Universitat de Vic
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Grade's final project

Orthologous and heterologous expression of a protein from *Saccharomyces cerevisiae*: Optimization for a biotechnological application

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Biothecnology grade

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FINAL PROJECT ABSTRACT

BIOTECHNOLOGY DEGREE

Title: *Orthologous and heterologous expression of a protein from Saccharomyces cerevisiae: Optimization for a biotechnological application*
Key words: *bacteria, yeast, purification, recombinant protein*
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In this thesis (TFG) the results of the comparison between different methods to obtain a recombinant protein, by orthologous and heterologous expression, are exposed. This study will help us to identify the best way to express and purify a recombinant protein that will be used for biotechnology applications.

In the first part of the project the goal was to find the best expression and purification system to obtain the recombinant protein of interest. To achieve this objective, a system expression in bacteria and in yeast was designed. The DNA was cloned into two different expression vectors to create a fusion protein with two different tags, and the expression of the protein was induced by IPTG or glucose. Additionally, in yeast, two promoters where used to express the protein, the one corresponding to the same protein (orthologous expression), and the ENO2 promoter (heterologous expression). The protein of interest is a NAD-dependent enzyme so, in a second time, its specific activity was evaluated by coenzyme conversion.

The results of the TFG suggest that, comparing the model organisms, bacteria are more efficient than yeast because the quantity of protein obtained is higher and better purified. Regarding yeast, comparing the two expression mechanisms that were designed, heterologous expression works much better than the orthologous expression, so in case that we want to use yeast as expression model for the protein of interest, ENO2 will be the best option. Finally, the enzymatic assays, done to compare the effectiveness of the different expression mechanisms respect to the protein activity, revealed that the protein purified in yeast had more activity in converting the NAD coenzyme.

2- Introduction

Protein biotechnology is involved in the isolation, production and improvement of biological properties from specific proteins obtained from natural sources like plants, animals or microorganisms for subsequent use in various applications. Recombinant DNA technology has played an important role in the development of protein biotechnology. [1]
A recombinant protein, or heterologous protein, is one whose synthesis is performed in another organism distinct from the native one. [2]

Nowadays, multiple systems to obtain recombinant proteins for biotechnology applications have been developed.[3] Their production is made in expression systems commercially available or in designed ones according to specific needs. An expression system is formed by a host organism and an expression vector or DNA fragments that contain genetic elements necessaries for the transcription and translation in this organism, and also receive the recombinant DNA molecules.

For selecting a suitable expression system for the synthesis of a recombinant protein, there are some considerations that we have to take into account: biological origin, chemical and biological properties from the protein of interest, subsequent application and bioprocesses that will be used for his production.

A variety of hosts that are currently used, ranging from unicellular organism such as bacteria, fungus or, yeast, and mammals cell lines, insects or plants, till complete organisms like animals or transgenic plants.

Among of the prokaryotes we can find bacteria, of which *Escherichia coli (E.coli)* has been the most used, although *Bacillus subtilis, Caulobacter crescentus, Pseudomonas sp, Lactobacillus lactis*, etc, have been used also.
However, expression systems of recombinant proteins based in yeast, which is an eukaryote, have proved to be an efficient and economical source of proteins of industrial interest. In yeast group, *Saccharomyces cerevisiae (S.cerevisiae)*[4] has been used for many years as a mechanism for the expression of heterologous genes but, nowadays, new organisms are also used, like *Pichia pastoris*, *Schizosaccharomyces pombe* and *Hansenula polymorpha*. [5]

In this project, the main objective is to develop a stable protein overexpression method, above all in yeast, but to start in the first steps of this project we started investigating the effectiveness of that expression in two model organisms previously mentioned: *Escherichia coli* [6] and *Saccharomyces cerevisiae*.[7]

Proteins that are going to be overexpressed will be called BC1 and BC2 because, for confidentiality reasons we can't mention the real names.

Expression system in *E.coli*

Using *E.coli* as a model organism, the expression system that we used consist in cloning and expressing the BC1 and BC2 genes through plasmids and inducing them by Isopropyl β-D-1-thiogalactopyranoside (IPTG) who triggers transcription of the *lac* operon and induce then the protein expression that is under his control. [8]

We designed two different methods of fusion proteins for the heterologous protein expression. We fuse the gene of interest to a histidine tag and to a glutathione S-transferase (GST) tag provided by the vectors in which the gene was cloned, pET28a and pGEX-4T-3 respectively.[9] These tags can give us advantages and disadvantages in the purification step because the poly-histidine tag can be attached into a divalent metal matrix and be eluted by imidazole competition, but the tag or the imidazole can affect the protein properties. On the other hand, GST can be attached to a glutathione agarose and be eluted by glutathione, but the dimerization of the GST and the elution with glutathione can affect the protein properties also. Some tags can protect the proteins against intracellular proteases or help to its solubilisation.[3]

E.coli is the most used expression system for the expression of heterologous proteins due to its high growth rate, its capacity for continuous cultivation and low cost. As it is well studied, we have a lot of knowledge about its genetics, physiognomy and genome sequence, that's why many expression systems were designed in this model, for different applications and compatibilities. [2, 3]

Nevertheless, *E.coli* has some limitations, for example:

- Impossibility of post-transcriptional modifications that are necessaries for the correct conformation of the structure.
- Absence of appropriate chaperones for some proteins, which causes an incorrect folding.
- Codons preferences, which can degenerate the genetic code, so the translation and the expression yield decreases considerably.
- High proteases content that can affect the final product, impeding the expression of active proteins; that's why some strains, like BL21, are used, because are deficient in some proteases.
- Generate endotoxins that are unhealthy.
- Inefficiency to secrete proteins into the culture medium and tendency to precipitate as insoluble inclusion bodies in the cytoplasm.

Another disadvantage that we had in our project is that we express a eukaryotic gene in a prokaryotic organism.

Expression system in *S. cerevisiae*

Using yeast as a model organism, the expression system was done without using plasmids, but tagging the gene of interest directly in the genome.

In the yeast expression, a particular protein or enzyme may be of interest as a product itself (e.g. human alpha interferon), or may be of interest because it facilitates production by the host yeast of a desired metabolite (e.g. ethanol) or production of a food or beverage product (e.g. beer, wine, bread or spirits). To obtain high levels of expression, promoters are used because they increases the transcription, translation or mRNA stability of the gene if they are under appropriate growth conditions (e.g., if the promoter is inducible, in the presence of the inducing substance). [10,11]

In this project, two different promoters are used for the expression of the protein; one promoter corresponds to the specific one of the protein we want to express and the other corresponds to the ENO2 promoter, giving, in this way, an orthologous and heterologous expression respectively.

ENO2 promoter of *S. cerevisiae* have the advantage of being regulated: expression can be repressed 20-fold by growing the yeast on a non-fermentable carbon source, such as glycerol, lactate or ethanol, but the expression of the desired gene can be induced by addition of glucose to the growth medium. [12,13]

S. cerevisiae is economic and meets all biosafety regulations for human applications, is considered a GRAS (Generally Recognized as Safe) organism by the american FDA and the european EFSA. By sharing many molecular, genetic and biochemical characteristics with higher organisms, has been confirmed the commercial potential of yeast expression systems. Yeast can produce correctly folded and soluble recombinant proteins that have undergone all the essential post-translational modifications to be functional, which is an advantage in the production of eukaryotic proteins. Moreover, yeast is easy to handle and has rapid growth. In addition, *S. cerevisiae* is one of the best characterized eukaryotic organisms and its genome has been fully sequenced. [2, 3]

The disadvantages of this organism are: production of hyperglycosylations and low efficiency in heterologous protein secretion. In addition, limitations appear when we compare it with the non-conventional yeasts, which exhibit better yield as producers of certain heterologous proteins and are used in various processes developed on an industrial scale.

3- Objectives

In this project, two main objectives were considered:

1. Identify which is, between bacteria (*E.coli*) and yeast (*S. cerevisiae*), the best expression system to purify the recombinant protein object of this study.

    a. Compare the efficiency of the purification between 6*His-tag and GST-tag systems in *E. coli.*
    b. Compare the efficiency of the purification between bacterial model systems (using expression vectors) and yeast model system (protein tagged in the genome).
    c. Compare the efficiency rate between the production of orthologous and heterologous protein.
    d. Assay the enzymatic activity of the purified proteins.
    e. Compare the activity of the purified protein from *E. coli* and *S. cerevisiae.*

2. Develop a stable overexpression method in yeast that allows us to obtain a considerably amount of purified protein with a high productivity.

    a. Compare the expression efficiency of the promoter of the own protein of interest with the efficiency of ENO2 promoter.
    b. Increase glucose concentration of the yeast culture to improve the efficiency of ENO2 promoter.

4- Materials and methods

Materials

The reagents for polymerase chain reaction (PCR) and KOD Hot Start DNA polymerase were purchased from Novagen. Page Ruler Prestained Protein Ladder, GeneRuler 1kb DNA ladder and plasmid isolation kit named GeneJET Plasmid Miniprep Kit were from Thermo Scientific. DNA and protein gel electrophoresis was performed using Bio-Rad tanks. SYBR safe DNA gel stain and Nickel-nitrilotriacetic acid (Ni-NTA) superflow were from Invitrogen. Restriction enzymes were purchased from New England Biolabs. Illustra GFX PCR DNA and Gel Band Purification Kit was from GE Healthcare. Rapid DNA Ligation Kit was obtained from Roche. DFS-Taq DNA Polymerase was purchased from Bioron. Oligonucleotide primers, monoclonal anti-PolyHistidine and β-Nicotinamide adenine dinucleotide hydrate (NAD) were ordered from Sigma-Aldrich. HRP-conjugated anti-mouse antibody was bought from Thermo Scientific. Luminata™ HRP Chemiluminescence Detection Reagents were purchased from Millipore and Amersham ECL Prime Western Blotting Detection Reagent was from GE Healthcare. Salmon sperm DNA solution was from Life Technologies.

Strains and culture conditions

E. coli DH5-α cells were used as hosts for plasmid transformation and isolation, and *E. coli* BL21 were used as hosts for protein expression and purification. *E. coli* strains were grown at 37ºC in Luria-Bertani (LB) medium supplemented with ampicillin (100μg mL^{-1}) for pGEX-4T-3 and pFA6-KanMX6 vectors and with kanamycin (100μg mL^{-1}) for pET28a vector.

- Escherichia coli

DH5-α and BL21 cell lines

The DH5-α strain of E.coli is not pathogenic, and was used because it has multiple mutations that allow a high efficiency of transformation. Important mutations it contains are: *endA1*, allowing the degradation of endonuclease which ensures a higher speed of plasmid transfer and *recA1*, reducing homologous recombination leading to a greater stability of the insert. This strain already contains plasmids, and has the ability to accept the insertion of other plasmids exceptionally well. [14]

BL21 cell strains are convenient for transformation and protein expression. Upon addition of isopropyl-β-D-thiogalactopyranoside (IPTG), T7 RNA polymerase is expressed and it induces a high-level protein expression from T7 promoter driven expression vectors, like pET vector. E. coli BL21 (DE3) strain lacks both the *lon* protease and the *omp*T membrane protease, which may degrade expressed proteins.

- Saccharomyces cerevisiae

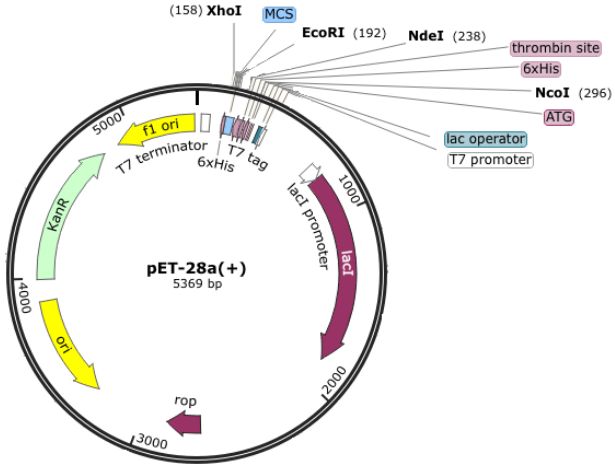
Saccharomyces cerevisiae was grown in Yeast extract Peptone Dextrose (YPD) broth medium at 30ºC. The wild type strain used was BY4741 which genotype is: MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0. When yeast cells were transformed with DNA fragments containing kanamycin resistance cassette, YPD-G418 antibiotic plates were used for positive colonies selection. [15]

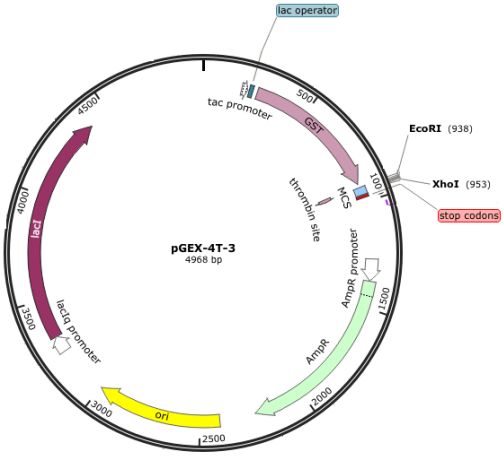
Plasmids

Expression vectors pET28a were provided by Novagen, pGEX-4T-3 from GE Healthcare and pFA6-KanMX6 was purchased from Addgene. The pET28a-BC1 and the DNA of BC2 were from our laboratory stock.

- pET28a vector

Contain: bacterial expression vector with T7lac promoter, adds N-terminal His tag, thrombin cleavage site, internal T7 epitope tag, C-terminal His tag; kanamycin resistance gene and multiple cloning site (MSC) for restriction enzyme cloning. Size: 5369bp.



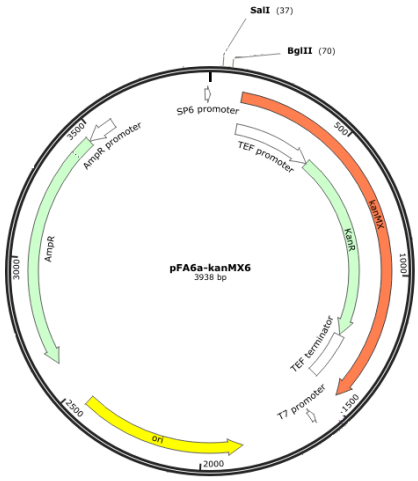


- pGEX-4T-3 vector

Contain: Bacterial expression vector with Tac promoter, GST tag, thrombin cleavage site; ampicillin resistance and multiple cloning site (MSC) for restriction enzyme cloning. Size: 4968bp.

- pFA6-KanMX6

Contain: Yeast genomic targeting, ampicillin resistance, kanamycin resistant for yeast; multiple cloning site (MSC) for restriction enzyme cloning. 5'sequencing primer: Sp6_primer and 3' sequencing primer: T7_promoter. Size: 3938bp.



- Isolation of plasmid DNA by miniprep

To isolate plasmid DNA we used the GeneJET Plasmid Miniprep Kit, which consists in centrifuging the cells, resuspending them in resuspension buffer and using SDS/alkaline lysis to liberate the plasmid DNA. The resulting lysate was neutralized to create appropriate conditions for the binding of the plasmid DNA to the silica membrane in the spin column. Cell debris and SDS precipitate were pelleted by centrifugation, and the supernatant containing the plasmid DNA was loaded onto the spin column membrane. The absorbed DNA was washed to remove contaminants, and was then eluted with small volume of elution buffer (10mM Tris-HCl, pH 8.5). After the purification of the plasmid, the concentration was measured using Nanodrop.

- Cloning of BC2 DNA into expression vectors

To clone the BC2 DNA into pET28a vector, we used PCR with BC2 in the plasmid BG1805 as a template and specific oligonucleotide primers were designed containing in the forward an EcoRI restriction site and in the reverse an XhoI restriction site. The amplification of the DNA was made by PCR using a KOD Polymerase with an annealing temperature of 60ºC for 10s during 35 cycles. (Not a single oligonucleotide will be shown for confidential motifs). [16]
The BC2 DNA fragment, obtained with the PCR, and the vectors were digested with restriction enzymes EcoRI and XhoI to create sticky ends in the DNA fragments and to open the expression vectors for the subsequent cloning, respectively. The BC2 DNA was ligated into the vectors using the Rapid DNA Ligation Kit to give pET28a-BC2 and pGEX-BC2.[17]
The ligations mixtures were then transformed into DH5-α by heat shock and plated on LB+Kan for pET28a and pFa6-KanMX6 and LB+Amp for pGEX-4T-3. The colonies obtained were checked by colony PCR and, from the positives ones, the plasmid DNA was extracted with the miniprep kit.

To clone His-BC2 into pFA6-KanMX6 vector, we amplified His-BC2 from pET28a-BC2 using specific oligonucleotide primers. SalI and BglII sites were incorporated into the forward and reverse primers, respectively, to clone into the expression vector.
The amplification of the DNA was made by PCR using a KOD Polymerase with an annealing temperature of 55ºC for 20s during 35 cycles.

The ligation mixture was then transformed into DH5-α by heat shock and plated on LB+Kan. A PCR of the colonies obtained from the transformation was made to check which ones contain the recombinant plasmid. In this case, the oligonucleotides used in the PCR of the colonies were: SP6 and a reverse primer designed; and the conditions were: DFS-Taq DNA polymerase, annealing temperature of 37ºC for 20s and 35 cycles.

- Transformation of *E.coli* cells with plasmid DNA

E.coli cells were defrosted on ice and DNA from the ligation reaction mix or pure plasmid DNA was added. Then, the E.coli were rested on ice for 30min, heat-shocked for 45s in a water bath at 42ºC and put on ice again for 5 min. Cells were resuspended with 1mL of medium and incubated at 37ºC for 1h at 250rpm. Finally, 100µL of culture were pipetted and plated onto plate with medium plus the required antibiotic and incubated at 37ºC for 24h.

- Colony PCR

Colony PCR was used to check which colonies from the transformation contain the vector with the inserted DNA fragment.
Primers used in this PCR were: T7 as forward and a designed one as a reverse for the pET28a vector, and pGEX 5' as a forward and a designed one as a reverse for the pGEX-4T-3. The conditions for the PCR used were: DFS-Taq DNA polymerase, annealing temperature of 55ºC for 20s and 35 cycles.

- Expression of BC1 and BC2 genes

To induce the expression of the proteins, BL21 cells were transformed with the plasmids previously obtained. Induction of protein expression was performed using 0.5mM of IPTG when the OD600 of the cultures was around 0.5. The induction was taken overnight at 20ºC shaking the cultures at 240rpm. The induced cells were harvested by centrifugation at 4º for 20 min at 9.000 x g, and stored at -20ºC. [18,19]

- Purification of BC1 and BC2 from BL21 *E.coli*.

To purify the recombinant BC1 and BC2, the E. coli cell pellets were resuspended in two bed volumes of lysis buffer (50 mM Tris pH 7.4, 150mM NaCl, 10% glycerol). Cell disruption was carried out by sonication at 4ºC for 2 min, and the lysate was centrifuged at 16.000 x g for 30 min at 4ºC to remove the cell debris. Supernatant was retained for purification. Ni-NTA beads were previously equilibrated with 10mM imidazole in lysis buffer and then applied onto the supernatant cell-free extract.
For 50 mL culture 200µL of Ni-NTA beads were used. The mixture was incubated at 4ºC rolling for 1h and washed first with 10 bed volumes of washing buffer (50mM Tris-HCl pH7.4, 25mM imidazole, 150mM NaCl and 10% glycerol), and finally with 10 bed volumes of the same buffer including 300mM NaCl.

The protein was eluted from beads by competition with imidazole following a gradient-step imidazole elution ranging from 50mM to 500mM at steps of 50mM in elution buffer (50mM Tris-HCl, 300mM NaCl and 10% glycerol). The mixture was incubated rolling at 4ºC for 15 minutes with each different amount of imidazole. A sample of each step was taken for protein quantification.

Enzyme fractions were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% gel and visualized by staining with Coomassie blue.[20]

- Protein quantification and determination of molecular mass

Protein concentrations were determined by Bradford using the spectrophotometer at 595nm and using bovine serum albumin as protein standard. The molecular weight was determined by SDS-PAGE under denaturing conditions, using a prestained marker as a reference. [21]

- His-BC2 DNA cassettes for yeast transformation

Taking pFA6-KanMX6-His-BC2 as a template, two PCRs were made in parallel to amplify the fragment of interest. The DNA cassette that is going to be inserted under the BC2 promoter was obtained using the oligonucleotides specially designed: His6-BC2-Kan-Fw, His6-BC2-Kan-Rv, while the one that is going to be inserted under the ENO2 promoter, was obtained using the oligonucleotides: His6-BC2-Kan-Fw-ENO2 and His6-BC2-Kan-Rv-ENO2 as primers. The amplification of the DNA was made by PCR using a KOD Polymerase with an annealing temperature of 60ºC for 10s during 35 cycles, and then the product was purified using the Illustra GFX PCR DNA and Gel Band Purification Kit. DNA concentration was measured with Nanodrop. [22,23]

- Yeast transformation

For the transformation we first inoculate 5 mL YPD with a single yeast colony and incubate the culture at 30ºC overnight. The day after, an appropriate dilution of the pre-culture was done, in order to have 100mL of culture to a final OD=0.5 the third day. 50mL of the culture was centrifuged at 1300rpm for 3min at RT and the pellet was resuspended in 1mL of sterile H2O.
Cells were spun at 2000rpm for 1min and the H2O was poured off. This wash step was repeated once. Next, the cells were washed once in TEL buffer (Tris EDTA Lithium acetate; 10mM Tris pH 7.7, 1mM EDTA, 100mM lithium acetate).

225µl of TEL was added and the cells were resuspended. Transformation was made with 50µl of cells in a 1.5mL eppendorf tube. 5µl of pre-boiled salmon sperm DNA were added together with 10µl of PCR product. We added 350µl TEL/PEG (10mM Tris pH 7.7, 1mM EDTA, 100mM lithium acetate, 40% PEG; polyethylene glycol) and mixed well the transformation mix. The mixture was incubated at 30ºC for 30min. Then, the tube was heat-shocked at 42ºC and incubated for 15min in the water bath.
Cells were spun down for 1min at 2000rpm and the TEL/PEG was removed. Cells were resuspended in 1mL of YPD and incubated for 3h at 30ºC shaking. Cells were centrifuged at 2000rpm for 1min and the YPD was removed completely. Finally, 50µl of sterile water was added to resuspend the cells and these were plated on YPD-G418 plates.

- SDS-PAGE and Western blotting

Colonies obtained from the transformation were put on a pre-culture. The OD of the pre-cultures was measured and the corresponding volume of the culture was taken to have the same concentration of cells in each case. To check if the His tag was inserted in the genome, a Western Blot anti-His was made after breaking the cells in a mechanical way by series of vortex/boil. The first antibody used was Monoclonal anti-PolyHistidine, which is produced in mouse and the secondary was HRP-conjugated anti-mouse antibody. The membrane was incubated in Luminata Classico Western HRP substrate.

- Purification of BC2 from *S. cerevisiae*

Strains containing BC2 tagged with 6His at N-terminus (S441 and S442) were used to purify the BC2 protein. A culture of 1L volume of each strain was grown in YPD medium at 200rpm for 2 days at 30ºC. Cell pellets were obtained from centrifugation at 4000rpm for 20min at 4ºC, the supernatant was eliminated and the pellets were resuspended with cold water. Pellet bottles were weighed before and after add the pellet to know how much it weighed. The pellet was obtained by centrifugation at 6000rpm for 15 min at 4º and stored at -80ºC.

For purification of the recombinant BC, the pellet cells were resuspended with two bed volumes of lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 10% glicerol). Lysis was carried out by cell disrupter at RBAR 2.7 pressure at 4ºC and the lysate was centrifuged at 9000 x g for 30 min at 4ºC to remove the cell debris. The resulting crude extract was retained for purification. Ni-NTA beads were previously equilibrated with 10mM imidazole in lysis buffer and then applied onto the supernatant cell-free extract.

For each 40 mL culture, 200µL of Ni-NTA beads were added. The next steps of protein purification (binding, washing and elution) follow the same protocol that the purification from *E.coli*.

- Spectrophotometric determination of enzyme activity

NAD-dependant enzymes are an important group of enzymes, which may be assayed rapidly by UV spectrophotometry. These enzymes use NAD^+ as their coenzyme which is reduced to NADH during the reaction that takes place. [24,25]

Reduced NAD^+ (NADH) exhibits strong UV absorption at 340nm whilst oxidised form has virtually no absorption at this wavelength. Therefore if one starts with a mixture of substrate, NAD^+ and enzyme in buffer, the reaction proceeds until equilibrium is established. The reaction may be followed by measuring the increase in absorbance of the solutions at 340nm as NADH is formed. [26]

Specific activity of an enzymatic preparation is defined as the number of micromoles of formed product per minute per microgram of protein or per microgram of enzyme. [27]

The BC2 specific activity was determined by a spectrophotometer assay at 340nm during 1 min. Substrate was used at 1M concentration, Tris pH 7.4 50mM, EDTA 1mM was used as a buffer and Nicotinamide Adenine Dinucleotide (NAD^+) was used as a cofactor at 50mM.

5- Results

In this project, we purified two different NAD dependent enzymes: for the privacy policy of the project we cannot tell the name of the enzymes so we will call it BC1 and BC2 in this document. For BC1 protein, we had the corresponding gene already cloned into a pET28a vector, so we just induced and purified it. However, for the BC2 protein, we had to clone the gene first in the expression vector to, then, purify the protein. To express BC2, we decided to clone the gene in two different expression vectors: pET28a and pGEX-4T-3. We planned, also, to employ different model organisms and compare the differences in expression and efficiency of the protein between the different hosts.

Cloning of BC2 gene in *E.coli*

To be able to purify the BC2 protein from *E.coli* we had to subclone the BC2 DNA into an expression vector. We decided to subclone into two expression vectors because we want to purify it with two different techniques.
With the pET28a vector, we wanted to express the BC2 protein fused to the 6*His tag that the vector contains, to purify it by Ni-NTA beads; with the pGEX-4T-3 vector the intention was to express the BC2 protein fused to the GST tag given by the vector, to purify it later with glutathione sepharose.

The BC2 gene was amplified from a BG1805 vector in which BC2 was previously cloned. In details, we first isolated the BG1805+BC2 vector, from our *E. coli* stock, using a miniprep kit as described in the materials and methods section. After the purification of the plasmid, we measure the concentration by using Nanodrop.

Vector (BG1805+BC2) → 420.2 ng/µL

Next, the BC2 gene was amplified by PCR using BG1805+BC2 vector as a template. As we wanted to clone BC2 in two different plasmids, we used two oligonucleotide primers for each vector.

EcoRI and XhoI sites were incorporated into the forward and reverse primers for cloning into the expression vectors (Fig.1). The samples were run in an agarose gel to confirm that the fragments were amplified (Fig.2).

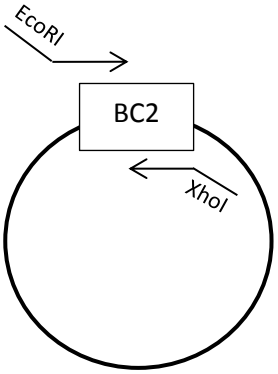


Fig.1. Scheme of the amplification process for vector pET28a and pGEX-4T-3.

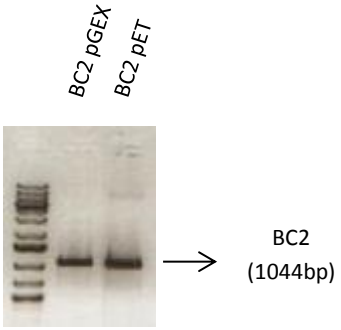


Fig.2. Agarose gel showing the PCR product.

The BC2 DNA fragment obtained with the PCR and the vectors were digested with restriction enzymes EcoRI and XhoI to create sticky ends in the DNA fragments and to open the expression vectors for the subsequent cloning, respectively. (Fig.3). Then, the product of the digestion was purified by column and run in the gel to check if the digestion worked properly (Fig.4). pGEX-4T-3 vector has a size of 4954bp and pET28a vector of 5369bp.

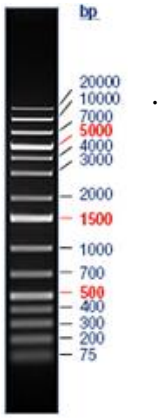


Fig.2.1. GenerRuler 1Kb Plus DNA Ladder. Thermo Scientific.

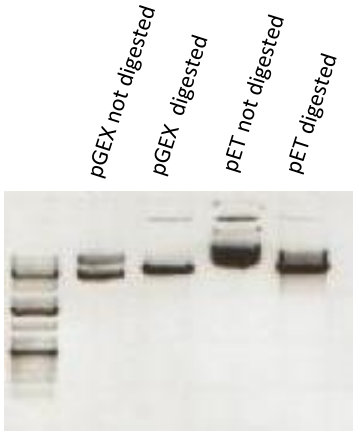


Fig.3. Agarose gel showing the vectors pET and pGEX not digested and digested.

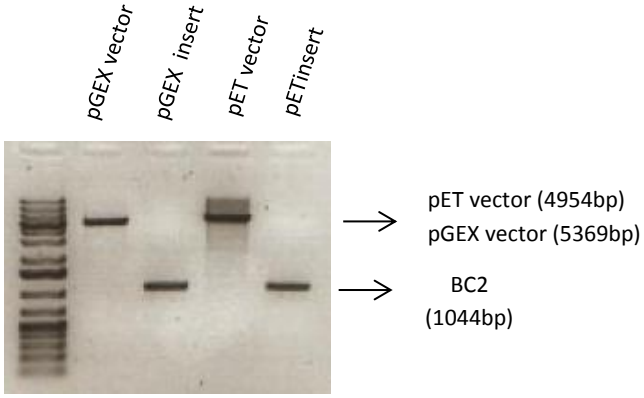


Fig.4. Agarose gel showing the vectors pET and pGEX and the inserts of BC2 digested.

The BC2 DNA fragment was ligated into the vectors to give pET28a-BC2 and pGEX-4T-3-BC2. The ligation mixtures were then transformed into DH5-α by heat shock and plated on LB+Kan and LB+Amp for pET28a and pGEX-4T-3 respectively. With the obtained colonies from the transformation, a colony PCR was made to check if the colony has the vector plus the insert, which means that the expected band should appear around 1242bp. The primers used for the PCR were the T7 primer and the reverse was specially designed.

As no clear band was obtained in the gel of the colony PCR, we decided to extract the plasmid DNA from different colonies and check them by digestion, using EcoRI and XhoI as a restriction enzymes (Fig.5).

Unfortunately, after several ligations with pGEX-4T-3 vector, we did not manage to get colonies containing the vector + insert, so we will try it again in the future (Fig.6).

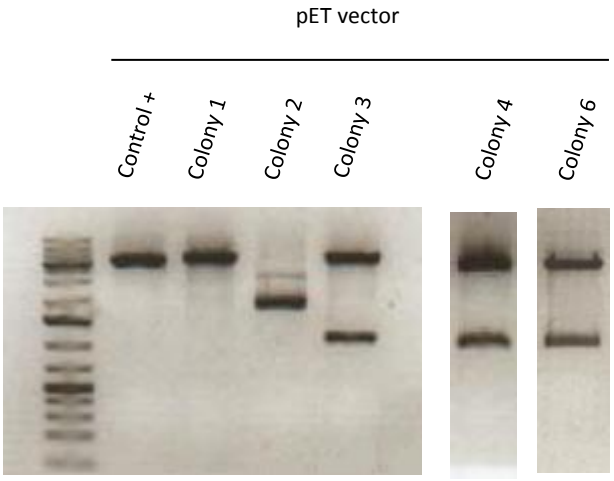


Fig.5. Cloning in pET28a: agarose gel showing the product of the digestion of plasmid DNA from several

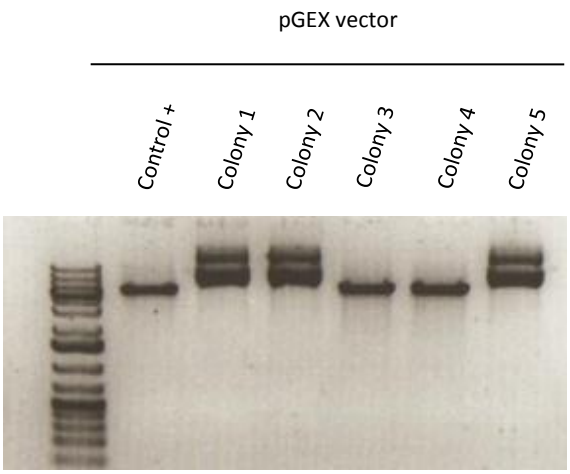


Fig.6. Cloning in pGEX-4T-3: agarose gel showing the product of the digestion of plasmid DNA from several colonies.

We could see that in colonies 3, 4 and 6 two distinct bands appeared which means that the colony contains the vector (5369bp) plus the insert (1044bp). We obtained the plasmid DNA from these three colonies using the plasmid miniprep kit.

To check that no mutations were inserted in the BC2 gene during the cloning, we decide to send them to sequence.

First, a quantification of the plasmid DNA was made:

Number of colony	Quantity of DNA (ng/µL)
Colony 3 (1)	61.6
Colony 4 (2)	104.6
Colony 6 (3)	83.2

Table1. Concentration of the DNA measured with Nanodrop.

Plasmid DNAs were sent to be sequenced to Centre for Research in Agricultural Genomics (CRAG) to make sure that no mutations occurred during the PCR.

The three positive colonies were from the ligation with pET28a vector, so we asked for the corresponding plasmid DNA to be sequenced using the T7 promoter as a forward, and the T7 terminal as a reverse primer.

The sequences obtained from CRAG were compared to the data base reference sequence of BC2 by alignment using the ApE program. (Images are not shown by confidential motifs).

We can see how in some of the alignments there are “red points” that indicate a difference from the BC2 reference sequence, but in all the cases, the differences shown in the alignment of the forward or reverse is counteracted by the other alignment. As the red points just appear close to the primer alignment site, they are sequencing inaccuracies, and we can conclude that no mutations occurred. Of the three positive vectors, we chose two to continue with the experiment. These vectors receive the name of SJR 254 colony 1 and SJR 254 colony 2.

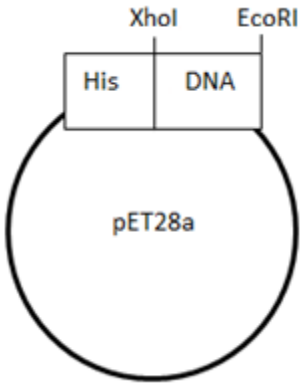


Fig.7. Scheme of the pET28a vector with the insertion of interest. His: histidine, DNA: BC1 or BC2. Restriction enzymes are shown.

Finally, the newly generated plasmids, SJR 254 colony 1 and SJR 254 colony 2, was used to transform *E*. coli BL21 cells and, at the same time, we did the same with pET28a-BC1, which was already cloned (Fig.7). The colonies that were grown contain the pET28a vector in which BC2 or BC1 gene are inserted. These genes are under the control of the T7 promoter and are expressed as a protein fused to 6*His tag at the N-terminus.

Heterologous expression of BC1

As was previously explained, two different proteins were purified in this project: BC1 and BC2. At the beginning of the project, we started with the purification of BC1, for which the DNA was already cloned into pET28a.

pET28a-BC1 vector was used to transform BL21 bacterial cells. From the resulted colonies, we picked up one to grow it in LB + Kanamycin liquid medium. Cell culture was induced with IPTG to express the plasmid, as described in material and methods section. After the induction, cell pellet was recovered and stored at -80ºC. Different volumes of protein extracts before and after induction were analysed by SDS-PAGE to check if there was expression of the BC1 protein after induction (Fig.8). A protein of 50KDa, a size consistent with the predicted molecular mass for the BC1 protein, was identified in soluble extracts only from cells harbouring pET28a-BC1 and induced by IPTG.

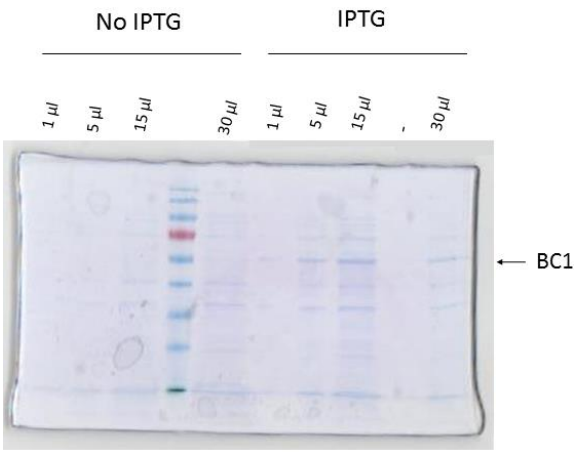


Fig.8. Checking the expression of BC1 by SDS-PAGE stained with Coomasie Blue. Lane 4: Molecular standard marker. Lane 1-5: Different volumes of *E.coli* extract without IPTG. Lane 6-10: Different volumes of *E.coli* extract after IPTG induction. An arrow indicates the protein of interest.

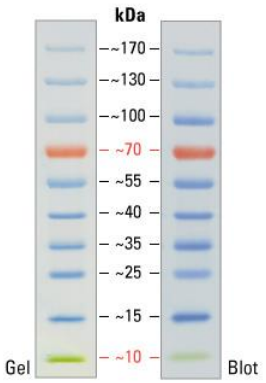


Fig.9. PageRuler Prestained Protein Ladder. Thermo Scientific.

We can see how the expression of the protein is overexpress in the IPTG lanes, which means that the induction gone well. Our protein of interest is the stronger band, but we can see other bands that correspond to other proteins present in the cell.

Purification of BC1

To purify the recombinant BC1, cells has to be broken by sonication and protein extract has to be treated with different buffers for the binding, washing and elution process (see Matherials and Methods section).

For the binding process, Ni-NTA beads were used. Ni-NTA Agarose is an affinity chromatography matrix for purifying recombinant proteins carrying a His tag. We used it in our experiment because the recombinant protein that was generated (pET28a-BC1) carries a His tag provided by pET28a vector. Histidine residues in the His tag bind to the vacant positions in the coordination sphere of the immobilized nickel ions with high specificity and affinity. Cleared cell lysates are loaded onto the matrix. His-tagged proteins are bound, and other proteins pass through the matrix in the washing steps.

A sample of each wash was loaded in an SDS-PAGE to see it efficiency (Fig.10).

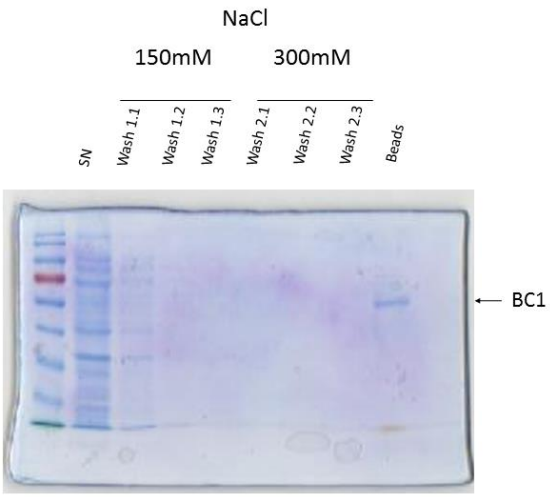


Fig10. SDS-PAGE showing the efficiency of washing. Lane 1: Molecular standard marker. Lane 2: Supernatant from the beads after binding and before washing. Lane 3-5: Washes at 150mM NaCl. Lane 6-8: Washes at 300mM NaCl. Lane 10: Beads after washing.

In the supernatant lane, we can see how all the proteins are in the supernatant excepting ours, which means that our protein was well attached to the beads.

As well as the concentration of salt increased in the washing steps, we can see how the other protein bands were disappearing, which means that the wash process was efficient.

After washing, His-tagged protein was eluted in buffer under native conditions. A gradient of imidazole was used because it acts by competition with histidine displacing the His-tag from nickel coordination, releasing the His-tagged protein.

Enzyme fractions, 20µL for each lane, were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), using a 12% gel, and visualized by staining with Coomassie blue (Fig.11).

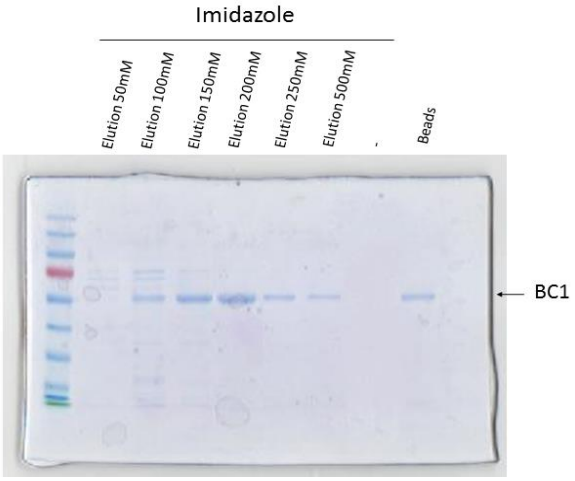


Fig11. Analysis of the eluted protein by SDS-PAGE and Coomassie blue staining. Lane1: Molecular standard marker. Lane 2-7: Samples of protein eluted at different concentrations of imidazole. Lane 9: Sample of the beads after the elution process.

We obtained the maximum elution using buffers with imidazole 150-200mM. The following elutions, with higher imidazole concentration, gave less protein amount because less quantity of the enzyme was still bounded to the beads. We can also see how, in the first lanes, apart from our protein of interest, there are other bands corresponding to other proteins, but in the following elutions these bands were disappearing and only BC1 is present. Finally, a sample of the beads was run and we can see a protein band, which indicates that the elution wasn't 100% efficient.

Protein quantification of BC1

Protein concentrations were determined by Bradford method using bovine serum albumin as a standard protein; three different line patterns were used because the result given by the average of the three ones will be more accurate (Table2).

$y = 0{,}516x + 0{,}0394 \quad R2 = 0.999$

$y = 0{,}5497x + 0{,}0061 \quad R2 = 0.998$

$y = 0{,}5639x - 0{,}0023 \quad R2 = 0.999$

Equation(1). Line patterns from bovine serum albumin.

Sample	OD 595nm	x = (y- 0,0394)/0,516	8/dilution factor	x = (y-0,0061)/0,5497	8/dilution factor	x = (y- 0,0023)/0,5639	8/dilution factor	Average Prot.Conc (µg/µL)
BC1 100mM	0,144	0,203	0,102	0,251	0,126	0,260	0,130	**0,12**
BC1 150mM	0,168	0,250	0,125	0,295	0,148	0,303	0,151	**0,14**
BC1 200mM	0,154	0,221	0,111	0,268	0,134	0,276	0,138	**0,13**
BC1 250mM	0,046	0,012	0,006	0,072	0,036	0,085	0,042	**0,03**
BC1 500mM	0,020	-0,038	-0,019*	0,025	0,013	0,040	0,020	**0,02**

Table2: Protein concentrations of BC1 in *E.coli*. *value discarded

Once obtained the concentrations, an estimation of the total quantity of purified protein was made, using the formulas written below (Equation (2)). First, we knew that we have approximately 300 µL of every elution, so we multiplied this quantity by the percentage obtained with the Bradford method. In a second step, we refer to the Coomassie stained samples (Fig. 11) to estimate which percentage of the Bradford quantification correspond to BC1 protein.

For example, in the first lane, in which other unspecific bands are present, we considered that just 60% of the quantity of protein estimated by Bradford corresponds to BC1. We applied the same calculation to all the samples.

Volume (µL) * Prot. Conc (µg/µL) = Total protein quantity (µg)
Prot. Quantity (µg)* % specific prot. in SDS-PAGE = Prot. Quantity BC1 (µg)

Equation(2). Formulas to calculate the quantity of a protein.

Elution 100mM

0.3*0.12=0.036mg = 36µg

36µg*0.6= 21.6µg

Elution 150mM

0.3*0.14=0.042mg = 42µg

42µg*0.9= 37.8µg

Elution 200mM

0.3*0.13=0.039mg = 39µg

39µg*0.95= 37.05µg

Elution 250mM

0.3*0.03=0.009mg = 9µg

9µg*1= 9µg

Elution 500mM

0.3*0.02=0.006mg = 6µg

6µg*1= 6µg

These theoretical results, obtained starting from the base that we had 300µL for each elution, were put it in a table to compare with the real volume we had. The results gave us the total quantity of BC1 protein we could purify from 50 mL of bacterial culture.

Sample Imidazole concentration	Theoretical volume (mL)	Theoretical Protein quantity (µg)	Real volume (mL)	Real Protein quantity (µg)	Total (µg obtained from 50 mL)
100mM	0,3	21,6	0,232	16,7	
150mM	0,3	37,8	0,232	29,23	
200mM	0,3	37,05	0,216	26,68	
250mM	0,3	9	0,248	7,44	
500mM	0,3	6	0,264	5,28	
					85,33

Table3: Protein quantification of BC1 in *E.coli*

We obtained a total of 85’33µg of BC1 protein from an initial culture of 50mL.We conclude that, expressing BC1 in a pET28a vector using BL21 bacterial cells is a quite efficient method to obtain a considerably pure BC1 protein. We had explained the process for the purification of BC1, and now we are going to see how this purification worked for BC2 protein.

Heterologous expression of BC2

Having generated the pET28-BC2 plasmid, we transformed this into BL21 as we explained in the cloning paragraph. The plasmids that were selected were the ones that come from the colonies number 1 and 2 (Table 1). With the colonies that result, a 5mL culture was grown in LB + Kan and after an overnight incubation, this culture was used to start a bigger 50 mL culture that was induced with IPTG when the OD600 was 0,762. The induced culture was grown overnight and the cell pellet was then recovered and stored at -80ºC until purification process.

Different amounts of bacteria before and after induction were analysed by SDS-PAGE to check if there was expression of the BC2 protein after induction (Fig.12). A protein of 37KDa, the size expected, was identified in soluble extracts only from E.coli induced by IPTG.

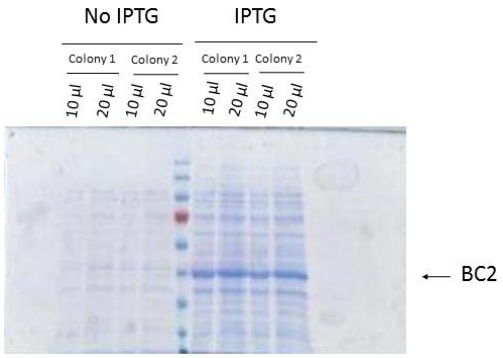


Fig12. Checking the expression of BC2 by SDS-PAGE. Lane 5: Molecular standard marker. Lane 1-4 *E.coli* extract without IPTG at different volumes and from different colonies. Lane 6-9: *E.coli* extract with IPTG at different volumes and from different colonies. An arrow indicates the protein of interest.

We can see clearly how only BC2 protein is shown in a more intense band in the IPTG part of the gel, which indicates that the induction has gone well and that our protein is well expressed and the most abundant in the mixture of the sample.

Purification of BC2

To purify the recombinant BC2 protein, first of all *E.coli* cells need to be treated with sonication to break them and extract the proteins that are inside. Then, the cell pellet was removed because contains the bacterial cell wall and the unbroken bacteria. Supernatant has to be treated with different buffers for the binding, washing, and elution process.

For the binding process, Ni-NTA beads are used, and the protein fused to the His tag provided by the pET vector are attached to nickel molecules on the beads. In the washing step, the buffer removes the other proteins that are not fixed so that mostly the recombinant protein that binds most strongly remains bound to the beads (Fig.13).

Supernatant from the two different colonies after binding was loaded on 12% SDS-PAGE gel to visualize, by staining with Coomassie blue, if all the protein has been removed by the beads.

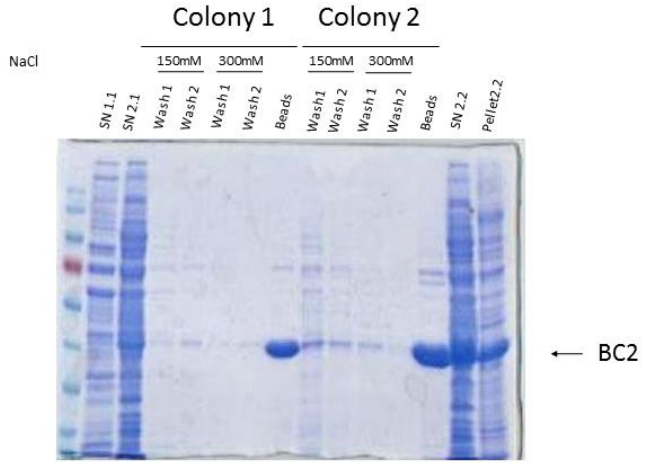


Fig13. SDS-PAGE showing the samples of the washing steps for two colonies. Lane 1: Molecular standard marker. Lane 2-3: Supernatant from the beads after binding and before washing. Lane 4-7: Washes at 150mM and 300mM NaCl concentration from colony 1. Lane 9-12: Washes at 150mM and 300mM NaCl concentration from colony 2. Lane 8 and 13: Beads after washing.

We can see how in the first two lanes, the ones that belong to supernatants, there are a lot of bands indicating that there are a lot of proteins but ours, which indicates that all our protein is well bounded to the beads. In the lanes containing the washes; we can see how all the other proteins, which we are not interested in, are removed, which indicate that the beads now just contain our protein of interest. Lane 8 and 13 are a sample of beads and we can see a huge clear band which belongs to our protein of interest. All these results let us think that the wash step gone well and all the BC2 protein rest in the beads attached. Lanes 14 and 15 are the supernatant and the pellet after breaking the cells to see which fraction is insoluble.

Next, the BC2 protein was eluted from the beads. This was done for the two colonies at different concentration of imidazole to see how the protein was eluted and checked that everything was removed from the beads (Fig.14).

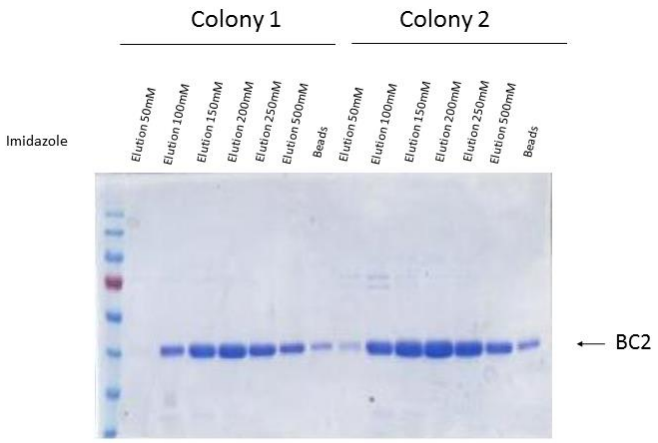


Fig.14. Determination of the eluted protein by SDS-PAGE. Lane1: Molecular standard marker. Lane 2-7: Samples of the elution at different concentrations of imidazole for colony 1. Lane 9-14: Samples of the elution at different concentrations of imidazole for colony 1. Lane 8 and 15: Sample of the beads after elution.

We can see how the stronger band has a size around 37KDa, which means that it is our protein of interest (BC). In addition, other bands appear in the gel but with a very low intensity indicating that our protein is well eluted and with a high level of purity. The stronger bands are shown when imidazole concentration is at 150-200mM indicating that the protein is well eluted at this range. However, protein is still attached to the beads as we can see in lanes 8 and 15, which indicates that the purification wasn't 100% efficient.

Protein quantification of BC2 from *E.coli*

Protein concentrations of the colonies 1 and 2 were determined by Bradford method as was previously described for BC1 (Equation (1)). The results are described below (Table 4 and 5).

Concentration of BC2 protein from colony 1								
Sample	**OD 595nm**	**x = (y- 0,0394)/0,516**	**8/dilution factor**	**x = (y-0,0061)/0,5497**	**8/dilution factor**	**x = (y- 0,0023)/0,5639**	**8/dilution factor**	**Average Prot.Conc(µg/µL)**
BC2 50mM	0,051	0,022	0,011	0,082	0,041	0,095	0,047	**0,03**
BC2 100mM	0,336	0,575	0,287	0,600	0,300	0,600	0,300	**0,30**
BC2 150mM	0,507	0,906	0,906	0,911	0,911	0,903	0,903	**0,91**
BC2 200mM	0,238	0,385	1,540	0,422	1,687	0,426	1,705	**1,64**
BC2 250mM	0,101	0,119	0,478	0,173	0,691	0,183	0,733	**0,63**
BC2 500mM	0,152	0,218	0,218	0,265	0,265	0,274	0,274	**0,25**
BC2 500 (B)mM	0,059	0,038	0,019	0,096	0,048	0,109	0,054	**0,04**

Table4: Protein concentrations of BC2 in *E.coli* for colony 1.

Concentration of BC2 protein from colony 2								
Sample	**OD 595nm**	**x = (y- 0,0394)/0,516**	**8/dilution factor**	**x = (y-0,0061)/0,5497**	**8/dilution factor**	**x = (y- 0,0023)/0,5639**	**8/dilution factor**	**Average Prot.Conc(μg/μL)**
BC2 50mM	0,097	0,112	0,056	0,165	0,083	0,176	0,088	**0,08**
BC2 100mM	0,739	1,356	0,678	1,333	0,667	1,315	0,657	**0,67**
BC2 150mM	0,285	0,476	1,904	0,507	2,029	0,509	2,038	**1,99**
BC2 200mM	0,349	0,600	2,400	0,624	2,495	0,623	2,492	**2,46**
BC2 250mM	0,208	0,327	1,307	0,367	1,469	0,373	1,492	**1,42**
BC2 500mM	0,297	0,499	0,499	0,529	0,529	0,531	0,531	**0,52**
BC2 500 (B)mM	0,141	0,197	0,098	0,245	0,123	0,254	0,127	**0,12**

Table5: Protein concentrations of BC2 in *E.coli* for colony 2.

As we did for BC1 protein, an estimation of the quantity of purified protein was made knowing that we initially had a volume of 300μL and 3μL of these was loaded in the gel (Fig.14). The same formula was used in all these cases (Equation (2)). Results are shown in the following table (Table6).

	Protein quantity (μg)	
Elution (mM)	Colony 1	Colony 2
50	1.8	12
100	72	161
150	262	507
200	479	665
250	189	426
500	75	156
500 (B)	36	36

Table6: Protein quantification of each elution for BC2

In the next table we can see the comparison between the theoretical result and the real one.

Protein quantification of colony 1					
Sample Imidazole concentration	Theoretical volume (mL)	Theoretical Protein quantity (µg)	Real volume (mL)	Real Protein quantity (µg)	Total (µg in 50 mL)
50 mM	0,3	1,8	0,284	1,70	
100mM	0,3	72	0,284	68,16	
150mM	0,3	262	0,276	241,11	
200mM	0,3	479	0,298	476,03	
250mM	0,3	189	0,298	187,74	
500mM	0,3	75	0,284	71	
500mM (2)	0,3	36	0,284	34,08	
					1079,82

Table7: Protein quantification of BC2 in *E.coli* for colony 1

Protein quantification of colony 2					
Sample Imidazole concentration	Theoretical volume (mL)	Theoretical Protein quantity (µg)	Real volume (mL)	Real Protein quantity (µg)	Total (µg in 50 mL)
50 mM	0,3	12	0,284	11,36	
100mM	0,3	161	0,284	152,22	
150mM	0,3	507	0,274	463,47	
200mM	0,3	665	0,298	659,77	
250mM	0,3	426	0,298	423,16	
500mM	0,3	156	0,284	147,68	
500mM (2)	0,3	36	0,284	34,08	
					1891,74

Table8: Protein quantification of BC2 in *E.coli* for colony 2

With these results, we can conclude that for 50mL of initial culture, we obtained 1079µg for the colony 1 and 1891µg for the colony 2, so we achieved more protein quantity of purified protein with the colony 2.

Once we had the BC1 and the BC2 protein purified by E.coli, we decided to clone and express BC2 also in yeast to compare how the expression levels and to know from which organism we could obtain the most protein.

Cloning and expression of BC2 gene in *Saccharomyces Cerevisiae*

Once observed that BC2 protein is soluble, we decided to check how this protein was purified from yeast, concretely, for Saccharomyces cerevisiae.

A PCR using the pET28a+BC2 (2) vector as a template was done to amplify the region of interest, which in this case was BC2 plus the His tag that the pET28a vector gives in the N-terminus.

The primers used had the sequence of SalI and BglII restriction enzymes that would be used in the digestion for the subsequent cloning (Fig.15). An agarose gel was run to confirm that the fragment was amplified (Fig.16).

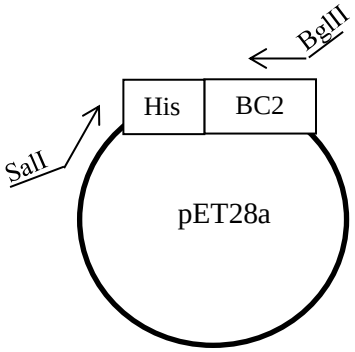


Fig.15. Scheme of the pET28a vector with the oligonucleotides aligned.

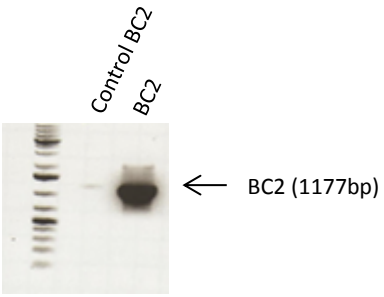


Fig.16. Agarose gel with the BC2 protein after amplification.

The fragment obtained from the PCR (His tag + BC2) and the pFA6-KanMX6 vector were digested with restriction enzymes SalI and BglII to create sticky ends in the DNA fragments and to open the expression vector for the subsequent cloning. The product of the digestion was purified by column and ran in an agarose gel to check if the digestion works well (Fig.17). pFA6 vector has a size of 3938bp and the insert (His tag+BC2) has a size of 1177bp.

The His tag + BC2 DNA fragment was ligated into the vector to give pFA6-His-BC2-Kan (Fig.18).

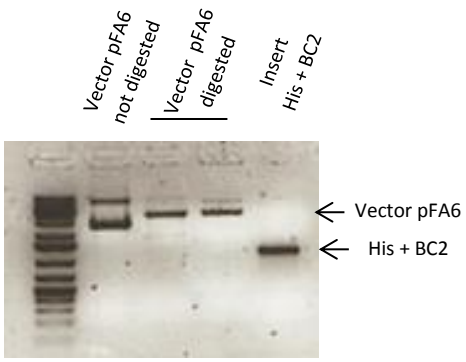


Fig.17. Samples of pFA6 vector were run in an agarose gel before (Lane1) and after (Lane 2 and 3) digestion. Insert of His+BC2 was also run.

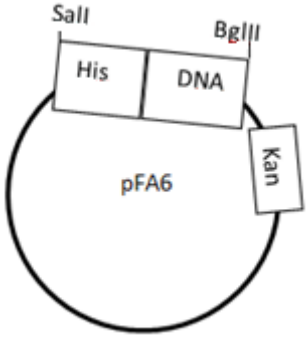


Fig.18. Scheme of the pFA6 vector after ligation. His: histidine, DNA: BC2 Restriction enzymes are shown.

We can see how the pFA6 vector was well digested because just one strong band appears in the gel in contrast with the not digested one.

The ligation mixture was transformed into DH5-α and plated on LB+Kan. Around 100 colonies grew after being overnight at 37ºC.

From these colonies, we selected 10 and a colony PCR was made to check if any of those colonies contained the vector plus the insert, which means that the size of the vector should be increased by around 1177bp. If the colonies didn't have the insert integrated, we expected to see a band of 200bp because this is the size between the two primer sites in the vector. The primers used for the PCR were SP6 as a forward and pFA6 control as a reverse.

The product of the colony PCR was run on an agarose gel to see the size of the bands and determine if the colonies contain the vector with the insert inside (Fig.19). As no band was clearly seen, another 8 colonies were selected and the same protocol was applied (results are shown together) (Fig.20).

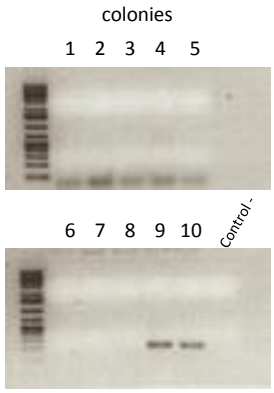


Fig.19. Products of PCR from 10 different colonies where run in the gel.

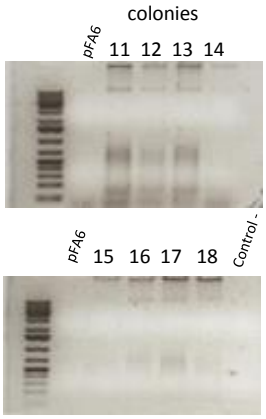


Fig.20. Products of PCR from 8 different colonies where run in the gel.

No band showed that the vector contained the insert, but in two colonies we could see the control band of 200bp which indicated that the colony was transformed with the vector.
However, we decided to make a second control growing a culture of the colonies, isolating plasmid DNA from these and measuring the concentration Nanodrop. The DNA was then digested with SalI and BglII because if the insert was present in the plasmid, two distinct bands should appear in the agarose gel (Table.9) (Fig 21; Fig 22).

Number of colony	Quantity of DNA (ng/µL)
1	263.1
2	160.9
3	242.5
4	137.5
5	218.1
6	175.4
7	193.1
8	214.7
9	176.1

Number of colony	Quantity of DNA (ng/µL)
10	189.5
11	227.7
12	218.5
13	227.7
14	210.2
15	229.2
16	171.9
17	223.1
18	219.8

Table 9. Concentration of Plasmid DNA obtained from different colonies.

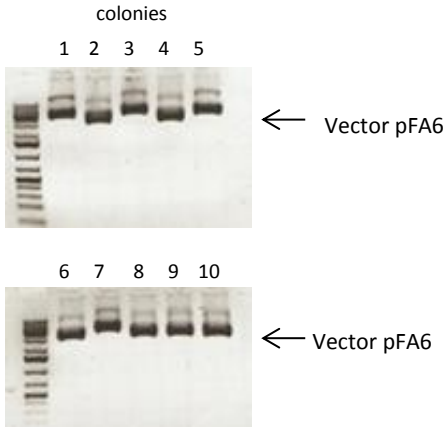


Fig.21. Products of digestion from the 10 colonies selected are run in the agarose gel.

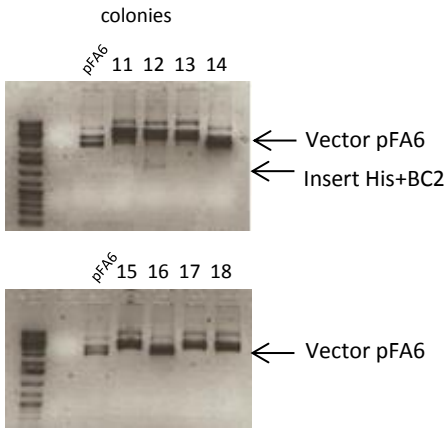


Fig.22. Products of digestion from the 8 colonies selected are run in the agarose gel.

In the first gel just one band was seen but the sizes vary between the colonies so we hypothesize that maybe the ones that are higher contain the insert and the problem was that the digestion didn't work well. Therefore, we decided to send the DNA from the first colony to be sequenced.

In the second gel the same pattern was shown but, in this case, it seems that in colonies 11 and 12 a second band appears at the size of insert (±1177) so, to confirm, we decided to digest again some samples but now using NdeI and NcoI as restriction enzymes (Fig.23). We chose these enzymes because they cut far from our insert. If no insert was present, we would see a band that confirmed that the digestion had gone well (Fig.24).

- In case that the vector does not contain the insert: a band of 540 bp plus a band of 3398bp.

- In case that the vector contain the insert: a band of 1717bp plus a band of 3938pb.

In the agarose gel we run the pFA6 vector not digested and digested to see in which size the band for the vectors without the insert appears. Three different colonies were run also based on the gel from the Fig 22. For the negative control, colony 14 was chosen because the band was lower, and colonies 11 and 12 were tested to confirm if the colonies with the higher band contains the insert

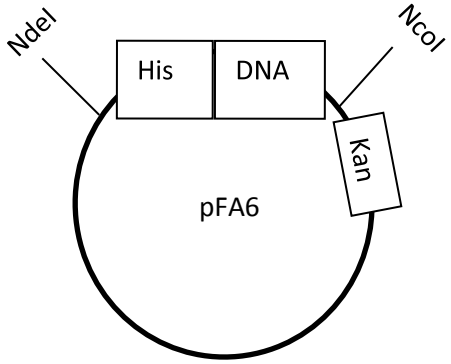


Fig.23. Scheme of the pFA6 vector with the insert on it. Restriction enzymes are shown.

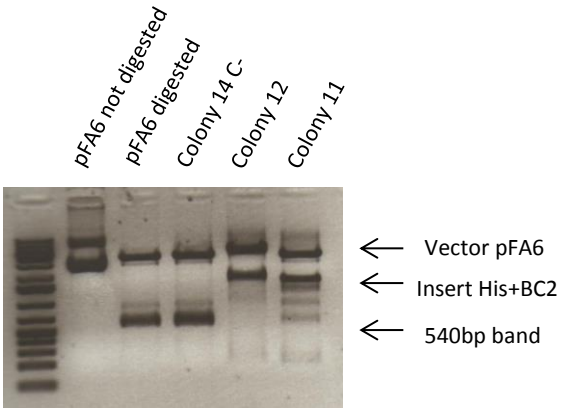


Fig.24. Products of the digestion were run in the agarose gel.

These results confirm that the colony 11 and 12 had the vector plus the insert, so we sent colony 12 together with colony 1 from the gel of Fig 21 because there were just 2 differentiated bands while in colony 11 it seems that some contaminants appear. Both colonies were sequenced using SP6 as a forward primer, and the pFA6 control as a reverse primer.

The sequences obtained from CRAG were compared with the reference sequence of BC2 by alignment using the ApE program. (Images are not shown by confidential motifs)
We can see how in some of the alignments there are "red points" that indicate a difference from the BC2 reference sequence, but in all the cases, the differences shown in the alignment of the forward or reverse is counteracted by the other alignment. As the red points just appear close to the primer they are sequencing inaccuracies, so we assume that no mutations occurred.

His-BC2 DNA cassettes for yeast transformation

As there were no mutations in the sequences, we used these plasmids for the purification of BC2 from yeast. First of all, a sample of wild type strain BY4741 was taken from our glycerol stock and scratched on YPD plate.
For the purification process, we decided to prepare two different constructs that aligns with two different promoters for the His-BC2 and check which one works better in the protein

expression. Both strategies used newly designed oligos: one for expression with the promoter of our protein (BC2) and the other with the promoter of Enolase (ENO2) (Fig.25).

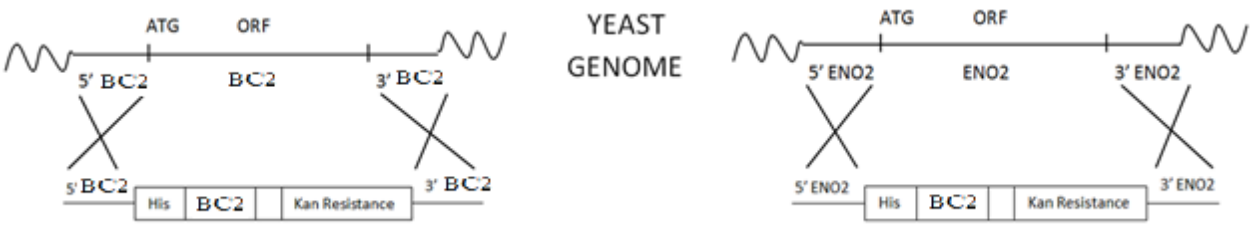


Fig.25. Scheme of the two constructs prepared and the recombination process.

An amplification of the His tag + BC2 + Kan was made by PCR from the colony 1 already sequenced using oligos specially designed that align with the BC2 promoter or with the ENO2 promoter (Fig 26; Fig 27).

pFA6 with promoter of BC2

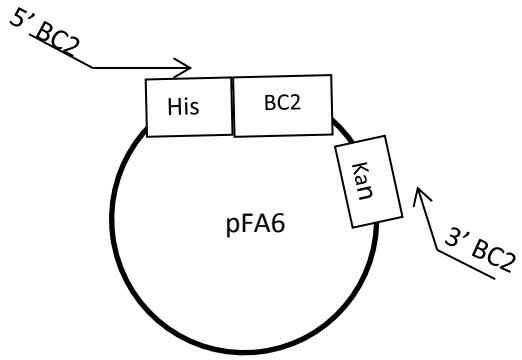


Fig.26. Scheme of the construct with the oligos containing the BC2 promoter aligned.

pFA6 with promoter of ENO2

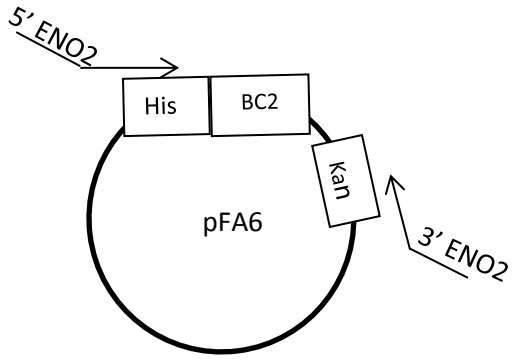


Fig.27. Scheme of the construct with the oligos containing the ENO2 promoter aligned.

The PCR product was purified by column and run on an agarose gel to make sure that the amplification worked well. The band expected was around 2459pb (Fig. 28).

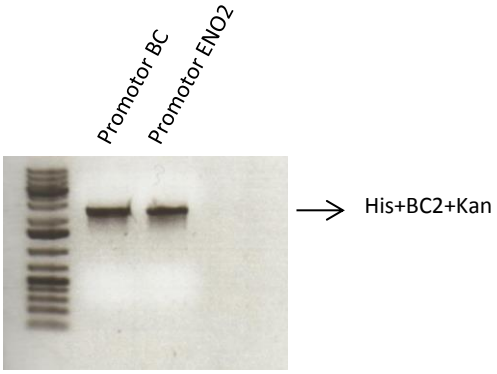


Fig.28. Product purified of the PCR from the two constructs was run on the agarose gel.

With the purified product, a yeast transformation was done with the culture prepared from the WT strain. The objective was to make a recombinant, changing the BC2 normal gene from the genome by the construct that we prepared that contains: His tag+BC2+Kan with the different promoters. The His-tag was used to purify the protein and the Kanamycin was useful for the selection of the colonies on YPD-G418 medium plates.

Checking the recombination

The BY4741 cells that were transformed and grown on the plates produced colonies that contain the Kanamycin cassette, but to check if either contains the His-tagged protein, a western blot was made with some of the colonies (Fig.29). For the ones that contain the ENO2 promoter, 8 colonies were taken, and from the BC2 promoter were taken 18 colonies.

Pre-cultures were made of the colonies, the OD was measured and the corresponding volume of the culture was taken to have the same amount of cells in each case. To check if the His tagged protein was inserted in the genome, a Western Blot with anti-His was made after breaking the cells in a mechanical way by series of vortex/boil. Protein fractions were analysed by SDS-PAGE using a 10% gel. Monoclonal anti-PolyHistidine, antibody produced in mouse was used as primary antibody and HRP-conjugated anti-mouse antibody was used as secondary. After the incubation with the two antibodies and the corresponding washes, the PVDF membrane was incubated in Luminata Classico Western HRP substrate, a chemiluminescence detection reagent.

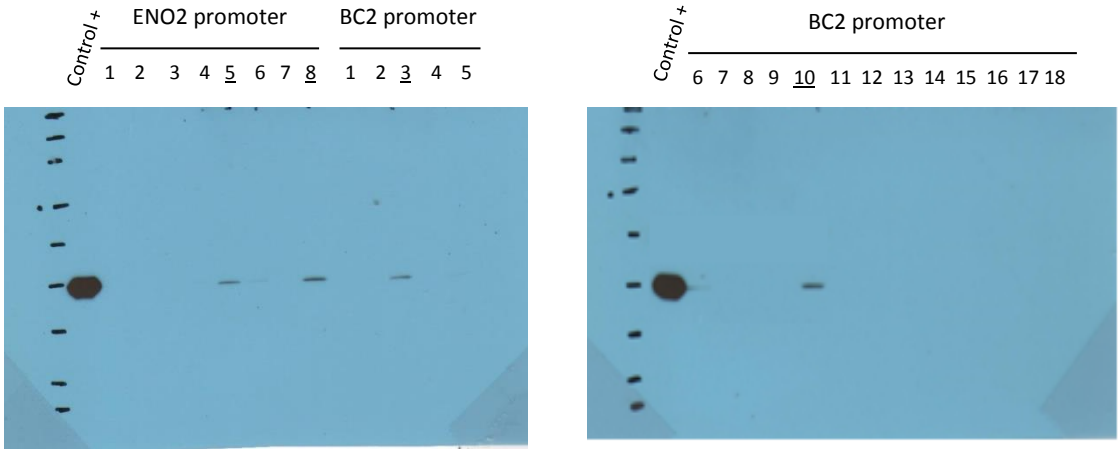


Fig.29. Cell extract from different colonies was analysed by SDS-PAGE analysis, following the histidine by immunoblotting against His.

On the film we can see how colony 5 and 8 from the ENO2 promoter and colony 3 and 10 from BC2 promoter contain the His-tagged BC2 in the genome. Colonies selected for the purification of BC2 were the number 8 from ENO2 promoter strain and the number 10 from BC2 promoter strain.

Purification of BC2 from *S. cerevisiae*

Two cultures of 1L YPD medium were prepared for growth of the yeast strains, one for the ENO2 promoter and the other for the BC2 promoter. After growing the cultures for two days, these were centrifuged and the cell pellet was weighted.

Pellet weight from BC2 with BC2 promoter → 15.65g
Pellet weight from BC2 with ENO2 promoter → 13.5g

The purification process used was the same as described for *E.coli* on "Purification from E.coli" section but with the difference that for breaking the cells, a cell disrupter was used. Total volume of the protein extract was 50mL. In the last step of the purification, elution was made and the samples at different imidazole concentrations were collected in a total volume of 200µL of elution buffer.

The OD of these samples was taken to see how much protein we had and to check if the elution process was working well. To continue, a sample of the supernatant was run on SDS-PAGE gel to observe how many proteins we had in our samples and if the BC2 protein was also present (Fig.30). BC2 has a size of 37 KDa, so we expected to see a bigger band in this zone because this would indicate that our protein is the majority and the elution step worked.

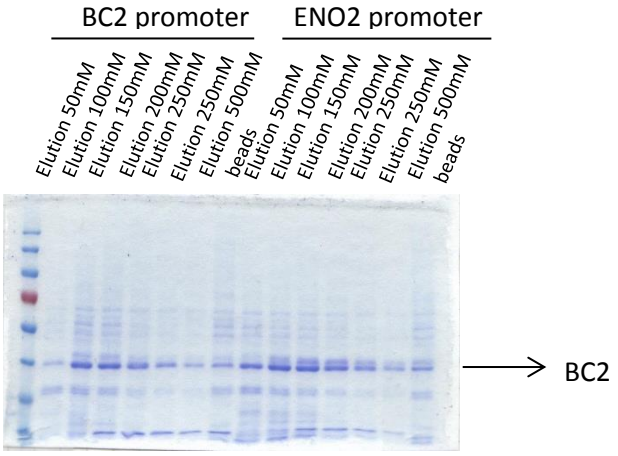


Fig.30. Determination of the eluted protein by SDS-PAGE. Lane1: Molecular standard marker. Lane 2-7: Samples of the elution at different concentrations of imidazole for BC2 promoter. Lane 9-14: Samples of the elution at different concentrations of imidazole for ENO2 promoter. Lane 8 and 15: Sample of the beads after elution.

In this gel we can see how we had the protein of interest in the sample. The maximum protein eluted was when the concentration of imidazole was at 100-200mM in both cases.
We can also see other bands that correspond to other proteins that were eluted together with our sample, and are also in the latest steps of the elution, so we can think that maybe are other proteins presents that have affinity with the beads and the purity of the sample was not as good as in the E.coli.

The 100mM elution samples were used to make a western blot with anti-his with the intention to check if the His tag was still remaining in the genome.

The same antibodies that were used for the previous western blot were used now, but membrane was incubated with the Amersham ECL Prime Western Blotting Detection Reagent (Fig.31).

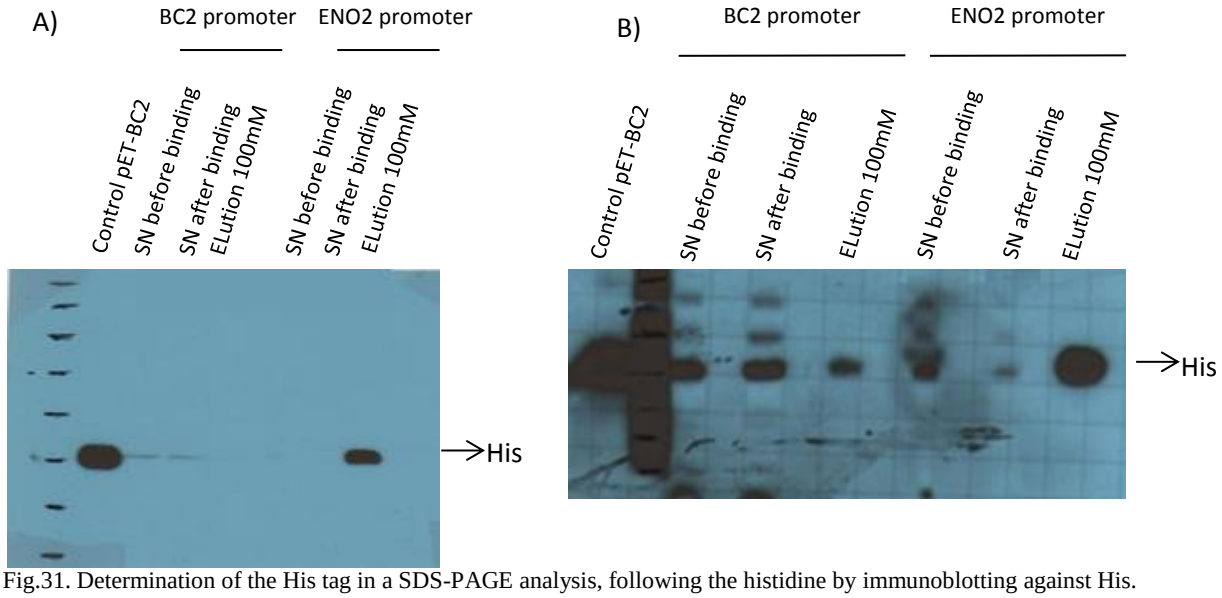


Fig.31. Determination of the His tag in a SDS-PAGE analysis, following the histidine by immunoblotting against His. SDS-PAGE gel was made at 10% of acrylamide. A) Time of exposure 5min. B) Time of exposure ON.

The Histidine control (control pET-BC2) was obtained from the elution sample from the colony 2 of *E.coli* purification at 100mM of imidazole. In the gel are run the supernatants before and after binding to check if the protein was well attached to the beads. Finally, an elution sample at 100mM obtained from this purification was added.

We cans see how in the long exposure film, a band appears in all the lanes. However, no band or very little band was expected in the SN after binding, and in the BC2 promoter region we can see how this band is equal to the supernatant before binding meaning that probably the amount of beads was not sufficient to remove all protein. Most importantly, we can see a band in the elution lane, so it means that we have purified protein.

We have obtained more protein from the ENO2 promoter strain than for the BC2 strain.

Protein quantification and determination of molecular mass

As we mentioned previously, colonies 8 and 10 were used to purify BC2 protein containing the ENO2 promoter and the BC2 promoter respectively.

Using the same method as previously was described for *E.coli*; we obtained the protein concentration for BC2 in *Saccharomyces cerevisiae* (Equation (1)) (Table 10 and 11).

Concentration of BC2 protein from colony 8 (ENO2 promoter)								
Sample	**OD 595nm**	**x = (y- 0,0394)/0,516**	**8/dilution factor**	**x = (y-0,0061)/0,5497**	**8/dilution factor**	**x = (y- 0,0023)/0,5639**	**8/dilution factor**	**Average Prot.Conc(µg/µL)**
PromoENO2 50mM	0,257	0,422	0,422	0,456	0,456	0,460	0,460	**0,45**
PromoENO2 100mM	0,295	0,495	0,495	0,526	0,526	0,527	0,527	**0,52**
PromoENO2 150mM	0,279	0,464	0,464	0,496	0,496	0,499	0,499	**0,49**
PromoENO2 200mM	0,345	0,592	0,296	0,617	0,308	0,616	0,308	**0,30**
PromoENO2 250mM	0,185	0,282	0,141	0,325	0,163	0,332	0,166	**0,16**
PromoENO2 500mM	0,108	0,133	0,066	0,185	0,093	0,196	0,098	**0,09**
PromoENO2 500 (B)mM	0,059	0,038	0,019	0,096	0,048	0,109	0,054	**0,04**

Table10: Protein concentrations of BC2 in *S. cerevisiae* for colony 8.

Concentration of BC2 protein from colony 10 (BC2 promoter)								
Sample	**OD 595nm**	**x = (y- 0,0394)/0,516**	**8/dilution factor**	**x = (y-0,0061)/0,5497**	**8/dilution factor**	**x = (y- 0,0023)/0,5639**	**8/dilution factor**	**Average Prot.Conc(µg/µL)**
PromoBC2 50mM	0,124	0,164	0,164	0,214	0,214	0,224	0,224	**0,20**
PromoBC2 100mM	0,279	0,464	0,464	0,496	0,496	0,499	0,499	**0,49**
PromoBC2 150mM	0,231	0,371	0,371	0,409	0,409	0,414	0,414	**0,40**
PromoBC2 200mM	0,339	0,581	0,290	0,606	0,303	0,605	0,303	**0,30**
PromoBC2 250mM	0,173	0,259	0,129	0,304	0,152	0,311	0,155	**0,15**
PromoBC2 500mM	0,126	0,168	0,084	0,218	0,109	0,228	0,114	**0,10**
PromoBC2 500 (B)mM	0,077	0,073	0,036	0,129	0,064	0,141	0,070	**0,06**

Table11: Protein concentrations of BC2 in *S. cerevisiae* for colony 10.

An estimation of the quantity of purified protein was made knowing that we initially had a volume of 200µL and 3µL of these was loaded in the gel (Fig.30) (Table 12).

The same formula was used in all these cases (Equation (2)).

	Colony 8	Colony 10
Elution (mM)	Protein quantity (µg)	
50	45	12
100	73	49
150	59	48
200	45	42
250	26	24
500	16	18
500 (B)	8	12

Table12: Protein quantification of each elution for BC2

In the next table we can see the comparison between the theoretical result and the real one (Table 13 and 14).

Protein quantification of colony 8 (ENO2 promoter)					
Sample Imidazole concentration	Theoretical volume (mL)	Theoretical Protein quantity (µg)	Real volume (mL)	Real Protein quantity (µg)	Total (µg in 50 mL)
50 mM	0,2	45	0,192	43,20	
100mM	0,2	73	0,192	69,89	
150mM	0,2	59	0,192	56,45	
200mM	0,2	45	0,184	41,40	
250mM	0,2	26	0,184	23,55	
500mM	0,2	16	0,184	14,90	
500mM (2)	0,2	8	0,184	7,36	
					256,75

Table13: Protein quantification of BC2 in *S. cerevisiae* for colony 8

Protein quantification of colony 10 (BC2 promoter)					
Sample Imidazole concentration	Theoretical volume (mL)	Theoretical Protein quantity (µg)	Real volume (mL)	Real Protein quantity (µg)	Total (µg in 50 mL)
50 mM	0,2	12	0,192	11,52	
100mM	0,2	49	0,192	47,04	
150mM	0,2	48	0,192	46,08	
200mM	0,2	42	0,184	38,64	
250mM	0,2	24	0,184	22,08	
500mM	0,2	18	0,184	16,56	
500mM (2)	0,2	12	0,184	11,04	
					192,96

Table14: Protein quantification of BC2 in *S. cerevisiae* for colony 10

With these results, we can conclude that for 50mL of initial culture, we obtained 257µg of BC2 protein for the strain with ENO2 promoter and 193µg for the strain with BC2 promoter, so we obtained more purified protein using the ENO2 promoter.
These two new strains were called: S441 for the BC2 promoter and S442 for the ENO2 promoter.

Spectrophotometric determination of enzyme activity

After the isolation and purification of the protein, an assay to determine the amount and purity of the enzyme and to assess its kinetic characteristics was performed. The measure of the conversion of substrate to product in a short period of time was done by spectrophotometry.

The activity assay is based on the detection of the absorbance difference between NADH and NAD^+. NADH absorbs at 340nm, while NAD^+ doesn't absorb at this wavelength. The enzyme activity in the samples will be detected as an increase in the absorbance at 340nm, due to the reaction in which NAD^+ is converted to its reduced form, NADH.

The BC2 specific activity was determined by a spectrophotometer assay at 340nm during 1 min. Substrate used was at1M concentration, Tris pH 7.4 50mM, EDTA 1mM was used as a buffer and Nicotinamida Adenine dinucleotide (NAD^+) was used as a cofactor at 50mM. The protein tested was the BC2 purified from E.coli and the one obtained from yeast with ENO2 promoter.

1.25mM NAD+
25mM substrate
X µg enzyme (see table 17- µg protein)
XµL buffer up to 800µL

Absorbance measurements were taken for different elutions obtained from the purifications described before (Equation (3). The measure of the concentration was calculated taking into account the difference of the absorbance in this time lapse (Table 15).

Δ Absorbance = Abs_f - Abs_i
$C = \Delta Abs/e*l$ (M^{-1}); (e= $6{,}22 \cdot 10^3 cm^{-1} M^{-1}$, l=$1cm^{-1}$)

Equation(3). Formula of the increment of absorbance (up) and Lambert-Beer formula (down)

	E. coli		*S. cerevisiae*	
Elution sample	Δ Absorbance	c= ΔAbs/(e*L) (M^{-1})	Δ Absorbance	c= ΔAbs/(e*L) (M^{-1})
50	0,2081	3,35E-05	0,2467	3,97E-05
100	0,1942	3,12E-05	0,2418	3,89E-05
150	0,2072	3,33E-05	0,2546	4,09E-05
200	0,2444	3,93E-05	0,2312	3,72E-05
250	0,101	1,62E-05	0,1934	3,11E-05
500	0,011	1,77E-06	0,0935	1,50E-05
500 (B)	0,011	1,77E-06	0,039	6,27E-06

Table 15. Measure of the concentration of the protein by the increasing of absorbance.

Specific activity of an enzymatic preparation is defined as the number of micromoles of formed product per minute per microgram of protein or per microgram of enzyme (Equation (4)).

Specific Activity = (µMols NADH/min)/µg enzyme

Equation(4). Specific activity formula

This result will tell us how efficient is our protein, in other words, how much substrate can transform to product in a short period of time.

We decided to calculate the activity in reference to seconds, hours and days to understand the results in a clearly way (Table 17).

Specific Activity of enzyme					
E. coli					
Sample	c= Δabs/(e*L) (M^{-1})	µg protein	Specific Act. (µMols/s)/µg)	Specific Act. (µMols/h)/µg	Specific Act. (µMols/d)/µg
50	3,35E-05	0,4	1,39	5018,49	120443,73
100	3,12E-05	3,35	0,16	559,20	13420,74
150	3,33E-05	9,95	0,06	200,88	4821,02
200	3,93E-05	12,3	0,05	191,67	4600,11
250	1,62E-05	7,1	0,04	137,22	3293,33
500	1,77E-06	2,6	0,01	40,81	979,47
500 (B)	1,77E-06	0,6	0,05	176,85	4244,37
S.Cerevisiae					
Sample	c= Δabs/(e*L) (M^{-1})	µg protein	Specific Act. (µMols/s)/µg	Specific Act. (µMols/h)/µg	Specific Act. (µMols/d)/µg
50	3,89E-05	2,25	0,29	1057,66	25383,92
100	4,09E-05	2,6	0,25	897,11	21530,55
150	3,72E-05	2,45	0,28	1002,43	24058,27
200	3,11E-05	1,5	0,41	1486,82	35683,60
250	1,50E-05	0,8	0,65	2331,99	55967,85
500	6,27E-06	0,45	0,56	2004,29	48102,89
500 (B)	3,97E-05	0,2	0,52	1881,03	45144,69

Table 17. Measure of the specific activity of the protein.

In this table we can see how *E.coli* and *S. cerevisiae* had obtained more or less the same concentration of protein in total, but *S. cerevisiae* is more efficient than *E.coli* because a higher specific activity was obtained having less quantity of purified protein, which means that the protein is more efficient when is grown in this model organism.

6- Discussion

In this project, the main goal was to find the best purification method to obtain pure protein with a high yield that can be used later in some biotechnology applications.

To achieve this goal, we first purified BC1 protein from *E.coli* to test the purification conditions for the selected method because the plasmid was already available in the laboratory. As an expression system, we used pET28a plasmid in which an N-terminal 6*His fusion protein of BC1 was generated that can be induced by IPTG so that the protein can be purified with Ni-beads.
From the results obtained, we can say that *E.coli* is a good host and it allows the expression of the protein of interest to obtain a high amount of it. Moreover, the purified product was very pure, so a His-tag will be a good option to use for the BC2 protein.

Nevertheless, the optimal purification system is different for every protein, that's why we also wanted to test how the GST purification will work. Sometimes GST tag works better but is much bigger than His-tag and is sometimes needed to get a soluble protein. However, in that case, the GST tag needs to be cleaved off subsequently. This is why we originally designed an expression vector containing the His-tag, but another one with the GST tag. Unfortunately, cloning with pGEX-4T-3 vector which contains the GST tag did not work, so we decided to continue just with the His-tagged protein that was expressed by the pET28a vector.
We studied the expression from two different colonies and the results obtained were more than the expected ones, because we obtained up to 20 times more purified protein than for BC1 and with a high purity.

At this point, we decided to express this protein also in yeast because it is already expressed in its genome, so we used *S. cerevisiae* as a host organism. In addition, we wanted to induce its expression by two different promoters, so we designed two mechanism of expression; one using the promoter belonging to our protein, and the other was from the enolase gen (ENO2). We chose ENO2 because it is one of the most studied and used promoters in yeast and its expression can be regulated by non-fermentable carbon sources or induced by glucose, that's why we hypothesize that with this one we could obtain more protein. The purification was done, as in *E.coli*, using the His-tag method.

Comparing the expression of the protein with the two promoters used, we could see how, as we expected, the ENO2 promoter works more efficiently than the BC2 promoter and that's why we have obtained more purified protein from the strain expressed by ENO2 promoter. However, the total quantity of the protein collected by *S. cerevisiae* was lower than that obtained from a similar amount of culture volume by *E.coli*.

Finally, an enzymatic assay was done comparing the BC2 product of the *E.coli* with the *S. cerevisiae* expressed from the ENO2 promoter. For the last one we observed higher activity, which means that a particular amount of BC2 protein purified from yeast converts more substrate into product in the same amount of time than a similar amount of BC2 purified from E.coli as we can see in the activity table, where from sample 500(B), for example, more quantity of protein was added from E.coli sample but more activity was obtained from S. cerevisiae.

To summarize, if we compare the two host organisms used in this experiment, we can see how *E. coli* is a better system for producing a lot of protein than *S. cerevisiae* because it grows faster and produce protein more quickly. However, the activity of BC2 obtained from yeast was higher than the one obtained from bacteria.
In our case, we will continue using yeast for the expression of BC2 protein, using ENO2 promoter for our production experiments but testing other conditions as well such as test with different glucose amounts in the medium.

7- Conclusions

After studying the results obtained in this project, we can say that the best expression system to obtain a purified protein is *Escherichia coli* because you can obtain the same product multiplied per 6.
If we focus on yeast, the best expression mechanism is use the glucose induction by ENO2 promoter because is a very stronger promoter.

This project is a part of a biggest project in which we are working in, and it helped us to develop the best method for a stable overexpression of a protein in yeast, and we will use it later for new purifications with other proteins. Looking forward, we also want to develop another method to compare it with these ones using more glucose in the induction of the protein expression in yeast with ENO2 promoter.

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