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Optimization of a Biotechnological Process for Production and Purification of Two Recombinant Proteins: Col G and Col H

IL DOTTORE
LORENZO VOLPE

IL COORDINATORE
PROF. CALOGERO CARUSO

IL TUTOR
PROF. GIULIO GHERSI

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Summary

Abstract

Different strategies can be used for increasing production of heterologous recombinant proteins in Escherichia coli. Protein size is often critical for obtaining the best quantity/quality ratio of recombinant protein expression. This study focuses on two recombinant proteins; Class I and class II Collagenases, namely Col G and Col H. Their size is about 150 kDa each. We have developed a method to obtain high levels of cell growth and intracellular expression of each Collagenases in recombinant *E. coli* BL21(DE3). Batch and Fed-batch fermentation procedures have been performed. Results show that Fed-batch technique was most effective in obtaining the highest cell density for each recombinant bacteria; 28 g/L. We also investigated how to optimize recombinant protein expression; best results were obtained when "multiple shot IPTG induction system" was chosen instead of canonical single shot. By applying a purification protocol based on the use of tangential flow filtration and affinity chromatography we were able to obtain the highest quantity of purified protein: about 13,2 g for Col G and about 12,6 for Col H fermentations. Moreover, by using a stainless steel cooling coil system, we have investigated the effects of low controlled temperature (7°C) during the whole purification process. This system, allowed us to improve the final enzymatic activity of both Collagenases, obtaining 2 fold increase values respect processes performed at room temperature, measured with Pz Grassmann assay. This study shows that, even when the size of a recombinant protein is limiting, is possible to apply a defined Fed-batch protocol to obtain a very high protein production. Moreover these results can be used as a scale up starting step for industrial production and purification of these kind of recombinant enzymes.

Introduction

The rise of biotechnology: The gene

More or less at the beginning of 1980 , the term “biotechnology “ was applied for the first time to describe any application of scientific and engineering principle to the processing of materials by biological agents to provide goods and services. The roots of Biotechnology have their beginnings in zymotechnology, which are the processes and techniques used for the production of beer. By the 1970s the term “genetic engineering” had its first use by science fiction writer Jack Williamson in his fiction novel *The Dragon Island* and after that, the expression became commonly used (Stablefort, 2004). In 1972 ,the first recombinant DNA molecule was produced by linking the DNA from the lambda virus and the SV40 virus. After this, Herbert Boyer and Stanley Cohen engineered the first transgenic organism by inserting antibiotic resistance genes into a plasmid of *E.coli.* (Arnold, 2009). Around 1983 Dr. Kary Mullis, when working as a DNA chemist for the Cetus corporation, gave a very strong contribution to the rise of recombinant protein production field of research, by improving and perfecting the already existing polymerase chain reaction (PCR) (Arnold, 2009). Instead of using temperature sensible DNA polymerase enzymes like those used by Khorana and Klepp, he chose and used the heat stable DNA polymerase enzyme, avoiding to add DNA polymerase enzyme after every heat cycle performed. This better crafted PCR method was based on the process of thermal cycling; basically on alternating cycles of repeated heating and cooling of reaction for DNA melting and enzymatic replication of the DNA molecule. Short DNA fragments defined as primers, being complementary in terms of sequence to the target region, will work as molecular graft along with a DNA polymerase. These key components enable selective and repeated amplification. As PCR goes on, the DNA newly synthesized is also employed as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR experiments can be widely modified to perform a broad array of genetic manipulation (Arnold, 2009). The research of Boyer and Cohen , as discussed above, using plasmids and restriction enzymes to manipulate DNA was the corner stone for what is now known as biotechnology (Cohen *et al.*, 1973).

Recombinant protein production

Recombinant proteins have become increasingly important as biological and pharmaceutical tools; their annual market growth is still increasing of 10-15% per annum. The combination of recombinant DNA technology and properly scaled culture processes has enabled the production of

these proteins in quantities that might otherwise have been difficult, if not impossible, to obtain from natural sources (Lee, S.Y. 1996). Among the many systems for heterologous protein production, the Gram-negative bacterium *Escherichia coli* is the standard choice system because of its ability to grow relatively quick and at high densities on cost-effective substrates, its well characterized genetics and the availability of an increasingly large numbers of cloning vectors and safe to use mutant host strains. Although there is little guarantee that a recombinant gene product will accumulate in *E. Coli* at high levels in full-length and biologically active form, quite a big amount of studies has been directed at improving the performance and versatility of this work-horse organism (Baneyx, 1999).

Collagenases

Collagens are the major components of the extracellular matrix. These proteins are especially abundant in mammals, constituting a quarter of their total weight. Nineteen different types of collagens are known to date and type I collagen is the major species in higher vertebrate. Collagenase, a metalloproteinase capable of cleaving native collagen types I, II, III, IV and V, is produced in large amount by *Clostridium hystoliticum* (Matsushita, 1994). *Clostridium histolyticum* is a anaerobic Gram positive bacterium, pathogenic to humans, involved in the formation of gangrene. It produces a variety of collagenases in large quantities, which efficiently degrade the collagen present in the connective tissue. The *clostridium histolyticum* collagenase are metalloproteases belonging to the M9 family, having collagenolytic activity (Matsushita, 1994). Different isoforms of collageases from clostridium are divided into two classes class I (col G) and class II (Col H). The various isoforms were determined by analyzing their relative activities against the native insoluble collagen and synthetic substrates. The enzymes of class I have high collagenase activity and a modest activity against peptides FALGPA and PZ, while the enzymes of class II have a modest activity against collagen and a high activity of the peptides FALGPA and PZ (Matsushita, 1994). Despite being a valuable tool in the laboratory, this kind of enzyme has found clinical applications in the treatment of third degree burns and decubitus, diabetic or arterial ulcers, transplant of human beta pancreatic islet (Volpe, 2016). Several cases of successful topical use of the crude enzyme in an ointment base are known to literature. Direct injection of a highly purified form of the enzyme has been proposed in the treatment of herniated discs and as an adjunct in vitrectomy (Mandl I.,1982). Consequently, in recent years its demand has strongly increased.

Abiel Col G and Col H

Abiel S.R.L.is a biotech company specialized in production and purification of proteolytic enzymes. In particular, two recombinant collagenases, a class I collagenase and a class II collagenase: namely Col G and Col H (Salamone M. *et al*., 2012), are produced. Each enzyme codifying sequence has been optimized to be expressed in *E. coli* BL21 (DE3) (Salamone M. *et al.,* 2012). Both Col G and Col H are chimeric enzymes, because they carry MBP (Maltose Binding Protein) fused in-frame at their C-terminal portion. The presence of MBP fused in-frame, causes a strong increase in molecular weight either for Col G (from 114 kDa to 146 kDa) and Col H (from 112 kDa to 144 kDa). Despite being produced in a foreign host and fused in-frame with a chimeric protein, each enzyme activity is not negatively affected and, moreover, their thermal stability is even increased in comparison with standard produced Col G and Col H.

Fermentation

Fermentation has been used since the very beginning of human civilization for producing both food and beverages, but more recently it has been employed in the manufacture of biological and medical products. Indeed man has harvested the energy obtained by fermentation to create new and important products, used not only in the field of medicine but also in bioremediation and agriculture (figure 1).

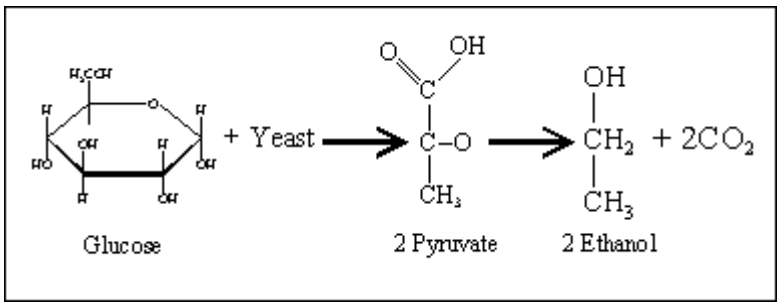


Figure 1. example of single sugar conversion to ethanol and carbon dioxide

The word “fermentation” has many distinct and slightly different meanings depending on the context. From a biotechnological point of view, we intend it as the use of a submerged liquid culture of specific strains of microorganisms , plant or animal cells, for the manufacture of some products or to gain insights into the physiology of those cell types. Today a fair amount of pharmaceuticals and proteins are indeed obtained through fermentation processes. Since the advent of high throughput sequencing era, the DNA in genomes of many organisms has been sequenced. This huge amount of new biological information has opened the possibility to improve and increase our knowledge about proteins structure and mechanism of action, rendering the development of new drugs for commercial and biomedical purposes even easier. Even antibodies with therapeutic application are now being obtained using recombinant expression host different from Chinese Hamster Ovary cells (CHO), such as yeast and *E. coli*. Significant advances have been made in antibody engineering design, with particular focus on fc engineering and glycol-engineering in order to improve their function, as well as cellular engineering for increased production of antibodies in yeast and bacterial hosts such *E. coli* (Cohen, *et al*., 1973).

Types of fermentation

There are three main modes of fermentation: batch, fed-batch and continuous. Batch and fed-batch fermentation techniques have been employed for the production of alcoholic beverages and fermented food since before 3000 BC in Egypt and Sumeria (McNeil, 2014). At the dawn of the Twentieth century, other industrial applications became widely spread, such as the production of acetone, butyl alcohol and ethyl alcohol, later still came the production of antibiotics by submerged culture of specific strains of filamentous bacteria and fungi. Each of these new types of products led to technological innovations within the fermentation industry, and, in particular, to new methods of bioprocess operation (McNeil, 2014). In human history, most of the time, fermentation processes were performed as batch, with fed-batch becoming commonly used only for the production of baker’s yeast and antibiotics. Continuous fermentation examples, in industry, are: vitamin C, propionic acid and Quorn production, for instance. Nevertheless, continuous fermentation are a good and well established laboratory tool for studying the physiology of microorganisms, metabolomics, proteomics, etc. In the pharmaceutical and biotechnology industry a huge amount of development work is necessary to define and optimize a full process of production of a desired product.

Bioreactors are the subject this very process, ranging from small-scale bench-top (≈1-2L), to pilot scale (20-100L) for feasibility studies, to large scale reactors that have the capability of cultivating thousands of litres of microbes, and more. In order to develop, perform and optimize each of these processes, is important to study the three main ways of performing a fermentation by describing and discussing each mode of operation and providing information regarding their advantages and disadvantages.

Batch culture

Batch fermentation is the simplest and most common mode of operation, and it is often used in the laboratory to obtain substantial amount of cells or products, such as proteins. A batch fermentation is a closed system, where all the elements necessary for the organism's growth and product formation are put within the vessel at the beginning of the process of fermentation. The vessel can be different in form and geometry, for example: shake flasks, single use disposable system, or, for an online control of such parameters like pH, agitation speed, Dissolved Oxygen transfer (DO) and temperature, etc., a bioreactor can be used (figure 2). This batch mode and nearly all types of fermentation are started by inoculation of a selected and specifically grown strain of microbe, animal or plant cell (seed culture) into sterilized vessel containing sterilized media. After seed culture injection, the organism is allowed to grow. The fermentation ends when one or more of the following circumstances have been obtained: (i) the process of growth has stopped because complete nutrients exhaustion or because the amount of toxic by-products is too high; (ii) after a specific amount of time as passed; (iii) the achievement of the desired amount of product been either cell cultured or recombinant protein expressed.

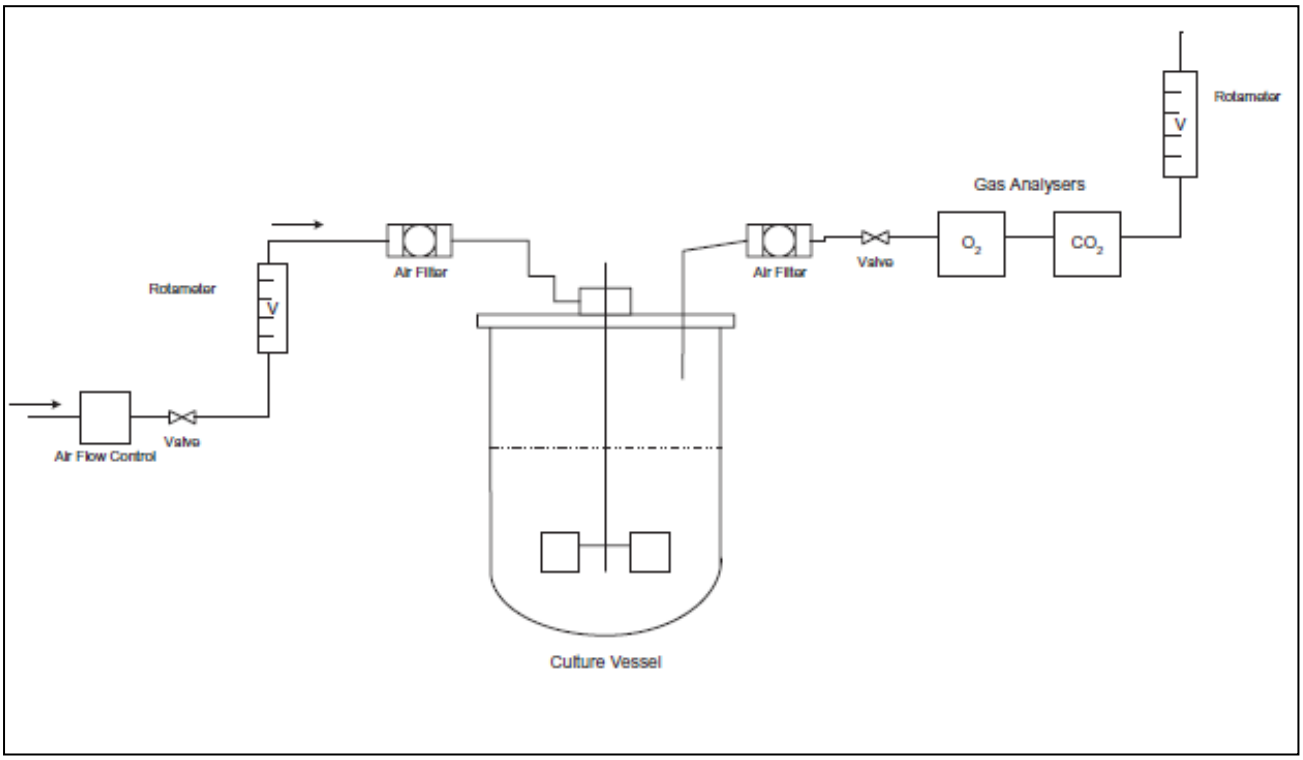


Figure 2. Graphic diagram of canonical batch fermentation. The system is closed, the only thing that comes out is air discharged.

Batch fermentation grow curve

Throughout the process of batch fermentation, cells will undergo through a number of distinct phases (figure 3). Usually the so called “lag phase”, during which there is little or no growth due to adaptation to different environment conditions, is the first phase that can be monitored during a batch grow curve. This step can be time consuming and so it would be better to minimize this gap of time. To do so is important that the seed culture is constituted at least by 5% in final fermentation volume of exponentially growing cells and more importantly that the parameters of the seed culture medium are to be the closest possible to those of the batch fermentation medium, minimizing both metabolic and environmental stresses. Once cells have adapted to the new environmental conditions, they start to grow and the exponential phase begins. Nutrient consumption and production of inhibitors (excreted products such as ethanol, lactic acid, acetic acid, and aromatic compounds) will slow down the process of cell growth. At this point cell will enter in stationary phase: when the rate of cell growth equals the rate of cell deaths. Eventually, the cells reach the cell

death phase during which the rate of cells that die is higher than the rate of cell that reproduce; clear indicators of the reach of death phase are: the drop in the culture's optical density and decreased level in biomass (figure 3).

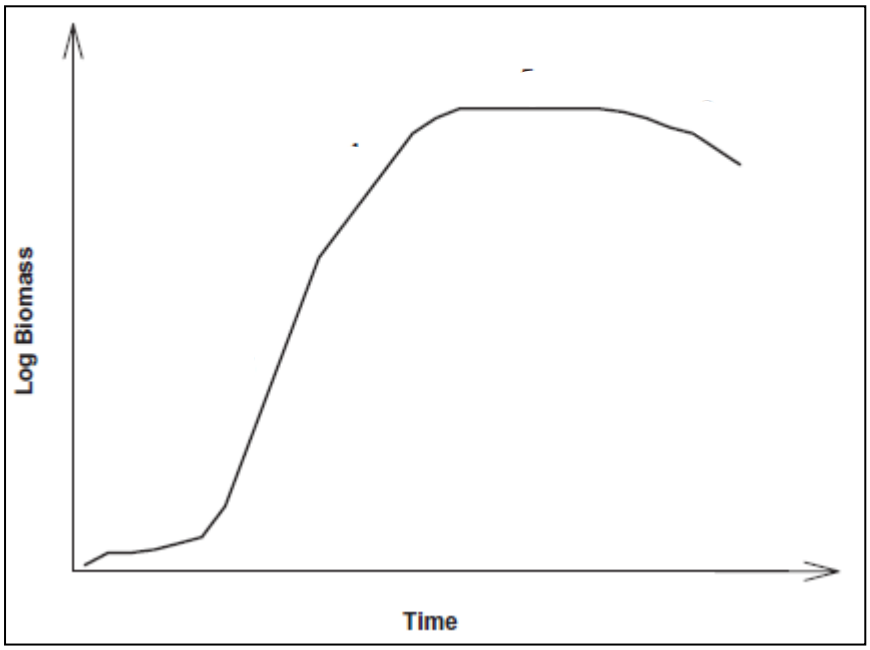


Figure 3. Typical growth curve of a microorganism cultured in batch mode.

Following the batch grow curve is possible to obtain a good indication of when to stop a fermentation. Primary metabolites or growth-associated products (for example riboflavin, citric acid) are usually produced throughout the exponential phase, diminishing when the growth is ceased. So, if the main interest is the production of a primary metabolite is good advice to stop the fermentation at the end of exponential growth phase just before entering stationary phase. This phase is sometimes defined "trophophase". On the other hand, when the fermentation is aimed at the production of a secondary metabolite or non growth associated products (for example antibiotics), the process can be interrupted just before cells enter death phase since these secondary metabolites have a very low rate of expression during growth associated phases but are strongly produced throughout stationary phase. In this case we can refer to the stationary phase as "idiophase". Of course is good to underline that these are generalizations and many times in actual practice operators have witness secondary products synthesized in great quantity during growth associated phases.

Advantages of batch culture

- Simply to perform process, almost plug and play system
- It happens in a short amount of time (usually less than 24 h)
- Less chances of contamination since all the necessary materials are within the vessel and together sterilized before the fermentation start
- Possibility to assign an unique batch number to each lot produced via batch fermentation so having the possibility to keep an highly regulated production environment.

Disadvantages of batch fermentation

- Absolute no control of the operator throughout the process of fermentation.
- Accumulation of toxic by-products negatively influencing both cell growth and product formation
- Batch to batch variability
- Non productive periods due to time employed for cleaning, re-sterilization, filling and cooling of the equipment.

Fed-batch culture

Fed-batch culture in many aspects is similar to batch culture, and most fed-batches start as batch processes. However, fed-batch cultures are not closed system. In fact at some specific point during fed-batch fermentation one or more specific nutrient/substrates are fed into the vessel. There are different ways of performing a fed-batch fermentation run (figure 4): fixed volume is one of those. It is based on the withdraw of a certain amount of fermentation volume at a specific time. This uptake is then counterbalanced by an input of the same amount of fresh medium volume (withdraw and refill). On the other hand, is possible to perform variable volume fed-batch fermentation. When performing this strategy nothing is taken from the bioreactor throughout the process, with cells and product staying within the vessel till the end of the operation and the sole input of fresh medium with the effect of increasing the final fermentation volume. This feeding strategy allows the cells to grow at a specific growth rate, minimizing by-product excretion and obtaining high cell densities and product concentration (table 1). The addiction of the feed solution can happen right after inoculation of the seed culture or later on, it can happen for short or continuous periods of time, can be incremental, linear or divided in legs. All of these feeding strategies are determined from

previous fermentation data or from the concentration of essential metabolites that constitute the fermentation medium.

Products	Host	Culture condition and carbon source	Productivity and characteristics
Insulin-like growth factor-2 (IGF-2)	*E. coli* BL21(DE3)	pH-stat, R medium, glucose	9.69 g/L, inclusion body
Single-chain antibody variable fragment	*E. coli* RV308	Exponential feeding, defined medium, glucose	1.2 g/L, PelB signal sequence
Human interferon-γ (hIFN-gamma)	*E. coli* BL21(DE3)	Exponential feeding, M9 modifed medium, glucose	2×10^7 U/mg protein, inclusion body
Human Interleukin-7	*E. coli* HMS174 (DE3)	Large scale batch fermentation (1000L), semi-defined medium, glycerol	46% of total proteins, inclusion body
Phytase	*E. coli* BL21(DE3)	DO-stat, glucose mineral salt medium	120 U/mL, *kil* gene coexpression
Carbamoylase	*E. coli* BL21(G2)	pH-stat, synthetic medium, glucose	14256 U/L, thermoreglulated T7 promoter
Human epidermal growth factor	*E. coli* HB101	Batch, semi-defined medium, glucose, lactose	242 mg/L
Human epidermal growth factor	*E. coli* JM101	pH-stat, MMBL medium, glucose	325 mg/L, OmpA signal sequence
Alkaline phosphatase	*E. coli* HB101	pH-stat, modified R medium, glucose, YE	5.2 g/L, Endoxylanase signal sequence.
Human granulocyte colony-stimulating factor (GCSF)	*E. coli* BL21(DE3)	pH-stat, modified R medium, glucose	22% of total proteins, Endoxylanase signal sequence
Protective antigen protein	*E. coli* DH5α	Batch, semi-defined medium, glycerol	125 mg/L, inclusion body, constitutive expression
Bone morphogenetic protein 2	*E. coli* TG1	Exponential feeding, defined medium, glucose	8.6 g/L, inclusion body.
Human mini-proinsulin	*E. coli* BL21(DE3)	pH-stat, semi-defined medium, glucose	7 g/L, two stage cyclic fed-batch culture
Human interferon-α	*E. coli* TG1	Exponential feeding, defined medium, glucose	4 g/L, inclusion body
Animolevulinate synthease	*E. coli* MG1655	Batch fermentation, LB medium, media optimized supplement	5.2 g/L
Annexin-V-hirudin chimeric protein	*E. coli* BL21(DE3)	DO-stat, LB medium, glucose	10 mg/L/OD_{600} (after purification)
Human Tissue-type plasminogen activator	*E. coli* SF110	Exponential feeding, semi-defined medium, glucose, casein, YE	180 µg/L (after purification), StII signal sequence, DsbC coexpression
Human necrosis factor-related apoptosis-inducing ligand	*E. coli* C600	Combined feeding using pH- and DO-stat, semi-defined medium, glucose	1.4 g/L soluble protein
Antifungal peptides	*E. coli*	DO-stat, glycerol minimal salt medium	40% of total proteins, PelB signal sequence, not secreted, inclusion body
Human necrosis factor-α	*E. coli* BL21(DE3)	Batch fermentation, semi-defined medium, glycerol	Constitutive production, P_{HCE} promoter
Bioadhesive protein	*E. coli* AS002	pH-stat, exponential, constant feeding, R medium, glucose	5.3 g/L
Human leptin	*E. coli* BL21(DE3)	pH-stat, modified R medium, glucose, YE	41% of total proteins, endoxylanase signal sequence, DsbA coexpression
Pectate lyase	*E. coli* BL21(DE3)	DO-stat, FB synthetic medium, glucose, casamino acid	2200 U/mL, PelB signal sequence
Human leptin	*E. coli* FMJ123	pH-stat, modified R medium, glucose, casamino acid	9.7 g/L, constitutive production, P_{HCE} promoter
Insulin-like growth factor-1 fusion protein	*E. coli* W3110	pH-stat, modified R medium, glycerol, YE	4.3 g/L, PrsA and GlpF coexpression

Table 1. Examples of proteins obtained by using high cell density culture fed-batch fermentation.

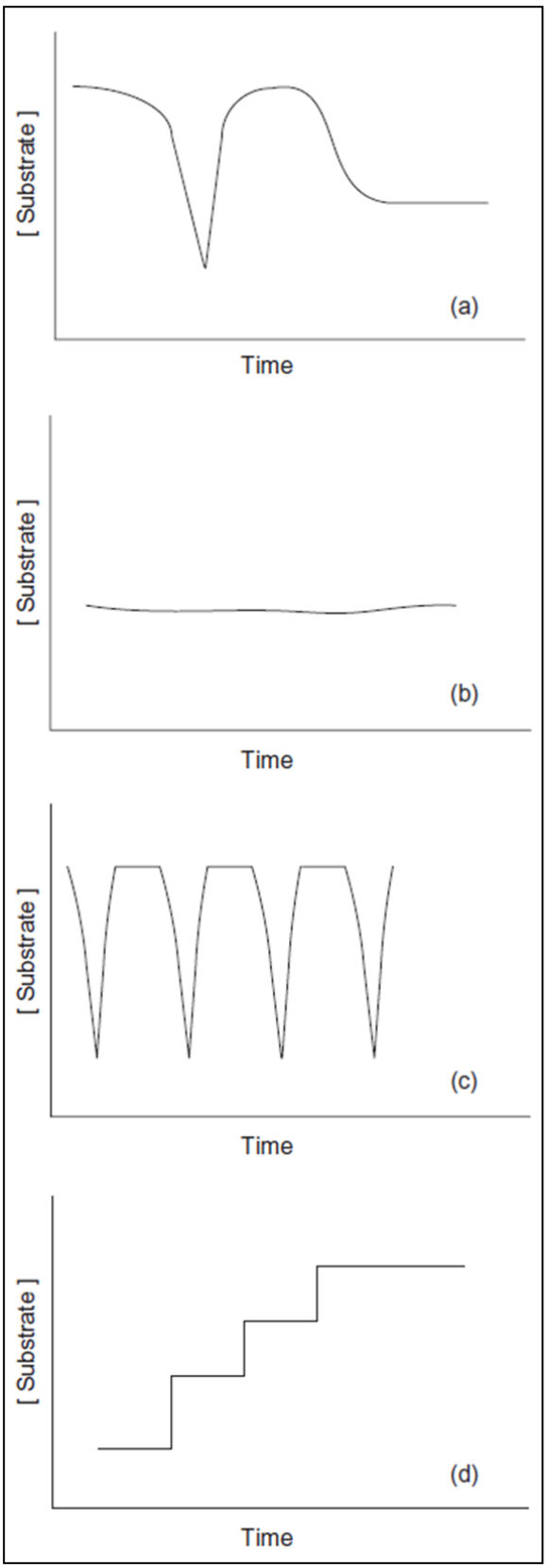


Figure 4. different feeding approaches in fed-batch fermentation: (a) variable feed regime, (b) continuous feeding regime, (c) intermitting feeding regime, (d) incremental feeding.

A good example is brought by *E. coli*, when grown aerobically in excess of the carbon source glucose its oxidative capability gets unbalanced and it excretes a significant amount of acetate (Eiteman, 2006). Is been proven that when acetate is excreted, at certain concentration it will inhibits both cell growth and recombinant proteins production in recombinant expression system (Eiteman, 2006). But, if the glucose feed rate is kept under a specific threshold, is possible to obtain bacterial growth at the highest rate, very low excretion of acetate and consequently remarkable production of desired recombinant protein. To summarize, fed-batch can be used to minimize nutrients overflow towards production of waste by-products, extending the productive phase of the fermentation process (figure 5).

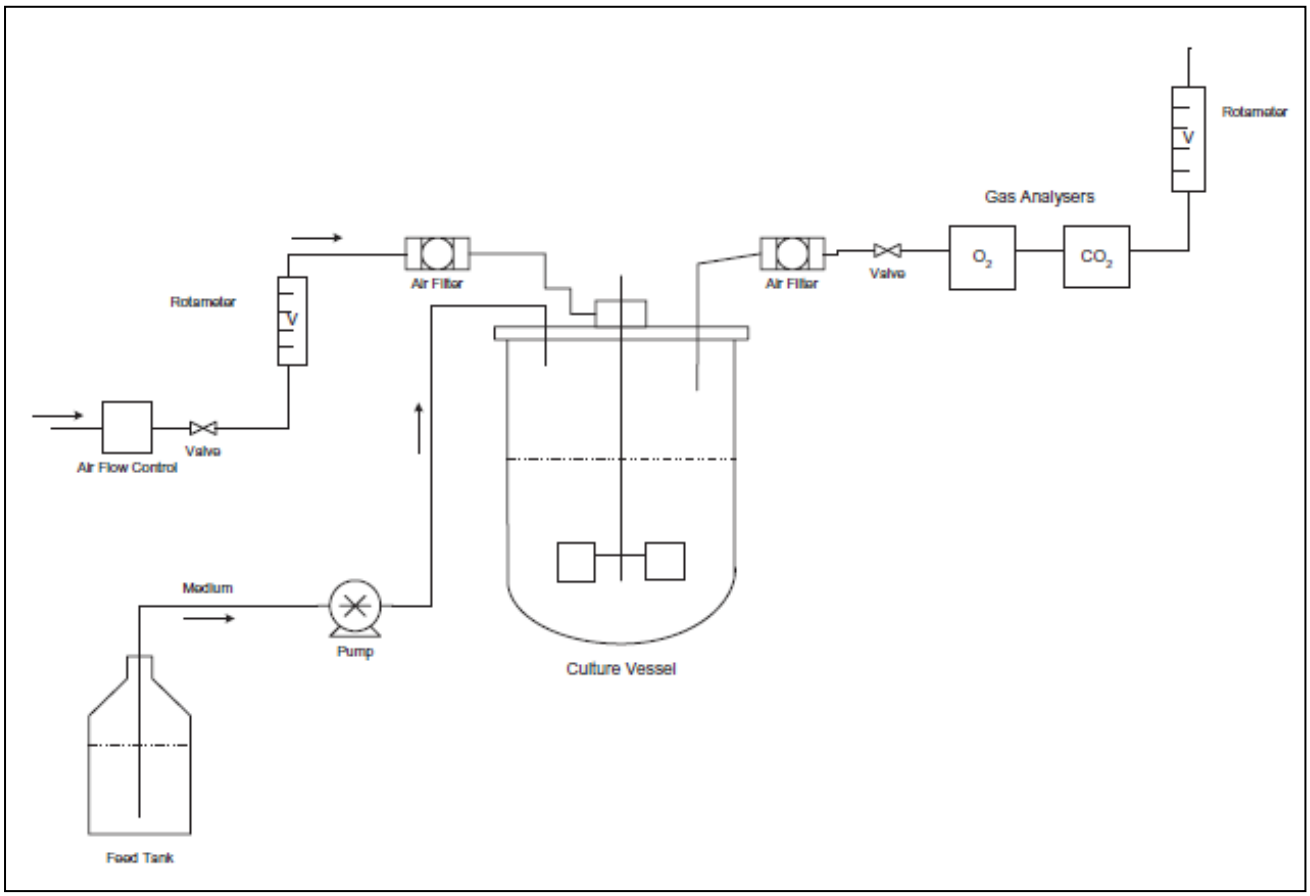


Figure 5. A typical fed-batch set up. The graphic is quite similar to a classic representation of batch fermentation process. Differences are that, often at the end of exponential growth phase, a super concentrated feed solution get pumped in the vessel with a specific strategy, allowing control on cell growth and carbon source consumption.

Techniques to control fed-batch process

When performing such a bioprocess like fed-batch fermentation, is mandatory to monitor the feeding strategy in order to gain optimal yield of desired product. Different strategies can be chosen depending on the operator's experience and the availability of equipment:

- No feedback monitoring: this is an open loop strategy in which the operator skills play a capital role. Using this control, the feeding rate has been predetermined by previous fermentation data and observations. This type of control is pretty simple but it has the big disadvantage that unexpected variation during the process cannot be predicted or taken in account.
- Direct feedback monitoring: this method is based on the calculation of the substrate's concentration in the culture medium either by online or offline measurements. These measurements can give accurate info having the possibility to best calibrate the feeding strategy. Another strong advantage is that every single unforeseen or unexpected variation throughout the bioprocess can be measured and so counterbalanced. The disadvantages are the limited availability and high cost of online monitoring systems.
- Indirect feedback monitoring: this strategy is based on the measurements of fermentation parameters that are not directly related to the substrate such as dissolved oxygen (DO), biomass, pH, oxygen uptake rate (respiratory quotient), etc. The strong advantage of using this strategy is the speed at which the measurement can be performed. The disadvantage is that these parameters are cell/bioprocess specific so previous info about must be already known.

Advantages of fed-batch technique

- Preventing negative effects of high substrate concentration and avoiding catabolite repression by controlling the limiting substrate's concentration
- Careful control of organism's growth rate and consequent oxygen consumption
- Achievement of higher cell densities than batch processes
- Decrease of broth viscosity

Disadvantages of fed batch technique

- Absence of trustworthy online sensor for precise substrate determination
- Profound knowledge of both organism's growth and product formation is essential
- The operator must be well trained and experienced

Continuous Cultures

Continuous processes are commonly used at industrial level for the production of vinegar, treatment of wastewater, single cell production, ethanol production, etc. Such a process is not commonly used at laboratory scale. The only laboratory application is where this process is used to study both physiology and growth characteristics of microorganisms. Basically continuous cultivation is a strategy aimed at elongating the exponential growth phase of a cell in batch culture, while keeping an environment that has minimum variation in nutrients, biomass and number of cells. This is defined as "steady state". The microorganisms are fed with fresh medium and at the same rate, exhausted medium and cells are removed from the system. This process allows that several parameters like culture volume, biomass, product and substrate concentration are kept in equilibrium, moreover also physical parameters like pH, temperature and dissolved oxygen are balanced too, throughout the whole operation (figure 6).

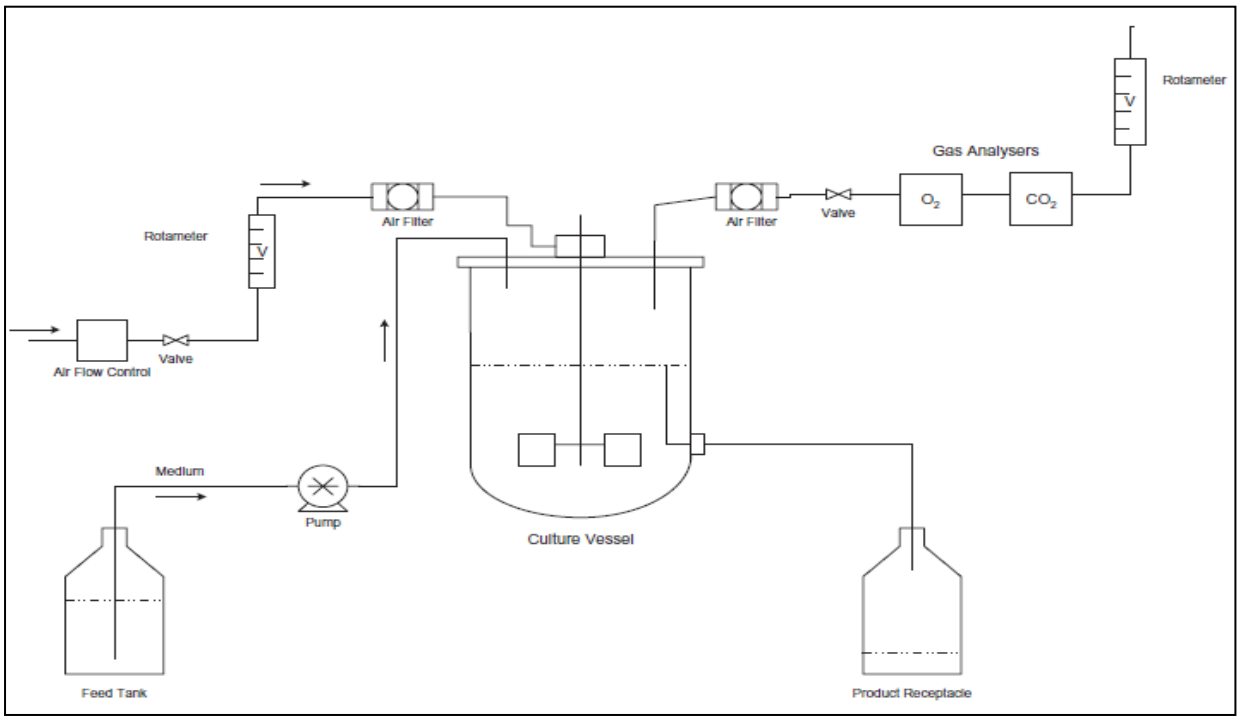


Figure 6. schematic representation of typical continuous fermentation set up.

E. coli

E. coli is a Gram-negative, rod shaped bacterium that is usually found in the intestines of warm-blooded organisms (figure 7). Most *E. coli* strains cause no harm to human health, but some serotypes can be the cause of serious food poisoning. The harmless strains are part of the normal microbiota of the gut, and they can give some benefit to their hosts; for instance by producing vitamin K2, and by protecting the intestine environment from the establishment of pathogenic bacteria.

E. coli was among the first organisms to have its genome sequenced with the complete genome sequence of *E. coli* K12 (Blattner *et al*., 1997). It consisted in 4.6 million base pairs in length, encoding 4288 protein genes, organized into 2584 operons. In studies of microbiology, *E. coli* has been employed to investigate metabolic pathways, cell division and mechanisms of cell death. In 1946, Lederberg and Tatum discovered bacterial conjugation using *E. coli* as a model bacterium (Lederberg and Tatum, 1946). Before the discovery of restriction enzymes in the 1970s, researchers did not have efficient ways to modify genetic material, such as what happens when a bacterium is infected by a bacteriophage or a foreign plasmid. And, with the discovery, characterization and isolation of the restriction enzyme Hind III in 1970 (Smith, et al., 1970, Robert, 2005) and the subsequent discovery and characterization of numerous restriction endonucleases (Danna, and Nathans, 1971) recombinant DNA technology and its application bloomed onto the scientific field. Indeed, one of the first and most important product crafted with the help of this new technology was the large-scale production of human insulin for diabetes treatment, using *E. coli* as the recombinant host. This initial success with recombinant technology brought many scientists to reckon that this new technology was theoretically limitless in its applications. But, despite having success in production of many recombinant enzymes, hormones and immunogens for vaccines, soon the scientific community realized that their expectations were too big and effort to produce such proteins were constantly being stymied. The fact was that some proteins made in *E. coli* recombinant system showed differences to the same proteins obtained from natural source and consequently those proteins were not safe for human use. Soon the scientific community realized that the use of recombinant *E. coli* had limitations: it couldn't be employed to produce large multimeric heterologous proteins or proteins that necessitate complex disulphide bonds formation or unpaired thiols or proteins that natively contain post-translational modifications.

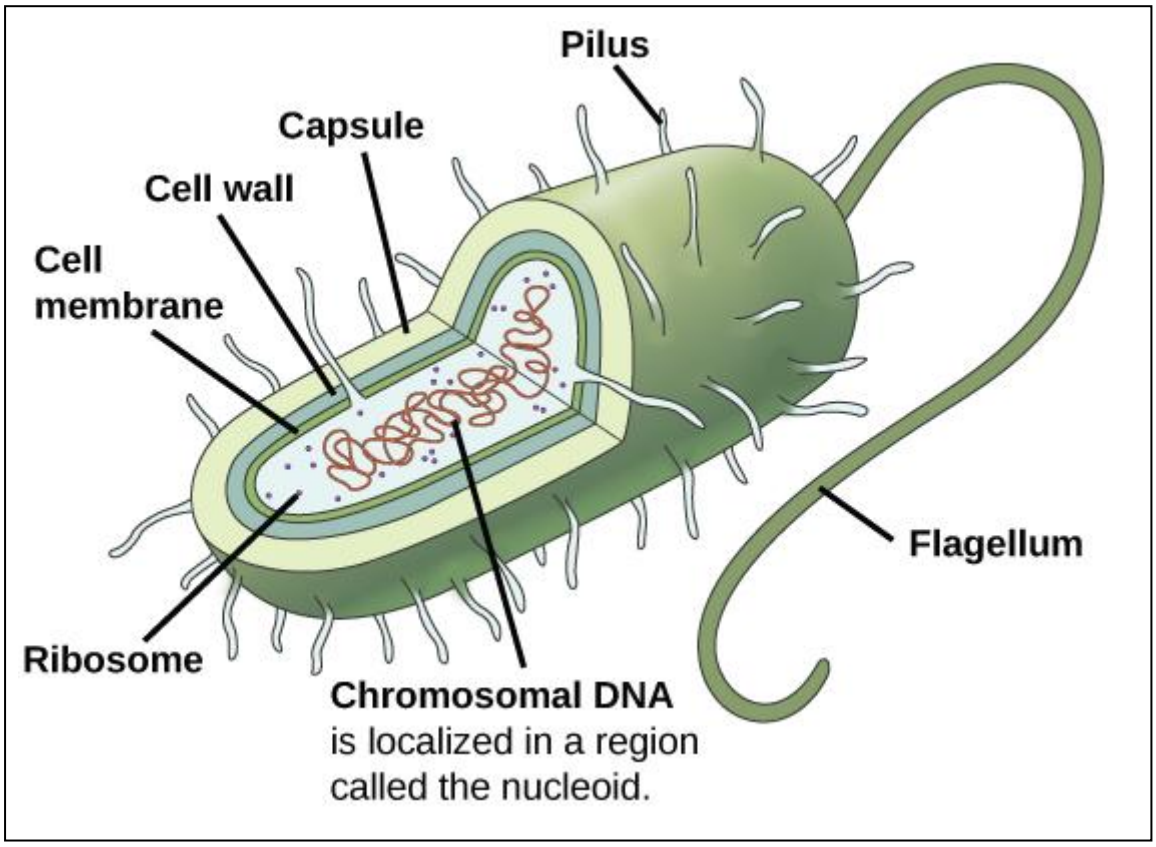
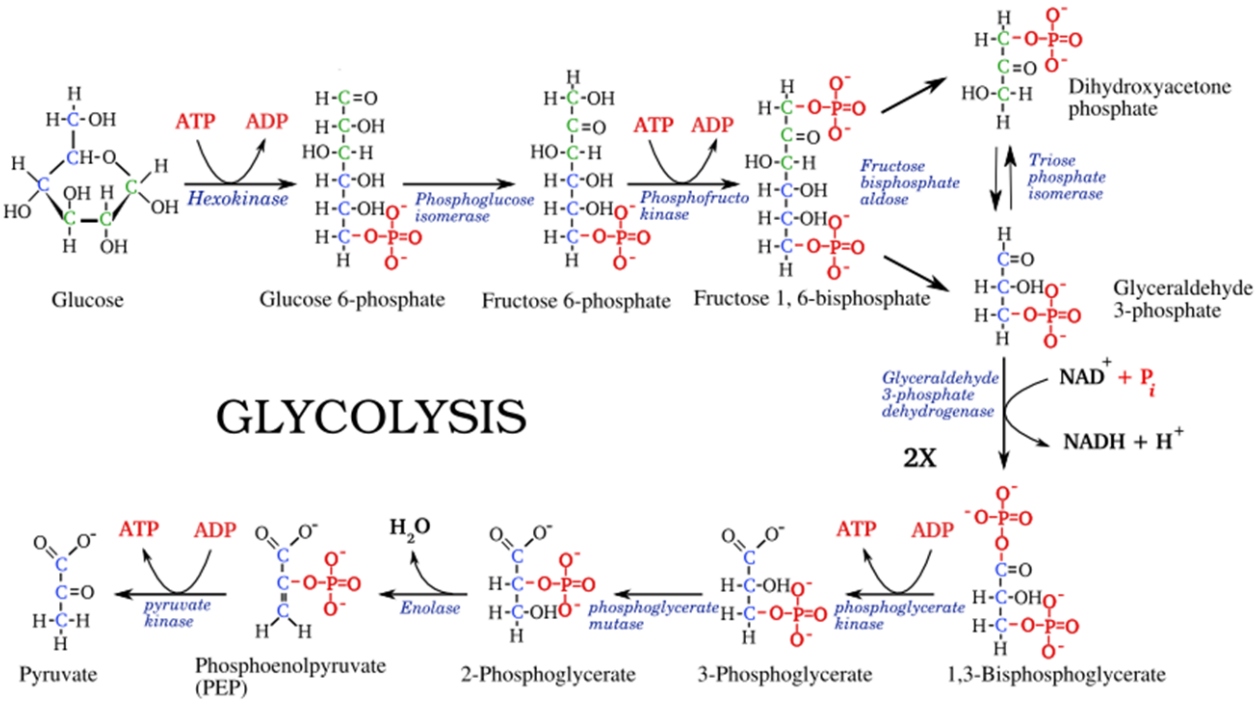


Figure 7. *E.coli* cell description.

Today a growing number of different strains of *E. coli* are subject of study and development. Among these, *E. coli* B (BL21) and *E. coli* K (JM109) are the ones where the research's focus has been concentred the most and probably to ones mostly used as recombinant system. THE K-12A strain was isolated from a stool sample of a patient and in 1922 was marked K-12 at the university of Stanford (Bachmann, 1972). In the 1940s, Charles Clifton studied the mechanisms of this strain for metabolism of nitrogen. Eduard Tatum also studied tryptophan biosynthesis using this K-12 strain. Today, K-12 are used with great results in recombinant protein production, in both research and manufacturing fields. Another typical laboratory *E.coli* strain is the B strain, which got named by Delbruck and Luria in 1942. It was discovered for the first time at the institute Pasteur by Felix d'Herelle in 1918. This strain moved quite a lot from one lab to another before falling into the hands of Delbruck and Luria, and eventually gave rise to more familiar strain known as BL21. Both B and K strains have been studied in depth and have been found to answer differently to glucose concentration in their growth medium, in particular when the glucose concentration is 10 g per litre or more (Lee, 1966). These diversifications in glucose's metabolism are expressed within the glycolytic pathway and the tri-carboxylic acid (TCA) cycle (figure 8).



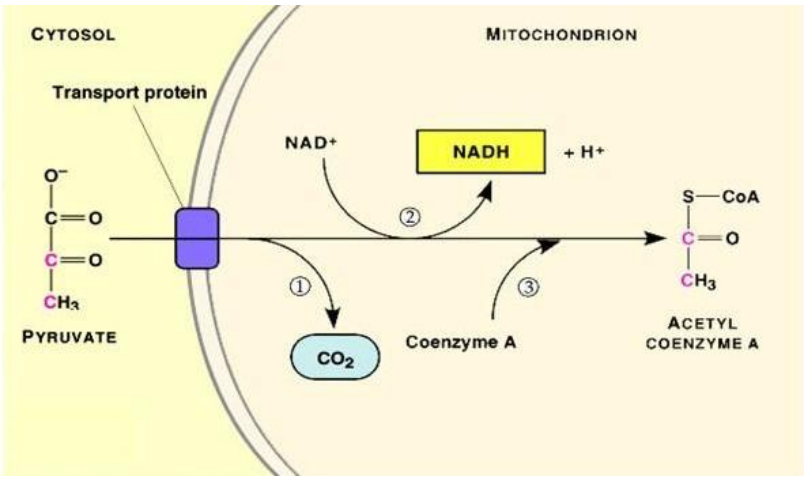


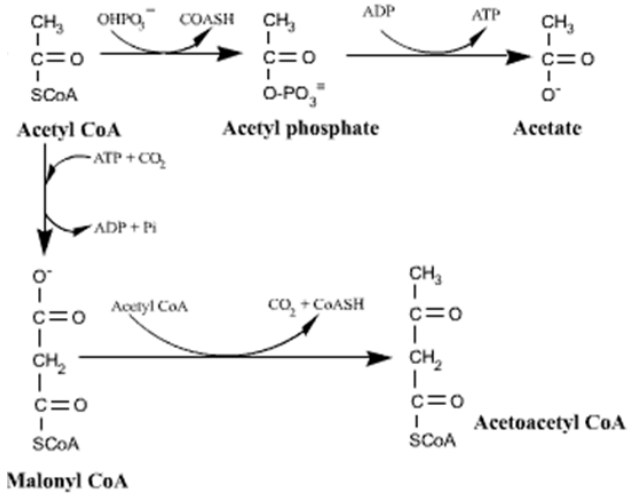
Figure 8. Glycolytic pathway and acetyl-CoA formation process

The B and K strains will metabolize glucose and the subsequent glycolytic product pyruvate at different rates and thus create different stresses that can generate more or less acetate production and consumption. This balance of acetate production/consumption is essential to the efficiency of carbon metabolism and ultimately to the growth and recombinant protein production in most types of culturing methods, especially fed-batch (Shiloach, 2009).

Effects of glucose metabolism and acetate accumulation *in E. coli.*

Under aerobic respiration, glucose is usually employed as the main carbon source and it is fed in a non-limiting way in order to reach high cell densities. Complications can appear when the culture keeps an high growth rate throughout the exponential phase of growth with the secretion of acetate into the culturing media. This biosynthetic process is exposing the bacterial culture to metabolic stress through their Central Carbon Metabolism (CCM) and it is tightly linked with higher amount of acetate secretion (Lee, 1966., Shiloach, 2009). At enough high concentration, acetate can inhibit cell growth and/or recombinant protein production (Eitemann and Altman, 2006). Acetate can also unbalance trans-membrane pH gradients, negatively influence amino acids synthesis, osmotic pressure and intracellular pH.

The effects of acetate accumulation have been studied in depth in the recent years (Moulton *et al*., 2010, Shiloach, 2005, De May *at al*., 2007). The principal interest is, when focusing on recombinant protein production, to address and define glucose feed rates and acetogenesis when a cell culture is growing on excess glucose . During this gap of aerobic growth on excess glucose, the respiration efficiency can strongly decline due to metabolic overflow. This is defined bacterial Crabtree effect, in which as much as 15% of glucose is excreted as acetate (Wolfe, 2005, Crabtree, 1929, Doelle *et al*., 1982, Rinas *et al*., 1989) The mechanism that give rise to this effect is not completely understood but most likely it involves repression of many TCA regulation factors like promoters and genes that encode enzymes that phosphorylate and transfer glucose to the intracellular matrix for processing. Another index of overflow metabolism is the acetogenesis of the *E. coli* caused by the excretion of acetate (figure 9). This is the output of an imbalance that rises between fast carbon flux into the central metabolism and the limited capacity of the TCA cycle (Holmes, 1986, Kadir *et al.*, 2010, Sooan *et al*., 2009) . So is very important, when designing a successful fermentation experiment, to take seriously account of these metabolic behaviours.



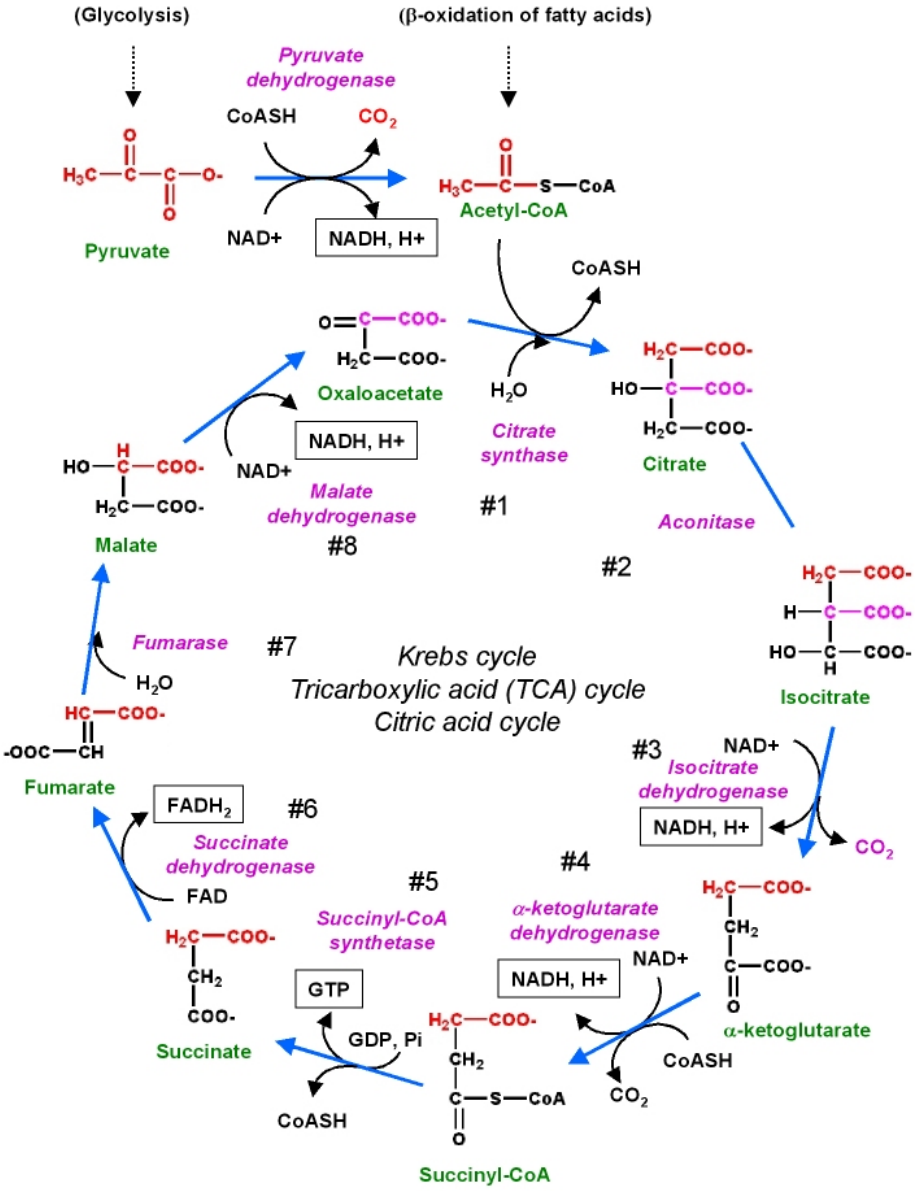


Figure 9. TCA cycle and the formation of Acetyl CoA from acetate.

Culture medium selection and preparation

Medium design is essential for successful laboratory investigations and also industrial bioprocess activities. What we define with the term medium? A specific nutrient solution containing all the necessary elements essential to cells growth and for expression of recombinant products. Is important to underline that medium choice can be the tipping point towards catastrophe of a research program or be the engine that skyrocket it. Defining a medium, in principle, should be quite a straight forward operation, the main thing would be providing to the microorganism or cell line all the necessary nutrients in a immediately usable form. Unfortunately many things must be taken in account: cost and availability of ingredients, preparation and storage, health and safety precautions. Ideally, the perfect medium should be made only by ingredients ready to be dissolved in water, unfortunately, in many cases and especially at industrial level, certain medium ingredients contain suspended solids or oily phases, adding difficulties to upstream processing.

Media can have different physical forms, they can be solid, semi-solid or liquid. Usually, most liquid media can be converted in solid by addiction of a specific gelling agent such as agar. Media employed in bioprocessing is mainly liquid, despite solid media used for starting plate cultures. Depending on their composition, we can have three main categories of bioprocessing media: synthetic, semi-synthetic and complex.

Synthetic media

These types of media are fully chemical defined. Every component is been predetermined. Specific concentration of each component is known. Such media are in principle quite simple, being composed by a carbon source, a nitrogen source, a variety of salts. However , depending on the cell line chosen, the variety of ingredients and consequently the media complexity can strongly increase. When experimental precision is essential, synthetic media are quite an useful tools in laboratory research. These types of media tend to be more expensive than others because some ingredients, like vitamins for instance, are supplied in pure form. One down side of synthetic media is that final cells yield values tend to be lower than those obtained with semi-synthetic or rich media, because the synthetic media usually do not match exactly the specific energetic cell line demands. On the other side, having the knowledge of exactly what components are in the media, is possible to investigate the influence of every single nutrient on both cell growth and secondary products formations. Moreover, since medium composition is strictly defined, it can be highly reproducible

and, in principle, fermentations performed with these media show less variability of results, both in terms of cell yield and product formation.

Semi-synthetic media

Semi-synthetic media are composed mostly by defined chemicals, with the exception of one or more components poorly characterized but present in defined quantity. Among these components, one good example is yeast extract. Such media are quite useful in laboratory research because the presence of one or more of these poorly characterized components can substitute the requirement for an ingredient too expensive to be supplied in pure form. Moreover the use of semi-synthetic media give good results, in terms of cell growth and product production, when the organism's specific metabolic requests are not well defined; this is due to the presence of a component like yeast extract, which is a rich source of many nutrients, and consequently being able to cover the lack of many minor nutrients requirement. In the past many different types of extract have been employed: microbial, animal, plant and fish extracts. Each and every one of those being a very good source of essential growth factors and vitamins. The current pharmacological legislation has switched the production flow in order to avoid the animal origin extracts and in particular those of bovine origin in consequence to the potential health risk associated with such products.

Is important to take in account that, when using semi-synthetic media, batch to batch reproducibility can be highly variable. So, if accurate batch reproducibility is the main target, is strongly advisable, despite being quite expensive and time consuming, to analyze the poorly characterized component in order to completely define what is contributing to cell growth or product formation or both.

Complex media

The composition of complex media is poorly characterized, usually are largely made of substances directly derived from animal or plant. We can tell that composition of each of these components can be different from year to year, location of origin can be different too and small changes in manufacturing can cause a high batch to batch variation when fermentations are performed using these media. They are relatively cheap, are usually supplied in bulk. Substances like cane and beet molasses, whey protein powders, hydrolyzed starch, soya bean powders and a wide range of oils belong to this category. Complex media are employed in many biotechnological processes, in

particular in those with a high volume/low value product ratio, like, for instance, the production of baker's yeast cells. One strong disadvantage of complex media is that foam production, due to the high concentration in amino acids, is far worse than in synthetic media, so the use of antifoam must taken in account when employing such media.

Small scale research rarely employ such media, unless a proper big scale industrial process has to be reproduced in laboratory conditions. Nevertheless high variability from experiment to experiment has to be taken in account and in order to obtain statistic significance in reproducibility, the operator must replicate fermentation for a longer period of time.

Medium components

Each medium, being synthetic, semi-synthetic or rich, has to reflect the elemental balance of the cell that the operator wants to cultivate (table 2). In light of the macromolecular composition of the typical cells employed in bioprocess science, no matter how particular a medium will be, it will be designed to include a carbon source, a nitrogen source, a phosphate source and trace elements.

Macromolecule	Bacteria (%)	Yeast (%)	Fungi (%)
Protein	55	40	32
Carbohydrate	9	38	49
Lipid	7	8	8
Nucleic acid	23	8	5
Ash	6	6	6

Table 2. Typical macromolecular composition of bacteria, yeasts and fungi.

Carbon source

A carbon source is the energetic coin that cells use both for growth processes and production of primary and secondary metabolites. There is a wide choice of carbon sources that can be utilized, this choice is usually driven not only depending on the organism needs but also thinking about the economics of the process. Indeed is very important, when designing a bioprocess with the future goal of scaling up, to think also about increasing costs of production. Here we will report some commonly used carbon sources underlining their advantages and disadvantages.

Glucose

Glucose is universally used with success for growth of most cell lines, either animal, plant or microbial. Supplied as powder, this substrate is reliable, cheap, immediately available, easy to handle and with zero health risks. These properties make glucose the first choice carbon source. Nevertheless there are some drawbacks when employing glucose, in particular: if the concentration of glucose is not calibrated or the glucose is over fed, the organism will suffer from "Crabtree effect" at the initial stage of growth. This situation can be easily avoided by careful monitoring of glucose feed.

Glycerol

Glycerol is also a typical carbon source employed in bioprocess experimentation. The advantages of using glycerol is that it does not tend to suffer from the same drawbacks of glucose, in particular if over fed, its presence will not cause any metabolic shift and no "Crabtree effect" will take place, so no acetate will be produced. The disadvantages are mainly cost related, in fact glycerol is much more expensive than glucose powder, this has to be taken in account when designing a bioprocess with the future intention of scaling it up. It is difficult to sterilize. When used pure its density can cause some problems when calibrating the feed pumps, flow rate must be double checked in order to ensure that the right amount of glycerol is pumped within the vessel.

Nitrogen source

Nitrogen plays a fundamental role on determining the final amount of biomass produced at the end of fermentation. It is necessary for synthesis of proteins and nucleic acids. There are various sources from which nitrogen can be obtained and their choice strongly depends on the type of final product that the operator wants to harvest.

Complex Nitrogen Source

On market there are a wide choice of complex nitrogen sources: soya beans meals (8% w/w nitrogen), yeast extract (13-14% w/w nitrogen) and corn steep liquor (4% w/w nitrogen). All contain a significant amount of nitrogen, plus they are also a good source of vitamins, micronutrients and minerals. Of course, when using complex nitrogen sources, is mandatory to take in account low batch to batch reproducibility. Consequently in order to obtain stable results, more experimentation is needed, consuming time and spending considerable amount of money on costly chemical analysis.

Nitrogen Based Salts

The most common and most employed nitrogen based salts are probably ammonium sulfate and ammonium chloride. Ammonium salts are used by many organisms, they are cheap and easy to use. No need of special expertise for their use, those can simply be storage at cool temperature. Thanks to ammonium probes, which are routinely used, is also very easy to measure the amount of ammonium present in a fermentation broth. Nevertheless, is important to remind that, dealing with salt, demands attention, in fact the wrong salt concentration in medium formulation or the combination with other salts can cause precipitation, possibly damaging the vessel or other bioreactor's parts.

Phosphorus source

Among those elements that are essential for the growth of microorganisms, phosphorus is probably one of the most important when the main target of production is the synthesis of primary and secondary metabolites. In fact, by providing the right amount of phosphorus, the processes of ATP generation and DNA biosynthesis are strongly enhanced. Moreover, using phosphorus sources like phosphate buffers, can contribute to pH balancing throughout the process of fermentation. Is important to remind that this element is usually found under the form of inorganic salts, so the operator must be careful when preparing media, especially rich complex media, because the high probability of salt precipitation due to combination with other salts contained in the rich nitrogen sources that compose typical riche media.

Trace Elements

Also known as micronutrients, trace elements are required in tiny amounts, usually few μg per liter. They have many functions within the cell, but most of the time their function is coupled with enzymatic activity, being a part of the active site's enzymes or functioning as a catalyst for the chemical reaction in which the enzyme is involved. When using complex or semi-synthetic media, the use of trace elements is not needed, as they may be already present. Micro nutrients are usually used when employing synthetic media or if a cell has an absolute demand of one or more of these specific elements. Typical trace elements are: iron, (Fe, very important function in cellular respiration), chromium (Cr), molybdenum (Mo), copper (Cu), manganese (Mn), cobalt (Co). they are administered through a trace element solution, which usually is prepared right before administration because the solution is highly susceptible to precipitation.

Purification tools

There is a wide choice of tools that can be employed to perform purification. Here, the term purification is referred to cells purification and also to proteins purification. Usually, when performing an upstream bioprocess such fermentations, the operator must take in account that both cells produced and then target proteins expressed must be purified. There are three main techniques that are applicable: centrifugation, filtration and chromatography.

Centrifugation

Centrifugation is, by definition, the process of separating lighter portions of a solution, mixture, or suspension from the heavier portions by centrifugal force. This process is widely used in all science laboratories around the world and when it comes to fermentation bioprocesses, centrifugation can play a pivotal role.

Conventional lab scaled centrifuges have the advantages of been easy to use and widespread. However, their main limitation is the bucket capacity which is most of the time under sized, with a maximum capacity of 1 liter. Although it may be sufficient to process small scale work, such capability isn't going to be able to keep up with purification of large quantities of cell broth since the process would be too much time consuming. There are some valid alternatives, in fact, when is needed to process relevant volumes of fermentation slurry (from 20 to 500 liters), continuous centrifuges are employed. These machines require a continuous flow of broth into their rotor enabled by hydrostatic pressure. Examples of continuous centrifuges are the "Heraeus Continuous Flow Centrifuge", ideal for small working volumes (10 liters max) and the "Tubular Bowl centrifuges", suitable for harvesting volumes from 30 to 500 liters (figure 10).

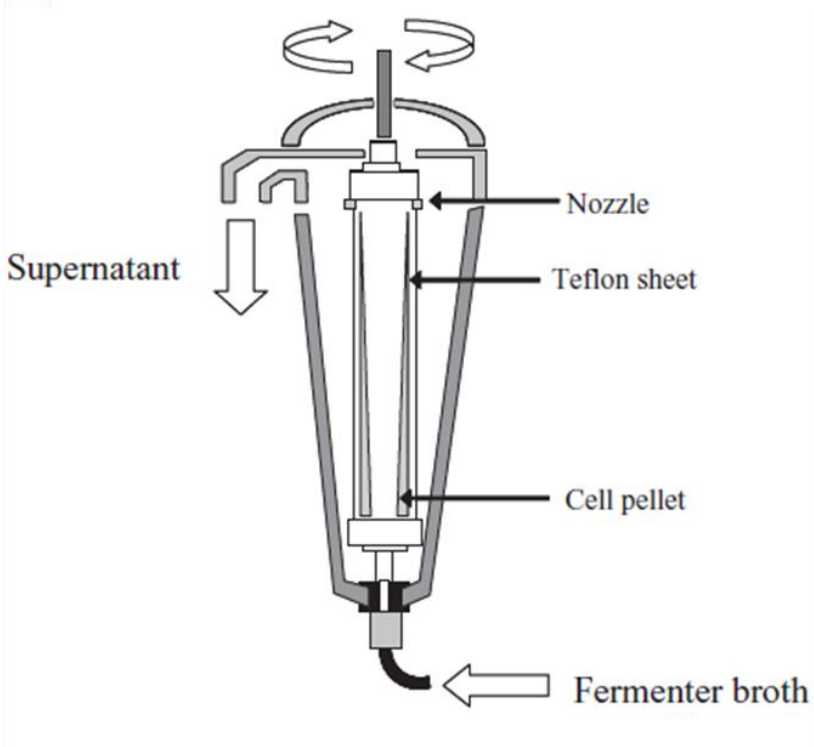


Figure 10. schematic description of a tubular centrifuge.

The process of centrifugation is not just suited for separating cells from culture medium, but such process can be essential for separation of cell debris from cytoplasm. Indeed if the product of interest is a protein produced at cytoplasm level, then centrifugation can be used to clarify the solution obtained after cell lysis. Some of those centrifugation machineries that have been described previously can be employed for both functions.

Filtration

Filtration is another separation technology that can be employed for various aspects of purification. It can be divided in two main branches: Single Pass dead end filtration and Tangential Flow Filtration (TFF). Dead end filtration can be defined as the separation of particles from a fluid (liquid or gas) by passage of the fluid through a permeable medium. At laboratory scale, this process is probably the most used, for example sterilization of small amount of solutions like media, antibiotics, buffers, etc., are performed using dead end filters. This process has the advantage of

been quick and easy to perform, with very low cost, but unfortunately, it can be used only for purification of small amount of solutions and not to separate cells from media (figure 11).

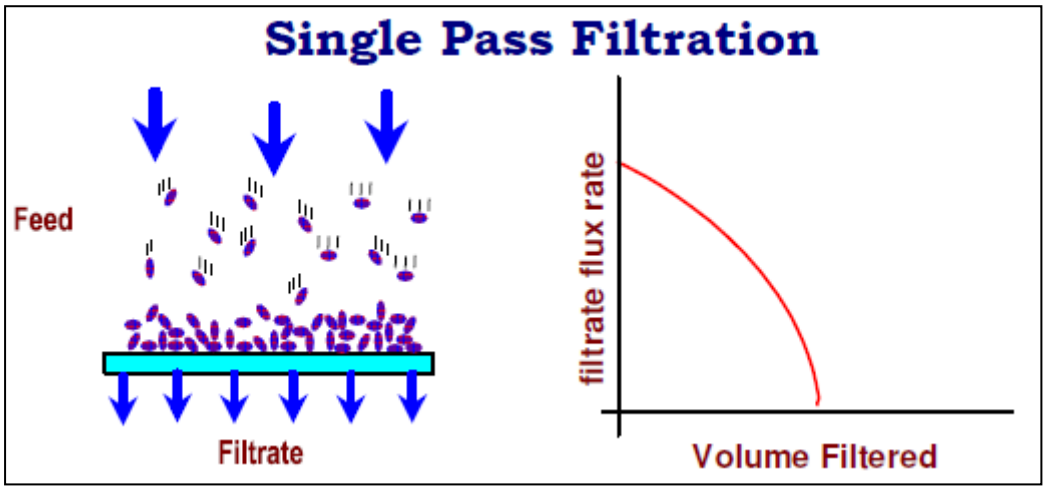


Figure 11. Graphical description of single pass filtration scheme of action.

TFF filtration, on the other hand, has many strong advantages: it can be used as an alternative to centrifugation for harvesting cells from culture media, it can be used to process a huge range of volumes, from few liters to hundreds, it can be employed for cell washing or buffer exchange and it can be used even for proteins concentration and separation. The principle behind the TFF machinery lies in the specific structure of the membranes utilized: the solution, that has to be filtered, passes through specific membrane's channels thanks to a pressure imposed. These channels have porous walls, the pores size and structure defining the specific membrane cut-off. A solution that contains different size particles, when gets through these channels, undergoes to a process of separation; if one particle has a size equal or bigger than the pores size, it will stay within those channels and will become the so called "concentrated" because it will be recycled in the initial solution repeatedly. On the contrary, those particles of size smaller of the membrane's pores cut-off will get through those very pores and channeled in the "filtrated" flux (figure 11). There is a very detailed range of membrane sizes and cut-offs, so, not just cell separation can be performed, but also protein solution concentration and separation. The only disadvantage is the initial cost of the whole apparatus, which is expensive, and the need of specialized operators.

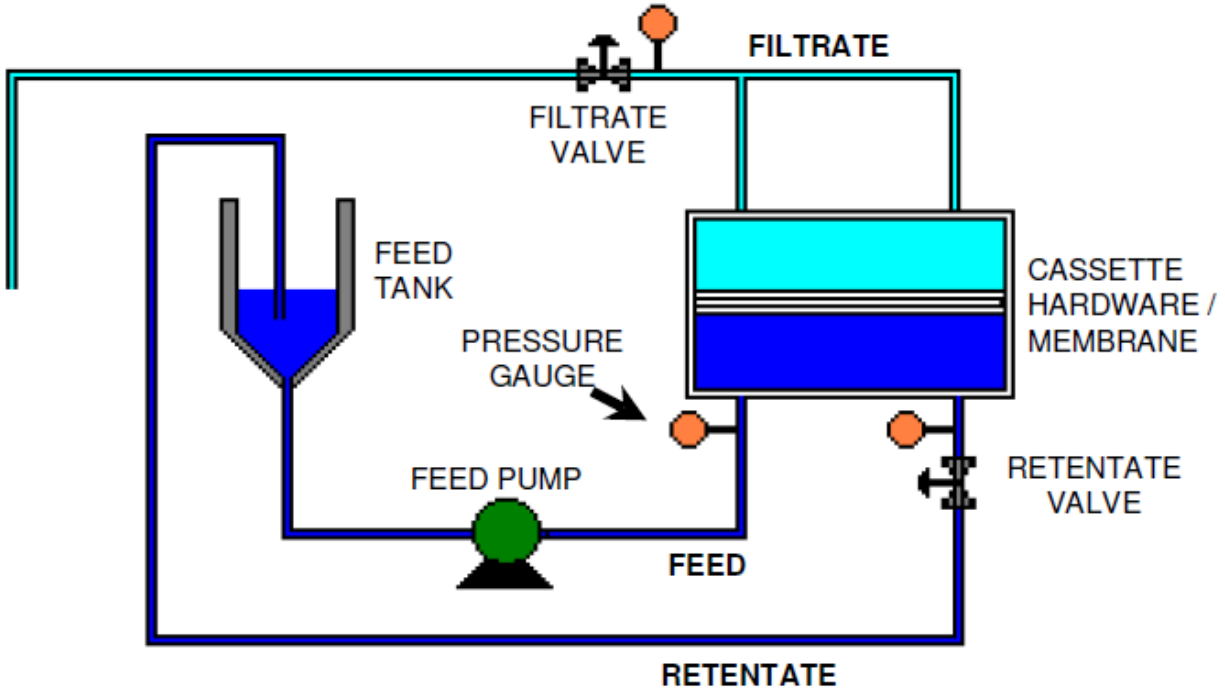


Figure 11. Tangential flow filtration system mode of operation.

Chromatography

Historically, liquid chromatography was first developed and put in use during the early 1900s. A glass cylinder was packed with finely separated powder, a sample was applied to the top part of the column and a solvent was poured directly onto the column (figure 12).

As the solvent gets down the column by gravity, the various components of the sample start to move at different velocity and became separated. At the beginning, samples were colored in order to visually observe the separation process. Then portions of the solvent coming out the column were collected, the solvent was removed by evaporation and the separated compounds were recovered for further analysis. Back then a brand new column was required for every new experiment and the whole process was performed manually from beginning to end. Even so, at that time, the scientists were willing to go through with all the labor necessary to perform such experiment because its unique capability, in comparison to other techniques, for analyzing chemical mixtures. Today, chromatography technology has strongly evolved in applications, automation and performances. The apex of this technology is High Performance Liquid Chromatography (HPLC). Its functioning

is simply explained: the operator starts by placing samples on a tray for automatic injection into the column, a pump or a sets of pumps ensure that the solvent is continually pumped through the column and the separated compounds are continually monitored by a specific detector as they are eluted from the column. The resulting detector signal, plotted against time, generates the so called *chromatogram* (figure 12).

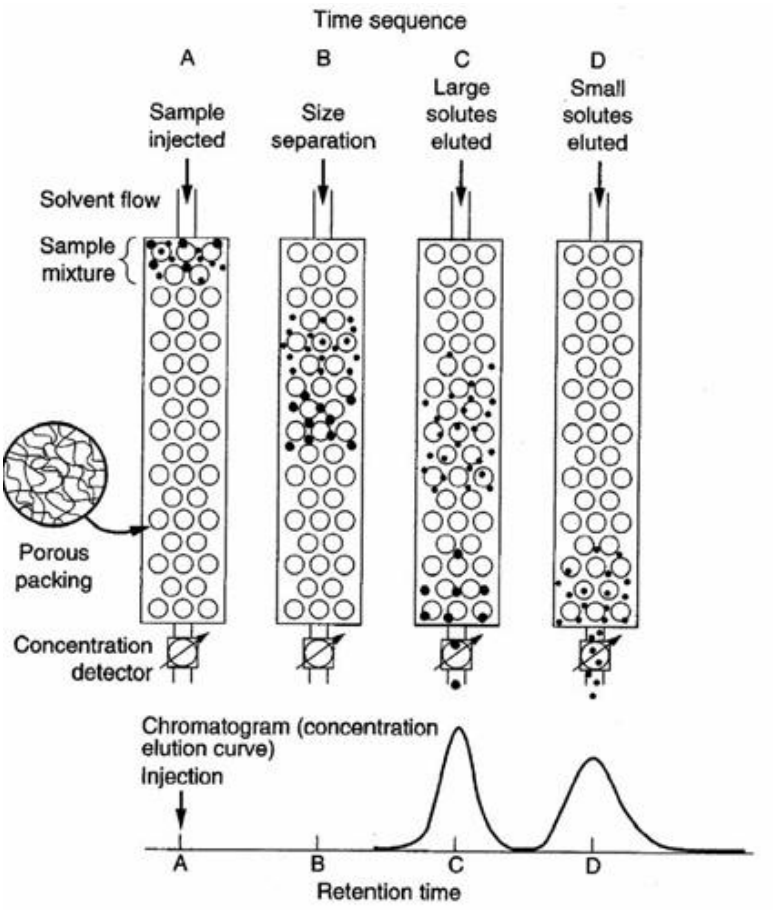


Figure 12. Stages in a process of chromatography and example of relative chromatogram.

The entire operation is under computer control, so the process is almost fully automated and this very computer can generate also specific reports for each run. Despite the automation, HPLC is differentiated from others chromatographic techniques because the use of high-pressure pumps that allow faster and enhanced separation, reuse of columns, better control of the process and very high reproducibility, is important to underline that HPLC is a analytic system, perfect to verify or identify the presence of specific compounds, but unfitted to perform production's purifications.

Here are going to be listed the most common HPLC separation modes:

- **Reverse phase chromatography (RPC)**: probably the most used method, it employs a nonpolar column and a mobile phase which is a mixture of organic solvent like acetonitrile and water
- **Normal phase chromatography (NCP)**: is used for non sample which are insoluble in water, preparative HPLC and isomer's separation
- **Non aqueous reverse phase chromatography (NARP)**: usually needed for very hydrophobic, water insoluble samples.
- **Hydrophilic interaction chromatography (HILIC)**: this technique gives best results for samples that are very polar. The column is made out of silica or amide-bonded phase (polar phase), and the mobile phase is usually a mix of water and polar solvent
- **Ion exchange chromatography (IEC)**: here the material, which the column is made of, contains charged groups that can and bind sample ions of opposite charge, the mobile phase is usually aqueous mixture of salts plus buffers; this technique is useful for separation of ionizable samples such bases and acids and even more for large bio-molecules like proteins and nucleic acids.
- **Size exclusion chromatography (SEC)**: the process of separation, in this case, is performed on the basis of molecular weight. An inert column is employed with either an aqueous or organic mobile phase; this technique is employed for large bio-molecules and synthetic polymers.

Now days the process of chromatographic separation can also be performed by using specific membranes instead of using traditional stationary phases like columns. Industrial research has focused more on scaling up such processes and the use of membranes with high retention capability is continuously increasing. Each membrane has the same possibility of a column; it can work as ion exchange, as size exclusion, etc. The major advantages of using membranes instead of columns is that the purification process is upgraded in terms of speed, volume of sample purified, quantity of separated sample and cost effectiveness. For these reasons, the use of membranes for separation is strongly advised when and industrial setup is to be developed.

Aim of the project

Production of big size recombinant proteins (90 kDa or more) in foreign expression hosts, has many drawbacks. Indeed, the bigger the protein the more will be the effort for its production, either in terms of time and energy consumed. Most of the times, these metabolic stresses will upset the protein production yield, both in quantity and quality, obtaining low amount of desired product, low functionality in terms of activity or both. So fine tuning an expression system, allowing significant production and efficient purification of such products, is highly desirable. The aim of this research is been focused on defining the most suitable protocol for production and purification of two recombinant collagenases: Abiel's Col G and Col H. Firstly is been investigated the best way to recover significant amount of each collagenase. After classic shake flask culturing, and definition of best growth medium, different volume batch fermentations have been performed. The data obtained from batch fermentation's experimentations is been used to gain info regarding metabolic growth and recombinant protein expression of each collagenase expressing bacteria. These info have been used to go further and develop a fed-batch fermentation strategy for both collagenase expression bacteria. Such choice was driven by the will of improving even more final cell density and consequently recombinant protein production. In order to define the most appropriate purification protocol for each lot of collagenase produced, we investigated the efficacy and efficiency of several downstream processes. Regarding the best way to treat and process both fermentation slurry solution and also crude extract proteins solutions, a cool temperature Tangential Flow Filtration (TFF) is been specifically customized. For better recombinant protein purification, an affinity chromatography was applied to separate each chimeric collagenase from the rest. Since one of our most important concerns was the quality of each protein of interest, many investigations have been performed: endotoxins measurements assays have been performed for each collagenase solution. Plus, to monitor the quantity of enzyme recovered from each fermentation run, HPLC chromatography is been employed. In the end, is been also experimented the enzymatic activity of each lot of collagenase produced. The means of such experimental protocol were aimed at defining the best parameters for both production and purification of such peculiar recombinant products like Col G and Col H. After this work is possible to state that, not just is possible to use a stable protocol for production and purification of big size proteases, but also this very protocol is indeed fully scalable both in terms of production and purification, consequently, this research can be a step forward in the field of up and downstream processing science, contributing to the advance of industrial research regarding production of big size recombinant proteins.

Materials and methods

Bacterial Strains and Plasmids

Each strain used was an ampicillin-resistant recombinant *E. coli* BL21 (DE3); one developed for over-expression of Col G encoding gene and one for Col H, respectively. The over-expression of each cloned gene is under the regulation of T7 polymerase responsive promotor and a lac operator in a pMAL-c5X expression vector. Also, the recombinant plasmid contained the ampicillin resistance gene for selection of plasmid containing bacterial clones. Each stab was maintained in 40 % sterile glycerol at minus 80 °C.

Cultivation medium

Different media formulations have been employed throughout the course of this research. In particular, after a series of shake flask culture experimentations, during which M-9 synthetic medium, M-9 yeast extract enriched semi-synthetic medium, LB (Luria-Bertani) broth medium and Terrific broth medium have been tested either for bacterial cells growth and collagenases production, Terrific broth was the one which gave the best results both in terms of bacterial cell growth and recombinant protein production. Consequently, is been chosen to use the animal derived complex medium Terrific broth, both for the first series of batch fermentation runs and fed-batch fermentations (Lessard ,2013). In a second phase, in obedience to new upstream bioprocess regulations, no animal derived medium components have been employed to perform any kind of fermentation. It was decided to redesign Terrific broth using alternative complex nitrogen sources, 100% vegetable (vegetable terrific broth). In particular tryptone vegetable (Sigma Aldrich) and vegetable yeast extract (Merck). Glycerol, 99% pure and bi-distilled (VWR) was added at concentration of 4 ml per liter both in batch and fed-batch fermentations media. Both batch media and fed-batch solutions, with the exception of glycerol, have been heat sterilized at 121°C for 15 min. Filter sterilized ampicillin was added at a final concentration of 100 mM in all cases. The inducer, isopropyl-b-D-thiogalactopyranoside (IPTG) was also filter-sterilized and added to the culture when required at specifics final concentration. Fed-batch solutions were two and consisted in: Nitrogen feed solution and Carbon feed solution.

Nitrogen plus phosphate feed solution

This solution is composed by vegetable tryptone and vegetable yeast extract in a 1/1 ratio. After heat sterilization at 121°C for 15 min, a solution of K_2HPO_4 12g/100ml concentrated, is then aseptically added to the Nitrogen solution via 0.22μm filter injection.

Carbon feed solution

This solution is made of sterile glycerol, 99% pure and bi-distilled. Since evident problems of heat stability, this solution could not be heat sterilized nor filtered with a 0.22μm filter. It had to be purchased already sterile and opened under the hood every time it is employed.

Inoculum development

A loopful of frozen glycerol stock (kept at minus 80 °C) was streaked on a LB agar plate containing ampicillin, 100 mM concentrated, and incubated at 37 °C over-night. A single isolated colony was then harvested and transferred to LB vegetable medium, incubated on a rotary shaker at 31 °C and 180 rpm for 16 h. This pre-inoculum was then transferred at a rate of 50 % (v/v) to the main inoculum medium (vegetable terrific broth) and incubated for 7 h at 37 °C, stirring at 220 rpm.

Bioreactor cultivation

Batch and fed-batch culture experiments were conducted in a 10 L Bioflo celligen benchtop bioreactor (New Brunswick Scientific Co., USA) (figure 13). pH was set and maintained at 7.2, 5 N NaOH was used to stabilize pH. Temperature was kept at 37 °C throughout batch an fed-batch fermentations processes. Dissolved Oxygen Concentration (DOC) was monitored using a polarographic steam sterilizable oxygen electrode (Mettler–Toledo International Inc., Switzerland), and reported as percentage of air saturation. The DOC was maintained at specified values by varying airflow and impeller speed.

Figure 13. Eppendorf Bio-flow 13 Celligene bench top bioreactors .

Batch cultivation

First series of batch fermentation were performed with a final volume of 4 liters. Second series, with a final working volume of 10 liters. For both series, sterilization was performed by using a 155 liters working volume steam autoclave (VWR). Sterilization parameters were the same for 4 liters fermentations and 10 liters fermentations, 121°C for 15 min. Each Col G ad Col H batch fermentations were induced when the OD_{600nm} reached a value of 5/5.5 (5 h after inoculum injection). Induction was performed using IPTG (isopropyl β-D-thiogalactoside) (USB chemicals) 1M initial concentrated. DO was maintained at 20 % (unless otherwise stated) by cascading agitation rate (280–900 rpm) with DO concentration at constant aeration rate.

Fed-batch cultivation

Fed-batch experiments for each collagenase expressing bacteria started as batch experiments; the initial batch cultures were started by injection of 250 ml of an overnight-growth seed culture. Foam production was kept under control by adding 1,5 ml of Poly Propylene Glycol (PPG). After exhaustion of glycerol, visualized with the simple Dissolved Oxygen stat method (Lee J. et al.,1999), cultivation was begun by pumping ultra pure sterile glycerol within the vessel following a specific exponential criteria. It was carried on for 8 h. Feeding start point corresponded to a common OD_{600nm} of 12/13 for both recombinant strains. Induction was done at this very point applying different strategies of induction. DO concentration was kept at a minimum of 20 % saturation throughout each process by cascading impeller speed and increasing inlet air volume per minute.

Exponential feeding was performed applying equation 1, which was obtained from a mass balance on cell and substrate, and by assuming a quasi-steady state (babaeipour *et al*., 2008).

$$M_s(t) = F(t)S_0 = [\mu(t)/Y_{x/s} + m]X_{t_0}V_{t_0}\exp[\int_0^t \mu(t)dt] \quad \textbf{(1)}$$

Where $M_s(t)$ is the mass feeding rate of glucose ($g \cdot h^{-1}$), V_{t0} is the medium volume in the bioreactor (litres), X_{t0} is the biomass concentration at the start of the feeding step ($g \cdot litre^{-1}$), t is the time (h), $\mu(t)$ is μ as a function of process time (h^{-1}), S_0 is the glucose concentration ($g \cdot litre^{-1}$) in the feeding solution, F(t) is the feeding rate ($litres \cdot h^{-1}$), and $Y_{x/s}$ is the yield of biomass/substrate ($g \cdot g^{-1}$), t_0 is the time at the start of feeding (h) and m is the specific maintenance coefficient ($g \cdot h^{-1} \cdot g^{-1}$). The yield coefficient ($Y_{x/s}$) and the maintenance coefficient (m) were set at specific constant values for all experiments.

Induction system

Different approaches have been chosen in order to obtain best induction of collagenase production in each collagenase's expressing bacteria. The inductor IPTG (isopropyl β-D-thiogalactoside) (USB chemicals), is been administered following a single shot method, a "steady multiple shot induction system", and then a " variable multiple shot induction system". The first approach is the canonical one; when the operator reckons that time to induce is approaching, a single injection of inducer solution is administered (figure 14). The second approach "steady multiple shot system" is based on dividing the same amount of a single inductor solution in multiple and equal volume and concentration mini shots, separating each shot from another by a constant gap of time. This process is been designed in order to get a better coverage and avoiding possible growth inhibition by high concentration of inducer. The last method: "variable multiple shot induction" is an evolution of the second approach, in this case each shot concentration is balanced depending on the OD (optical density units) reached by the fermentation slurry at each selected time. So, even if the gap of time between a shot and the other is constant, the concentration varies depending on the bacterium growth curve.

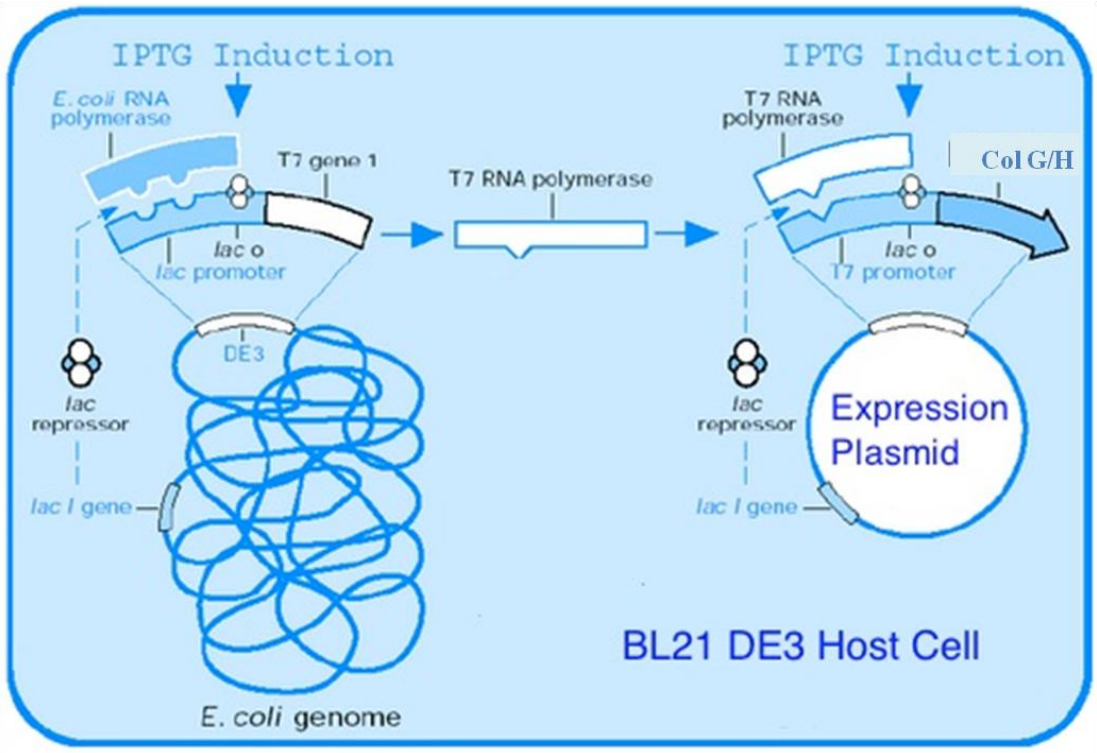


Figure 14. IPTG molecule induction system workflow.

Bacterial purification

Each fermentation broth, no matter how it was obtained (batch or fed-batch) was purified by means of tangential flow filtration (TFF). In particular, a "CM500S" ultra filtration device (Pall corp.) is been employed. Bacteria concentration and dia-filtration were performed using a 300 kDa cut-off TFF membranes (Pall corp.) Purified bacteria, were lysed by means of mechanical shear, using a 1000 bar French press (Niro Soavi Panda). Separation between cell debris ad crude protein solution was obtained by centrifugation (Beckman Cultures).

Analytical methods

Crude extract protein concentration was measured by means of Bradford assay (Bradford, 1976). Protein samples obtained from each fermentation experiment were loaded onto a SDS PAGE 7.5 % gel (Sambrook et al.,1989), in order to assess the level of collagenases induction obtained. The expression of each collagenase was quantified by densitometric analysis of the Commassie stained bands and BSA standards (Image J software). Affinity chromatography was used to purify each collagenase; indeed, each enzyme produced, carries a maltose binding protein tag at its C-terminus. Previous studies demonstrated that the tagging does not affect catalytic activity. Each enzyme solution, obtained from affinity chromatography, was dia-filtered and concentrated using yet again tangential flow filtration. In this case is been employed a 50 kDa TFF membrane cassette (Pall corp.). This purification step was carried both at room temperature and at 7°C using a stainless steel cooling coil system (custom made). Final enzymatic activities were evaluated using partial modified Grassmann Pz activity assay (Grassmann et al., 1960). At the end of purification process, both Col G and Col H collagensases purified solutions have been lyophilized (using a Martin Christ Epsilon 1.4D instrument) for best preservation.

HPLC

Each collagenase sample, after lyophilisation, is been rehydrated with a solution of Tris 1 mM obtaining a concentration of 1 mg/ml. Each solution is been analysed in order to test the final purity in terms of percentage of enzymes, through High Pressure Liquid Chromatography (Hitachi Chromaster). The column used for this purpose is a Size-Exclusion Chromatography (SEC) column (Shodex PROTEIN KW403-4F). Here an inert column is employed either with an aqueous mobile phase or an organic one, providing separation on the basis molecular weight. This procedure is applied for the study of large biomolecules or synthetic polymers. So, through SEC HPLC, each lot of Col G and Col H is been validated to be pure in a concentration up to 98% via isocratic elution, using Tris 1 mM as solvent (figure 15).

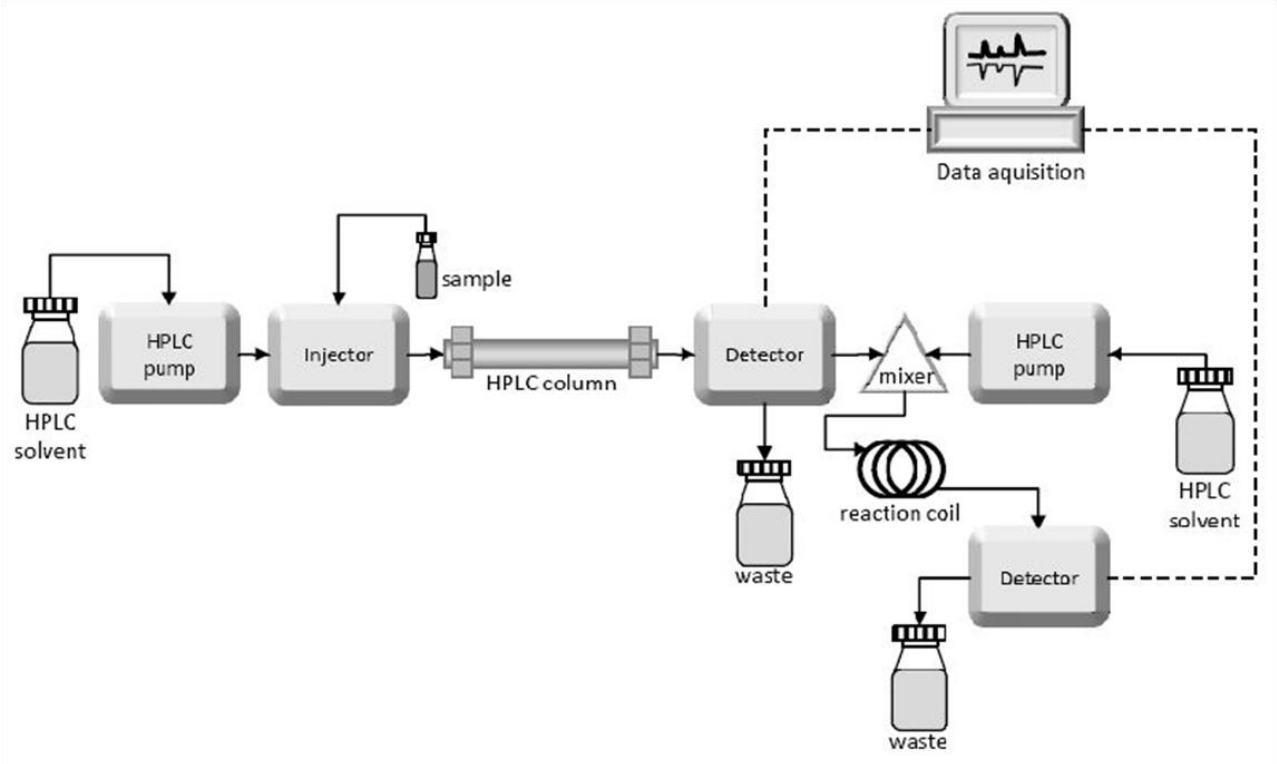


Figure 15. schematic representation of HPLC- SEC workflow for analysing Col G and Col H.

Results and Discussion

In order to define the most profitable protocol for producing and purifying Col G and Col H recombinant proteins, different production approaches have been put to test. Batch and fed-batch fermentations strategies have been performed and customized. The resulting fermentation slurries were then employed in purification studies aimed at obtaining maximum recovery and best enzymatic quality, in terms of final activity measured.

Culture medium choice

Defining the best medium is not an easy task, usually it consumes relevant amount of time, but if the process of medium selection is been done properly then the time is well spent because the bio-process will be optimized from the start.

When planning on choosing which medium to employ, is essential to focus on the final target of the culturing experiment; what the process is about? Production of cells, primary metabolite, secondary metabolite, recombinant product.

One of the subjects of this PhD project was to optimize upstream production of two recombinant proteases: Col G and Col H. Since each collagenase has been engineered to be produced by a commercial strain of *E. coli,* firstly several shake flask culture experiments runs were performed. Each run of shake flask cultures was necessary to test the ability of several putative media formulations both for growth of each collagenase expressing bacteria and subsequent recombinant protein expression (figure 16). Three types of media have been selected for shake flask culture experiments: a minimal synthetic medium (M9), a semi-synthetic medium (M9 Modified) and a complex medium (Terrific Broth).

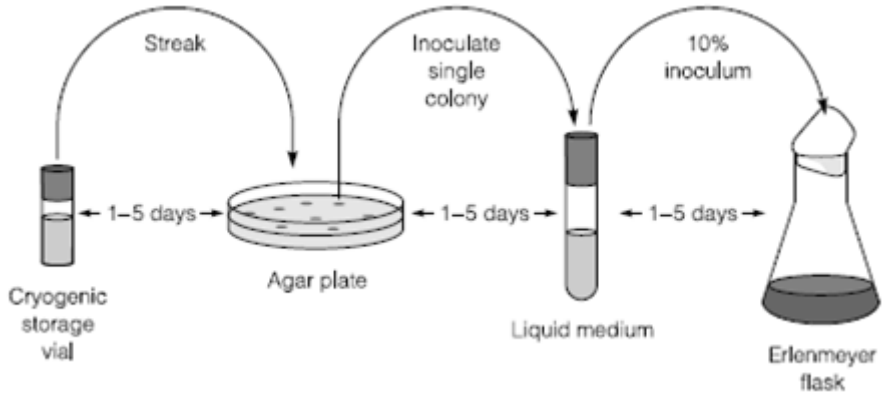


Figure 16. shake flask culturing workflow, from stab to fermentation's seed culture.

M9 minimal synthetic medium shake flask cultures

M9 is a synthetic medium 100% defined. Its ingredients, for 1 liter solution, are: $Na_2HPO_4 \cdot 7H_2O$ 12.8g *OR* Na_2HPO_4 (anhydrous) 6g, KH_2PO_4 3 g, NaCl 0.5g, NH_4Cl 1g, a carbon source (glycerol) to 0.2% (v/v) final volume, pH to 7.4 with NaOH and autoclaved for 15 min at 121°C and then addiction of sterile micronutrient components to a final concentration of:

Stock Concentration	Micronutrient	Final Concentration
1 M	$MgSO_4$	1 mM
1 M	$CaCl_2$	100 μM
3 mM	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	$3x10^{-9}$ M
400 mM	H_3BO_3	$4x10^{-7}$ M
30 mM	$CoCl_2 \cdot 6H_2O$	$3x10^{-8}$ M
10 mM	$CuSO_4 \cdot 5H_2O$	$1x10^{-8}$ M
80 mM	$MnCl_2 \cdot 4H_2O$	$8x10^{-8}$ M
10 mM	$ZnSO_4 \cdot 7H_2O$	$1x10^{-8}$ M
5 mM	$FeSO_4 \cdot 7H_2O$§	$1x10^{-6}$ M

After having batched up some litres of M9 medium, several runs of shake flask cultures were performed. For each run is been decided to employ 1 liter Erlenmeyer flasks, 5 for each run. Culturing was performed by adding 200 ml of sterile M9 medium to each flask, injecting filter sterilized ampicillin to a final concentration of 100 mM, adding 20 ml of Col G/H over-night starting inoculums and by incubating each flask at 37 °C for 8 h at 240 rpm in a orbital shake incubator (Sartorius Stedim). Throughout the process of culture, samples were taken aseptically in order to monitor Optical Density Units (ODs). This was performed by reads at 600 nm using spectrophotometric analysis. After 8 h of growth, each culture reached a maximum OD value of 1,8. During this first run no inductor was added, but only cell growth was monitored. A second run was

prepared and incubated at the same conditions but for a longer period of time (16 h). In this case, each culture was able to reach a maximum growth of 2.4 OD. Then is been decided to study, despite the not encouraging growth results, if the M9 medium was fit for expression of Col G and/or Col H. Another run of shake flask culture was performed, and when cells OD reached 1.1, an injection of IPTG inductor at final concentration 100mM was administered to each culture. The whole process of growth went on for 8 h and induction was carried on for 3 h before cells were harvested. Final OD in this case reached a maximum value of 1,5. Protein extracts were recovered from each induced culture and analyzed for the level of induction of recombinant product (Col G expressing bacteria cultures and Col H expressing bacteria cultures, respectively). SDS-PAGE analysis (7,5% gels) revealed a very low level of induction both for Col G and Col H protein extracts, meaning ultimately that this medium was neither fit for cell proliferation and neither for supporting recombinant protein production. Each run was performed in triplicate in order to confirm the validity of results.

M9 modified semi-synthetic medium shake flask cultures

After having used M9 minimal medium, is been decided to try and add yeast extract. Basically is been investigate if the addiction of a complex nitrogen source to M9 medium would cause an increase in final optical cell density and better recombinant protein expression. The classic M9 medium recipe is been modified by the addiction of 18 g/L of yeast extract. Again, same procedure employed for testing M9 synthetic medium is also been chosen for this new formula. First series of cultures, using the new formula, were performed in order to measure the capability of improving final cell concentration; unfortunately after 8 h of incubation at 37 °C, the maximum OD measured at 600 nm had the value of 3,8.

Another series of cultures was tested to measure the capability of this medium in supporting recombinant protein expression. Unfortunately, adding yeast extract did not bring better result, in fact induction, carried on for 3 h, did not increase but only basal recombinant enzyme expression was observed. Is important to remark that all culturing parameters were maintained the same as those used when classic M9 medium was employed and also in this case, each culture run was performed in triplicate.

Complex medium: Terrific Broth

The need for a medium which would allow consistent cell growth and favour recombinant protein expression has pushed this research towards complex media. After careful literature studies, is been decided to try out Terrific broth (TB). Such medium is a mixture of yeast extract, tryptone, glycerol and phosphate salts. In particular, its composition for liter is: 24 g of yeast extract, 12 g of tryptone, 2.31 g of KH_2PO_4 and 12.5 g of K_2HPO_4 and 4 ml of pure glycerol. First series of cultures were performed as previously stated for both M9 and M9 semi-synthetic media experiments. After having cultured over-night a starter culture of Col G and Col H separately, 20 ml of each starter culture were added to 200 ml of autoclaved TB. Cultures were incubated for 8 h. OD increase was monitored with the use of a spectrophotometer , by readings at 600 nm. Each shake flask culture, at the end point, had reached a value oscillating between 7.5 and 8.2 OD. These results were pretty encouraging and so the values reached were now acceptable for further experimentations. Second step, is been to investigate if such medium was capable of sustain acceptable levels of recombinant protein production (both Col G and H). Same culturing conditions were applied. IPTG was injected at a final concentration of 100 mM (same value of previous experimentations). After 3 h of induction, cells were harvested and protein extracted. Each protein extract, loaded onto a 7.5% SDS-PAGE gel showed a remarkable induction band. The band intensity was measured using the software Image J and then compared to the intensity of other bands from the same pattern of proteins extracted. This analysis underlined that when both Col G and Col H were produced in TB they represented more than 25% of total protein produced, confirming that TB is not just fit for cell growth but also for supporting recombinant protein expression.

Complex medium: Vegetable Terrific Broth

According to a new and stricter pharmaceutical legislation, the use of animal derived media for production of drugs, is banned. Only animal free media are now allowed in the production of cells or human related drugs. Consequently, is been decided that Abiel's Col G and Col H recombinant proteases would have been produced through the utilization of such media or media components. The precious information gained through previous experimentation was a considerable help in choosing how to define the brand new animal free medium. The target was to replicate the rich concentrations of nitrogen and phosphate that allowed Col G and Col H expressing bacteria to grow up to 8 OD and express relevant amount of recombinant products; basically a vegetable terrific broth had to be developed. To do so, is been decided to employ vegetable yeast extract (Merck) and

vegetable tryptone (Sigma) instead of the animal derived analogs. It was no easy job since is been discovered that solubility of vegetable tryptone was less than the typical animal derived tryptone. In the end 24 g of vegetable yeast extract, 12 g of vegetable tryptone, 13 g of K_2HPO_4, 2 g of KH_2PO_4 and 4 ml of pure glycerol were employed to define 1 liter of vegetable terrific broth. Same series of experiments already used for other media experimentations were carried out to test both cell growth efficiency and recombinant protein expression. When using vegetable terrific broth in shake flask cultures, both for Col G and Col H expressing bacteria, the maximum OD registered after 8 h of incubation at 37 °C was 7,4. Induction tests demonstrated that such formulation did not diminish the overall induction intensity, obtaining a value of 25% when SDS-PAGE gels were analyzed with Image-J software. The use of such medium will take place in this research only at the late stage development of fed-batch fermentation process; the reason was indeed the change in market legislation.

First stage batch fermentation

First series of batch fermentations, both for Col G and Col H expressing bacteria, were carried out using 10 liter working volume. In light of previous shake flasks culturing experimentations, we decided to employ TB as fermentation medium. In preliminary fermentation experiments, M9 and M9 modified were also employed, despite unfitting results obtained with shake flasks culturing, but each batch fermentation carried either with M9 or M9 modified was unsuccessful in terms of cell growth.

After Sterilization Out Place (SOP), performed at 121°C for 15 min., the vessel filled with medium was cooled by cold water. The cooling process stopped when temperature reached 37 °C. This temperature was kept constant for the rest of fermentation by using a specific heating jacket wrapped around the vessel itself. Each batch fermentation was carried out for 25 h. Just before injecting the seeding culture, 10 ml of ampicillin 100mM and 1 ml of Poly propylene glycol (antifoam agent) were added. Each starter seeding culture, either of Col G and Col H, had a volume of 60 ml and an optical cell density of 7.5 OD. After inoculum injection, the fermentation started; air saturation was set at 10% minimum by cascading with impeller mixing speed (value preset between 200 and 900 RPM).

Induction Timing

Induction was performed by single injection of 10 ml IPTG 1M, this injection was administered when OD culture reached a value of 4,5/5. Choosing 4,5 or 5 OD as induction point was determined by previous shake flasks induction experiments. Col G and Col H recombinant proteins should be classified as " non growth-associated products" like secondary metabolites, for example. Usually "non growth-associated products" possess a negligible rate of formation during active cell growth. These secondary metabolites can be produced in big quantity when cells enter stationary phase. This can be also defined as idiophase. Most antibiotics are produced as secondary metabolites. In this case, induction can be performed when entering stationary phase and after that the fermentation can then be terminated just before the cells enter the death phase. Peculiarly enough is the fact that the expression of both Col G and Col H recombinant proteases, in each induction test performed, could be classified as the production of "growth-associated products". Growth-associated products (primary metabolites) are produced in big quantities during the exponential phase with their formation decreasing when cell growth ceases. Typically the rate of product formation directly relates to the rate of growth (Figure 17). The fermentation can be stopped at the end of the exponential growth phase before entering stationary phase. This growth phase is sometimes referred to as the trophophase. Consequently, Col G and Col H expression had to begin at the start of exponential phase, in this case 4,5/5 OD units.

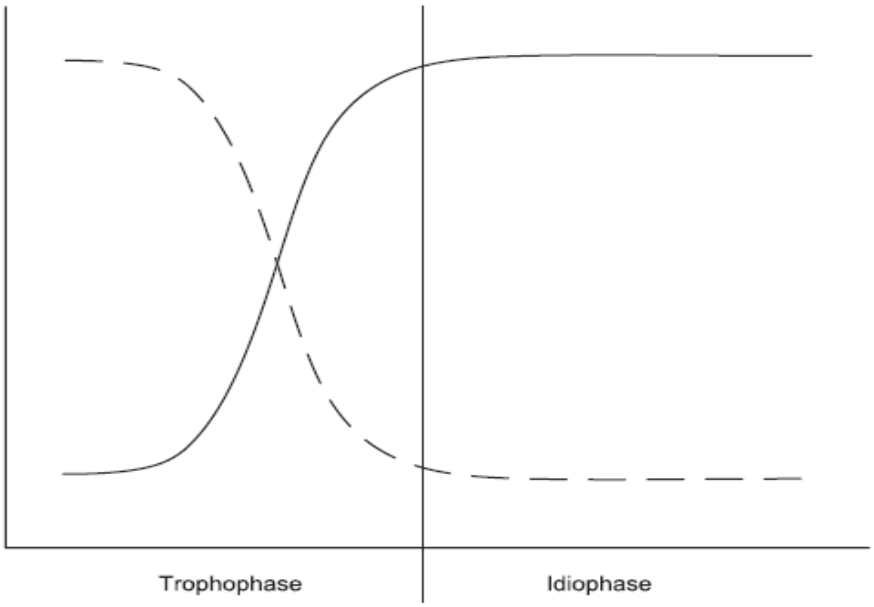


Figure 17. Trophophase and idiophase production within a classic cell colture.

Batch grow curve and recombinant protein expression

Each fermentation had a final output of 18 optical cell density units (Figure 18), corresponding about to 2 g of Col G and 1,8 g of Col H produced per fermentation (10 liter).

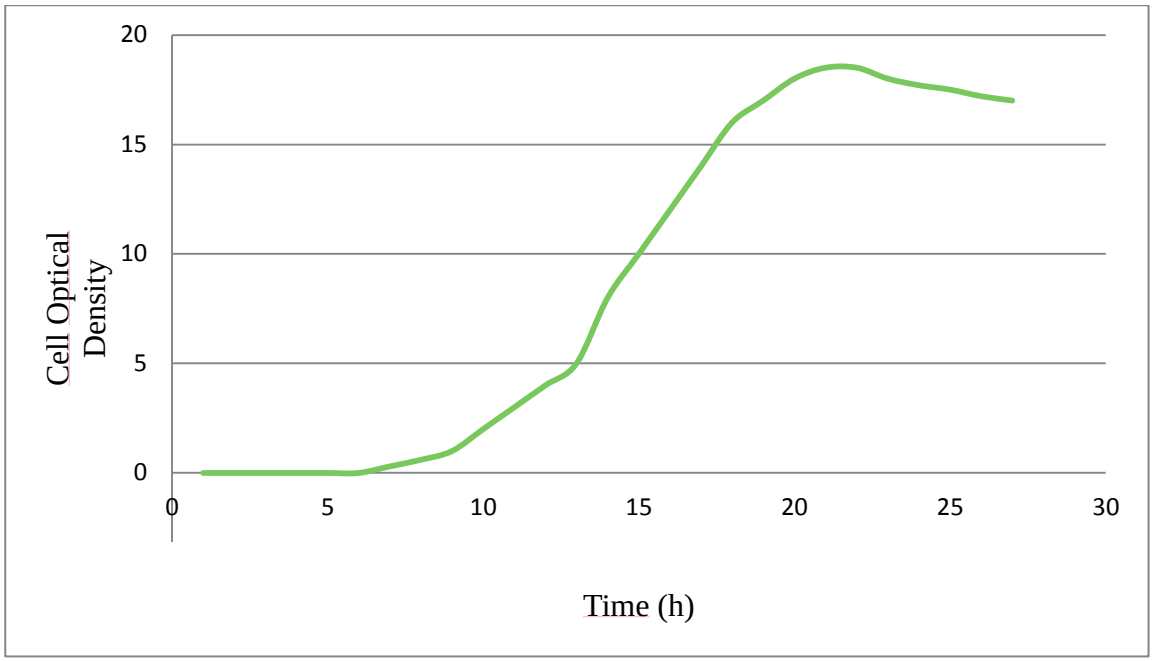


Figure 18. Optical cell density plotted against time during batch fermentation.

These early results, either looking at final cell growth and also at final quantity of recombinant protein obtained, were pretty disappointing. Consequently, it was clear that one or more growth/induction parameters were to be modified. First, is been decided to increase Minimum DO concentration from 10 to 20 % (Hopkins *et al.*,1987). Since there were no data obtainable from shake flask culture experimentations regarding each collagenase expressing bacteria oxygen's consumption, is been decided to start the first series of batch fermentations by keeping air saturation at 10% minimum. This value was chosen after studying a series of related scientific publications. But, after deeper analysis of specific scientific literature and in light of poor results, is been decided to increase minimum quantity of air saturation to 20% throughout the process of fermentation. Moreover the induction strategy is also been changed. In order to obtain better coverage of exponential growth phase, induction is been switched from a single shot injection to a "steady multiple shot induction system".

Steady Multiple Shot Induction System

The reason behind employing a different induction strategy was related to the fact that both Col G and Col H behave just like primary metabolites and are produced during the exponential growth phase. So is been reckoned that by administrating 1/6 of the original amount of inductor in 6 injections separated by 30 min one after another, it was possible to obtain a better coverage of the whole exponential phase growth curve and consequently having a better induction (figure 19).

Results showed that by applying these changes, the detection of a strong increase in terms of final cell concentration wan not obtained; in fact optical density units only increased by a value of 4 (from 18 to 22 OD), but the whole process was much more productive in terms of recombinant protein expression since it was now possible to obtain 0,6 g/L of both classes of collagenases for each batch fermentation. These results evidenced that the increase of air saturation percentage and a better calibrated timing of induction can be key factors for strong improvement in Col G and Col H fermentations output.

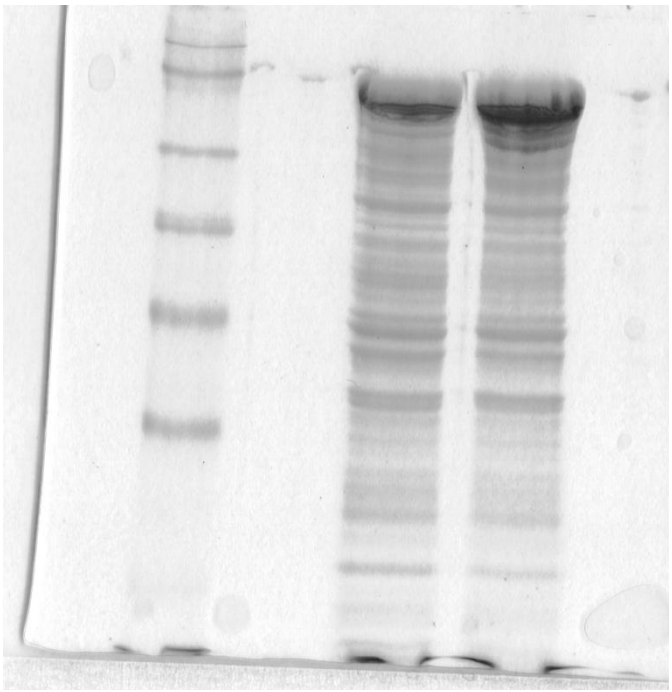


Figure 19. 7.5% SDS PAGE Gel showing: the marker “SeeBlue Plus 2” (Life Technologies) (first lane from the left), single shot induction system (second lane from left side) compared to multiple shots induction system (third lane).

Fed-batch fermentation

In light of batch fermentation results, and considering this research focused on improving the overall quantity of Col G and Col H production, is been reckoned that a fed-batch strategy could be also defined both for Col G and Col H expressing bacteria.

In general, the main advantages of high-density cultivation such fed-batch fermentation are: reduced fermentation volume, improved space time yield (volumetric productivity), reduced medium costs, reduced volume in primary downstream processing, frequent omission of concentration steps, and reduced plant and operating costs. But limitation and/or inhibition of substrates, limited capacity for oxygen supply, and formation of metabolic by-products, are all problems that can arise in fed batch cultures (Volpe et al. 2016).

Using the data obtained from batch fermentation, is been decided to try and develop a 4 liter fed-batch protocol. Consequently, for both Col G and Col H expressing bacteria, TB was chosen as culture broth. Even though the fermentation final volume was 4 liter, is been decided to keep the same amount of inoculum volume used for batch experiments: 60 ml of starter seeding culture per fermentation. When fed-batch fermentation has to be developed, is important to take in account several factors. First, is essential to choose the right carbon source for supporting the process of growth without metabolic unbalance. In the first stage of fed-batch experimentations, is been chosen the most common, used and cheap carbon source there is: glucose.

As is been said before, the first part of a fed-batch fermentation is just a batch fermentation process; after growth limiting substrate total exhaustion, the process of cell growth is carried on by application of a specific amount of a feeding substance in a specific manner through time. Each fed-batch fermentation process was carried on for 28 h. A feed solution of glucose 400 g/liter concentrated was filter sterilized in order to avoid high temperature carbon degradation. After 15 h of batch growth, it was possible to witness a sharp increase in dissolved oxygen percentage in the vessel, clear symptom of total consumption of carbon source. At this point the fed-batch phase could begin. Is been decided to employ an exponential feeding strategy and, to define the right amount of feed to be administered time after time, equation 1 was used. This equation is just the simplification of a simple mass balance applied to the cell growth limiting substrate, indeed the carbon source. By applying this equation is possible to choose, and govern the coefficient growth rate of cultivated cells.

The process of feed was carried on for 13 h, induction was performed by “steady multiple shots” of IPTG, cell growth was monitored by off-line measurements using a spectrophotometer at 600 nm of λ. Air saturation was kept at a minimum of 20% until possible. Agitation speed upper limit was increased to 1100 rpm in order to increase dissolved oxygen volume when culture viscosity increased. Induction was started when OD reached the value of 14. At the end of the process OD reached a value of 46, both for Col G and Col H expressing bacteria fermentations.

From a cell density point of view, these results were pretty encouraging, indeed the process of fed-batch could be applied for production of Col G and Col H expressing bacteria. Unfortunately the output in terms of recombinant protein expression was extremely disappointing. Only 1.2 g of Col G and 1 g of Col H were recovered from each experiment. There were several factors that could be responsible for such a poor recombinant protein expression. First of all, the coefficient growth rate chosen could have been too high, causing a metabolic unbalance that promoted cell growth but inhibited recombinant protein expression(Luo *et al.*, 2006). The pH value, despite being controlled by a base pump (NaOH 5N solution) and being set at 7.2, showed strong tendency to acidification, especially during the feeding phase, forcing the system to pump considerable amounts of base into the vessel. Is also being speculated that the sole carbon source would suffice just for the energy necessary for cell reproduction, but for sustaining the expression of high amount of each recombinant collagenases the feed solution had to be implemented also with a supplemental nitrogen source (Goyal, *et al.*, 2009). Indeed is important to remind that each recombinant enzyme has a molecular size of almost 150 kDa, so, is been thought that maybe fed-batch processes would not couple with the production of such big products. But the experimentation did go on.

Implemented Fed-Batch Fermentation

After having focused on what could be responsible for such an incomplete fed-batch process, is been decided to apply a different approach. Is been decided to use pure glycerol instead of glucose as feeding solution; to implement the concentration of nitrogen source, a specific nitrogen rich solution was pumped during the feed into the vessel, and a different calibration of the coefficient grow rate was chosen. At the end, when each variation was applied, it was possible to obtain a final value of optical cell density of 46 OD units after 28 h of cultivation (figure 20). Regarding recombinant protein expression, it was able to reach 1,5 g/l of Col G and 1,5 g/L of Col H, purifying form each fed-batch fermentation 6 g of Col G and 6 g of Col H.

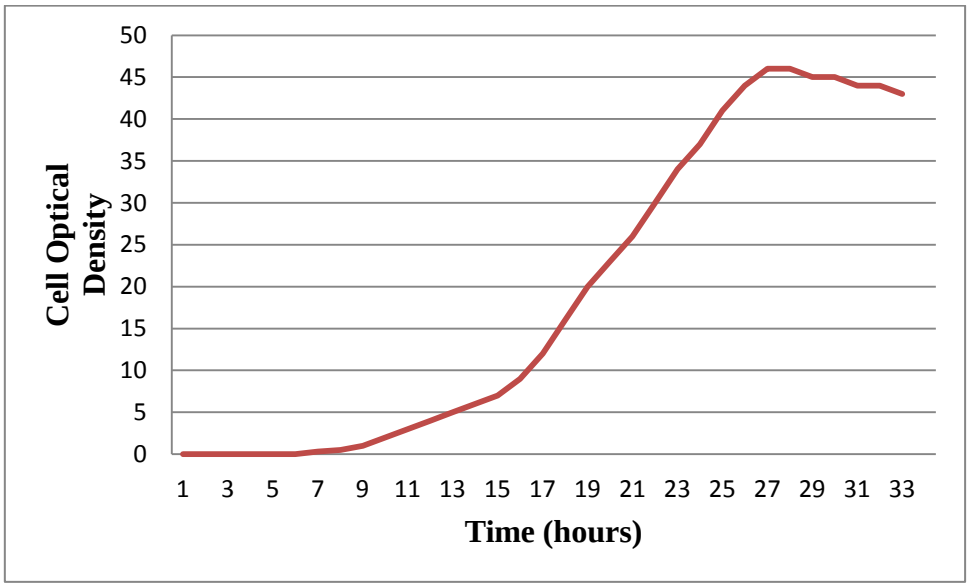


Figure 20. Optical cell density versus time in 4 liter fed-batch fermentation experiments

Glycerol versus Glucose

The importance of choosing the right carbon source is been evidenced previously in materials and methods. The fermentation of metabolic by-products (acetate, ethanol, D-lactate, t-glutamic acid) during aerobic growth in media containing glucose, pyruvate and/or complex components might become a serious problem if the by-products accumulate to concentrations inhibitory to growth. This occurs if the fluxes of carbon sources into the central pathways do not exactly match the biosynthetic demands and energy generation. Of all possible carbon sources glycerol is been chosen to substitute glucose, in the next fed-batch experimentation series, because it is the most advantageous from a by-product formation point of view (figure 21). Indeed the reduced maximum growth rate of *E. coli* in glycerol compared with glucose is less of a problem in fed-batch processes, and recent results have demonstrated that glycerol is superior to glucose for reduced acetate production and increased recombinant protein formation. The same simple mass balance equation is been applied to our brand new limiting substrate. Is been taken in account that pure glycerol is indeed more expensive than glucose, but the potential metabolic and biosynthesis advantages that the use of such component could bring would definitely make up for its higher costs. Results obtained when glycerol feed was employed instead of glucose were more positive: the final OD of each fermentation slurry was increased to 46. Plus, recombinant collagenase expression was increased too: 0,84 g/L of Col G and 0,8 g/L of Col H per fermentation.

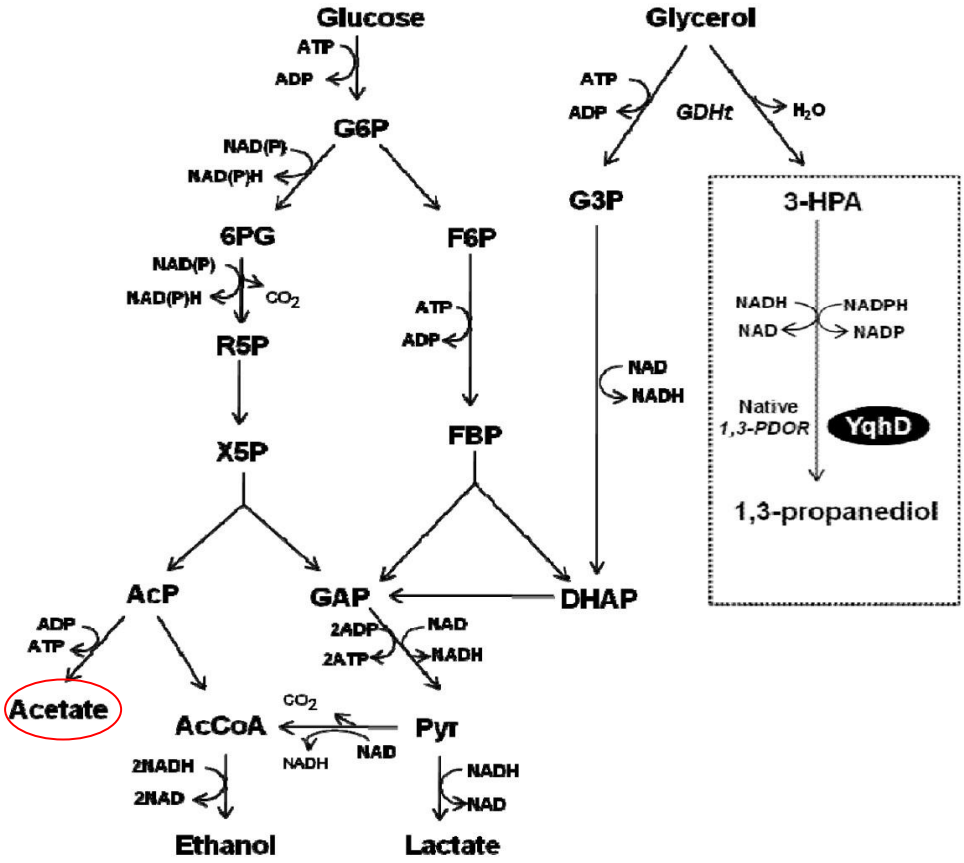


Figure 21. Pathways of acetate formation staring from different carbon sources: glucose and glycerol

Nitrogen Source

Having considered that the expression of each recombinant protein of interest could be a huge metabolic burden for cells through the process of fed-batch fermentation, is been decided to study the effects of supplying a nitrogen source solution throughout the feeding stage. Scientific literature presented several examples of nitrogen source supplementation. In particular the use in combination of yeast extract and tryptone were those which gave best result for increased production of streptokinase (Goyal, *et al.*, 2009). These complex and rich nitrogen sources are known to result in increased recombinant protein expression either due to the availability of biosynthetic precursors (Zabriskie et al., 1987) or increase in plasmid stability (Matsui *et al.*, 1990) and cell mass (Li *et al.*, 1990; Goyal, *et al.*, 2009). Consequently is been though that, by supplying such a rich nitrogen source solution, the process of cell growth would be supported, and more importantly each

collagenase expressing bacteria would have, in theory, the possibility to be sustained also for the process of recombinant protein expression.

Different complex nitrogen sources were tested, either alone or in combination. The choice to use yeast extract and tryptone was immediate. Using yeast extract solution, as nitrogen feed, brought to obtain 0,9 g/L of Col G and 0,87 g/L of Col H per fed-batch fermentation. Employing tryptone as the sole nitrogen source solution brought to produce 1,1 g/L of Col G and 1 g/L of Col H per experiment. Taken together these results were positive, it was indeed a big step forward towards the optimization of Col G and Col H expression. But the best results were obtained when yeast extract and tryptone were used in combination. A solution (1 liter) in which were dissolved 120 g of yeast extract and 120 g of tryptone was the nitrogen source feed which gave best results: 1,6 g/l of Col G and 1,5 g/L of Col H. The hypothesis is that the increase of collagenase yield was due to amino acids and vitamins supplied by these nutrients, which reduced the cellular burden for their expression.

Considerations on Dissolved Oxygen concentration (DO)

The importance of dissolved oxygen concentration is paramount for obtaining best results when performing fed-batch fermentations. There was a previous report on drastic decline (close to 99%) in plasmid stability within 90 min of a single drop in DOC to 5% during the exponential growth phase of *E. coli* (Hopkins et al., 1987). Keeping the right amount of dissolved oxygen is key to maintain a perfect metabolic balance within each cultivated bacteria. In light of this knowledge, each process of fed-batch fermentation was performed by keeping DO at 20% minimum saturation. Unfortunately, in the late stage of exponential feed, minimum saturation was recorded to drop to 5%. Is not clear if this drop has affected the performance of each fed-batch fermentation, since the good results recorded in terms of expression. Probably each expression system behaves differently also in terms of oxygen consumption, but further experimentation with pure oxygen supply could reveal more about it.

8 liter fed-batch fermentation

The result obtained with 4 liter fed-batch fermentations, were a more that positive starting point to improve even more the outcome of Col G and Col H expressing bacteria fed-batch fermentation experiments. The idea was simple: to increase starting volume from 4 to 8 liter with the intention of obtaining more liters of fermentation slurry, more bacterial cells and consequently more recombinant proteins to be produced. Basically this was the very first step for a possible upstream process scale up.

Every choice and strategy adopted in the execution of the 4 liter fed-batch protocol was applied also for this series of fermentations. Carbon feed was continued until each fermentation was terminated, as well was the feed of nitrogen source. The only parameters that varied were the glycerol feed solution pumping speed and the volume of starting seeding culture. Induction was started when OD reached a value of 18, same induction method adopted for the previous fed-batch fermentations. This process was carried on for 30 h instead of 28 h, the reason was to try and elongate the exponential phase even more. Thanks to the development of this brand new protocol it was possible to reach 65 OD units (figure 22) per fermentation and to recover a total of 13,2 g of Col G and 12,6 g of Col H, with yield of 1,65 g/L of Col G and 1,57 g/L of Col H. This results were most likely due to increased starting volume and as a consequence to a bigger quantity of cells present at the beginning of culture.

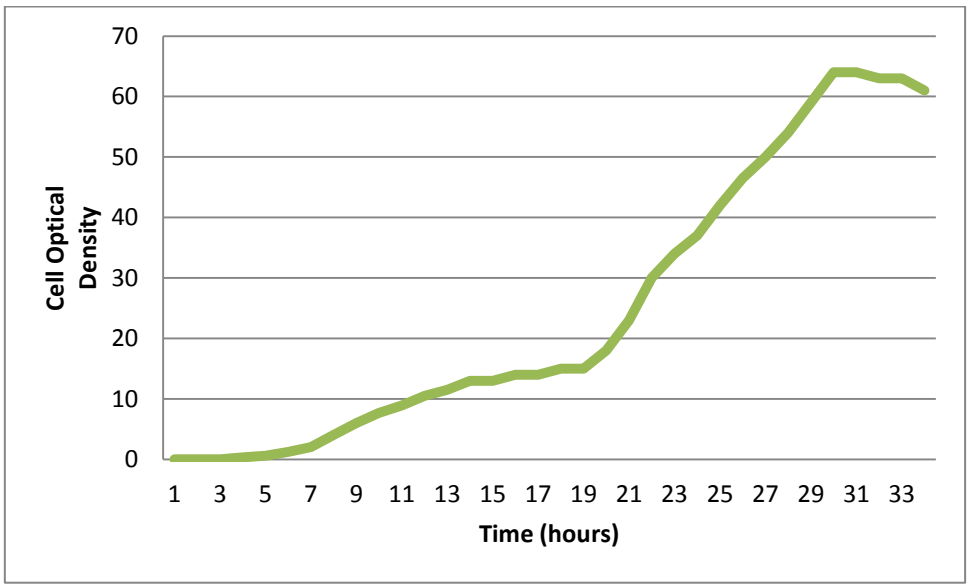


Figure 22. Curve that represent optical cell density plotted against time in 8 liter fed-batch fermentation.

Carbon feed calibration

The change in the quantity of glycerol pumped per unit of time was just consequent to the different initial volume used in this series of experiments. Indeed this value is a component of the mass balance equation 1, therefore a different calculation had to be performed to obtain the same exponential feeding strategy adopted in the 4 liter fed-batch fermentation experiments.

8 liter animal free fed-batch fermentation

Despite having defined a straight forward and highly productive fed-batch protocol for production of each recombinant protease of our interest, the will to produce higher quality products and the necessity to bid new international standard regulations regarding drugs production, has risen the necessity to switch both fermentation medium and nitrogen feed solution with animal free alternatives. Knowing that changing culture medium is a great challenge and also a risk at this point, it was just necessary for the reasons previously stated.

Since positive results were obtained with the use of a complex rich medium such terrific broth, is been decided to try and redesign this very medium but using animal free components: vegetable tryptone (Sigma Aldrich) and vegetable yeast extract (BD). The brand new animal free medium composition for one liter was: 12 g of vegetable tryptone, 24 g of vegetable yeast extract, 13 g of K_2HPO_4, 2 g of KH_2PO_4, 4 ml of pure glycerol.

After a quick series of shake flask culture and induction tests, it was clear enough that this brand new animal free medium had the same possibilities of the old one: in 1 liter shake flask culture, 200 ml of AFTB (animal free terrific broth) inoculated with 20 ml of overnight starting culture, reached 7.4 OD units after 8 h of culture at 37 °C at 220 rpm. These results were valid both per Col G and Col H expressing bacteria. Induction was tested also and the intensity of stained Col G and Col H bands was comparable to those obtained when classic terrific broth was used (25-30% of band intensity, Image-J software).

These results were the green light for using AFTB as new medium for the recently developed 8 liter fed-batch protocol. Almost all parameters were exactly the same as those of classic 8 liter fed-batch fermentation, the only things that changed were indeed the medium of culture and the nitrogen source feed solution. Regarding nitrogen source feed, because its components were all of animal origin, such solution had to be redesigned also; in this case is been chosen to substitute, just like is

been done for AFTB medium, classic yeast extract and tryptone with their vegetable analogs. Nitrogen source composition for 1 liter solution was 120 g of vegetable yeast extract and 120 g of vegetable tryptone, this solution was vapor sterilized for 15 min. at 120°C and not for 20 min., this choice was taken in order to not degrade each component because their increased thermo sensibility.

Each fed-batch fermentation, performed with alternative medium and different nitrogen feed, had these results: the final cell concentration in terms of OD units, was 60 for both Col G and Col H fermentations, the final quantities of recombinant enzyme recovered from each fermentation were 12,5 g of Col G and 12,1 g of Col H. Taken together, these results confirms the effectiveness of both AFTB and vegetable nitrogen feed in reaching high density cell cultures of Col G and Col H expressing bacteria and in sustaining the expression of significant amount of each recombinant collagenase. Even though the final quantity of collagenses produced per experiment was slightly inferior to the quantity obtained when employing animal derived medium and classical nitrogen feed solution, these outputs clearly indicate that animal free components and medium can be used with remarkable success for production of recombinant proteins like Col G and Col H.

First Stage Purification

In order to recover, separate and purify each recombinant collagense, Col G and Col H expressing bacteria's fermentation slurries had to undergo to a series of sequential steps of purification.

The first purification strategy employed was based on the use of such processes like centrifugation, homogenization, affinity chromatography, filtration and lyophilisation. This so called "first stage purification" protocol was used to purify slurries obtained with batch fermentations only. Later on, another strategic step was added to improve the overall efficiency of purification, this step was always used to perform each downstream purification.

Centrifugation

At the end of each batch fermentation, slurries were collected in 5 liter glass bottles (Schott), each solution was then cold centrifuged in order to obtain separation between cells and culture medium. Is been decided to employ a J 24 AVANTI (Beckmann Culture) to perform such separation. Each fermentation slurry was aliquot in 250 ml PVC bottles (Nalgene), 6 bottles were then centrifuged at 10000 rpm for 15 min at 4°C.

After being centrifuged, each bottle was then emptied of the supernatant and each pellet was consequently suspended once more in Column buffer (20 mM Tris-HCl pH 7.4, 0.2 M NaCl, 1 mM EDTA). This process was carried for the entire volume of each fermentation slurry produced. At the end, the whole amount of pellets obtained from a single fermentation slurry, were re-suspended in 2 liter of column buffer solution.

Homogenization

All lots of re-suspended cells were then homogenized by using an high pressure machinery (GEA Niro Soavi PANDA). Such machine uses efficient high pressure energy to break bacterial cells present in fluids, down to nanometer range (figure 23 B). This process creates a stable emulsion or dispersion by forcing the product through a specifically engineered homogenizing valve under high density fluid-dynamic energy conditions thus improving product shelf life and performance. Consequently, bacteria dissolved in column buffer solution, were broken up by a single passage through such machinery when pressure was set at 1000 bar for the whole process of brakeage. Since

the process of homogenization generated a significant amount of heat, each homogenized solution was collected in a specific ice cooled recipient, in order to protect recombinant proteases from being denatured by high temperature.

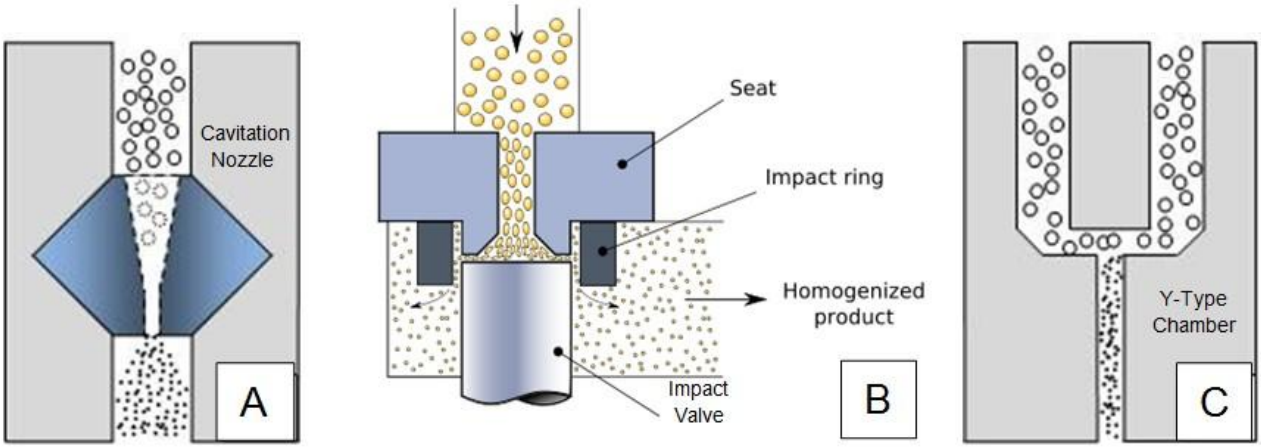


Figure 23. different types of homogenization: (A) cavitation nozzle, is suitable for high viscosity liquid, suspension or paste, but usually has a tendency of degrading pharmaceutical emulsion, (B) is defined the mid way between A and C, (C) Y-type chamber, is suitable for pharmaceutical emulsions but has not enough strength for high viscosity liquids or suspension.

2nd centrifugation

The same centrifugation apparatus employed to separate cells from culture medium, was then used to separate cells debris from the soluble solution released after homogenization. Such solution is usually defined as Crude Extract. Because recombinant collagenases have a cytoplasmatic localization, such solution was now most important since contained the product of interest. Homogenized solution was centrifuged at 12000 rpm for 30 min. at 4°C. Supernatant solution was then collected and then immediately storage at 4°C. After this step of separation was over, the final amount of crude extract obtained per batch was about 1,6 liter.

Affinity chromatography

After having analyzed protein concentration through Bradford assay (Bradford, 1976), each crude extract obtained, both form Col G and Col H expressing Bacteria fermentations, was subject to affinity chromatography purification. Col G and Col H carry fused in frame at their c-terminus the Maltose Binding Protein (MBP). Consequently, by employing a specific amylose resin cross-linked with maltose (NEB), it was possible to purify each recombinant protease of interest (figure 24). The advantage of using such technique was given by the high purity of each collagenase eluted, unfortunately such process had some weak points too: the whole process was time consuming, and resin high costs.

4 columns packed with 300 ml of amylose resin were employed to purify a 1,6 liter solution of crude extract. To perform the whole process of binding, washing and elution, were necessary 5 days. Washing solution was column buffer (see material and methods), and elution solution was column buffer plus maltose 10mM concentrated. The output of such process was a 3 liter solution of column buffer plus maltose and the protein of interest, either Col G or Col H. Washing and elution progresses were monitored by spectrophotometric reading at 280 nm.

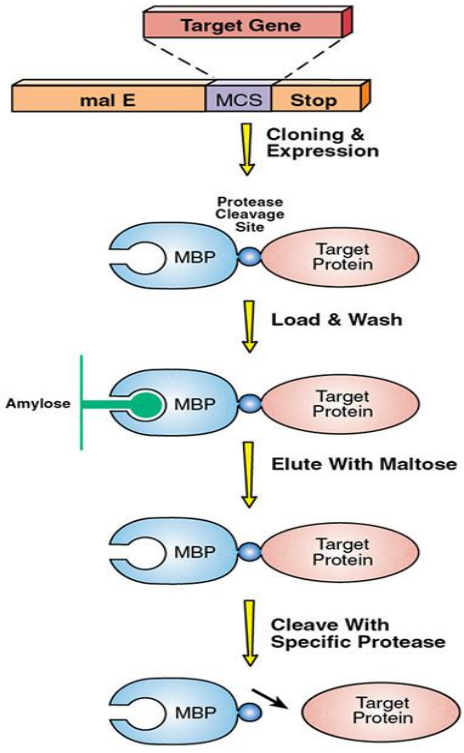


Figure 24. Col G and Col H chimeric proteins workflow of affinity chromatography purification.

Filtration and concentration

At the beginning of purification protocol development, a 200 kDa cut-off filtration membrane (insert brand) was used to concentrate the affinity chromatography eluted product in order to obtain 10 mg/ml of collagenase solution, then by applying a 3 days process of manual dialysis, column buffer plus maltose was substituted by a solution buffered with Tris-HCl 1 mM pH 7.5. This purification step was in use for purification of collagenases obtained from batch fermentations only. When fed-batch fermentations increased the final yield of both Col G and Col H production, the need to process more product and in a shorter amount of time promoted the employment of a more efficient and effective purification approach.

Tangential Flow Filtration (TFF)

The use of a TFF apparatus was a great step forward in terms of purification time optimization and increase in volume treated. The whole purification process was redesigned around it. By employing 300 kDa cut-off filtration cassettes, it was possible to substitute centrifugation for medium broth separation and cell dia-filtration. Each fermentation slurry was first concentrated to a working volume of 1 liter, secondly such concentrated volume underwent to 7 washing passages using 2 liter solution of Tris-HCl 20 mM pH 7.5 and EDTA 1mM for each passage. Dia-filtration was carried on until all fermentation medium was removed and cells were totally immersed in Tris-EDTA solution. This 1 liter solution was then completed by addiction of the remaining component of column buffer solution, to a final volume of 2 liter. At this point the solution was ready to be homogenized (same parameters reported above). The output of homogenized solution was then collected and immediately centrifuged at 4°C in order to obtain separation of cell debris from crude extract. Each protein solution was purified by the same affinity chromatography process described above. Eluted proteins were then concentrated by employing TFF process, in particular with the help of a 50 kDa filtration cassette. First, the eluted solution was concentrated to a working volume of 1 liter. Subsequently, 7 steps of diaflitration performed with 2,5 liter of Tris-HCl 1 mM pH 7.5 per passage were executed in order to remove the almost totality of elution buffer. Is important to underline that during the concentration step, protein concentration was monitored by off line spectrophotometric reading at 280 nm of absorbance. This was necessary to determine the desired protein concentration in terms of mg/ml and so better defining the process of lyophilisation. Depending on the final quantity of collagenase produced the desired protein concentration was between 10 and 15 mg/ml. All together, the use of TFF has strongly improved the process of purification of each recombinant

collagenase, in fact by decreasing the amount of down-stream purification time, it was possible to reach higher standards, in terms of enzymatic quantity and quality.

Enzymatic Activity

Is important to underline that, when trying to produce enzymes like collagenases, is necessary to consider not just the final amount of enzyme produced but also, and maybe even more important, the final activity that each enzyme produced can express. Because if, at the end of purification, the enzymes obtained will show low or no activity, their value would be almost worthless. As a consequence of that, after being concentrated and dia-filtered each batch of Col G and Col H produced both through batch and fed batch methods, was tested for its enzymatic activity using a modified Grassmann Pz activity assay. It is a colorimetric assay based on the enzyme's capacity to cleave a synthetic specific peptide.

Room Temperature Purification versus Cool Temperature

After having performed a series of enzymatic activity assays for Col G and Col H purified at room temperature and knowing that collagenases become active if exposed for long periods of time at 25/30°C, in order to prevent auto-proteolysis activity and with the aim of best preserve enzyme's structure and activity, is been chosen to employ a customized stainless steel cooling coil system to be coupled to TFF machinery. Initial experimentations led to define the lowest utilizable cold temperature that did not interfere with TFF sensors. Such temperature was 7 °C.

When enzymatic activity of each recombinant protein was measured from batches purified at room temperature (25°C), results gave a medium value of 2 Pz unit/mg for Col G and 14 Pz unit/mg for Col H. On the other hand, when purification was carried at 7 °C, it was measured a strong increase in each collagenase activity: 4 Pz unit/mg for Col G and 38 Pz unit/mg for Col H. These findings proved that keeping cool temperature during each TFF step of purification was critical to protect both collagenases enzymatic activity at their best (table 3).

Purification At 25 °C	Purification At 7 °C
Col G: 2 Col H: 14	Col G: 4 Col H: 38

Table 3. Col G and Col H enzymatic activity (Pz unit/mg)

Effects of Vegetable Medium on Collagenases Activity

Throughout the whole process of upstream process development, a major change has happened to such protocol: the substitution of animal derived medium with 100% animal free culture medium. Such substitution slightly affected the final quantity of recombinant protein obtained per fermentation. Consequently is been tested if also the activities of both Col G and Col H were in some way affected after such drastic change.

Enzymatic activities of several Col G and Col H lots purified from animal derived fermentation were compared with batches obtained from 100% animal free fermentations. Results were quite interesting; while Col G activity was neither negatively or positively affected by such change in

culture medium, Col H, on the other hand, showed a strong increase in terms of enzymatic activity measured. The enzymatic activity of Col H was now settled at a value between 58 and 65 Pz units/mg (table 4). Such findings were very positive news, in fact the activity of Col G was unaffected by medium change and it remained the same, but surprisingly Col H activity almost doubled. The reason for such increase is unclear, considering that every production and purification steps was unvaried, one possible explanation can be that the new animal free culture medium contains certain micronutrients that strongly favour the biosynthesis of Col H active site. Another explanation could be that animal free components

Purification At 25 °C	Purification At 7 °C	Purification At 7 °C from animal free production
Col G: 2 Col H: 14	Col G: 4 Col H: 38	Col G: 4 Col H: 64

Table 4. Col G and Col H enzymatic activity (Pz unit/mg)

Lyophilisation

Col G and Col H purified solutions (filtered on 20 μm to sterilize), after being tested for enzymatic activity, were subjected to lyophilisation. Each recombinant protein solution was divided in glass vials and put inside the lyophilisation machinery. The process of division was based on Pz units/mg measured; basically each vial was filled with a specific amount of volume corresponding to a certain number of enzymatic units. This was necessary for best use of lyophilised collageanses.

The process of lyophilisation was articulated in 3 steps: minus 40 °C freezing step, main drying at 0.630 bar, and final drying at 0,010 bar. The time necessary to complete such process was influenced by the volume of solution to be lyophilized. Usually a final purification volume of 600 ml of Col G or either Col H took 5 days to be fully lyophilized: half day for minus 40 °C freezing, one day for main drying at minus 30 °C, one day for main drying at minus 20 °C, one at minus 10 °C, one and an half at 0 °C and 3 to 4 h for final drying.

HPLC Analysis

After lyophilisation, each sample, either Col G or H, was then tested for its capability of re-solubilisation. This test was performed by injecting a specific amount of ultra pure H_2O, calibrated in relation with enzymatic units numbers, inside a random lyophilised vial. After 30 min. the solubilisation of lyophilised proteins was total. Such experiment is been repeated for each purification performed, both per Col G and Col H.

For every solubilisation experiment, a small amount of protein solution is been used to analyse the overall purity grade of each purification. In this case, purity is intended as the percentage of recombinant protein of interest within 1 mg/ml of solution. To investigate such purity, size exclusion HPLC experiments were performed. Chromatography parameters were the same for both Col G and Col H analysis. Samples were first quantified in terms of proteins concentration by 280 nm spectrophotometric readings. Samples, 1 mg/ml concentrated, were then filtered and then directly put in mini vials HPLC specifics. HPLC pumps speeds and time of analysis were defined by following SEC column best use criteria (Shodex PROTEIN KW403-4F). Analysis calibration was done by performing chromatography runs with known standards, both in size and concentration. A solution of Tris-HCl 1 mM pH 7,5 was used for the elution process. Col G and Col H chromatograms clearly demonstrated that each solution analysed was pure up to 98% (figures 25 and 26).

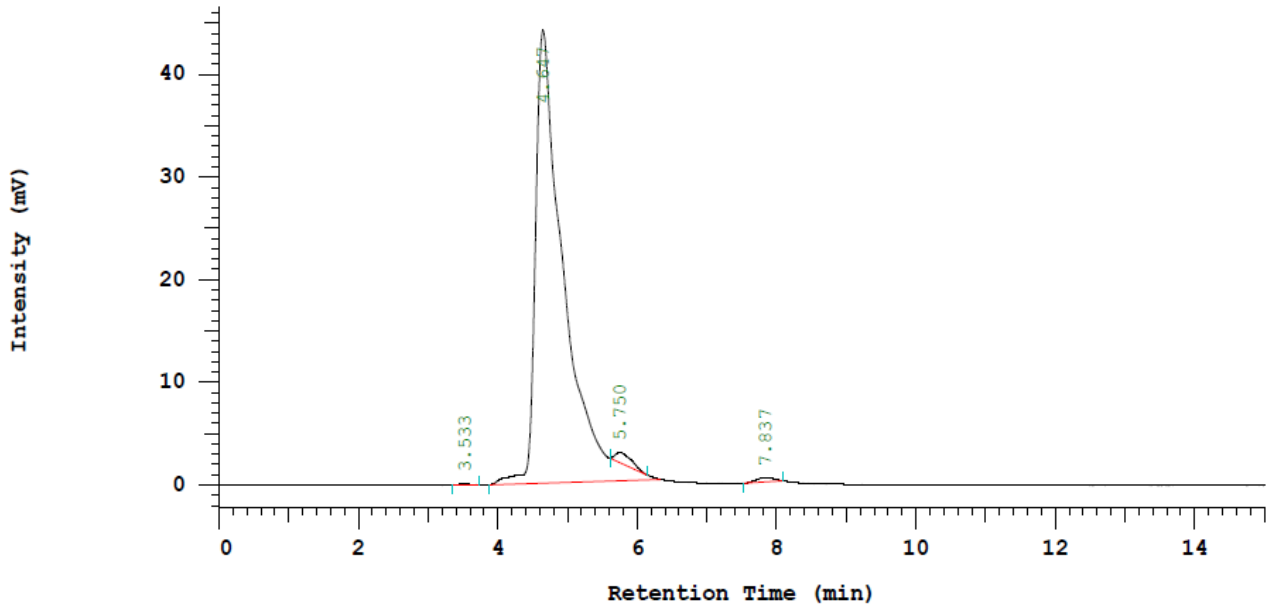


Figure 25. Col G SEC HPLC chromatogram at the end of all steps of purification.

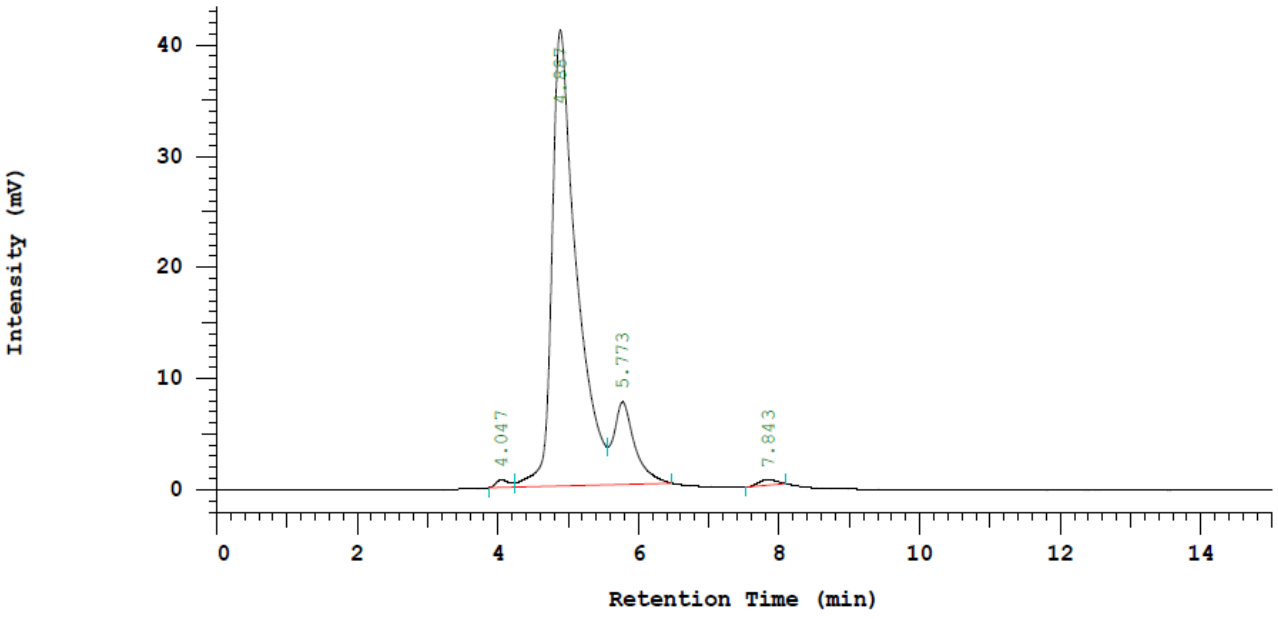


Figure 26. Col H SEC HPLC chromatogram at the end of all steps of purification.

Conclusions

Production of recombinant proteins is now days a wide spread strategy to obtain useful components for medical and research applications, food industry and in the field of agriculture. But producing a recombinant protein is not a standardized procedure. Every recombinant protein production protocol is an unique story; molecular size, ion charge, folding, intracellular localization, system of expression are just some of the many variables that concur to make such process different from protein to protein.

In this context, this PhD project is been performed with the aim of developing an efficient and effective upstream production strategy for Abiel's recombinant proteases: Col G and Col H. Moreover, is been decided to go further and define an appropriate downstream purification protocol with the intention of pursuing not just enzyme's quantity but also quality.

First, is been investigated which culture medium was the most suited for both Col G and Col H expressing bacteria in terms of growth demands and recombinant protein expression. Shake flask culture results demonstrated that, for supporting both cell growth and recombinant protein expression, terrific broth medium was the only one among those tried that was efficient enough. The reason has to be attributed to the fact that terrific broth is a complex rich medium and, even though its components are poorly characterized, is the only one capable of giving all necessary nutrients for cell replication and recombinant protein expression.

Batch culture experiments demonstrated that, even for recombinant proteins of size superior to 100 kDa like Col G and Col H, it was possible to obtain 0,6 g/L of each enzyme per fermentation. Moreover, in this work is been evidenced that the calibration of induction strategy has shown great influence on the whole performance. These results were used as a starting point to set up a theoretically more profitable process: fed-batch fermentation.

Fed-batch fermentations final outputs definitively confirmed that a complete control on growth and fully scalable approach like fed-batch strategy was not just feasible but also more convenient in terms of final concentration of Col G and Col H expressed per experiment. Final OD cell concentration was above 60 units both per Col G and Col H expressing bacteria fermentations. The final yield of recombinant enzyme produced was risen to 1,65 g/L of Col G and 1,57 g/L of Col H when fermentations were performed with animal derived terrific broth medium. Interestingly, the necessity to switch culture medium only slightly affected fed-batch final enzymatic yield outputs. More importantly enzymatic activity for Col H, produced with animal free terrific broth medium, resulted to be much higher than the activity of the same recombinant enzyme produced with animal derived medium. All together these information indicate that fed-batch fermentation is so far the

most profitable process to produce relevant amount of Col G and Col H recombinant proteases. Such protocol could be used to scale up production and establish a medium to large scale industrial manufacturing process.

The second part of this PhD project regarded the process of purification. Such study is been focused on finding and employing the most productive strategies and tools to maximize each recombinant protein final quantity and at the same time to preserve and possibly enhance their quality in terms of enzymatic activity.

The use of tangential flow filtration, throughout the process, demonstrated to be both more efficient and much less time consuming that centrifugation. Plus, by running each step of tangential flow filtration at 7 °C, it was possible to evidence that the enzymatic activity of both Col G and Col H showed a two fold increase when compared with the same enzymes, of the same lot of production, but purified at 25 °C.

All together these results clearly evidence that is possible to produce and purify recombinant proteases of molecular size of almost 150 kDa. Such protocol could be further improved by substitution of affinity chromatography purification with a different and, more importantly, less time consuming process. This research has showed that, when working with proteolytic enzymes, timing is essential. The more time will pass from production to final stage purification the more are the probability that both Col G and Col H activity will be negatively affected. Even though affinity chromatography has an high purification yield, it takes usually 5 days to complete such process, consequently a more rapid way of purification could be perfected. At the moment, the idea is to employ strong anion exchange filtration processing. One of the advantages of such substitution would be the gain in time. Preliminary results showed that the same amount of volume that is usually purified in 5 days by affinity chromatography, would only take no more than 3 h to be completely purified by ion exchange filtration. Is likely that employing anion exchange filtration instead of affinity chromatography would bring another strong increase in final enzymatic activity by avoiding auto-degradation. Unfortunately during this PhD experience only preliminary results were obtained regarding such possible purification improvement, consequently these data are not reported. Nevertheless the protocol here presented could be readily used as it is as a platform for high standard bigger scale collagenase's manufacturing.

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