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OPTIMIZATION STRATEGIES AND SCALE-UP THE PRODUCTION OF A RECOMBINANT PROTEIN IN A METHYLOTROPIC YEAST *PICHIA PASTORIS* FROM EGGSHELLS

Sorin Septimiu COMAN

Universidad Autonoma de Barcelona-Ecola de Doctorat

Corresponding author email: comansorin.innovas@gmail.com

Abstract

The goal of achieving a high quality final product of Collagen type 1 suitable for both cosmetical and pharmaceutical industry is a constant battle between the production cost and maintaining a superior improved standard working protocol.
The aim of this study is to optimize the production of Collagen type 1 from different waste materials such as eggshells and methanol by using a fermentation protocol with genetic modified Pichia pastoris yeast.
In our first experiment we used as carbon sources a mixture of eggshells and a glycerol and methanol feeding solution and a fermentation procedure with oxygen limited strategy. The intermediary product was lyophilized and then capped in sterile bottles.
The production method that we tried to optimize regarding the profitability and also the obtaining of high quality final product of Collagen type 1 was successfully achieved. Taking into considerations this result, we believe that the productions costs could be further lowered by adding as a carbon source from marine Black sea algae such as Ulva familae, Porphyra leucostica or Punctaria latifolia.

Key words: *Collagen type 1, Pichia pastoris.*

INTRODUCTION

Collagen is a fibrous protein that strengthens and supports many tissues in the human body In this direction collagen has a number of biomedical uses in plastic surgery like prosthetic implants, tissue replacement, angioplasty sleeves even in cornea repair surgery. Collagen is a fibrous protein found in 20 - 30% of the human body and contains 20 amino acids. The most important amino acid is Gly - glycine about 33% of the collagen molecule, prolyne - Pro and hydroxiprolyne about 22%, alanine 11% and aspargic and glutamic acid 12%. As an important observation specific only for collagen molecule is hydroxiproline which is about 10%. (Traub 1969, Kuhn 1987, Buehler 2006, Fratzl 2008) The goal of our project is to obtain undenaturated *collagen type 1* with a fermentation method and from such waste materials as eggshell and Black Sea marine algae. Marine algae do have an unusual metabolism breath which results in nutritional qualities, including protein and peptides, minerals and vitamins (Kalpa S. and You-Jin J. 2012). They are useful research tools and their natural products are practicable in the treatment of the human disease. Marine algae might be good candidates for harvesting bioactive peptides against cancers. Recently (Chen et.al 2011) it was reported that the protein waste from C. Vulgaris derived peptides has been shown to inhibit UVB-ultraviolet solar B. This can be used for the cosmetic industry as well. In another study has been proved the antitumor activities of C. Vulgaris. For the food industry, in red and green seaweeds - macroalgae that for example Prphyra tenera there have been found about 21 up to 47 grams of protein per 100 grams of dry weight and Ulva perusa form 20-26 grams of protein per 100 grams of dry weight . In the pharmaceutical industry there is a permanent research of the utilization of the marine algae with respect to their bioactive peptides. With new isolation techniques under optimized conditions this could become a time and cost effective process. The therapeutic proprieties of the eggshell and marine algae together with the fact that are toxically free and present a high bioactivity and also bio specificity for

the research and clinical trials are also the reason of the present project.

The basic principle is that we use those waste materials each proven to be useful for the collagen production and with available biotechnology techniques. We also consider optimization of the process in the future post research studies.

For the fermentation we use oxygen limited method and also *Pichia pastoris* yeast for the intracellular collagen production.

Pichia pastoris as a methilotropic yeast was used in many research projects, also in some industrial projects. The efficiency of *Pichia* is also due to it being easily manipulated. (Lin C.Y. and Cregg J.M. 2000)

We use the *Pichia pastoris* strain expressing *Collagen type I* (Project Innovas - Bioingenium S.L). The development of the strain expressing 4-hydroxylase has subunits alpha and beta *Collagen type I* subunits A1 and A2. The process was conducted for the intracellular expression so until a specific technological stage we will use the pellet resulted from the centrifugation process.

In a first stage encoding genes prolyl 4-hydroxylasse subunits alpha and beta were cloned into 2 independent *P. pastoris* shuttle expression vectors and later were transformed into wild-type of *Pichia pastoris* strain. This dual integration was confirmed for isolated clones by genomic PCR and was screened by expression analysis of both prolyl 4-hydroxylasse subunits by western blot. After selecting a prolyl 4-hydroxylase producing clone, an expression vector encoding both subunits of *collagen type 1* (alpha1 alpha2) was transformed into it. Resistant clones were isolated and screened by expression analysis of both *collagen type 1* subunits by western blot. We separate by size and transfer them to solid membrane support with a specific antibody for a better visualization.

We use as carbon sources a mixture between of eggshells, methanol and glycerol feeding solutions in a fermentation process using a 5 L Braun bioreactor. For fermentation we used an oxygen limited procedure and for methanol addition we used a method with feed-back control. (Shiova 1992, Carrondo 2005, Inan 2000).For that reason we monitories the DO - dissolved oxygen (in percent feeding), pH, capacitance (in microfarad) - in relation with turbidity, temperature and the foam level of course. For optimization of the entire process during the cultivation phase we use as carbon first source glycerol and second phase methanol. We supplement the mixture with eggshell initially treated in base solution for 24 hours. In the future-summer season - we will use also specific Black Sea algae, because of the rich glycine and hydroxi-proline composition. As we know from literature hydroxiproline is characteristic only for collagen and it confers stability because of the hydrogen linkages. The initial alkaline treatment of the mixture wills action through molecular linkage without destroying them.

Maximization of the protein production is one of the objectives of the research and post research studies as well. We determine a specific fermentation moment, when viable cell density was measured by capacitance and optical density by spectrophotometer. At an optical density of 300 (OD=300) and a measured capacitance of 32 microfarad we start methanol addition. This is the proper moment for methanol feeding program. From now on we measure the methanol concen-tration by Raven Biotech Me OH sensor online and with our own software made by Innovas Waste Technologies. The calibration methanol sensor was made by initial fermen-tation volume and the methanol feeding percentage as per manufacturer indications.

The methanol addition was performed in 1% shots from culture volume. Special attention must be taken because any exceed of that concentration could become toxic for the culture.(Heisey 1997)

Our specific work is that we use as a multiple carbon and nitrogen source a mixture of eggshell and Black Sea marine algae together with the glycerol and methanol feeding solutions. Our study is about the collagen production technology with waste material like eggshells and marine algae as a carbon and nitrogen source and we believe that after our completed research studies our type 1 collagen will be with basic triple helical structure (tropocolagen) suitable for the pharmaceutical and cosmetic industry.

MATERIALS AND METHODS

Pichia pastoris strain expressing *Collagen type 1* (project Innovas - Bioingenium S.L)
5 litre Braun bioreactor, type 880
Raven Biotech online methanol sensor and control
Cole Parmer peristaltic pumps SDS electrophoresis unit
Lyophiliser unit
In that study we used a series of laboratory equipment and materials provided by Innovas Waste Technologies

Culture maintenance

Pichia pastoris was grown on YPD agar medium plates containing 1% yeast extract, 2% peptone, 2% dextrose and 2% agar. *Pichia* yeast master plate must be stored at 4°C for a one two months use. For long term the cultivated plates should be stored at -80° C, according to the Invitrogen recommendations for medium store.Source of the strain was the project Innovas-Bioingenium S.L

Inoculum Preparation

With 24 hours before fermentation we will use a pre-inoculum in shake flasks from a fresh agar plate containing the strain. We achieve an optical density of 1 and we use 150 ml shake flask incubated at 30°C at 250 rpm with BMGY complex medium (7 g yeast extract, 14 g peptone, potassium phosphate buffer, YNB stock and glycerol) from Sigma Aldrich.

Medium preparation

We prepare trace salts and biotin solutions and store at 4°C. We prepare also fermentation basal salts medium for our bioreactor.
For each litre of fermentation basal salts we will use 4.35 mL of trace salts. We start from 3 (three litre) according to our bioreactor volume.
We prepare the mixtures from eggshell and marine Black Sea algae with alkaline water.

Bioreactor setup and sterilization

For this operation we use a vertical autoclave so that we can completely sterilize the whole bioreactor. The same procedure was applied to all air filters and pipes that were mounted to be sterilized.
We protect the filters and sensors with aluminium foil. We introduce the assembled bioreactor with the medium inside the vessel into the autoclave for 20 min to 121°C.

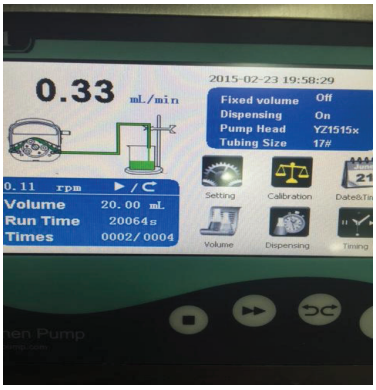
Fermentation procedures

We use an oxygen limited strategy fermentation for improving production. The oxygen is controlled and consumed in connection with the liquid gas transfer and with the methanol additions. (Picture 1, Picture 2)
After autoclaving we connect the cooling system and we add the prepared mixture and we set all the parameters as following. The cultivation conditions were stirring rate 1000 rpm, temperature 30°C and dissolved oxygen in a limit of 20%. Initial working volume was 3L. All medium salts were autoclaved or filter sterilized by 0.2 micrometre Millipore filter. Air outlet was secured also by 0.22 micrometre PTFE filter. We adjust the pH to 5-6 with a base and with an acid. We used NaOH, ammonium hydroxide as a base pH adjustment and HCl or acetic acid for the acid pH. For the level controlling pH we use peristaltic pumps from Master Flex and Tygoon rubber connections. We add trace salts through a sterile septum.
All sensors must be calibrated so we can start polarization of the dissolved oxygen sensor. For that sensor we use one or two point calibration. All prepared so the entire controllers could be started. Special attention must be taken about the feeding with oxygen or air to be sterile as well. Special filters with pharmaceutical quality standards must be used in gas line connection.
We measure the optical density from the fermenter and we calculate the necessary volume of inoculum to assure an OD of 1. By a sterile septum or an inoculum bottle we inoculate the bioreactor fermentation medium. When we check the dissolved oxygen and capacitance and we detect a rapid increase of those values we start with glycerol fed-batch phase. Glycerol feed pump must be connected and a special addition program will be implemented to add flow rate from 20 up to 100 mL per hour. We measure optical density

for each 30 minutes and when we will have a value of 350 we stop the glycerol addition.
After that phase we start methanol feeding stage in shots of maximum 1% of the bioreactor volume. We observe the online methanol measurement and the dissolved oxygen values also. A sudden peak is observed when methanol is depleted. We measure the desired protein concentration and when we realize it is steady we decide to end the fermentation. In all the methanol addition phase, because our process is exothermal we must strictly control the temperature. (Picture 3)
Once the process is finished the fermentation culture is stopped by the aid of controllers, sensors and stirring. This could be observed by no increasing of the desired protein concentration observed.
We collect the bioreactor by draining valve in a sterile bottle by a sterile connection.

Picture 1 - Computer control and software for on line monitoring from Innovas Waste Technologies

Picture 2 - Obtained centrifugation pellets



Picture 3 - Glycerol and methanol feeding

Picture 4 - Innovas fermentation software

Early downstream

We initiate early downstream to recover and purify the fermentation product.

The obtained product from the fermentation process was centrifuged at 6000-8000 g for about 20-30 min.

The supernatant will be used for extracellular production or cell pellet for intracellular production mode. We add pepsin solution 0.2 mg/ml according to Nokelainen et al., 2001 and we gently stir. A washing step was done for cell pellet in Phosphate Buffer Solution from Sigma Aldrich at 4^0C and we centrifuge again at 6000-8000 g for 25-30 min. The obtained pellet we re-suspend in a suitable binding buffer for the purification step. The intracellular product must to be disrupted by one of the available known techniques such as chemical treatment, enzymatic digestion treatment or French press, sonication, glass beds etc. We used French Press because *Pichia pastoris* has a strong cellular membrane.

In that stage some protease inhibitors were used to prevent the protein to be proteolysis. After the disruption the lysed solution was centrifuged at 10000g for 15 min. With the obtained supernatant we start filtration procedures. As a filtration steps we first used is a macro filtration by a 30 micrometre filter, then a filtration with 5-10 micrometre filter and a clarification or sterilization by 0.45 - 0.2 micrometre filter. The concentrate the product by ultrafiltration treatment Sartocom cassettes of 100 kDa was performed. The intermediary product was lyophilized and then capped in sterile bottles.

Late downstream stages

For characterization we use SDS-page electrophoresis and UV-VIS analysis for concentrate protein.

RESULTS AND DISCUSSIONS

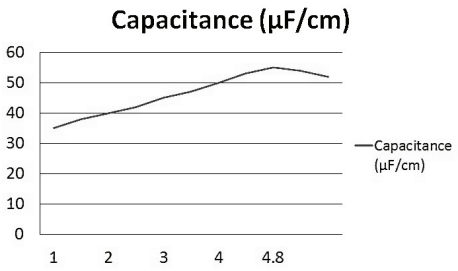


Figure 2. Capacitance variations by the number of fermentation days

Capacitance online measurements were very useful for our project, taking into consideration that the methanol addition should be started when a few parameters like optical density OD or turbidity reach the values of 300 respectively 35. The proper moment for the methanol feeding is very important to be accurately determined.

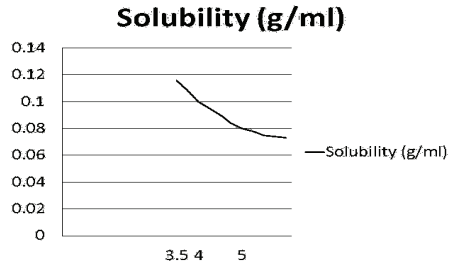


Figure 3. Variation of solubility at different pH values

The pellet obtained after the second centrifugation must be treated with acid for lowering the pH. We have found that in alkaline pH collagen solution incline to precipitate. So for any pepsin treatment or another filtering operation we must be sure that the solution is as liquid as it is possible.

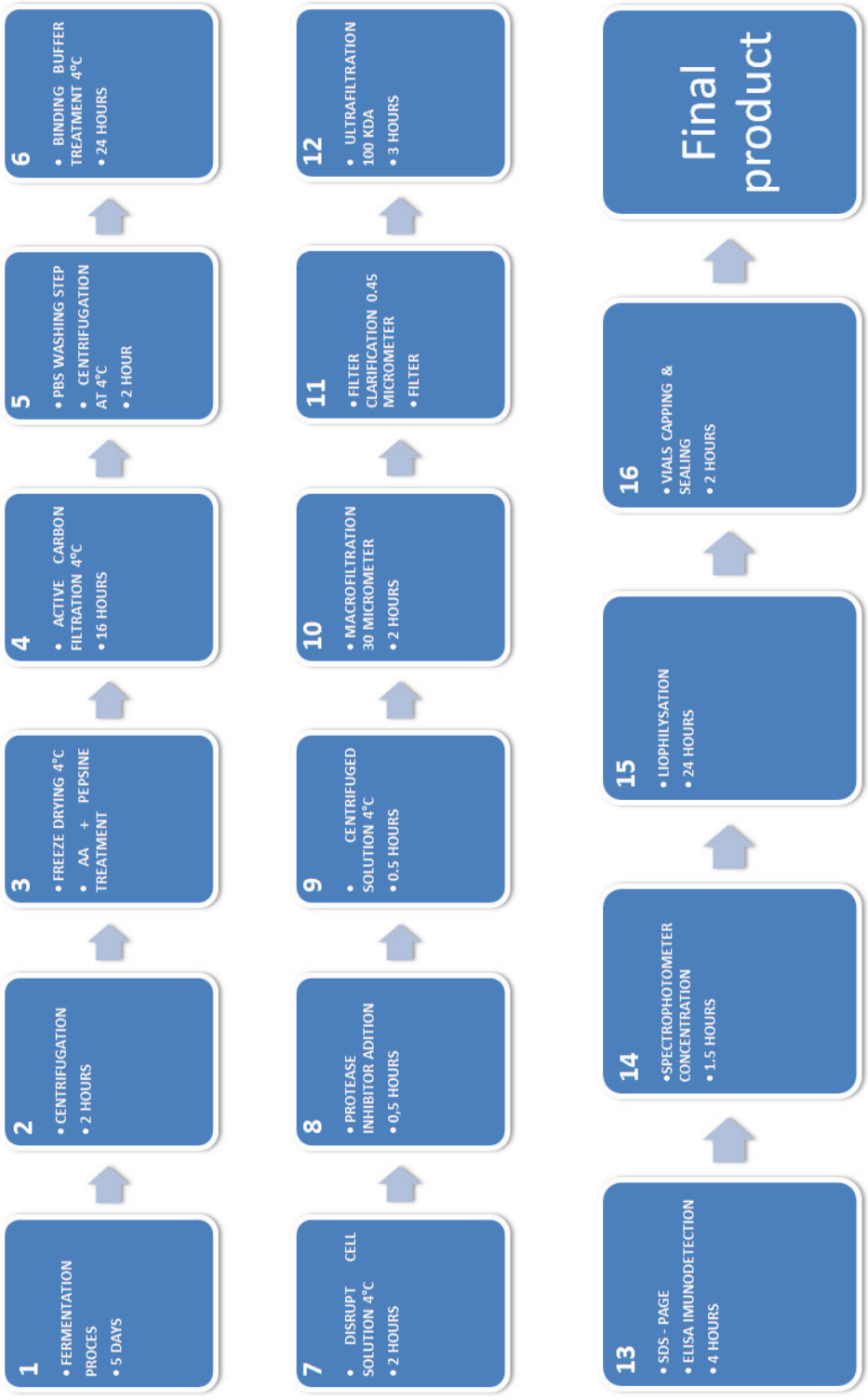


Figure 1. Methods plan with operations time calculation

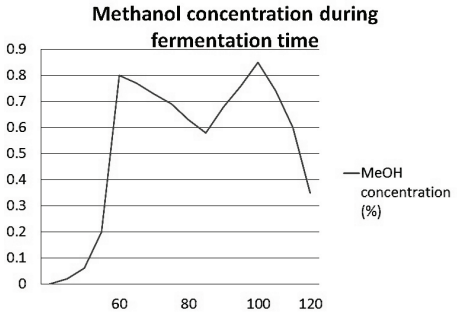


Figure 4. Methanol concentration during fermentation time (min)

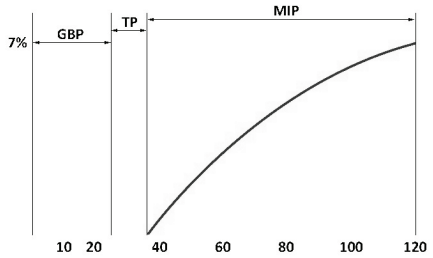


Figure 5. Fermentation phases yield during fermentation time. GBP=Glycerol base phase, TP=Transition phase, MIP=Methanol induction phase

Methanol concentration is very important to be checked online and controlled if possible because of the potential toxic concentration value which can affect the *Pichia pastoris*. During the fermentation phases in GBP (glycerol base phase) we have no methanol addition. In TP (transition phase) we must wait until the glycerol is completely depleted and in approximately 40 hours from the fermentation we started MIP (methanol induction phase). The feeding rate should be less than 1% from the existing bioreactor volume in that time.

CONCLUSIONS

During the present project there have been a lot of modifications and variations of the results. These could be from the eggshell composition, from algae composition or from different fermentation condition despite our computer monitoring. For a small industrial scale we are looking in the future to scale up the production in order to create a production platform that is time and cost efficient. We also have the intention to optimize the screening, purification, filtration and product transfer and finally to make comprehensive analysis accepted by the pharmaceutical industry and also by the cosmetically and food industry as well.

The proposed collagen production was obtained in small quantities based on the production strategy and from the proposed mixture. The online methanol control in concordance with the capacitance sensor was very useful to observe the most important moments of the fermentation process and on the other hand to avoid any negative effects for the protein production.

We strongly believe that such waste materials have the potential for the mentioned industries taking into account that there are clean and prolific sources from the biochemical and ecological point of view.

However the results need to be improved for any future commercial use, taking into consideration the productivity and rentability.

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