

**High Cell Density Cultivation to
Produce Heterologous Proteins by *S.*
cerevisiae Strains: a Holistic Approach
to Investigate and Optimize the System**

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Unione Europea



*Ministero dell'Istruzione,
dell'Università e della Ricerca*



UNIVERSITÀ DEGLI
STUDI DI SALERNO

FONDO SOCIALE EUROPEO
Programma Operativo Nazionale 2000/2006
“Ricerca Scientifica, Sviluppo Tecnologico, Alta Formazione”
Regioni dell’Obiettivo 1 – Misura III.4
“Formazione superiore ed universitaria”

Department of Industrial Engineering

*Ph.D. Course in Chemical Engineering
(XII Cycle-New Series)*

**High Cell Density Cultivation to Produce Heterologous
Proteins by *S. cerevisiae* Strains: a Holistic Approach to
Investigate and Optimize the System**

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Acknowledgements

I would like, firstly, to thank my supervisor, Prof. Palma Parascandola, for her passionate support. In addition, I would like to thank the members of the Scientific Committee, Prof. Elisabetta de Alteriis and Prof. Francesco Giannino for their precious assistance.

Special acknowledgements are for Prof. Jesus Zueco and Prof. Stefano Mazzoleni for their contribution to this work.

A special thanks to the researchers of "System and Synthetic Biology Lab" of Chalmers University, directed by Prof. Jens Nielsen, with which I have spent a wonderful professional experience.

This work was supported by the project “Sicurezza, sostenibilità e competitività nelle produzioni agroalimentari campane” (CARINA)

During the PhD work the following paper have been written:

Paciello L, **Landi C**, Zueco J, Parascandola P. 2013. Production in fed-batch reactor of *Bacillus subtilis* lipaseA immobilized on its own producer *Saccharomyces cerevisiae* cells. *Chemical Engineering Transactions*. **32**, 907-9012.

Paciello L, Falco FC, **Landi C**, Parascandola P. 2013. Strengths and weaknesses in the determination of *Saccharomyces cerevisiae* cell viability by ATP-based bioluminescence assay. *Enzyme and Microbial Technology*. **52**, 157-162.

Paciello L, **Landi C**, de Alteriis E and Parascandola P. 2012. Mathematical modeling as a tool to describe and optimize heterologous protein production by yeast cells in aerated fed-batch reactor. *Chemical Engineering Transactions*. **27**, 79-84.

Landi C, Paciello L, de Alteriis E, Brambilla L, Parascandola P. 2011. Effect of auxotrophies on yeast performance in aerated fed-batch reactor. *Biochemical and Biophysical Research Communications*. **414**, 604-611.

Landi C, Paciello L, de Alteriis E, Brambilla L, Parascandola P. High Cell Density Culture with *S. cerevisiae* CEN.PK113-5D for IL-1 β production: modeling, optimization and physiological aspects. *Process Biochemistry* (submitted).

Landi C, Paciello L, Zueco J., Parascandola P. On the fermentative behavior of auxotrophic strains of *Saccharomyces cerevisiae*. *Journal of Microbiology and Biotechnology* (submitted).

Falco FC, **Landi C**, Paciello L, Zueco J, Parascandola P. Fed-Batch Production of Endoglucanase with a Recombinant Industrial Strain of the Yeast *Saccharomyces cerevisiae*. *Chemical Engineering Transactions* (submitted).

Proceedings of the conferences:

Landi C, Mazzoleni S, Paciello L, Giannino F, de Alteriis E, Carteni F, Parascandola P. A novel system dynamics model for the growth of

Saccharomyces cerevisiae in batch and fed-batch reactors. 30th SIMGBM Meeting, 2013, 18-21 September. Ischia, Italy.

Martinez JL, Huang M, Liu L, Bao J, **Landi C**, Petranovic D, Nielsen J. Recombinant protein production by yeast: establishing *Saccharomyces cerevisiae* as a robust cell factory. *Science and Technology Day 2013*. April 16, 2013 – Goteborg (Sweden).

Landi C, Paciello L, Bellofatto G, Zueco J, Parascandola P. Production of Lipase A from *Bacillus subtilis* expressed in *Saccharomyces cerevisiae* strains: a preliminary approach to the feasibility of the bioprocess. Oral presentation - XII FISV Congress - Rome September 24-27, 2012

Landi C, Paciello L, de Alteriis E, Brambilla L, Parascandola P. *Saccharomyces cerevisiae* CEN.PK *ura⁻* strain as host for heterologous protein production: a systematic investigation to optimize the aerated fed-batch culture. 29th SIMGBM Meeting, 2011, 21-23 September 2011, Pisa, Italy.

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Abstract

The research of this PhD thesis has been focused on the study and realization of high cell density cultivation (HCDC) systems with auxotrophic strains of the yeast *Saccharomyces cerevisiae*, these latter to be used as hosts for heterologous proteins (HEPs) production.

Considering the complexity of the HCDC systems and that their performance depends on the strong interactions between biological and environmental determinants, the study of such systems was carried out by means an holistic approach or by considering the cultivation system as a whole and its behavior explicable on the basis of the intimate interconnection of its parts. In this light, the work has been aimed not only at improving the HCDC performance but also elucidating the physiology expressed by yeast when it proliferates in this type of system.

The HCDC study has been carried out in an aerated fed-batch reactor which allows the proliferating biomass to be accumulated so as to achieve high cell density (HCDC). This is possible due to the extension of working time and the control of by-products, or catabolite repression effects through controlled conditions for the substrate supply.

Initially, the experimental activity has been directed to find out the host for heterologous proteins expression. For this purpose a systematic investigation has been made considering six strains belonging to the CEN.PK family of the yeast *S. cerevisiae*, characterized by different types and number of auxotrophies. This preliminary investigation gave a clear result on the best candidate to be used as host for recombinant protein production. Indeed, the selected strain, CEN.PK113-5D, produced an amount of biomass which was, approximately, three-fold higher than the other strains tested, and similar to that obtained with *S. cerevisiae* industrial strains which did not carry any auxotrophy.

Based on this result, the operative conditions of the bioprocess were optimized. Particularly the selected host has been tested in different operative conditions to find those values of specific growth rate ensured high volumetric productivity and biomass yield.

Therefore, CEN.PK113-5D was transformed for the production of interleukin-1 β , used as a model protein and subsequently, for two proteins of great interest for the agri-food field: Lipase A from *Bacillus subtilis* and an endoglucanase from *Paenibacillus barcinonensis*.

Fed-batch tests were combined with physiological studies to assess Reactive Oxygen Species, catalase, cell viability and presence of by-product into the culture medium during fed-batch runs.

The results of this work were implemented by expressing in CEN.PK 113-5D three human proteins: hemoglobin, myoglobin and neuroglobin. In this way, the effects of the production of a wide range of recombinant proteins of different origin, on the yeast growth, has been investigated. It is worth noticing here the special interest, from an applied point of view, of the positive effect obtained with the expression of human myoglobin and neuroglobin on the growth characteristics of the recombinant yeast strains and its possible relationship with a better oxygen transfer. This part of the work has been performed at the Chalmers University in Goteborg (Sweden).

During the work, particular interest was reserved to the study concerning the limits encountered in the cultivation of microorganisms at high cell densities. Indeed, fermentation runs of the *S. cerevisiae* strains investigated in fed-batch, were characterized by a peculiar decay in the specific growth rate and metabolic fluctuation with a limit of cell density hardly to be overcome, that for CEN.PK 113-5D was about 100 g l^{-1} . Further, it has been highlighted that growth decay phenomena were related to the accumulation in the medium of not yet identified, inhibitory compounds.

The experimental approach of this thesis work has been implemented with the development of an innovative mathematical model built up on the System Dynamic principles. This model was capable to represent the growth dynamics of *S. cerevisiae* strains in batch and fed-batch reactors. The model has been developed through the explicit representation of the two main pathways of the glucose catabolism in yeast, respiration and fermentation. Pyruvate was identified as the key intermediate of sugar metabolism in yeast. Moreover, other components exerting negative feedback effect on cell proliferation were modeled.

Model results highlighted that in order to obtain a good fitting between the simulation curves and the experimental data, it was essential to consider a negative feedback effector represented by the secretion of inhibitory compounds along the fermentation runs and as such capable to describe entirely the yeast growth in the fed-batch reactor.

Preface

High cell density cultivations (HCDC) are defined as microbial cultures with an high value of final biomass concentration.

HCDC are powerful systems largely employed for both biomass and heterologous protein (HEP) production whereas auxotrophic strains are widely used as hosts for HEP production due to the stable maintenance of expression vectors.

Productivity, *i.e.* the amount of product formed per volume unit per time unit is a function of cell density so increasing the cell density, productivity increases as well. Increasing of productivity is the major objective of fermentation in research and industry and, as mentioned by Riesenber and Guthke (1999), it is a prerequisite to maximize the amount of product in a given volume within a certain time. This is not possible in conventional batch and continuous processes but only in semicontinuous (fed-batch) which allow the proliferating biomass to be accumulated. However push the proliferation of living organisms, albeit of microbial nature *i.e.* yeast, to achieve HCDC is not a trivial challenge, and this requires the study of the bioprocesses under a global view.

Although yeast has been used by mankind since ancient times, it has had, surprisingly, little application as an organism to be exploited for production of therapeutic proteins, fine chemicals or any other industrially relevant sector (Schmidt, 2004). This is surprising as research publications on yeasts (Gerngross, 2004) demonstrate its considerable potential in several areas of application and also document particular examples of its commercial use in addition to those employed in traditional nutrition and food products (e.g. oxido-reduction and/or enantio- and regioselective reactions in organic chemistry), production of technical enzymes such as lipases and esterases, and low cost products, for instance feed enzymes (Mayer *et al.*, 1999). Furthermore, yeast cell material can be a source of several substances which are beneficial to health or can be used in cosmetic applications (e.g. glycans, ceramides).

The commercial utilization of yeast, a unicellular organism which combines the advantages of the eukaryotic expression system with those of a bacterial one, is supported by the following arguments:

Preface

- ✓ as a fast-growing organism, yeast can be easily cultivated to high cell densities (HCDC) in non-expensive mineral media and is therefore free from retroviral contaminations;
- ✓ in contrast to e.g. *Escherichia coli*, components of yeast cells do not cause endotoxic (i.e. pyrogenic) reactions in humans;
- ✓ the ‘food’ yeasts are highly acceptable for the production of pharmaceuticals having GRAS affirmation (i.e. ‘Generally Recognized as Safe');
- ✓ yeast usually expresses heterologous genes using a natively strong and tightly regulated promoter;
- ✓ yeast is able to secrete large amounts of functional protein into the culture broth, i.e. it performs many of the posttranslational modifications typically associated with higher eukaryots;
- ✓ yeast is easy to handle at large scale and during product recovery.

During the period of my PhD, the main objectives of my investigation were to set-up an entire turnkey bioprocess in a fed-batch reactor with auxotrophic glucose-sensitive yeast (*Saccharomyces cerevisiae*) as hosts for production of HEPs such as the enzymes endoglucanase from *Paenibacillus barcinonensis* and lipase A from *Bacillus subtilis* to be employed in agri-food industry.

An holistic approach has been chosen to pursue the aim of this thesis, indeed HCDCs are complex systems originated from the interactions of biological and environmental determinants. This means emphasizing the importance of the whole system and the interdependence of its parts. More specifically, several factors such as the influence of growth environment with particular attention to the feed rate and metabolic fluctuation during bioprocess related to the glucose repression phenomena, have been taken into consideration.

Before to test the effect of the heterologous protein expression, a strain belonging to the CEN.PK family of the yeast *S. cerevisiae* has been identified as the most suitable strain to be used as host for heterologous expression. Particularly six isogenic haploid strains were characterized in an aerated fed-batch reactor. The choice to perform productions with *S. cerevisiae* CEN.PK yeasts strain derives from the fact that these strains were selected as yeasts with a good aptitude to display a respiratory metabolism, moreover they exhibit a high grow rate in continuous and fed-batch culture systems (van Dijken *et al.*, 2000).

After host selection, in view of the optimization of the bioprocess, the chosen strain was tested in different operative conditions to find the value of specific growth rate, which ensured high volumetric productivity and high biomass yield developing a bioprocess as economically as possible.

The selected yeast strain, was subsequently transformed, with the expression of a model protein in order to confirm the optimization protocol adopted.

Great attention was given to the kind and the nature of the HEP expressed by yeast cell. To this purpose several HEPs were tested including also proteins of human origin (human hemoglobins and Interleukin-1 β). As far as this, part of the investigation has been carried out at Chalmers University (Goteborg – Sweden) where the HEPs expression has been studied also with the aim of exploiting the heterologous expression to increase the performance of the yeast introducing a protein that facilitates oxygen transport (Liu *et al.*, 2013).

In parallel with experimental activities, a novel model based on the most significant metabolic pathways has been developed according to the principles of the Dynamic Systems (Forrester, 1971). The proposed model has been capable to describe growth dynamics of yeast in complex culture systems as fed-batch reactor. In particular, the model has been built up considering pyruvate as the key metabolite capable to regulate the two catabolic pathways of yeast metabolism, respiration and fermentation. Moreover the negative effect of toxic compounds, produced during cell proliferation has been considered. Model has been assembled in a flexible and modular structure, using the system dynamic software Simile[®].

Chapter I

Strategies to obtain high cell density cultures

I.1 Culture system overview

The major objective of fermentation in both research and industry is to maximize the amount of a certain product in a given volume within a certain time (Riesenberg and Guthke, 1999) that is to maximize the productivity. To this purpose, high cell density cultivation (HCDC), represents a powerful technique to produce biomass or other products (also heterologous proteins) because productivity is a function of both cell density and specific productivity *i.e.* the productivity per cell mass unit.

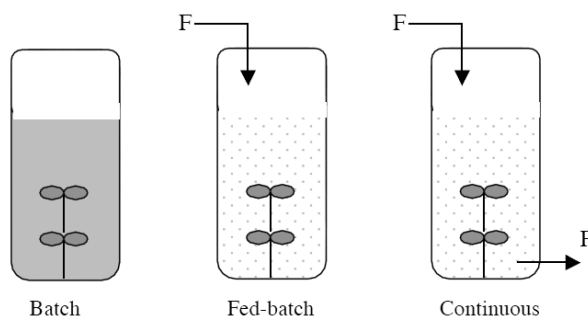


Fig. I.1 Layout of the three main types of fermentation modes: batch, fed-batch and continuous to set up submerged cultures.

Regard to the design of cultivation systems for cells proliferation it should be considered that yeast can be grown in either liquid medium or on the surface of a solid agar plate, however for large scale application the only feasible way is to build up a submerged culture or a liquid culture subjected to continuous and vigorous agitation. Submerged culture are set-up in a

chemical reactor commonly known as fermenter. The fermenter is the central component of the system in which the organism is grown under optimum conditions for product formation. Particularly a controlled environment is realized especially as regards pH, temperature, dissolved oxygen, etc.

The fermenter can work in three different modes *i.e* discontinuous, continuous and semi-continuous which correspond to batch, chemostat and fed-batch cultivation system, respectively.

The following sections (I.2, I.3, I.4) are an overview on the different solutions

I.2 Batch culture

The batch culture is a discontinuous system and can be considered as a closed system except for oxygen supply needed in the case of aerobic microbial growth (e.g. yeast growth). The only liquid additions made during the batch process are acid/base for pH control and antifoam for foaming control. In this culture system, biomass is loaded to the reactor containing substrates (carbon and energy source and other nutrients) at a suitable initial concentration. The cells grow using these substrates until their exhaustion. Therefore, nutrient concentrations are not controlled, as well as cell density and products concentration. So these important process parameters are not stable and vary with time. As previously said, the only optimization parameter of the batch fermenter is the initial medium composition that cannot be controlled over time as well as the metabolism of the microorganism.

I.3 Continuous culture

In a continuous operation mode, one or more feed streams containing the necessary nutrients are fed continuously to the fermenter, while the effluent stream containing the cells, products, and residuals is continuously removed. A steady state is established by maintaining an equal volumetric flow rate for the feed and effluent streams. In so doing, the culture volume is kept constant, and all nutrient concentrations remain at constant steady state values. Continuous reactor operations are common in chemical industries whereas, with the exception of single-cell protein production (Sajc et al., 2000) certain beer production (Brányik et al., 2005) and municipal waste treatment processes, continuous cultures have not yet been widely adopted by the industry. This depends primarily on the difficulty of maintaining sterility (absence of any living organism, except for the producer organism)

and protecting the culture from phage attacks or mutations. Anyway, there is a clear resistance from the industry to convert plants due to the belief, not yet fully understood, that steady state operations are found to yield poorer results than dynamic operations.

I.4 Fed-batch culture

A fed-batch culture is a culture carried out in a semi-batch operation mode that is the nutrients necessary for cell growth and product formation are fed to the fermenter via one or more feed streams during the course of an otherwise batch operation. The broth culture is harvested only at the end of the operational period, either fully or partially (the remainder serving as the inoculum for the next repeated run). This process may be repeated (repeated fed-batch) a number of times if the cells are fully viable and productive. Thus, there are one or more feed streams but no effluent during the course of operations. Sources of carbon, nitrogen, sulfur and other elements, precursors, or inducers are supplied to the fermenter planning a well-defined feeding strategy that can be changed, if necessary, during the fermentation run. Therefore, the culture volume increases during the course of operation until the fermenter volume is full. Thereafter, a batch mode of operation might be used to attain the final results. Thus, the fed-batch culture is a dynamic operation. By manipulating the feed rates, the concentrations of limiting nutrients in the culture can be manipulated either to remain at a constant level or to follow a predetermined optimal profile in order to maximize the concentration of the desired product or the yield of product. This type of operation was first called a fed-batch culture or fed-batch fermentation by Burrows, 1970 and Yamane, 1978. In environmental engineering dealing with toxic waste, this type of operation is known as a fill and draw operation or as a sequencing batch reactor. In biomedical engineering, the breathing process in and out of the lung is known as stick and balloon, as the volume of the lung increases as we inhale and decreases as we exhale, which is a form of fed-batch process.

Fed-batch cultures are frequently used in laboratories, pilot plants, and industrial plants for production of various products such as baker's yeast, antibiotics, amino acids, fine organic acids, enzymes, solvents, recombinant DNA products, etc.

1.4.1 Advantages of Fed-Batch Cultures

The fed-batch culture has been practiced since the early 1900s, when it was recognized, during yeast production from malt wort, that the malt concentration in the medium had to be kept low enough to suppress alcohol formation and maximize the yield (White, 1954) of yeast cells. Indeed, high malt concentration would accelerate the cell growth, which in turn would cause anaerobic conditions that favored ethanol formation and lowered the yield of yeast cells. So, additional wort was added at a rate that was always less than the maximum rate at which the yeast cells could use it. Thereafter, Intermittent or incremental feeding of nutrients to an initially dilute medium was introduced in large-scale yeast production to improve the yeast yields while obviating the production of ethanol (Reed and Pepler, 1973).

Through the manipulation of one or more feed rates, the fed-batch operation can provide unique means of regulating the concentration of compounds that control the key reaction rates and, therefore, can provide a definite advantage over the batch or continuous operation. If it is advantageous to control independently the concentration of more than one nutritional component, more than one feed stream may be used.

In developing a fed-batch, the most important questions to ask are what compounds should be fed and how they should be added. The answers depend on the characteristics of the organism used. The primary candidate in the list of compounds that may be fed during the course of the fermentation run includes the limiting substrate (the substrate whose concentration limits the growth rate), carbon, nitrogen, sulfur, phosphorus sources, inducers, precursors, and other nutrients. The feeding patterns are open loop or feedback controlled to maintain some key variables at constant optimum values such as the specific growth rate, respiratory quotient, pH, partial pressure of carbon dioxide, dissolved oxygen, substrate concentration, and some metabolite concentrations.

Regarding *S. cerevisiae*, the yeast investigated in this work for heterologous proteins production, it must be taken into account that it is a "glucose sensitive" yeast (Lievens and Lim, 1982) and its metabolism is strongly influenced by glucose concentration in the medium, that is the main carbon and energy source. This latter is responsible for the carbon source flux throughout the metabolic pathways and, consequently, of specific metabolic rates. Further, depending on glucose concentration in the medium, yeast displays two different metabolisms: fermentative metabolism characterized by low values of biomass yield and respiratory metabolism that allows high biomass yields to be achieved. The shift from fermentative to respiratory metabolism is dictated by a threshold value of 0.1-0.2 mg l⁻¹ glucose concentration (Enfors, 2001). Above 0.2 g l⁻¹ glucose concentration, yeast metabolism is fully fermentative.

When *S. cerevisiae* is grown limited on glucose with ammonia as nitrogen source, approximately 50% glucose is used to produce energy, and the rest for anabolism. In this case the metabolism is respiratory and the carbon source devoted to produce energy is completely oxidized to carbon dioxide and water. Differently, under conditions of high glycolytic flux, part of glucose is diverted from respiratory pathway (due to limited respiratory capacity and/or catabolite repression of respiratory enzymes) to convert excess pyruvate into by-products (ethanol and some acetaldehyde and acetate) in the so called over-flow metabolism (Postma *et al.*, 1998).

Production of over-flow by-products means lowering of biomass yield (0.1 – 0.2 vs 0.5 g cells g⁻¹ glucose) in the *S. cerevisiae* cultures.

It is evident that the over-flow metabolism must be avoided by controlling the specific glucose uptake rates.

Stirred batch reactor configuration does not allow the control of specific growth rate and so this solution is unwrapped. On the contrary, continuous stirred tank reactor (chemostat) allows the glucose specific uptake to be regulated by suitable feeding but, as appear evident, at low specific growth rates, this system is not profitable. Fed-batch cultivation which has been proved to be an advanced technique (Pham, 1999), remains the unique cultivation system which permits both glucose limited growth and high cell density for yeast cultivation.

I.5 Fed-batch reactor: the ideal system to achieve HCDC

Fed-batch represents a suitable strategy to achieve high cell density cultures due to (1) extension of working time (particularly important in the production of growth-associated products), (2) controlled conditions for the provision of substrates during fermentation and (3) control of by-products and catabolite repression effects, due to limited provision of those substrates which are solely required for product formation.

Feeding strategy is the most important aspect for the outcome of a fed-batch process. Different feeding strategies, including constant-rate feeding, stepwise increase of the feeding rate, and exponential feeding have been used to obtain fed-batch cultures with high cell densities (Lee, 1996; Shiloach and Fass, 2005).

The design of an optimized fed-batch protocol depends, to a great extent, on the physiological parameters of the strain chosen. In this concern, the major points to consider, regard if:

- 1) the producer strain is Crabtree positive or Crabtree negative on the limiting substrate
- 2) the expression of the product is constitutive or regulated and, if regulated, in what way.

As said in the par. I.4.1, the metabolism of *S. cerevisiae* is affected by glucose concentration in the medium, this means that if it is a Crabtree positive strain, therefore to obtain high biomass yield, it is necessary to limit carbon metabolism by limiting the feed rate. This is possible if the inlet substrate feed rate does not exceed the critical substrate consumption rate according to:

$$\frac{F(t)}{V(t)} S_R \leq \frac{\mu_{\text{crit.}}}{Y_{X/S}} X(t) \quad \text{I.5 - 1}$$

where F is the flow rate (ml h^{-1}), V is the broth culture volume (ml), S_R is the carbon source concentration (mg ml^{-1}) in the inlet, μ_{crit} (h^{-1}) is the critical μ value above which fermentative metabolism takes place, X is the cell density (mg ml^{-1}), and $Y_{X/S}$ is the biomass yield on glucose. An important parameter is μ_{crit} that is the value of specific growth rate below which the metabolism is fully respiratory. In the literature, some data (Postma *et al.*, 1998 ; Enfors, 2001) show that this value is about $0.6 \cdot \mu_{\text{max}}$ where μ_{max} is the maximum μ value according Monod equation (Monod, 1949).

According to the equation I.5-1, the simplest working condition may be a constant flow rate which would ensure high biomass yield but also a long duration of the fermentation process, given the continuous decrease in the specific growth rate, μ . Being the volumetric productivity (the amount of product per volume and time unit), one of the most important parameters in the bioprocess design (once defined the costs for using the fermentation plant), this solution is not practicable or, better, is not feasible for the entire duration of the bioprocess.

The typical solution to work at a high productivity level, is an exponential feeding, to allow cells to grow at constant μ value. This strategy also presents the advantage that acetate production, a serious problem associated with the process, can be minimized by setting the specific growth rate at a value below the critical one in correspondence of which acetate begins to be produced. Exponential feeding is a simple but efficient method that has been successfully used for high cell density cultivation of several recombinant and non-recombinant microorganisms.

Maintaining of the specific growth rate close to an appropriate value, could provide a desirable metabolic condition and result in maximum productivity. Therefore, exponential feeding can be used as a convenient method to both avoid by-product formation and obtain maximum attainable cell density but, the details of such feeding are still a matter of debate and new researches aim at optimizing the feeding method.

The exponential feeding strategy is not adoptable for long times due to the occurrence of the same engineering limitation in the fermenter or physiological changes of the growing cells. The typical feeding strategy adopted to bypass this obstacle is that to follow a constant supply to the exponential. This strategy permits to combine high productivity with low

ethanol formation in the production of baker's yeast, and it is a strategy used also for *E. coli* processes to avoid excessive acetate formation at the beginning (Enfors, 2011).

1.5.1 Design of feeding profile to allow yeast growth at a constant μ

To achieve a constant specific growth rate (μ) during the production phase in the fermenter, the exponential profile of flow rate $F(t)$, was obtained from the mass balance on limiting substrate, S .

$$\frac{dS}{dt} = \frac{F}{V}(S_R - S) - q_S X \quad \text{I. 5.1 - 1}$$

To avoid overflow metabolism, the optimal strategy for the fed-batch fermentation was to feed the growth-limiting substrate (glucose) at the same rate ($q_G X$) the growing biomass utilizes ($q_G X$), to grow at a constant μ value below the critical one.

If growth-limiting substrate is fed at the same rate with which it is utilised by the microorganism, residual glucose concentration in the medium, S , would be null, and $dS/dt = 0$. In these conditions, the rearrangement of the mass balance for $F(t)$, gave the following equation:

$$F(t) = \frac{q_S (XV)_t}{S_R} \quad \text{I. 5.1 - 2}$$

Since the biomass quantity, $(X \cdot V)_t$ had to increase exponentially with time to give constant specific growth rate (μ), according to

$$(XV)_t = (XV)_0 e^{\mu t} \quad \text{I. 5.1 - 3}$$

also the feed rate had to increase exponentially with time. Combining the eqs (I.5.1-2) and (I.5.1-3), the time dependent feed flow could be written as:

$$F(t) = \frac{q_G (XV)_0 e^{\mu t}}{S_R} \quad \text{I. 5.1 - 4}$$

Because $q_G = \mu/Y_{X/G}$, equation (I.5.1-4) became

$$F(t) = \frac{\mu (XV)_0 e^{\mu t}}{Y_{X/G} S_R} \quad \text{I. 5.1 - 5}$$

and F_0 , the initial feed rate, was given by

$$F_0 = \frac{\mu (XV)_0}{Y_{X/G} S_R} \quad \text{I. 5.1 - 6}$$

I.6 Oxygen transport from the air to the yeast cell

In the liquid medium, oxygen is transferred from a rising gas bubble. The study of oxygen transfer from air bubbles, through the liquid medium, to microbial cells is of great importance for the design of the bioprocess.

The cultural system as a whole must guarantee that the oxygen transfer rate in the liquid phase OTR ($\text{Kg m}^{-3} \text{s}^{-1}$) is equal to the oxygen uptake by the growing biomass R_{VO_2} ($\text{Kg m}^{-3} \text{s}^{-1}$). Therefore the operative condition to achieve a steady state, regarding to oxygen concentration, is given by the following equation:

$$R_{\text{VO}_2} = \text{OTR} \quad \text{I.6 - 1}$$

The oxygen uptake depends on both the growing microorganism and its growth rate value while OTR is directly proportional to both the driving force (ΔC) and the area available for transfer (Charles and Wilson, 1994):

$$\text{OTR} = K_L a \cdot (C_s - C_l) \quad \text{I.6 - 2}$$

where:

K_L = liquid-phase mass-transfer volumetric coefficient (m h^{-1})

a = surface area available for mass transfer per culture volume unit (m^{-1})

ΔC = concentration gradient driving force (Kg l^{-1})

For the design of bioreactors with predictable oxygen transfer performances and for the evaluation of existing oxygen transfer equipment, a knowledge of the product $K_L a$ is essential.

The product $K_L a$ is strongly influenced, by all other parameters such as temperature, cell density, pressure of inlet gas, characteristic design of the bioreactor and agitation.

Control of dissolved oxygen levels is done by varying the agitator speed. This is the traditional approach used to enhance the oxygen transfer rate since more agitation produces more gas dispersion, and more gas dispersion produces more mass transfer.

In theory, molecular O_2 can be seen as a common substrate, but hardly soluble in the liquid phase. It can be toxic to all aerobic organism (Li *et al.*, 2009).

I.7 Growth medium

The composition of the cell growth medium must be carefully formulated and monitored, because it may have significant metabolic effects on both the cells and protein production. There are three types of media: defined, complex and semi-defined. Defined media are generally used to obtain high-cell density, as the nutrient concentrations are known and can be controlled during cultivation. Nutrients in complex media, such as peptone and yeast extract, can vary in composition and quality, which makes fermentation with

these complex media less reproducible. However, semi-defined complex media are sometimes necessary to boost product formation. To grow cells to a high density, it is necessary to design a balanced nutrient medium that contains all the necessary components for supporting cell growth, while avoiding inhibition. It is desirable to make the feed-solution as simple as possible by including sufficient non-carbon and non-nitrogen nutrients in the starting medium. In the case of auxotrophic yeast strains the proper aminoacidic concentration is of basic importance as shown by Paciello et al. (2009). The development of semi-defined and complex media require empirical and trial-and-error-based processes. The optimization of any fermentation medium is a labor-intensive process, owing to the large number of nutrient combinations to be tested.

I.8 Fed-batch cultivation mode gives rise to a stressful environment

During fed-batch runs, the operative conditions imposed to promote microbial growth and high biomass yield can lead to stressful environmental conditions.

It is common understanding today that stress situations, and stress reactions, of the host cells can largely influence the yield and productivity of the cultural system. The possible environmental stressors that can occur are: temperature, pH, aeration and agitation and osmotic stress. The first two stress factors can be ignored because the fermenter as a cultural system, ensures no stressful values of pH and temperature.

As regards the stress due to aeration and agitation, it has to be considered that fed-batch operations are generally carried out under conditions of vigorous and continuous agitation and aeration to suitably supply the growing biomass with oxygen, so that shear and oxidative stress are almost unavoidable. About the oxidative stress, during respiration, O_2 is progressively reduced to two molecules of water because oxygen works as the final acceptor of electrons coming from the electron transport chain (Jamieson, 1998). During this electron transport inside the chain, chemical entities could be formed that are still potent oxidants, such as $O_2^{\cdot-}$ (the superoxide anion), H_2O_2 (hydrogen peroxide), and HO^{\cdot} (the hydroxyl radical). These species are called with the acronym ROS (Reactive Oxygen Species) and may cause cellular damage, and DNA damage, can elicit either cell survival or apoptosis mechanisms (Finkel and Holbrook, 2000). The final result is a decrease in the performance of the producer microorganism. In order to optimize the bioprocess to obtain high values of biomass and product of interest, it would be interesting to study the interactions between

process parameters and the occurrence of oxidative stress conditions. For example, it would be interesting to understand how the formation of ROS in a complex system, such as the aerated fed-batch reactor, is influenced by the value of dissolved oxygen concentration (outside the cell of microorganism). Other important process parameter which can affect ROS production is the consumption rate of oxygen that is linked to the growth rate imposed.

Hyperosmotic stress is regarded as a typical problem connected with high cell density fermentations, as the media formulation requires high salt concentration in such systems. Batch media may have an osmolality around 1000 mOsmol/l, which is comparable to a 0.5M NaCl solution (Mattanovich *et al.*, 2003), as frequently utilized to induce osmotic stress in yeasts. Due to the uptake of nutrients during growth, the osmolality will decrease, and the final osmolality at the end of a fed batch process will strongly depend on how well balanced the media were with respect to the cell demand.

I.9 *Saccharomyces cerevisiae*: the central carbon metabolism

All the yeasts taken into consideration during the work for the PhD thesis are *S. cerevisiae* strains. The knowledge of their metabolism is a prerequisite to successfully develop a fed-batch reactor.

The term central carbon metabolism refers to the set of anabolic and catabolic reactions needed by the cell for the biosynthesis of precursor and to generate energy. A schematic representation of *S. cerevisiae* central carbon metabolism can be found in Fig. I.9-1. In *S. cerevisiae*, the central carbon metabolism encompasses both catabolic and anabolic pathways. Among the catabolic ones we have a) glycolysis, essential for the breakdown of sugar, generating ATP, and metabolic precursors, b) pentose-phosphate pathway (PPP) used by the cell to generate NADPH, the reducing power, used in the anabolic pathways) tricarboxylic acid cycle (TCA) or Krebs cycle, which is an amphybolic pathway and needed to produce pyruvate, generating CO₂, FADH₂ and NADH and needed to produce pyruvate for the gluconeogenesis. Linked to the Krebs cycle there is d) the glyoxylate cycle which has an anaplerotic role in sustaining the replenishment of the cycle with the intermediate oxalacetate which is required for both the correct operation of the TCA cycle and growth on C₂ and C₃ compounds. The reduced electron carriers (coenzymes) generated through the TCA cycle, NADH and FADH₂, are oxidized via the e) electron transport chain, located on the inner mitochondrial membrane. The electron transport chain pumps protons out of the inner mitochondrial membrane, creating a proton gradient used by the ATPase to synthesize ATP through a process called oxidative phosphorylation. f) Gluconeogenesis is one of the main anabolic, pathway

used by the cell to generate hexose phosphates during growth on C2 and C3 substrate.

All the pathways in the central carbon metabolism contribute to supply biomass with precursors in order to produce new biomass, *de novo* synthesized. In this regard, the different pathways are finely tuned to meet the exact need for building blocks and Gibbs free energy. This is because all the pathways interact among each other as they share cofactors (such as ATP, ADP and redox equivalents, substrates/products (metabolites) and compartments, leading to a tight regulation of the cell metabolism. Despite this regulation, *S. cerevisiae* shows “metabolic un-coupling”: the lack of coupling between anabolic requirements and catabolic energy production, meaning that catabolic activity is higher than what required for growth (Larsson *et al.*, 1995); this phenomenon has been observed both during glucose-excess and under steady state conditions and brings in an additional level of complication when describing cell phenotypes.

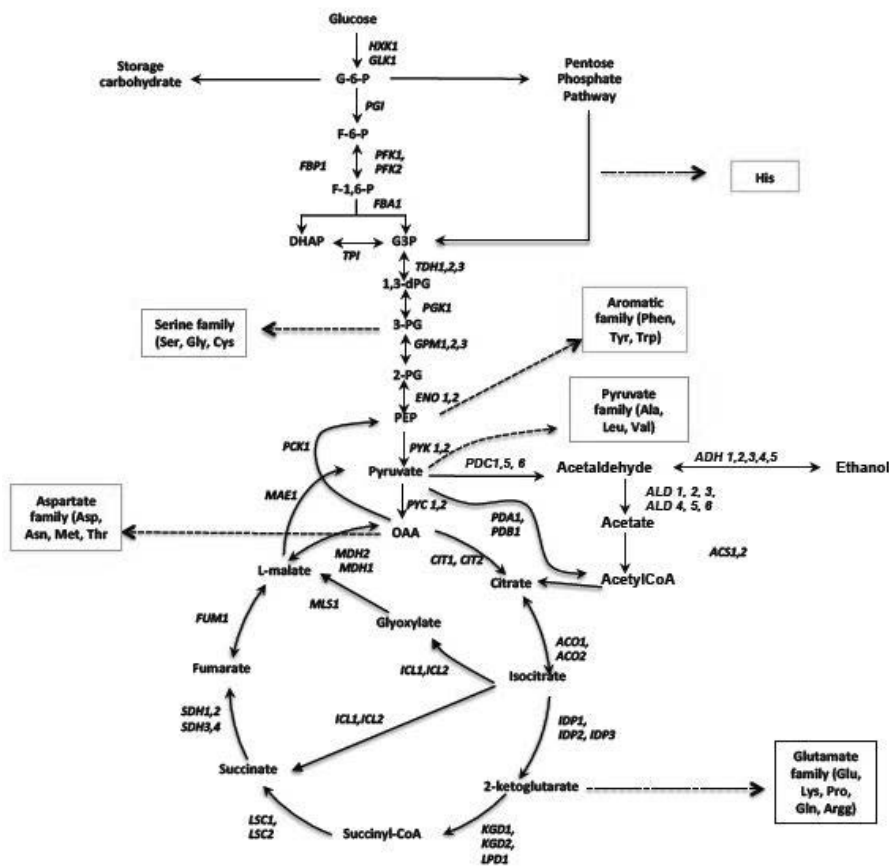


Fig. I.9-1. Schematic representation of *S. cerevisiae* central carbon metabolism

What is extremely intriguing and most fascinating about the yeast metabolism, is the fact that different pathways are activated or down-regulated by the cell to satisfy its needs according to different environmental conditions, reflecting different metabolic states. At this point the question arises about how, when and why a certain pathway is active, introducing the concept of metabolic regulation. It is worth to mention that we are still far from having a global understanding of the interconnection of regulatory mechanisms by which the cell machinery finely tunes its metabolism however, combining approaches giving detailed snapshots together with studies aiming at providing a global view of the cellular processes can contribute to gain a deeper insight into these mechanisms and lead to a more complete understanding.

1.9.1 Fermentation, respiration and regulatory phenomena

S. cerevisiae is a Crabtree positive yeast, able to perform alcoholic fermentation of glucose under fully aerobic conditions. When growing aerobically, *S. cerevisiae* can both respire and ferment. Glucose can be fully oxidized (at least the aliquot of substrate for ATP production), under respiratory conditions, to biomass, CO₂ and water or, under respiro-fermentative conditions, primarily to CO₂ and ethanol. As the growth of *S. cerevisiae* can be supported by mitochondrial oxidative phosphorylation or by substrate-level phosphorylation leading to ethanol fermentation, the activation of pathways contributing to respiro-fermentative or respiratory metabolism will depend on environmental factors such as the carbon source (and the amount) available (e.g. repressing/non-repressing and fermentable/non-fermentable sugars) and/or on the presence or absence of oxygen. On the contrary, anaerobic growth is supported exclusively by fermentative processes through substrate-level phosphorylation; under this condition, since no oxygen is present, the activation of specific “hypoxic” genes is required. As mentioned above, the metabolic state of *S. cerevisiae* is dependent on the sugar present in the cultivation medium (raffinose and ethanol are, for instance, non-fermentable carbon sources) and, for glucose and other fermentable carbon source (such as fructose, maltose, mannose and galactose), the fermentation rate is dependent on the amount of glucose present. The consumption of different carbon sources is differently regulated and leads to different metabolic conditions (e.g. raffinose and ethanol can be only respired, whereas glucose is both respired and fermented); additionally, some fermentable sugars, such as mannose and galactose, show higher respiration rates, compared to glucose, probably as results of inhibition of fermentation (Kappeli 1986).

It is well established that during aerobic growth on medium containing glucose having concentrations above a certain threshold, 0.5-0.8 mM (Verduyn *et al.*, 1992), *S. cerevisiae* produces ethanol as a result of the Crabtree effect. This phenomenon is accompanied by an increase in the production of CO₂ and the secretion, in smaller quantities, of other metabolites such as acetate, pyruvic acid and glycerol, needed to maintain the redox balance. Dissimilation of glucose to pyruvate through glycolysis is stoichiometrically linked to NADH formation, which is then re-oxidized through ethanol fermentation. Alcoholic fermentation of glucose is used by yeast as a way to re-oxidize excess of cytosolic NADH and it has recently been shown that when *S. cerevisiae* is supplied with an alternative heterologous oxidase, reduced aerobic ethanol fermentation is observed (Vemuri *et al.*, 2007), thus suggesting that, in *S. cerevisiae*, the onset of fermentation is a consequence of the limited respiratory capacity.

Under anaerobic conditions, the only source of ATP generation is substrate-level phosphorylation. Anaerobically, *S. cerevisiae* shows fully fermentative metabolism and ethanol is the main product found, however formation of glycerol, carbon dioxide and smaller amount of others by-products (acetate, pyruvic acid) are observed. The energy gain from alcoholic fermentation is lower than that obtained by respiratory dissimilation of glucose and the cell respond to a lower ATP yield under fermentative conditions by increasing glycolytic fluxes.

During fully respiratory growth on glucose, the pyruvate generated through the glycolytic pathway is oxidized to acetyl-CoA and further through the tricarboxylic acid cycle (TCA) to produce CO₂ and H₂O. Electrons generated in the oxidation process are captured by FADH₂ and NADH which are reoxidized through mitochondrial respiration, leading to the generation of additional ATP. Several studies demonstrated that the respiratory capacity of *S. cerevisiae* is lower than other microorganisms and the theoretical P/O ratio (number of ADP molecules phosphorylated per pair of electron transferred to oxygen) is approximately 2 (Famili *et al.*, 2003), but the operational P/O ratio is lower and probably in the range of 1-1.5 (Verduyn *et al.*, 1991). The fully respiratory dissimilation of glucose yields more than 10 ATP per glucose, which can support a biomass yield of 0.5 g biomass per g glucose. The aerobic ATP yield is higher than what can be theoretically obtained through ethanol fermentation (Bakker *et al.*, 2001) and the fermentation of glucose to ethanol and CO₂ brings a biomass yield of 0.1 g biomass per g glucose. An interesting parameter used to determine the metabolic state of *S. cerevisiae* is the respiratory quotient (RQ) defined as the moles of CO₂ produced per mole of O₂ consumed. Under fully respiratory conditions, the RQ is around 1, whereas when shifting towards respiro-fermentative metabolism, the specific oxygen uptake rate becomes smaller and the RQ assumes values greater than 1. To study the metabolism of *S. cerevisiae* under respiratory or fermentative conditions, different tools

to keep the cell in the respiro-fermentative or in the respiratory state have been developed. During batch cultivations in the first phase of growth, respiration is repressed and the glucose consumed is fermented to ethanol and smaller amounts of by-products such as glycerol, acetate and pyruvate. When glucose is depleted, *S. cerevisiae* is able to efficiently adapt its metabolism and consume the ethanol produced in the first phase to gain energy, a phenomenon called diauxic growth. It was shown (Ephrussi *et al.*, 1956) that the slow respiration rate of glucose observed during batch cultures is due to inhibition of the activity and/or the repression of the synthesis of the respiratory enzymes *cytochrome a*, *b* and *c* complex while the higher respiratory rates of sugars such as galactose and mannose are consequences of the de-repression of cytochrome complex synthesis (Fiechter, 1975). During chemostat cultivations at low dilution rates, when the glucose concentration is low (<100 mg l⁻¹), *S. cerevisiae* shows fully respiratory metabolism. It was suggested that when glucose is fed to the culture at low rates, its breakdown occurs oxidatively (Kappeli, 1986) and, under these conditions, the cells have enough time to adapt to respiratory conditions (Fiechter, 1975) (Barford and Hall, 1979) which results in a purely respiratory metabolism, yielding high amount of biomass with no production of other metabolites. When the dilution rate is increased above the critical one, fermentation is used in addition to respiration as *S. cerevisiae* shift its metabolism to respiro-fermentative mode until reaching the wash-out.

Several regulatory phenomena are present in yeast species to respond to environmental changes such as the presence of oxygen or glucose. The mechanisms regulating the response to a certain condition took the name from the scientist who discovered and characterized them. Differences in facultative fermentative yeasts with respect to the onset of ethanol fermentation due to the presence of oxygen, are ascribed to the phenomena known as *Custer Effect* and *Pasteur Effect*. The Pasteur effect can be described as the decrease of fermentation efficiency in presence of air (Wyman, 2000), while the Custer effect (not observable in *S. cerevisiae*) indicates inhibition of fermentation by anaerobiosis.

In the field of yeast physiology, there has always been a constant interest in understanding what determines the onset of fermentative metabolism and numerous efforts have been focused on characterizing in details the metabolic states of *S. cerevisiae*. The metabolic shift from fermentative to oxidative growth has been investigated through different methods, from traditional physiological studies to microarrays-based works, as well as through metabolome and flux distribution analysis. Transcript levels during fermentative and respiratory conditions have been characterized based on expression arrays (De Risi *et al.*, 1997), showing differential expression of a wide number of gene families (over 400 transcripts changed by more than 2-fold). The metabolic shift observed when *S. cerevisiae* cells are transferred from a fermentable to a non-fermentable carbon source has also been

addressed through microarray experiments (Kuhn *et al.*, 2001), showing a decrease in translation efficiency. Transcriptome studies demonstrated to be a useful tool to compare expression patterns between different conditions however, applying microarray studies to look at gene expression provide limited biological in-sights of regulatory phenomena. As regards this, analysis of intracellular fluxes has proven to be a valid tool in characterizing cellular phenotypes as they reflect the final outcome of the regulation of the cell. Analysis of intracellular metabolic fluxes has been applied to characterize the shift towards fermentative conditions, providing significant information about how the fluxes are distributed in the cell as a result of regulatory phenomena. In a work by Frick and Wittman, the authors describe the re-arrangement in metabolic fluxes as a response to different dilution rates, through ¹³C glucose labeling (Frick and Wittmann, 2005). *S. cerevisiae* was cultivated in chemostat and the dilution rate increased until the critical one, when yeast switches its metabolism from fully respiratory towards the respiro-fermentative. The shift towards respiro-fermentative conditions is characterized by a 5-fold increase in the glucose uptake rate, together with a decrease in the pentose phosphate pathway (PPP) flux and decrease in the fluxes through the tricarboxylic acid cycle (TCA). As the PPP is required for NADPH generation, the finding of a reduced flux through the PPP well correlates with the reduced biomass yield, suggesting that, under respiratory conditions, the PPP pathway is enough to sustain anabolic NADPH demand. An increased flux through the pyruvate carboxylase (PYC) was reported, indicating an increased formation of oxaloacetate (OAA) supply. Interestingly, the flux through pyruvate decarboxylase (PDC) was found to assume significant values also during fully oxidative conditions, probably indicating the fundamental role of this reaction in providing acetyl-CoA supply; this finding is in agreement with the increased acetyl-CoA transport in the mitochondria observed. However, the flux through the acetyl-CoA synthase (ACS) remains constant even at high dilution rates when acetate secretion increased.

The capability of shifting from fermentation to respiration under aerobic conditions, is one of the most fascinating and studied phenomenon of *S. cerevisiae* metabolism, however a complete understanding of the regulation systems and mechanisms underlying the rearrangement of flux distribution and gene expression analysis is still under discussion.

Chapter II

HEP production with Auxotrophic yeast

II.1 Heterologous Protein Production

High level production of proteins from engineered organisms provides an alternative to protein extraction from natural sources. Natural sources of proteins are often limited, and furthermore the concentration of the desired product is generally low, making its extraction very cost-intensive or even impossible. Besides, extraction might bear the danger of toxic or infectious contamination depending on the natural origin of the protein. With the advent of molecular cloning in the late 70's using the bacterium *Escherichia coli* as a genetically and metabolically well-known host, it became possible to produce foreign proteins in new hosts. Recombinant DNA (rDNA) technologies (genetic, protein, and metabolic engineering) allow the production of a wide range of peptides and proteins from naturally non producing cells. The first recombinant product on the world market made by means of rDNA was insulin in 1982 (Humulin by Eli Lilly). Since then, more biopharmaceutical proteins such as interferons, erythropoietin, vaccines, and more recently monoclonal antibodies as well as industrial enzymes (for example, used for the treatment of food, feed, detergents, paper-pulp, and health care) followed. Today, recombinant protein production is a multibillion dollar market. The global sales for biopharmaceutical proteins was 87 billion USD in 2008 and is expected to rise further, while the market for industrial enzymes is continuously increasing and in 2009 reached 5.1 billion USD. Indeed, the growing demand in the animal feed and biofuels market, in addition to pharmaceutical enzymes, requires high production of industrial enzymes such as proteases, lipases, and carbohydrases. In 2009, out of the 151 approved recombinant biopharmaceutical products, 29 monoclonal

antibodies contributed to more than 40% of the revenues, followed by vaccines, TNF blockers, hormones such as insulin and erythropoietin. Around 20% of biopharmaceutical proteins are produced in yeast hosts, 30% in *E. coli*, and 50% in higher eukaryotic cells, mainly mammalian cells and hybridomas (Fig. II.1-1).

Currently, nearly all yeast-derived products on the market are produced in *Saccharomyces cerevisiae*; in 2009 the first biopharmaceutical protein produced in a non-*Saccharomyces* yeast, approved by the FDA (Food and Drug Administration), was Kallikrein inhibitor (Kalbitor by Dynax Inc.) produced in *Pichia pastoris*.

Regarding the production of industrial enzymes, the share is almost total of microbial hosts especially *E. coli* (mostly recombinant), *Bacillus subtilis* (natural producer), yeasts (mostly recombinant secretion), and filamentous fungi (often native producers). Especially for the recombinant production of fungal enzymes, secretory expression in yeasts is often the best choice. Despite the fact that a large number of protein drugs are produced in higher eukaryotic cells, there is still a strong interest of biopharmaceutical industry in developing improved microbial production platforms (mainly *E. coli* and yeast systems).

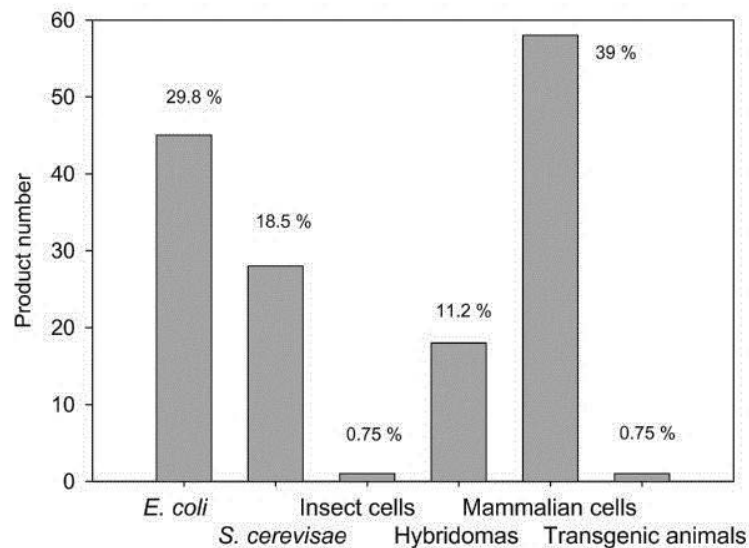


Fig. II.1-1 Hosts for heterologous proteins production (Ferrer-Miralles et al., 2009)

This indicates that microbial cells represent convenient and powerful tools for recombinant protein production. In this respect, the dominance of *E. coli* as host for the production of heterologous proteins (HEPs) is clearly a reflection of the quantity and quality of the information available about its genetics, molecular biology, biochemical, physiological, and fermentation

technologies. Indeed, recombinant protein fraction can reach up to 50% of total biomass and protocols for high cell density cultivation (HCDC) are established, reaching up to 100 g biomass per liter. But, it has to be noted that bacterial hosts lack the ability of correct post-translational protein processing and, in a lot of cases, heterologous proteins aggregate as inclusion bodies inside the bacterial cells, often causing the proteins to lose their enzymatic activity/3D structure. Due to their incorrect structure any use of such proteins in humans is also excluded, unless they can be correctly refolded in vitro. However, the dogma that *E. coli* cannot secrete or glycosylate recombinant proteins became obsolete due to extensive genetic engineering efforts: both extracellular protein production as well as glycosylation has been reported also for prokaryotic hosts. However, the yields achieved (up to maximum 100 mg/L secreted protein), or the authenticity of the glycans are far from the eukaryotic systems.

II.2 Why to use the yeast *Saccharomyces cerevisiae* as host to produce HEPs

Among the microbial eukaryotic hosts systems, yeasts combine the advantages of unicellular organisms (i.e., ease of genetic manipulation and growth) with the capability of a protein processing typical for eukaryotic organisms (i.e., protein folding, assembly, and post-translational modifications), together with the absence of endotoxins as well as oncogenic or viral DNA. Moreover the use of yeast permits the possibility to secrete the heterologous protein directly in the culture media with remarkable advantages in the down-stream process.

Starting from the early 1980s, the majority of recombinant proteins produced in yeasts have been expressed using *Saccharomyces cerevisiae*. Indeed, due to the long application of *S. cerevisiae* there are some advantages associated with the use of this microorganism in a new process: much knowledge of the organism has accumulated, and the approval process is considered to be easier/cheaper than when applying a new unknown organism. Furthermore, *S. cerevisiae* is capable of growth in a cheap medium and it can perform some posttranslational modifications. Moreover this organisms have been extensively used in the pharmaceutical industry for the production of HEP and most companies have developed large-scale fermentation capacity since *S. cerevisiae* has obtained a GRAS (generally regarded as-safe) status by FDA. This is a major advantage for applications in the food or pharmaceutical industry since less extensive trials are required by the regulatory authorities (Hensing *et al.*, 1995).

II.3 Role of auxotrophic yeast in HEPs production

The expression of a protein in yeast consists of, first, cloning of a foreign protein coding DNA sequence within an expression cassette containing a yeast promoter and transcriptional termination sequences; secondly, transformation and stable maintenance of this DNA fusion in the host. Efficiency of transformation is clearly strain dependent and detailed studies should be carried out when a high efficiency of transformation is required. During process transformation is required selectable marker genes (Tab. II.3-1) for efficient detection and selection of transformed cells (Botstein and Davis, 1982). Several marker genes used in yeast genetics confer resistance against antibiotics or other toxic compounds (Van den Berg and Steensma, 1997). Selection for strains that carry such marker genes requires the addition of these toxic compounds to the growth media. In addition to their toxicity, the price of many of these compounds precludes their use in large-scale processes. Moreover, even in resistant strains, the presence of antibiotics may affect cellular function. An alternative is the use of marker genes that complement specific nutritional requirements. Some of the most commonly applied marker genes are wild type alleles of yeast genes that encode key enzymes in the metabolic pathways towards essential monomers used in biosynthesis. An example is the *URA3* gene, which encodes an essential enzyme in pyrimidine biosynthesis in *S. cerevisiae*. Similarly, the *HIS3*, *LEU2*, *TRP1*, and *MET15* marker genes encode essential enzymes for de novo synthesis of the amino acids L-histidine, L-leucine, L-tryptophan, and L-methionine, respectively (Botstein and Davis, 1982; Cost and Boeke, 1996).

Use of these genes as markers is restricted to host strains that are auxotrophic for the nutrient in question due to the absence of a functional chromosomal copy of the marker gene. Unless transformed with the marker gene, auxotrophic yeast strains can be propagated only in media that contain the appropriate growth factor(s). The ease with which auxotrophic yeast strains and the corresponding auxotrophy-complementing genes can be manipulated and the low cost of the chemicals involved have contributed to the development of numerous auxotrophic laboratory strains of yeast to be transformed for the production of heterologous proteins (Pronk, 2002).

Table II.3-1 Examples of selection markers

<i>Selection method</i>	<i>Selection principle</i>	<i>Examples</i>	<i>Advantages /disadvantages</i>
Antibiotic resistance	Cells containing plasmid which confers resistance to an antibiotic are able to grow in medium containing antibiotic whereas cells which does not contain the plasmid cannot grow	G418 (geneticin), methotrexate and chloramphenicol	Not suited for large scale production due to environmental concerns
Auxotrophic selection	Genes necessary for formation of a component essential for growth is deleted and the plasmid contains genes which make it possible to produce the missing component	Deletion of <i>LEU2</i> , <i>TRP1</i> , <i>URA3</i> or <i>HIS3</i> .	Defined growth medium is required. Risk of cross-feeding
Autoselection	A gene encoding an essential enzyme activity is deleted and the activity is restored by a gene encoded on the plasmid	Deletion of <i>TPH1</i> and restoring the activity of the enzyme by including the gene encoding the enzyme from <i>Schizosaccharomyces pombe</i>	Does not require defined growth medium. No risk of cross-feeding

II.4 HEP expression in *Saccharomyces cerevisiae* CEN.PK 113-5D

In this thesis, the auxotrophic *S. cerevisiae* CEN.PK 113-5D strain was used as host to express the Pir4-IL1 β fusion protein by using the signal peptide and pro domain of the Pir4 cell wall protein (Moukadiri *et al*, 1999) to drive its secretion to the culture. The original strategy of expression (Andrès *et al*, 2005) has been used to obtain in addition to Interleukin-1 β employed as model protein, two enzymes, a lipase from *Bacillus subtilis* and an endoglucanase from *Poenibacillus barcinonensis*. The details of these latter are reported in the following section. The expression strategy above mentioned gives the possibility or less to release the protein from the cell wall, with the protein naturally immobilized on the yeast cell wall or

secreted in the broth-culture with a simplification of the down-stream process. The possibility of producing a protein linked to the cell-wall could be fascinating due to all the consequent advantages of an immobilized enzyme (Bickerstaff, 1996; Messing, 1975).

II.4.1 PIR4-HEP coding sequence: three different fusion strategies for retention or secretion of HEPs into the culture medium

The gene fusion has been performed by inserting the coding sequence of the HEP considered between the naturally occurring restriction sites Bgl II and Sal I of the coding sequence of the Pir4 cell wall protein of *S. cerevisiae* (Moukadiri *et al.*, 1999). The first step was the insertion of the gene sequence in the vector YEPlac 195 containing the complete sequence of *PIR4* i.e. its promoter and terminator in addition to URA as selectable marker (i.e. the gene introduced into the yeast cell to confer the trait suitable for artificial selection). Pir4 signal peptide, is processed at the endoplasmic reticulum, and the pro-peptide (Subunit I) is processed at the Golgi by the Kex2 protease. The mature protein (Subunit II) includes a 19 aminoacid repetitive domain and a very conserved carboxy-terminus that contains four cysteine residues at fixed positions, which, considering the extractability of some PIR-CWPs by reducing agents (Moukadiri *et al.*, 1999; Moukadiri and Zueco, 2001), should be responsible for cell wall retention.

The way in which the genetic information for the heterologous expression is inserted, determines or not the cell wall retention of the HEP.

The structure of the *PIR4*, as outlined above and that of a generic *HEP* gene is shown in Fig. II.4.1-1, together with a schematic representation of the three different fusion strategies used. The first two (L1 and L2) consisted of inserting all the coding sequence of the HEP gene, minus the 5' fragment coding the leader peptide, in the naturally occurring Bgl II or Sal I restriction sites of the coding sequence of *PIR4*. These two constructions were designed so that the Pir4-HEP fusion could be retained in the yeast cell wall so as to be able to use the cells as a source of immobilized enzyme for technological applications.

Enzyme construction L3 (Fig. 2.1) involved the substitution of fragments of *PIR4* by the coding sequence of the *HEP* gene. In this case, the HEP coding sequence was subcloned in the Bgl II-Sal I sites of *PIR4*, with the loss of the carboxyterminal fragment of Subunit II of Pir4 that contains three of the four very conserved cysteine residues that are responsible for cell wall retention. Accordingly, this construction was designed to lead to the secretion of the HEP fusion protein to the growth medium.

The original expression strategy was developed in collaboration with the group of the Prof. Zueco of University of Valencia (Spain) and has been employed for the expression of several HEPs: xylanase, interleukin-1 β , endoglucanase and lipase (Andrés *et al.*, 2005; Mormoneo *et al.*, 2008 ; Paciello *et al.*, 2009; Mormoneo *et al.*, 2012).

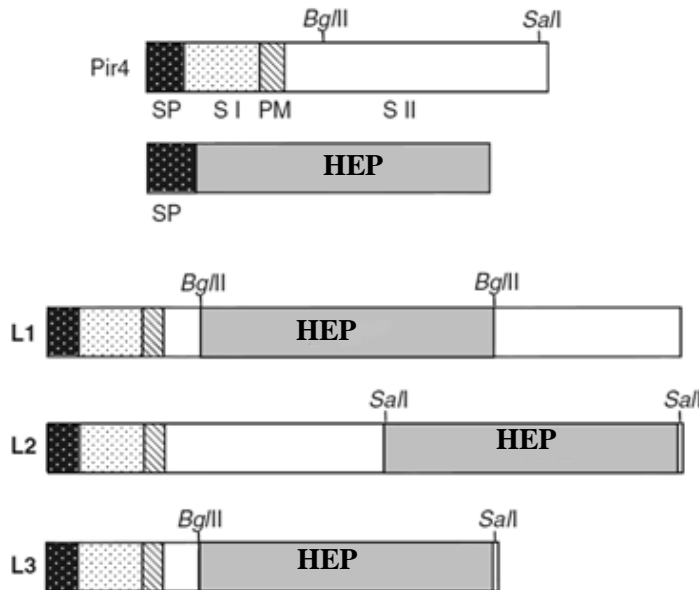


Fig. II.4.1-1 Schematic representation of a generic HEP and PIR4 genes together with the different Pir4-HEP gene fusions. SP, signal peptide; S I, subunit I; PM, Pir motive; S II, subunit II; LP, leader peptide. Mature Pir4 corresponds to subunit II after removal of subunit I by Kex2 protease at the Golgi

II.5 HEPs expressed in this work

HEPs expressed in this thesis work, using *Saccharomyces cerevisiae* as host, are described in the paragraph below. Interleukin-1 β is a protein of human origin that has a role in the immune response, the other two proteins are enzymes of bacterial origin, LipaseA from *B. subtilis* and an Endoglucanase. Interleukin-1 β will be used as a model protein to study interactions host-protein-growth environment. These studies were used to set up and optimize a bioprocess on laboratory scale to produce a Lipase A naturally immobilized on yeast producer cells and an Endoglucanase. Both proteins are of agri-food field interest.

Another section of this work concerns the study of the expression on hemoglobin of human origin in order to evaluate the effect of the expression of these specific proteins on yeast.

II.5.1 Interleukin-1 β as a model HEP

Interleukin-1 β (IL-1 β) was the first protein expressed in this thesis work using the *S. cerevisiae* CEN.PK 113-5D strain as host. This protein has to be considered as a model protein used to set-up the HCDC system for HEPs production and compare with the other protein productions.

Interleukins are a group of cytokines (secreted signaling molecules) that were first seen to be expressed by white blood cells (leukocytes, hence the *-leukin*) as a means of communication (*inter-*). The name is something of a relic though (the term was coined by Dr. Paetkau, University of Victoria-USA); it has since been found that interleukins are produced by a wide variety of bodily cells. The function of the immune system depends in a large part on interleukins, and rare deficiencies of a number of them have been described, all featuring autoimmune diseases or immune deficiency. IL-1 β is one of the first cytokines ever described. Its initial discovery was as a factor that could induce fever, control lymphocytes, increase the number of bone marrow cells and cause degeneration of bone joints.

At this time, IL-1 β was known under several other names including endogenous pyrogen, lymphocyte activating factor, haemopoetin-1 and mononuclear cell factor, amongst others. It was around 1984-1985 when scientists confirmed that IL-1 β was actually composed of two distinct proteins, now called IL-1 α and IL-1 β (Dinarello, 1994).

The original members of the IL-1 superfamily are IL-1 α , IL-1 β , and the IL-1 Receptor antagonist (IL-1RA).

- IL-1 α and - β are pro-inflammatory cytokines involved in immune defense against infection.
- The IL-1RA is a molecule that competes for receptor binding with IL-1 α and IL-1 β , blocking their role in immune activation.

II.5.2 Lipase A naturally immobilized on yeast cell

Lipases (EC 3.1.1.3) are enzymes which constitute a special class of carboxylic esterases. They are one of the most important group of biocatalysts and have a wide industrial application in the oleo-chemistry, detergent formulation, organic synthesis and nutrition. This great interest in

lipases is mainly due to their properties in terms of enantioselectivity, regioselectivity and broad substrate specificity.

The physiologic role of lipases is to hydrolyze triglycerides into diglycerides, monoglycerides, fatty acids, and glycerol. In addition to their hydrolytic activity on triglycerides, they can catalyze other reactions such as esterification, interesterification, acidolysis, alcoholysis, and aminolysis (Fig. II.5.2-1).

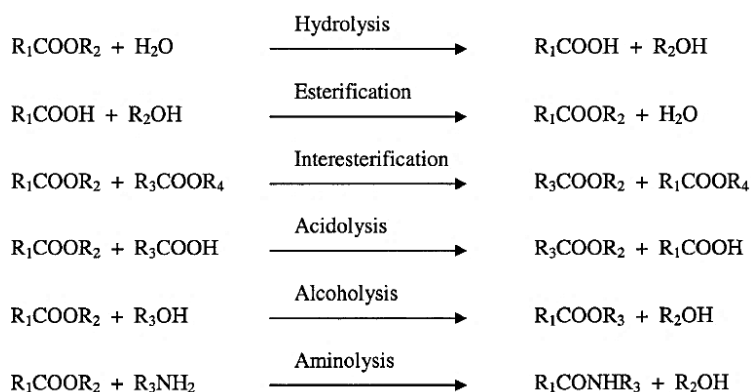


Fig. II.5.2-1 Different reaction catalyzed by lipase (Houde *et al.*, 2004)

Lipases are widely distributed throughout the plant and animal kingdoms, as well as in molds and bacteria. As regard these latter, several lipases of bacterial origin have been isolated and characterized. Lipase A from *Bacillus subtilis* is one of the smallest lipases known (19 KDa) shows good hydrolytic activity in medium-length substrate and is used for biotechnological applications including bread making or preparation of technological additives.

In this work a lipase A from *B. subtilis* has been produced as naturally immobilized on yeast cells, using the genetic construction that permits to retain the HEP on the yeast cell wall of the *S. cerevisiae* strain chosen as host.

Immobilization permits multiple uses of the lipases and often enhances its thermal and chemical stability, thus leading to predictable decay rates. It also enhances opportunities for better control of both the process and product quality. In fact, the incremental costs of using an immobilized biocatalyst in a continuous process are more than 20 times lower than with a traditional one, arising primarily from the cost of the relatively large amount of non-reusable enzyme required by the latter process (Murty *et al.*, 2002). An application of immobilized lipase was developed by Akon and Yee (1997) to reduce the energy content of fatty food by interesterification of tristearin (C18:0) with either tricaprin (C10:0) or tricapyrin (C8:0).

II.5.3 Endoglucanase

Endoglucanase is an enzyme that is part of the enzyme complex of cellulases. Particularly endo- β -1,4-glucanases (E.C. 3.2.1.4) together with exo β -1,4-glucanases (E.C. 3.2.1.91) or cellobiohydrolases, ensure efficient hydrolysis of cellulose. Endo- β 1,4- glucanase catalyzes the endohydrolysis of β -1,4-D glucosidic linkages in cellulose, hydrolyzing cellulose to cellooligosaccharides. Simultaneously, cellobiohydrolases, remain attached to the substrate and progressively release cellobiose from one (reducing or non reducing) of the two chain ends (Bèguin and Aubert 1999 ; Teeri, 1997).

S. cerevisiae, known to produce ethanol from fermentable sugars, has a high tolerance towards this alcohol. This contributes to enhance the yeast robustness which is required for its use in industrial processes. However, *S. cerevisiae* cannot naturally utilize cellulosic material to produce ethanol. As a matter of fact, preliminary treatment of this material is needed to yield fermentable sugars (Lynd *et al.*, 2005). This drawback has led scientists to get strains of *S. cerevisiae*, expressing recombinant cellulases, that possess the ability to enzymatically degrade lignocellulose and ferment the resulting sugars to ethanol in a single step, in a process that has been named consolidated bioprocessing (CBP). Synergistic expression of cellulase enzymes for degradation of cellulosic substrates has been demonstrated by several studies (Dan Haan *et al.*, 2007; Lee *et al.*, 2007; Du Plessis *et al.*, 2010; Wen *et al.*, 2010; Nakatani *et al.*, 2013) with some of the strains created being able to grow on acid-swollen cellulose. Other potential, and more traditional, fields of application of *S. cerevisiae* strains expressing recombinant cellulases are those of wine-making (Galante *et al.*, 1998; Singh *et al.*, 2007) and beer brewing (Bamforth, 1994). This enzyme indeed improve color extraction, skin maceration, must clarification, filtration, so the quality and stability of the final product, moreover in the case of beer brewing endoglucanase acts during malting and fermentation process.

Endoglucanase employed in this thesis work and coded by *CELA* (GenBank Access No. Y12512) from *Paenibacillus barcinonensis* (Sánchez *et al.*, 2005), is an enzyme with good characteristics for application on paper manufacture from agricultural fibers. This enzyme comes from a newly identified *Paenibacillus* species, originally isolated from a rice field, that shows a multiple enzyme β -glycanase system correlated with its high polysaccharide degrading potential (Blanco *et al.*, 1999; Pastor *et al.*, 2001; Sánchez *et al.*, 2003). Endoglucanase was expressed in *S. cerevisiae* (Mormoneo *et al.*, 2012), transformed with the shuttle vector pIA1 (Andrés *et al.* 2005), carrying the translational fusion of endoglucanase to the Pir4 cell wall protein, so as to drive the secretion of the recombinant product to the culture medium.

II.5.4 Human hemoglobins

An aspect of great interest, in the field of recombinant proteins is the expression of human proteins in order to study the effect on the microorganism and find further relation, on what happens in the humans e.g. disease studies. In this way yeast is one of the most attractive hosts because around 60% of the yeast genes show sequence homology to those of humans (Bostein *et al.*, 1997), and of the human disease-related genes, over 25% have a close homologue in yeast (Basset *et al.*, 1996). Importantly, yeast and human cells share fundamental aspects of eukaryotic cell biology. In many cases, yeast has even been the model system where these cellular processes and the genetic components comprising them have been elucidated. All this together with the easily of effecting genetic manipulations without incurring in ethical issues, make the yeast, and in particular *S. cerevisiae* the most widely used model organism to date.

In relation to this thesis work, the period spent and the experience made in the laboratory of “System and Synthetic Biology” of Chalmers University of Goteborg-Sweden was an unforgettable moment to understand that it is not possible to ignore that during a bioprocess carried out with microorganisms, a close relationship is created in the inner space of the fermenter between the environmental and biological components and this relation can give rise to an emergent property.

The strain CEN.PK 113-5D has been employed to assess the effect of the expression of human hemoglobins. The hemoglobins involved in this study were neuroglobin (Ngb) (Burmester *et al.*, 2000) an human globin expressed in the brain whose function is not yet completely clear, human hemoglobin (Hb) (Hünefeld, 1840) and myoglobin (Mb) (Kendrew *et al.*, 1958).

Generally, globins are a large family of proteins traditionally associated with the storage and transport of oxygen, therefore the study of their function may facilitate the understanding of oxidative stress, hypoxia and other adnexa phenomena to them such as cancer.

In this thesis we have studied the influence of the expression of these specific proteins essentially on the growth parameters. This work is part of a project developed under the direction of Prof J. Nielsen.

Human Hemoglobin

The human Hemoglobin (Hb) is a globular metalloprotein present in the red blood cells of all vertebrates. It has the function to ferry oxygen (O₂) and carbon dioxide (CO₂) molecules throughout the body (Sadava *et al.*, 2008). Hemoglobin tetramers are comprised of the four subunits, two α -globin chains and two β -globin chains all of which take the form of alpha helices (Sadava *et al.*, 2008). The α and β chains are constituted by 141 and 146 amino acids, respectively with a molecular weight of 64.0 KDa. Found in each chain is a non-protein heme group, which is an assembly of cyclic ring

structures surrounding an iron, Fe^{2+} , ion that is tethered by nitrogen atoms (Perutz, 1990).

The iron ions permit the bonding between globin and oxygen molecules. Oxygen works as a cooperative substrate promoting the oxygen circulation through the body in red blood cells. In this way the oxygen is delivered to tissues to be used, as final electron acceptor, in the respiration. Alternatively, globin can bind to carbon dioxide, so favoring its transport to the lungs (to be exhaled as waste). Therefore, hemoglobin is present in two forms: oxyhemoglobin which is the form of hemoglobin whose heme group binds to oxygen; deoxyhemoglobin which is that without oxygen. The oxygenation of hemoglobin is accompanied by structural changes in the subunits determining an allosteric mechanism for the kinetics of this enzyme (Perutz, 1970).

Myoglobin

Myoglobin (Mb) is a single chain globular protein of 153 amino acids containing a heme group with a molecular weight of 16.7 kDa. Myoglobin is expressed solely in cardiac myocytes and oxidative skeletal muscle fibers and has the function to reversibly bind oxygen by its heme residue, facilitates O_2 diffusion and NO detoxification (Wittenberg, 1970; Flogel *et al.*, 2001; Ordway and Garry, 2004). More precisely myoglobin produces and scavenges nitric oxide (NO) in deoxygenated and oxygenated conditions, respectively. This process is activated in cardiac muscle cells under hypoxic stress, where it suppresses the production of damaging reactive oxygen species (ROS) in mitochondria, so protecting the muscle cells from damage. For this reasons Myoglobin is a sensitive marker for muscle injury, making it a potential marker for heart attack in patients with chest pain.

Neuroglobin

Neuroglobin (Ngb) is a single chain globular protein expressed in the central and peripheral nervous system of vertebrates. It is composed by 150 amino acids with a molecular weight of 16.0 kDa. Neuroglobin was first, recently identified by Thorsten Burmester *et al.* in 2000. Since its discovery it has generally been accepted that Ngb has a function similar to that of Hb and Mb, either storing O_2 for hypoxic phases (e.g. in diving mammals) or facilitating O_2 diffusion from the capillaries to the respiratory chain in the mitochondria. However, within the past ten years it has become evident that this globin may carry out various other functions (Fig. II.5.4-1) (Pesce *et al.*, 2003; Burmester and Hankeln, 2009; Brittain, 2012):

- May enhance O_2 supply to the mitochondria of the metabolically active neurons
- May counter the harmful effect of reactive oxygen or nitrogen species (ROS/RNS)
- May detoxify harmful excess of nitric oxide (NO) to nitrate (NO_3^-)

- May be part of a redox process of Cytochrome c preventing the apoptosis

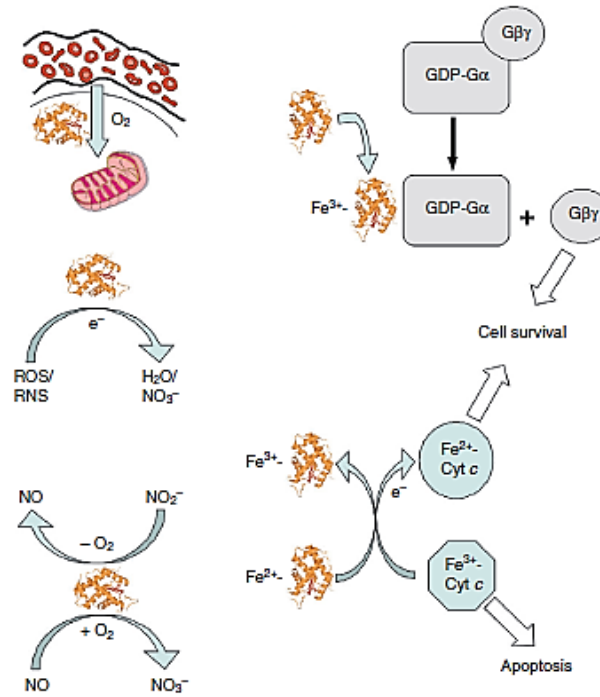


Fig. II.5.4-1 Some functions of Neuroglobin (Burmester and Hankeln, 2009)

Chapter III

Materials and Methods

III.1 Yeast strains

The strains used in this thesis are all GRAS (Generally Regarded as Safe) *Saccharomyces cerevisiae* yeasts. The strains used more frequently belong to the CEN.PK family and were purchased at the EUROSCARF (www.uni-frankfurt.de/fb15/mikro/euroscarf).

Table III.1-1 *Saccharomyces cerevisiae* strains used in this work

<i>Strain</i>	<i>Genotype</i>	<i>Auxotrophy</i>
	<i>MATa URA3 HIS3, LEU2 TRP1 MAL2-8c SUC2</i>	-----
CEN.PK 113-5D	<i>MATa ura3-52 HIS3 LEU2 TRP1 MAL2-8c SUC2</i>	Uracil
CEN.PK111-32D	<i>MATa URA3 HIS3 leu2-3,112 TRP1 MAL2-8c SUC2</i>	Leucine
CEN.PK102-3A	<i>MATa ura3-52 HIS3 leu2-3,112 TRP1 MAL2-8c SUC2</i>	uracil and leucine
CEN.PK102-5B	<i>MATa ura3-52 his3-D1 leu2-3,112 TRP1 MAL2-8c SUC2</i>	uracil, histidine and leucine
CEN.PK2-1C	<i>MATa ura3-52 his3-D1 leu2-3,112 trp1-289 MAL2-8c SUC2</i>	uracil, histidine, leucine and tryptophan
BY4741	<i>MATa ura3Δ0 leu2Δ0 met15Δ0 his3Δ1</i>	uracil, histidine, leucine and methionine
Y306	<i>Mata/MATa ade2/ade2 leu2/leu2 his3/his3 ura3/ura3 trp1/trp1 NBP35/nbp35: :HIS3</i>	Tryptophan

Moreover were used *S. cerevisiae* BY4741 and *S. cerevisiae* Y306, this latter is used at industrial level and kindly provided only for research purposes by Prof. Jesus Zueco from the Department of Microbiology University of Valencia (Spain).

All the *S. cerevisiae* strains employed in this work, their genotype and auxotrophies are listed in Table III.1-1.

The strains were kept at -80 °C in 12.5 % v/v glycerol containing a complementing auxotrophy suitable mineral medium.

III.2 Shake flask cultures

Growth in shake-flask cultures was performed in 500 ml flasks containing 100 ml of a defined mineral medium prepared according to Verduyn *et al.* (1992) and containing vitamins and trace elements. For all the strains examined, the medium was supplemented with 1% w/v casamino acids (BD Bacto™ Casamino Acids, BectonDickinson & Co., Sparks, MD 21152 USA) as auxotrophy-complementing amino acid source, and, when there was the need, also uracil (7.5 ml of a 0.2% w/v stock solution) or tryptophan (0.8 ml of a 0.5% w/v stock solution) was added. The addition of tryptophan was necessary due to the low tryptophan content of casamino acids. The amount of casamino acids in the medium was determined on the basis of its aminoacidic composition (BectonDickinson, technical handbook), and the recommendations on the nutritional requirements obtained from the literature (Hägerdal *et al.*, 2005).

The initial glucose concentration was 2% w/v. Each inoculum (an aliquot of cell suspension resulting in 0.2 initial optical density at 590 nm, O.D.₅₉₀) was prepared from an exponential pre-culture. This latter contained the same medium of the shake flask cultures and was inoculated with an aliquot of a frozen stock culture (-80 °C in 12.5 % v/v glycerol) to give a 0.2 initial O.D.₅₉₀. Pre-cultures and cultures were incubated at 30 °C, and 220 rpm (Stuart Scientific S150 Orbital Incubator). Specific growth rates were calculated from optical density measurements at 590 nm.

III.3 Fed-batch cultures

As regards fed-batch cultures, these were performed in a 2.5 l working volume of a stirred fermenter, Bioflo 110 (New Brunswick Scientific). For all the strains examined, the bioreactor initially contained 1 liter of a medium having the same composition of that of shake flask experiments. The

bioreactor, so prepared, was inoculated with an adequate aliquot of an exponential pre-culture prepared in the same conditions of shake flask cultures, to give an initial O.D.₅₉₀ of 0.04.

Fed-batch cultures were performed in two steps. First, the reactor was allowed to proceed in a batch mode (1 l volume) overnight. The second step corresponding to the exponential feeding phase, started when glucose of the batch phase was exhausted. At that point, an exponentially increasing feed was applied to allow the biomass to increase with a constant specific growth rate whose value was always chosen below a critical one corresponding to 60 % of the μ_{\max} value (Enfors, 2001).

The feeding solutions contained glucose (50 % w/v), salts, trace elements, glutamic acid, vitamins, and the auxotrophy-complementing amino acids. Final salt concentrations per litre were: KH_2PO_4 15.70 g, KCl 5 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5.83 g, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.20 g, NaCl 0.44 g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 250 mg. Final trace elements concentration per liter was: $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 50 mg, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 2 mg, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 40 mg, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 50 mg, and final glutamic acid concentration per liter was 1 g (Porro *et al.*, 1991). Filter-sterilized vitamins were added after heat sterilization. Final vitamin concentrations per litre were: biotin 4 mg, calcium-pantothenate 40 mg, nicotinamide 90 mg, myo-inositol 50 mg, thiamine HCl 100 mg, pyridoxine HCl 20 mg (Porro *et al.*, 1991). The overall concentration of each auxotrophy-complementing amino acid was calculated taking into account the value of biomass yield for the given amino acid in aerobic conditions and considering an excess factor of ca. 25% (Pronk, 2002). Feeding of the substrate solution was ensured by a peristaltic pump controlled via computer.

Oxygen was supplied by sparging the bioreactor with air at a flow rate of 1 and 1.5 vvm in the batch and exponential feeding phase, respectively, the cascade system acted with the agitation speed automatically increasing or decreasing in the range 50-900 rpm, until the DOT set-point (30% air saturation) was reached. When stirring did not ensure the maintenance of DOT set-point, air enrichment with oxygen was automatically accomplished. The culture pH was maintained at 5.00 by automatic addition of 2 N KOH during batch phase, whereas ammonium supply and control of pH were ensured by automatic addition of 10% v/v NH_4OH during exponential phase. The foam level in the bioreactor was controlled by the automatic addition of the antifoam B (Sigma Aldrich) (dil. 1:10).

III.4 Chemostat cultures

Aerobic chemostat cultivations were carried out in the laboratories of System and Synthetic Biology of Chalmers University (Goteborg, SE). They were performed, in duplicate, at 28°C in laboratory fermenters of 1 liter

working volume (DASGIP[®], Eppendorf), containing a synthetic mineral medium with 1% w/v glucose, mixed at a constant stirrer speed of 600 rpm. First, the reactor was allowed to proceed in a batch mode (0.4 liter volume) overnight until carbon source exhaustion. The next step corresponded to a chemostat cultivation that was performed with a dilution rate (D , equal to specific growth rate in steady-state cultures) of 0.10 h^{-1} feeding fresh medium with 2 % w/v of glucose. A steady state was defined as the situation in which, after at least five residence times, corresponding to five volume changes passed after the last change in growth conditions, the biomass concentration, as well as the specific rates of carbon dioxide production and oxygen consumption remained constant (<2% variation). The working volume of the cultures was kept constant (0.4 liter) by a peristaltic effluent pump having the intake tube placed at suitable height and working at a flow rate higher than the inlet. This set-up ensured that under all growth conditions, biomass concentrations in samples taken directly from the culture, differed by <1% from biomass concentrations in samples taken from the effluent line. The dissolved oxygen concentration was continuously monitored and remained above 30% air saturation. Steady-state data are reported for cultures without detectable oscillations in oxygen consumption and carbon dioxide production rates. Chemostat cultures were routinely checked for purity by microscopy.

III.5 Biomass determination

Biomass was determined by optical density at 590 nm (O.D.₅₉₀) and dry weight determination. The calibration curve relating O.D.₅₉₀ values to biomass density provided a correlation factor of 2.30 O.D.₅₉₀ per g l⁻¹. Cell viability during fed-batch runs was determined by viable count on SDM agar plates, incubated at 30 °C for 48 h. Death kinetics was evaluated by plotting the ratio CFU/OD₅₉₀ vs. time, where CFU corresponded to the colony forming units originated from viable cells, and OD₅₉₀, the culture optical density, corresponded to the total amount of cells (viable and dead cells) in the medium. In a growing culture, the decrease in CFU/OD₅₉₀ allowed death kinetics to be monitored. An unchanged value of the ratio CFU/OD₅₉₀ corresponded to a culture where cell death did not occur. The viable biomass concentrations during bioprocess runs have been obtained employing the relation (III.5-1) below and assuming that, at the beginning of bioprocess (time 0) all the cells of the yeast population were viable.

$$X_v(t) = \frac{X_{v0} \frac{UFC}{ml_t}}{\frac{UFC}{ml_0}} \quad \text{III.5 - 1}$$

III.6 Enzymatic assays

III.6.1 Catalase assay

The catalase assay is based on monitoring the disappearance of the H₂O₂, than is the enzyme substrate, by the absorbance decrease at 240 nm (wavelength), using the hydrogen peroxide extinction coefficient, $\epsilon = 39.4 \text{ mol l}^{-1} \text{ cm}^{-1}$, to determine its concentration.

Catalase activity was determined in the supernatants coming from cell extracts. To obtain cell extracts, yeast cells were washed twice with 20 mM phosphate buffer, pH 7.0 and resuspended in 200 μl of the same buffer containing 1 mM EDTA and 0.1 mM PMSF (phenylmethanesulphonyl fluoride) to avoid catalase digestion by yeast proteases. Cells were disrupted with 0.4 mm diameter Ballottini glass beads for 20-30 min (shaking for 1 min and placing the sample on ice for another 1 min, alternatively). Then, the cell homogenate was centrifuged (4000 rpm, 15 min) and the supernatant was used for catalase assays.

The readings were carried out in cuvettes against the blank that does not contain cell extract. The specific catalase activity (A.S., expressed in μmol of substrate transformed in one minute per mg of total proteins) of the yeast extract, was calculated dividing the slope of the straight line obtained by plotting the decreases in OD₂₄₀ against time, by the protein content of the extract, P_{tot}, and by the molar extinction coefficient ϵ , according to the equation:

$$\text{A. S.} = \frac{d(\text{OD})}{dt} \cdot \frac{1}{\epsilon \cdot P_{\text{tot}}} \quad \text{III.6.1 - 1}$$

The protein content of the extract is determined using the Bradford method (Bradford, 1976).

III.6.2 Lipolytic activity assay

Lipolytic activity quantification was performed by a previously described colorimetric assay (Prim *et al.*, 2000) in which the release of para-nitrophenol (pNP) from pNP-butyrate was measured. One international unit of activity (U) was defined as the amount of enzyme necessary to release 1 μmol of pNP per hour under the assay conditions described.

III.6.3 Endoglucanase activity assay

Endoglucanase (EG) activity was evaluated using a reducing sugar assay to measure the amount of glucose released from a soluble cellulose derivative with a high degree of polymerization (DP) such as carboxymethyl cellulose (CMC) see Fig. III.6.3-1.

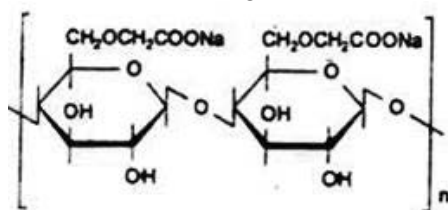


Fig. III.6.3-1 Carboxymethyl cellulose (CMC)

Carboxymethyl cellulase (CMCase) activity was mainly evaluated accordingly to the procedure described by Mandels *et al.*, (1976). In this method, CMCase activity was measured by determining reducing sugars released, at 45°C, after 15 min of enzyme reaction with 0.5% CMC w/v at pH 4.4 (acetate buffer). One unit (U) of EG activity was defined as the amount of enzyme that liberates 1 μ mol of glucose per minute under assay conditions. Reducing sugars have been estimated by applying the Somogyi-Nelson method (Somogyi, 1952).

III.7 Interleukin-1 β determination

Interleukin-1 β was determined in quadruplicate by immuno-blot analysis in Bio-Dot[®] Microfiltration Apparatus (Bio-Rad, Hercules, CA, USA). After microfiltration of 200 μ l of samples, the nitrocellulose membranes were treated with a TBS (Tris/HCl 100 mM pH 7.5 in 2.5 M NaCl) blocking solution containing 0.05 % v/v Tween 20 (TBST) and 5% w/v dry skimmed milk and then probed with primary antibody (Rabbit polyclonal IgG IL-1 β -Santa Cruz Biotechnology) diluted to 1:200 in TBS for 1 h. After washings in TBST, the membranes were treated with a secondary antibody conjugated with alkaline phosphatase (Goat anti-rabbit IgG-AP), (Bio-Rad) diluted to 1:4000 in TBS and incubated overnight at 4 °C. Color development was obtained with NBT/BCIP system (Bio-Rad), containing the substrates for alkaline phosphatase. IL-1 β was quantified by densitometric analysis (MultiAnalyst, Bio-Rad) using the human EuroClone IL-1 β as a standard.

III.8 Glucose and ethanol analysis

Samples were quickly withdrawn from batch and fed-batch cultures, filtered on 0.45 μm GF/A filters (Millipore, Bedford, MA USA) and filtrates analyzed to determine residual glucose and ethanol concentrations. Residual glucose (g l^{-1}) in the medium was determined by enzymatic D-Glucose assay (GOPOD - Megazyme International, Ireland Ltd). Ethanol production was evaluated with the enzymatic kit from Megazyme. All the samples were analyzed in triplicate showing a standard deviation always lower than 5%.

III.9 Staining tests

Staining tests were carried out by collecting samples of approximately $2 \cdot 10^7$. The cells were washed in PBS (phosphate buffered saline) buffer and centrifuged. Immediately after, the pellets were treated, according to standardized protocols (see below), with dyes and observed at fluorescence microscope Leica Microsystems[®] to evidence mitochondria, DNA, Reactive Oxygen Species (ROS) and cell viability. The captured images were elaborated with LAS AF software.

III.9.1 Staining of yeast mitochondria

Cells were resuspended in 1 ml of 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer, pH 7.4, containing 5% w/v of glucose. At this suspension, 1 μl of 100 μM MitoTracker[®] Green FM was added. Samples were incubated in the dark for 15 min at room temperature, further, after centrifugation, supernatant was discarded and cells analyzed at fluorescence microscope. The excitation emission wavelength was 516 nm. MitoTracker[®] Green FM passively diffuses across the plasma membrane and accumulates in active mitochondria evidencing a green fluorescence. Typical cases of mitochondrial morphology are reported in Fig. III.9.1-1

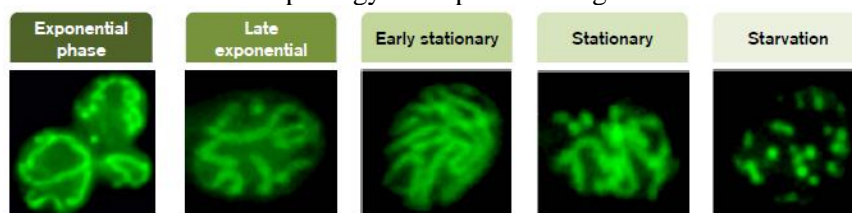


Fig. III.9.1-1 *Some observations of mitochondria at fluorescent microscopy at the different phases of microbial growth*

III.9.2 Staining of DNA

Cells were resuspended in 1 ml of 10 mM HEPES buffer, pH 7.4, containing 5% w/v of glucose. 1 μ l of 10 μ M Propidium Iodide (PI) was added to this suspension. Then the cells were incubated in the dark for 15 min at room temperature. Then the cells were centrifugated, the supernatant discarded and the cells analyzed at fluorescence microscope. The excitation emission wavelength was 605 nm. Cells that appear to have damaged nucleus, and therefore a DNA dispersion, appeared stained due the fluorescence intensity of PI bound to DNA.

III.9.3 Staining of live and dead cells

Cells were washed in 1 ml of 10 mM HEPES buffer, pH 7.2, containing 2% w/v of glucose and resuspended in 250 μ l of the same buffer adding 0.375 μ l of 10 mM FUN[®]1. Samples were incubated in the dark for 30 min at 30 °C. Then they were centrifuged, the supernatant discarded and the cells analyzed at fluorescence microscope. The excitation emission wavelengths were 516 and 605 nm. Only metabolically active cells are marked clearly with fluorescent intravacuolar structures, while dead cells exhibit extremely bright, diffuse, green-yellow fluorescence. Cells with intact membranes but with little or no metabolic activity have a diffuse green cytoplasmic fluorescence and lack fluorescent intravacuolar bodies.

III.9.4 Staining of reactive oxygen species (ROS)

Cells were resuspended in 1 ml of 50 mM sodium citrate buffer, pH 5.0, containing 2% w/v of glucose. 1 μ l of 50 mM of dihydrorhodamine 123 was added to this suspension. Samples were incubated in the dark for 15 min at room temperature. Then, after centrifugation, the supernatants were discarded and the cells observed at fluorescence microscope. The excitation emission wavelength was 543 nm. The occurrence of this fluorescence at the level of single cells was indicative of an high amount of ROS.

Chapter IV

Mathematical Model

IV.1 Overview on the modeling of microbial growth

Mathematical modelling of kinetics of microbial cell growth continues to be an important topic of biochemical engineering research (Nilsen and Villadsen, 1994). The potential impact of predictive models on bioprocess simulation, design, optimization and control is substantial. As compared to conventional chemical reactors, bioreactors are particularly difficult to model due to the complexity of the biochemical reactions occurring inside the reactor itself, the unique characteristics of individual cells and the lack of key process measurements. The consumption of substrates and production of metabolites result from hundreds of coupled biochemical reactions (Mauch *et al.*, 1997). Construction and dynamic modelling of these complex reaction networks is a very challenging problem. While they often are viewed as homogeneous mixture of identical cells, microbial cultures actually are comprised of heterogeneous mixtures of cells that differ with regard to size, mass and intracellular concentrations of proteins, DNA and other chemical constituents (Srienc and Dien, 1992). Accurate modelling of cell growth and metabolite formation kinetics may require that individual cells be differentiated based on these characteristics.

The development of a mathematical model for aerated fed-batch cultivation could be of great importance in an attempt to:

- describe the real phenomena occurring in the bioprocess, to investigate important questions about the variables observed and to explain this, to check hypothesis and to make predictions about the behavior of the producer strain in a complex environment such as that arising in a fed-batch reactor by varying some process variables. (Clementsich and Bayer, 2005).
- describe and predict some process variables such as the degree of substrate conversion, the concentration of products and byproduct by varying operating conditions

- characterize the physiology of the host whose phenotype is the results of both biological and operative process conditions especially when the metabolic fluxes cannot be directly evaluated

During the years several models for *S. cerevisiae* growth were proposed, since the beginning of the last century when Richards (1928) performed the mathematical analysis of the growth of the yeast. He devised that the growth of yeast *Saccharomyces cerevisiae* has an asymmetrical S-shape. He also measured the effect of temperature by van't Hoff Arrhenius equation.

Monod (1949) implemented a mathematical description of bacterial growth based on enzyme kinetics. He assumed that it was limitation of nutrients and not cell death that ended the exponential phase of growth, and that growth is proportional to the concentration of the nutrient when looking at individual cells. To model this it is necessary to evaluate how the growth rate varies with respect to the concentration of limiting nutrient.

Models based on Monod kinetics are able to describe only balanced growth conditions and so are not suited for modelling transient phenomena. Then in addition to the Monod equation, several other functional dependencies of growth rate for a single limiting substrate have been proposed (Jannasch and Egli, 1993). Many of these models are very similar to the Monod model, but might contain additional parameters (Moser, 1958 ; Contois, 1959).

Specifically for *S. cerevisiae* growth it is of particular relevance the description and modelization of the limited capacity of the respiratory metabolism that has been studied by Sonnleitner and Kappeli (1986) with an unstructured and unsegregated model. This model assumes that there is a finite substrate flux available to the oxidative pathways, with glucose being the preferred substrate.

The model is formulated by using the stoichiometric growth equations for pure oxidative and reductive (fermentative) glucose and ethanol metabolism. It is based on the fact that glucose is metabolized part oxidatively and part reductively, with ethanol being the end product of reductive energy metabolism. The corresponding metabolic state is designated oxido-reductive. Ethanol can be used oxidatively only. Maximum rates of oxidative glucose and ethanol degradation are governed by the respiratory capacity of the cells. The latter model has further developed by Lei *et al.* (2001) that proposed a structured model. The model is designed to describe the onset of aerobic alcoholic fermentation during steady-state as well as under dynamical conditions, by triggering an increase in the glycolytic flux using a key signalling component which is assumed to be closely related to acetaldehyde.

Overflow metabolism has subsequently been described by Pham *et al.* (1998, 1999). They assumed that concentrations of glucose, biomass and ethanol are controlled by the substrate feed rate. The substrate, is assumed first to be channeled into the oxidative metabolism, which is divided into

different fluxes used for anabolism and for energy metabolism in which part of the latter flux is used for the maintenance.

Other assumption have been suggested to explain respiro-fermentative physiology of *S. cerevisiae*. Hanegraaf et al. (2000) proposed a mechanistic model assuming the presence of multiple types of glucose carriers and multiple assimilation pathways with different affinity of the carriers.

The dynamics of *S. cerevisiae* growth on multiple substrates has been studied with cybernetic models (Ramkrishna, 1982) that describe enzyme synthesis and activity based on the hypothesis that microorganisms optimize utilization of available substrates to maximize their growth rate at all times (Jones et Kompala, 1999).

A stoichiometric model describing the anaerobic metabolism of *Saccharomyces cerevisiae* during growth on a defined medium was used to calculate intracellular fluxes based on measurements of the uptake of substrates from the medium, the secretion of products from the cells, and of the rate of biomass formation (Nissen *et al.*, 1997). According to the subsequent genetic studies *S. cerevisiae* is the first eukaryotic genome that was fully sequenced (Goffeau, 1997), several models that take into account genetic information have been developed. These models are aimed at studying the metabolic network of the yeast cell englobing genomic, biochemical, and physiological information, defining a new modeling approach that is part of the systems biology (Forster *et al.*, 2003; Castrillo *et al.*, 2007).

IV.2 Modeling approach for High Cell Density Culture

Modeling of high-cell density cultures and recombinant protein production needs to be continuously developed and improved, considering the peculiar environment represented by the aerated fed-batch reactor. Mathematical modeling can have different levels of complexity. The first level is that which considers the cell as a black box and therefore the structuring of the cell is not taken into consideration, in this case it comes of “unstructured models”. The black box models are easy to implement and can clearly describe the process but often result not very flexible and consequently unsuitable for simulation under different process conditions. However, models with a high degree of structuring may be very complex and so could benot applicable in industrial bioprocesses. For this purpose, it was decided to implement the mathematical models developed in the past by the group of “Industrial Microbiology ” of the “University of Salerno”. This mathematical model was unstructured and non-segregated . The simple approach, in which all the cellular reactions are lumped into a single overall

reaction, described, quite well, the trend of viable cellular mass, residual glucose and ethanol. However, this type of model always considers the metabolic processes outside the cell and only two specific glucose consumption rate, one which refers to glucose spent for growth and one that refers to the remaining glucose that is spent for all processes of maintenance (Paciello *et al.*, 2012; Landi *et al.*, 2011). To better understand the dynamic response of the glucose sensitive yeast *S. cerevisiae* when it grows in a complex environment such as the bioreactor, it was necessary to enhance the detail level of the mathematical model. This can be done by defining key processes in the network of metabolic pathways, that are deemed to be those that influence an emergent property of the system. So a model not overly complex and capable to retain a good level of flexibility could be set up. To do this, the Dynamic Systems, formulated in the 60s by Jay Forrester at the Massachusetts Institute of Technology (Forrester, 1961; Forrester, 1971) were taken into consideration. The dynamic systems are a particular aspect of system theory that allows us to understand the dynamic behavior of complex systems. Fundamentals of this method resides in the recognition that understanding the behavior of any system is important to know both the structure as well as its individual components. Indeed using this approach it is possible to highlight emerging properties of the system that cannot be found between the properties of its components. This highlights how the behavior of the whole cannot be explained by the behavior of its parts.

The quantitative modeling was carried out with Simile®, modeling software for complex dynamical systems developed by the company Simulistics of Edinburgh (UK). Simile® uses a declarative modeling approach that allows to build graphically the conceptual framework of the model. In this way it is possible to avoid the implementation of the model with programming languages such as “Fortran” or “C”.

The main advantage is that the model is constructed in a manner more intuitive, and requires no extensive mathematical and computer skills. Further this model maintains a graphical structure that lets you view the elements and processes clearly.

IV.3 Simile® an useful tool for yeast growth modeling

Simile is billed as a “visual modeling environment”, meaning that model is developed diagrammatically (as opposed to writing lines of text, as in a programming language or a simulation language). However, a more fundamental feature of Simile is that it is a declarative modeling environment. In declarative modeling, we represent a model not as a series of assignment and control statements, but as a set of facts that are true about

the model. The order in which we present the facts is (unlike a procedural program) irrelevant.

There are significant advantages in adopting a declarative approach. First, there is no risk of the description of the model failing to match the implementation of the model: the description is the implementation. Second, once a model is represented declaratively, one can do many things with it as well as just simulating its behavior: for example, generate descriptions in a variety of formats, interrogate its structure, compare its structure with that of another model, or transform it into a simpler or more complex model. Third, one can generate runnable versions of the model in a variety of languages (including languages for parallel computers). Finally, the adoption of a declarative modeling approach encourages the development of common standards for representing models, the distributed development of modeling tools, and the sustainability of the effort put into developing models.

The key elements that make up a similar model are as follows (Fig. IV.3-1):

- *Compartments*: also called stock or levels, representing the value (or state) of a certain variable over time. Mathematically, a compartment is a state variable whose behavior is defined by a differential equation. Requires that the initial value is defined, generally a numeric constant (initial condition).
- *Flow arrows*: represent a rate of increase or decrease of the compartment value. Mathematically they are the positive elements (arrows in entry) and negative (arrows out) of the differential equation of the state variable which they are associated. The value associated to a flow can be a constant or a function.

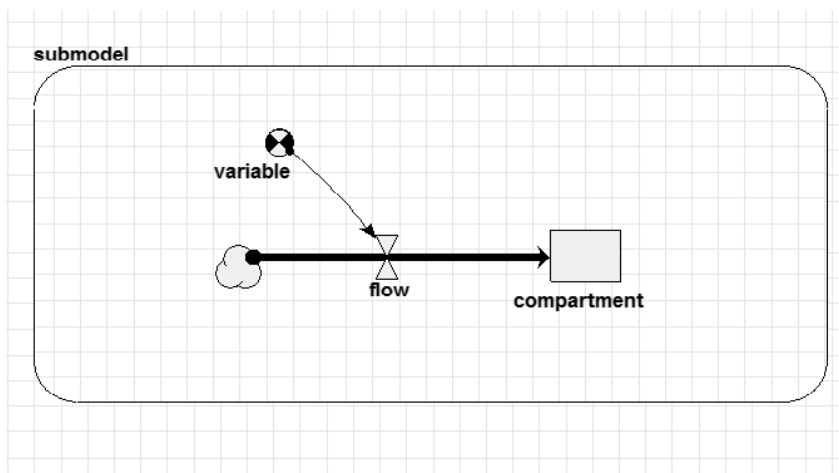


Fig. IV.3-1 Main elements of a Simile[®] model

- *Variables*: are any quantity whose value is constant or calculated as a function of other quantities. In terms of modeling, these elements represent parameters, exogenous quantities, intermediate variables or output.
- *Arrows of influence*: are connection elements which represent the fact that a quantity is used to calculate another
- *Submodels*: are containers that contain one or more elements among those mentioned above. In modeling terms they can have different meanings: to isolate conceptually different parts of a complex model, to separate sections that have different time scales, to reproduce a certain number of times the structure of elements contained in it.

IV.4 Map of *S. cerevisiae* model

The purpose of the model is to simulate the behavior of the yeast growing in aerated fed-batch reactor modeling the variables that best describe the bioprocess on macroscopic scale (microbial mass, ethanol and residual glucose). This is done with a simple and flexible schematization of the yeast cell functioning. The objective is to highlight physiological changes that have a strong influence on the performance of yeast in fed-batch bioreactor. In particular the aim is to describe the phenomena of reduction of proliferative capacity occurring during fed-batch yeast cultivation and that are strictly linked to metabolic fluctuations.

The first step of the model building was to create a structure that allows, with a relative simplicity, to describe the metabolism of the yeast.

From literature studies on metabolic fluxes (Frick and Wittmann, 2005; Christen and Sauer, 2010) can be seen that the metabolism of *S. cerevisiae*, considering glucose as the only limiting carbon and energy source, consists in many steps (Fig. IV.4-1); therefore describing all compounds and any flow that takes place exists within the cell, would be a difficult task with the risk that the mathematical model built on this approach is too complex and difficult to apply.

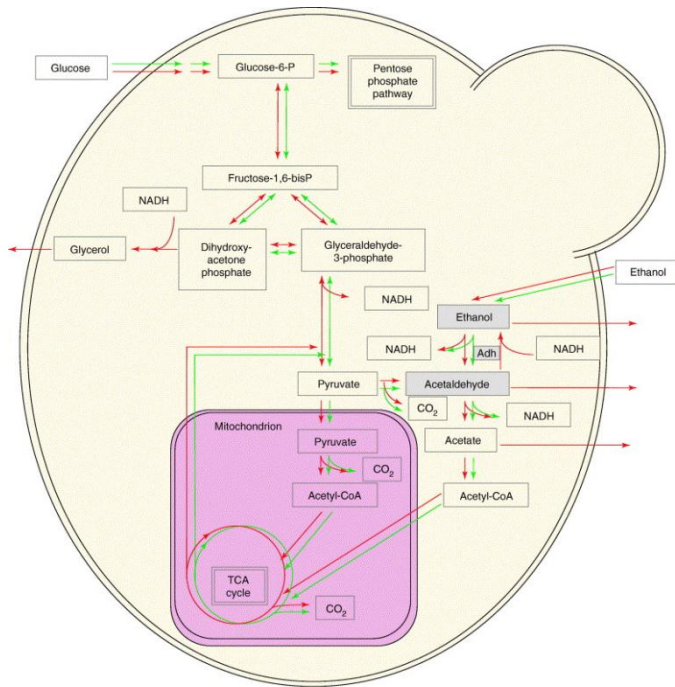


Fig. IV.4-1 Scheme of metabolic pathways involved in glucose and ethanol assimilation for *S. cerevisiae* (Piškur et al., 2006).

In order to synthesize the pattern of the metabolic fluxes, pyruvate was identified as the crucial metabolic intermediates and the central hub for all the metabolic pathways which lead from glucose (carbon and energy source) to biomass.

Another aspect very relevant of the logic of modelization adopted in this work, was the developing of a pathway that negatively regulates yeast growth by reducing the rates of the metabolic fluxes modeled and causing cell death. The negative effect on growth is considered to evaluate presence of ethanol and other identified (e.g. acetate) or unidentified metabolites that could inhibit the growth as shown schematically in Fig. IV.4-2. So this principle is based on negative feedback logic since the products of the process influence the process itself in such a way as to reduce changes.

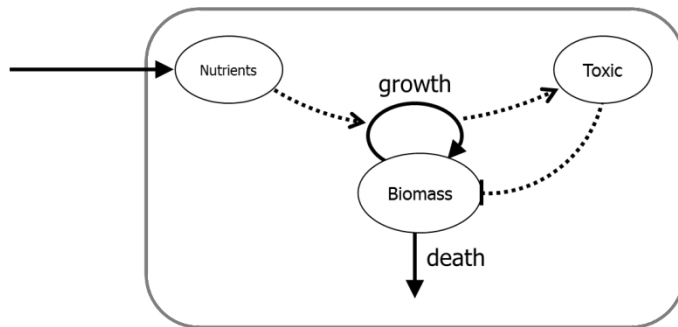


Fig. IV.4-2. Schematic representation of microbial growth with negative feedback influence

The diagram of the model proposed in this work is showed in Fig. IV.4-3. More specifically, cell metabolism is simplified with explicit representation of pyruvate as key metabolite and three major metabolic pathways: Glycolysis, Respiration and Fermentation. These latter are the ways through which glucose is channeled to generate carbon skeletons.

The metabolic reactions that exist between these steps cause the variation of the compartment values over time.

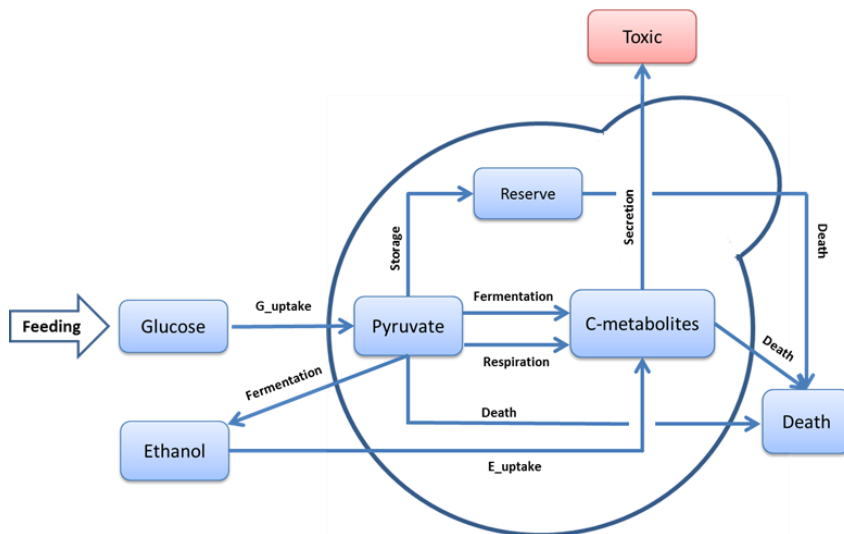


Fig. IV.4-3 Schematic diagram of yeast growing on glucose

All pathways have pyruvate as hub metabolite, glycolysis is the first step common to all metabolic pathways and consisted in the Pyruvate formation by feedstock (Glucose). Pyruvate, once formed, can follow two paths depending on its concentration within the cell: may be breathed, forming biomass (respiratory metabolism) or fermented with the consequent production of ethanol and a low amount of biomass. The preference of a pathway over another is given by the pyruvate accumulation inside the yeast

cells. This approach is reasonable and is endorsed by several studies in literature (Pham *et al.*, 1998; Pronk *et al.*, 1996; Sonnleitner and Hahnemann, 1994).

The model takes into account the possibility to metabolize ethanol, by yeast cells, as a substrate. The formation of carbon skeletons that leads to the production of new cellular material and thus determine the increase of biomass has been represented with C-metabolites. Furthermore the metabolic pathways that provide for the production of reserve material (eg glycogen) and the loss of metabolic active material (cell death) was considered. Finally great importance in the modelization was given to the exudation of a toxic substance as growth inhibitor.

IV.5 Model Implementation

Mathematical model implementation was made using the software Simile[®] in order to achieve maximum stability and expandability a modular approach has been preferred, and conceptual distinction among compartments has been emphasized by the use of submodels, characterized as:

- Environment includes the macroscopic variables that describe a common fed-batch bioprocess (liquid volume, ethanol, glucose, toxic inhibitor and cell death amount) and the microbial mass.
- Yeast is a submodel of environment, describes the life of microorganism considering the crucial metabolite pyruvate. This compound, together with “C-metabolites” and “Reserve” material represented the dry weight of the yeast, in particular the compartment “C-metabolites”, in which the products of the reactions of formation of carbon skeletons of yeast cells converge.

The overall diagram of the mathematical model compiled with Simile[®] is shown in Fig. IV.5-1 and the description of the compartments is reported in Table IV.5-1.

The values over time of each compartment depend on metabolic fluxes which are regulated by enzyme kinetics and by a term which takes into account the limitation of the maximum value of the metabolic intermediates acetyl and pyruvate. Therefore the general kinetic expression for each flow/volumetric rate is :

$$v = v_{\max} \frac{[C]_i}{k + [C]_i} \left(1 - \frac{[C]_j}{[C]_{\max j}} \right) \quad \text{IV.5 - 1}$$

where v represents the flow rate, v_{\max} is the maximum specific flow rate, $[C]$ is a generic concentration and k is an affinity or saturation constant.

Table IV.5-1 Model compartment description

Compartment	Interpretation	Unit
Volume	Volume of the brothculture	l
Death	Death cell or metabolically inactive material	g
Toxic	Growth inhibitor in the brothculture	g/g
Glucose	Glucose in the brothculture	g/g
Ethanol	Ethanol in the brothculture	g/g
Pyruvate	Pyruvate in the microbial mass	g/g
Reserve	Reserve material of the microbial mass	g/g
C-metabolites	Carbon skeletons and main compounds of the microbial mass as dry weight	g

A crucial parameter of the modeling is the metabolic switch (MS) which regulates the type of metabolism of the yeast cells. More specifically, its value is 0 when the metabolism is fully fermentative and 1 when the metabolism is fully respiratory. Further has been assumed that this parameter depends on the concentration of pyruvate in accordance with the following logistic expression:

$$MS = \frac{1}{(1 + a \cdot e^{b \cdot [P]})} \quad \text{IV.5 - 2}$$

Where “a” and “b” are fitting parameters and [P] is the pyruvate concentration inside the cells.

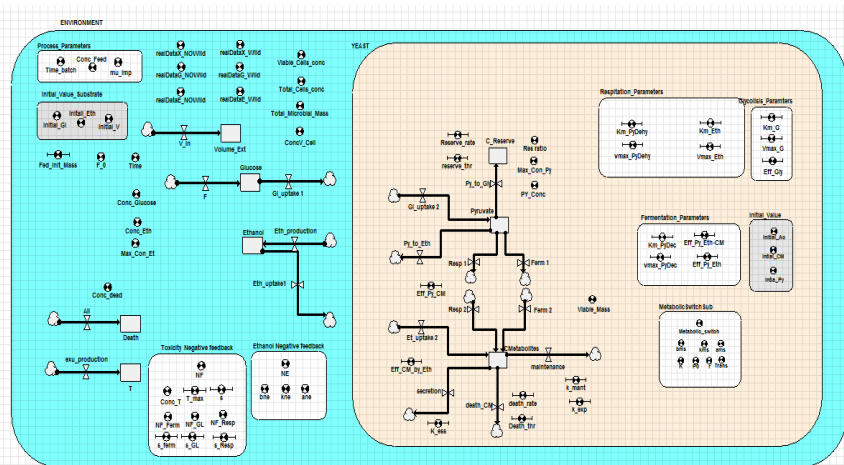


Fig. IV.5-1 Structure in Simile® of the mathematical model

Cell population growth depends on anabolic processes associated with respiration of pyruvate and ethanol and fermentation of pyruvate, according to specific efficiencies η_{RG} , η_{RE} and η_F . Population mass decrease depends on cell death and secretion of inhibitory compounds. The balance equation of

the carbon skeletons that can be assumed as the biomass (M) balance can be written as:

$$\frac{\partial M}{\partial t} = \eta_R \cdot \text{Respiration} + \eta_F \cdot \text{Fermentation} + \eta_{RE} \cdot E_uptake - \text{Secretion} - \text{Death}_M \quad \text{IV.5 - 3}$$

where:

$$\text{Respiration} = v_R \frac{[P]}{k_R + [P]} VM(1 - NT) \cdot (1 - NE) MS \quad \text{IV.5 - 4}$$

$$E_uptake = v_E \frac{[E]}{k_E + [G]} VM(1 - NE) \cdot (1 - NT) MS \quad \text{IV.5 - 5}$$

$$\text{Fermentation} = v_F \frac{[P]}{k_F + [P]} VM(1 - NT) \cdot (1 - NE) \cdot (1 - MS) \quad \text{IV.5 - 6}$$

with v_R , v_E and v_F representing the maximum specific flow rates, k_R , k_E and k_F the saturation constants of the three reactions while VM is the total viable mass. NE and NT account for the negative effect that high concentrations of E and T , respectively, have on respiration and fermentation processes and are formulated as follows:

$$NE = \sigma_E \frac{[E]}{[E]_{max}} \quad \text{IV.5 - 7}$$

$$NT = \sigma_T \frac{[T]}{[T]_{max}} \quad \text{IV.5 - 7}$$

where σ_E and σ_T are species-specific parameters representing the sensitivity of cells to the two negative effects; $[E]_{max}$ and $[T]_{max}$ are the concentrations where the maximum negative feedbacks are exerted;

Furthermore, the secretion of inhibitory compounds is assumed to be a constant proportion (ρ) of the anabolic activity of the cell population and is formulated as:

$$\text{Secretion} = \rho(\eta_R \cdot \text{Respiration} + \eta_F \cdot \text{Fermentation}) \quad \text{IV.5 - 8}$$

Cell population mortality is due to a constant rate δ and is assumed to be triggered by high pyruvate concentrations as follows:

$$\text{Death}_M = d \cdot \delta \cdot M \quad \text{IV.5 - 9}$$

with

$$d = \begin{cases} 0, & [P] \leq \tau - NT \\ 1, & [P] > \tau - NT \end{cases} \quad \text{IV.5 - 10}$$

where τ is the threshold value of pyruvate concentration that induces cell death and it is assumed to be lowered by the inhibitory factors.

Intracellular pyruvate changes in time as a function of synthesis, consumption and cell death. Pyruvate increases depending on external

glucose uptake and is consumed as a function of respiration, fermentation and accumulation of reserve compounds. So, we can write:

$$\frac{\partial P}{\partial t} = \eta_G \cdot G_uptake - Respiration - Fermentation - Storage - Death_p \quad \text{IV.5 - 11}$$

Uptake depends on glycolysis efficiency η_G and is a function of both external glucose and internal pyruvate concentrations formulated as follows:

$$G_uptake = v_G \frac{[G]}{k_G + [G]} VM \left(1 - \frac{[P]}{[P]_{max}} \right) \cdot (1 - NE) \quad \text{IV.5 - 12}$$

where v_G and k_G are the maximum reaction velocity and the saturation constant respectively, and $[P]_{max}$ is the maximum concentration of pyruvate.

Accumulation of reserve compounds depends on pyruvate and reserve concentrations as follows:

$$Storage = v_S \frac{[P]}{k_R + [P]} VM \left(1 - \frac{[R]}{[R]_{max}} \right) (1 - NE) \cdot (1 - MS) \quad \text{IV.5 - 13}$$

with

$$[R] = \frac{R}{P + M + R} \cdot c \quad \text{IV.5 - 14}$$

where v_S and k_R are the maximum reaction velocity and the saturation constant respectively, and $[R]_{max}$ is the maximum concentration of reserves.

To summarize, the system of all the differential equations, which characterizes the model, is shown in table IV.5-2. The balance equations of flows are explained in table IV.5-3 while the respective intermediate variables of the fluxes are reported in table IV.5-4. The parameters used for the modelization and their description are reported in table IV.5-5.

Parameter estimation was formulated as an unconstrained nonlinear optimization of the sum of the squared errors between simulated and experimental values of three state variables (B , G and E). The estimation procedure has been performed using the software MATLAB R2012b (MathWorks Inc.).

Table IV.5-2 Balance equations

Variable	Balance equation
Volume	$\frac{\partial V}{\partial t} = \text{Feeding}$
Glucose	$\frac{\partial G}{\partial t} = C_F \cdot \text{Feeding} - G_{\text{uptake}}$
Ethanol	$\frac{\partial E}{\partial t} = \eta_E \cdot \text{Fermentation} - E_{\text{uptake}}$
Pyruvate	$\frac{\partial P}{\partial t} = \eta_G \cdot G_{\text{uptake}} - \text{Respiration}$ $- \text{Fermentation} - \text{Storage}$ $- \text{Death}_P$
CMetabolites	$\frac{\partial M}{\partial t} = \eta_R \cdot \text{Respiration} + \eta_F \cdot \text{Fermentation} +$ $\eta_{RE} \cdot E_{\text{uptake}} - \text{Secretion} - \text{Death}_M$
Reserves	$\frac{\partial R}{\partial t} = \eta_S \cdot \text{Storage} - \text{Death}_R$
Death	$\frac{\partial D}{\partial t} = \text{Death}_P + \text{Death}_M + \text{Death}_R$
Toxic	$\frac{\partial T}{\partial t} = \text{Secretion}$

Table IV.5-3 Equations of fluxes considered in the model

Flux	Equation
Feeding	$F_0 e^{\mu \cdot t}$
G_{uptake}	$v_G \frac{[G]}{k_G + [G]} VM \left(1 - \frac{[P]}{[P]_{\max}} \right) \cdot (1 - NE)$
E_{uptake}	$v_E \frac{[E]}{k_E + [G]} VM (1 - NE) \cdot (1 - NT) MS$
Fermentation	$v_F \frac{[P]}{k_F + [P]} VM (1 - NT) \cdot (1 - NE) \cdot (1 - MS)$
Respiration	$v_R \frac{[P]}{k_R + [P]} VM (1 - NT) \cdot (1 - NE) MS$
Storage	$v_S \frac{[P]}{k_R + [P]} VM \left(1 - \frac{[R]}{[R]_{\max}} \right) (1 - NE) \cdot (1 - MS)$
Secretion	$\rho (\eta_R \cdot \text{Respiration} + \eta_F \cdot \text{Fermentation})$

<i>Flux</i>	<i>Equation</i>
$Death_P$	$DS \cdot \delta \cdot P$
$Death_M$	$DS \cdot \delta \cdot M$
$Death_R$	$DS \cdot \delta \cdot R$

Table IV.5-4 Equation of the intermediate variables

<i>Intermediate Variable</i>	<i>Equation</i>
[G]	$\frac{G}{V}$
[E]	$\frac{E}{V}$
[T]	$\frac{T}{V}$
[P]	$\frac{P}{P + M + R} \cdot c$
[R]	$\frac{R}{P + M + R} \cdot c$
NE	$\sigma_E \frac{[E]}{[E]_{max}}$
NT	$\sigma_T \frac{[T]}{[T]_{max}}$
MS	$\frac{1}{1 + a \cdot e^{b \cdot [P]}}$
DS	$\begin{cases} 0, & \frac{[P]}{[P]_{max}} + NT \leq \tau \\ 1, & \frac{[P]}{[P]_{max}} + NT > \tau \end{cases}$
F_0	$\begin{cases} 0, & t < t_B \\ \frac{VM_0 \cdot \mu}{C_F \cdot Y_R}, & t \geq t_B \end{cases}$

Table IV.5-5 Model parameter description

<i>Parameter</i>	<i>Unit</i>	<i>Description</i>
Process parameters		
t_B	h	Batch phase duration
C_F	g l ⁻¹	Glucose concentration of feeding
μ	h ⁻¹	Specific growth rate imposed
F_0	l h ⁻¹	Initial volumetric feed rate
Y_R	--	Theoretical biomass yield on glucose
Glucose uptake parameters		
v_G	h ⁻¹	Maximum specific rate of glucose uptake
k_G	g l ⁻¹	Affinity constant of glucose uptake
η_G	--	Efficiency of pyruvate formation by glucose
Respiration parameters		
v_R	h ⁻¹	Maximum specific rate of pyruvate consumption through respiration process
k_R	g l ⁻¹	Affinity constant of pyruvate consumption through respiration process
v_E	h ⁻¹	Maximum specific rate of ethanol uptake
k_E	g l ⁻¹	Affinity constant of ethanol uptake
η_R	--	Efficiency of carbon skeletons formation by glucose respiration
η_{RE}	--	Efficiency of carbon skeletons formation by ethanol respiration
Fermentation parameters		
v_F	h ⁻¹	Maximum specific rate of pyruvate consumption through fermentation process
k_F	g l ⁻¹	Affinity constant of pyruvate consumption through fermentation process
v_S	h ⁻¹	Maximum specific rate of reserve material formation
k_S	g l ⁻¹	Affinity constant of reserve material formation
η_F	--	Efficiency of carbon skeletons formation by glucose fermentation
η_S	--	Efficiency of reserve material formation
$[R]_{max}$	g l ⁻¹	Maximum reserve material concentration inside yeast cell
Metabolic regulation parameters		
a	--	Pre-exponential factor of metabolic switch function
b	l g ⁻¹	exponent of metabolic switch function
$[P]_{max}$	g l ⁻¹	Maximum pyruvate concentration inside cells

<i>Parameter</i>	<i>Unit</i>	<i>Description</i>
Death and toxicity control parameters		
δ	h^{-1}	Specific death rate
ρ	--	Toxic secretion coefficient
σ_E	--	Sensitivity to ethanol toxicity
$[E]_{max}$	g l^{-1}	Tolerance threshold of ethanol
σ_T	--	Sensitivity to the toxicity of a generic substance
$[T]_{max}$	g l^{-1}	Tolerance threshold of generic toxic substance
τ	--	Threshold value for death kinetic activation

Chapter V

Results

V.1 Yeast strain selection for Heterologous Protein Production

Initially a screening of different auxotrophic yeast belonging to the *S. cerevisiae* CEN.PK family was done. This analysis was made in both shake flask and fed-batch reactor and was aimed at selecting the best host to produce the heterologous protein, interleukin-1 β , used as model protein. The yeast strains tested as hosts differed in one prototroph and five auxotrophs, were taken into consideration and characterized.

First, the characterization consisted in the determination of maximum specific growth rate of the strains considered then these strains were tested in aerated fed-batch reactor which represents, on laboratory scale, the reactor where heterologous protein should be produced for next industrial applications.

Long-term operations carried out in the fed-batch reactor promote the onset of a peculiar physical environment which might exert a significant influence on the behavior of the producer strain. As a consequence, the phenotype of the producer microorganism can be considered as the result of the interaction between biological determinants (genotype *in primis*) from one side and chemical-physical or environmental determinants (temperature, pH, medium composition, etc) on the other side.

V.1.1 Shake-flask experiments: evaluation of the maximum specific growth rate

To evaluate the maximum specific growth rate of the strains under investigation, yeast growth was carried out in shake-flask. Particularly, the growth was realized and monitored under conditions of substrate saturation.

As shown in the following Tab. V.1.1-1, μ_{\max} value of the prototrophic strain resulted to be 0.54 h^{-1} , whereas all μ_{\max} values of the auxotrophic strains were slightly lower, around $0.50 \pm 0.02 \text{ h}^{-1}$, as if the type and number of auxotrophies did not exert a strong negative influence on their performance.

Data relative to μ_{\max} don't give information on the most suitable host for the production among those tested, however, they provide important information for the set-up of the production process. Indeed if we consider that μ_{critical} (μ value in correspondence of which ethanol begins to be produced) is 60 % of μ_{\max} (Enfors, 2001), we can build up the exponential feeding profile for the aerated fed-batch reactor by choosing a value of 0.16 h^{-1} , well below 0.50 h^{-1} .

Table V.1.1-1 Maximum specific growth rate of *S. cerevisiae* CEN.PK prototrophic and auxotrophic strains

<i>Strain</i>	<i>Auxotrophy</i>	$\mu_{\max} \text{ h}^{-1}$
CEN.PK 113-7D	---	0.54
CEN.PK 113-5D	uracil	0.52
CEN.PK 111-32D	leucine	0.48
CEN.PK 102-3A	uracil and leucine	0.48
CEN.PK 102-5B	uracil, leucine and histidine	0.50
CEN.PK 2-1C	uracil, leucine, histidine and tryptophan	0.52

V.1.2 Experiments in fed-batch reactor: performance of the prototrophic S. cerevisiae CEN.PK 113-7D strain

To set up the fed-batch process and study the behavior of CEN.PK strains proliferating inside the reactor, the CEN.PK prototrophic strain of *S. cerevisiae*, i.e. the family member without nutritional needs, was taken into consideration as reference strain. Indeed this strain is not suitable as host to produce HEPs since it does not have the selection marker (the auxotrophy), but it is a good reference strain to study yeast behavior during fed-batch runs, being the parental strain of all the potential host strains.

As shown in Fig. V.1.2-1, the prototrophic yeast was capable to sustain the exponential growth during the entire feed phase (25 h). The average experimental value for specific growth rate was very close to that selected to build up the feeding profile (0.16 h^{-1}). The total biomass obtained at the end

of the fermentation run was 250 g corresponding, in a 2.3 l working volume, to a cell density of 110 g l^{-1} , similar to that reported in the literature for industrial yeast strains (van Hoek *et al.*, 2000) and for HCDC.

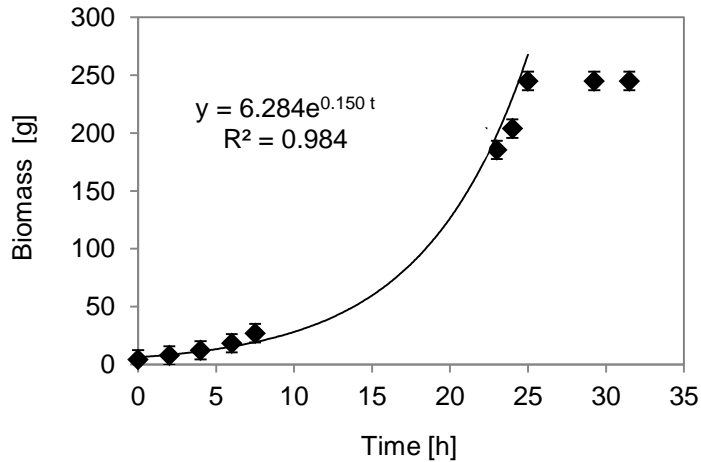


Fig. V.1.2-1 Growth in aerated fed-batch reactor of *S. cerevisiae* CEN.PK 113-7D (prototrophic strain) - feeding time duration 25 h.

V.1.3 Investigation in fed-batch reactor: influence of the type of auxotrophy

Having in mind to examine the possible influence that the type of auxotrophy can have on the performance of the strain selected as host for HEP production, two CEN.PK strains bearing a single auxotrophy, *S. cerevisiae* CENPK 113-5D and CEN.PK 111-32D, for uracil and leucine respectively, were taken into consideration and cultivated in aerated fed-batch reactor.

A remarkable difference existed between the two strains examined (see Fig. V.1.3-1), since the total biomass produced by CEN.PK 113-5D was approximately three-fold higher than that achieved by CEN.PK 111-32D. CEN.PK 113-5D, achieved a significant total biomass (about 190 g), slightly lower than that of the prototrophic strain (see section V.1.2).

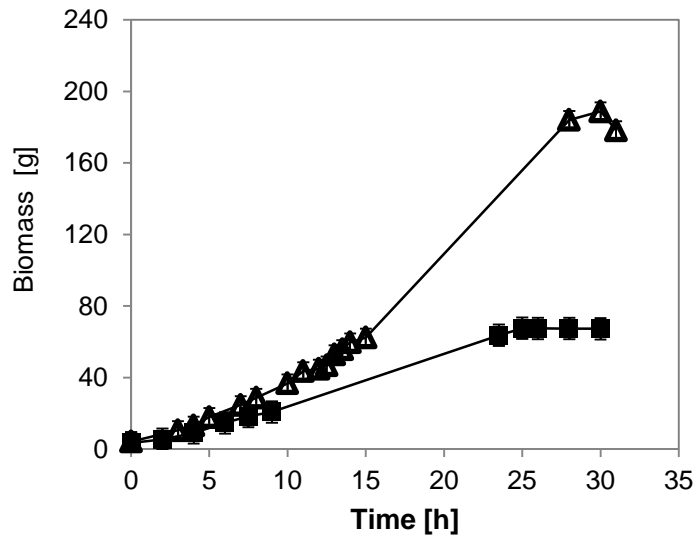


Fig. V.1.3-1. Growth in aerated fed-batch reactor of *S. cerevisiae* CEN.PK 113-5D (empty triangle), and *S. cerevisiae* CEN.PK 111-32D (full square).

V.1.4 The fed-batch reactor: influence of the number of the auxotrophies

To complete the study on auxotrophic yeast strains and evaluate the effect of the number of auxotrophies on the performance of *S. cerevisiae* CEN.PK strains in aerated fed-batch reactor, three strains, characterized by an increasing number of auxotrophies, CEN.PK 102-3A (two auxotrophies for uracil and leucine), CEN.PK 102-5B (three auxotrophies for uracil, leucine and histidine), and CEN.PK 2-1C (four auxotrophies for uracil, leucine, histidine and tryptophan), were taken into consideration.

The strain performance, expressed in terms of total biomass obtained, decreased with the number of auxotrophies (see Fig. V.1.4-1), even though the differences in total biomass among the three strains were not as great as that observed between strains bearing one autotrophy (see section V.1.3).

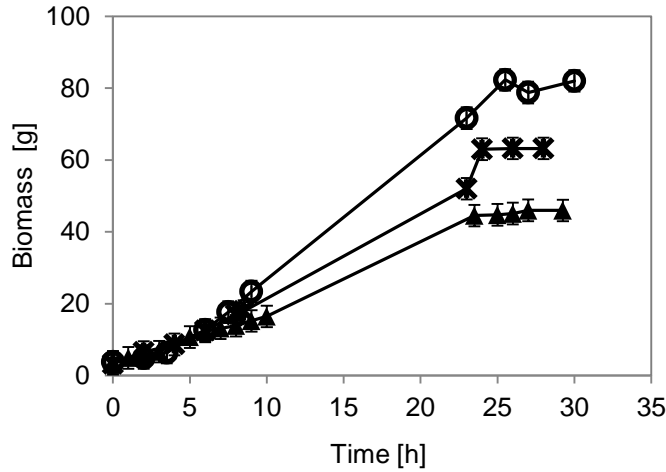


Fig. V.1.4. Growth in aerated fed-batch reactor of *S. cerevisiae* CEN.PK 102-3A (empty circle), *S. cerevisiae* CEN.PK 102-5B (star) and CEN.PK 2-1C (full triangle).

The most important design parameters of the bioprocess, the average biomass yield on glucose ($Y_{X/G}$), and the volumetric productivity (P_X) together with the final total biomass (X_{tot}), are reported in the table V.4.1-1 to summarize the results obtained with all the auxotrophic strains tested. These data were evaluated over the entire feeding phase (25 h). The highest value of each parameter was obtained from the strain carrying the single auxotrophy for uracil. Total biomass, biomass yield and biomass volumetric productivity decreased even more when the auxotrophy for leucine was introduced and dropped in the case of the strain carrying four auxotrophies.

Table V.1.4-1. Quantitative design parameters of the auxotrophic strains

	Total Biomass X_{tot} [g]	Biomass Yield $Y_{X/G}$	Volumetric Productivity P_X [$g\ l^{-1}\ h^{-1}$]
CEN.PK 113-5D	189	0.410	3.24
CEN.PK 111-32D	67.2	0.140	1.35
CEN.PK 102-3A	70.0	0.146	1.39
CEN.PK 102-5B	63.2	0.131	1.56
CEN.PK 2-1C	46.6	0.093	0.95

On the basis of the above results it is possible to conclude that the best producer strain is that bearing one auxotrophy, more precisely for uracil (*S.*

cerevisiae CEN.PK 113-5D). This strain should be was to design and optimize the entire production process.

V.2 Procedures for bioprocess optimization

In the section V.1.4, it was highlighted that the best east performer east strain to employ for HEP production is CEN.PK 113-5D. The next step for the bioprocess design was to set up an efficient optimization procedure of fed-batch culture with this strain. So, the optimal values of the chemical-physical parameters for the cell cultivation (T, pH, composition of the culture medium) were chosen and particular relevance was given to the basilar aspects of the bioprocess such as the issue regarding aeration.

Once fixed pivotal conditions, the work aiming at process optimization for the selected strain, began. Optimization of biomass productivity in fed-batch reactor, requires an increase in both the specific growth rate and the biomass yield to achieve the highest value possible under sugar-limited cultivation. Generally, the growth rate profile during fed-batch cultivation was controlled primarily by feed rate of the carbohydrate feedstock (Beudeker *et al.*, 1990). If the only objective is to maximize the biomass in the shortest time as possible, it is necessary to grow cells at a rate as close as possible to the critical growth rate (μ_{crit}), which depends exclusively on the yeast strain (Valentinotti *et al.*, 2002), avoiding ethanol and acetate formation.

The basic work made to search for the optimal growth rate profile, was combined with the study of the yeast behavior in the fed-batch reactor, considering the production system as a whole that is a system whose properties cannot be considered simply the sum of their components but so approaching with an holistic approach. This type of investigation considered the presence of both ethanol and glucose in the culture medium, the determination of the level of catalase activity in the cell lysate, the determination of the presence of oxidative stress through the ROS evaluation and the determination of the cell vitality/viability.

Importance was given to the presence of ethanol in the culture medium. Ethanol is an index, on macroscopic scale, of the switch from respiratory to the fermentative metabolism (Pronk *et al.*, 1996) that has been repeatedly observed for glucose sensitive yeast cultivation in fed-batch reactor (Landi *et al.*, 2011; Paciello *et al.*, 2009; Paciello 2009).

In order to gain a deeper insight in the phenomenon, therefore other significant metabolic intermediates were considered and determined. Glycerol and acetate accumulation was monitored in the different conditions, and the extent of cellular stress was assessed determining catalase activity, ROS formation and cellular vitality. Glycerol formation is a redox sink when

the mitochondrial reoxidation of cytoplasmic NADH is impaired, therefore we looked at glycerol as a marker of poor oxygenation. Catalase instead, is a complex of enzymes produced by the microorganisms during respiration to defend itself against ROS (Aebi, 1984 ; Martins *et al.*, 2005). The increase in catalase activity in the cell lysate can be viewed either as an increase in oxidative stress (such as due to an overproduction of ROS) or as an index of a fully respiratory metabolism of yeast growth.

In the following paragraphs are shown the results of experiments carried out first on the host strain and then on the strain modified by the insertion of the plasmid for the expression of IL-1 β used as model protein.

V.2.1 μ value optimization with *S. cerevisiae* CEN.PK 113-5D

In view of optimizing the bioprocess, the CEN.PK 113-5D strain was assayed during fed-batch runs which differed for the exponentially increasing carbon supply to the fermenter, so that the strain grew at three different values (0.1, 0.16 and 0.2 h⁻¹) of specific velocity (μ). To evaluate the performance of the CEN.PK 113-5D strain in aerated fed-batch, biomass profile (Fig. V.2.1-1A), biomass yield and biomass volumetric productivity (Fig. V.2.1-1B), the most important parameters in a bioprocess, were taken into consideration.

The run carried out at $\mu = 0.16$ h⁻¹ (25 h duration) resulted to be a good compromise between the longest-term run at 0.1 h⁻¹ (40 h duration) characterized by the highest biomass yield and the shortest term-run at μ 0.2 h⁻¹ (20 h duration). This concept is well displayed by plotting the product of biomass yield per volumetric productivity in function of the μ value (Fig. V.2.1-1C). This product in fact, is maximum in correspondence of $\mu = 0.16$ h⁻¹.

As already mentioned important parameters to evaluate the performance of the bioprocess are residual glucose and ethanol production.

Residual glucose (Fig. V.2.1-2A) is always almost zero for all the values of specific feeding rate assayed. This means that the producer strain has a good ability to metabolize this carbon source and no limitation occurs during the entire feeding run.

Instead, regarding ethanol (Fig. V.2.1-2B) there were differences depending on the specific feeding rate. At $\mu=0.1$ h⁻¹, residual ethanol coming from the batch phase was completely consumed and no accumulation of this metabolite was observed. Ethanol accumulation was observed with higher value of feed rate (μ 0.16 and 0.20 h⁻¹).

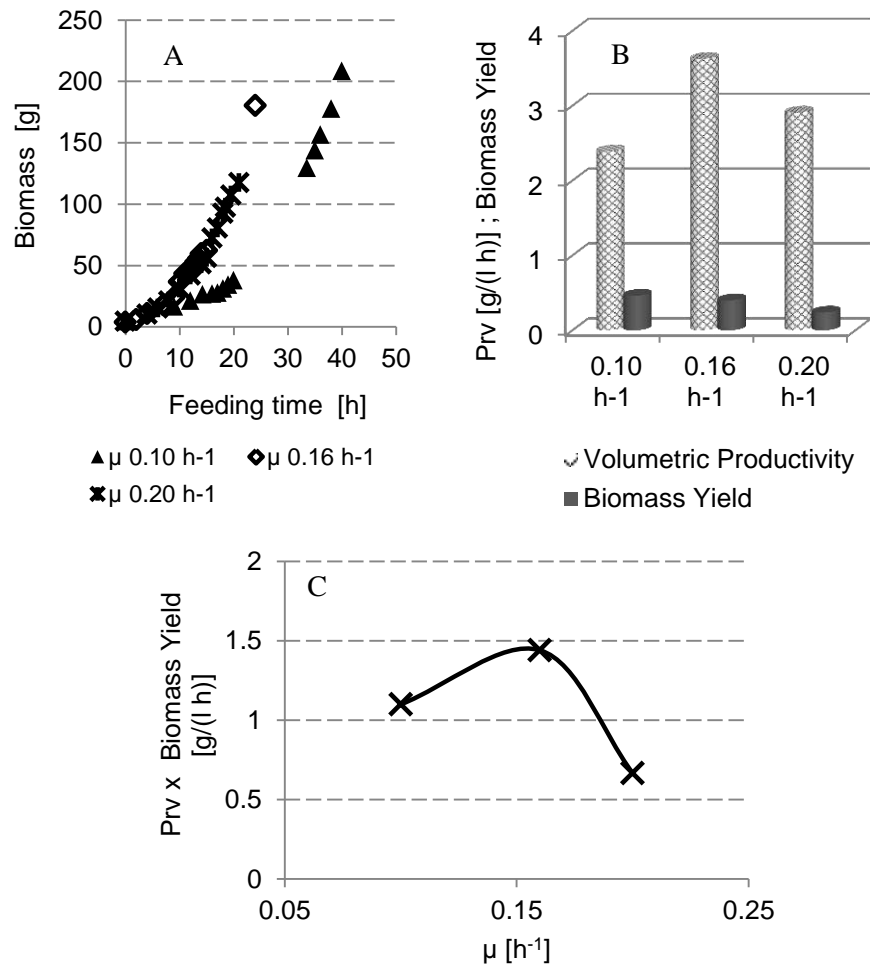


Fig. V.2.1-1 Biomass profile (A); biomass volumetric productivity and biomass yield (B); product of biomass productivity per biomass yield (C) of *S. cerevisiae* CEN.PK 113-5D growing in fed-batch reactor at different μ value: 0.10, 0.16 and 0.20 h⁻¹

The phenomenon of ethanol production, does not occurs immediately, but after a certain time of the run depending on the μ value imposed with the feeding profile. Particularly it was seen that there is a difficulty by yeast to grow through a respiratory way for long times of the run; this difficulty occurs in ever shorter time increasing the value of feeding rate.

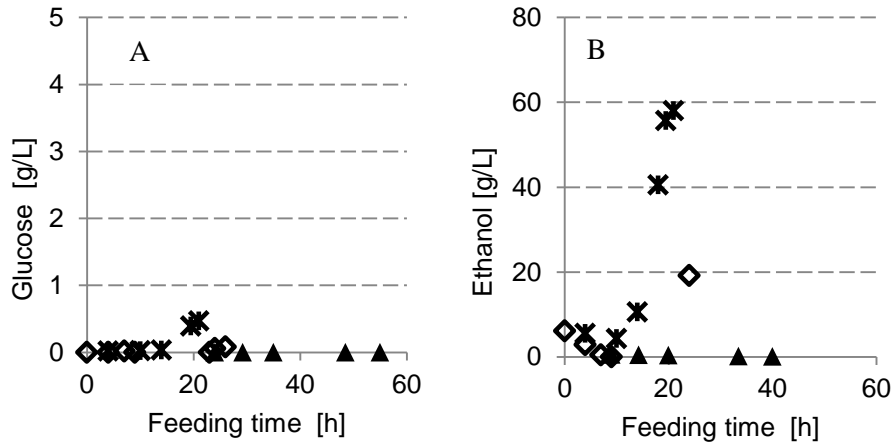


Fig. V.2.1-2 Concentration of residual glucose (A) and ethanol (B) in the culture medium of *S. cerevisiae* CEN.PK 113-5D growing in fed-batch reactor at different μ value: 0.10 h^{-1} (full triangle), 0.16 h^{-1} (empty rhombus) and 0.20 h^{-1} (star).

V.2.2 Validation of the μ value optimization procedure with *S. cerevisiae* CEN.PK 113-5D transformed for IL-1 β production

The μ value optimization procedure, carried out with the non-producer strain, was validate expressing IL-1 β as a model protein. As regard IL-1 β quantification performed with immuno-blot analysis, was observed that the amount of IL-1 β per biomass dry weight produced was almost constant (Fig. V.2.2-1A) and this is plausible since the heterologous product was expressed under a constitutive promoter. The value of the interleukin yield was around $0.08 \text{ (mg IL-1}\beta\text{)/(g d.w.)}$.

These considerations confirmed that all the assessments on the non-transformed strain were applicable also for the producer strain. Indeed was possible verify, that the optimal value of the feed rate to the bioreactor, in order to guarantee the maximization of the product between the volumetric productivity and IL-1 β yield on substrate (glucose), was that to set a specific growth rate of the microorganism of 0.16 h^{-1} constant (Fig. V.2.2-1B).

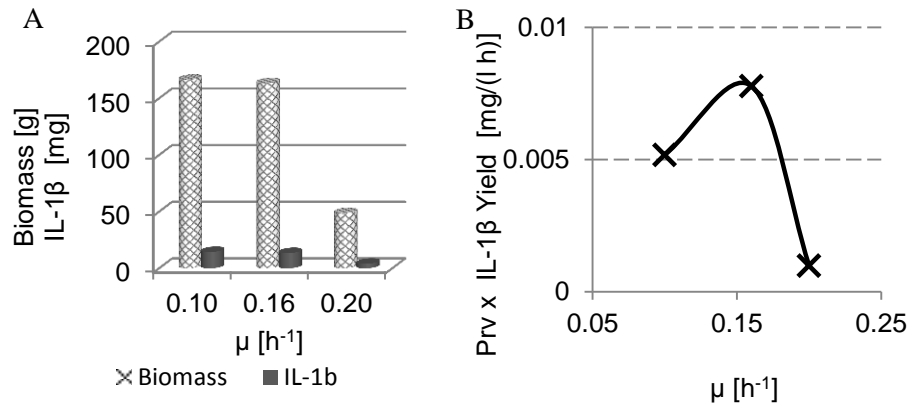


Fig. V.2.2-1 Biomass and IL-1 β production at the end of fermentation run with *S. cerevisiae* CEN.PK 113-5D [IL-1 β] for three different μ values imposed (A). Product between IL-1 β productivity and IL-1 β yield at different μ values (B).

V.2.3 Metabolic burden and physiological evaluations

The occurrence of metabolic burden, in *S. cerevisiae* CEN.PK 113-5D, was investigated considering the expression of IL-1 β according to the strategy described in section II.4.1 in the case that the HEP was expressed directly in the culture medium.

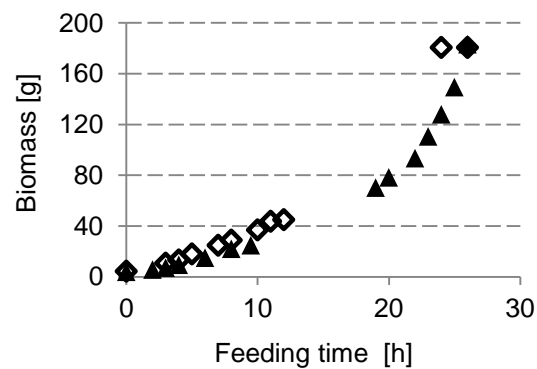


Fig. V.2.3-1 Growth in fed-batch reactor of *S. cerevisiae* CEN.PK 113-5D at a μ value of $0.16 h^{-1}$ (empty rhombus) and *S. cerevisiae* CEN.PK 113-5D [IL-1 β] at the same μ value (full triangle).

Growth curves of both the transformed and non-transformed strains are compared in Fig. V.2.3-1. The two biomass profiles almost overlap and so

for this HEP no metabolic burden was observed comparing only this parameter at the optimal μ value of 0.16 h^{-1} .

To have a wider view on the dynamics that regulate the metabolic fluctuation during yeast growth in the fermenter the catalase activity inside the cells was evaluated, assaying the lysates of the growing cells.

For the non-transformed strain CEN.PK 113-5D, catalase activity (Fig. 3.2) remains significantly high throughout the fed-batch run performed at $\mu 0.1 \text{ h}^{-1}$, indicating that only in this case the metabolism of yeast was always fully respiratory. At $\mu 0.16 \text{ h}^{-1}$, catalase activity decreased after the first 15 hours of run. At $\mu 0.2 \text{ h}^{-1}$, catalase activity was significantly lower. Differently, in the transformed strain, catalase was produced at lower levels (Fig. V.2.3-2), independently of the feeding profile used and the enzyme activity amounted, more or less, on the same value along the entire fermentation run.

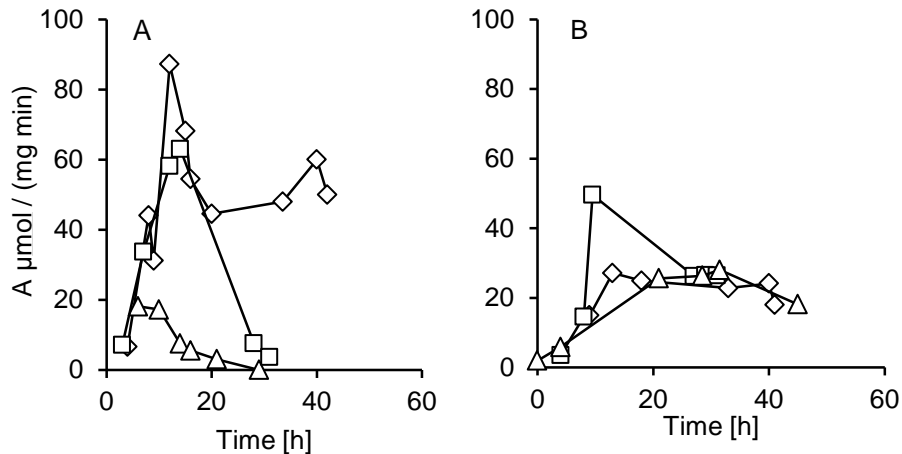


Fig. V.2.3-2. Catalase activity of *S. cerevisiae* CEN.PK 113-5D (A) and CEN.PK 113-5D [IL-1 β] cells growing in fed-batch reactor at different μ value: 0.10 h^{-1} (rhombus), 0.16 h^{-1} (square) and 0.20 h^{-1} (triangle).

Further information came from the analysis of the culture broth by HPLC (High Pressure Liquid Chromatography) for the determination of acetate and glycerol and with the contribution of the flow cytometry. Flow cytometry is a laser based, biophysical technology employed in cell counting, sorting, biomarker detection and protein engineering, by suspending cells in a stream of fluid and passing them by an electronic detection apparatus (Fig. V.2.3-3). The angular dependence of scattered light provides further information on the nature of the scattering particles, but, more importantly, appropriate fluorophores may be added to the cell suspension. These may be stains which bind to (or react with) particular molecules such as DNA, RNA, or protein allowing simultaneous multiparametric analysis of the physical and/or chemical characteristics.

Flow cytometry has been used extensively to monitor many different properties of yeasts; the information thereby obtained enables determination of the population distribution with respect to that property.

For our purposes flow cytometry has revealed an useful tool for the measurement of viable cells fraction, and ROS quantification during the growth in aerated fed-batch reactor.

As regard HPLC analysis, table V.2.3-1 reports glycerol and acetate concentrations in the samples collected just after ethanol appearance in the various experiments.

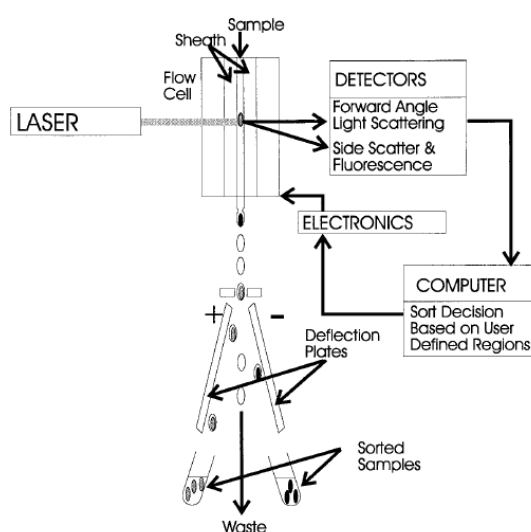


Fig. V.2.4-2 Flow cytometer diagram (Davey and Kell, 1996)

As concern glycerol, its accumulation was observed only for transformed strains (table V.2.3-1), with the highest concentration (about 4 g/l) observed for cells growing at a feeding rate of 0.2 h^{-1} . Since similar ethanol was detected productions in the non transformed strain (Fig. V.2.1-2) without a corresponding glycerol accumulation, it was possible to conclude that a poor oxygenation was not the main cause of metabolic shift from respiratory to fermentative metabolism.

In all occasions the total acetate concentration resulted quite low, with the highest value (350 mg/l) reached for the transformed strain (CEN.PK 113-5D [IL-1 β]) grown at a μ of 0.2 h^{-1} . Based on the literature data (Verduyn *et al.*, 1990) it is presumable that the amount of acetate present at the time of ethanol detection is too low to be considered responsible for the phenomenon. Moreover, acetate concentration didn't increase significantly in the following samples (data not shown).

Further indirect confirmation of the little toxicity of acetate in the experimental conditions adopted, come from the analysis of ROS formation and cellular vitality.

The monitoring of ROS evolution has been obtained at the single cell level in any condition along all the feeding, comparing the levels of fluorescence of the population under examination, obtained with a 123-DHR staining against the fluorescence obtained in a wealthy population of cells growing in exponential balanced phase on glucose. As reported in Table V.2.3-1, in any condition we could not measure relevant increase in the ROS formation, since the far large part of the population displayed fluorescence levels comparable to the control population. Finally as regard cell viability only a slightly decrease of the percentage of viable cell was observed in the transformed strain for IL-1 β expression.

Table V.2.3-1 Glycerol and acetate concentration in the supernatant evaluated by HPLC analysis and results of cytometry analysis at different μ values. ROS values are expressed as a percentage with respect to a signal obtained by stimulating the cells with hydrogen peroxide for 75 min.

		CEN.PK 113-5D			CEN.PK 113-5D [IL-1 β]		
		μ	0.1	0.16	0.20	0.1	0.16
μ	h^{-1}						
	g l^{-1}	<0.01	<0.01	<0.01	0.26	0.52	4.03
acetate	g l^{-1}	na	<0.015	0.160	<0.015	0.025	0.351
ROS	%	9.9	14.2	17.3	11.2	16.4	21.0
Viable cells	%	99.9	93.5	91.2	93.8	92.6	88.4

V.3 Expression of human globins in yeast: a preliminary study on growth parameters

The effect of heterologous expression of human globins in *S. cerevisiae* CEN.PK 113-5D was evaluated setting batch fermentation with an initial glucose concentration of 20 g l^{-1} , and monitoring the growth in the exponential phase until entering in the stationary phase after consumption of ethanol and glycerol, produced during glucose fermentation.

An easy comparison is shown in Fig. V.3-1 where the CO₂ profile obtained when the yeast strain was producing the globins, has been compared to the negative control represented by CEN.PK 113-5D transformed with the empty plasmid. Monitoring of this metabolite to describe and model yeast growth is widely used in experimental practice (van Urk *et al.*, 1988 ; van Hoek *et al.*, 1998).

By monitoring CO₂ profile of the transformed strain, in the case of the expression of Ngb and Myo (Fig. 5.3-1), an increase of the metabolic activity in the exponential phase, was observed, if it was compared to the reference strain. On the contrary, in the case of Hb expression, a lower CO₂ production was found. This could be related to the fact that this protein has a size four times higher than the other two (64 vs 17 KDa), and therefore the occurrence of metabolic burden can be hypothesized. The increasing of the metabolic capacity in the case of the strain expressing Mb and Ngb is also found after the glucose consumption (see CO₂ drop in Fig V.3-1) since after this time there was a more rapid attainment of the stationary phase in which the profile of CO₂ flattens.

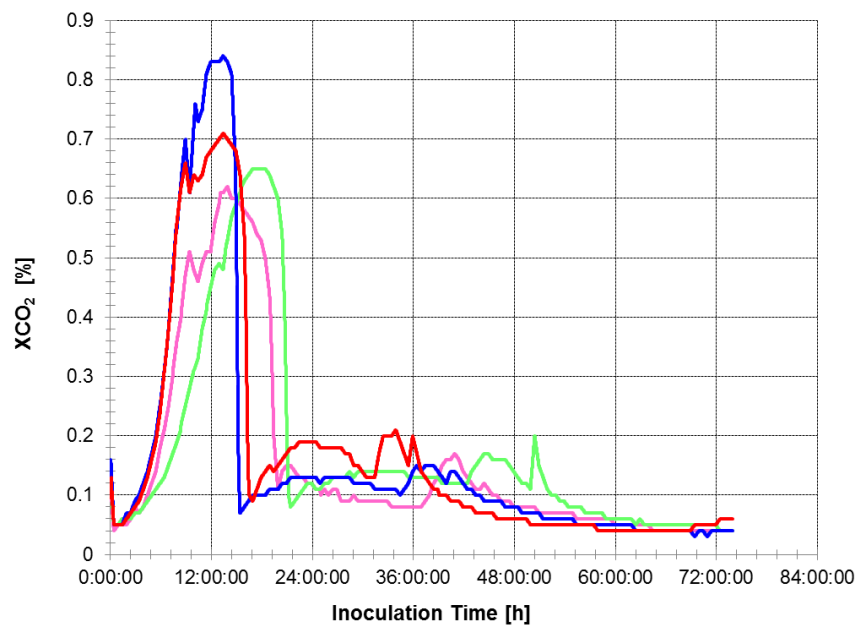


Fig. V.3-1 CO₂ profile of batch fermentation of CEN.PK 113-5D producing different human globins. Hb – green line; Mb – blue line; Ngb – red line; control – pink line.

In table V.3-1 the main important growth parameters are reported. An increase of specific growth rate of 7-12 % with Ngb and Myo expression (by comparison with the reference strain) was detected. Moreover Ngb seems to produce a slightly higher amount of biomass after 24 h but the biomass yield is not affected greatly by the globin expression.

Table V.3-1 Growth parameters of of *CEN.PK 113-5D* producing different human globins: maximum specific growth rate, cell density and yield on glucose after 24 h.

<i>HEP</i>	μ_{max} h^{-1}	$X_{24 h}$ $g l^{-1}$	$Y_{X/G 24 h}$
Hemoglobin	0.214	2.92	0.131
Myoglobin	0.385	3.6	0.155
Neuroglobin	0.402	4.11	0.178
Control	0.360	3.68	0.176

To better investigate the expression of hemoglobins in yeast, the mitochondrial morphology at the fluorescence microscope (Fig. V.3-2) was studied. Indeed, these structures have a key role in the respiratory metabolism because they generate most of the cell's supply of adenosine triphosphate (ATP), used as a source of chemical energy.

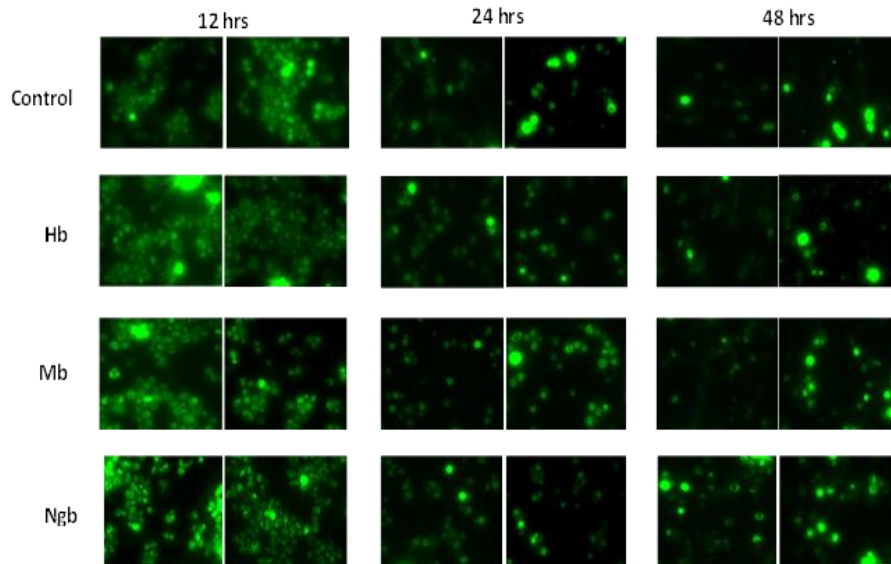


Fig. V.3-2 Batch cultures: mitochondrial morphology observed at fluorescence microscopy with Mitotraker Green[®] FM.

In the light of the results obtained, no substantial differences were highlighted looking at the morphology of mitochondria at different times of the batch process, expressing Mb or Ngb (see Fig. V.3-2).

V.3.1 Expression of human globins in yeast: physiological study in chemostat

In order to better investigate the effects of the expression of human globins a continuous culture or chemostat was prepared. This cultivation strategy is frequently employed to carry physiological studies in order to ensure the steady state conditions eliminating the problems of metabolic fluctuations during the time occurring with other culture systems (batch or fed-batch), as seen also in this work.

The dilution rate chosen was 0.1 h^{-1} , value commonly used for research study with *S. cerevisiae* (van Dijken et al., 1993; van Maris et al., 2004) that ensures yeast proliferation with a fully respiratory metabolism, without ethanol production.

The preparation of a chemostat culture was useful to perform staining test at the fluorescence microscope to evaluate ROS (Reactive Oxygen Species), responsible for DNA damages, mitochondrial morphology and the metabolic activity of the yeast cells.

Being that the expressed proteins have a role in the transport of oxygen inside the cell, two experiments were carried out by using two different concentrations of dissolved oxygen in the broth culture: 30% of air saturation, value normally used for yeast cultivation in bioreactor and a concentration significantly lower equal to 6%.

At this step, the analysis was developed considering only the expression of single chain globins (Ngb and Mb) compared to the reference strain in order to eliminate the influence of metabolic burden that occurs with expression of Hb. The analysis consisted in staining tests (see Material and Methods section) of samples collected and their observation at fluorescence optical microscope.

Looking at the mitochondrial network morphology highlighted by a fluorescent dye (Mitotraker Green® FM) significant differences are not detectable among control strain and those producing Mb and Ngb (Fig. V.3.1-1). Therefore, the heterologous expression of a single globin chain seems not to affect the mitochondria conformation.

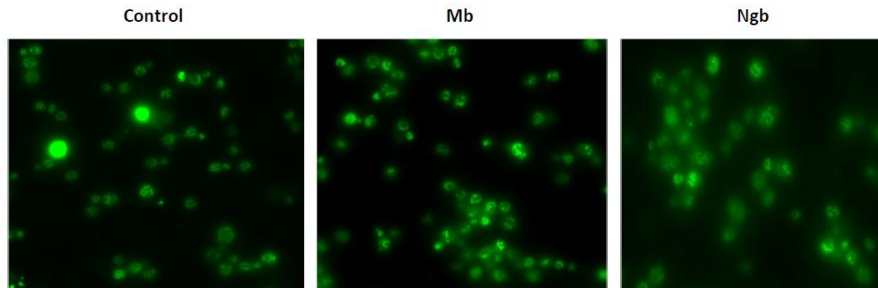


Fig. V.3.1-1. Chemostat cultures with a dilution rate of $0.1\ h^{-1}$: mitochondrial morphology (observed at fluorescence microscopy with Mitotraker Green® FM).

In Fig. V.3.1-2 are reported the results of the staining test for chemostat culture working at value of 6% dissolved oxygen. Generally, no stress conditions were observed also in the case of low oxygenation. Indeed the fraction of cell population in which ROS were detected, that is metabolically inactive and damaged at DNA level, is very low. Moreover, no relevant differences were found in the case of myoglobin and neuroglobin expression if compared to the behavior of the control strain.

An interesting thing is that in the case of the cultures performed in standard conditions of oxygenation (30% air saturation), it was observed a greater number of cells metabolically inactive in the case of the strains expressing Mb and Ngb (Fig. V.3.1-2B) but this behaviour was not confirmed at lower values of dissolved oxygen.

In conclusion with regard to the experiments carried out in chemostat with the operating conditions described above, it has not been well identified the influence of these particular proteins. However, the strains expressing Mb and Ngb exhibited, in batch culture, an increase of the specific growth rate, compared to non-producer strain. This behavior could lead to increases of the yeast performance when it is cultivated for bioproductions.

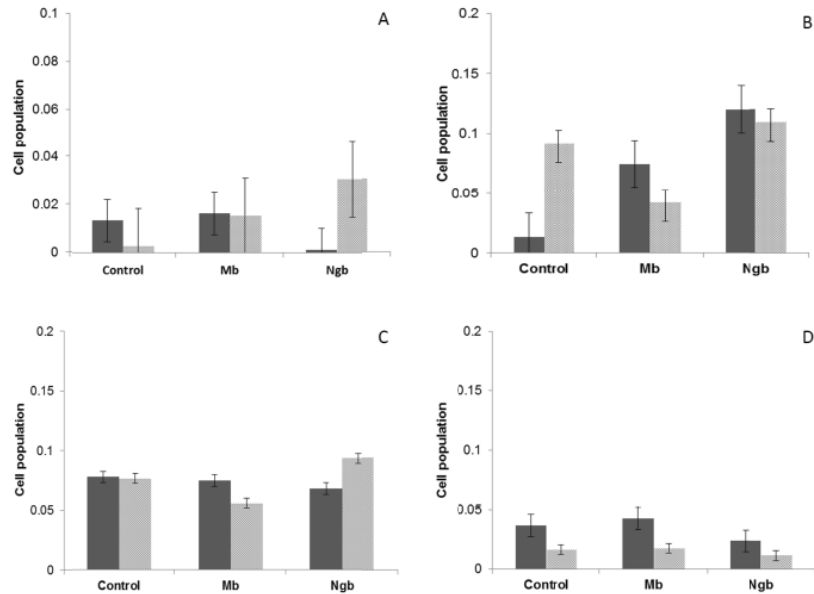


Fig. V.3.1-2 Chemostat cultures at 6% (grey) and 30 % (dark-grey) of air saturation and a dilution rate of 0.1 h^{-1} . Staining test to identify cells with presence of ROS (A), metabolically inactive (B), with abnormal mitochondrial morphology (D), with damaged DNA (D).

Therefore, this work can be interpreted as a development of studies aimed at the enhancement of the growth and respiratory characteristics of the yeast expressing hemoglobins especially of bacterial origin (Chen et al., 1994; Wei and Chen, 2008; Liu et al., 2010).

V.4 Lipase A production

The heterologous protein Lipase A was produced in shake-flask in order to confirm the lipolytic activity and its distribution in the broth-culture. After that, production tests were carried out in fermenter with the optimized conditions find for the non-transformed strain *S. cerevisie* CEN.PK 113-5D (see par. V.2).

The results of the first investigation shows that the enzyme activity was almost completely confined on microbial mass being negligible in the supernatant (table V.4-1).

Table V.4-1. Lipase activity and distribution in the broth-culture for *S. cerevisiae* CEN.PK 113-5D [LipA] growing in shake-flask for 24 h. one International Unit of activity (U) was defined as the amount of enzyme necessary to release 1 μmol of *p*-nitrophenol per minute

Cells d.w. [g/l]	Volumetric Activity [U/l]		Specific Activity [U/g]	
	Supernatant	Microbial mass	Supernatant	Microbial mass
3.43	17.0 \pm 0.8	431 \pm 20	5.0 \pm 0.2	125 \pm 6

To confirm the lipase activity distribution, two tests were carried out. In the first one, yeast cells were broken with Ballottini beads (ϕ 425÷600 μm) but no increasing of enzyme activity was observed in the supernatant so that cytoplasmic localization can be excluded (Fig. V.4-1). Cells were also treated with dithiothreitol (DTT) a reducing agent of disulphide bridges. This treatment should cause the detachment of the enzyme from the cells by reducing disulphide bridges. Contrary to the expectations no increasing of enzyme activity was observed in the supernatant after treatment of microbial cells with the reducing agent (DTT – 10 mM ; 1 h) (Fig. V.4-1). This is in contrast with the hypothesis that the enzyme was linked by disulphides bridges to the cell wall.

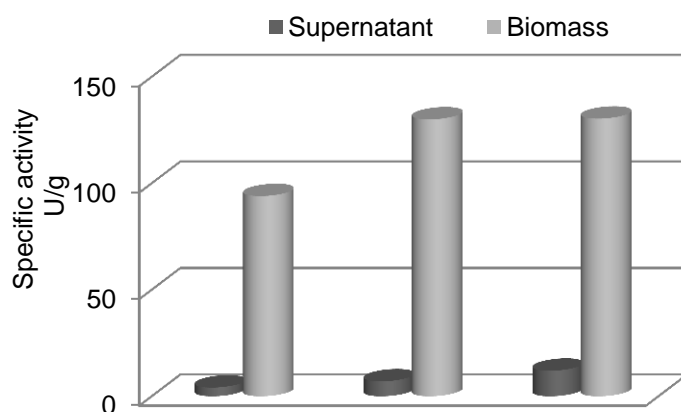


Fig. V.4-1 Lipase activities of supernatant and biomass after cell breakage and DTT treatments.

In short, we can say that the enzyme activity resulted to be associated to the microbial mass, but to date it is not clear yet the nature of the enzyme bond to *S. cerevisiae* CEN.PK cells.

Concerning the production of Lipase A in aerated fed-batch reactor this was carried out in the same growing conditions taken for *S. cerevisiae* CEN.PK 113-5D and *S. cerevisiae* CEN.PK 113-5D [IL-1 β] at the optimized μ value of 0.16 h⁻¹.

The growth results obtained with *S. cerevisiae* CEN.PK 113-5D [LipA] are far from those expected (Fig. V.4-2) and in contrast to that seen with the expression of interleukin. Particularly, in the case of lipase A expression, a strong inhibition of growth was detected since the early hours of feeding phase with the consequent failure in reaching the target to obtain a microbial culture with high cell density. In fact, the final cell density obtained in fed-batch reactor with *S. cerevisiae* CEN.PK 113-5D [LipA] was about 20 g/l vs. 90 g/l obtained with the non transformed parental strain.

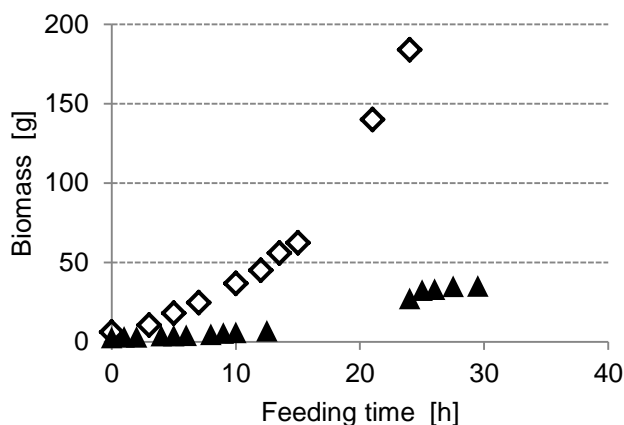


Fig. V.4-2 Growth in fed-batch reactor of *S. cerevisiae* CEN.PK 113-5D at a μ value of 0.16 h⁻¹ (empty rhombus) and *S. cerevisiae* CEN.PK ura⁻ [LipA] at the same μ value (full triangle).

From what has been observed experimentally, may be assumed that lipase expression determines a deleterious influence on yeast growth. Further, during proliferation, morphological changes in the transformed strains were observed with significant elongation of the cells (Fig. V-4-3). This effect may be due to the deleterious action caused by the heterologous enzyme on the cell-wall structure.

To confirm these data, *S. cerevisiae* CEN.PK 113-5D as well as, other two strains transformed with the lipase A expression immobilized on yeast cell wall were assayed. They are two *S. cerevisiae* strains: *S. cerevisiae* Y306 and *S. cerevisiae* BY4741. The first is an industrial diploid strain, Y306, and the second is a haploid laboratory strain.

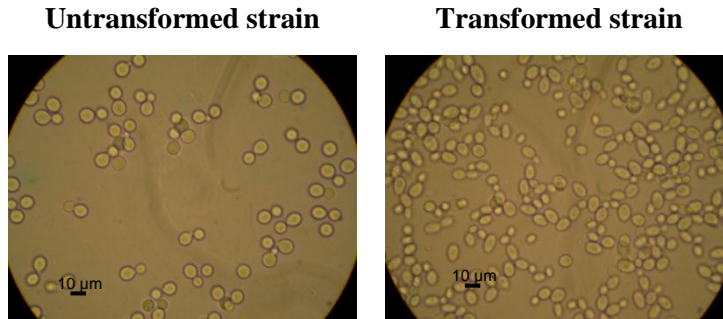


Fig. V.4-3 Observations at optical microscope (100X) of *S. cerevisiae* CEN.PK 113-5D non transformed and transformed strain with the lipase expression, during the fed-batch proliferation.

The growth in fed-batch reactor, of the three producer strains assayed was similar and always lower than expected (Fig. V.4-4), confirming the difficulty to produce immobilized lipase with *S. cerevisiae* yeast strains.

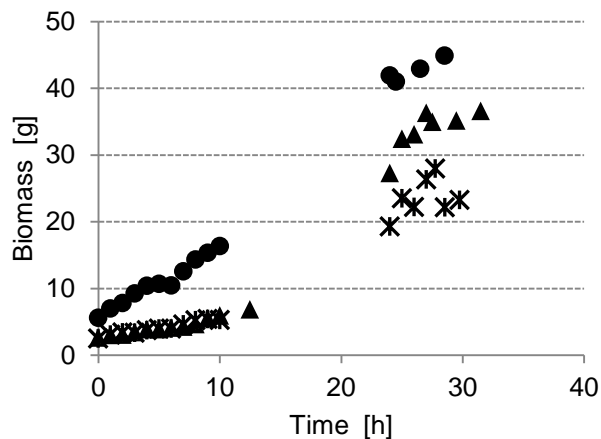


Fig. V.4-4 Growth in fed-batch reactor of *S. cerevisiae* CEN.PK 113-5D [LipA] (full triangle), *S. cerevisiae* BY4741 [LipA] (star) and *S. cerevisiae* Y306 [LipA] (full circle) at a μ value of 0.16 h^{-1} .

A complete evaluation of the production of lipase A can be made only by comparing the results of enzymatic activity of the brothculture. To summarize these results, they are reported in Fig. V-4-5 as the values of specific activity and volumetric activity at the end of feeding phase and at an intermediate time of the production process.

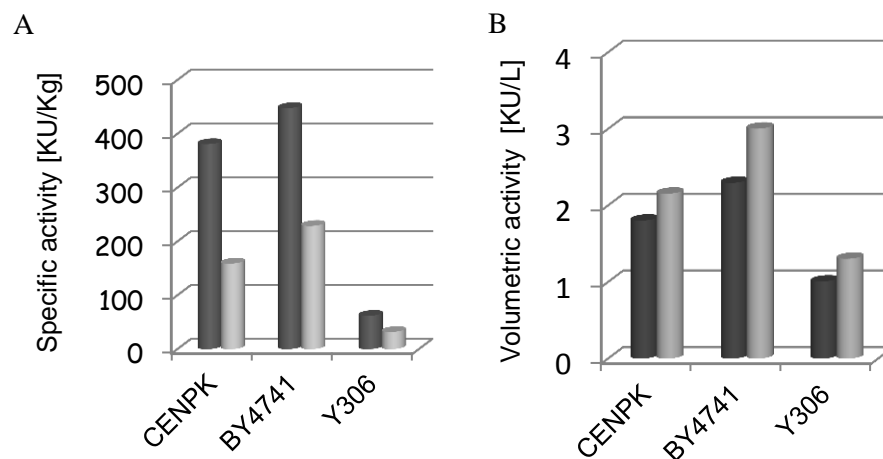


Fig. V.4-5. Specific activity (A) and volumetric activity (B) of three producer yeasts of heterologous lipase. The results are referred to the growth in fed-batch reactor at 10 (dark grey) and 25 (grey) h of feeding with a $\mu = 0.16 \text{ h}^{-1}$

As shown in Fig.V.4-5A *S. cerevisiae* strains produce different amounts of lipase activity per unit of cell mass (d.w.) during fed-batch run. This activity decreased after 10 h and reduced almost to the half at the end of run (25 h).

Considering the volumetric activity, its maximum value was achieved at the end of the fermentation run (25 h) and it was slightly higher than that obtained after 10 h. Therefore it was convenient the interruption of the fermentation after 10 h of feeding with a considerable saving of the carbon source.

V.5 Endoglucanase Production

Production of endoglucanase coded by CelA (EG-CelA) was carried out using both CEN.PK113-5D and Y306 strains of the yeast *S. cerevisiae*.

EG-CelA production was performed in bioreactor by setting up fed-batch cultivation at constant specific growth rate, being the specific growth rate value suitably chosen according to the optimization procedure (see Par. V.2).

More precisely, to produce EG-CelA, *S. cerevisiae* CEN.PK113-5D [EG-CelA] and *S. cerevisiae* Y306 [EG-CelA] have been cultured in aerated fed-batch fermenter supplied with a feeding profile set up to allow the exponential growth of the yeast strains at a constant μ value of 0.16 h^{-1} .

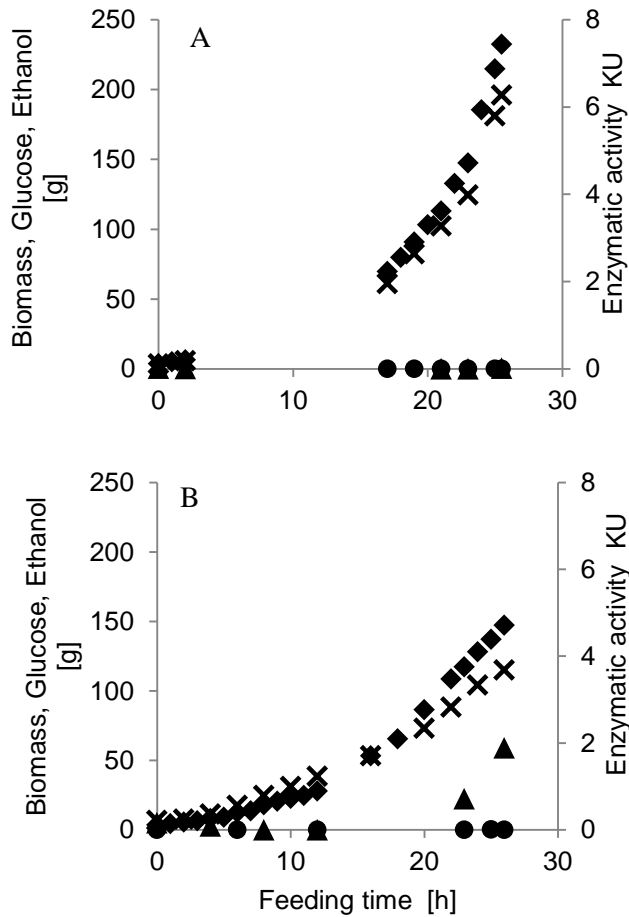


Fig. V.5-1 Growth in fed-batch reactor at $\mu=0.16 \text{ h}^{-1}$ of *S. cerevisiae* CEN.PK 113-5D [EG-CelA] (A) and *S. cerevisiae* Y306 [EG-CelA] (B). Biomass (rhombus), residual glucose (circle), ethanol produced (triangle), EG-CelA activity (star).

As regard CEN.PK113-5D [EG-CelA] (Fig. V.5-1A) total microbial mass, produced during the fed-batch only during the phase, grown following the established profile, only in the first 10 hours of exponential feeding. After this time, the specific growth rate started to gradually decrease and the biomass profile did not follow anymore the theoretical path (data not shown). The total microbial mass value reached at the end of the fermentation was significantly lower than the expected notwithstanding the metabolism of the strain was kept fully respiratory during the entire time-course of the cultivation (Fig. V.5-1A). This is also highlighted by ethanol production which leads the accumulation of this metabolite into the culture medium (Fig. V.5-1A) The product of interest, that is the recombinant

enzyme activity, increased during nearly the entire cultivation period reaching a plateau value during the last four hours as shown in Fig..

Regarding *S. cerevisiae* Y306 [EG-CelA] (Fig. V.5-1B), the total microbial mass produced during the fed-batch phase followed the established profile during the entire time course (data not shown). The value of total microbial mass, reached at the end of the fermentation, was substantially identical to the expected one (Fig. V.5-1B). Relatively to Y306 producing endoglucanase, it is possible to conclude that the metabolism of the strain was kept fully respiratory during the entire course of cultivation. In fact, ethanol did not accumulate in the medium at the end of the fed-batch run and no residual glucose was found in the culture medium (Fig. V.5-1B). The recombinant enzyme activity increased during the entire cultivation period reaching its maximum value at the end of the fed-batch run as shown in Fig..

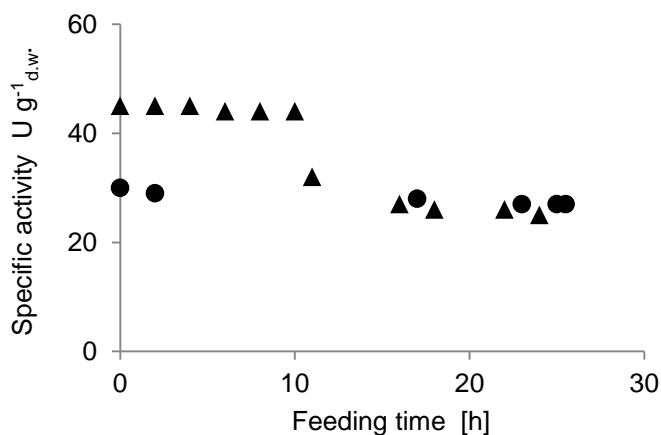


Fig. V.5-2 EG-CelA activity per biomass dry weight of CEN.PK 113-5D [EG-CelA] (triangle) and Y306 [EG-CelA] (circle) during fed-batch run

Looking at the product yield on biomass ($Y_{P/X}$), expressed as unit of enzymatic activity per cell mass unit, this was kept nearly constant over the entire time-course of fermentation only in the case of *S. cerevisiae* Y306 [EG-CelA] (Fig. V.5-2). Indeed, the EG was efficiently secreted into the culture medium along the entire time course of growth confirming that the recombinant enzyme was a growth linked product. This behavior was not present in *S. cerevisiae* CEN.PK 113-5D [EG-CelA], on the contrary it was characterized by a progressive decrease in enzyme activity per biomass unit (Fig. V.5-2). It should be noted that in the early hours of fed phase the specific enzymatic activity of *S. cerevisiae* CEN.PK 113-5D [EG-CelA] was higher than *S. cerevisiae* CEN.PK Y306 [EG-CelA], however it diminished after about 10 hours of run (Fig. V.5-2). This phenomenon could be associated to that observed in the strains transformed with *B. subtilis* Lipase A which resulted to be characterized by a reduction of cell percentage

holding the plasmid vector with the genetic information for the expression of heterologous protein.

The performance in the aerated fed-batch of the two producer strains proliferating at constant specific growth rate, has been compared in terms of final endoglucanase activity, productivity and yield on carbon source (Table V.5-1).

Summarizing the results obtained, it can be affirmed that *S. cerevisiae* Y306 [EG-CelA] was the best producer strain if compared to CEN.PK 113-5D [EG-CelA] when it is cultivated in a fed-batch reactor at the same constant specific growth rate; inasmuch Y306, exhibited the best performance at the end of bioprocess in terms of enzymatic activity, volumetric productivity and product yield on carbon source.

Table V.5-1 EG-CelA activities, productivity and glucose yield at the end of the fed-batch run at $\mu=0.16\text{ h}^{-1}$ feeding 450 g of glucose

	Specific activity $U\text{ g}_{d.w.}^{-1}$	Vol. activity $U\text{ ml}^{-1}$	Vol. productivity $U\text{ l}^{-1}\text{ h}^{-1}$	Yield on glucose $U\text{ g}_{gluc}^{-1}$
CEN.PK 113-5D [EG-CelA]	25.0	1.76	67.6	8.16
Y306 [EG-CelA]	27.0	2.58	101	13.9

V.6 Investigations on growth limitations to obtain HCDC

In this section is reported an overview of the investigations-researches that are aimed at studying the limitations in the achievement of HCDC.

Theoretically, fed-batch system should ensure, even if for an infinite feeding time, achievement of a cell density value equal to the product between biomass yield and substrate concentration. This means that feeding the fermenter with a glucose concentration of 500 g l^{-1} , it is possible to hypnotize to reach with *S. cerevisiae* a biomass concentration near to $250\text{ g}_{dw}\text{ l}^{-1}$ being the biomass yield on glucose 0.5 (under respiratory metabolism conditions). Actually, as widely discussed in Chapter I, only for a successful bioproduction with *S. cerevisiae* it is possible to overcome the limit of $100\text{ g}_{dw}\text{ l}^{-1}$. Indeed during presentation of the experimental results shown so far, it has repeatedly found that *S. cerevisiae* strains, when cultured in an aerated fed-batch reactor showed a reduced proliferation capacity, notwithstanding proper nutrients was supplied. This reduced capacity could not be exhaustively explained. As seen, this phenomenon is amplified by the

presence of the auxotrophy (see par. V.1.3) or by the expression of a peculiar HEP (see par. V.4) but first or later, its occurrence becomes evident as shown below. In Fig. V.6-1 are reported the trends of the main process variables (biomass, ethanol and glucose concentration) for a fed-batch run with the prototrophic strain *S. cerevisiae* CEN.PK 113-7D. This experiment differs from that shown in par. V.1.2 since feeding time was prolonged to observe the growth decay phase, even in the case of the stronger performer tested in fed-batch reactor in this work.

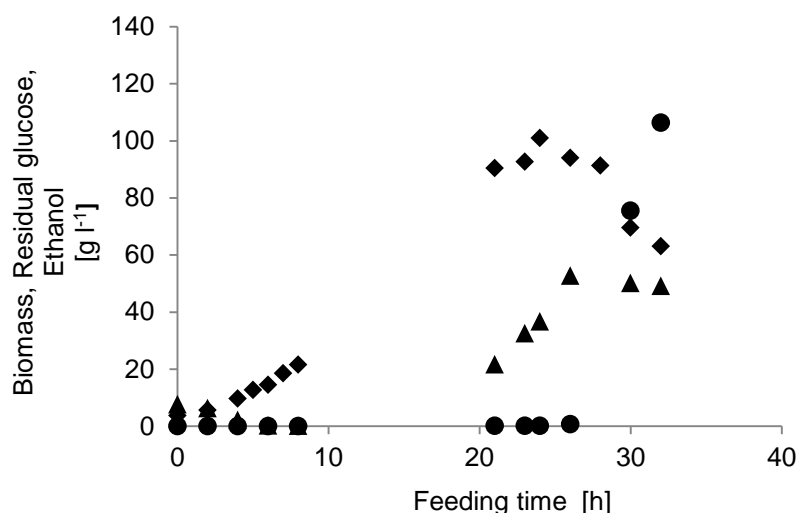


Fig. V.6-1 Growth in fed-batch reactor at $\mu=0.16\text{ h}^{-1}$ of *S. cerevisiae* CEN.PK 113-7D for prolonged time. Biomass (rhombus), residual glucose (circle) and ethanol (triangle) concentration.

Indeed, it was found that after achievement of a cell density slightly higher than 100 g l^{-1} growth dramatically decreases. This phenomenon was accompanied, as frequently observed, by a metabolic shift to fermentative metabolism with ethanol production. A later stage of decay was characterized by the inability to metabolize glucose fed to brothculture with its accumulation in the media and further growth depletion (Fig. V.6-1).

At this point, assumptions on the causes of this depletion were made. The first hypothesis was that the growth deficiency could be caused by occurrence of damages to the microbial mass, given stressful growth conditions. But this seems to be disproved by the experimental results of each stress factors investigations (see par. V.2.3 and V.3.1); Moreover it has been repeatedly observed that by proliferating the microbial mass subjected to growth depletion, in a new growth environment it takes again its initial vigor (data not shown). It is therefore subsequently hypothesized that damage could be of the culture medium (and not of the microorganism) or due to

substance/substances accumulation in the medium that cause microbial growth inhibition.

Fig. V.6-2 shows how growth on the supernatant of a strain (*S. cerevisiae* CEN.PK 2-1C), which exhibited a poor performance in fed-bach reactor (see par V.1.4), is totally inhibited. This phenomenon is not so pronounced (Fig. V.6-2) in the case of CEN.PK 113-5D, that is the best performer among the auxotrophic strains tested in this work. It would also be specified that greater is the strain proliferation in the medium greater should be the impoverishment of the nutrients necessary for growth, despite this the growth is more vigorous than in the case of a medium in which less biomass has proliferated.

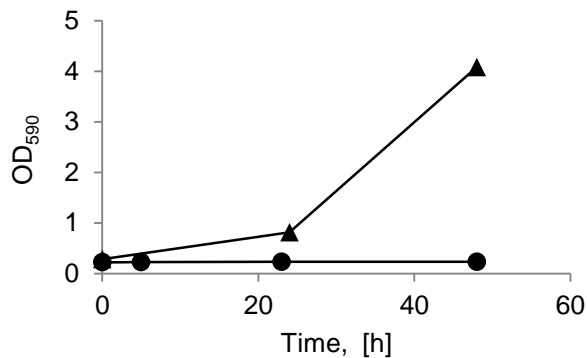


Fig. V.6-2. Growth in shake flasks in supernatant coming from fed-batch run of 24 h duration at μ of 0.16 h^{-1} for *S. cerevisiae* CEN.PK 113-5D (triangle) and *S. cerevisiae* CEN.PK 2-1C (circle). The medium was prepared supplementing the supernatant with vitamins and traces according to a typical growth in flask (see Materials and Methods) with 2 % w/v initial glucose. Inoculum was constituted by a fresh preculture of the same strain used in the fed-batch culture.

Fig. V.6-3 shows the percentage of growth inhibition for growth in a medium constituted by the supernatant of fed-batch run of CEN.PK 113-5D. The supernatant was subjected to different treatments to investigate the nature of the possible inhibitory compound. First ethanol accumulated in the culture medium was removed showing that the recovery on the inhibition of growth, was very modest (Fig. V.6-3) excluding that this metabolite is the only one to give a negative effect and growth depletion. The same supernatant was thermally treated at $121 \text{ }^\circ\text{C}$ for 20 and 40 min showing reduction of its inhibitory capacity of more than 50 %, despite the fact that it is well-known the damaging of nutrients in the culture medium after heat treatments (Bridson and Brecker, 1970). On the basis of the results obtained

it can be assumed that during the fed-batch run, the yeast exuded inhibitory substance/substances. Most likely were heat-labile compounds.

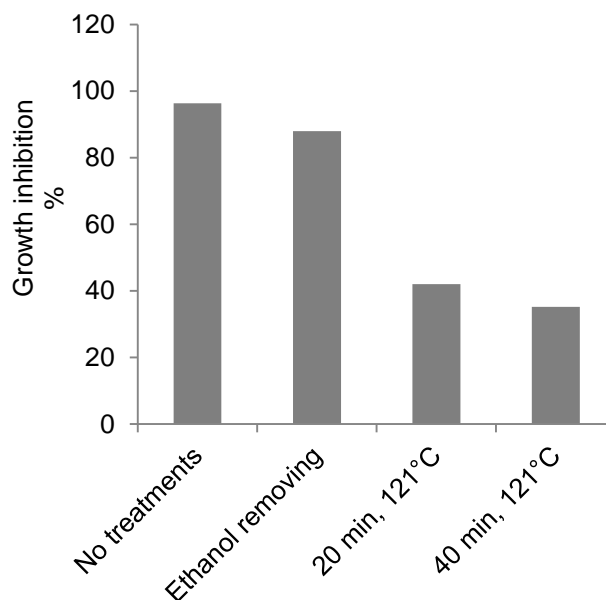


Fig. V.6-3 Percentage of growth inhibition on exhausted medium coming from fed-batch run of 24 h duration at μ of 0.16 h^{-1} for *S. cerevisiae* CEN.PK 2-1C. The supernatant of fed-batch growth was submitted at different treatments: complete ethanol removal under vacuum at 38°C and heat treatments in autoclave a 121°C for 20 and 40 min. The medium was prepared supplementing the supernatant, after treatments, with vitamins and traces according to a typical growth in flask (see Materials and Methods). The growth was compared to that on a fresh SDM after 24 h.

V.7 Yeast growth model results

In this section are reported the results referred to the mathematical modelling of the bioprocess described in Chapter IV. As shown in Fig. V.7-1, the parameter regulating the negative feed-back of the toxic compound, σ_T , have a crucial rule. Indeed, only taking into account the effect of toxic compound (Fig. V.7-1B), a good fitting of either biomass glucose and ethanol was reached. Indeed, without taking into account the effect of toxic compound (Fig. V.7-1A), the fitting of biomass glucose and ethanol was not achieved. More precisely an acceptable fitting, with $\sigma_T=0$, was realized only for the batch and for the early hours of bioprocess but without toxic compound influences model was not able to describe growth decay,

metabolic shift with ethanol production and glucose accumulation occurring in the late stage of fed-batch run (Fig. V.7-1A).

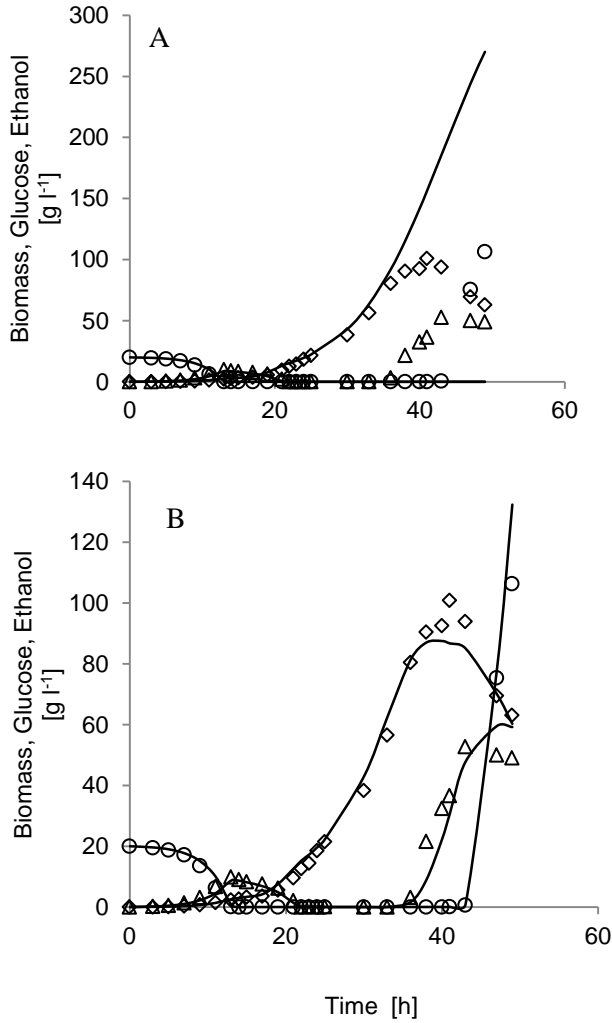


Fig. V.7-1 Experimental data vs. simulated data of CEN.PK 113-7D growth in fed-batch experiment for biomass (rhombus), ethanol (triangles) and glucose (circles) concentrations. Lines represent simulation results for $\sigma_T = 0$ (A) and with inhibitor negative feedback, $\sigma_T = 1$ (B). R^2 values referred to the fitting of the graphs B are 0.989, 0.989 and 0.935 for biomass glucose and ethanol respectively.

On the contrary, when the effect of toxic compound was considered, mathematical model well described trends of process variables considered of both batch and fed-batch phase (Fig. V.7-1B).

Values of model parameters are reported in table V.7-1, they are processes parameter, come from literature indication or calibrated. Parameter calibration was formulated as an unconstrained nonlinear optimization of the sum of the squared errors between simulated and experimental values of three state variables (B , G and E) using the software MATLAB R2012b.

Table V.7-1 Model parameter estimation

Parameter	unit	Source	Value
Process parameters			
t_B	h		15
C_F	g l ⁻¹		500
μ	h ⁻¹		0.16
Y_R	--	<i>Lievense and Lim, 1982</i>	0.50
Glucose uptake parameters			
v_G	h ⁻¹	<i>Heyland et. al, 2009</i>	3.64
k_G	g l ⁻¹	<i>Weusthuis et al. 1994</i>	0.18
η_G	--	<i>Christen and Sauer, 2011</i>	0.92
Respiration parameters			
v_R	h ⁻¹	<i>Calibrated</i>	1.5
k_R	g l ⁻¹	<i>Calibrated</i>	0.075
v_E	h ⁻¹	<i>Sonnleitner and Hahnemann, 1994</i>	0.22
k_E	g l ⁻¹	<i>Sonnleitner and Kappelli, 1986</i>	0.10
η_R	--	<i>Calibrated</i>	0.83
η_{RE}	--	<i>Paalme et al., 1997</i>	0.68
Fermentation parameters			
v_F	h ⁻¹	<i>Calibrated</i>	4.88
k_F	g l ⁻¹	<i>Calibrated</i>	0.10
v_S	h ⁻¹	<i>Calibrated</i>	5.0
k_S	g l ⁻¹	<i>Calibrated</i>	1.0
η_F	--	<i>Calibrated</i>	0.14
η_S	--	<i>Calibrated</i>	0.2
$[R]_{max}$	g l ⁻¹	<i>Experimental</i>	40
Metabolic regulation parameters			
a	--	<i>Calibrated</i>	$2 \cdot 10^{-4}$
b	l g ⁻¹	<i>Calibrated</i>	195
$[P]_{max}$	g l ⁻¹	<i>Literature indications (Yang et al., 2001; Diderich et al., 2001)</i>	1.0
Death and toxicity control parameters			
δ	h ⁻¹	<i>Calibrated</i>	0.03
ρ	--	<i>Calibrated</i>	0.01
σ_E	--	<i>Calibrated</i>	1.4
$[E]_{max}$	g l ⁻¹	<i>Literature indications (Walker, 1998)</i>	100
σ_T	--	<i>Calibrated</i>	1.0
$[T]_{max}$	g l ⁻¹	<i>Fixed</i>	1.0
τ	--	<i>Calibrated</i>	0.8

Discussion and Conclusions

The research project that I developed during the period of my PhD was aimed, at studying the setting-up and optimization of high-cell density cultivation (HCDC) systems to produce heterologous proteins by using *Saccharomyces cerevisiae* as host for protein expression and developing a suitable mathematical model through a dynamic structure capable to simulate yeast behavior in batch and fed-batch reactor

At the beginning of my research project, being already available an expression system well proven (Andrès *et al.*, 2005) and a suitable formulation of the culture medium for batch and fed-batch (semi-continuous) cultures, I proceeded systematically to i) set-up the reactor in which to realize the HCDC, ii) choose the yeast to use as host of heterologous expression and iii) establish operative conditions to be applied during the process to achieve the maximum productivity.

Productivity is a function of both cell density and specific productivity, so it increases by increasing the cell density and specific productivity. Increasing productivity is the major objective of fermentation in research and industry and as mentioned by Riesenber and Guthke (1999), HCDCs are a prerequisite to maximize the amount of product in a given volume within a certain time.

To achieve HCDC, many cultivation methods have been described (Bunch, 1994). They fall into two groups, those that are a modification of the stirred tank reactor such as semi-continuous stirred tank reactor and those that are developed around the “steady-state” concept, which include both freely suspended and immobilized, continuous cell cultures. The former type of fermenter is attractive because need fairly minor modifications to conventional batch, whereas continuous cultures have never been employed extensively for reasons related both to the working practices of industry and to biological properties of microorganisms which can often show unstable productivity in continuous systems.

Fed-batch fermenter can be considered as the most successful culture system for increasing the volumetric productivity and biomass yield especially for processes carried out with glucose-sensitive yeast such as *S. cerevisiae*. In *S. cerevisiae*, alcoholic fermentation and respiration occur

simultaneously when the sugar concentration in aerobic cultures exceed a critical value (De Deken, 1966; Petrik *et al.*, 1983). Occurrence of alcoholic fermentation has to be avoided during heterologous protein production because it negatively affects biomass yield, being ATP yield from fermentation much lower than from respiratory sugar dissimilation (van Hoek *et al.*, 1998). In fed-batch fermenter, aerobic ethanol production can be avoided since sugar concentration and consequently yeast specific growth rate can be controlled by supplying the fermenter with glucose at a proper feed rate (Beudeker *et al.*, 1990). Oxygen demand in HCDC is high and oxygen limitation has to be avoided because it results, in formation of several metabolites such as, acetate, lactate, ethanol, and hydrogen, as regards yeasts in ethanol, acetate and glycerol, which are undesirable and decrease the productivity (Castan *et al.*, 2002; Enfors *et al.*, 2001). Moreover, when oxygen enriched air or pure oxygen is used to achieve high feed rate, it should be considered that oxygen itself is potentially toxic. In the literature, oxygen supply has been reported to be increased in several different manners even by resorting to devices such as pressurized conditions (Belo and Mota, 1998). In the present work, oxygen has been supplied to the reactor by air sparging, keeping the dissolved oxygen tension (DOT) at 30% air saturation by the cascade system which acted, at first, on impeller speed, and when this latter reached its maximum value (1000 rpm), on air enrichment with pure oxygen through a valve with automatic opening.

As regards the microorganism to be utilized as host for the heterologous protein expression, nowadays researchers seem to be oriented, by using the powerful tools of genetics and cellular engineering, to build up a better host (Jena and Deb, 2005) or redirecting the metabolic pathway to the massive synthesis of a given product (Hou *et al.*, 2012). A large number of auxotrophic strains of the yeast *S. cerevisiae* have been developed and extensively used to produce heterologous proteins (Russo *et al.*, 1995) because they ensure the maintenance of plasmids with selectable markers. In the present work, aiming at selecting the host that would provide the best performance in a fed-batch culture, six isogenic haploid strains belonging to the CEN.PK family of the yeast *S. cerevisiae*, one prototroph and five auxotrophs, have been considered with the purpose to carry a systematic investigation on the effect of auxotrophies on their growth in aerated fed-batch reactor. The strains belonging to the CEN.PK family have been considered as reference strains in both basic and applied research on yeast (van Dijken *et al.*, 2000). Four of the strains were characterized by an increasing (up to four) number of auxotrophies, two of them differed only for the type of auxotrophy (uracil or leucine). The feeding strategy used to achieve HCDC consisted in an exponentially increasing feed covering the entire run so yeast strains grew with a constant specific growth rate. The auxotrophic yeast strains were compared on the basis of total biomass, biomass yield on sugar and volumetric biomass productivity, the most

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important quantitative design parameters considered in the industrial cultivation of *S. cerevisiae*. In terms of total biomass obtained, it resulted that the yeast strain performance decreased with the number of auxotrophies, and glucose supplied to the reactor was completely consumed during almost the entire runs except for CEN.PK bearing four auxotrophies which accumulated ethanol in the medium at the end of the feeding phase. Further, a deviation from the ideal behavior was evidenced for all the auxotrophic CEN.PK strains examined, it occurred after about 7 h of the feeding phase and consisted in a sudden decrease in the specific growth rate (μ), which remained constant from that time on. The decrease in the μ value was greater the higher the number of auxotrophies.

The results of this work identified *S. cerevisiae* CEN.PK113-5D as the best performer that is the strain which should be preferably selected as host for the expression of a heterologous protein, in terms of final biomass, biomass yield on glucose, volumetric productivity and the capacity of maintaining the specific growth rate as near as possible to the μ value imposed. Indeed, this latter declines with the increase in the number of auxotrophies.

Also the origin of auxotrophy has been highlighted to exert a profound effect on the performance of the auxotrophic strains as shown by the strong deleterious effect of the auxotrophy for leucine, in accordance with other observations regarding leucine auxotrophic strains (Choen *et al.* 2007). Only in the case of CEN.PK 2-1C bearing four auxotrophies, the intracellular accumulation of reactive oxygen species (ROS) during the run was indicative of an oxidative stress induced presumably by the exposure of the cells to the peculiar conditions of the aerated fed-batch cultivation. As a matter of fact, fed-batch operations are generally carried out, as previously mentioned, under conditions of vigorous and continuous aeration, so that oxygen stress is almost unavoidable. It is known that ROS accumulation is responsible for serious damages to biomolecules and cellular structures (Costa *et al.* 2001) and, therefore ROS could be evoked for the loss in cell viability encountered during the process. Apparently, CENPK and the other auxotrophic strains, have been resulted unable to face the vigorous aeration applied during the fermentation run to maintain the oxygen concentration at saturating levels.

On the whole, the results obtained by Landi *et al.* (2011) have highlighted that the behavior of the yeast strains tested could be considered as the result of the interaction between biological (genotype, metabolic properties, etc.) and environmental (operative conditions) determinants and that the dynamics of metabolic interactions which characterize the system would deserve a deeper insight, through the study of the networks that originate yeast behavior in the fed-batch.

Once selected as the best performer, in view of the optimization of the bioprocess, *S. cerevisiae* CEN.PK 113-5D, carrying one auxotrophy (for

uracil), has been tested in different operative conditions to find those values of specific growth rate which ensured high volumetric productivity and high biomass yield in order to develop an economically suitable bioprocess. CEN.PK 113-5D strain was tested as such (non-transformed) and once transformed (recombinant) to express human interleukin-1 β (IL-1 β), used as model protein in this work.

The recombinant strain has been obtained after transformation of *S. cerevisiae* CEN.PK113-5D with the shuttle vector pIA1 (Andrès *et al.*, 2005) carrying the translational fusion between the gene of human interleukin-1 β (IL-1 β) and the gene of the Pir4 cell-wall mannoprotein (Moukadiri *et al.*, 1999), so as to drive the secretion of IL-1 β to the growth medium (Paciello *et al.*, 2010).

The choice to test *S. cerevisiae* CEN.PK113-5D strain, as either non-transformed or recombinant, has been founded on the need to investigate the influence of recombinant protein expression on the metabolic behavior of the host. In the perspective to optimize IL-1 β production, the performance has been studied by varying the feeding rate of the carbon source to the fermenter.

As regards the feeding strategy, it has been chosen to supply the fermenter with an exponentially increasing feeding as in the previous investigation to allow cells to grow at constant specific growth rate. Further exponential feeding provides the advantage of controlling the specific growth rate below the critical value and so avoiding by-product formation.

On the basis of the results obtained, it has been possible to identify a suitable value of specific growth (0.16 h⁻¹) in correspondence of which maximum volumetric productivity was achieved.

Anyway in all the fermentation runs realized, it was evidenced again that yeast growth diminished over and then arrested notwithstanding the feeding went on. By considering, in a holistic approach, the behavior of the proliferating yeast in the HCDC system as the result of influence exerted by both the biological and the environmental components, the occurrence of stress phenomena have been taken into consideration because they might be considered as responsible for the observed behavior. Oxidative stress has been evaluated by determining the levels of both catalase activity, and reactive oxygen species (ROS) as toxic by-product formation. The overflow metabolites ethanol, acetate, glycerol have also been tested. In the light of the results obtained, CEN.PK113.5D the *S. cerevisiae* strain chosen for heterologous protein production, did not result to be affected by oxidative stress phenomena nor exposed to the action of substances generally recognized as toxic (e.g. acetate) and resulted to have a good performance as long as it was provided with a suitable feed rate in correspondence of which a compromise between volumetric productivity and biomass yield could be reached. Also in the case of CEN:PK113-5D and the parental strain the same

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growth decay and growth arrest phenomena were observed even if it was necessary to prolong the feeding up to 45 h.

The period of study and work spent, as part of my PhD, at the laboratory of Systems Biology in Chalmers University, headed by Prof. J. Nielsen, has been useful to deepen my knowledge on other production systems and some aspects of CEN.PK113-5D the microbial cell factory used in Goteborg too and so enlarge my cultural background. There, the topic of my work has been to study the effect of the protein expression on the kinetic parameters of the growth of CEN:PK113-5D the same strain that I have used for the other productions of this investigation. Other proteins of human origin, in addition to IL-1 β , have been tested there with the purpose to evaluate the influence of their expression in yeast. Particularly, I had the possibility to study the effect of the expression of human globins on the specific growth rate of *S. cerevisiae* CEN.PK 113-5D. These proteins, involved in oxygen transport phenomena inside the cell, have been tested in batch and continuous stirred reactor or chemostat. The results of this investigation have indicated that *S. cerevisiae* CEN.PK 113-5D expressing single chain human globins (myoglobin and neuroglobin), exhibited, in batch culture, an increase of the specific growth rate, if compared to the non-producer strain. This behavior could lead to an increase of the yeast performance whenever it was cultivated for bioproductions. The results obtained can be interpreted as a development of studies aimed at the enhancement of the growth and respiratory characteristics of the yeast expressing hemoglobins especially of bacterial origin (Chen *et al.*, 1994; Wei and Chen, 2008; Liu *et al.*, 2010).

Another objective of this thesis work was to achieve high cell density cultivation in view to produce heterologous proteins of great industrial interest. For this purpose, in particular, two enzymes of bacterial origin and of industrial interest have been considered, Lipase A (EC 3.1.1.3) from *Bacillus subtilis* a lipolytic enzyme and endoglucanase (EG-CelA) (EC 3.2.1.4) an enzyme of cellulase complex from *Paenibacillus barcinonensis*.

Lipase A has been produced as an enzyme immobilized on its own yeast producing cells. In this case, the immobilization was obtained by inserting the coding sequence of *Bacillus subtilis lipA* gene in the *BglIII* site of *PIR4* (the gene coding for the cell-wall mannoprotein) so retaining the carboxy-terminal region of *PIR4* that contains four cysteine residues responsible for the anchorage of the mannoprotein protein Pir4 and consequently of the protein fusion to the cell-wall by disulphide linkages. The so naturally immobilized enzyme has been successfully produced, but contrary to expectations the performance in the fermenter in terms of biomass was much lower if compared to that of the non-transformed strain and of the interleukin producing strain. These conflicting results have led to test the production of immobilized lipase A also with other *S. cerevisiae* strains. In particular, in addition to *S. cerevisiae* CEN.PK 113-5D, the immobilized lipase was produced also with an industrial strain (*S. cerevisiae* Y306) and a laboratory

strain (*S. cerevisiae* BY4741). All experiments, carried out in the fermenter, suggested a deleterious action of the expression of lipase on *S. cerevisiae*. This deleterious action has not been found expressing EG-CelA. Indeed, in this case, the sequence of *P. barcinonensis celA* gene was inserted in frame between two restriction sites *Sal I* and *BglII* of *PIR4*. This type of insertion implied the substitution of the carboxy-terminal fragment of *PIR4* with *celA* sequence and, therefore, the lost of the carboxy-terminal region of *PIR4* needed for the anchorage of Pir4 to the cell-wall with consequent release of the protein fusion into the culture medium. In this case a good performance was obtained in terms of yields and volumetric productivity.

As mentioned previously, fermentation runs of most of the *S. cerevisiae* strains investigated in fed-batch were characterized by a peculiar decay in the growth rate clearly observable since 7 hours of feeding onwards. So, another significant part of my investigation was aimed at studying there are limits in the achievement of high cell densities in fed-batch reactor. It was highlighted that there is a limit of cell density hard to be overcome. A cell density slightly higher than 100 g l^{-1} can be assumed as the threshold value above which CEN.PK113-5D yeast performance in terms of growth dramatically decreases. This phenomenon was accompanied, as frequently observed, by a metabolic shift to fermentative metabolism with ethanol production.

It has been hypothesized that growth decay might be related to the secretion of inhibitory compounds that made the culture medium, during fed-batch run, no longer suitable for yeast proliferation. These inhibiting compounds have not yet been identified, but preliminary experiments have shown that they are heat-labile compounds.

In parallel with the experimental activity a novel mathematical model has been developed on the basis of the principles of System Dynamics by representing the complex system of yeast cell metabolism by means of flows, stocks and feedback loops, and taking into consideration the experimental data collected during the research activity of these three years, combining them with literature indications.

In the model the two main pathways regarding glucose catabolism, respiration and fermentation, have been taken into consideration, and identified as capable to have an influence on system variables. Pyruvate has been considered the key compound in the two catabolic processes, capable to drive glucose to new cell mass (C-metabolites) through either respiration or fermentation according to its concentration level in the cell. Further, the production of inhibitory metabolites capable to regulate growth dynamics through a negative feed-back has been represented in the model. The inhibitory compound/compounds would be released along the entire time-course of growth. In the model, each variable (broth culture volume, glucose, ethanol, etc.) is a function of the bioprocess time and is regulated by flows and efficiencies, being efficiencies mass yields.

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In the mathematical expression of intracellular flows, these are regulated by Monod kinetics and/or saturation kinetics. An important flow-regulating parameter is MS (metabolic switch) which is a partition coefficient between respiration and fermentation flows, modeled as a logistic function of the intracellular pyruvate concentration.

In the light of the results obtained during the period of my PhD thesis, I can draw the following conclusions:

- The selection of the most suitable host for heterologous protein expression has been accomplished among the components of the CEN.PK family of the yeast *S. cerevisiae*. The strain CEN.PK113-5D has been identified as the most robust strain to be utilized during the fed batch runs, capable to cope the stressful environmental condition arising during fermentation runs.
- Optimization protocols to set up HCDC in aerated fed batch reactor have been obtained by using the selected yeast strain CEN.PK113-5D transformed for interleukin-1 β expression, and varying the feeding rate to find a compromise in order to achieve both high volumetric productivity and product yield.
- Two enzymes, endoglucanase from *Paenibacillus barcinonensis* and lipase A from *B. subtilis* have been produced according to the protocol previously optimized in fed-batch reactors. Expressing these protein in CEN.PK113-5D, the achievement of HCDC was realized only in the case of endoglucanase. Indeed, we have revealed negative influences when the yeast expresses LipaseA that cause reduction of yeast performance in fed-batch.
- The expression of human globins in *S. cerevisiae* amplifies the fan of HEPs expressed in yeast and confirms the various effect that their expression can have on yeast growth.
- During yeast proliferation in aerated fed-batch reactor a decay in specific growth rate is observable over time, accompanied by a shift from respiratory to fermentative metabolism. This phenomenon depends on a number of factors such as the feeding rate, the heterologous protein expression, and the yeast genetic background. These factors can anticipate or defer the growth decay, that unavoidably occurs. The growth decay has been ascribed to the accumulation of secreted toxic compounds in the culture medium.

- The search for the specific toxic compound responsible for this behavior has not yet been completed. At the moment, it seems to be a thermolabile compound of unknown chemical structure.

- The mathematical model developed through a dynamic structure able to simulate yeast behavior in batch and fed-batch reactor represents the ultimate expression of the holistic approach to the comprehension of microbial growth because looks at the host behavior as the result of the interaction between biological and environmental determinants. The model has highlighted that it is essential to consider a negative feedback effector represented by the secretion of inhibitory compounds during the fed-batch process to describe the global behavior of yeast growth in this peculiar environment.

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