount of target protein retained intracellularly in order to compare the effects caused by different cultivation conditions, as well as along the bioprocesses.

Different buffers (A, B, C; described in Materials and Methods section) were selected from the bibliography [40–42] and compared for extraction efficiency when combined with 1% of three different detergents (Tween20, Triton X-100 and CHAPS). Previous studies used SDS as detergent, but this component was discarded as it interfered with the Fab detection system (data not shown). In Figure 5.2 it can be observed that the best detergent was CHAPS, a zwitterionic detergent, being Buffer B the one leading to the best levels of Fab extraction. It must be taken into a account that the extraction step is a protein dependent process, where the optimal detergent would change depending on protein characteristics (i.e. hepatitis B surface antigen is better extracted by 1.5-2% Tween 20 [42], while G-protein-coupled receptors prefer decylglucopyranoside [41], both non-ionic detergents).

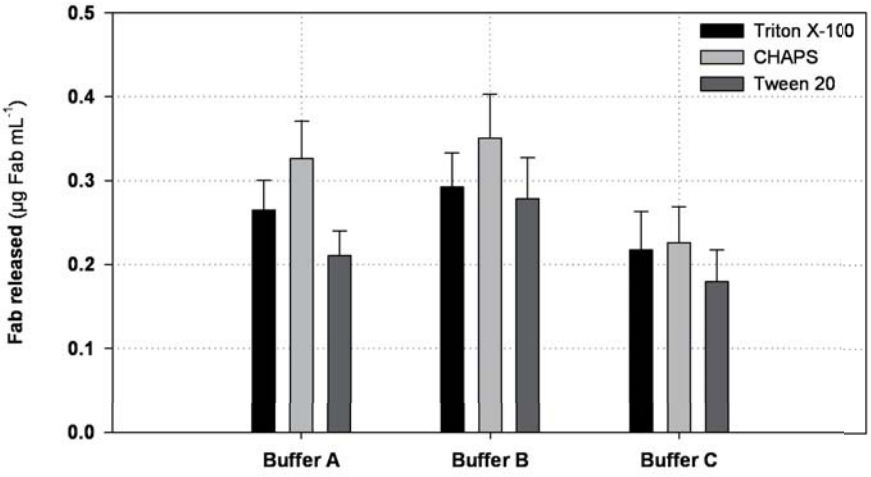

Figure 5.2: Release of 2F5 Fab obtained by using the buffers and detergents compared. Error bars indicate standard deviation.

Different CHAPS concentrations and different incubation times at 4 ºC were tested in order to improve Fab extraction. The amounts of solubilised Fab are shown in Figure 5.3. It was observed that incubation time had an important impact on Fab extraction, where long incubation times (overnight incubation) resulted in 2.5-fold increase of Fab determination. The tested concentrations of CHAPS did not have a significant impact on Fab extraction. Thus, the final selected extraction conditions were 1% CHAPS with Buffer B incubated for 16 h (overnight) at 4 ºC.

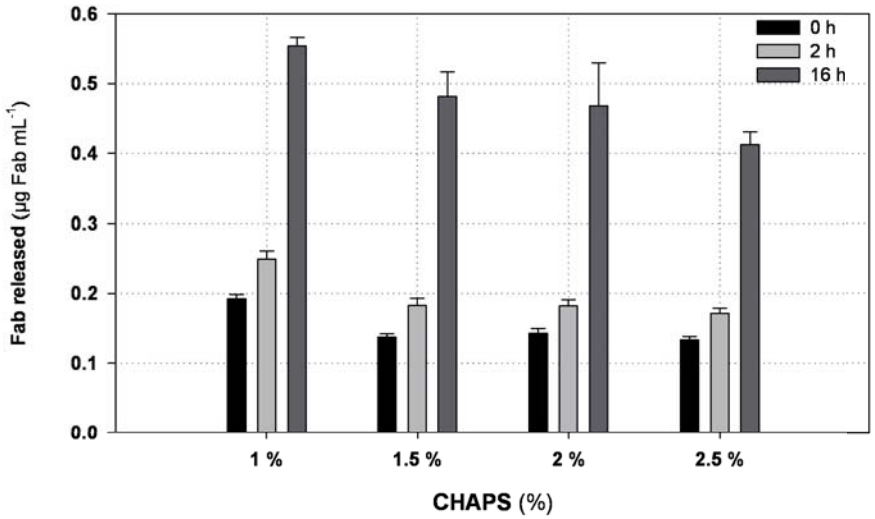

Figure 5.3: 2F5 Fab obtained by using different concentrations of CHAPS and incubation times. Error bars indicate standard deviation.

Thereby, it can be concluded that the developed method improves specifically the overall Fab quantification and recovery by the reliable extraction of protein retained intracellularly, both soluble and insoluble fraction.

5.4.2 Effect of the specific growth rate on the protein retained intracellularly

A set of carbon-limited chemostat cultivations of the recombinant strain growing at different specific growth rates between 0.025 and 0.15 h^{-1} was carried out. Data was obtained from samples taken after, at least, 5 residence times, from when it is considered that the steady state of the culture is reached. The closure of carbon and redox balances calculated for all the culture conditions compared in the present work were always above 95%, additionally results were validated using the standard data consistency check and reconciliation procedures described in materials and methods section. These tests confirm the robustness and the reliability of the results obtained from the chemostat cultivations performed.

The main specific rates of the cultivation are plotted in Figure 5.4A. The parameters related to cell growth (q_{Glu}, q_{O2} and q_{CO2}) presented a similar behaviour; they increased according to the μ. In contrast, RQ remained constant since the proportion between q_{O2} and q_{CO2} was rather constant. The amount of the 2F5 Fab and its distribution in the different fractions studied, as fermentation broth as well as SCF and IMF of the cell lysates, were compared between the different culture conditions tested (Figure 5.4B). As described by other authors, q_P presented a rather linear increase as μ was also increasing; for the highest, it was observed up to an 8-fold increase respect to the lowest. In contrast, no important effect of the specific growth rate was observed in Fab distribution among the different fractions studied; for all the cases, around 80 % of the Fab was secreted, 15% and 5% was detected respectively in the SCF and the IMF. It is also important to mention a significant decrease of the overall biomass yield at low μ

(accordingly to q_{Glu} variation on μ shown in Figure 5.4A) which is due to the higher proportion of maintenance energy requirements respect the total energy available for growth [43].

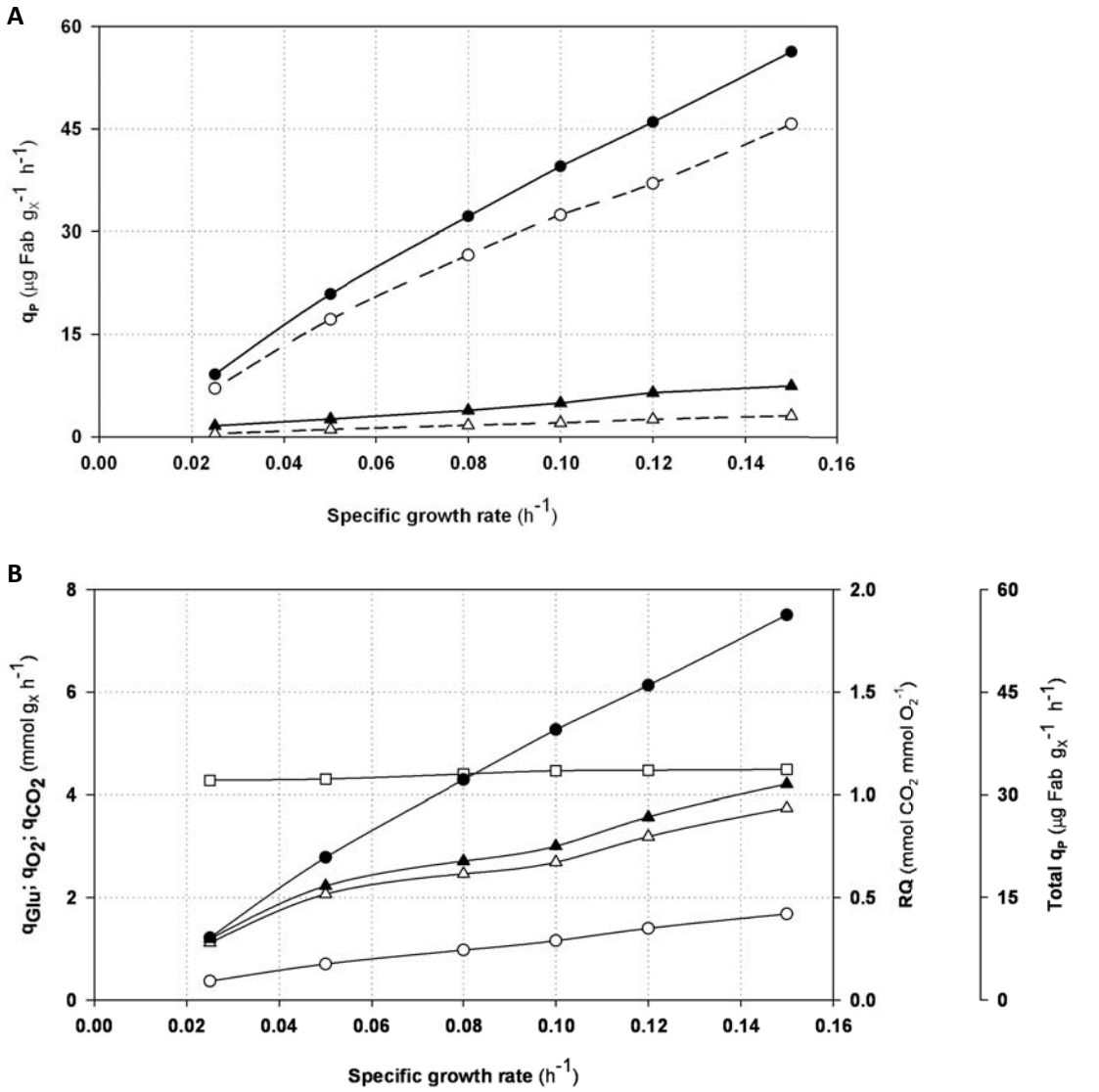

Figure 5.4: Steady states of chemostat cultures at different specific growth rates. A: Main growth parameters and rates: glucose uptake rate (q_{Glu}; ○); oxygen uptake rate (q_{O2}; △); carbon dioxide production rate (q_{CO2}; ▲); respiratory quotient (RQ; □) and total Fab production rate (q_P, ●). B: Fab distribution among the different fractions studied: Total 2F5 synthetized (●); 2F5 Fab secreted to the broth (○); 2F5 present in the soluble cytosolic fraction (SCF; ▲); 2F5 present in the insoluble membrane fraction (IMF; △).

The cause of this important q_P increase have been discussed by other authors, who attributed this effect to several factors. As described Stadlmayr et al. [36], since the expression of the target protein is driven by the glycolytic *GAP* promoter and its transcription levels are directly related with the glycolytic flux, and thus specific growth rate, an important increase of recombinant protein transcription levels takes place at increasing μ. In addition, Rebnegger et al. [26] studied the effect of different specific growth rates on the transcriptome of *P. pastoris*, describing significant changes in the regulation of important groups of genes at high μ that also contributes to the positive effect in the q_P. Specifically, upregulation of translational and UPR genes such those implied in the translocation to ER, the enhance protein folding in ER and

cytosolic chaperones; and downregulation of proteolytic degradation of proteins in the secretory pathway and exocytosis related genes. Thus, although important genes of the secretory pathway are upregulated at high μ, the exocytosis processes may constitute, in fact, the real important bottleneck for the protein secretion. However, from the results presented in this section, there is not a significant intracellular accumulation of protein of interest regardless the specific growth rate, even at high μ's, when the secretory pathways are supposed to be saturated. It may be due to proteolytic degradation processes that take place in the proteasome, considered an important sink for recombinant protein in *P. pastoris* [40,44].

Therefore, it can be concluded that the specific growth rate has an important impact on the total recombinant protein production of this expression system. This parameter regulates the transcriptional levels of several groups of genes that promote the synthesis of proteins expressed under P_{GAP} at high μ's. However, no significant effect could be observed in the balance intra- and extracellular of the product. Therefore, the low fraction of Fab retained intracellularly in any of the conditions tested does not justify the implementation of the recovery of the non-secreted proteins in a real bioprocess in order to improve its efficiency.

5.4.3 Implementation of carbon-starving periods within carbon-limited fed-batch processes

The previously described correlation between q_P and μ has been widely exploited in order to reach either the maximum protein titres and/or productivities in fed-batch cultures by the implementation of feeding profiles that allows achieving optimal specific growth rate [11,13,45]. From the different strategies proposed, the pre-programmed exponential feeding rate profile for substrate addition to maintain a very high constant μ in carbon-limiting conditions lead to the best results in terms q_P. Furthermore, it has the advantage to be the simplest to carry out in any standard fed-batch cultivation system.

On the other hand, as mentioned in the introduction section, Kern et al. [27] described an important productivity increase of proteins regulated by P_{GAP} for a short-time after the depletion of glucose. However, the work did not hypothesize about the causes that could lead to the rapid increase of protein synthesis during short periods and the potential applications on bioreactor cultures. Thus, this work aimed to study the implementation of short carbon-starving periods in fed-batch cultivations based on pre-exponential feeding rate profiles at high specific growth rates and its effect on the recombinant protein production rates and yields, as well as its distribution among the different cell fractions analysed.

Figure 5.5 presents the basic features of the implementation of 30 min carbon-starving periods every 3 h of standard pre-exponential feeding profile. In Figure 5.5A the innovative profile is

set side by side with the conventional strategy. In addition, q_P profiles are also compared, from which it can be observed that during the periods when the feeding is stopped, and thus the cells are subjected to carbon-starving conditions, there is a very important increase of the specific production rate, the effect that was aimed to be exploited in this work. In Figure 5.5B the time profile of fed glucose and Fab titration are compared showing that the total amount of glucose added in to the system is equivalent, but in a longer time; in contrast, the production of Fab is significantly higher, which supposes a very important increase in the substrate to product yield.

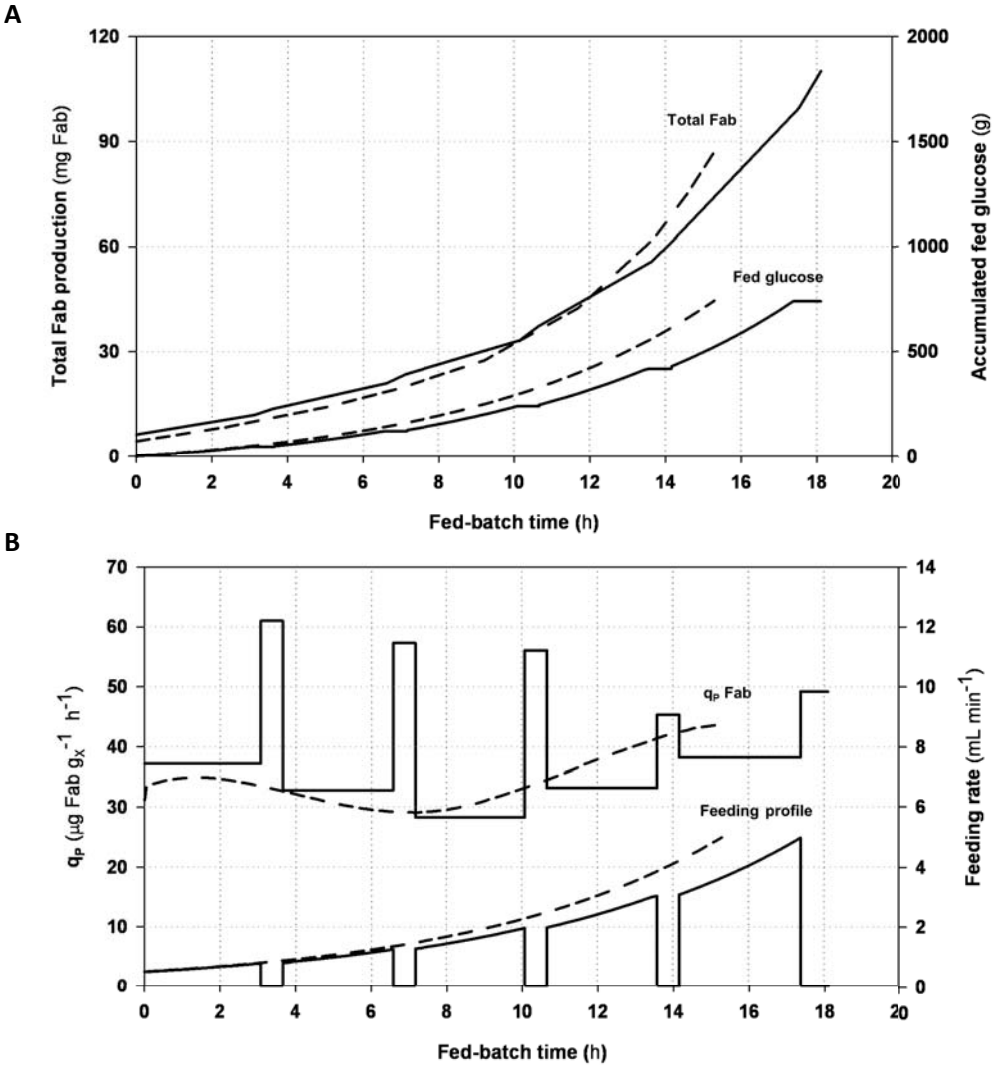

Figure 5.5: Main features of the implementation of 30' carbon-starving periods every 3 h of exponential feeding respect to the standard exponential feeding profiles in fed-batch cultivations at nominal specific growth rate μ= 0.15 (h^{-1}). A: Time evolution of the specific production rate of 2F5 Fab (q_P) versus the feeding rate time profile. Solid lines indicate mean specific rates calculated within feeding and starving periods. B: Comparison of total 2F5 Fab produced and fed glucose time profile.

Therefore, the objective was to compare different combinations of carbon feeding and starvation periods in order to determine the strategy that leads to the maximum increase production at the end the fed-batch. The different feeding strategies carried out are presented

in Table 5.1, which compared feeding periods of 1.5 and 3 h, and also carbon-starving periods of 0.5 and 1 h.

Table 5.1: Summary of the different feeding strategies implemented in fed-batch cultivations grown at nominal specific growth rate μ = 0.15 (h^{-1}).

Strategy	Feeding time periods (h)	Starving time periods (h)	Total fed-batch time (h)
Standard	-	.	15.5
A	3	1	20.4
B	3	0.5	18.1
C	1.5	0.5	20.4

For all the cases, it was considered that the cultivations end when the total amount of glucose was fed into the system, which was kept constant around 800 g. The different feeding strategies did not affect to the growth parameters of *P. pastoris*. After the starving periods the yeast started to consume the glucose added immediately and the substrate to biomass yield was not affected, thus the final amount of biomass achieved was always around 100 g DCW L^{-1}.

In Figure 5.6 are plotted the secreted Fab production time profiles of the different strategies tested, main production parameters of the system are also presented in Table 2. All the new strategies implemented achieved important increases of Fab production in terms of product titration in the broth, as well as in product to substrate ($Y_{P/S}$) and product to biomass ($Y_{P/X}$) yields. The range of increases was between 15% for the strategy A and more than 40% for the strategy C. However, in terms of productivities, the results obtained applying the innovative feeding strategy were not always better respect to the conventional pre-exponential feeding profile. The strategies B and C lead to moderate productivity enhancements, between 5 and 10%. On the other hand, for the strategy concerning the longest carbon-starving periods (Strategy A, 1 h of starvation), even though the total Fab produced was significantly higher, its productivity rates were significantly lower. This fact is due to increased bioprocess times because of the carbon-starving periods. When cell productivity during the carbon-starving periods is not higher than the growing periods, the overall bioprocess productivity levels will end up being lower.

Considering all the compared production parameters, strategy C, based on the combination of 1.5 h of feeding followed by 0.5 h of starvation, lead to the best results. The production increment presented can be considered as an important operational improvement for the P_{GAP} -based expression system. A similar intermittent feeding strategy was previously proposed by Heo et al. [46] for fed-batch cultivations of *Hansenula polymorpha* presenting positive results respect to the conventional feeding profiles.

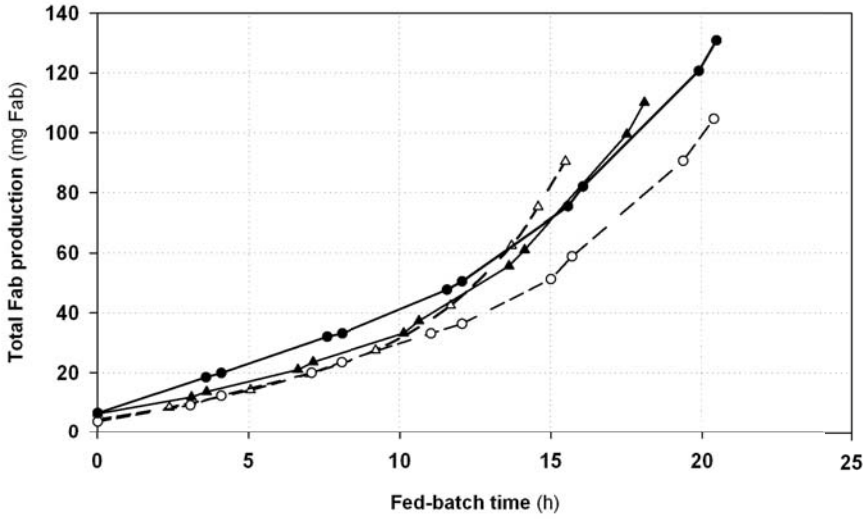

Figure 5.6: Time profile of the total amount of 2F5 Fab secreted to the fermentation broth by using the different feeding strategies compared. Standard strategy (△); Strategy A (○); Strategy B (▲) and Strategy C (●).

Table 2: Main production parameters obtained by applying the different feeding strategies compared.

Strategy	Standard	A	B	C
Feeding time (h)	15.5	20.4	18.1	20.4
Fab production (mg Fab)	90.4	105	110	131
Fab production increase (%)	-	15.7%	21.8%	44.8%
q_P (µg Fab DCW^{-1} h^{-1})	35.2	31.4	37.1	36.8
q_P increase (%)	-	-10.9%	5.40%	4.55%
Q_P (mg Fab h^{-1})	5.83	5.13	6.08	6.42
Q_P increase (%)	-	-12.1%	4.30%	10.0%
$Y_{P/S}$ (mg Fab g_S^{-1})	0.122	0.146	0.152	0.173
$Y_{P/S}$ increase (%)	-	19.6%	24.7%	41.9%
$Y_{P/X}$ (mg Fab DCW^{-1})	0.245	0.282	0.294	0.359
$Y_{P/X}$ increase (%)	-	15.0%	20.0%	46.6%

In addition, the amount of Fab secreted to the cultivation broth during the carbon-starving periods was monitored in order to be able to describe the evolution of product increase along the period within glucose is depleted. This monitoring was performed during all the starving periods of the fed-batch cultivation in which the feeding strategy A was implemented, thus non-fed periods of 1 h. The average of the time-evolution increase of Fab secreted is presented in Figure 5.7, where the increment every 20 min is represented in percentage respect to the total increase of secreted Fab during the studied period. The observed curve can be fitted to a first-order kinetics in which an important fraction of the total secreted Fab during the whole period is released during the first 20 min, corresponding to the time constant of the first-order system. Consequently, in order to achieve the best results for all the productivity rates, the carbon-starving periods longer than 30 min should be avoided since the increase of production after 30 min of no feeding is relatively small.

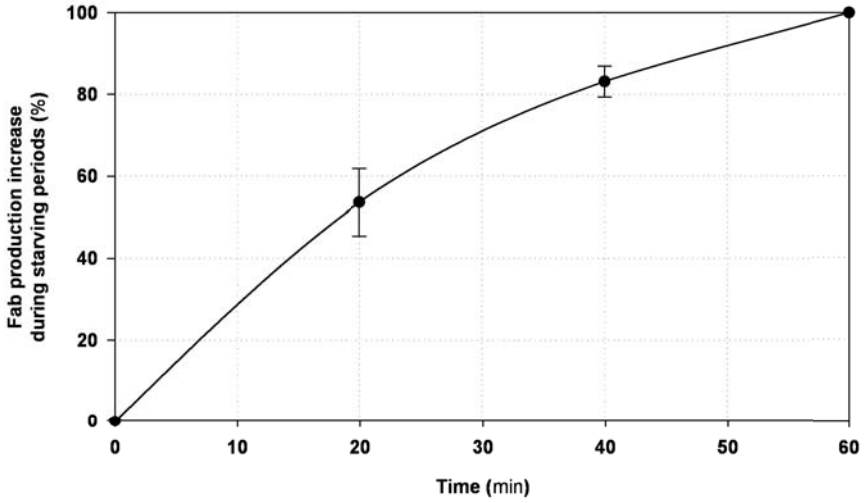

Figure 5.7: Time evolution of the 2F5 Fab secreted during 60 min carbon-starving periods.

The distribution of the Fab expressed by the recombinant *P. pastoris* strain among the different cell fraction mentioned above in previous sections (SCF and IMF) was also determined for fed-batch cultures implementing this innovative strategy. In this case the aim was to determine the cause of the secreted Fab increase in order to identify if the Fab increase detected in the broth during the non-fed period is due to the synthesis of new protein, or to the release of protein previously expressed during the growing phase but retained intracellularly as a result of a possible saturation of the secretory pathways. The distribution of Fab among the different cell fractions and the gradual increase of Fab levels along the successive carbon-starving periods of the fed-batch cultivation performed with feeding strategy C is shown in Figure 5.8. Although a moderate increase of Fab levels were detected after the starving period, no significant differences were observed on the extra/intracellular distribution of Fab.

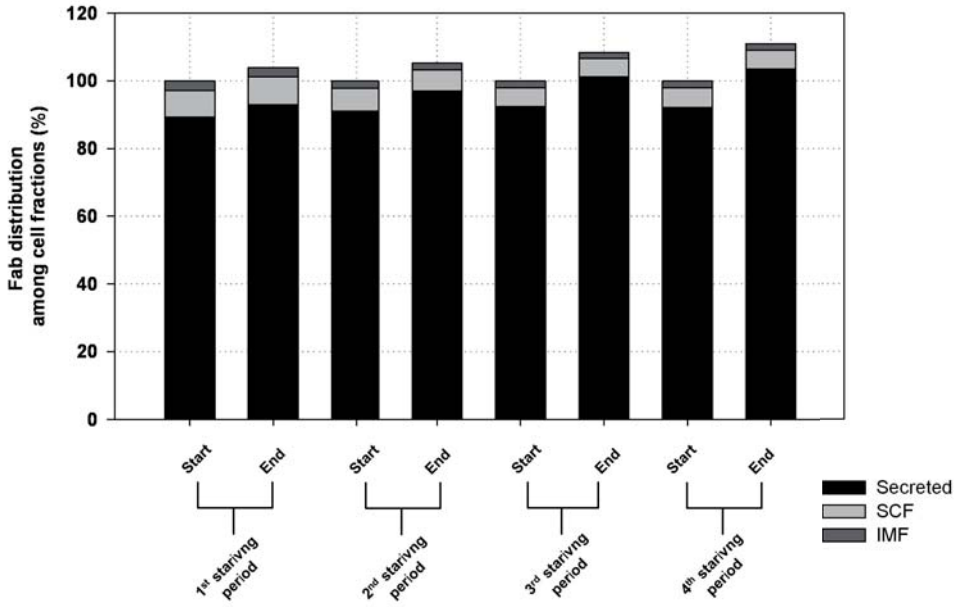

Figure 5.8: Cell fraction distribution of the 2F5 Fab produced before and after 30 min carbon-starving periods.

Therefore, it could be concluded that the increase of secreted Fab is due to the fact that during the non-fed period the yeast is still able to synthetize recombinant proteins driven by P_{GAP} upon short-time depletion of glucose. It has been widely reported that yeasts are sensitive to the level of nutrients such carbon and nitrogen sources, which allows them to adapt readily to changing nutritional states [47,48]. Early studies in the field described an overall decrease of protein synthesis levels for carbon-starving conditions due to a global inhibition of the translational capacity of the cells [49]. Nevertheless, recent works have concluded that, although the overall expression levels of proteins is reduced, the transcription of many genes whose products promote adaptation to low glucose environments is upregulated [50,51]. Thus, as was described by Baumann et al. under hypoxic conditions [52,53], transcription and translation levels of the glycolytic genes could be upregulated as an adaptation response to an environment lacking of important essential nutrients for yeast growth. Accordingly, since the glycolytic *GAP* promoter regulates the expression of the product, the productivity of the recombinant protein could be also upregulated in carbon-starving conditions.

As a conclusion, the implementation of carbon-starving periods in fed-batch cultivations can be considered as a truly step forward in the optimization of recombinant protein production processes using *P. pastoris*. In general, the optimization of these bioprocesses depends mainly on the relationship between product formation and biomass growth. The interdependence of these factors is of key importance regardless of the selected criterion for the optimization, both maximizing yield and productivity [7].

In large-scale industrial fermentation processes, biomass is frequently considered as an unavoidable waste product but essential, due to most of microbial production processes are growth-associated. Furthermore, as in the case described in this work, very often the higher specific production rates are obtained at higher specific growth rates [54]. In contrast, it must also be taken into account that the biomass growth in a bioprocess is limited, which may be due to biological and different physical restrictions, mainly heat and mass transfer. Often oxygen availability is the most important limitation for aerobic processes [55]. Thus, lower growth rates lead to longer fermentation times, and therefore, more product may be generated before the process is stopped due to system limitations, whenever the specific production rate do not decrease drastically within the operation range for μ. In fact, a compromise between higher q_P and lower μ arises when productivity and/or product yield are the performance indexes to be optimized. High values for these indexes result in the reduction of capital and operating costs. The trade-off between yield and productivity is key in the design of a bioprocess and its optimal performance.

Consequently, some authors have proposed different approaches to deal with this scenario. For the system based on P_{GAP}, Maurer et al. [13] implemented an optimal trajectory for feeding rate that, controlling the specific growth rate, maximizes the volumetric productivity with also suitable product yield. Alternatively, Buchetics et al [25], aimed to improve the ratio between q_P and μ, especially for lower μ, by engineering the producer strain. On the other hand, for processes based on the *AOX1* promoter, which have been extensively used, different alternatives for optimizing the production of some recombinant proteins can also be found in the literature [56–58].

In the present work it has been proposed a non-complex fermentation strategy that makes possible at the same time the growth of the yeast at very high specific growth rates and to prolong the bioprocess. Initially, significant increase of the q_P has been described during short carbon-starving periods respect to fed phases. Therefore, by exploiting this effect, important overall increases both in productivity and, especially, in total amount of product formation and yields ($Y_{P/S}$, $Y_{P/X}$) have been described for the bioprocess. This innovative strategy can be considered as an important operational improvement for the P_{GAP}-based expression system. In addition, it has been proposed that the rise of recombinant protein production achieved with this innovative strategy is due to the higher synthesis rate of protein regulated by the *GAP* promoter during short periods of carbon starvation, and not to effects related with the saturation of the secretory pathways.

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6

A versatile approach to implement oxygen-limiting conditions for improving recombinant protein production in *Pichia pastoris*

Chapter submitted as research article to Applied Microbiology and Biotechnology

Garcia-Ortega X, Valero F, Montesinos JL. A versatile approach to implement oxygen-limiting conditions for improving recombinant protein production in *Pichia pastoris*. Submitted to Applied Microbiology and Biotechnology in September 2015.

Table of content

6.1 Abstract

The yeast *Pichia pastoris* is widely used as a production platform of secreted recombinant protein production. It is recently known that the application of oxygen-limiting conditions leads to an important increase on protein specific productivity driven by the *GAP* promoter. The physiological and metabolic adaptation of recombinant *P. pastoris* to a wide range of oxygen availability conditions has been systematically studied in glucose-limited chemostat cultivations producing an antibody Fab fragment (Fab). Besides the important increase on both Fab yield and productivity, as a consequence of the metabolic shift from respiratory to respiro-fermentative pathways, an important decrease on biomass yield and generation of several amounts of secreted by-products have been observed, which increases as the oxygen availability is being reduced. The reliable study of the main specific rates of the bioprocess, as well as the detailed monitoring of the physiological state of the cells by means of flow cytometry analysis, leads to a deep characterization of the system. This has allowed the determination of the optimal oxygen-limiting conditions for enhancing the recombinant protein production. An important increase of up to 3-fold of the specific Fab production rate (q_{Fab}) has been achieved for the optimal conditions. Finally, this work presents a versatile approach based on the physiological behavior of the yeast that could be used to implement the desired oxygen-limiting conditions to fermentations set-ups with different oxygen transfer capacities, alternative operating modes, and also for the production of others proteins of interest.

6.2 Introduction

In the last years the recombinant protein industry has been growing rapidly and bringing innovative products to the market [1,2]. In these production processes, genetic engineering, microbial physiology and bioprocess engineering including up and downstream must be combined with the objective of increasing the specific production rate of the desired recombinant proteins. Since there is often a lack of knowledge about the production pathway and its dynamic profile in the producing cells, detailed physiological studies are required for optimizing the overall bioprocess [3].

Pichia pastoris is one of the most effective and versatile expression systems. This yeast is being widely and successfully used for the production of heterologous proteins [4–6]. The combination of traits that makes *P. pastoris* a suitable expression system have been broadly reviewed in the literature [7–9]. Although the use of the *AOX1* promoter (P_{AOX1}), which is induced in the presence of methanol, is extensively used [10,11]; in the last decade, the glycolytic *GAP* promoter (P_{GAP}) has become to an efficient alternative as a strategy to produce heterologous proteins on glucose or glycerol constitutively [12,13]. The main advantages of P_{GAP} in front of P_{AOX1} can be found elsewhere [14]. In the last years different works have been

published describing the behavior of the P_{GAP}-based *P. pastoris* expression system for heterologous proteins in chemostat as well in fed-batch operational mode [14–17].

The impact of oxygen supply on the heterologous production has been studied for different recombinant production hosts due to that the oxygen transfer rate is usually one of the most limiting bottlenecks for high cell density cultivations of microorganisms [18]. In *Escherichia coli* cultivations, oxygen limitation leads to a stress response and by-product formation such acetate, which inhibits the growth and recombinant protein production [19,20]. The impact of the oxygen limitation was also studied in *Saccharomyces cerevisiae* observing the production of ethanol and glycerol as by-products of the cultivation [21].

In *P. pastoris* cultures expressing a human antigen-binding fragment (Fab), an important increase of the specific productivity (q_P) was described at low oxygen supply [22]. This work studied three different oxygen-limiting conditions in chemostat cultivations observing increases up to 2.5-fold in the q_P, but also a decrease in the biomass production, and the generation of ethanol as a by-product. In addition, a primary strategy of fed-batch cultivation under hypoxic conditions was carried out also showing an important increase in the volumetric productivity, Q_P. Following studies including transcriptomic, proteomic and metabolomics analyses were developed with the same expressing strain under similar hypoxic conditions in order to extent the knowledge of the metabolic response of the cells for some specific conditions [23–25]. However, no further studies have been published in order to identify the optimal culture conditions that lead to maximal productivities and yields for the protein of interest.

In the previously cited works, the different culture conditions in terms of oxygen availability for the cells have been indirectly related to the O_2 composition in the inlet gas phase. This approach does not allow a proper comparison of the results among experimental set-ups with different oxygen transfer capabilities because of its particular k_La [26]. Thus, a systematic methodology that lets working with equivalent conditions of oxygen availability to the cells using different bioreactor configuration is needed in order to satisfactorily apply this cultivation strategy to other fermentation system. In a previous work with *E.coli* growing under hypoxic conditions an innovative indirect reporting parameter for oxygen availability was presented. It was based on the determination of the minimal oxygen supply rate needed in each particular fermentation system for fully oxidative metabolism, in which no by-products are generated [27]. This approach is based on the physiological behavior of the culture rather than on cultivation settings itself.

A strain expressing the human 2F5 Fab, which is slightly different than previously cited, 3H6 [22,24]**,** has been used as model protein. Fabs have a wide range of applicability as therapeutic

agents [28] and are complex proteins composed by different domains connected via disulfide bonds [29,30], which makes them a suitable model protein for studying the efficiency of recombinant protein production processes.

In the present work, a thorough study of a wide range of oxygen-limited *P. pastoris* chemostat cultivations has been performed searching for the best conditions to improve yields and productivities. The determination of the key specific rates of the bioprocess, including a detailed characterization of the by-products generated, was carried out identifying new extracellular metabolites produced respect to the previous works of *P. pastoris* growing in hypoxic conditions. In addition, cell viability and reactive oxygen species (ROS) analysis were also performed by flow cytometry in order to monitor the oxygen limitation effect on the physiological state of the cells. As main relevant outcome, a versatile methodology based on the control of physiological parameters like some specific by-products rate or respiratory quotient is proposed. It can be applied to *P. pastoris* cultures in order to work under equivalent oxygen-limiting conditions for different cultivation set-ups although may differ in their oxygen transfer capabilities. An extra value of the proposed approach is that the concept can be implemented for different operating modes, continuous or fed-batch, and even for other proteins of interest that could be positively affected by oxygen limiting conditions.

6.3 Materials and methods

6.3.1 Strain and cultivation conditions

The *P. pastoris* strain X-33 P_{GAP}ZαA-Fab2F5 used expresses both light and heavy chain genes of the human Fab 2F5 under control of the constitutive *GAP* promoter. Using the *Saccharomyces cerevisiae* α-mating factor signal sequence the Fab is secreted to the medium. The construction of the strain was described previously [29].

The preparation of the inoculum cultures for bioreactors cultures were performed as described by Garcia-Ortega *et al.* [14]

Chemostat cultivations were carried out in a 2 L Biostat B Bioreactor (Braun Biotech, Melsungen, Germany) at a working volume of 1 L. Cells were grown under carbon-limiting conditions at a dilution rate (D) of 0.10 h^{-1}, with different oxygen composition in the bioreactor inlet gas in order to apply different oxygen-limiting conditions. The cultivation were performed using the batch and chemostat medium compositions detailed elsewhere [15]. Minor differences were applied to the cited compositions, which are detailed below. Glucose concentration was 50 g L^{-1}; Biotin 0.02% (1 mL), PTM1 (1.6 mL) trace salts stock solution (also described in [15]) and antifoam Glanapon 2000kz (0.2 mL; Bussetti & Co GmbH, Vienna, Austria) were added per liter of chemostat medium.

Culture conditions were monitored and controlled at the following values: temperature, 25 °C; pH, 5.0 with addition of 15% (v/v) ammonium hydroxide; the pressure in the culture vessel was maintained at 1.2 kbars using a pressure valve (GO Inc, Spartanburg, SC, USA); pO_2, above 20% saturation by controlling the stirring rate between 600 and 900 rpm during the batch phase, in the continuous phase it was kept constant at 700 rpm; the total gas flow was kept constant for all experiments at 0.8 vvm. In order to apply different controlled oxygen-limiting conditions and to keep constant the hydrodynamic behavior of the system, air was partially replaced with and equivalent flow of nitrogen in the gas inlet. Different hypoxic conditions were changed from high to low air fraction set points using mixtures of the gases by means of thermal mass-flow controllers (TMFC; Bronkhorst Hi-Tech, Ruurlo, The Netherlands). An exhaust gas condenser with cooling water at 4 ºC minimizes mass loses by water evaporation and other volatile compounds. In all the experiments, the continuous cultivation was performed for at least five residence times (τ) before taking samples and shifting the inlet air-flow to the next set point.

6.3.2 Analytical methods

6.3.2.1 Biomass determination by dry cell weight (DCW)

P. pastoris biomass concentration of the cultivations for each steady state was determined as DCW using the method described elsewhere [31]. Determinations were performed by triplicate and the relative standard deviation (RSD) was about 4%.

6.3.2.2 Biomass analysis

Biomass samples for the determination of the elemental composition, as well as the ash content, were prepared and analyzed as described by Carnicer et al. [24].

6.3.2.3 Product quantification

The amount of 2F5 human Fab produced was quantified by ELISA as previously described [32]. Determinations were performed by triplicate and the RSD was about 4%.

6.3.2.4 Carbon source and by-products quantification

The concentrations of the substrates and common by-products obtained such glucose, arabitol, glycerol and ethanol were determined by HPLC with a HP 1050 liquid chromatograph (Dionex Corporation, Sunnyvale, CA, USA) using an ICSep ICE COREGEL 87H3 column (Transgenomic Inc., Omaha, NE, USA). The mobile phase was 8 mM sulphuric acid. Injection volume was 20 μL. Data was quantified by Chromeleon 6.80 Software (Dionex Corporation, Sunnyvale, CA, USA). The estimated RSD was below 1% for all the analytes.

The concentrations of the newly identified by-products at oxygen-limiting culture conditions, such α-ketoglutarate and succinate, were determined by means of LC-MS. The analysis of

filtered supernatants were performed on a Shimadzu Prominence HPLC with a UV/VIS detector coupled to a Mass Spectrometry detector Shimadzu 2010A also coupled to an Electro Spray Ionization (ESI) interface operating at a wavelength of 210 nm. Metabolite compounds were separated on an ICSep ICE COREGEL 87H3 column (Transgenomic Inc., Omaha, NE, USA) using 15 mM formic acid in milliQ water. The analyses were performed at room temperature using a 20 µl injection volume. The estimated RSD was about 3% for all the analytes.

6.3.2.5 Off-gas analysis

A quadrupole mass spectrometer (Balzers Quadstar 422, Pfeiffer-Vacuum, Asslar, Germany) was used for on-line exhaust gas analysis. Exhaust gas humidity was reduced by using a condenser (water at 4 ºC) and two silica gel columns. The Faraday cup detector was used for its simplicity, stability, and reliability, determining responses of *m/z* corresponding to the major gas peaks (N_2: 28, O_2: 32, CO_2: 44, Ar: 40). Normalized mass spectrometer signals were used to reduce errors caused by variations on operating conditions such pressure and temperature as well as others that can generate some drift and noise of signals. Multivariate calibration was performed by ordinary least squares (OLS) minimization with suitable standard calibration mixtures according to the components to be analyzed and its concentration range.

The total humid off-gas flow rate was not measured directly and it was calculated by inert balance around the reactor. To properly estimate this flow rate, determination of its water composition is necessary. It can be calculated from the quotient between the off-gas O_2 composition without bioreaction, due to water stripping, and the corresponding inlet O_2 molar fraction. Inlet air composition was obtained from a 12 h measurement average before inoculating.

Thus, through O_2 and CO_2 balances accurate estimation of oxygen uptake rate (OUR), carbon dioxide production rate (CPR), and respiratory quotient (RQ) were carried out [33].

6.3.3 Flow cytometry measures and analysis

Cell counting, viability and measure of the stress caused by intracellular radical oxygen species (ROS) were determined by means of flow cytometry assays using the Guava EasyCyte™ Mini cytometer (Millipore, Hayward, CA, USA). All samples were always previously briefly sonicated in order to avoid the presence of cell clumps.

Viability assays were performed by means of the propidium iodide (PI) staining procedure as described elsewhere [34,35]. The accumulation of ROS was also monitored since it has been described as an important factor that induces the mechanisms of apoptotic death of yeasts [36]. For ROS determination, intracellular superoxide anions were measured by using dihydroethidium (DHE) and dihydrorhodamine 123 (DHR), as previously described [37–39].

6.3.4 Process parameters determination, consistency check and data reconciliation

6.3.4.1 Mass balance and stoichiometric equations

The oxidative and oxidoreductive growth can be described on a C-molar basis by a single overall reaction, a so-called Black Box model, which is a simplification of all the biochemical reactions involved:

$$S + Y^*_{O_2/s}\, O_2 \rightarrow Y^*_{x/s}\, X + Y^*_{CO_2/s}\, CO_2 + Y^*_{p/s}\, P \qquad (1)$$

where *S* denotes one single limiting substrate as the carbon and energy source; O_2, oxygen; *X*, biomass; CO_2, carbon dioxide; *P,* products. $Y^*_{i/s}$ are stoichiometric coefficients that can also be called overall "i" component-substrate yields.

Specific rates (q_i) and yields are parameters of capital importance for comparing different culture conditions and allow the identification of changes in the physiological cell state that can impact into product quality [40]. Their calculation is based on the conversion rates (r_i) determined in the general mass balance of the cultivation. Specific rates are typically conversions rates related to the biomass concentration (equation 2). Yields are defined as ratios between rates (equation 3) and positive.

$$q_i = \frac{r_i}{X} \qquad (2)$$

$$Y_{i/j} = \frac{r_i}{r_j} = \frac{q_i}{q_j} \qquad (3)$$

For an ideal stirred tank-reactor, considering conversion rates of biomass formation, substrate uptake and product formation, the following mass balance equations for the continuous operation at steady state can be formulated according equation 4.

$$\begin{bmatrix} \mu \\ q_S \\ q_P \\ q_{O_2} \\ q_{CO_2} \end{bmatrix} XV = \begin{bmatrix} F_{out}\, X \\ -F\, S_0 + F_{out}\, S \\ F_{out}\, P \\ OUR\, V \\ CPR\, V \end{bmatrix} \qquad (4)$$

where μ is the specific growth rate (h^{-1}); q_S, specific substrate uptake rate (g g^{-1} h^{-1}); q_P, specific production rate (U g^{-1} h^{-1}); q_{O2}, specific oxygen uptake rate (mol g^{-1} h^{-1}); q_{CO2}, specific carbon dioxide production rate (mol g^{-1} h^{-1}); *F*, substrate feeding rate (L h^{-1}); F_{out}, outlet flow rate (L h^{-1}); *V*, volume of broth in the reactor (L); S_0, substrate feeding concentration (g L^{-1}); *OUR*, oxygen uptake rate (mol L^{-1} h^{-1}); *CPR,* carbon dioxide production rate (mol L^{-1} h^{-1}). F_{out}, can be obtained by the total mass balance for an ideal stirred tank reactor in continuous operation at steady state (equation 5).

$$F_{out} = \frac{\rho_{Feed}F - \rho_{H2O}F_{Evap} + \rho_{Base}F_{Base} - \rho_{Broth}F_O + M_{GAS}}{\rho_{Broth}} \quad (5)$$

where F_{Evap} is the water evaporation rate (L h^{-1}); F_{Base}, base feeding rate (L h^{-1}); F_O, withdrawal rate (L h^{-1}); M_{GAS}, net mass gas flow rate (g h^{-1}); ρ_{Feed}, substrate feed density (g L^{-1}); ρ_{H2O}, water density (g L^{-1}); ρ_{Base}, base density (g L^{-1}); ρ_{Broth}, mean broth density (g L^{-1}). The net mass gas flow rate is calculated with the equation (6):

$$M_{GAS} = -(W_{O2}OUR\ V + W_{CO2}CPR\ V) \quad (6)$$

where W_{O2} is the oxygen molar mass (g mol^{-1}); W_{CO2}, carbon dioxide molar mass (g mol^{-1}). In case of product stripping for ethanol or any other compound, an additional term is included in equation (4) in order to not underestimate its corresponding specific rate. Substrate and product concentrations were referred to the whole medium, including biomass volume [41].

6.3.4.2 Consistency check and data reconciliation

Specific rates and yields can be affected by random errors, drifts and even gross errors. Besides the propagation of random measurement errors, gross errors such as analyzer miscalibration and drifts can alter their values. Mean values or moving average method is normally used to reduce random noise. Generally, applicable constraints such as elemental balances can remove measurement error by using very little prior knowledge [42].

The consistency of the measurements was checked by standard statistical tests considering elemental balances as constraints [43]. With the current experimental set-up it was possible the measurement of the five key specific rates in the black-box process model: biomass generation (μ), glucose uptake (q_s), product formation (q_p), oxygen uptake (q_{O2}), and carbon dioxide production (q_{CO2}). In this work, the carbon balance and the redox balance were used as constraints and protein production considered negligible in these balances. So, the system is overdetermined and the degree of redundancy is the same as the number of constraints. This fact can be used to check the measurements for gross errors or unidentified metabolites, respecting the covariance for each measurement [44,45], and to improve the accuracy of the measured conversion rates by data reconciliation methods [46]. The *h* value given by the sum of the weighted squares of the residuals ε is the output of the statistical test for the presence of gross errors or neglected components.

$$h = \varepsilon P^{-1} \varepsilon \quad (7)$$

If *h* exceeds the threshold value that depends on the significance level α (0.95 in this work) and degree of redundancy according to the χ^2 distribution, it is concluded that there are significant errors in the measurements or any compound has not been taken into account in the black

box process model. The variances of all specific rate measurements were considered uncorrelated and estimated by replicates and/or error propagation.

The χ^2-test performed for all the experimental data obtained from chemostat cultivations showed the measurements satisfied mostly the stoichiometric model and hence, both C-balance and e-balance.

Data reconciliation procedures are also based on the use of elemental balances according to a black box reaction scheme to improve the accuracy of the measured specific rates or yields and also to determinate the unknown specific rates [47]. A measurement error vector δ is found by using a least squares approach to calculate the reconciled vector which includes the best estimates of reaction rates to fit all constraints imposed.

6.4 Results

6.4.1 Process variables

Chemostat cultivations with different oxygen supplies were performed in order to describe the effect of oxygen-limiting conditions on the physiology of *P. pastoris* as well as on different productivity parameters of the bioprocess. A wide range of oxygen-limiting conditions, achieving different steady-state set points by applying stepwise reductions of the oxygen mole fraction in the inlet gas, were compared leading to a deep study of the hypoxic conditions effect on P_{GAP}-based recombinant protein production processes with *P. pastoris*. The studied range was defined from 21% to 4% of oxygen mole fraction in the inlet air. The closure of carbon and redox balances calculated for all the culture conditions compared in the present work were always above 90%, additionally results were validated using the standard data consistency check and reconciliation procedures described in materials and methods section. These tests confirm the robustness and the reliability of the results obtained from the chemostat cultivations carried out.

From the main process variables of the cultivations plotted in Figure 6.1A, a separation in areas among different glucose and oxygen limiting conditions that presents similar behaviors can be proposed. The first group is comprised of set points in which the cultivation is only limited by the carbon source. The non-limitation by oxygen is evidenced by the positive values of pO_2 determined by the oxygen sensor. The non-accumulation of glucose, as well as the non-production of ethanol as a by-product, both indicates that cells are growing in fully aerobic glucose metabolism. In this area, the DCW is rather constant among the different set points, however a slight increase on Fab production can be observed, close to the transition area where oxygen also starts to limit the growth faintly.

A second pool of oxygen-limitation set points can be grouped in an area in which glucose and oxygen are both limiting simultaneously the cultivation. For these culture conditions pO_2 is always 0%, which indicates that there is not an excess of dissolved oxygen in the culture broth. Within this area of set points, the total amount of oxygen that is being transferred to cultivation is being consumed by the biomass. However pO_2 is always 0%, different oxygen-limitation steady states set points can be achieved by supplying different mixtures of air. Within this area, as the oxygen limitation becomes more severe, DCW clearly decreases and ethanol production, as a main by-product of the cultivation, is produced up to concentrations of 9 g L^{-1}, which indicates a shift to a respiro-fermentative metabolism. The remaining glucose in the broth for all these conditions is always undetectable, thus one can conclude that glucose-limitation is still being the main limiting factor. Among these points also an important peak of Fab titration can be observed.

Finally, a third area can be defined for the strictest oxygen-limiting cultivation condition. For this set point, since glucose can be detected, it can be assumed that oxygen is the only factor that is truly limiting the process. The DCW determined is the lowest, and ethanol production the highest, following the same trend from the previous conditions. For the Fab titration, an important decrease is observed in comparison with less-restrictive oxygen conditions.

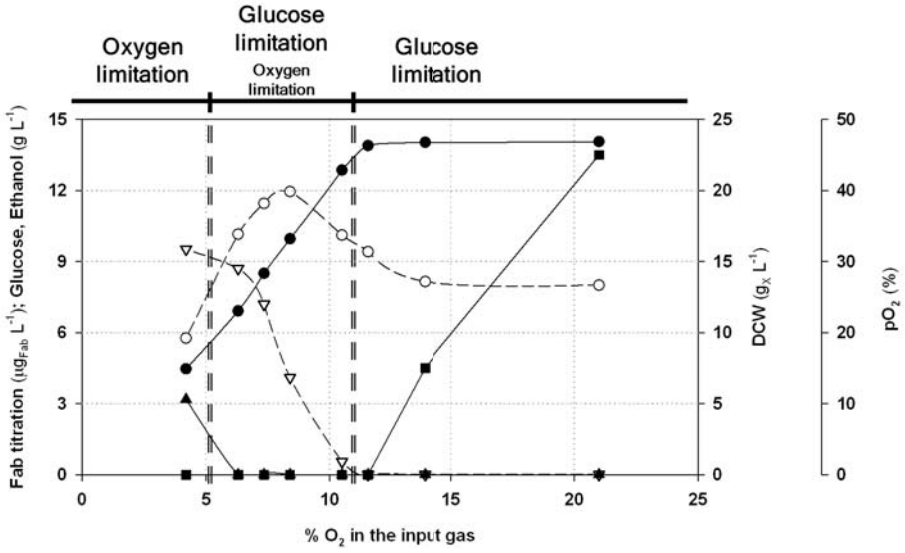

Figure 6.1A: Main cultivation parameters at different oxygen supply levels: dry cell weight (DCW, ●); 2F5 Fab titration (○); glucose concentration (▲); ethanol concentration (▽); and dissolved oxygen (DO or pO_2,■).

Since the biomass yield is not constant among all the conditions compared, in order to analyze the process parameters studied in this work, DCW must be taken into account for determining the specific rates of each parameter. Thus, the parameters plotted in Figure 6.1A are also shown in terms of specific rates in Figure 6.1B. Although the main trends are similar, in the most restrictive oxygen conditions when the DCW amount is significantly lower, important differences between plots are observed. In terms of specific rates, the increase of ethanol production rate ($q_{ethanol}$) becomes rather linear and the peak of maximal Fab production is shift

to stricter oxygen-limiting conditions. In this peak, q_{Fab} achieves a 3-fold increase upon non-limiting conditions. In addition, since most of cultivations conditions are carbon-limited, the decrease of biomass yield under hypoxic conditions results in an important rise of specific glucose uptake rate ($q_{glucose}$), which reaches up to 2-fold increase at low oxygen supplies. However, no significant differences were observed between the consumption rates of oxygen (q_{O2}) of the different oxygen-limiting set points compared.

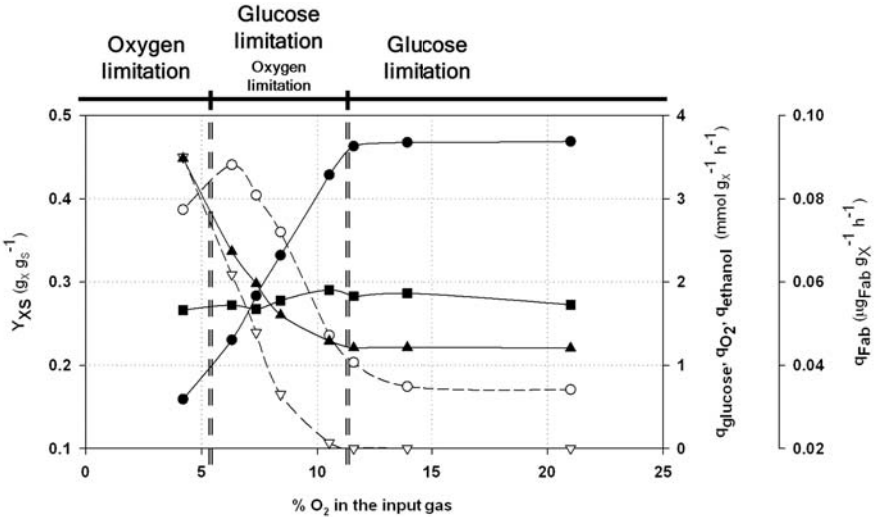

Figure 6.1B: Biomass yield and main specific rates of the cultivation at different molar fraction of oxygen in the inlet air: biomass yield (Y_{XS}, ●); specific 2F5 Fab production rate (q_{Fab}, ○); specific glucose uptake rate ($q_{glucose}$, ▲); specific ethanol production rate ($q_{ethanol}$,▽); and specific oxygen uptake rate (q_{O2},■).

In Figure 6.1C the respiration parameters of the cultivations are presented. As was commented in the previous paragraph, no significant differences were observed in q_{O2}. However, a very important increase can be observed in the specific production of CO_2 (q_{CO2}) when oxygen-limiting conditions turn stricter. Consequently, a significant increase is also observed in the respiratory quotient (RQ). In the most severe conditions, both parameters can even double the values determined at normoxic conditions.

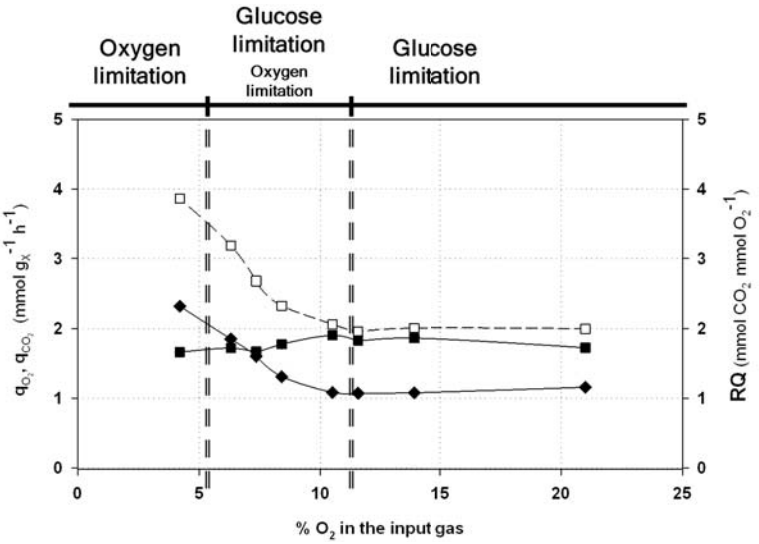

Figure 6.1C: Oxygen uptake rate (OUR, ■); carbon dioxide production rate (CPR, □); and respiratory quotient (RQ,◆) at different molar fraction of oxygen in the inlet air.

6.4.2 By-products observed at the oxygen-limiting conditions

One of the major impacts of the reduced oxygen supply on *P. pastoris* cultures growing on glucose is the generation of secreted fermentation by-products, which reflects the adaptation from a respiratory to a respiro-fermentative metabolism.

While carbon limitation is the only acting on the system, no by-products can be detected. However, for oxygen-limiting conditions in which the O_2 in the input gas is below 11% mole fraction and pO_2 values are 0%, different by-products were determined. Ethanol is the main extracellular metabolite, reaching concentrations up to 10 g L^{-1} in the most restrictive conditions. Arabitol, a C5 sugar alcohol related to the pentose phosphate pathway, was also detected at concentrations significantly lower than ethanol. Both metabolites were previously described as by-products of *P. pastoris* during fermentation at low oxygen supply [23,24]. Thus, both components were easily determined by HPLC analysis. In addition, other significant peaks that could be related to other unknown metabolites were observed in the HPLC chromatograms. This fact, coupled with the significant carbon balance mismatches determined in hypoxic conditions lead to think about other missing compounds are being generated as a fermentation by-products. Carrying out LC-MS coupled with HPLC analysis, two major peaks were identified from the molecular weights detected in the unknown chromatogram peaks. The new compounds identified were succinate (MW=118) and α-ketoglutarate (MW=146), both related with the Tricarboxylic Acids (TCA) cycle.

In Figure 6.2 the specific production rates of the extracellular metabolites detected in oxygen-limiting fermentation samples are shown. The generation of ethanol is notably higher than the others by-products, and has a rather linear increase accordingly to the reduction on oxygen supply. This fact makes the specific production of ethanol an interesting indirect reporting parameter of the oxygen availability for the cells, which is required for the implementation of oxygen-limiting conditions to different cultivations set-ups and operational modes. The rest of by-products have a similar behavior between them, low specific production rates while glucose and oxygen limitations are both acting on the cultivation, however, an important increase in the specific production rates is triggered for the most restrictive oxygen-limiting condition.

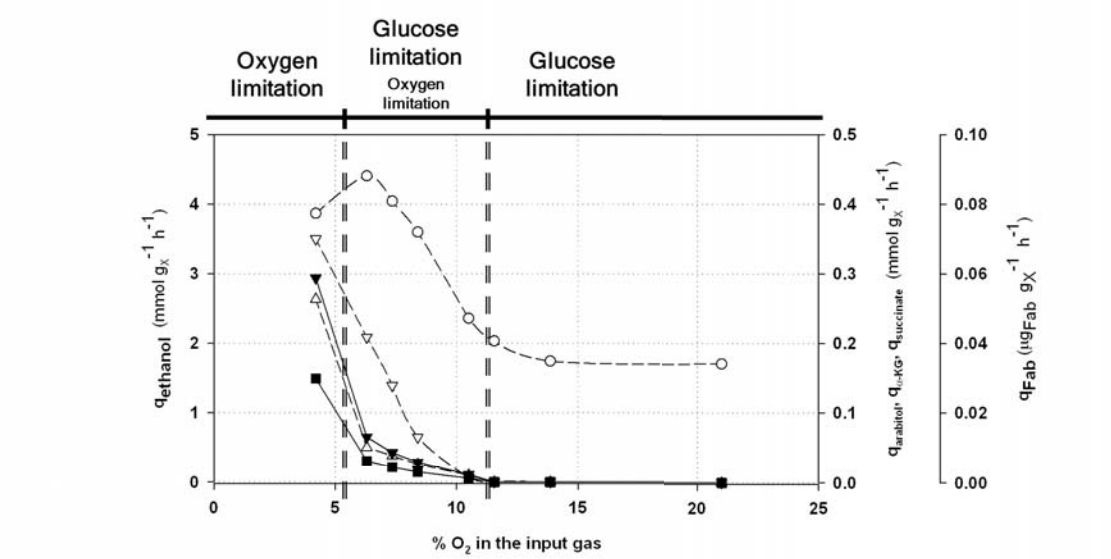

Figure 6.2: Comparison of the specific production rates of the 2F5 Fab and the by-products monitored at different molar fraction of oxygen in the inlet air: specific 2F5 Fab production rate (q_{Fab}, O); specific ethanol production rate ($q_{ethanol}$,▽); specific arabitol production rate ($q_{arabitol}$, △); specific α-ketoglutarate production rate ($q_{a\text{-}KG}$,▼); and specific succinate production rate ($q_{succinate}$, ■).

6.4.3 Physiology study based on flow cytometry analysis

Flow cytometry is a powerful tool that enables to determine the physiological state of the cells growing in a culture with high accuracy and reproducibility. Since in this work the effect of an important limiting factor such oxygen availability in the *P. pastoris* growth is studied, these analyses provide valuable information about how the physiology of the yeast can be affected. These results are shown in Figure 6.3, in which also are indicated the same areas defined by the limitations that are being applied to the cultivation.

For the viability determination, it is considered that propidium iodide (PI) stained cells are dead, thus will not further participate in cell growth and product formation. No important differences were observed in the ratio of cell viability up to O_2 set point in the input of 6% mole fraction, viability results were always rather constant above 95%. For the most severe hypoxic conditions a significant drop of cell viability to a ratio under 90% was observed. In the flow cytometry protocols carried out to determine the presence of ROS, DHR and DHE were used to monitor the stress effects on the cells caused by the oxygen-limiting conditions. In the different set points of normoxic conditions, no stained cells were detected by using neither DHE nor DHR protocols, thus was considered that ROS stress was not affecting the cells in these growing conditions. When oxygen and glucose limitation were both applied to the system, a significant rise in the level of ROS was observed, which was increasing progressively as the oxygen supply was being reduced. DHR protocol detected fractions of stressed cells by ROS between 15 and 20%; DHE protocol determined that the fractions of stressed cells were between 25% and 35%. In contrast, as other parameters commented previously, an abrupt change was observed for the most severe oxygen-limiting conditions, in this set point, the

fraction of stressed cells by ROS was up to 30% and 50%, by applying the DHR and DHE procedures respectively.

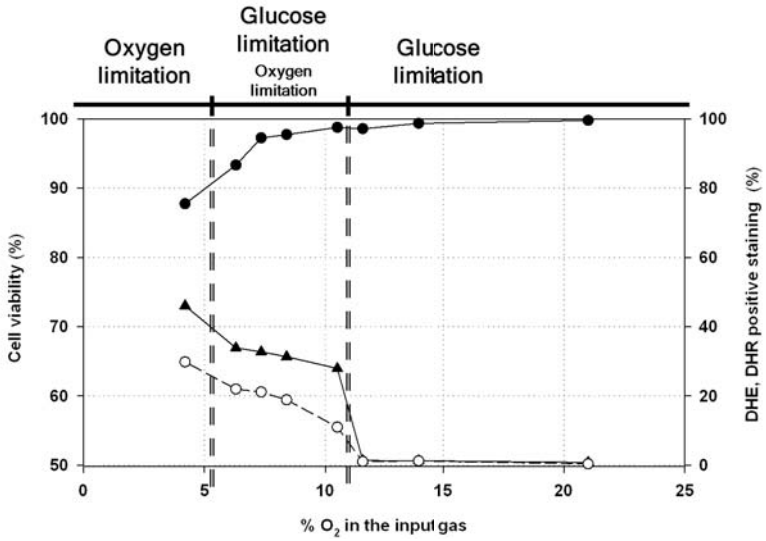

Figure 6.3: Cell viability monitored with PI (●), ROS monitored with DHE (▲) and ROS monitored with DHR (○) at different molar fraction of oxygen in the inlet air.

6.5 Discussion

In this work a thorough study on the global adaptive response of recombinant *Pichia pastoris* to a wide range of oxygen availability has been carried out. As was described by some other authors, a very strong positive effect of oxygen-limiting conditions on specific productivity of some recombinant proteins was observed [22–24]. Nevertheless, in the mentioned works only two limiting conditions for specific set-up cultivations were characterized. In contrast, in this work an important number of different degrees of oxygen availability have been compared in order to deeply characterize the system describing accurately the effect of the oxygen limitation on the physiology and the metabolism at macromolecular level of the yeast, and also to determine the specific conditions that lead to the maximum productivity of the process. In addition, alternative strategies to implement equivalent oxygen-limiting conditions to different cultivation set-ups, operating modes and key proteins have been proposed.

The main causes that lead to the important increase of specific recombinant protein production under oxygen-limiting conditions were extensively discussed in a previous work [23], in which transcriptomic, proteomic and metabolic fluxes analyses were integrated to understand the adaptation of cellular mechanisms to low oxygen availability in a recombinant *P. pastoris* strain. This study hypothesized that important increment of specific productivity may be as a result of increased transcriptional levels of genes involved in the glycolytic pathway, hence genes under the control of glycolytic promoter such as P_{GAP}. In addition, this work also described other effects due to hypoxia conditions such changes in membrane fluidity and increased transcription of genes related with the unfolded protein responses (UPR), e.g.

PDI1, *Ero1* and *Hac1*, which may also contribute to enhance specific productivity of secreted recombinant proteins [48].

Besides the important increase of specific productivity on recombinant protein production, most of the adaptation effects to low oxygen supply on *P. pastoris* cultivations are caused by the metabolic shift from a respiratory to a respiro-fermentative pathways, which leads to a decrease in the biomass yields, generation of secreted by-products (ethanol, arabitol, α-ketoglutarate and succinate), increment of the specific uptake rate of the carbon source ($q_{glucose}$), as well as q_{CO2} and RQ. These metabolic effects increased progressively as the oxygen availability decreased. In contrast, q_{O2} is rather constant among the different conditions of oxygen limitation. Thus, while in normoxic conditions all the carbon provided by glucose are directed to biomass and CO_2 formation, in oxygen-limiting conditions an important fraction of carbon goes to ethanol, arabitol, α-ketoglutarate and succinate that are secreted into the fermentation broth. The different C-distribution in function of the oxygen supply is shown in Figure 6.4.

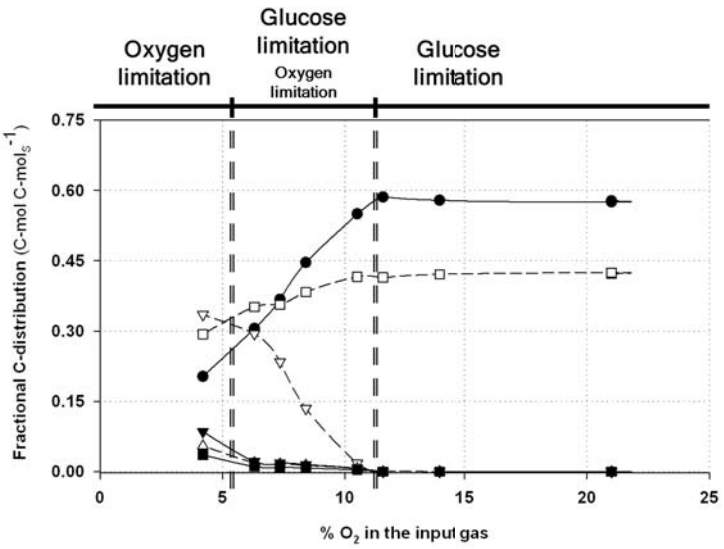

Figure 6.4: C-mol distribution at different molar fraction of oxygen in the inlet air: biomass (●); CO_2 (□); ethanol (▽); arabitol (△); α-ketoglutarate (▼) and succinate (■).

The reduced oxygen supply leads to a strong transcriptional induction of glycolysis and fermentative pathways as well as the downregulation of the pentose phosphate pathways (PPP) and the tricarboxylic acid (TCA) cycle [23]. The generation of ethanol, the main by-product in the culture, was clearly defined as a metabolic swift in the pyruvate breaching point from the pyruvate dehydrogenase pathway, the respiratory flux through the TCA cycle, to the pyruvate decarboxylase pathway, which leads to the ethanol production.

The formation of the other by-products should be related to the adaptation towards a fermentative metabolism in which cells have to remove the excess redox equivalents that accumulates during biomass synthesis and excretion of oxidized metabolites [49]. Previous

works described the generation of arabitol as a mechanism to maintain the redox balance during the fermentative growth and as a kind of protection to osmotic stress [24,50]. The generation of succinate during growth under oxygen-limiting conditions in yeasts has been widely described, especially those related with wine production [51,52]. This formation was also related with the need to maintain the redox balance in hypoxic conditions [53,54]. The production and secretion of α-ketoglutarate, also another important intermediate in the TCA cycle as succinate, as a fermentation by-product of bacteria and yeasts cultures including *Pichia* species was reviewed by Otto *et al.* [55]. This formation may be related with the decrease of carbon flux through the TCA cycle due to the limitation of oxygen availability, as well as the growth in presence of significant concentrations of ethanol [56,57].

Small fractions of all the mentioned metabolites could also be detected in glucose limited chemostat cultivations of *P. pastoris* and *S. cerevisiae* in previous works [58,59]. Other extracellular central metabolites described in these studies might also be present in the cultivation broth of the oxygen limited cultivations of the present work; however their concentration levels might be under detection limit of the analytical techniques used. Interestingly, different from other authors that described the formation of acetate under non-limited glucose conditions [60,61], in this work non detectable amounts of this metabolite could be detected by means of none of the analytical techniques detailed in the materials and methods sections, neither using enzymatic kits or gas chromatography analysis.

The application of flow cytometry analysis enabled a more thorough understanding of the oxygen availability effect on the physiology of *P. pastoris* producing recombinant proteins. By comparing the viability and the accumulation of ROS among samples of steady-state chemostat cultures at different set points of oxygen supply it was possible to determine the stress effects on cells caused by oxygen-limiting conditions. From the results, it was shown that the percentage of viable cell that are growing in glucose-limited chemostat is near to 100%, which is in accordance with other results published [17]. Only when very low oxygen fractions were supplied to the cultivations, a significant decrease on the viability up to around 12% could be detected.

On the other hand, from the very beginning of the application of non-severe oxygen-limiting conditions it induced significant levels of cell stress that caused a relevant accumulation of ROS. This accumulation was progressively increasing as the oxygen availability was being reduced. However, for the most restrictive condition, the accumulation was triggered to levels significantly higher, thus indicating important changes on the physiology in which cells were exposed to an important oxidative stress. Although the significant quantitative differences observed between both reporting indicators use for each method, DHE and DHE, the similar behavior observed between them leads conclude that both are valid for the qualitative

detection of ROS accumulation. Nevertheless, in order to improve the accuracy for a quantitative determination the procedures should be revised and improved.

In the literature the higher vitality of cells has been described in continuous cultures respect to batch and fed-batch processes [34,62]. It was attributed to the absence of accumulated substances that, unlike non-continuous cultivations, are continuously washed out as well as the aging phenomenon of fed-batch processes, what makes the cells more sensitive to stress [40]. Thus, the important effects observed in flux cytometry analysis even though the cells were grown in a chemostat set-up, leads to conclude that oxygen-limiting conditions causes an important stress on the physiological state of *P. pastoris*.

By the integration of the results from the different analysis carried out for the study of the adaptation of *P. pastoris* to different degrees of oxygen availabilities in glucose-limited cultures, it was able to determine the optimal conditions that maximize the productivity of recombinant proteins. As was described above, the conditions that maximize q_{Fab} are the most severe oxygen-limiting while glucose limitation is still the major limiting factor of the culture. Thus, similar physiological states should be achieved in order to reach the maximum levels of protein production. When oxygen is the biggest limiting factor, besides a significant decrease of q_{Fab}, also has been observed an important rise of oxidative stress that leads to an increase of cell mortality and accumulation of ROS, as well as an important metabolic shift that triggers the generation of big amounts of culture by-products, which could be caused by to the collapse of the respirative pathways due to the very low levels of oxygen supply.

As it is stated in the introduction section, a methodology that allows applying equivalent oxygen-limiting conditions to experimental set-ups with different oxygen transfer capabilities is required. Otherwise, the full study relating the O_2 molar fraction in the input gas with the real oxygen available for the culture and its effects should be carried out for every fermentation system and operating mode used for implementing this cultivation strategy. Some of the parameters studied in this work could be selected as a reference of the degree of oxygen limitation applied to the *P. pastoris* cultivations.
As it was mentioned previously, the rather linear increase of $q_{ethanol}$ as the oxygen supply decreases makes it possible that this specific rate can be properly used as an indirect reporting parameter of the oxygen availability for the cells. In contrast, other specific rates of metabolites generated as by-products are not suitable to be of reference because their lower production and non-linear dependence on oxygen limitation of them all. Thus, in Figure 6.5 are presented the behavior of the main parameters studied in this work in function of $q_{ethanol}$. In this plot there are only shown the areas in which oxygen limitation is affecting the cell growth, otherwise the ethanol would not be generated. Interestingly, the plot also shows a linear correlation between the RQ and $q_{ethanol}$. Consequently, RQ could be also a useful reporting

parameter of the oxygen availability for the cells with its own pros and cons. One of the major advantages is RQ gathers information associated to q_{O2} and q_{CO2} into one single parameter not dependent on biomass concentration. The determination of biomass is not straight forward, because both cell physiology and metabolism can vary significantly due to the effects of the oxygen limitation and the biological burden caused by recombinant protein expression.

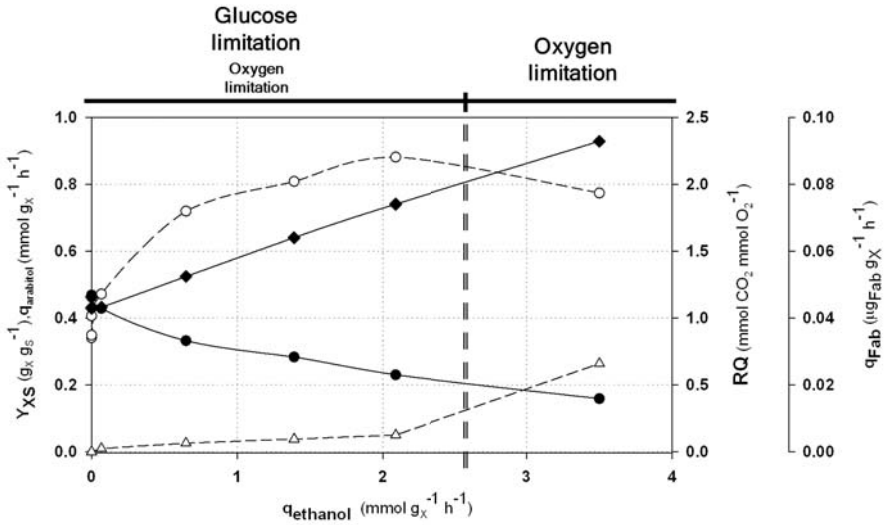

Figure 6.5: Biomass yield (Y_{XS}, ●); specific 2F5 Fab production rate (q_{Fab}, ○); respiratory quotient (RQ,◆); and specific arabitol production rate ($q_{arabitol}$, △) respect to specific ethanol production rate ($q_{ethanol}$).

The application of determined oxygen-limiting conditions into different set-ups working on continuous mode, which advantages have been recently reviewed for industrial bioprocesses [2,63], can be carried out by using as a reporter indicator either the $q_{ethanol}$ or RQ. Since this cultivation mode works ideally in steady-state conditions the process variables are rather constant and the implementation should be straightforward. Ethanol concentration could be determined off-line, by using HPLC or equivalent analytical methods [61], or on-line, by NIRS [64], MS [65] or sensors able to monitor either methanol or ethanol, which are commonly used in *P. pastoris* processes that use methanol [66]. RQ could be easily determined from off-gas data analysis. Therefore, according to the reporting parameters selected, the manipulated variable would be the molar fraction of O_2 supply required for applying the desired oxygen-limiting conditions in the system. Due to the specific characteristics of the chemostat cultures, working at stead-state conditions, this operating condition would be kept constant in time, in order to obtain a maximal Fab production.

The implementation of this cultivation strategy to other operational mode different from continuous mode broadens the versatility of the proposed approach. In this sense, fed-batch cultivation, in which the process parameters are time dependent, should be considered significantly more challenging. Opposite to the continuous mode, along fed-batch cultivations the molar fraction of O_2 supply should be continuously modified by means of reliable monitoring and control strategies. It would allow maintaining key oxygen availability to the

cells throughout the process in which the amount of biomass and its requirements are continuously changing.

In a previous work by Baumann *et al.* [22] different approach was implemented to take advantage of the increase on specific and volumetric productivity of recombinant proteins with hypoxic conditions. In this case, the concentration of ethanol in the culture was kept rather constant always below approximately 1.0% (v v^{-1}) by a regulated addition of feed medium following a feedback control scheme. This strategy do not assure to keep $q_{ethanol}$ constant, because only in chemostat when maintaining the concentration of a component for a given dilution rate at steady-state the corresponding specific rate and productivity are kept constant. In the new proposed approach in this work it is intended to mimic continuous conditions to give it high versatility, not only to be portable from other fermentation systems but also between different operating modes. The proposed fermentation strategy aims to achieve pseudo-steady-state conditions for cell growth (μ) and substrate uptake (q_S) as reached in continuous mode. A pre-programmed exponential feeding rate profile for substrate addition derived from mass balance equations to maintain a constant specific growth rate (μ) would be implemented [14].

As it was described for continuous mode, again either $q_{ethanol}$ or RQ could be selected as reporting parameters of the degree of oxygen limitation. The control of $q_{ethanol}$ would require the estimation of both ethanol production rate and biomass concentration. From the measurements of ethanol concentration and application of mass balances the production rate of ethanol can be obtained. On the contrary, there is not currently available a reliable standard method for the on-line determination of biomass, as each available technique has its own advantages and disadvantages [65]. Alternatively, real-time determination of biomass can be conducted by means of different estimation algorithms and techniques [33,67], but always incorporating some complexity and even instability in the system. Control of RQ is *a priori* not so complex, because its on-line determination is commonly carried out from off-gas analysis and mass balancing of CO_2 and O_2.

As summary, process efficiency is strongly affected by changes in the cellular state, which should be monitored, and properly manipulated. A generic methodology has been discussed and allows the control of the physiological state of the cell related to specific metabolic rates reported here by specific by-products rate or respiratory quotient in different *P. pastoris* culture systems. The versatility of the proposed approach has been discussed for three scenarios. First, in a more general way it could be applied to work under equivalent oxygen-limiting conditions for different cultivation set-ups although may differ in their oxygen transfer capabilities. Second, the understanding of the physiological state of the cell gained from continuous mode could be migrated to fed-batch operation, which is intrinsically time variant. Third, the whole system could be applied for the production of other recombinant proteins to which a positive effect of oxygen limiting conditions has been previously described.

6.6 References

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7

Overview

Table of content

7.1 Introduction

This section provides a brief outlook of the most illustrative results obtained during the present thesis. Since a detailed description and analysis of the results can be found in the previous sections, which correspond to published journal articles and/or submitted manuscripts, extended discussions and not essential citations have been avoided.

This overview aims to integrate the findings of the different sections towards broaden horizons in the development of innovative recombinant protein production processes from a bioprocess engineering point of view. The results of the present thesis contribute to this process development at different levels, which may not correspond strictly with the previous sections. The chapters have been determined in terms of publications. Consequently, to expose this overall discussion, a new structure is defined in order to match with the different fields of study in bioprocess engineering.

7.2 Reliable recovery and quantification of intracellular protein of interest

In *P. pastoris* processes, the intracellular accumulation and the degradation of a protein of interest that should be secreted is often considered a very important bottleneck that limits the efficiency of the bioprocess. Consequently, this work initially aimed to evaluate the impact of this bottleneck and the possibility to implement recovery processes of the non-secreted recombinant proteins in order to improve the overall efficiency of bioprocesses.

The reliable quantification and recovery of intracellular components and product accumulated intracellularly should be considered of capital interest working with a host expression system with a limited secretion capacity reported. However, no efficient disruption methods for *P. pastoris* were have been defined previously due to severe methods that requires this yeast and the lack of attention that historically have had the optimization of cell disruption methods.

Since the presence of methanol in the cultivation leads to and important widening of the cell thickness of *P. pastoris,* more severe methods should be expected for an efficient disruption comparing with *E. coli* and *S. cerevisiae* as model cell factories [1]. Therefore, initially the study was performed with cells of a wild-type *P. pastoris* strain grown on this substrate. By comparing different cell disruption methods, high-pressure homogenisation was determined as the most efficient procedure. Subsequently, by defining a Key Performance Indicator (KPI) based on three reporting indicator (OD decrease, and release of total protein and alkaline phosphatase activity) the cell disruption performance of a homogeniser was optimized respect to number of passes and biomass concentration. These two factors, which were analysed by design of experiments (DOE), have been reported to have a high impact on the cell disruption performance efficiency using high-pressure homogenisation methods [2].

These disruption settings for high-pressure homogenisation were later on successfully applied to the *P. pastoris* strain disruption when producing constitutively the 2F5 Fab. An important amount of the protein of interest was expected to be retained through the secretory pathway including cell membranes, ER, Golgi and other organelles. However, in the standard intracellular protein quantification procedures usually described in the literature, the fraction of protein retained in these organelles is normally being discarded as an insoluble fraction of the cell lysate. It is considered that it can lead to an important underestimation of the target product. Therefore, in order to increase the accuracy quantification for the protein of interest, a method for properly extracting it from the insoluble fraction was also developed. Specifically, different extraction buffers, detergents, detergent concentration and incubation time were screened to determine the best protein extraction procedure. However, it must be taken into account that the protein extraction procedure is protein dependent and the settings could change depending on protein characteristics.

The combination of the optimized cell disruption and protein extraction procedures lead to a very significant accuracy improvement towards a more reliable quantification of intracellular components and proteins of interest retained intracellularly. The reliability of this determination is essential to properly evaluate a possible underestimation of the overall bioprocess yield that may be caused by a fraction of non-quantified target protein retained intracellularly.

7.3 Effect of the carbon source at the different phases of fed-batch cultivations

As said in the state of the art section, at the initial point of the present thesis only few works were published performing fed-batch cultivations for recombinant protein production processes using *P. pastoris* under the regulation of the constitutive *GAP* promoter. Consequently there was lack of knowledge of the impact of some basic operational parameters on the cultivations and its performance. One of those was the effect of the carbon source used at both phases of fed-batch cultures.

In *P. pastoris* production processes driven by P_{GAP} different carbon sources leads to the constitutive expression of the protein of interest. Between them, stand out glycerol and glucose with similar yields [3]. Both were being used for both phases of a fed-batch cultivation, however, to our knowledge, there was not any specific work comparing the effect on a cultivation of using either carbon sources.

In terms of recombinant protein of interest, the end-of-process yields and productivities obtained in both phases by using alternatively the carbon sources did not differ significantly. However, important differences were observed in other cultivation parameters, which were used to determine the optimal carbon sources for each fed-batch phase.

For the batch phase, the production of biomass growing on glucose at standard operational conditions was importantly lower than using glycerol. At the end of the batch phase significant amounts of fermentative by-products were observed. This fact indicates a metabolic shift from fully respirative to respire-fermentative, even under fully aerobic (standard conditions of dissolved oxygen concentration around 30% air saturation). This metabolic change could explain the important reduction of biomass yield, the alteration in the oxygen consumption profile and the generation of significant amounts of fermentation by-products also reported by other authors [4,5]. In addition, the mentioned effects had a negative impact in the performance of the subsequent fed-batch phase regardless the carbon source used in the second phase. Product titers and yields were around 30% lower when using glucose in the batch phase. Consequently glycerol, which leads to a fully respirative metabolism regardless the dissolved oxygen concentration, was selected as the optimal carbon source for batch cultivations.

In contrast, in the fed-batch phase no metabolic shifts were observed for any of the carbon source alternatives compared. It is probably due to the fact that the carbon source is limiting the growth during this phase. Therefore the criterion selected for determining which was the best alternative was based in oxygen requirements and heat productions, two parameters that are often considered important limiting factors in large-scale cultivations. Both were significantly higher for cultures growing on glycerol. Consequently, glucose was selected as the best carbon source for the fed-batch phase of the cultivation.

First fed-batch cultivations of *P. pastoris* expressing constitutively a recombinant protein were used to develop the main fermentation procedures and calculations that afterwards would be used around the project thesis. In addition, important physiological effects of the carbon source were described for both cultivation phases, which lead to select different carbon sources for the each fed-batch cultivation phase: glycerol for the batch phase, and glucose for the fed-batch phase.

7.4 Effect of the specific growth rate on the productivity

As it is widely described in the introduction section of the present thesis, since the specific growth rate reflect to a certain extent the metabolic state of the cell, correlations between the specific growth rate (μ) of the host cell system and the specific production rate (q_P) can be found. Actually, the relationship between μ and q_P, also sometimes termed as "production kinetics", is considered crucial in the design of a bioprocess [6]. Different trends have been reported for different recombinant production platforms using several hosts. For *P. pastoris,* since the current knowledge does not allow predicting it *a priori,* it has to be determined empirically.

As a reference prior to the beginning of this thesis, Maurer *et al.* [7] described the production kinetics of the strain used in this work (*P. pastoris* expressing the human 2F5 regulated by the *GAP* promoter) by means of performing several continuous cultivation at different μ. Product formation was found to increase with specific growth rates almost up to μ_{max}. Initially in this work, once the best carbon source combination was determined, this production kinetics was aimed to be reproduced in fed-batch cultivations growing at different nominal μ. As it was expected, the production kinetics determined was the same; the best results regarding yields and productivity were also obtained for the highest μ carried out. The best results obtained were, in turn, compared with others published for the same strain with different fed-batch cultivation strategies; constant feed rate [8] and customized feed rate by optimization procedures [7]. In this work In terms of both specific production rate (q_P) and volumetric productivity (Q_P), the production of protein of interest was increased about 4-fold respect to constant feed rate [8] and about 25% respect to customized feed rate [7].

The important increment of productivity observed at high μ has been related by some authors to a potential effect of the μ on the capacity of the secretory pathways to release the recombinant proteins [9]. Consequently, it was hypothesised that if the secretion processes were the only limiting factor for protein production at low μ, in these growing conditions a very important amount of protein synthetized could be retained intracellularly. Therefore, the recovery of the protein of interest retained intracellularly could increase significantly the efficiency of the bioprocess.

Accordingly, in order to study the effect of the μ in the secretion capacity, and thus also the balance intra- and extracellular of the product, continuous cultivations growing at different μ were carried out. The development of procedures that allows the reliable recovery and quantification of protein of interest was essential for carrying out this work. The Fab distribution determined between intracellular and extracellular fractions was rather constant regardless the μ, being the intracellular fraction importantly lower than the extracellular.

Consequently, it was considered that the secretion capacity at different μ could not explain differences in protein production.
The bases of the production kinetics observed have been afterwards explained by means of systems biology studies. Since the expression of the target protein is driven by the glycolytic *GAP* promoter and its transcription levels are directly related with the glycolytic flux, and thus μ, an important increase of recombinant protein transcription levels takes place at increasing μ [10]. In addition, significant changes in the regulation of important groups of genes that also contributes to the positive effect in the q_P have been reported at high μ [11].

The production kinetics for the constitutive expression human 2F5 Fab in *P. pastoris* has been determined both in fed-batch and continuous cultivations. Higher production rates were obtained at increasing μ. q_P presented a rather linear increment as μ was also increasing; for the highest, it was observed up to an 8-fold increase respect to the lowest. This production increment is considered associated to an increment of the glycolytic flux at high μ rather than a possible effect of the μ in the secretory capacity of the host.

7.5 Environmental stress conditions to enhance protein production

In the last years, several studies have been reported in which cell stress responses are coupled with the overexpression of the protein of interest. Hence, bioprocess engineering is aiming to exploit the effects of different cellular stresses in order to increase yields and productivities of recombinant protein production processes.

In the present thesis the impact of two important environmental stresses on the production of proteins regulated by *GAP* promoter have been studied, carbon-starving and oxygen-limiting conditions. Therefore, different innovative cultivation strategies have been proposed in order to maximize the positive effect of the stress conditions.

7.5.1 Carbon-starvation

The starting point of this research was the empirical observation of an important production increase of proteins regulated by P_{GAP} for a short-time after the depletion of glucose. This fact was also reported by Kern *et al*. [12], however their work did not hypothesize about the causes that could lead to the rapid increase of protein synthesis during short periods and the potential applications on bioreactor cultures.

The present work was aimed to study the implementation of short carbon-starving periods in fed-batch cultivations at high μ and its effect on the recombinant protein production rates and yields. Several cultivation strategies combining the implementation of carbon-starving periods of different time duration and frequency with an exponential feeding profile commonly used to carry out fed-batch cultivations at constant μ. All the new implemented strategies achieved

important increases up to 40% of Fab production in terms of product titration as well as product to substrate ($Y_{P/S}$) and product to biomass ($Y_{P/X}$) yields. In contrast, in terms of productivities, increases obtained applying the innovative feeding strategy were more moderate (up to 10%). The best results were obtained for the shortest carbon-starving periods implemented with the highest frequency.

In addition, it was also aimed to elucidate if the increase of protein production during the carbon-starving periods was due to the secretion of protein of interest that was previously synthetized but retained intracellular, or to *de novo* protein synthesis. Once again, the balance intra- and extracellular of the product was studied for samples taken prior and at the end of the carbon-starving periods. No significant differences were observed in the Fab distribution between the samples. In addition, the total amount of Fab determined taking into account all the fractions were significantly higher after the carbon-starving periods, which lead to conclude that the increase of product is due to a *de novo* synthesis of protein of interest under glucose depletion conditions.

The implementation of carbon-starving conditions into an exponential feeding profile at high μ can be therefore considered a non-complex operational improvement in fed-batch fermentations. By the exploitation of an environmental effect stress in the host cells, bioprocess engineering approach allowed to achieve very important increase of recombinant protein productions.

7.5.2 Implementing oxygen-limiting conditions

The impact of oxygen supply on the heterologous production has been studied for different recombinant production hosts due to that the oxygen transfer rate is usually one of the most important technical limitation for high cell density cultivations of microorganisms at large-scale.

For *P. pastoris* producing constitutively the human 2F5 Fab, an important increase of q_P was described at low oxygen supply [13]. This work studied the impact of few oxygen-limiting conditions in continuous cultivations and also presented a primary strategy of fed-batch cultivation under hypoxic conditions achieving significant increases of product formation. However, no further studies have been published in order to identify the optimal degree of limitation. None operational approaches that allows implementing the desired oxygen-limiting conditions to fermentations set-ups with different oxygen transfer capacities and also alternative operating modes have been proposed at the moment.

In this work, the physiological and metabolic adaptation of recombinant *P. pastoris* to a wide range of oxygen availability conditions has been systematically studied in glucose-limited chemostat cultivations. This has allowed the determination of the optimal oxygen-limiting

conditions for enhancing the recombinant protein production and to evaluate their macromolecular physiological effects on *P. pastoris* such generation of fermentation by-products, decrease of cell viability and intracellular accumulation of reactive oxygen species (ROS). The maximum levels of protein production have been achieved for the most severe oxygen-limiting conditions while glucose limitation is still the major limiting factor of the culture. In contrast, an excessive oxygen limitation could collapse the respirative pathways leading into lower productivities. The increase of protein production has been associated to a strong transcriptional up-regulation of genes related to the glycolysis pathway at reduced oxygen supply [14].

In order to exploit the positive impact of the oxygen-limitation in different fermentation systems, since the operational settings are dependent of the oxygen transfer capabilities of the set-up, physiological parameters of the culture should be selected as reporting parameters of the degree of oxygen limitation for the cells. Different parameters studied in this work could be selected in *P. pastoris* cultivations. Among them, stand out the specific production rate of ethanol and RQ, which present a rather linear increase as the oxygen supply decreases.

A systematic study of the impact of oxygen supply in *P. pastoris* cultivations has been carried out in order evaluate the physiological effects in the host and to determine the optimal oxygen-limiting conditions that allows maximizing the production of recombinant proteins. In turn, the use of reporting parameters such specific production rate of ethanol and RQ has been proposed to replicate the desired oxygen conditions in fermentations set-ups with different oxygen transfer capacities and also alternative operating modes.

7.5.3 Lack of essential nutrients

Both alternatives exposed in this section present a common factor, which is the induction of an environmental stress to the cells caused by the lack of essential nutrients for the growth.

It has been widely reported that yeasts are sensitive to the level of nutrients such carbon, nitrogen and oxygen sources, which allows them to adapt readily to changing nutritional states. This adaptation is the result of multiple interconnected signalling networks, which allow the yeast to adjust their metabolism, transcriptional profile and developmental program to the new environment [15]. The recombinant expression system studied in this thesis is based on the use of a glycolytic promoter (P_{GAP}), which natively regulates the Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), a key NAD-binding enzyme in the glycolysis and gluconeogenesis pathways, and therefore the central carbon metabolism (Figure 7.1). The adaptation responses of the cell to an environmental stress is expected to regulate the central carbon metabolism of the yeast, thus of the metabolic fluxes through the glycolysis pathway, and, in turn, the transcription of P_{GAP}-regulated genes. It is therefore not surprising that environmental stress conditions have a direct impact in the levels of recombinant protein expression.

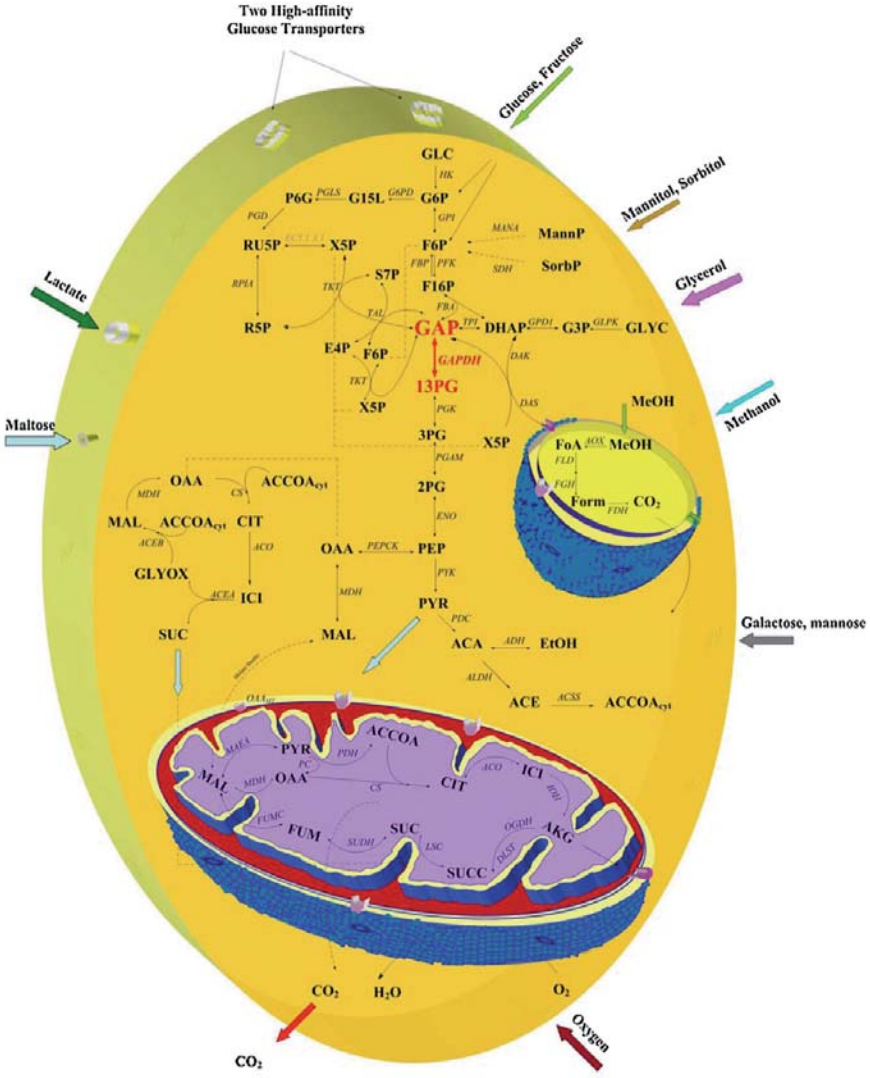

Figure 7.1: Central carbon metabolism of *P. pastoris*. Figure taken from [16].

For oxygen-limiting conditions, a work in systems biology described integrating different –omics technologies (transcriptomic, proteomic and metabolic fluxes analyses) hypothesized that important the increment of specific productivity observed is mainly the result of increased transcriptional levels of genes involved in the glycolytic pathway [14]. Accordingly, it can be also hypothesized that similar cellular response could take place in *P. pastoris* cells to adapt their metabolism to the environmental stress caused by carbon-starving conditions.

However, in this work it has been also described that an excessive impact of both environmental stress conditions studied can even lead to a detrimental effect in the product formation. Therefore, deep and systematic studies to characterize the environmental stress conditions effect in the host cell physiology and its productivity are essential to exploit the effect of environmental stress conditions. In turn, bioprocess engineering will aim to design, to develop and to implement new operational cultivation strategies that allow maximizing the recombinant production yields and product.

7.6 References

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8

Conclusions and future propsects

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8.1 Conclusions

A deep understanding of the host cell system physiology has enabled an important progress towards the development of cultivation strategies that enhances the recombinant protein production processes. Currently, the development of improvements in the production processes is often rationally designed from the physiological characterisation of the strain. In this respect, the present thesis have been focused on the recombinant protein production using the yeast *P. pastoris* under the regulation of the *GAP* promoter, which offers important operational advantages in industrial scale production respect to the widely used methanol-based production processes.

The fermentation technology for this expression system at the beginning of this thesis was considered in a very early stage of development. In this work, the impact of several factors affecting the protein production have been studied in aiming to design, develop and implement innovative cultivation strategies that allow to achieve significant progresses in these production processes.

For his purpose the human 2F5 antigen-binding fragment (Fab), has been used as model protein. Currently Fabs have a wide applicability as therapeutic and diagnostic agents. The Fab structure is composed by four subunits, which requires the formation of disulphide bonds between them. Its structure becomes the Fab into very complex proteins, and thus a very important challenge for *P. pastoris* in terms of protein expression, folding and secretion. It is expected that the bioprocess advances developed for this model protein are also applicable to the protein of other recombinant proteins in *P. pastoris* driven by P_{GAP}.

One of the major bottlenecks of *P. pastoris* as a host for recombinant protein expression is considered the limited secretory capacity. Therefore, a reliable procedure is essential to evaluate properly the effect of this bottleneck on the bioprocess production under different cultivation conditions. In this work, a cell disruption method by high-pressure homogenisation and a protein extraction protocol have been developed and optimized achieving an important increase in the accuracy of intracellular product quantification.

In fed-batch cultures, glycerol and glucose as alternative carbon sources have been compared for both phases of the cultivation. Although the results in term of yields and productivity were rather similar, the important physiological effects observed lead to select different carbon sources for each fed-batch phase: glycerol for the batch phase, and glucose for the fed-batch phase.

The effect of the specific growth rate (μ) on the specific production rate (q_P) has been studied both in fed-batch and continuous cultivations. Higher production rates were obtained at

increasing μ. A very important q_P increase of up to 8-fold was obtained at the highest μ respect to the lowest. This increment of the production is considered associated to an increment of the glycolytic flux at high μ rather than a possible effect of the μ in the secretory capacity of the host. Therefore, the relationship between μ and the q_P has been confirmed to be crucial in the design of a bioprocess.

It has been demonstrated that taking advantage of the stress caused in the host cells by certain environmental conditions is a successful strategy for improving the recombinant protein production. In this work, important production increases have been achieved by carrying out the cultivations at carbon-starving and oxygen-limiting conditions. For both cases, the increments have been related to a hypothetic up-regulation of the glycolytic pathways enzymes as a cell adaptation response to new conditions. In order to maximize the positive effect of the stress on the production rates, the impact of the environmental stress on the cell factory must be carefully characterised prior to the design and implementation of novel cultivation strategies.

8.2 Future prospects

As summary, substantial enhancements have been certainly achieved on the recombinant production processes of *P. pastoris* driven by the *GAP* promoter. Different promising relationships between the host cell physiology and product formation have been identified, studied and then rationally exploited in innovative cultivation strategies. However, at the same, there still remains a long way to go towards the optimization of the cultivation strategies presented.

One particular issue is related with the implementation of the optimal oxygen-conditions in fed-batch cultivations, therefore in a dynamic system. Although in this work either $q_{ethanol}$ or RQ has been identified as promising reporting parameters of the degree of oxygen limitation, the development of control strategies that allows maintaining the desired set-point is expected to be very challenging.

Further studies, for example, will also be required to elucidate the carbon-starving effects on the host cell transcriptome, proteome, metabolome and fluxome. In turn, innovative cultivation feeding profile could be developed for maximizing even more the production yields obtained.

Finally, other operational strategies that allow increase the flux through the glycolytic pathway should be identified and exploited in order to also increase the expression of recombinant proteins regulated by the *GAP* promoter. The implementation of the proposed bioprocess engineering developments should be complementary to the genetic and host cell engineering strategies in order to enhance the recombinant protein production processes.