

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

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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 140 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; 14/20 gr/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 8.2 gr for Col G and about 7.2 for Col H fermentations. Moreover, by using a stainless steel cooling coil system, we have investigated the effects of low 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 when 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

1. Introduction

Collagenase, a metalloproteinase capable of cleaving native collagen types I, II, III, IV and V, is produced in large amount by *Clostridium histolyticum*. 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. Several cases of successful topical use of the crude enzyme in an ointment base are known to literature. More recently 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. Unfortunately, direct production from *Clostridium histolyticum* is, because its pathogenicity, in many cases considered to be not well suited for large scale production, moreover, new pharmaceutical standards require the use of a safer host; *E. coli*.

Although a fair amount of scientific literature is available regarding heterologous proteins production in *E. coli*, it should be carefully considered that each expression system is unique in terms of promoter system, host-vector interactions, sequence and characteristics of recombinant product and the effect of the expressed foreign protein on host cell physiology. Hence the optimum requirements for growth and product formation for sure will be different from case to case. The importance of growth parameters can be deduced from the fact that although parameters like stable maintenance of recombinant plasmid, plasmid copy number, protease degradation of the recombinant product and inclusion body formation are primarily a function of genetic

makeup of the host and vector system, these are also known to be greatly affected by the cultivation conditions and media composition (Lee, S.Y., 1996; Yee, L., Blanch, H.W., 1992; Zabriskie, D.W., Arcuri, E.J., 1986). Hence, the production or upstream processing of any recombinant product from *E. coli* requires detailed study of the effect of different cultivation conditions and media constituents so that key parameters affecting product yield can be optimized.

This study focuses on the production's optimization of two recombinant collagenases, a type-I collagenase and a type-II collagenase: namely Col G (145 kDa) and Col H (149 kDa) (Salamone M. et al., 2012). Each enzyme coding sequence has been optimized to be expressed in *E. coli* BL21 (DE3) (Salamone M. et al., 2012). Here we describe our method for production of each collagenases through batch fermentations, moreover, we report that a fed batch approach is also feasible and it is even more advantageous for increasing each recombinant protein final yield.

2. Methods

2.1 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 promoter and a lac operator in a pET-series 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

2.2 Cultivation medium

Being this work a first step investigation on possible first scale production of big size recombinant proteins, our choice was to use Terrific broth medium for both batch and fed batch experiments (Lessard JC.,2013). In fed batch we used ultra pure, sterilized glycerol as feeding solution. 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 initial concentration of 1 M.

2.3 Inoculum development

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

2.4 Bioreactor cultivation

Batch and fed-batch culture experiments were conducted in a 10 L Bioflo celligen benchtop bioreactor (New Brunswick Scientific Co., USA) at pH of 7.2 and 37 °C. 5 N NaOH was used to control pH. 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.

2.5 Batch cultivation

Each Col G and Col H batch fermentations were induced when the OD_{600nm} reached a value of 5/5.5 (5 h after inoculum injection). DO was maintained at 20 % (unless otherwise stated) by cascading agitation rate (280–900 rpm) with DO concentration at constant aeration rate.

2.6 Fed batch cultivation

Fed batch experiments for each collagenase expressing bacteria started as batch experiments; after exhaustion of glycerol, visualized with the simple Dissolved Oxygen stat method (Lee J. et al.,1999), we began our cultivation 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 a specific steady multiple shot induction system. DO concentration was kept at a minimum of 20 % saturation throughout each process by cascading impeller speed and increasing inlet air volume per minute.

2.7 Bacteria purification

Each fermentation slurry, no matter how it was obtained (batch or fed batch) was purified by means of ultra filtration. In particular we used a "CM500S" ultra filtration device (Pall corp.). Bacteria concentration and diafiltration were performed using a 300 KDa TFF membrane (Pall corp.)

3. Analytical methods

Bacterial cell concentration was evaluated off-line by optical density at 600 nm (OD_{600nm}) in a spectrophotometer (BMG specktrostar). Dry cell weight samples were obtained by overnight heating at 65°C. Purified bacteria, were lysed by means of mechanical shear (Niro Soavi Panda). Separation between cell debris and crude protein solution was obtained by centrifugation (Beckman). Crude extract protein concentration was measured by means of Bradford assay (Bradford, M.M., 1976). Protein samples obtained from each fermentation experiment were loaded onto a SDS PAGE 7.5 % gel (Sambrook J. 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 we produce carries a maltose binding protein tag at its carboxy terminus. Previous studies demonstrated that the tagging does not affect catalytic activity. Each enzyme solution, obtained from affinity chromatography, was diafiltered and concentrated using yet again tangential flow filtration. In this case we used 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 W. et al., 1960).

4. Results and Discussion

4.1 Batch fermentation

Each recombinant *E. coli* BL21 strain was initially evaluated in shake flasks cultures and collagenases expression levels in LB and TB medium were compared. TB was a better medium for both bacterial growth and recombinant protein expression. TB, despite being not a defined medium was the best choice because its richness in nitrogen source; indeed it was necessary for producing recombinant proteins of big size like our collagenases. Different formulations of defined media were also tested, but no one suited our bacteria energy demands like TB.

Batch bioreactor cultivations were carried out using 6 L medium volume. In early experimental stage, we were able to obtain, after 25 h of batch growth and 3 h of induction, 18 optical cell density units (Figure 1) corresponding about to 2 gr of Col G and 1,8 gr of Col H per fermentation. Dissolved oxygen concentration was kept at 10 % minimum.

Minimum DO concentration was increased from 10 to 20 % (Hopkins, D.J., et al., 1987) plus we changed our induction mode. In order to obtain better coverage of exponential growth phase, we switched from a single shot induction to a multiple shot induction system. By applying these changes we were able to obtain far better result; we did not detect a strong increase in terms of final cell concentration (22 OD units), but we were able to produce 14 gr/L of drycell weight, corresponding to 6 gr of both classes of collagenases per batch.

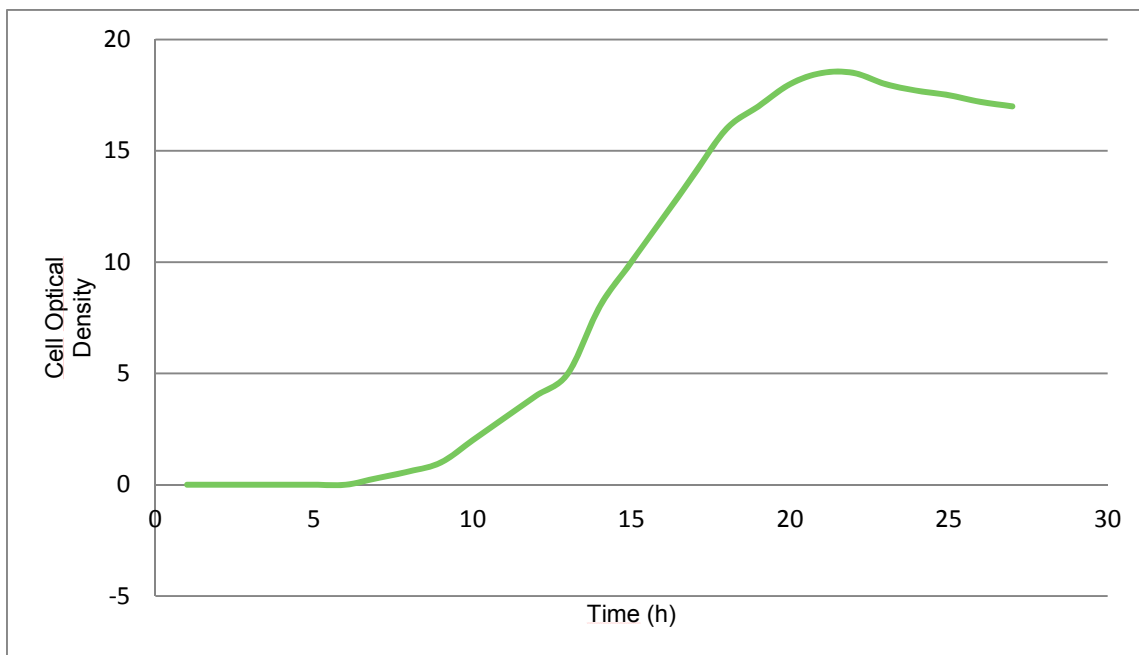


Figure 1: Growth profiles for batch fermentation process

4.2 Fed batch fermentation

In light of our batch fermentation results, we tried to increase the overall quantity of bacteria produced per L/medium and to optimize production we opted for a fed batch fermentation application. In general, the main advantages of high-density cultivation such fed batch fermentation are: reduced fermentor 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. 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 flux of carbon sources into the central pathways do not exactly match the biosynthetic demands and energy generation. Of all possible carbon sources we chose glycerol because it is the most advantageous from a by-product formation point of view. 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. We applied a simple mass balance equation to our limiting substrate, glycerol. This scenario was a bit tricky because we have been used a not defined medium like TB as fermentation medium. After first phase of batch growth (overnight), we noticed a sharp increase in DO concentration; a clear symptom of residual carbon source full consumption. At this point, we applied an exponential fed batch (Cheng L.C, 2002) based on pure glycerol. The process went on for 10 h and induction was started when OD units were about 8. It was carried on for 3 h divided in 6 shot each one with 1 M concentrated IPTG. Result showed that with this procedure we were able to increase final cell concentration from 18 to 34 OD (Figure 2) and since there is a direct correlation between number of cell produced and recombinant protein obtained, we were capable to obtain 20 gr/L dry weight cell, containing 8,2 gr of purified Col G and 7,2 gr of purified Col H per experiment.

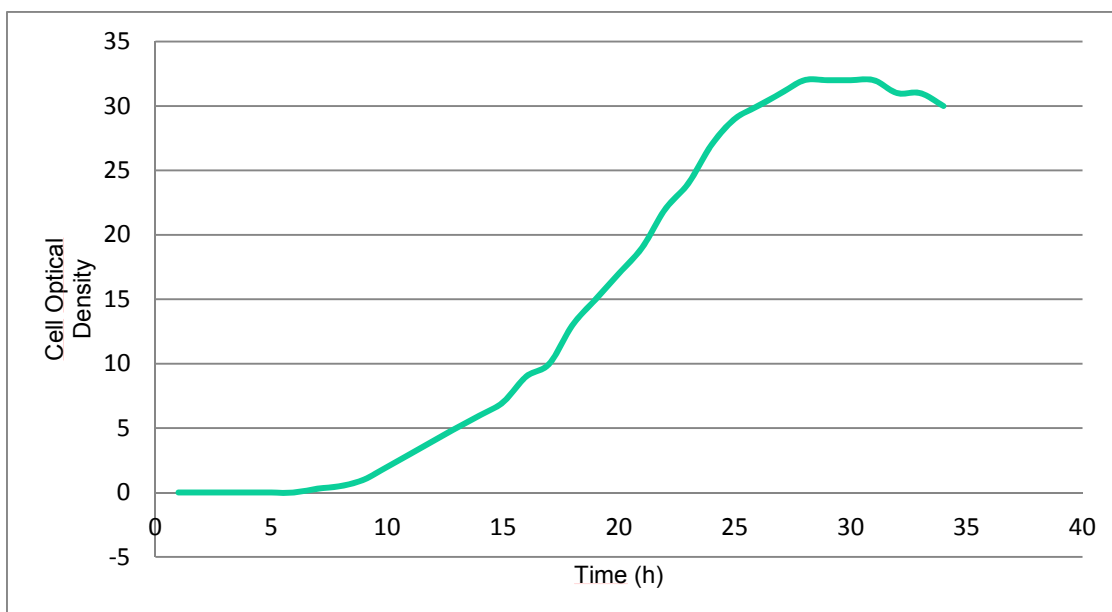


Figure 2: Growth profile for fed batch fermentation process

4.3 Cold temperature purification

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. When enzymatic activity of both our proteins was measured from batches purified at room temperature (25°C) we obtained 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 we measured a strong increase in each collagenase activity; 4 Pz unit/mg for Col G and 28 Pz unit/mg for Col H. These findings prove that cool temperature during purification processes is critical to protect both collagenases enzymatic activity at their best (Table 1).

Table 1: Col G and Col H enzymatic activity (Pz unit/mg)

Purification At 25 °C	Purification At 7 °C
Col G: 2	Col G: 4
Col H: 14	Col H: 28

5. Conclusions

This study demonstrate that, even for proteins of size far superior to 100 KDa, is possible to apply not just batch but also fed batch strategies for their production. Moreover it shows that when purification is carried out at low temperature is possible to better protect enzymatic activity for both classes of collagenases. These findings could be used as a platform to develop an industrial protocol for each enzyme production. Next step will be to further develop the fed batch strategy in order to obtain even higher quantity of each recombinant protein, plus we will investigate if a different purification protocol can be developed. The idea is to choose such a purification strategy that will allow the operator to perform it in a much shorter gap of time than classic affinity chromatography. By doing so we aim at optimizing the overall amount of time of each enzyme production/purification and, more importantly, we probably could increase each recombinant enzyme final activity by avoiding the time consuming process of chromatography and instead choosing a much quicker strategy like, for example, ion exchange membrane filtration.

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