



**Bárbara Martins Paiva da Cunha**

Degree in Biochemistry

**Expression of the human carboxylesterase 2 enzyme  
(CES2) in mammalian cells**

Dissertation to obtain a Master Degree in Biotechnology

Supervisor: Ana Luísa Simplício, Ph.D, IBET/ITQB-UNL

Co-Supervisor: Ana Sofia Coroadinha, Ph.D, IBET/ITQB-UNL

Júri:

Presidente: Prof. Doutor Rui Manuel Freitas de Oliveira

Arguente: Prof<sup>ª</sup>. Doutora Alexandra Fernandes

Vogal: Doutora Ana Luísa Ferreira Simplício

**September 2011**



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CIÊNCIAS E TECNOLOGIA  
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"Expression of the human carboxylesterase 2 enzyme (CES2) in mammalian cells".

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## Abstract

The carboxylesterase 2 enzyme (CES2), the main carboxylesterase (CES) expressed in human intestine, has a key role in the metabolism of ester containing xenobiotics including the activation of several prodrugs and therefore, it can influence significantly the bioavailability of these compounds. CES2 is receiving an increasing attention due to its potential application in anti-cancer combined therapies, for the treatment of different pathologies like colon adenocarcinoma and malignant glioma.

Unlike carboxylesterase 1 (CES1), the structure of this enzyme is not yet known. Since the knowledge of the structure of this protein will be crucial for the understanding of its properties, it becomes relevant the establishment of a process for the production and purification at the milligram level.

The development and optimization of human recombinant CES2 production process, using human embryonic kidney cells (HEK-293T cells), is described in this thesis. First, the C-terminal 10xHistidine tag CES2 (CES2-10xHis) was produced using adherent cultures, and then the process was transferred to suspension cultures with serum-free media and finally scaled up to a five-litre stirred tank bioreactor. An affinity chromatography purification process was applied to obtain the CES2-10xHis protein, with a high purity grade.

Despite being an Endoplasmic Reticulum (ER) anchored protein, secretion of CES2 to serum free media was achieved due to the presence of the in frame C-terminal 10xHistidine tag. With this elegant procedure we avoided the addition of extra N-terminal signaling sequences or the mutation or deletion of the C-terminal Histidine - Threonine - Glutamic acid - Leucine (HTEL) motif responsible for anchoring the protein in the lumen of the ER.

Secretion contributed to a good production yield; however, the global process yield was quite low, due to difficulties in the purification step, which we attributed to protein aggregation.

**Keywords:** carboxylesterase 2, manufacturing, secretion, mammalian cells, scale-up



## Resumo

O enzima carboxilesterase 2 (CES2), o carboxilesterase (CES) maioritário no intestino humano, tem um papel fundamental no metabolismo de xenobióticos que contenham grupos éster e também na activação de diversos pró-fármacos, podendo influenciar significativamente a sua biodisponibilidade. CES2 tem vindo a receber uma atenção constante e crescente devido ao seu potencial papel em terapias combinadas anti-cancerígenas para o tratamento de diferentes patologias, tal como adenocarcinoma do cólon ou ainda glioma maligno.

A estrutura deste enzima ainda não é conhecida, ao contrário da do carboxilesterase 1 (CES1). O conhecimento da sua estrutura poderá ser crucial para compreender as suas propriedades, pelo que se torna relevante o estabelecimento de um processo para a sua produção e purificação (ao nível de miligrama).

Nesta tese de mestrado, é descrito o desenvolvimento e optimização do processo de produção de CES2 recombinante humano, utilizando células derivadas de rim humano (Human Embryonic Kidney cells, HEK-293T cells). Numa primeira fase, a proteína com um tag de dez histidinas a C-terminal (CES2-10xHis) foi produzida em cultura aderente, tendo posteriormente o processo sido transferido para culturas em suspensão com meio sem soro, finalizando com o aumento de escala para 5 L em bioreactor de tanque agitado. Foi aplicado um processo de purificação com cromatografia de afinidade para obter a proteína CES2-10xHis, com um elevado grau de pureza.

Embora esta proteína esteja ancorada ao retículo endoplasmático (ER), a sua secreção para meio sem soro foi alcançada através da presença do *tag in frame* de dez histidinas a C-terminal. Com este elegante procedimento, evitou-se a adição de sequências extra de sinalização a N-terminal ou mutar ou delectar o motivo HTEL a C-terminal, responsável pela ancoragem da proteína ao lúmen do ER.

A secreção contribuiu para um bom rendimento na produção, contudo, o rendimento do processo global foi baixo, devido a dificuldades no passo de purificação, que foi atribuído a agregação da proteína.

**Palavras chave:** carboxilesterase 2, produção, secreção, células de mamífero, *scale-up*



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## Abbreviations

- 10xHis** - Ten Histidine Tag
- 4-MUB** - 4-methylumbelliferone
- 4-MUBA** - 4-methylumbelliferyl acetate
- Abs 260nm** - UV absorbance read at 260 nm wavelength
- Abs 280nm** - UV absorbance read at 280 nm wavelength
- AMP** - Adenosine monophosphate
- Amp** - Ampicillin
- Anti-CES2** - Antibody raised to recognize a CES2 epitope
- Anti-His** - Antibody raised to recognize Histidine tagged proteins
- Anti-mouse** - Antibody raised to recognize a primary antibody produced in mice
- Anti-rabbit** - Antibody raised to recognize a primary antibody produced in rabbit
- ATCC** - American Type Culture Collection
- ATP** - Adenosine Triphosphate
- BCA** - Bicinchoninic acid
- BHK** - Baby hamster kidney cells
- bp** - Base Pairs
- BSA** - Bovine Serum Albumin
- BuChE** - Butyrylcholinesterase
- CaPi** - Calcium Phosphate
- CES** - Carboxylesterases
- CES1** - Carboxylesterase 1
- CES2** - Carboxylesterase 2
- CES2-10xHis** - Carboxylesterase 2 with a C-terminal ten-histidine tag
- CES3** - Carboxylesterase 3
- CES5** - Carboxylesterase 5 (cauxin)
- CES6** - Carboxylesterase 6
- CHO** - Cells from Chinese hamster ovary
- CMV** - Cytomegalovirus
- COS-7** - Kidney fibroblast cell line from African Green Monkey
- CPT-11** - Irinotecan or Camptothecin-11
- DMEM** - Dulbecco's Modified Eagle's Medium
- DMSO** - Dimethyl sulfoxide
- DNA** - Deoxyribonucleic acid
- DSMZ** - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
- ECACC** - European Collection of Animal Cell Cultures
- ECL** - Enhanced chemiluminescence
- ECL-HRP (HRP)** - Horseradish peroxidase conjugated antibodies to allow ECL detection

***E. coli*** - *Escherichia coli*

**EDTA** - Ethylenediaminetetraacetic acid

**ER** - Endoplasmic reticulum

**Erl** - Erlenmeyer

**FBS** - Fetal Bovine Serum

**FW** - Forward

**Glu** - Glutamic acid

**HEK-293 (T)** - Human embryonic kidney cells

**HeLa** - Human cervical carcinoma cells

**HIEL** - Histidine - Isoleucine - Glutamic acid - Leucine aminoacid sequence

**His** - Histidine

**HTEL** - Histidine - Threonine - Glutamic acid - Leucine aminoacid sequence

**HTIEL** - Histidine - Threonine - Isoleucine - Glutamic acid - Leucine aminoacid sequence

**IMAC** - Immobilized metal-affinity chromatography

**KDEL** - Lysine - Aspartic acid - Glutamic acid - Leucine aminoacid sequence

**LB** - *Luria Bertani*

**MRC-5** - Human fetal lung fibroblast cells

**mRNA** - Messenger Ribonucleic acid

**NAG** - N-acetylglucosamines

**NBT/BCIP** - The combination of NBT (nitro-blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt)

**NSO** - Murine myeloma cells

**OD** - Optic density

**PBS** - Phosphate Buffer Saline

**pCI-neo-CES2** - pCI-neo plasmid with the *CES2* gene

**pCI-neo-CES2-10xHis** - pCI-neo plasmid with the *CES2-10xHis* gene

**PEI** - polyethylenimine

**PER.C61** - Cells derived from a single human cell

**p-NPA** - p-nitrophenyl acetate

**PVDF** - Polyvinylidene Fluoride

**QEDL** - Glutamine - Glutamic acid - Aspartic acid - Leucine aminoacid sequence

**RT** - Room temperature

**RV** - Reverse

**SDS-PAGE** - Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

**Ser** - Serine

**Sf21** - Insect cell line from *Spodoptera frugiperda*

**Sf9** - Insect cell line from *Spodoptera frugiperda*

**SIA** - Sialic Acids

**SN-38** - 7-Ethyl-10-Hydroxycamptothecin

**SP2/0** - Murine myeloma spleen cells

**SV40** - Simian virus 40

**T-25** - T-flask with 25 cm<sup>2</sup>

**TAE** - Tris-acetate-EDTA

**TB** - *Terrific Broth*

**TCA** - tricarboxylic acid cycle

**tPA** - Human tissue plasminogen activator

**TTBS** - Tris buffered saline with 0.05% Tween20

**UV** - Ultraviolet radiation

**vvm** - Gas volume flow per media volume per minute

**Wi-38** - Human fetal lung fibroblast cells



## 1. Introduction

### 1.1 - Animal Cell culture

Animal cell culture appeared more than a hundred years ago, but it was the initial need for human viral vaccines in the 1950s that accelerated the design of large-scale bioprocesses with mammalian cells (1). The enhanced interest in mammalian cell culture bioprocesses has also been associated with recombinant protein development in the 1970s and 1980s. In 1986, human tissue plasminogen activator (tPA, Activase; Genentech) (2) became the first therapeutic protein produced from mammalian cells (CHO cells) to obtain market approval. Today, 60-70% of all therapeutical recombinant proteins are produced in mammalian cells (3).

Animal cell cultures require a high level of laboratory material and continuous maintenance, which may lead to morphological and functional changes in cellular growth and in their karyotype (4). Authenticated stocks of continuous cell lines (more homogenous, more stable and more reproducible) can be acquired from a recognized animal cell culture repositories, such as the American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Germany, the Riken Gene Bank in Japan, or the European Collection of Animal Cell Cultures (ECACC) in the United Kingdom (5). The cells or tissues may be freshly isolated from animal or human donors (primary cells) or may comprise a laboratory-adapted strain or line that has been serially propagated and maintained in continuous culture (cell line) (5).

Depending on their applications, animal cells can also be grouped as (6):

- (i) Cell producing proteins employed in the production of complex therapeutics, subunit vaccines, and diagnostic products, such as CHO, BHK, HEK-293, WI-38, MRC-5, SP2/0, NS0, and insect cells (7);
- (ii) Cells producing viruses used in gene therapy and viral gene vaccines (for instance, Vero, HEK-293, and PER.C61 cells);
- (iii) Normal cells, tumor cells, and stem cells used in research and development, specifically in the discovery of new products and for *in vitro* study and toxicology models (e.g. nerve cells, fibroblasts, Caco-2, MRC-5, and endothelial cells);
- (iv) Human cells for subsequent use in cell therapy and regenerative medicine (e.g. embryonic and adult stem cells);

The animal cell culture has become an indispensable technology in many science areas. It allows the study of inaccessible phenomena in intact cellular tissues, as well as to obtain cells with good homogeneity and characterization and economy of reagents, time and animal sacrifice due to, for instance, control of the chemical (pH, temperature, oxygen concentration) and physiological conditions. Mammalian cells have several advantages for production of recombinant proteins, due to their capability to elaborate post-translational modifications (glycosylation) and therefore a higher probability of obtaining biologically active and fully functional human proteins (4).

Animal cell culture techniques are similar to those employed for bacteria, fungi, and yeast, although there are some differences. In general, animal cells are more vulnerable to mechanical damage and present lower growth rates, in some cases lower productivities and require more complex culture media and special substrates (8). Also, cell culture has to be performed under rigorous aseptic conditions, since animal cells grow more slowly than most usual microorganisms, such as bacteria and fungi. *In vitro*, animal cell growth is dependent on several factors, such as pH, temperature, osmolality and gas concentration, mainly oxygen (O<sub>2</sub>) and carbon dioxide (CO<sub>2</sub>) (9, 10). Culture medium needs to be buffered to compensate for CO<sub>2</sub> and lactic acid derived from glucose metabolism.

In animal cells, glucose, a monosaccharide sugar that functions as an important energy source for metabolism, may be stored (as a polysaccharide such as glycogen or starch), oxidized to pyruvate via glycolysis to provide ATP and metabolic intermediates, or oxidized via the pentose phosphate pathway with the oxidation of NADH and FADH<sub>2</sub> from tricarboxylic acid cycle (TCA), being the last the major metabolic pathway involved in energy production (11).

Cells in culture have a modified metabolism when compared with the same cell in the organism of origin, showing a high glycolytic flux and being unable to completely oxidize glucose, generating high amounts of lactate as an end product, leading to a rise of osmolality and decay of the pH, which combined lead to cellular growth inhibition, even under fully aerobic conditions (11). These high consumption levels of glucose and also in glutamine (major source of energy, carbon, and nitrogen for mammalian cells), due to their inefficient utilization and consequent production of toxic metabolites such as lactate and ammonia, are a clear evidence of the unregulated metabolism. These toxic metabolites interfere in the cellular growth and in the expression of recombinant proteins (11-13). As the productivity of mammalian cell lines is some times lower compared with that of other production systems (bacteria, insect cells, etc.), it is desirable to improve the productivity per cell or the total cell yield per unit volume of culture. The knowledge of mammalian cell metabolism can help in the development of strategies for productivity enhancement, as changes in the culture conditions can affect both metabolism and productivity (12).

To help monitor pH variation (cell culture usually from pH values between 7.0 and 7.4) some commercial media formulations have incorporated a pH indicator, such as phenol red, that enables immediate visual inspection of media pH – phenol red is rose colored at more basic pH values (7.8) becoming red, orange and yellow with the increase of the acidic conditions (7.4, 7.0 and 6.5 respectively) (14). For the control of the pH in *in vitro* culture systems, sodium bicarbonate is usually used in media formulation, to maintain physiological pH.

Regardless the scale or culture method (T-flask, Schott bottle, Erlenmeyer, spinner flask, or bioreactor), the temperature of the culture medium is always a fundamental variable, since it interferes not only with cellular growth and the production process, but it also affects the solubility of various medium components, especially gases such as CO<sub>2</sub> and O<sub>2</sub>, which have low solubilities (15). Most mammalian cells have optimal growth rates within the range of 35–37 °C.

Most cell lines present a wide tolerance range to osmotic pressure. Metabolic transformations that occur during cell culture can cause osmolality changes. Additionally, culture medium osmolality can

also increase due to evaporation, since culture flasks are generally not sealed (to allow equilibrium between culture medium and the CO<sub>2</sub>-air gas mixture) (4).

The most important components of the gaseous phase are CO<sub>2</sub> and O<sub>2</sub>. Monitoring and controlling these gases in the culture medium are essential procedures for the success of *in vitro* animal cell culturing. O<sub>2</sub> is frequently the first component to limit achieving high cell densities due to its low solubility in aqueous medium; therefore, ideally it needs to be supplied continuously to the culture medium (15).

### 1.1.1 - Bioprocessing

To bring to the market a product of pharmaceutical interest successfully produced in the laboratory, further process development is needed (1), as well as the development and optimization of methods and techniques for the separation and purification of these biological macromolecules. A successful bioprocess is the one where upstream (production) and downstream (purification) find and complete each other leading to the production of the desired biopharmaceutical product with high purity and quality at a cost-effective price.

#### 1.1.1.1 - Upstream: relevant culturing systems and process modes

Laboratory scale processes are great platforms for process development and optimization. Normally the process starts with small flasks (with adherent cells) and then it is transferred to spinner flasks or erlenmeyers (attempting to adapt the cells to grow in suspension) (1), but ultimately the process needs to be scaled-up to bioreactor, to assure it is transferable to industrial scale.

The production processes must be cost-effective and need to be well controlled in order to produce a consistent product. To obtain a high quality product, cell proliferation and product biosynthesis have to be efficient. As a practical consequence of these requirements, several types of equipment designed to control environmental culture conditions have been developed, giving rise to controlled bioreactor systems, currently well established and used in the biopharmaceutical industry (1, 3, 16), such as stirred tank (most employed), Roller Bottles, Cell Factories, Wave™, among others.

An ideal culture system has to meet requirements such as (a) control of the temperature and of the acid-base equilibrium of the culture medium; (b) to provide gas exchange by supplying O<sub>2</sub> to the cells and promote CO<sub>2</sub> stripping (removal of the excess of CO<sub>2</sub>), which alters the pH of the media; (c) allow an adequate supply of nutrients through the use of specifically designed formulations of culture media (9, 17); and (d) should maintain aseptic conditions, therefore avoiding contamination by microorganisms, viruses, or other cells (1, 6).

Although many different culture modes can be adopted for bioreactors, the most general is the one that considers the following operation modes: batch (discontinuous mode), fed-batch (semi-continuous), continuous, and perfusion (which is a continuous mode with cell retention) (14). The batch operation mode is the simplest to carry out, and therefore it is widely employed (1).

In this work, the production of human carboxylesterase 2 (CES2) was made in Erlenmeyer flasks and also in stirred tank bioreactor, operating in batch mode. Batch is a discontinuous operation mode,

and cell growth occurs without any additional supplementation of nutrients after inoculation of cells. While substrates are metabolized, the cell population grows, forming the product and other metabolites. The volume is maintained constant throughout the whole process. Due to the low solubility of oxygen, this gas must be supplied continuously, instead of just at the start of the culture (1). Control of pH is carried out through addition of base and by varying the CO<sub>2</sub> concentration in the gas phase, since most animal cell culture media contain sodium bicarbonate, in order to allow this type of buffering control of the medium pH (6).

#### 1.1.1.2 - Downstream: recombinant protein purification

For certain applications, biological products can be used as crude extracts with little or no purification at all (11). However, biopharmaceuticals typically require exceptional purity, making downstream processing a critical step of the overall bioprocess. Currently, proteins are the most important biopharmaceuticals (18).

Purity requirements are different for each therapeutic agent, since it depends on the intended use of the biopharmaceutical - the dose, the risk–benefit ratio, etc. The most common methods for preparative purification of proteins involve chromatography, since (a) it provides very high separation efficiencies, which allow the resolution of complex mixtures with very similar molecular properties; (b) chromatography columns packed with high capacity adsorbents are ideal for capturing molecules from the dilute solutions encountered in bioprocessing; (c) can be performed in an almost closed system and the stationary phase can be easily regenerated; and (d) chromatographic methods are well established in many practical biopharmaceutical manufacturing processes and suitable equipment and packing materials are readily available and are suitable for scale-up (18, 19).

Different methods can be applied in chromatography process depending on different protein properties (Table 1.1), and allow to separate according to differences between the properties of the protein to be purified and the properties of other substances in the sample.

**Table 1.1:** Chromatographic methods applied according to differences in protein properties.

Protein Property	Method
<b>Specific ligand recognition</b>	Affinity chromatography (AC)
<b>Metal ion binding</b>	Immobilized metal ion affinity chromatography (IMAC)
<b>Charge</b>	Ion exchange chromatography (IEX)
<b>Size</b>	Gel filtration (GF)
<b>Hydrophobicity</b>	Hydrophobic interaction chromatography (HIC) or Reversed phase chromatography (RPC)
<b>Isoelectric point</b>	Chromatofocusing

In this work, IMAC strategy was used, since it is appropriate to purify recombinant proteins containing an affinity tag consisting of polyhistidine residues (19), such as the CES2-10xHis, which will allow its strong binding among all the proteins in a crude sample extract, while other cellular proteins

will not bind or will bind weakly. IMAC is a versatile method that can be utilized to rapidly purify polyhistidine affinity-tagged proteins with high yields and up to 95% of purity (20). This method is based on the interactions between a transition metal ion ( $\text{Co}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ) immobilized on a matrix and specific amino acid side chains (20, 21). These matrices securely coordinate metal ions through four coordination sites while leaving two of the transition metal coordination sites exposed to interact with histidine residues in the affinity tag (20, 22).

### 1.1.2 - Cell line

In order to obtain high amounts of a desirable recombinant protein, the choice of the host cell line is crucial - it should be susceptible to transfection, be able to transcribe, translate, fold, and process the protein and, if possible, to secrete it to the culture medium (7). On the other hand, it is advisable that the selected cell line grows in a serum-free media, since recovery of the recombinant protein from culture media with a low protein content is simpler (23) and the risk of contamination with animal pathogens is smaller, reducing the burden of quality control analytics and the approval process simpler (24). Post-translational modifications, such as glycosylation, are fundamental to insure the full biological activity of proteins (11). Eukaryotic cells have the metabolic capability to perform these modifications and so, mammalian cells have become the host cells of choice to produce recombinant proteins with complex post-translational modifications (16, 25).

One of the most used cell lines in industry for recombinant protein is human embryonic kidney cell line (HEK-293). HEK-293 cell line exhibits epithelial morphology and is derived from human embryonic kidney cells, by the transformation of these cells with a fragment of adenovirus type 5 DNA.

This cell line is well suited for large-scale production and has desirable features such as its quick and easy reproduction and maintenance, amenability to transfection using a wide variety of methods, high efficiency of transfection and protein production, faithful translation and processing of proteins (26), growing easily in suspension and being adaptable to serum-free medium (7, 26). They also stably express the adenovirus 13-S-E1a protein that has been shown to significantly enhance transcription from the cytomegalovirus (CMV) promoter (further discussed in Section 1.1.4) (27).

The HEK-293T cell line has been explored for viral vector production for gene therapy and for obtaining human recombinant proteins with normal glycosylation profiles (4). In addition, this cell line is a highly transfectable derivative of the HEK-293 cell line into which the temperature sensitive gene for SV40 T-antigen was inserted (28), which allow episomal replication of transfected plasmids containing the SV40 origin of replication.

### 1.1.3 - Protein processing

Many proteins require post-translational modifications to insure the full biological activity that is essential for their use as efficient biopharmaceuticals. The most widely recognized form of post-translational modification is glycosylation that requires an extensive sequence of processing in the endoplasmic reticulum (ER) and Golgi complex, which insures structural stability but also biological and clinical activity.

In eukaryotes, proteins are synthesized on ribosomes in the cytosol and then are directed to their cellular destinations. This process uses a short sequence of amino acids called a signal sequence, whose function is to direct a protein to its appropriate location in the cell and, for many proteins, is removed during transport or after the protein has reached its final destination. For instance, most lysosomal, membrane, or secreted proteins have an amino-terminal signal sequence that marks them for translocation into the lumen of the endoplasmic reticulum. The signal sequence has a cleavage site where proteases remove the sequence after the protein is imported into the ER (11).

In the ER lumen, newly synthesized proteins are further modified in several ways, following the removal of signal sequences, polypeptides folding, disulfide bonds formation, and many proteins glycosylated to form glycoproteins. Proteins travel from the ER to the Golgi complex in transport vesicles and once there, the Golgi complex also sorts proteins and sends them to their final destinations (29).

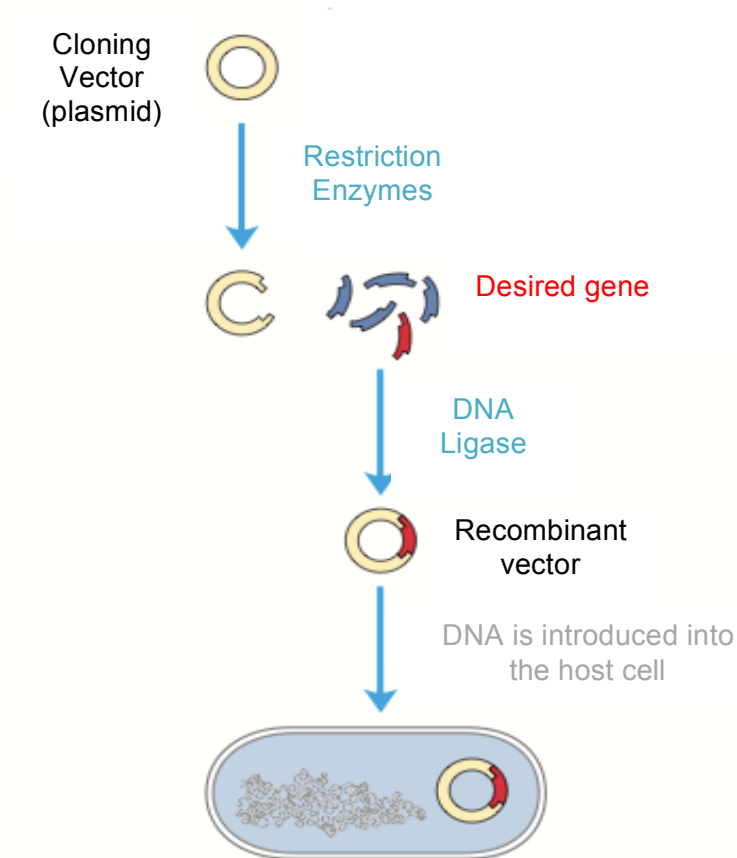
### 1.1.4 - Expressing heterologous proteins in mammalian cells

Cell lines can be generated following delivery of the gene of interest and the selection gene into host cells by transfection (30). In fact, to develop a successful production process, several aspects need to be optimized, namely, correct selection of the expression vector, host cell, and culture medium and also the deoxyribonucleic (DNA) acid quality, the transfection vehicle and the culture medium (4, 7, 28). Moreover, the genetic manipulation of the first two and the choice of the appropriate method for transfection and genetic amplification may influence positively the specific cellular productivity. An increase in the specific productivity could involve enhancement at the genetic level by gene amplification or by the addition of an inducer to enhance the transcription of a gene.

Cloning (Figure 1.1 (11, 31)) is the basic procedure in molecular biology required to move inserts from one vector to another to gain the desired functionality and thus creating a functional vector with the desired gene in it, which later will be transferred to the cells (31).

For the expression of heterologous proteins, it is not enough just to have the sequence of DNA encoding for the protein of interest. The presence of sequences that regulate gene expression are essential. The promoter is the sequence located upstream from the beginning of the transcription, whose function is also to regulate the initiation of the transcription of the adjacent gene (32). The CMV promoter allows for strong, constitutive expression in a variety of cell types (7). Also, the presence of introns (non-coding sequences further excised from the precursor mRNA by specific cleavage in a process known as splicing) (32) significantly improves the efficiency of transcription and thus the expression of recombinant proteins, by increasing mRNA stability and export from the nucleus (7).

The pCI-neo vector, used in this work, has a CMV enhancer and promoter, an intron, and also contains the SV40 origin of replication (SV40 Ori T), which contains the SV40 enhancer and early promoter region upstream of the neomycin phosphotransferase gene (33), which will induce transient, episomal replication of the pCI-neo vector in cells expressing the SV40 large T antigen.



**Figure 1.1:** Illustrative scheme of the DNA cloning process. Adapted from (11).

Nowadays, physical, biological and chemical methods are applied to introduce genetic material into the cells and this process may comprise temporary introduction of DNA into the host cell (transient transfection) or permanent integration into the genome (stable transfection) with clone selection through the use of antibiotics. Transfection may be performed by physical methods such as electroporation or microinjection, which require special instruments and their application is often restricted to special cell lines or tissues (4); by biological systems for nucleic acid delivery, which often employ viruses. Viral transfection methods use genetically modified viruses that are no longer pathogenic, however their application is limited by viral-related immunogenicity and the size limitation of the transgene. This system uses complex methods that are not easy to use for general applications (4). The most widely applied transfection method (transient) is the chemical one using cationic lipids, cationic polymers or Calcium Phosphate (CaPi) precipitates. These vehicles should allow the DNA condensation, promote its binding to the cell membrane and facilitate its entry into the cell and nucleus (7). Available cationic lipids may be applied, which result in high expression levels, although CaPi and the polycation polyethylenimine (PEI) are currently the two most cost-effective and efficient transfection vehicles used. These methods are easy to use and do not require any additional laboratory instruments. PEI offers additional advantages over CaPi, since it is simpler to use, has compatibility with serum-free medium (7), and is also efficient in suspension cultures (34).

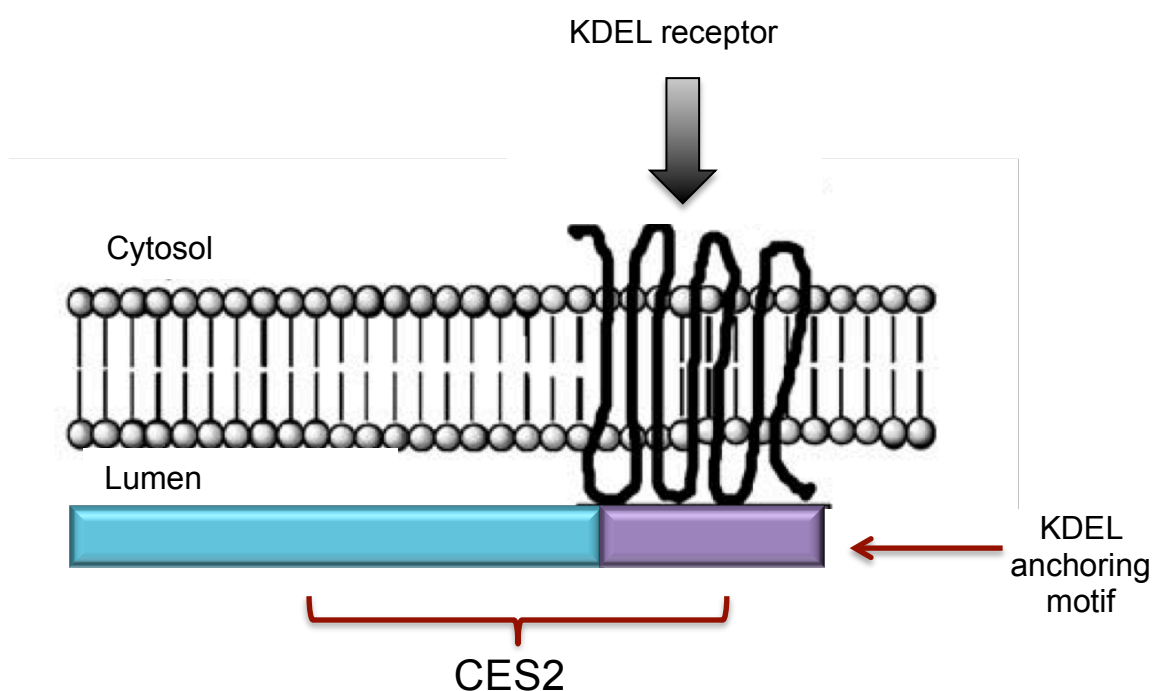
## 1.2 - Carboxylesterases

Carboxylesterases (CES) comprise a multigene superfamily (35) and are categorized as phase-I drug-metabolizing enzymes, since is their responsibility the detoxification of a wide a range of ester containing xenobiotics (biotransformation of these compounds to polar products to facilitate their elimination) (36), such as heroin and cocaine (37, 38), but also drugs and prodrugs (39) as well as insecticides, such as pyrethroids (40, 41). They are also involved in several lipid metabolic reactions (42, 43) and may also be connected to the assembly of low-density lipoprotein particles in the liver (44). An increased interest in the CES field arise due to their application to design herbicides with selective toxicity (45, 46), to their potential use for treatment of drug overdose, addiction, and chemical warfare, as well as cancer prodrug combined therapy (47).

CES are a subset of esterases (48) (EC 3.1.1.1) (38), which are hydrolytic enzymes that catalyze the conversion of carboxylic esters to their corresponding alcohols and carboxylic acids. Thus, drugs such as heroin, cocaine, irinotecan, capecitabine, oseltamivir (Tamiflu), lidocaine, and meperidine (Demerol) are all hydrolyzed by CES (49). Besides these, several other clinically used compounds are esterified, since this is an approach commonly used by the pharmaceutical industry to improve the water solubility of molecules, and thus are also hydrolyzed by CES.

Mammalian CES genes usually contain 12-14 exons of DNA encoding CES enzyme sequences, which may be shuffled during mRNA synthesis, generating several CES transcripts and enzymes encoded by each of the CES genes (36). CES were initially classified by their substrate specificity and isoelectric point (pI) (50). However, this classification is ambiguous in overlapping substrate specificities, for instance, a single esterolytic reaction can be mediated by several kinds of enzymes. Since CES comprises a superfamily of genes, identification of homology and similarity of characteristics are acceptable factors to classify these enzymes. Thus, mammalian CES nomenclature has been recently reviewed (51) and gives (a) a name for each human (CES) or mouse and rat (Ces) gene; (b) names and identifies the gene family of origin for identified CES pseudogenes; and (c) provides a system for naming transcript isoforms derived from each of the CES genes. This new classification for mammalian CES comprises at least five gene families including carboxylesterase 1 (CES1), the major liver enzyme (42, 52), CES2, the major intestinal enzyme (53, 54), CES3, expressed in the brain, liver, and colon (55), CES5 (also called CES7 or cauxin), a major urinary protein of the domestic cat also present in human tissues (56), and CES6, a predicted CES-like enzyme in the brain (57).

These enzymes can be found both intra and extracellularly (36, 51), however, unlike previously stated (50), human CES are not found freely in the plasma (58). They are located intracellularly due to the existence of a C-terminal retention sequence composed of four conserved aminoacid residues: Histidine - Threonine - Glutamic acid - Leucine (HTEL), Glutamine - Glutamic acid - Aspartic acid - Leucine (QEDL) and Histidine - Isoleucine - Glutamic acid - Leucine (HIEL) for CES2, carboxylesterase 3 (CES3) and CES1, respectively (51). This motif enables the anchoring of CES in the lumen of ER (36, 51) (Figure 1.2).



**Figure 1.2:** Illustrative representation of the internal localization of CES2 protein. Adapted from (36).

### 1.2.1 - CES characterization

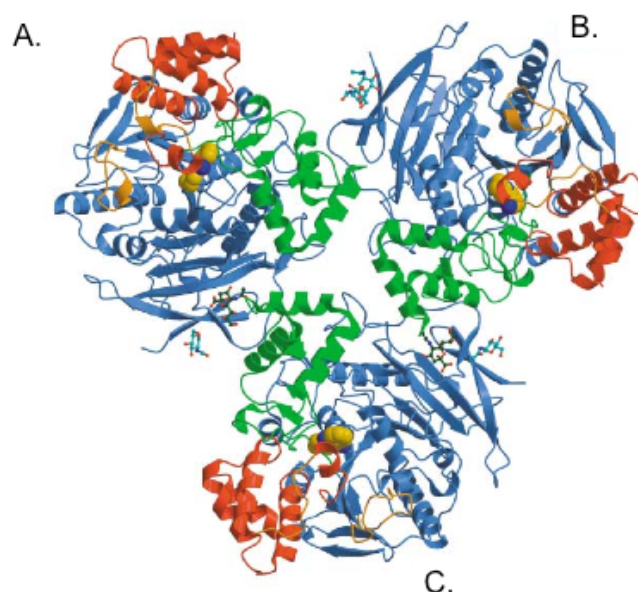
The two major expressed human CES are CES1 and CES2. CES1 is highly expressed in the liver and also observed in macrophages, human lung epithelia, heart, testis and other tissues (59). CES2 is present in the small intestine, colon, kidney, liver, heart, brain and testis (36). CES1 and CES2 are two different gene products and the two genes have only 48% amino acid sequence homology (41, 59).

Mammalian liver is predominantly responsible for drug detoxification (where CES1 is present in higher amounts than CES2), with CES1 and CES2 playing major roles, following absorption of drugs into the circulation (37, 59), while mammalian intestine (where CES2 is present in higher amounts than CES1), has a key role in first-pass clearance of several drugs, predominantly via CES2 in the ileum and jejunum (51).

To date, from human carboxylesterases, only the CES1 crystallographic structure is available (60) (Figure 1.3), confirming that this enzyme is found in trimers composed of approximately 60 kDa monomers. This enzyme has also been described to occur as hexamers (60), existing in a trimer-hexamer equilibrium that could be shifted towards trimer or hexamer, depending on the substrate present (60).

The three-dimensional structural analysis of CES1 (Figure 1.3), revealed that this enzyme exhibits the  $\alpha/\beta$ -hydrolase-fold, typical of serine esterases, but also contains a large substrate binding area with both rigid and flexible pockets (60). In fact, it pointed to three major ligand binding sites, including the broad-specificity active site (which is lined largely by hydrophobic residues which surround a serine esterase catalytic triad composed of Serine-221, Histidine-468, and Glutamic acid-354), the

"side door" and the "Z-site", where substrates, fatty acids and cholesterol analogs, respectively, are bound (60).



**Figure 1.3:** Illustrative representation of the tridimensional structure of the human carboxylesterase 1 trimer (A, B and C monomers) complexed with tacrine (potent inhibitor of human acetylcholinesterase). The catalytic domains,  $\alpha/\beta$  domains, and regulatory domains of each monomer are in blue, green, and red, respectively, the novel  $\Omega$  loops are in orange, the N-acetylglucosamines (NAG) are in cyan, and the sialic acids (SIA) are in dark green (60).

A N-glycosylation site was described for CES1, contributing to its stability and maintenance of catalytic efficiency (61), also potential N-glycosylation sites were observed although in different positions for other CES enzymes and may perform similar functions as to the one on CES1 (51).

Mammalian CES families show promiscuity toward a wide range of substrates, exhibiting a broad substrate specificity, which leads to difficulties in establishing specific roles for these enzymes (36, 51). CES1 preferentially catalyzes the hydrolysis of compounds esterified with a small alcohol and a large acyl group such as clopidogrel, while CES2 hydrolyzes compounds with a large alcohol group and a small acyl group (59), such as cocaine, 4-methyumbelliferyl acetate, heroin and 6-monoacetylmorphine (37).

CES2 is described as being a very unstable (62) 60 kDa monomeric enzyme (37), and is receiving a continuously increase in attention due to its potential role in anti-cancer combined therapies, with specific drugs such as Irinotecan (CPT-11) (63), for the treatment of different pathologies such as colon carcinoma (39) and malignant glioma (64). These therapies enable the dosage reduction of the administered drug and consequently, the reduction of its severe adverse side effects (65), due to tumour specific activation (39). CES2 converts CPT-11 to its active metabolite, SN-38 (CES2 has a higher affinity and conversion velocity for CPT-11 than CES1 (63)), which has at least a 100-fold greater in vitro cytotoxic effect than CPT-11, at equimolar concentrations (66). Moreover, it has been reported to be advisable to monitor CES2 expression levels before the treatment of human lung cancer with Irinotecan (36).

### 1.2.2 - CES2 manufacturing in different systems

Recombinant active CES expression has already been reported in the literature, for several mammalian (rat, rabbit, porcine, human) CES, in different cell types, such as yeasts (67, 68), insects (68-71) and mammalian cells (65, 68, 72). Purification using bacterial strains has proved to be unsuccessful since despite significant amounts of recombinant CES were expressed, however very low level of enzyme activity was observed (68). For enzymes that are secreted from or sequestered within the endoplasmic reticulum of mammalian cells, expression in bacteria may yield inappropriately folded and/or glycosylated proteins (68).

The expression of recombinant CES is mainly reported intracellularly (68-70, 72). However, the secretion of CES has been previously reported in yeast (73), insect (68) and mammalian cells (39), always comprising the modification/deletion of the retention signal and/or the addition of signalling N-terminus sequences. An alternative pig liver esterase containing the  $\alpha$ -factor signal sequence of *Saccharomyces cerevisiae* and missing the five last C-terminus amino acid residues was produced in liquid cell culturing of *Pichia pastoris* (73). A rabbit liver carboxylesterase without the six last C-terminus amino acid residues was produced in serum-free Sf21 insect cells (68) and human CES2 was reported to be produced, with 60% purity grade, also in a secreted form in Sf9 insect cells (71), and in Sf21 cells, with 98% purity grade (38). A secreted form of human CES2 was reported in COS-7 cells, without the C-terminal ER anchoring motif and with an Ig  $\kappa$ -chain leader sequence (directs proteins to the secretory pathway) (39) and in human cervical carcinoma (HeLa) cells, without the KDEL C-terminal amino acid residues (65).

Previous reports of CES purification produced in insect cells show a wide range on protein yield and purity. Human CES2 produced intracellularly in Sf9 insect cells was purified with a 0.8% yield (70). Rabbit liver carboxylesterase, produced extracellularly using Sf21 insect cells originated an active protein with, according to the authors, 98% purity grade and 61.6% yield (68).

CES2 is known to be highly unstable (62), thus, a fast purification process that minimizes the exposure of the protein to conditions that could hamper CES2 stability and consequently its activity is highly desirable. Expression in mammalian cells has been used for innumerable proteins, although purification of enzymes derived from these extracts can be tedious and time consuming and can result in low yields (68). Furthermore, different purification strategies have been applied (68, 70) for protein purification, being one of the most rapid and specific, the immobilized metal-affinity chromatography (IMAC) (20, 46, 74). The majority of the reported CES purification processes either for the purification of recombinant CES (68, 70) or the purification of native CES (63, 75-77) involve several steps, being time consuming. Affinity chromatography for CES purification has been performed (46) being an alternative to these processes.

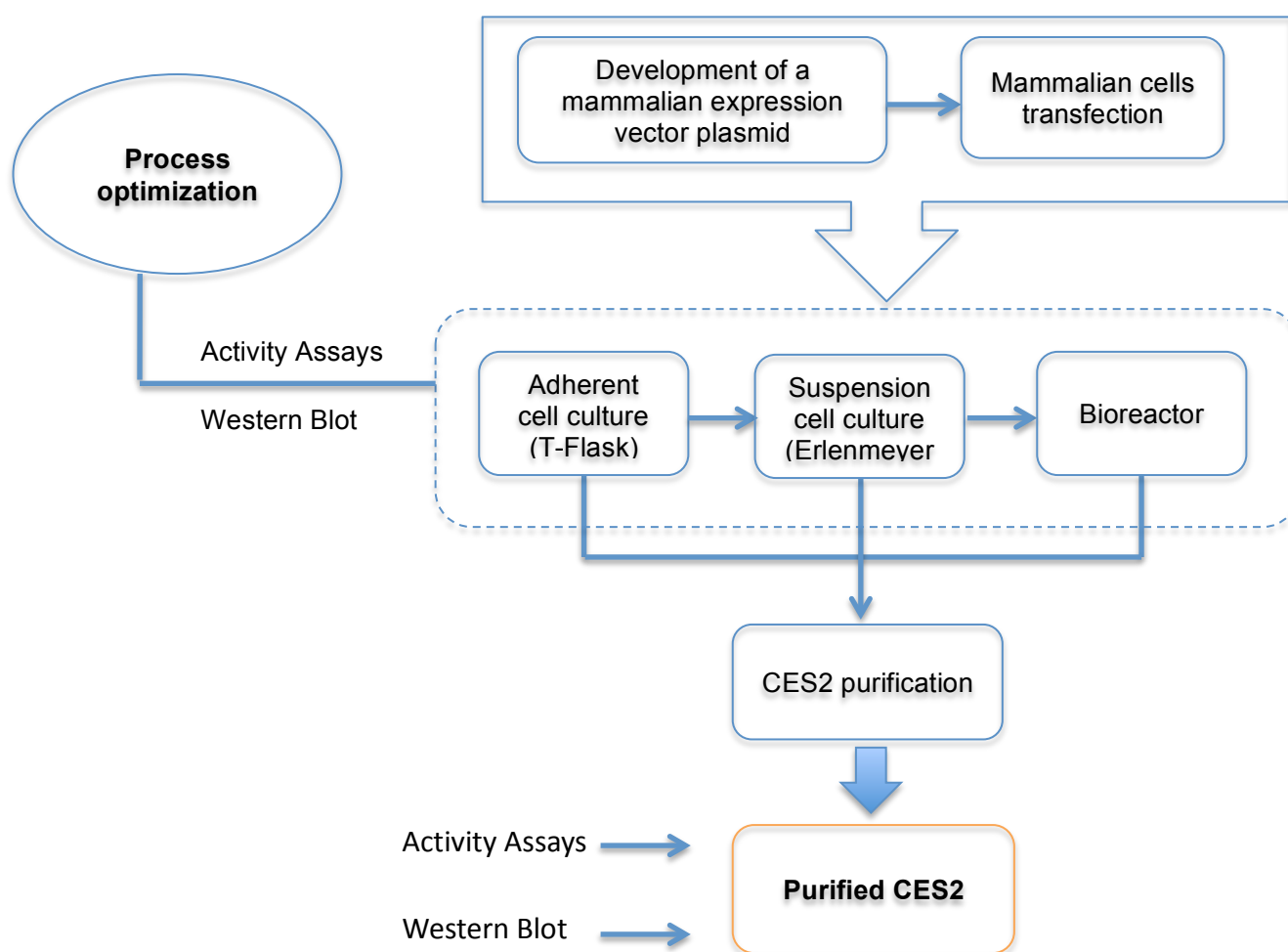


## 2. Goals

The main goal of this project was the development and optimization of a process for the production and purification of the human carboxylesterase enzyme (CES2).

This protein has a key role in the metabolism of ester containing xenobiotics (41) and also in the activation of several prodrugs (49). Therefore, it can influence significantly their bioavailability or contribute to the reduction of their secondary effects (65).

The knowledge of this protein structure will be crucial for the understanding of its properties, as well as to further understand the well described similarities and differences between CES1 and CES2 in their behaviour towards different substrates and inhibitors (60, 78). However, one of the main bottlenecks for these studies is the unavailability of purified CES2, whose structure is not yet known, unlike CES1. Therefore, it becomes relevant the establishment of a robust platform for the production of this protein (the workflow is represented in Figure 2.1), in order to have the enough protein amount (1 - 10 mg of highly pure protein) to proceed for the crystallographic studies.



**Figure 2.1:** Flow diagram for the expression and purification of CES2.

The followed strategy for the development and optimization of the CES2 manufacturing process consisted in four major points - Development of a mammalian expression vector plasmid with the *CES2* gene, recombinant CES2 expression in mammalian cells (in adherent and suspension cultures), CES2 purification and development of a scalable bioprocess for CES2 production.

### 3. Materials and Methods

#### 3.1 Biological Material and Culture Media

##### 3.1.1 Bacterial Strain

*Escherichia coli* (*E. coli*) DH5- $\alpha$  Library Efficiency (Invitrogen, Carlsbad, USA) competent cells were used for the production of the DNA plasmids. The bacteria transformation was performed following the manufacturer's instructions. Working banks of transformed bacteria for each used plasmid were created - the bacteria were grown until optical density approximately of 2 was reached at 600 nm (OD 600 nm). The bacteria suspension was mixed with 15% of glycerol, aliquoted and frozen at -80 °C.

The bacterial culture was performed with *Luria Bertani* (LB) (NZYTech, Lisboa, Portugal) medium, supplemented with 100  $\mu$ g/mL of Ampicillin (Sigma, St. Louis, U.S.A.) and with *Terrific Broth* (TB) (Fast-Media® Amp TB from Invivogen, California, U.S.A.). LB medium was sterilized for 30 minutes at 121 °C and 1.2 bar, and afterwards supplemented with ampicillin. This medium was used for low cell density culture of transformed bacteria. Sterile TB medium was used for high cell density culture of transformed bacteria. Both media were prepared accordingly the manufacturer's instructions and used for extraction of high quantity and quality of DNA plasmids. For the preparation of both media, ultrapure water (Millipore, Billerica, U.S.A.) was used.

After thawing or after transformation, bacteria were spread in agar plates. The agar plates (with ampicillin) were prepared with Fast-Media® Amp Agar (Invivogen) accordingly to the manufacturer's instructions. After inoculation, bacteria were grown at 37 °C for 15 - 16 hours (h). Single and well-individualized colonies were picked from the previously inoculated agar plates. Each colony was grown in a small volume of liquid media (TB or LB) (3-4 mL in 13 mL tubes) at 37 °C for 12-16 h in an orbital shaker at 180 rpm. For a laboratory scale production of the plasmids, the bacterial culture previously grown in 3 - 4 mL, were grown in Erlenmeyers (Erl) (Shott, Mainz, Germany) with a working volume of 400 mL in an orbital shaker at 180 rpm, 37 °C for 12 - 16 h. For larger scale production of pCI-neo-CES2-10xHis plasmid, a 10 litre working volume bioreactor (BIOSTAT ED-10, Sartorius Stedim Biotech, Aubagne, France) was inoculated with OD 600 nm of 0.5 from a pre-inoculum of *E. coli* DH5- $\alpha$  Library Efficiency transformed with pCI-neo-CES2-10xHis, previously grown in TB medium at 37 °C and 150 rpm, in 100 mL in Erlenmeyer (Corning, Amsterdam, The Netherlands). The bioreactor was operated in batch mode for 8 h and was equipped with two 6-D Rushton impellers. The  $pO_2$  (partial pressure of oxygen) was set at 50% of air saturation and sequentially controlled varying the agitation rate (100 to 1000 rpm). Culture gassing was performed using a ring sparger at a constant gassing rate of 1 vvm. The temperature was kept at 37°C by water steam recirculation in the vessel jacket. The Universal Bio-Process Control System, UBICON (Electronic System Design, Hanover, Germany), was used for data acquisition and process control.

### 3.1.2 Mammalian cell line

HEK-293T cell line is derived from HEK-293 cell line in which the SV40 T-antigen gene was inserted (7). This cell line was purchased from ATCC (CRL-11268) and used in this work.

In a first stage of this work, HEK-293T cell line was cultured in adherent monolayers in T-flasks with 25, 75 and 175 cm<sup>2</sup> (BD, New Jersey, U.S.A.) using high glucose DMEM media (Dulbecco's Modified Eagle's Medium, Gibco®, Grand Island, U.S.A.), containing 0.11 g/L of pyruvate, 4.5 g/L of glucose, 0.58 g/L of L-glutamine and 3.7 g/L of sodium bicarbonate (NaHCO<sub>3</sub>), supplemented with 10% Fetal Bovine Serum (Gibco) (FBS) at 37 °C with 5 % CO<sub>2</sub>. Cells were detached from T-flask surface with Trypsin 0.05%- EDTA (Gibco), and were split twice a week after reaching 70 - 80% confluence.

Afterwards, in-house suspension adapted HEK-293T were cultured in Freestyle™ 293 (Gibco) serum-free, animal origin-free, chemically defined culture media formulated with Glutamax™-I (contains a dipeptide, L-alanyl-L-glutamine, a stabilized form of L-glutamine, Invitrogen). This medium was specifically developed for the high-density, suspension culture and transfection of 293 cells grown in suspension conditions and was stored, protected from light, at 4 °C.

The cells were sub-cultured when their density was approximately 2 - 3 x 10<sup>6</sup> viable cell/mL, typically every 3 - 4 days. Briefly, cellular suspension was centrifuged at 200 g for 10 minutes (min) at room temperature (RT) and the sediment was carefully resuspended with fresh Freestyle™ 293 medium. The obtained cellular suspension was used to inoculate new Erlenmeyers with a cellular concentration of 0.3 - 0.5 x10<sup>6</sup> cell/mL. The centrifuging step was performed in every 2 - 3 cell passages. Cells were maintained either in 125 or 500 mL polycarbonate, disposable, sterile Erlenmeyer flask (Corning) or glass, sterile Erlenmeyer flasks (Shott), containing 20 to 25 mL or 80 to 90 mL total working volume of cell suspension, respectively. The erlenmeyer flasks were incubated at 37 °C in a humidified atmosphere of 8 % CO<sub>2</sub>, using orbital agitation (130 rpm). The assays for optimization of the transfection conditions were performed in Erlenmeyers with 90 mL (working volume).

For larger scale production, a five-litre working volume bioreactor (BIOSTAT DCU-3, Sartorius Stedim Biotech) was inoculated with 0.4 - 0.5 x10<sup>6</sup> cell/mL of HEK-293T cells previously grown in FreeStyle™ Expression medium, at 37 °C and 130 rpm, in 90 mL working volume Erlenmeyers. Transient transfection was performed as described in section 3.2.2, using 5 µg/mL of pCI-neo-CES2-10xHis plasmid. The bioreactor was operated in batch mode, equipped with two 6-D Rushton impellers. The pO<sub>2</sub> was set at 40% of air saturation and sequentially controlled varying the agitation rate (60 to 210 rpm) and the oxygen partial pressure in the gas inlet (0 - 100%). pH was controlled at 7.2 using CO<sub>2</sub> and with 1 M NaHCO<sub>3</sub> addition. Culture gassing was performed using a ring sparger at a constant gassing rate of 0.01 vvm. The temperature was kept at 37 °C by water recirculation in the vessel jacket. Data acquisition and process control were performed using MFCS/Win (Multi Fermenter Control Supervisory for Windows) control and data acquisition software (SCADA; Sartorius Stedim Biotech).

Mammalian cells, when continuously cultured in vitro, may suffer morphological and functional changes in their growth pattern or even in their karyotype (4). When properly frozen, the cells may be preserved for a long-term period of time without suffering these changes. Thus, a cell bank with cells properly frozen and preserved allows to repeat the cell culture, from a certain cell lot, with identical characteristics.

Adherent HEK-293T cells were frozen with a concentration of  $2 \times 10^6$  cell/mL per cryovial. A specific freezing solution was used, constituted by 95% of FBS and 5% of dimethyl sulfoxide (DMSO, Sigma). DMSO is a conventional cryoprotector that is toxic at RT (79), reason why the cell freezing process needs to be done at low temperatures (achieved by using cooled down cryovials and freezing solutions). Cells were frozen at a  $-1$  °C/minute cooling rate using 5100 Cryo 1 °C Freezing Container, "Mr. Frosty" (Nalgene Nunc, New York, U.S.A.). Briefly, the cells grew until the exponential phase was reached and then they were sedimented by centrifugation at 200 g for 10 min at 4 °C. The cells were carefully resuspended in the freezing solution. The cellular suspension was aliquot in cryovials (Nalgene Nunc) and remained for 20 minutes at 4 °C. The cryovials were then transferred to the "Mr. Frosty" at  $-80$  °C. After 24 h, one cryovial was thawed to confirm the freezing efficiency. The thawed cryovial was incubated at 37 °C and immediately after thawing the cellular suspension was carefully added to fresh medium and to a T-Flask. After 3 h, the medium from the T-Flask was removed (containing DMSO) and replaced by fresh medium.

The HEK-293T suspension cells were frozen with an average concentration of  $10 \times 10^6$  cell/mL or  $4.5 \times 10^7$  cell/mL to thaw directly into a 125 mL or to a 500 mL Erlenmeyers, respectively. The cells grew until the exponential phase was reached and then they were sedimented by centrifugation at 200 g for 10 min at 4°C. The cell pellet was carefully resuspended in the freezing solution CryoStor™CS10 (STEMCELL Technologies, Grenoble, France). The cellular suspension was aliquoted in cryovials (Nalgene Nunc) and remained for 20 minutes at 4 °C. The cryovials were then transferred to the "Mr. Frosty" at  $-80$  °C. After 24 h, one cryovial was thawed to confirm the freezing efficiency. The thawed cryovial was incubated at 37 °C and immediately after thawing the cellular suspension was carefully added to fresh medium and centrifuged at 200 g for 10 min at 4 °C. Due to DMSO's toxicity to the cells at RT, its quick removal from the cell suspension is critical. The cell sediment is then resuspended in fresh medium and then added to the Erlenmeyer.

### 3.1.3 Mammalian Expression vectors

For the generation of pCI-neo-CES2-10xHis mammalian expression vector, human recombinant *CES2* gene (geneID 8824) synthetically synthesized with a C-terminal 10xHis tag (GeneArt, Regensburg, Germany) was cloned into pCI-neo plasmid (Promega, Madison, U.S.A.) using *SalI* and *NotI* restriction endonucleases (New England Biolabs, Ipswich, U.S.A.). For the formation of a full transcript containing the C-terminal His tag, the stop codon of *CES2* gene was deleted. The ligation process was performed with T4 DNA Ligase (New England Biolabs). At the same time, a plasmid containing *CES2* gene without the His-tag was generated (pCI-neo-CES2). This plasmid is similar to the pCI-neo-CES2-10xHis, but it only has the *CES2* gene without any other features added to the plasmid. Both constructs were fully sequenced to verify the integrity of *CES2* gene.

During this project, working DNA banks for each of these plasmids were generated and stored at -20 °C.

Plasmid purification was performed in different scales. Small-scale purification (yields around 20 µg of DNA) was performed with QIAprep® Miniprep (Qiagen, Hilden, Germany). A larger scale purification (yields around 500 µg of DNA) was performed with Genopure Plasmid Maxi Kit (Roche, Basel, Switzerland) following manufacturer's instructions. Large-scale purification was performed with QIAprep® Gigaprep (Qiagen) (yield of 25 mg of DNA) following the manufacturer's instructions. The isolation procedures are based on a modified alkaline lysis protocol followed by absorption of DNA onto silica in the presence of high salt. The bacteria are partially lysed, allowing the plasmid DNA to escape the cell wall into the supernatant, while the larger *E. coli* chromosomal DNA is trapped in the cell wall. Then the lysate is cleared of cellular debris by centrifugation and the plasmid DNA containing fraction is added to a column. The bound plasmid DNA is washed to remove contaminating bacterial components and then is eluted in a low-salt buffer. The DNA is eluted and precipitated to remove salt and to concentrate the eluate.

### 3.2 Transfection assays

The transfection assays were carried out with the polycation polymer polyethylenimine (PEI, Linear 25 kDa from Polysciences, Eppelheim, Germany) at 1:3 ratio of DNA:PEI, based on previous studies (80). For the preparation of the transfection solution, which contains fresh serum-free-media, DNA and PEI, it was used a PEI stock solution with 1 mg/mL. This solution was prepared by the addition of the medium, DNA and PEI. This solution was vigorously stirred and incubated for 10 min at RT before being added drop wise to the cell culture. HEK-293T cells were transiently transfected with pCI-neo-CES2 or pCI-neo-CES2-10xHis plasmids.

#### 3.2.1 Static culture

HEK-293T cells were seeded in 100 mm sterile Petri dishes, with a seeding inoculum of  $5 \times 10^4$  cell/cm<sup>2</sup> and transfected 24 h after cell seeding. Transfection was performed using 30 µg (16.6 µg/mL) of plasmid DNA (pCI-neo-CES2-10xHis) and 90 µg of PEI in a final volume of 1.8 mL per plate. Cells were harvested (by centrifugation at 200 g, 10 min, 4 °C) 48 h after transfection and chemically lysed with 750 µL of M-PER® Mammalian Extraction Reagent (Pierce Biotechnology, Rockford, U.S.A.). After lysis the lysate was centrifuged at 12000 g at 4°C for 15 min and the supernatant was stored at -20 °C.

#### 3.2.2 Suspension culture

Three days before transfection, the cells were inoculated at a concentration of  $0.5 \times 10^6$  cell/mL and they grew until cellular concentration of  $1.8 - 2 \times 10^6$  cell/mL was reached. On the transfection day, the cellular suspension was diluted to a concentration of  $0.8 - 1.2 \times 10^6$  cell/mL and after 3 - 6 h the transfection was performed. The transfection mixture volume corresponds to 10% of the cellular

suspension volume. Different plasmid DNA concentrations were tested: 2, 5, 10 and 20 µg/mL to optimize the transfection assay. Cell growth and viability was followed throughout a culture time period of 96 h. In each time point (24, 48, 72 and 96 h post-transfection) a sample of 10 mL was taken from the Erlenmeyer, centrifuged at 200 *g*, at 4 °C for 10 min. The supernatant was stored at -20 °C and the sedimented cell pellet was lysed with 200 µL of M-PER® Mammalian Extraction Reagent (Pierce Biotechnology) and further clarified at 12000 *g* for 10 min at 4 °C. The cells extracts were stored at -20 °C. No protease inhibitors were added for both samples.

After optimizing the conditions, all the transfections assays were performed only with 5 µg/mL DNA concentration. Cells and supernatant were harvested after 96 h.

### 3.3 Brefeldin A assay

Inhibition of protein secretion was performed with brefeldin A antibiotic (99.9%, Aldrich, Steinheim, Germany), as described before (81), with some modifications. HEK-293T suspension cells were transiently transfected with 5 µg/mL of pCI-neo-CES2-10xHis or pCI-neo-CES2 expression vectors, as described in section 3.2.2. At 24, 48, 72 and 96 h post-transfection, two samples of 10 mL from each transfection type were centrifuged at 200 *g*, for 10 min. The cellular pellet was resuspended in FreeStyle™ Expression medium or in the same medium, but containing 10 µg/mL of brefeldin A and were incubated, for 4 h. Cells extracts and supernatants were processed as described above.

### 3.4 Protein Purification

For the purification of CES2-10xHistidine, platform ÄKTAexplorer™ 10S System (GE Healthcare, Little Chalfont, U.K.), a purification platform based on chromatographic analysis and separation, was used, at 4 °C.

#### 3.4.1 CES2 purification from cell extracts of transfected HEK-293T adherent cells

This purification was performed with 1.5 mL of cell extract from transiently transfected HEK-293T cells and with 1.5 mL of cell extract from non-transfected HEK-293T using HiTrap™ Chelating HP Column of 1 mL (GE Healthcare). The cells extracts were obtained as previously described in Section 3.2.1. The samples were applied in the Chelating Column, charged with 10 mM NiSO<sub>4</sub> (extra pure, Merck, Darmstadt, Germany), at a low flow rate (0.5 mL/min). The column was first equilibrated with Phosphate buffer (20 mM Na<sub>2</sub>HPO<sub>4</sub> (≥ 99.5%, Merck), buffer and 500 mM NaCl (≥ 99.5%, Merck), pH 7.4) and after sample loading, the column was washed with Wash buffer (20 mM Na<sub>2</sub>HPO<sub>4</sub> buffer, 500 mM of NaCl, 20 mM Imidazole (≥ 99.5%, Sigma), pH 7.4). Elution was performed with a gradient from Elution buffer (20 mM Na<sub>2</sub>HPO<sub>4</sub> buffer to 500 mM NaCl, 500 mM Imidazole, pH 7.4) at a flow rate of 3 mL/min.

Purified samples were loaded into a desalting column (Sephadex™ G-25 desalting column HiPrep™ 26/10, GE Healthcare) to remove imidazole (since it damages the enzyme activity). Samples were eluted in Storage Buffer (20 mM CH<sub>3</sub>COONa (≥ 99.0%, Fluka, Seelze, Germany), 600 mM NaCl pH 5.0), and then filtered under sterile conditions with 0.2 µm filters (low protein binding, PALL Corporation, New York, U.S.A.). For proper storage it was added 20% of sterile glycerol (Sigma) to each sample and the samples were stored at -80 °C.

### 3.4.2 CES2 purification from cell extracts of transfected HEK-293T suspension cells

Culture supernatant containing the soluble human recombinant CES2-10xHis protein was analysed and stored at -20 °C until the beginning of the purification process.

Purification of human recombinant CES2-10xHis was performed using a 5 mL HiTrap Chelating High Performance column (GE Healthcare), charged with 100 mM of nickel sulphate and equilibrated with 20 mM sodium phosphate buffer, pH 7.4, containing 10 mM imidazole and 500 mM sodium chloride. 210 mL of cultures supernatants, obtained as described above and further clarified by centrifugation at 10000 *g*, for 10 min at 4 °C, were diluted in an equal volume of 20 mM sodium phosphate buffer, pH 7.4, containing 20 mM imidazole and 500 mM NaCl, and loaded onto the column. A washing step was performed with the same buffer until stabilization of the baseline. Elution of the His-tagged bound proteins was performed with a two-step imidazole gradient, from 10 to 500 mM. The first slower step was performed until 250 mM of imidazole with 10 column volumes. The following step, until 500 mM of imidazole was performed with 5 column volumes. A flow rate of 5 mL/min was used. Buffer exchange and concentration with 20 mM sodium acetate buffer, pH 5.0, containing 600 mM sodium chloride (Storage Buffer), was performed to all eluted fractions using Vivaflow cassettes (Sartorius Stedim Biotech) with a membrane cut off of 30 kDa. After imidazole removal, all samples were sterilized by filtration (0.2 µm filters (low protein binding, PALL)). After the addition of 20 % glycerol, all samples were aliquoted and stored at -80 °C. All fractions were analysed by SDS-PAGE and western blot.

## 3.5 Analytical tests

### 3.5.1 Quality of the purified plasmids

The DNA concentration was determined by the Lambert-Beer law ( $A = \epsilon \times l \times C$ , where  $\epsilon$  is the extinction coefficient,  $l$  is the path length and  $C$  the sample concentration) with absorbance measurements made in a spectrophotometer (*Biophotometer*, Eppendorf AG, Hamburg, Germany). The quality of the purified plasmids was determined by the  $Abs_{260nm}/Abs_{280nm}$  ratio and by the assessment of plasmid integrity with and without enzymatic restriction assays in Agarose gels (Lonza, Basel, Switzerland) 0.8% (w/v). The agarose gels were performed in a horizontal apparatus with Tris-acetate-EDTA (TAE) 1x (40 mM Tris-Acetate, 1 mM EDTA) (Qiagen) as running buffer. Benchtop 1 kb DNA Ladder (Promega) with DNA fragments between 250-10000 base pairs (bp) was used. Gels were stained by in-gel incorporation of GelRed (Biotium, California, U.S.A.) 1:20000, less toxic than

Ethidium Bromide, and visualized under UV light (in a transilluminator from Uvitec, Cambridge, England).

Restriction analysis was performed with restriction enzymes from New England Biolabs - *Sall*, *NotI* and *XhoI*. In short, all the necessary components - nuclease-free water (Promega), 10X restriction buffer, Bovine Serum Albumin (BSA), DNA and enzyme(s) - were mixed by pipetting. These reactions were performed at 37 °C for 3 h.

### 3.5.2 Determination of cell concentration and viability

Cell concentration and viability were determined by the trypan blue exclusion method using a 0.1% (v/v) solution prepared in Phosphate Buffer Saline (PBS, Gibco) and counting cells in a Fuchs-Rosenthal haemocytometer (Brand, Wertheim, Germany). Each counting was performed at least twice and a 15% error percentage was assumed.

### 3.5.3 Metabolite quantification

Glucose and lactate were quantified in an automated analyzer (YSI 7100 Multiparameter Bioanalytical System (Dayton, Ohio, USA). These measurements have a 5% error associated.

### 3.5.4 Protein Quantification

Total protein quantification in cell extracts was performed by the bicinchoninic acid (BCA) assay with BCA™ Protein Assay Kit (Pierce Biotechnology), using BSA as standard, according to the manufacturer's instructions. The BCA Protein Assay is a detergent-compatible formulation based on bicinchoninic acid for the colorimetric detection and quantitation of total protein. This method combines the well-known reduction of  $\text{Cu}^{2+}$  to  $\text{Cu}^+$  by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation ( $\text{Cu}^+$ ) using a reagent containing bicinchoninic acid. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over a broad working range (20 - 2000  $\mu\text{g}/\text{mL}$ ).

In some cases, it was used the Micro BCA™ Kit (Pierce Biotechnology) to quantify more dilute protein samples (0.5 - 20  $\mu\text{g}/\text{mL}$ ), following the manufacturer's instructions.

### 3.5.5 Enzymatic Activity Assay

Enzymatic kinetic profiles were spectrophotometrically determined (at 350 nm, using a plate spectrophotometer (SPECTRAMax™340, Molecular Devices, Sunnyvale, U.S.A.)), following the hydrolysis of 4-methyl-umbelliferyl acetate (4-MUBA,  $\geq 98\%$ , Sigma) to 4-methyl-umbelliferone (4-MUB,  $\geq 98\%$ , Sigma), at 37 °C (37). All the components were added in ice to a final reaction volume of 250  $\mu\text{L}$  per well and the reactions were undertaken at 37 °C under substrate saturation conditions. In short, 96-well plates were used to evaluate carboxylesterase activity: 4-MUBA was dissolved in

absolute ethanol (99.5%, Panreac, Barcelona, Spain) to a concentration of 15 mM and then diluted in reaction buffer to a final concentration of 7.5 mM (organic solvent percentage in the final reaction volume never exceeded 5% (v/v)); the substrate concentration used in each assay was 0.5 mM; 90 mM  $\text{KH}_2\text{PO}_4$ , 40 mM KCl solution, at pH 7.3, was used as the reaction buffer; each cell extract was used at a final concentration of 0.2  $\mu\text{g}/\mu\text{L}$  (10  $\mu\text{g}$ ) (dilution was made in Tris-HCl Buffer with Trizma hydrochloride (> 99%, Sigma) and Trizma base (> 99.9%, Sigma)). For all supernatants, 50  $\mu\text{L}$  were applied (it is not possible to determine their concentration due to interferences in the BCA assay). Absorbance was measured in 30 seconds intervals, for 15 minutes. An extinction coefficient of 12.2  $\text{mM}^{-1}\cdot\text{cm}^{-1}$  was considered for 4-MUB (37). Non-enzymatic hydrolysis was subtracted to each enzymatic reaction. Results are presented as CES activity per volume unit ( $\mu\text{mol}\cdot\text{min}^{-1}\text{mL}^{-1}$ ), as CES total activity, determined for the total volume of used supernatant ( $\mu\text{mol}\cdot\text{min}^{-1}$ ) or as CES specific activity ( $\mu\text{mol}\cdot\text{min}^{-1}\text{mg}^{-1}$ ). For most cases, results represent the average and standard deviation of three independent assays, each of which performed in triplicate.

### 3.5.6 Electrophoresis

The electrophoresis for protein separation and detection was performed under denaturing conditions (SDS-PAGE), following the manufacturer's instructions. It was used a Novex® NuPAGE® SDS-PAGE Gel System (Invitrogen) with NuPAGE Bis-Tris 4-12% gels (Invitrogen). This system is a neutral pH, precast, discontinuous SDS-PAGE mini-gel (10 cm x 10 cm) system providing maximum stability of both proteins and gel matrix during electrophoresis, and better band resolution than other gel systems (82). NuPAGE® SDS-PAGE gels are polyacrylamide gels made with Bis-Tris/HCl buffer (pH 6.4). During separation, the gels operate close to pH 7.0.

When suitable, i.e. when the sample concentration was far too low and thus, the necessary volume to be applied overcame 20  $\mu\text{L}$ , the samples were precipitated with ice-cold absolute ethanol at -20 °C - the ratio was always 1:4 (sample:ethanol).

All samples were denatured and reduced for 10 min at 70 °C and prepared according to the manufacturer's instructions. Electrophoresis was performed in XCell SureLock mini-cell system (Invitrogen) using as running buffer either NuPAGE® MES SDS Running Buffer (Invitrogen) (separation range of 2 – 200 kDa) or NuPAGE® MOPS SDS Running Buffer (Invitrogen) (separation range of 14 – 200 kDa).

Depending on the protein amount loaded, gels were stained with SimplyBlue™ SafeStain Coomassie® blue G250 (Invitrogen) or with InstantBlue™ (Expedon, California, U.S.A.), or with SilverXpress® Silver Staining Kit (Invitrogen) (for a more sensitive staining of proteins) according to each manufacturer's instructions.

### 3.5.7 Western Blot

After electrophoresis, the gels were transferred to the membranes with a semi-dry system (Amersham Hoefer TE 70 transfer unit (GE Healthcare)), blocked overnight with 5% milk (skim milk for

microbiology, Merck) in 0.05% Tween (tween 20 (for synthesis), Merck), Tris buffered saline (TTBS) (Merck) solution and then incubated with the antibodies. The primary antibodies used (dilution with TTBS and albumin fraction V ( $\geq 98.0\%$ , Merck) were anti-His primary antibody (1:100, Santa Cruz), anti-CES2 primary antibody (1:200, Sigma) while anti-mouse alkaline phosphatase conjugated secondary antibody (1:2000, Sigma) (for colorimetric detection), anti-rabbit alkaline phosphatase conjugated secondary antibody (1:2000, Sigma) (for colorimetric detection) and anti-rabbit ECL HRP (horseradish peroxidase) conjugated secondary antibody (1:20000, GE Healthcare) were used as secondary antibodies.

Two types of detection were made in western blot assays and in each case following the manufacturer's instructions - the colorimetric detection using NBT/BCIP reagent (Pierce Biotechnology) in a nitrocellulose membrane (GE Healthcare), or a chemiluminescent detection using Amersham ECL Plus™ Western Blotting Detection Reagent (GE Healthcare) in a PVDF (Polyvinylidene Fluoride) membrane (Millipore), for more sensitive detections. Image acquisition was performed with ChemiDoc and quantified using ImageJ (<http://rsbweb.nih.gov/ij>). At least three images, acquired with different exposure times, without saturation of the obtained signal were used for quantification.

To simplify the comprehension of the presented Western Blots, the showed range corresponds to the area where CES2 protein may be (range from 62 to 49 kDa from the SeeBlue® Plus2 Ladder).



## 4. Results and Discussion

### 4.1 Development of a mammalian expression vector plasmid

The first step of this work was to develop a mammalian expression vector suitable for the expression of *CES2* gene with a 10x Histidine tag (for purification purposes) localized at the C-terminus of the gene (and subcontract its construction), in HEK-293T cells.

*CES2* (GeneID: 8824) gene is localized in the chromosome 16q22.1 (55, 56, 83) and its sequence is available in public databases (such as Entrez Gene (NCBI) (<http://www.ncbi.nlm.nih.gov/gene>), Ensembl (<http://www.ensembl.org/index.html>), UniProt (<http://www.uniprot.org/>), etc.). Its codifying sequence is present in table 4.1:

**Table 4.1:** *CES2* codifying gene sequence (Entrez Gene database)

CES2 codifying gene sequence
<p><b>atg</b>actgctcagtcccgtctcctaccacacccaccttcccggccaagccagcgcaccccgtgactccctgccagtccaaactccaagg  ctgggcaaggcactgatccactgctggacagaccggggcagcctctgggtgaacagcagcgtgtccgccggcagcgaaccgagaccag  cgagccgaccatgaggctgcacagactctgtgctggctgagcggctggcctgtggctctgtctctgtccggggccagggccagga  ctcagccagtcccatccggaccacacacacggggcaggtgctggggagtctgtccatgtgaagggcgccaatgccggggccaacctcc  tgggaattccattgccaagccactctaggtccgctgctgattgcaccccctgagccccctgaatctggagtggtgtgagggatggaaccac  catccggccatgtgtctacaggacctaccgcagtgaggctcagagttcttagccagttcaacatgacctccctccgactccatgtctgaggact  gcctgtacctcagcatctacacgccggccatagccatgaaggctctaacctgccggtgatgggtggatccacgggtggcgtgttttggca  tggcttctgtatgatggttccatgctggctgctggagaacgtgggtggatcatccagtaccgctgggtgctggcttctcagcactg  gagacaagcacgcaaccggcaactggggctacctggaccaagtggctgactacgctgggtccagcagaatatgcccactttggaggca  acctgaccgtgtcaccattttggcgagtctggggtggcagagtgcttctgctgtgtccccatataccaaggactctccacggagcc  atcatggagagtggtggcctcctgcccggcctcattgccagctcagctgatgtcatctccacgggtggccaacctgtcctgtgacca  agttgactctgaggccctggtggctgctgccccggaagagtaaagaggagattctgcaattaacaagccttcaagatgacccggagtg  gtggatgggtcttctgcccaggcaccaccaggagctgctggcctctccgacttcagcctgtccctagcattgttggttcaacaacaatgaa  ttcggctggctcatcccaaggctatgaggatctatgataccagaaggaatggacagagaggcctcccaggctgctctgcagaaaatgta  acgctgctgatgtgctcctacatttggtgacctgctgaggaggagtacattggggacaatggggatccccagaccctccaagcgcagttcc  aggagatgatggcgactccatgtttgatccctgactccaagtagcacatttctcagttccggggcccctgtgtactctacagattccagcat  cagcccagctggctcaagaacatcaggccaccgcacatgaaggcagaccatggtgatgagcttctttgtttcagaagtttcttgggggcaa  ctacattaaattcactgaggaagaggagcagctaagcaggaagatgatgaagtactggccaactttgagagaaatgggaacccaatggc  gagggtctgccacactggccgctgttcgaccaggaggagcaatactgcagctgaacctacagcctgctggggccgggctctgaaggccc  acaggctccagttctggaagaaggcgtgccccaaaagatccaggagctcgaggagcctgaagagagacacacagagctgtag</p>

This gene originates two protein isoforms (1 and 2) produced by alternative splicing (51). *CES2* isoform 1 protein is the longest one and it differs from *CES2* isoform 2 sequence in the aminoacids “GDELPVFRSFFGGNYI” which are replaced by the aminoacid “V” (valine), as it can be seen in the

alignment of both sequences in the freeware software BioEdit (www.mbio.ncsu.edu/bioedit/bioedit.html) - Figure 4.1.1.



Figure 4.1.1: Sequence alignment of CES2 protein isoforms 1 and 2 (NCBI database).

Also, there are some differences regarding CES2 protein sequence lengths between NCBI (longer protein sequences) and Uniprot databases for both isoforms (Table 4.2). These differences affect only the beginning of the reading frame (in the UniProt database, the sequence begins with the start codon "ATG", while in the NCBI database there are several nucleotides before the start codon), being the codifying sequence the same, for both cases.

In the literature, the commonly found protein weight for CES2 protein is around 60 kDa. This protein weight usually results from SDS-PAGE estimation (41).

**Table 4.2:** Available data present in two different databases regarding the two CES2 isoforms

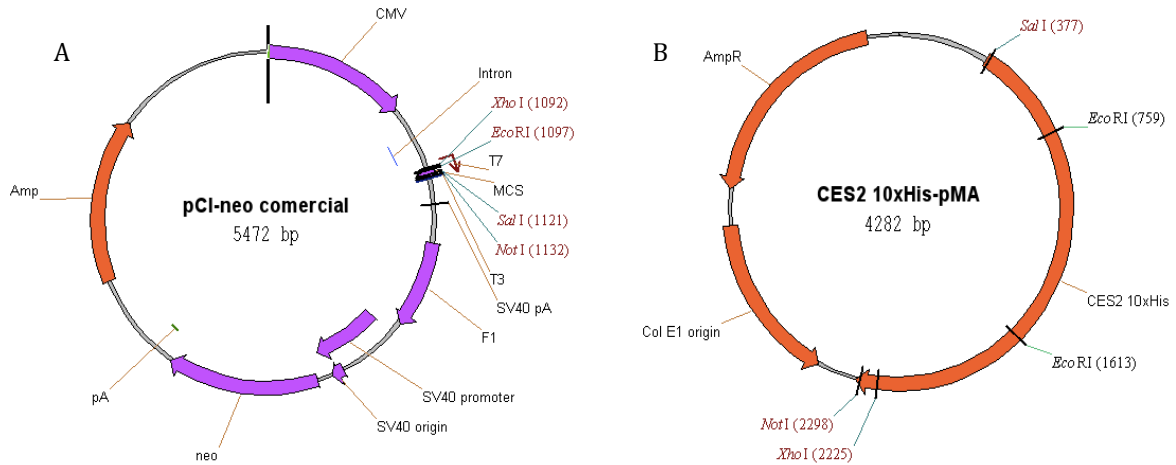
	Database	ID	Aminoacid sequence
			length
<b>CES2 isoform 1</b>	NCBI	NP_003860	623
	UniProt	O00748-1	559
<b>CES2 isoform 2</b>	NCBI	NP_932327	607
	UniProt	O00748-2	543

After searching commercially available plasmids with C-terminally localized 10xHistidine tag suitable for high protein expression levels in mammalian cells, it became evident that the easiest and more economical path was to synthesize the gene with the tag and clone the sequence into a mammalian expression vector, in this case, pCI-neo mammalian expression vector, since it is a vector for studying constitutive expression of genes in mammalian cells. This high-copy vector carries the human cytomegalovirus (CMV) enhancer and promoter region (allows for strong, constitutive expression in a variety of mammalian cell types), which promotes constitutive expression of cloned DNA inserts in mammalian cells. The pCI-neo vector also contains the SV40 enhancer and early promoter region upstream of the neomycin phosphotransferase gene. The SV40 early promoter contains the SV40 origin of replication, which will induce transient, episomal replication of the pCI-neo vector in cells expressing the SV40 large-T antigen.

The histidine tag was placed in C-terminal due to the presence of a signal peptide in the N-terminus. Thus, for the construction of the vector, the *CES2* gene was synthesized by GeneArt® (Life Technologies Corporation) with new features, such as:

- Encoding sequence for a 10xHistidine Tag, to perform affinity chromatography for the protein purification;
- Kozak consensus sequence (“GCCACC”), since it plays a major role in the initiation of the translation process in eukaryotic cells;
- *AfeI* restriction site between the end of *CES2* gene (without the stop codon) and the 10xHis tag; this restriction site will enable if desired, in the future, to add additional features between the gene and the tag (such as a proline arm and/or a protease recognition sequence);
- A stop codon after the 10xHis tag;

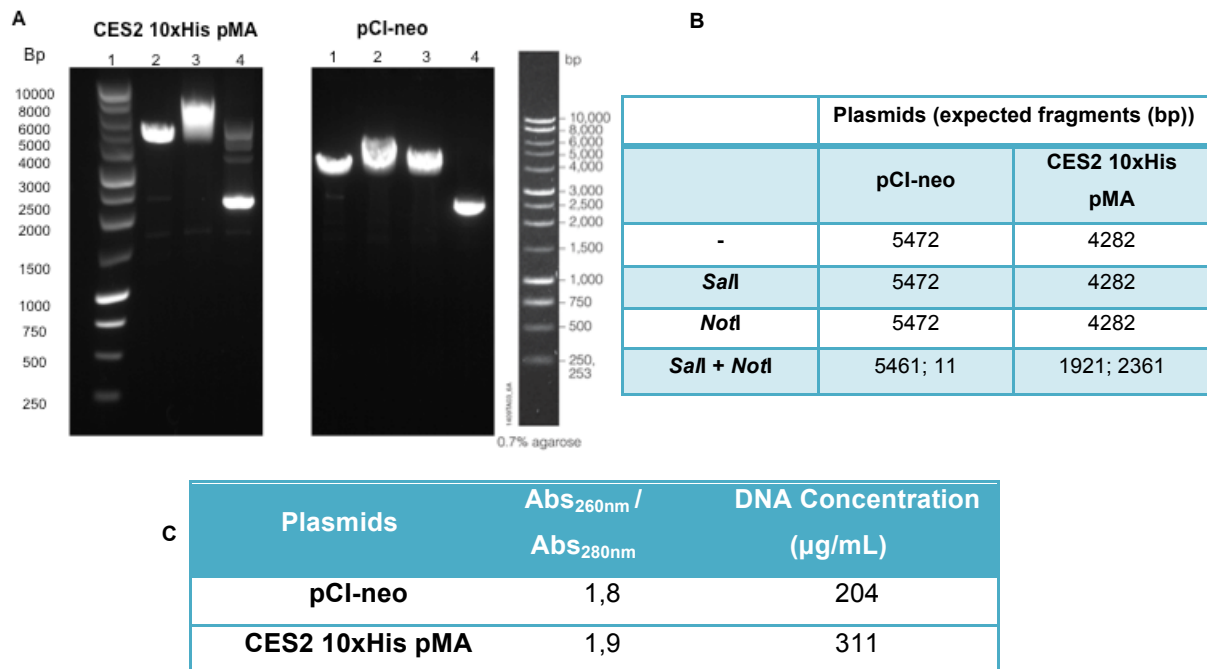
Also, the gene has a *SaI*I recognition sequence added to the 5' region and a *NotI* recognition sequence in the C-terminus, to clone this gene into pCI-neo (Figure 4.1.2 A). The synthesized gene was inserted in the pMA plasmid (Figure 4.1.2 B) for replication in bacteria only.



**Figure 4.1.2:** Schematic representation of commercial pCI-neo (A) and CES2-10xHis-pMA (B) plasmids.

Transformation of DH5- $\alpha$  Library Efficiency bacteria with the mentioned plasmids was performed in order to have a working plasmid bank. 3-4 mL of transformed bacteria were grown in TB/LB medium and the purification of the plasmids was performed from these bacteria with QIAprep<sup>®</sup> Miniprep, following the manufacturer's instructions.

The quality and presence of both *SalI* and *NotI* recognition sequences in the pCI-neo and CES2-10xHis-pMA plasmids (Figure 4.1.3) were assessed. Also, enzymatic restriction assays were performed using specific restriction enzymes - *SalI* and *NotI* - in order to confirm the plasmid sequence, and the resulting fragments were analyzed by DNA gel electrophoresis, using 0.8% (w/v) agarose gels, as previously described in Materials and Methods Section (Figure 4.1.3 A). The results are shown in Figure 4.1.3. The overall plasmid purity, assessed by the  $Abs_{260nm}/Abs_{280nm}$  is high (to assess protein contamination), as well as the plasmid concentration (Figure 4.1.3 C). The DNA fragments obtained after gel electrophoresis correspond to the predicted weights (Figure 4.1.3 B), confirming the high purity and composition of both plasmids.



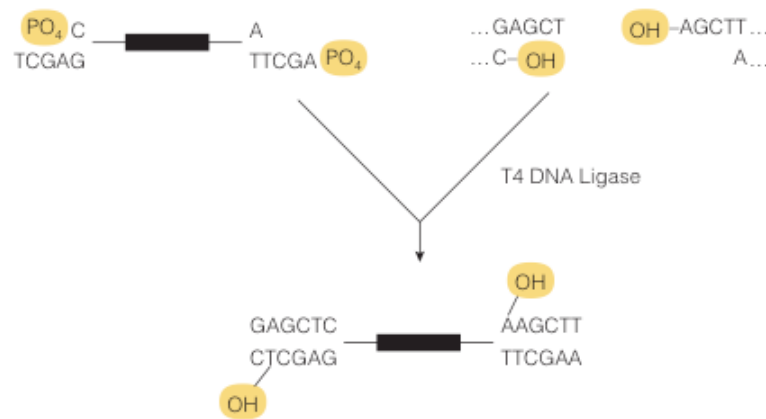
**Figure 4.1.3:** pCI-neo and CES2-10xHis-pMA characterization. **A.** Gel electrophoresis of the enzymatic digested products of pCI-neo and CES2-10xHis-pMA purified plasmids. Both plasmids were digested with *SaI* and *NotI* restriction enzymes. Lanes for CES2-10xHis-pMA: 1. BenchTop 1 Kb DNA Ladder; 2. Plasmid digested with *SaI* restriction enzyme 3. Plasmid digested with *NotI* restriction enzyme; 4. Plasmid digested with both *SaI* and *NotI* restriction enzymes; Lanes for pCI-neo: 1. Plasmid digested with *SaI* restriction enzyme 2. Plasmid digested with *NotI* restriction enzyme; 3. Plasmid digested with both *SaI* and *NotI* restriction enzymes; 4. Non-digested plasmid; **B.** Expected fragment lengths, in base pairs (bp) after enzymatic restriction. **C.** Purity and concentration for both plasmids.

Once the purity and the quality were assessed, enzymatic restriction assays (with *SaI* and *NotI* restriction enzymes) were performed in both plasmids (pCI-neo and CES2-10xHis-pMA), in order to clone the *CES2* gene in the pCI-neo vector. Preventing vector self-ligation is critical for reducing subcloning background (31). The efficiency of ligating of the plasmid to itself is far higher than ligating a separate piece of DNA into the vector and is the favored reaction, thus removing the 5' phosphates of the linearized vector will prevent T4 DNA Ligase from re-circularizing the vector (31). The Antarctic Phosphatase (New England Biolabs) enzyme was used to dephosphorylate the digested linear pCI-neo vector. After dephosphorylation, the enzyme and the 11 bp fragments were removed by a DNA purification system (illustra GFX PCR DNA and Gel Band Purification Kit, GE Healthcare) following the manufacturer's instructions.

The two fragments from CES2-10xHis-pMA plasmid originated by the double enzymatic restriction assay are far too big to be separated directly with this kit, so the approach for isolating the *CES2* gene had to be different than what was performed for pCI-neo. The resulting fragments from the enzymatic restriction assay with *SaI* and *NotI* were analyzed by DNA gel electrophoresis, as previously described in the Materials and Methods Section. The gel ran for a longer period of time to allow the

separation of the two fragments (collapsed in the lower band of the gel in Figure 4.1.3 A). The lightest band was removed from the gel and then purified using the DNA purification system (illustra GFX PCR DNA and Gel Band Purification Kit), following the manufacturer's instructions.

The ligation between pCI-neo and CES2-10xHis was performed with T4 DNA Ligase (New England Biolabs), which will catalyze the formation of a phosphodiester bond between adjacent nucleotides with the concomitant hydrolysis of ATP to AMP and inorganic phosphate (Figure 4.1.4). The ligation mechanism occurs in three stages. First is the formation of an enzyme-nucleotide intermediate through transfer of an adenylyl group (AMP) from ATP to the  $\epsilon$ -amine group of a lysine residue in the enzyme. This results in the release of pyrophosphate from ATP. Second, the adenylyl group is transferred from the enzyme to the 5'-phosphate of the DNA, thereby activating it. Finally, a phosphodiester bond is formed by nucleophilic attack of the 3'-hydroxyl group of the DNA with concomitant release of AMP.



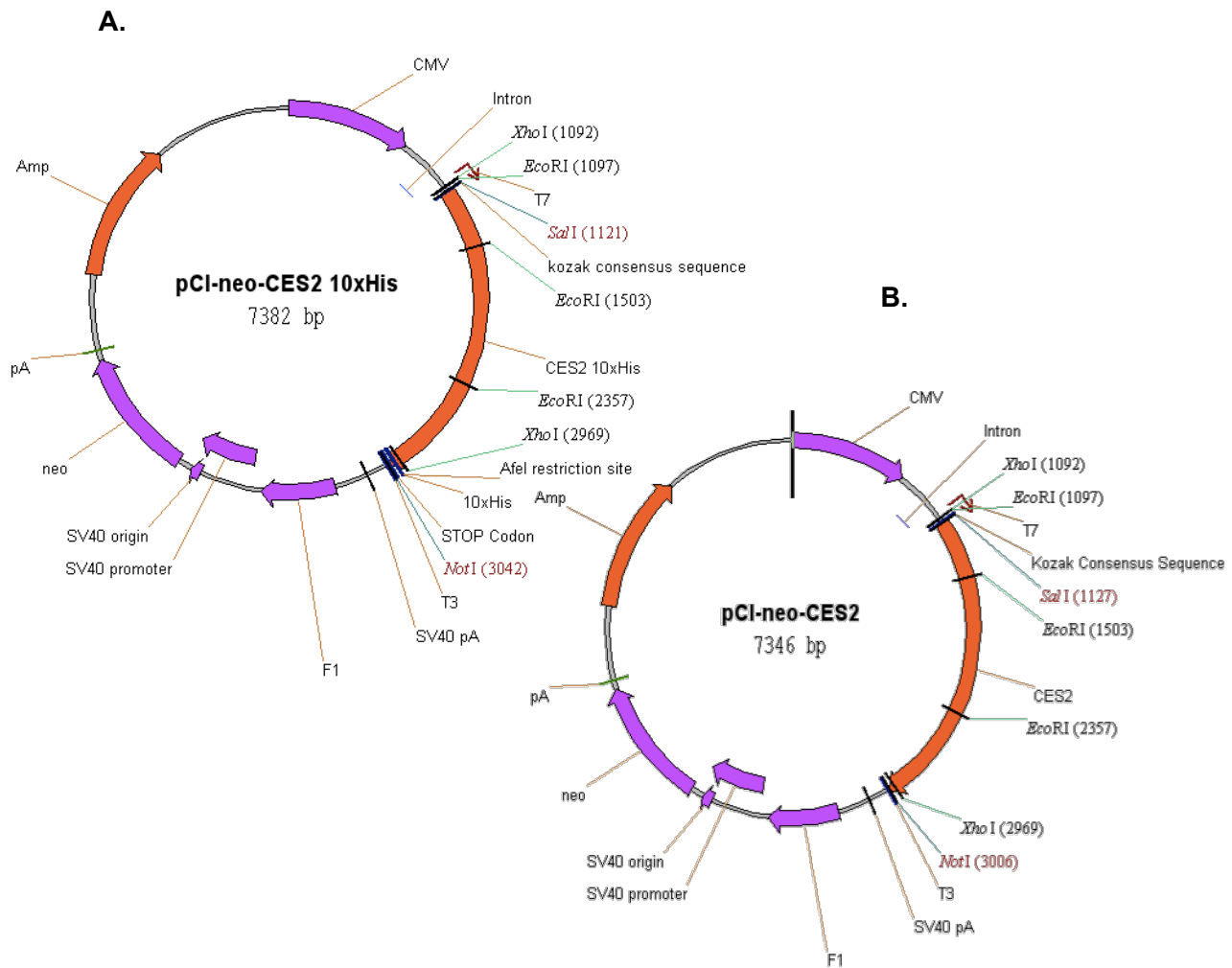
**Figure 4.1.4:** Illustrative representation of T4 DNA Ligase reaction mechanism. Adapted from (31).

Different molar ratios of vector:insert DNA were tested to clone the fragment into the plasmid vector - 1:1 and 1:3. 100 ng of vector were used and the amount of the insert was calculated accordingly to the following equation (31) (Equation 1):

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}} = \text{ng of insert} \quad (1)$$

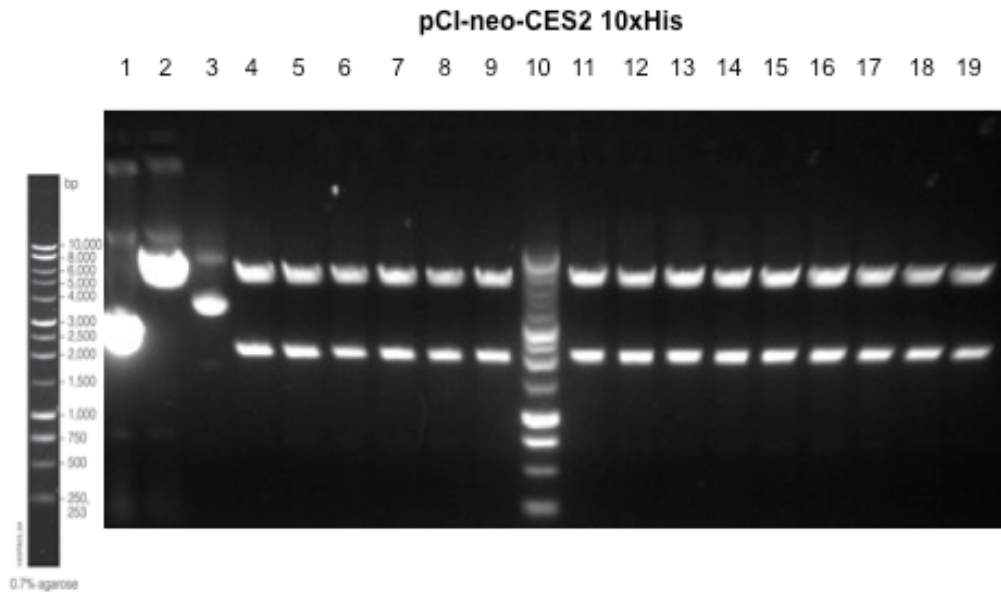
To confirm the success of the ligation process, an enzymatic restriction assay with *Xho*RI restriction enzyme was performed. This enzyme cleaves inside the *CES2-10xHis* gene and also in the pCI-neo vector (two recognition sequences in the *CES2-10xHis* gene, one in the pCI-neo plasmid and none in the pMA plasmid). The choice of the restriction enzyme is very important, because it is fundamental to distinguish the positive ligation product (pCI-neo-*CES2-10xHis* - Figure 4.1.5 A) from

the negative ones (pCI-neo-pCI-neo and pCI-neo-pMA). In this figure it is also presented the representation of pCI-neo-CES2 (Figure 4.1.5 B). pCI-neo-CES2 and pCI-neo-CES2-10xHis were further used in the subsequent studies.



**Figure 4.1.5:** Illustrative representation of pCI-neo-CES2-10xHis (A) and pCI-neo-CES2 (B) plasmids.

DH5- $\alpha$  bacteria Library Efficiency transformation with the ligation product was performed followed by an enzymatic restriction assay with purified plasmid from these bacteria (15 different clones were analyzed). The resulting fragments from the enzymatic restriction assay with *XhoI* were analyzed by DNA gel electrophoresis. The results are shown in Figure 4.1.6.



**Figure 4.1.6:** Confirmation of the ligation product (pCI-neo-CES2-10xHis). Gel electrophoresis of the enzymatic reaction products of purified DNA from 15 clones transformed with the ligation product. The DNA was digested with *XhoI* restriction enzyme. Non-digested and digestion with *XhoI* of pCI-neo plasmid are present in lane 1 and 2, respectively. Non-digested clone is present in lane 3. The 15 digested clones with *XhoI* restriction enzyme are in lanes 4 to 19.

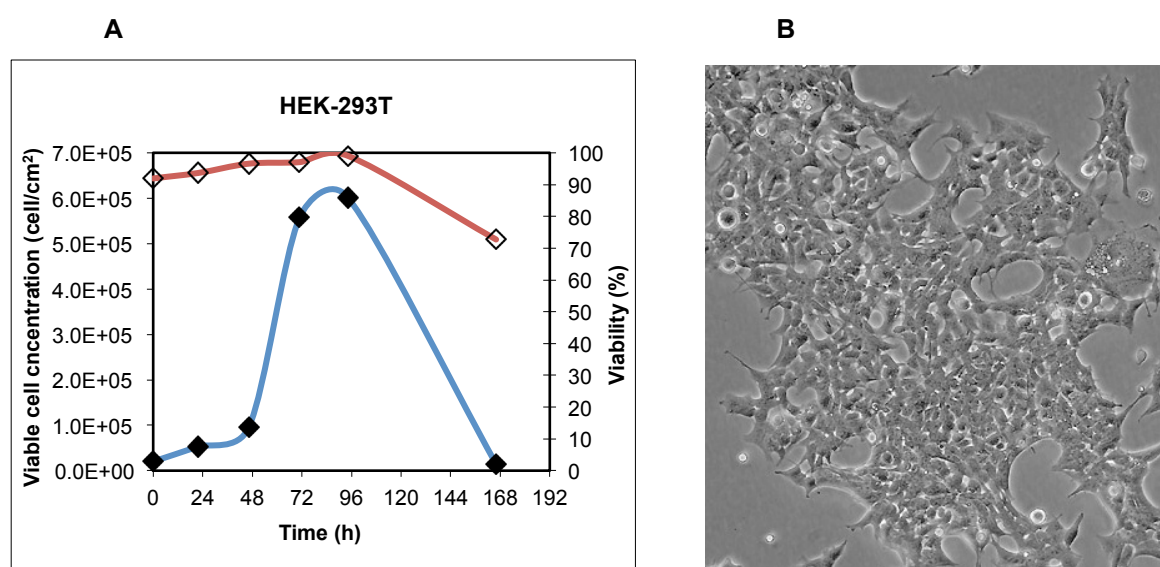
As it can be seen in Figure 4.1.6, all of the analyzed clones had a positive product ligation - pCI-neo-CES2-10xHis. *XhoI* restriction enzyme cleaves once in pCI-neo vector (thus, a ligation vector-vector that would correspond to a false positive, would give a band in the same size of pCI-neo vector digested with *XhoI* restriction enzyme - 5472 bp). *XhoI* restriction enzyme cleaves once in *CES2-10xHis* gene (and it does not cleave in the other fragment of the GeneArt Plasmid (pMA)). Thus, a true positive result would give two fragments with 5505 and 1877 bp.

Since the goal was to have the best plasmid as possible to proceed for testing in mammalian cells, the *CES2-10xHis* and *CES2* genes were both sequenced (sequencing performed by Macrogen, Seoul, Korea) to assure that during plasmid ligation and amplification no DNA mutations were introduced by the bacteria cellular mechanisms. The sequencing reactions were performed with 4 primers: RV T3 universal primer ("ATTAACCCTCACTAAAG", binds to T3 in pCI-neo), which is a reverse primer - the reaction result gives the final nucleotides from the gene (stop codon, histidine tag (for the pCI-neo-CES2-10xHis), etc.); the reaction with T7 FW primer ("AATACGACTCACTATAG", forward primer, binds to T7) gives the first nucleotides from the gene (Kozak consensus sequence, start codon), etc); *CES2*-RV ("ACATCAGCAGCGTTAACATTTTCTG" synthesized primer) and EBV-RP ("GTGGTTTGTCCAAACTCATC") primers give the rest of the nucleotide sequence. After aligning the theoretical sequence with the ones obtained by the sequencing reactions, it is possible to confirm that, in fact, all features were added to the *CES2* gene (*AfeI* restriction site, 10xHistidine tag, Kozak consensus sequence) for the pCI-neo-CES2-10xHis, and there were no mutations in both genes (*CES2* and *CES2-10xHis*).

## 4.2 CES2 production in transfected HEK-293T adherent cells

HEK-293T cells were selected because they are mammalian cells and therefore, are capable to perform elaborate post-translational modifications, such as glycosylation, that prokaryotes are not (25). In addition, they have desirable characteristics such as rapid growth and high transfectability. Other important aspects of this cell line are that it enables episomal replication of SV40 origin of replication containing plasmids and that they grow easily in suspension, thus enabling the scale-up of the production process (7).

Before performing a transfection with the previously described plasmids, first it was conducted a growth study (Figure 4.2.1) of HEK-293T adherent cells to better understand their growth. Briefly, T-25 flasks were inoculated with  $4 \times 10^4$  cell/cm<sup>2</sup> and each day an inoculated T-25 flask was removed to determine cells concentration and viability.



**Figure 4.2.1:** Adherent HEK-293T cell growth kinetics. **A.** HEK-293T viable adherent cell concentration (blue) and viability (red) throughout time **B.** HEK-293T cells (image from ATCC).

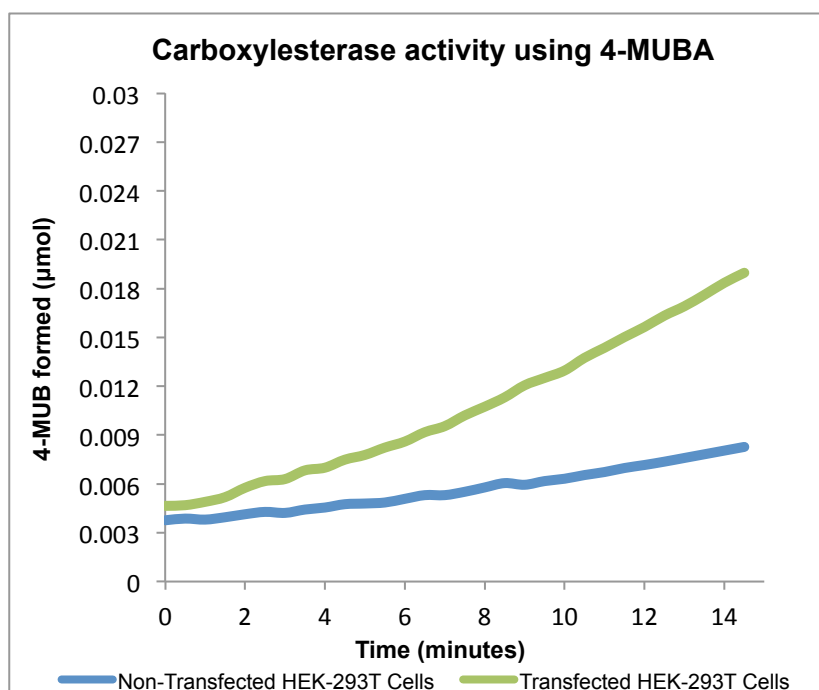
It can be observed, 72 - 96 h after cell inoculation the cells reach approximately 80% of confluence, which means that this is the ideal period of time for cell splitting. Also, after cells reached 100% confluence, adherent cells viability decreases as well as cell concentration.

After successful amplification and purification of the plasmids, transient transfection of HEK-293T cells was performed to express a functional recombinant CES2 protein. The transfections assays were carried out with pCI-neo-CES2-10xHis in order to have a recombinant protein to purify and with pCI-neo-CES2 to have a positive control sample for analytical assays. From all the available strategies to perform the transfection, as mentioned above, it was decided to adopt a non-viral gene delivery approach by using PEI due to its simplicity of use.

As described above, 48 h after transfection cells were harvested and lysed with M-PER® Mammalian Extraction Reagent (Pierce Biotechnology), since CES2 is anchored to the endoplasmic reticulum membrane, as previously mentioned in the Introduction Section. Total protein concentration in each extract was determined.

To assess if there was an increase in esterase hydrolytic activity in CES2 transiently transfected HEK-293T cells extracts, the enzymatic kinetic profiles were spectrophotometrically determined from the hydrolysis of 4-methyl-umbelliferyl acetate (4-MUBA) to 4-methyl-umbelliferone (4-MUB), at 37 °C and compared to the non-transfected HEK-293T cell extracts. 4-MUBA is a general CES substrate and was used during this work, since it is more stable than the classical CES substrate *p*-nitrophenyl acetate (*p*-NPA), and it is not hydrolyzed by cholinesterases, unlike *p*-NPA (84).

As it can be seen in Figure 4.2.2, transiently transfected cell extracts show increased carboxylesterase activity towards 4-MUBA, comparing with non-transfected cell extracts, validating the used transfection approach. The same conclusion can be reached by calculating the total activity in each sample - transfected cells have an activity of  $0.143 \mu\text{mol}\cdot\text{min}^{-1}$  against  $0.046 \mu\text{mol}\cdot\text{min}^{-1}$  of non-transfected cells. Also with these results, it's possible to observe that these cells have a basal activity towards 4-MUBA.

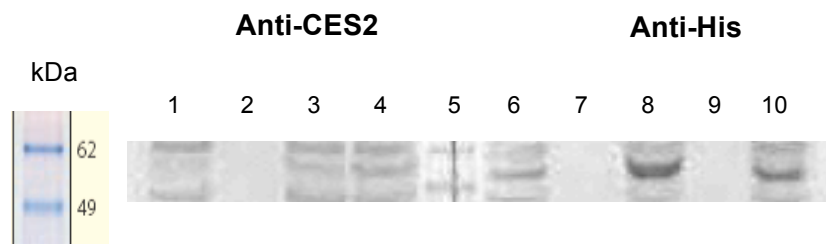


**Figure 4.2.2:** Carboxylesterase kinetic profiles in different cell extracts - transfected HEK-293T cells with pCI-neo-CES2-10xHis (green) and non-transfected cells (blue).

To further analyze the presence of CES2 protein in the cellular extracts, a western blot was also performed (Figure 4.2.3) with a SDS-PAGE electrophoresis (denaturing conditions), using NuPAGE® Bis-Tris 4-12% gels (Invitrogen). These two analytical tests (enzymatic assays and western blot) are complementary, since not always the increase in the amount of protein reflects also an increase in the

enzymatic activity, for CES proteins in particular (62). Protein precipitation was performed with ice-cold absolute ethanol at -20 °C, as previously described in the Materials and Methods section.

After electrophoresis, the gel was transferred to a nitrocellulose membrane (Amersham) and incubated with anti-CES2 (1:200, Sigma) and anti-rabbit alkaline phosphatase conjugated secondary antibody (1:2000, Sigma) - lanes 1 to 5 and with anti-His primary antibody (1:100, Santa Cruz) and anti-mouse alkaline phosphatase conjugated secondary antibody (1:2000, Sigma) - lanes 5 to 10. The use of these two antibodies allows for a double control in this process - for one hand it's possible to detect the protein itself and in another hand is possible to detect the histidine tag attached to the recombinant protein. Detection was performed by addition of NBT/BCIP reagent (Pierce Biotechnology). Since this work describes a process, colorimetric detection is useful in order to see major differences between samples.



**Figure 4.2.3:** Recombinant CES2 expression in cell extracts. Western Blot picture (range from 62 to 49 kDa from the SeeBlue® Plus2 Ladder, with 200 µg of total protein in all lanes. Lanes 1 to 4 were incubated with anti-CES2 while lanes from 6 to 10 were incubated with anti-His. Lanes: 1. Cell extract from non-transfected cells; 2 empty lane; 3. Cell extract from transfected cells with pCI-neo-CES2-10xHis; 4. Cell extract from transfected cells with pCI-neo-CES2; 5. SeeBlue® Plus2 Ladder (Invitrogen); 6. Cell extract from non-transfected cells; 7. Empty lane; 8. Cell extract from transfected cells with pCI-neo-CES2-10xHis; 9. Empty lane; 10. Cell extract from transfected cells with pCI-neo-CES2.

As it can be seen in Figure 4.2.3, the CES2 recombinant protein is in fact being produced in transfected HEK-293T cell extracts (lanes 3 and 8). As described before, protein weight for CES2 protein is around 60 kDa. Also, it is possible to see that recombinant CES2 is present in the cell extracts from HEK-293T transfected with both pCI-neo-CES2-10xHis (lane 3) and pCI-neo-CES2 (lane 4) but not in the cell extracts from non-transfect HEK-293T cells (lane 1). Incubation with anti-His antibody reveals that CES2-10xHis has in fact the histidine tag (lane 8), since this antibody was raised against His-tagged (polyhistidine expression) recombinant proteins, unlike the regular recombinant CES2 (lane 10) and non-transfected cells (lane 6). Also, bands corresponding to unspecific binding of the antibody (lanes 6 and 10) are present; however, these interactions are less intense than the interaction with CES2-10xHis.

### 4.3 CES2 purification from transfected HEK-293T adherent cell extracts

To perform the purification of recombinant CES2 protein, cell extracts from transfected HEK-293T cells and from non-transfected cells (as a negative control) were used.

As the recombinant CES2 protein has a C-terminal Histidine tag, a metal ion affinity strategy can be used in order to ease the purification process. ÄKTAexplorer™ 10S System, a purification platform based on chromatographic analysis and separation, and HiTrap™ Chelating HP Column of 1 mL at 4 °C were used to purify the protein. The system perform absorbance readings at 280 nm, which allows to follow the process, since proteins absorb ultraviolet light at this wavelength (85).

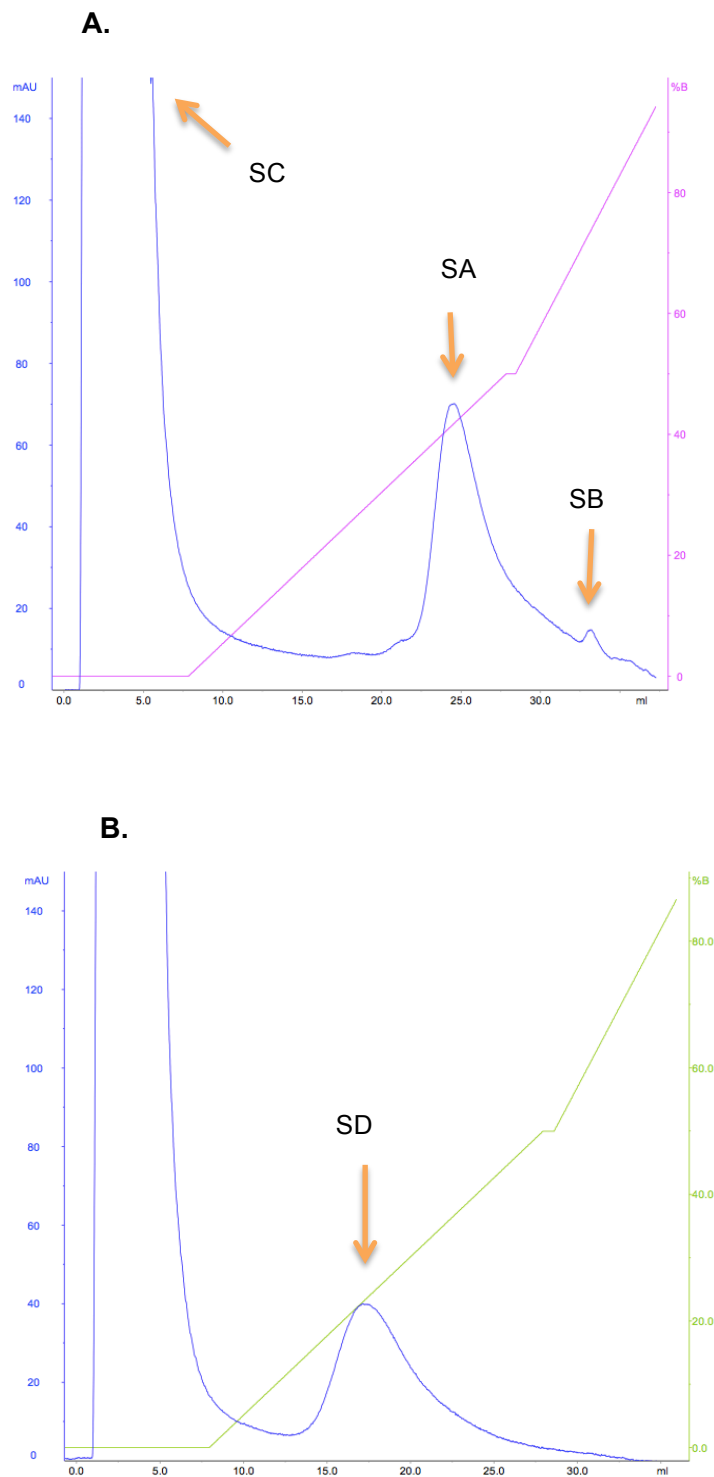
As it can be seen in Figure 4.3.1 A, three main peaks could be noticed in the chromatogram of the purification of cell extract from transfected HEK-293T cells (marked by orange arrows):

- The first fraction (the larger one), eluted with Wash buffer, corresponding to the flowthrough - sample that did not bind to the column (SC);
- A second fraction (medium), corresponding to the first eluted peak - sample eluted with 225 mM of imidazole (SA);
- A third fraction (the smaller one), corresponding to the second eluted peak (SB) - sample eluted with 350 mM of imidazole.

All of the purified samples were loaded into a desalting column (Sephadex™ G-25) to remove the imidazole present in the elution buffer. This is a critical step, since this compound damages the CES2 enzyme activity. Samples were eluted in Storage Buffer, filtered in sterile conditions and properly stored (with 20% glycerol at -80 °C), as described in Materials and Methods section.

Only two peaks could be noticed in the chromatogram of the purification of cell extract from non-transfected HEK-293T cells (Figure 4.3.1 B): the first one (the larger one), eluted with the addition of the wash buffer and the second one eluted in the beginning of the gradient with Elution buffer (with 125 mM of imidazole).

Only the sample corresponding to the eluted peak (with 125 mM of imidazole) (marked by an orange arrow in figure 4.3.1 B) was collected (SD) during this purification process. This sample was injected into the desalting column to remove imidazole and it was stored at -20 °C without the addition of glycerol.



**Figure 4.3.1:** Purification chromatograms of recombinant CES2 purification. Imidazole gradient is represented in purple (v/v) (%) while in blue is the absorbance at 280nm (mAU). The collected samples are indicated with oranges arrows. **A.** CES2 purification from cell extracts of transfected HEK-293T adherent cells with pCI-neo-CES2-10xHis; **B.** CES2 purification from cell extracts of non-transfected HEK-293T adherent cells.

Total protein concentration of each sample was determined using the BCA™ Kit for samples SC and SA and with the Micro BCA™ Kit to quantify the samples SB and SD. The results are presented in Table 4.3.

**Table 4.3:** Total protein concentration of the collected samples during the protein purification process.

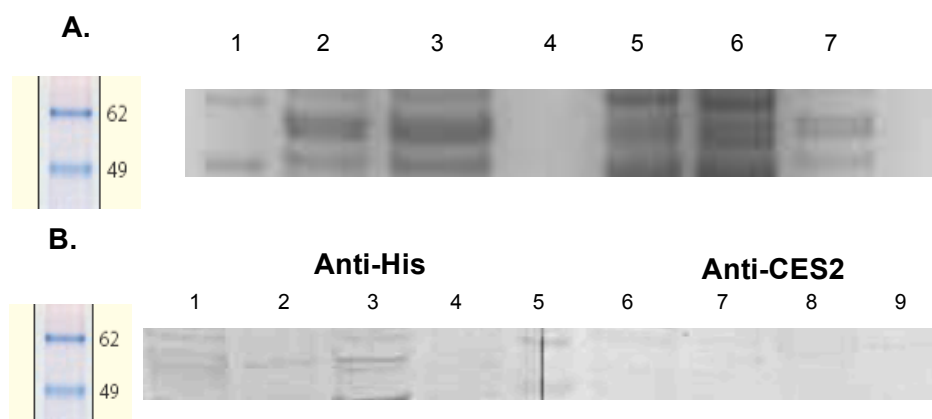
Sample ID	Sample Description	Sample Concentration (µg/µL)
SA	First peak eluted during the gradient of Elution buffer (desalted)	0.045 ± 0.001
SB	Second (smaller) peak eluted during the gradient of Elution buffer (desalted)	- *
SC	First (bigger) peak eluted after addition of Wash buffer (Flowthrough) (desalted)	0.4468 ± 0.0003
SD	Peak eluted during the gradient of Elution buffer (from non-transfected cells) (desalted)	0.014 ± 0.002
-	Cell extract from non-transfected cells	4.93 ± 0.06
-	Cell extract from transiently transfected cells	5.10 ± 0.50

\*The sample was not quantified due to low protein amount (lower than the inferior detection limit of the method).

The data presented on table 4.3 already takes into account for the 20% dilution performed by the addition of glycerol (in the Final buffer). Due to low protein amount in sample B (SB) and consequent impossibility to quantify the total protein amount, for further testing it was assumed that it had the lowest value of the detection limit of the method - 0.0005 µg/µL (over-estimation), since calculations had to be made in order to calculate the required volume to apply in the analytical tests (gel and western blot).

All samples were analyzed by SDS-PAGE electrophoresis, using NuPAGE Bis-Tris 4-12% gels, followed by Coomassie staining (Figure 4.3.2 A). 30 and 60 µg of each sample were loaded into the gels (working above the separation range and detection limit of the gel); however, as some samples (SB and SD) had very low protein content and due to sample limitation, only 10 µg of SD and 0.5 µg of SB were used. Protein precipitation was performed with ice-cold absolute ethanol at -20 °C, as previously described in the Materials and Methods section. The re-suspension of the dried samples was not trivial and in some cases not completely achieved, which may have lead to losses in the loaded amount. Using the same approach as before, all samples were analyzed by Western Blot (Figure 4.3.2 B), using anti-His primary antibody (1:100,) with anti-mouse alkaline phosphatase conjugated secondary antibody (1:2000) and anti-CES2 primary antibody (1:200), with anti-rabbit

alkaline phosphatase conjugated secondary antibody (1:2000). Detection was performed by addition of NBT/BCIP reagent.



**Figure 4.3.2:** Analysis of purified fractions. Gel range from 62 to 49 kDa from the SeeBlue® Plus2 Ladder. **A.** Coomassie stained gel. Lanes: 1. SeeBlue® Plus2 Ladder; 2. 30 µg of SA; 3. 60 µg of SA; 4. 0.5 µg of SB; 5. 30 µg of SC; 6. 60 µg of SC; 7. 10 µg of SD; **B.** Western Blot. Lanes: 1. SA incubated with anti-his primary antibody; 2. SB incubated with anti-his primary antibody; 3. SC incubated with anti-his primary antibody; 4. SD incubated with anti-his primary antibody; 5. SeeBlue® Plus2 Ladder; 6. SA incubated with anti-CES2 primary antibody; 7. SB incubated with anti-CES2 primary antibody; 8. SC incubated with anti-CES2 primary antibody; 9. SD incubated with anti-CES2 primary antibody.

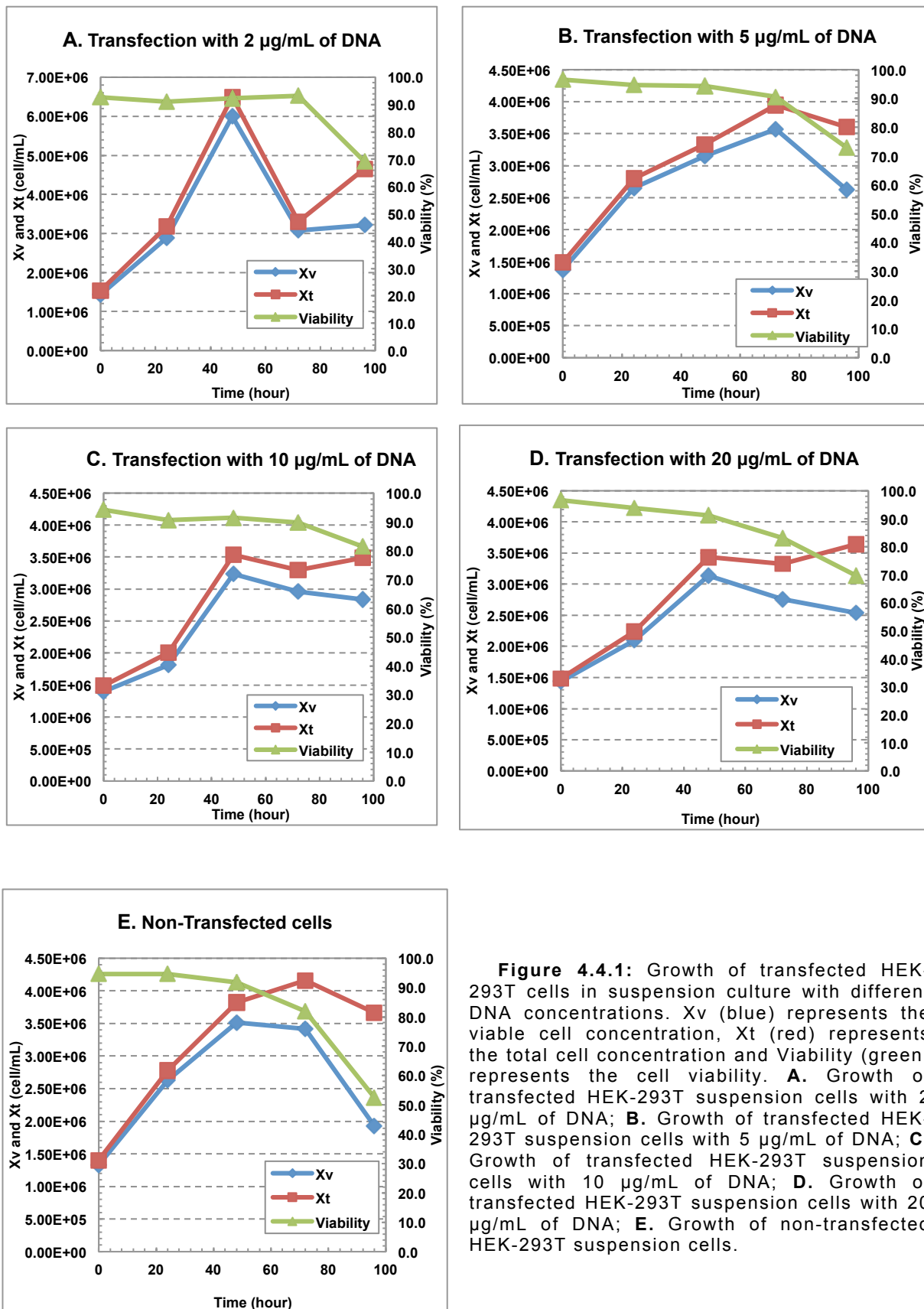
It was not possible to unequivocally detect a protein band corresponding to recombinant CES2 in the Coomassie stained gel. In the western blot (Figure 4.3.2 B), even with a low amount loaded for SB it was possible to detect a faint band that appears to correspond to recombinant CES2. Enzymatic assays were not performed because there was not enough sample left to perform this assay.

With these results, it was obvious that it was necessary to scale up the process in order to purify the amount required of recombinant CES2 protein to proceed to the crystallographic studies and to unequivocally detect it with the analytical assays.

The majority of mammalian cells need to be attached to a solid surface in order to grow, which makes the production process harder, limiting its scalability. Thus, it was chosen to proceed with the process with in-house HEK-293T adapted cells to grow in suspension. The aim was to have the CES2 production in large amounts, and a culture in bioreactor allows achieving the type of the required production. Also, it allows to monitor and control the reaction conditions and parameters, and at the same time minimize aseptic operations, maximize productivity at a lower cost for production in comparison to static conditions (6, 8, 9, 23).

#### 4.4 CES2 production in transfected HEK-293T suspension cells

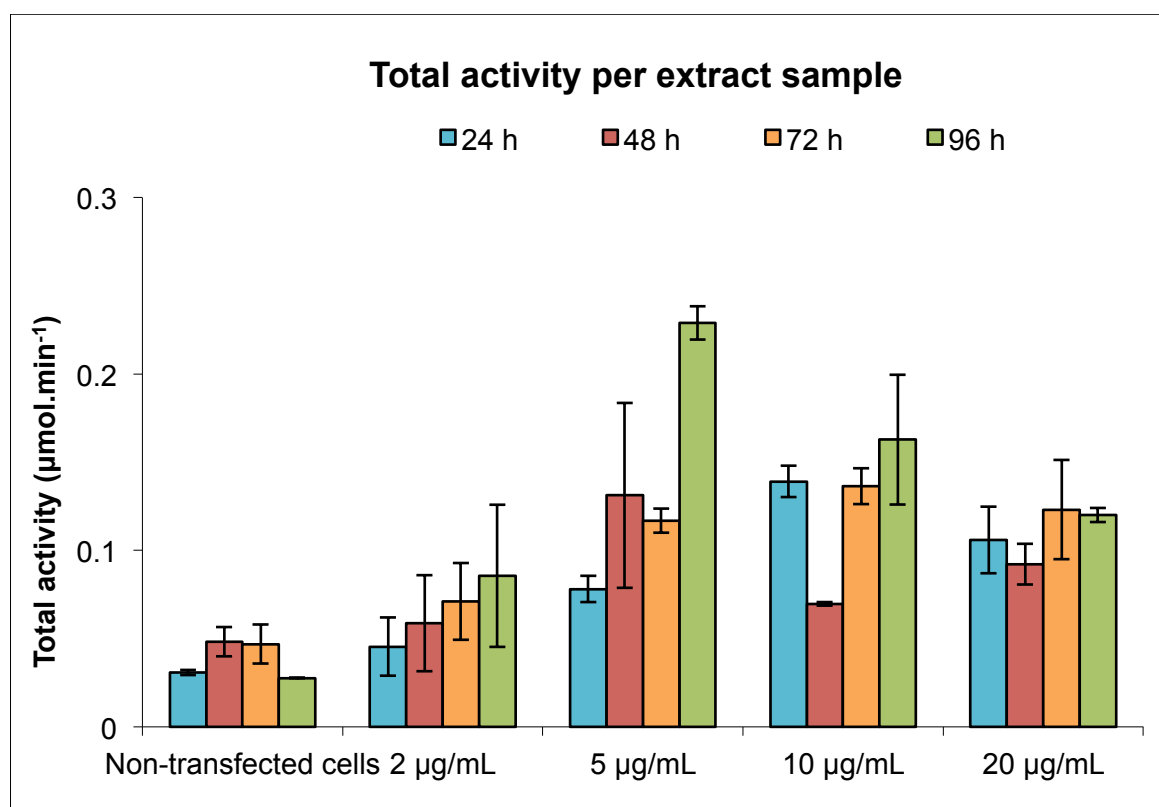
Before performing the culture in bioreactor, it was necessary to optimize the production conditions, namely the DNA concentration to perform the transfection, as well as the culture time to harvest the cells. Optimizing these conditions in bioreactor would be too expensive, so these parameters were optimized in a smaller suspension culture (Erlenmeyer, Figure 4.4.1).



**Figure 4.4.1:** Growth of transfected HEK-293T cells in suspension culture with different DNA concentrations. Xv (blue) represents the viable cell concentration, Xt (red) represents the total cell concentration and Viability (green) represents the cell viability. **A.** Growth of transfected HEK-293T suspension cells with 2 µg/mL of DNA; **B.** Growth of transfected HEK-293T suspension cells with 5 µg/mL of DNA; **C.** Growth of transfected HEK-293T suspension cells with 10 µg/mL of DNA; **D.** Growth of transfected HEK-293T suspension cells with 20 µg/mL of DNA; **E.** Growth of non-transfected HEK-293T suspension cells.

Four different DNA concentrations - 2, 5, 10 and 20  $\mu\text{g/mL}$  - were studied to optimize this parameter. The goal was to test a concentration range between the usual concentration used with these cells, already optimized for monoclonal antibodies production (2  $\mu\text{g/mL}$ ) (80), and the concentration used in the adherent cell transfection (approximately 20  $\mu\text{g/mL}$ ), in order to understand and use, in the future, the best production condition. The growth profile of the transfected cells was followed over time (Figure 4.4.1). Samples were taken in different time points 24, 48, 72 and 96 h, in order to choose the best DNA concentration and culture time where the CES2 production has reached its maximum.

Samples were taken in each time point as described in Materials and Methods Section. The evaluation of the best production conditions of an active recombinant protein was performed by analysing their kinetic enzymatic profile (Figure 4.4.2) and also by western blot (Figure 4.4.3).



**Figure 4.4.2:** Total enzymatic activity of transfected HEK-293T suspension cells with different DNA concentrations (0, 2, 5, 10 and 20  $\mu\text{g/mL}$ ) in different time points (24, 48, 72 and 96 h) for cellular extracts. The represented error bars correspond to three independent activity measurements.

As it can be seen in Figure 4.4.2, from all the tested conditions, 5  $\mu\text{g/mL}$  of DNA at 96 h (transfected cells have an activity of  $0.23 \pm 0.01 \mu\text{mol}\cdot\text{min}^{-1}$  against  $0.048 \pm 0.08 \mu\text{mol}\cdot\text{min}^{-1}$  of non-transfected cells (average  $\pm$  standard error of the mean)), is the condition where the maximum productivity is achieved. The total activity lowers as the amount of DNA increases for 10 and 20  $\mu\text{g/mL}$  of DNA, but is still more than the one obtained with 2  $\mu\text{g/mL}$  of DNA.

Comparing the specific activity (5 µg/mL of DNA at 96 h post-transfection, 0.0577 µmol.min<sup>-1</sup>mg<sup>-1</sup>) with the ones obtained with adherent cells (transfected cells have an activity of 0.0265 µmol.min<sup>-1</sup>mg<sup>-1</sup> against 0.0068 µmol.min<sup>-1</sup>mg<sup>-1</sup> of non-transfected cells), it is possible to point that the total activity appears to increase in the suspension culture assay.

In order to confirm these assessments, the most promising conditions - 5 and 10 µg/mL of DNA - were analysed by western blot (Figure 4.4.3) to check if in fact the observed activity corresponds to CES2 protein. The 24 h time point was not analysed by western blot because it was not a very promising time point (looking to the obtained results in the enzymatic assays). To perform this western blot, anti-His primary antibody (1:100), anti-mouse alkaline phosphatase conjugated secondary antibody (1:2000), anti-CES2 (1:200) and anti-rabbit alkaline phosphatase conjugated secondary antibody (1:2000) were used. Detection was performed by addition of NBT/BCIP reagent.



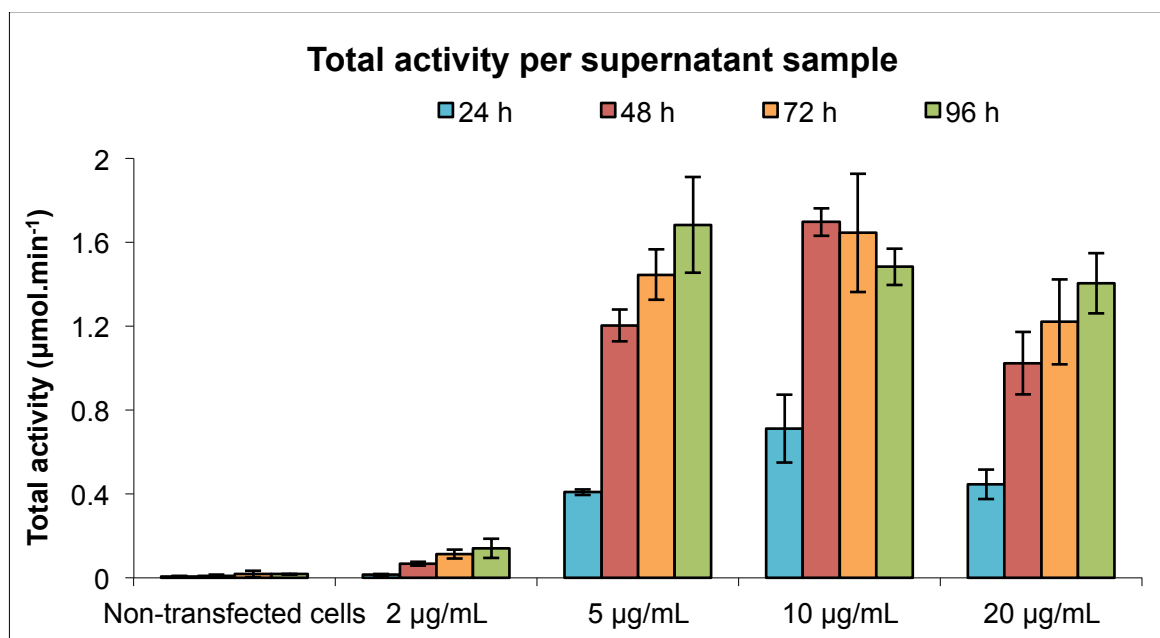
**Figure 4.4.3:** Recombinant CES2 production in cell extracts from HEK-293T cells in suspension. Western Blot gels (range from 62 to 49 kDa from the Ladder) Lanes 1-4 were processed by incubation with anti-His antibody; Lanes 6-9 were processed by incubation with anti-CES2 antibody **A.** Samples obtained from transfection with 5 µg/mL of DNA, 100 µg of total protein applied in all lanes. Lanes: 1. Cell extract from transfected (96 h time point) with pCI-neo-CES2-10xHis; 2 Cell extract from transfected cells (72 h time point) with pCI-neo-CES2-10xHis; 3. Cell extract from transfected cells (48 h time point) with pCI-neo-CES2-10xHis; 4. Cell extract from non-transfected cells; 5. SeeBlue® Plus2 Ladder; 6. Cell extract from transfected cells (96 h time point) with pCI-neo-CES2-10xHis; 7. Cell extract from transfected cells (72 h time point) with pCI-neo-CES2-10xHis; 8. Cell extract from transfected cells (48 h time point) with pCI-neo-CES2-10xHis; 9. Cell extract from non-transfected cells (incubation with anti-CES2); **B.** Samples obtained from transfection with 10 µg/mL of DNA, 50 µg of total protein applied in all lanes. Lanes: 1. Cell extract from non-transfected cells; 2 Cell extract from transfected cells (48 h time point) with pCI-neo-CES2-10xHis; 3. Cell extract from transfected cells (72 h time point) with pCI-neo-CES2-10xHis; 4. Cell extract from transfected cells (96 h time point) with pCI-neo-CES2-10xHis; 5. SeeBlue® Plus2 Ladder; 6. Cell extract from non-transfected cells; 7. Cell extract from transfected cells (48 h time point) with pCI-neo-CES2-10xHis; 8. Cell extract from transfected cells (72 h time point) with pCI-neo-CES2-10xHis; 9. Cell extract from transfected cells (96 h time point) with pCI-neo-CES2-10xHis.

From Figure 4.4.3, it is possible to confirm that the CES2 recombinant protein is in fact being produced in transfected HEK-293T cell extracts, since bands with molecular weight around 60 kDa are present in all lanes except the ones from non-transfected cells. With the incubation with anti-His antibody, it is possible to see that CES2-10xHis has in fact the histidine tag.

Unlike what was performed for adherent cells, for this set of experiments the supernatant was also kept in all time points, in order to check if the protein is being secreted due to masking the KDEL anchoring motif in the C-terminus region. When the *CES2* gene was designed, the histidine tag was positioned in C-terminal, after the signal for permanent retention of proteins in the endoplasmic reticulum (HTEL) (86) (Figure 4.1.5). Thus, the histidine tag may be masking HTEL motif. If the protein

was being secreted, it could justify the low activities obtained in the cell extracts for both adherent and suspension cells production assays.

As previously, the samples were characterized by their kinetic enzymatic profile (Figure 4.4.4) and also by western blot (Figure 4.4.5) to check if the protein was being secreted and if so, in what culture and transfection conditions are the maximum production and activity achieved.

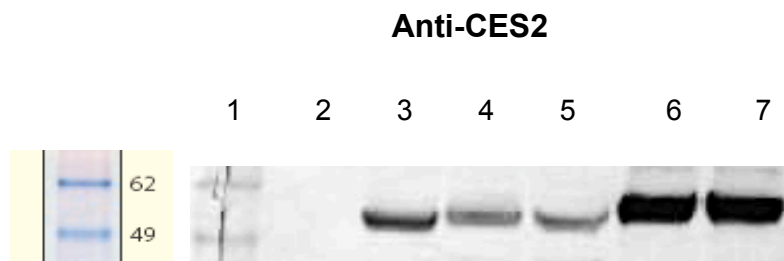


**Figure 4.4.4:** Total enzymatic activity of transfected HEK-293T cells in suspension with different DNA concentrations (0, 2, 5, 10 and 20 µg/mL) in different time points (24, 48, 72 and 96 h) for supernatant samples. The represented error bars correspond to three independent activity measurements.

This figure 4.4.4 it is represented the CES total activity detected in the supernatant and it is possible to state that the protein is mostly being secreted since the kinetic profiles obtained for the supernatant samples are 7-fold higher than the ones obtained for the cellular extracts ( $1.68 \pm 0.23 \mu\text{mol}\cdot\text{min}^{-1}$  (5 µg/mL, 96 h), versus  $0.23 \pm 0.01 \mu\text{mol}\cdot\text{min}^{-1}$  (5 µg/mL, 96 h) (average  $\pm$  standard error of the mean)).

There is a clear productivity increase as DNA concentration is increased from 2 µg/mL to 5 µg/mL in the supernatant samples. The two best conditions are 5 µg/mL and 10 µg/mL. Cell concentration and viability were also evaluated for these two expression vector concentrations (Figure 4.4.1) in order to evaluate differences between these samples and the non-transfected HEK-293T cells, and it was confirmed that there was no decrease in cells concentration in transfected vs. non-transfected cells. However, at 96 h post-transfection, both in transfected and non-transfected cells, there was a decrease in cell viability (Figure 4.4.1). For the cellular extracts, although the tendency is the same as the one observed extracellularly for the different DNA concentrations over time, the higher observed productivity was for the transfection with 5 µg/mL, 96 h.

Thus, samples from the two most promising conditions (5  $\mu\text{g}/\text{mL}$ , 96 h and 10  $\mu\text{g}/\text{mL}$ , 48 h) were analysed by western blot (Figure 4.4.5). However, and since it is not possible to quantify these extracellular samples with the BCA assay (due to interferences in the process), to overcome the detection limit of the process, these samples were concentrated with Vivaspin 15 mL centrifugal concentrators, or centricons, (Sartorius stedim biotech) with a cut-off membrane of 30 kDa). This western blot was made with anti-CES2 (1:200) and anti-rabbit alkaline phosphatase conjugated secondary antibody (1:2000) and detected by the addition of NBT/BCIP reagent.

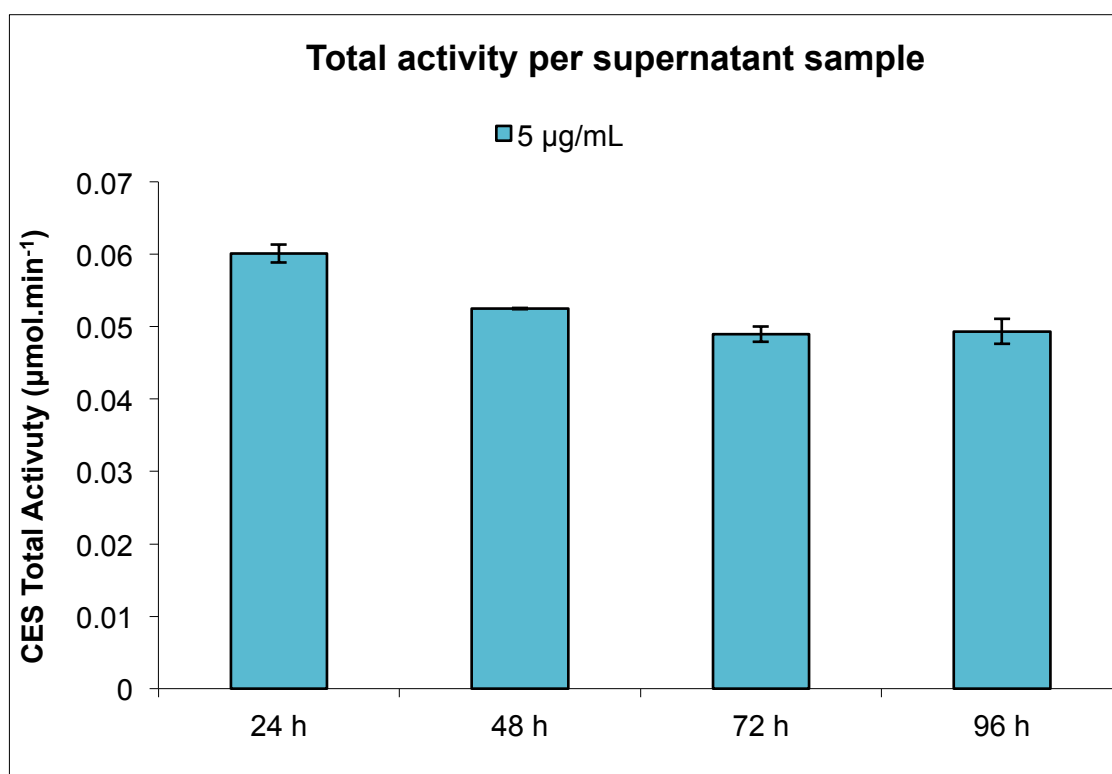


**Figure 4.4.5:** Recombinant CES2 production in the supernatant and cellular extracts from HEK-293T cells in suspension. Western Blot (range from 62 to 49 kDa) of samples obtained from transfection (with pCI-neo-CES2 and pCI-neo-CES2-10xHis) with 5 and 10  $\mu\text{g}/\text{mL}$  of DNA, for both cellular extracts and supernatant samples (incubation with anti-CES2). 50  $\mu\text{g}$  of total protein was applied in lanes 2 to 5, and 10  $\mu\text{L}$  of concentrated sample was applied in lanes 6 and 7. Lanes: 1. SeeBlue® Plus2 Ladder; 2 Cell extract from non-transfected cells; 3. Cell extract from transfected cells with 5  $\mu\text{g}/\text{mL}$  of pCI-neo-CES2 (96 h); 4. Cell extract from transfected cells with 5  $\mu\text{g}/\text{mL}$  of pCI-neo-CES2-10xHis (96 h); 5. Cell extract from transfected cells with 10  $\mu\text{g}/\text{mL}$  of pCI-neo-CES2-10xHis (48 h); 6. Concentrated supernatant from transfected cells (96 h) with 5 $\mu\text{g}/\text{mL}$  of pCI-neo-CES2-10xHis; 7. Concentrated supernatant from transfected cells (48 h) with 5 $\mu\text{g}/\text{mL}$  of pCI-neo-CES2-10xHis.

Different samples were tested, namely cellular extracts from transfected cells with pCI-neo-CES2 and pCI-neo-CES2-10xHis and supernatants from transfected cells with pCI-neo-CES2-10xHis (Figure 4.4.5). With this assay, it is possible to observe that the suspension cells are producing the recombinant CES2, with and without the histidine tag. And moreover, that the CES2-10xHis is present in the supernatant samples (lanes 6 and 7).

Since the protein is being unequivocally secreted, the impact of the culture media and agitation in the CES2-10xHis enzyme activity and stability were studied.

HEK-293T cells were transiently transfected with 5  $\mu\text{g}/\text{mL}$  of pCI-neo-CES2-10xHis and 24 h after the cells were removed. The decrease in the activity of the remaining supernatant was followed spectrophotometrically, as described before (Figure 4.4.6).



**Figure 4.4.6:** CES activity towards 4-MUBA evaluated spectrophotometrically, as described in Materials and Methods. 24 h post-transfection the supernatant from transfected cells with 5 µg/mL of DNA was separated from the cells and incubated for 48, 72 and 96 h. Error bars represent the standard deviation of three independent analytical assays.

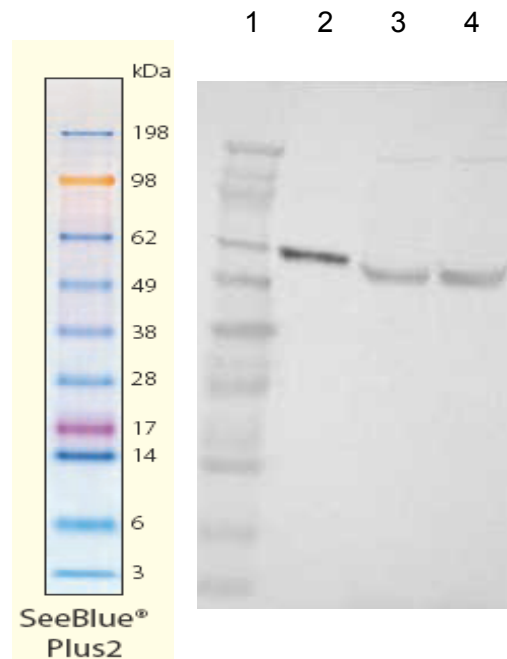
These results point to a decrease in CES activity from 24 to 48 h after transfection in the supernatant. From 48 h onwards, there is no significant decrease.

It had been previously seen that during production, the levels of CES2 activity increased throughout time (Figure 4.4.4) since the activity in the supernatant of pCI-neo-CES2-10xHis transiently transfected HEK-293T cells was more than 15 times higher at 96 h comparing to 24 h after transfection (Figure 4.5.1 B). This result corroborates the fact that no evident decrease in CES2 activity occurs from 48 to 96 h after transfection (Figure 4.4.6), also indicating that the cells are producing and excreting CES2-10xHis at a fairly constant rate and the protein is being accumulated extracellularly, in an active form. The enzyme activity seems to be stable in the culture media.

With these results, the 5 µg/mL of pCI-neo-CES2-10xHis at 96 h post-transfection was the chosen as the best condition (best expression vector concentration and culture time to harvest the product) to collect the highest amount of active human recombinant CES2-10xHis in the culture media of HEK-293T transiently transfected cells, without compromising enzyme activity or cell viability. Although the 10 µg/mL DNA concentration at 48 or 72 h post-transfection would also be suitable, the chosen condition was 5 µg/mL at 96 h, since it allowed to use less DNA and PEI in the transfection process (both are toxic for the cells).

Surprisingly in all the performed gels, intracellular CES2 protein appeared to have a lower molecular weight, when compared to the extracellular CES2 protein, and both bands are lighter than the one obtained from the commercial enzyme (Figure 4.5.1 A). To evaluate if this was a simple matrix

effect, another western blot was performed (Figure 4.4.7), by applying in the same gel extracellular and intracellular samples in different lanes but also a mixture of the two in the same lane.

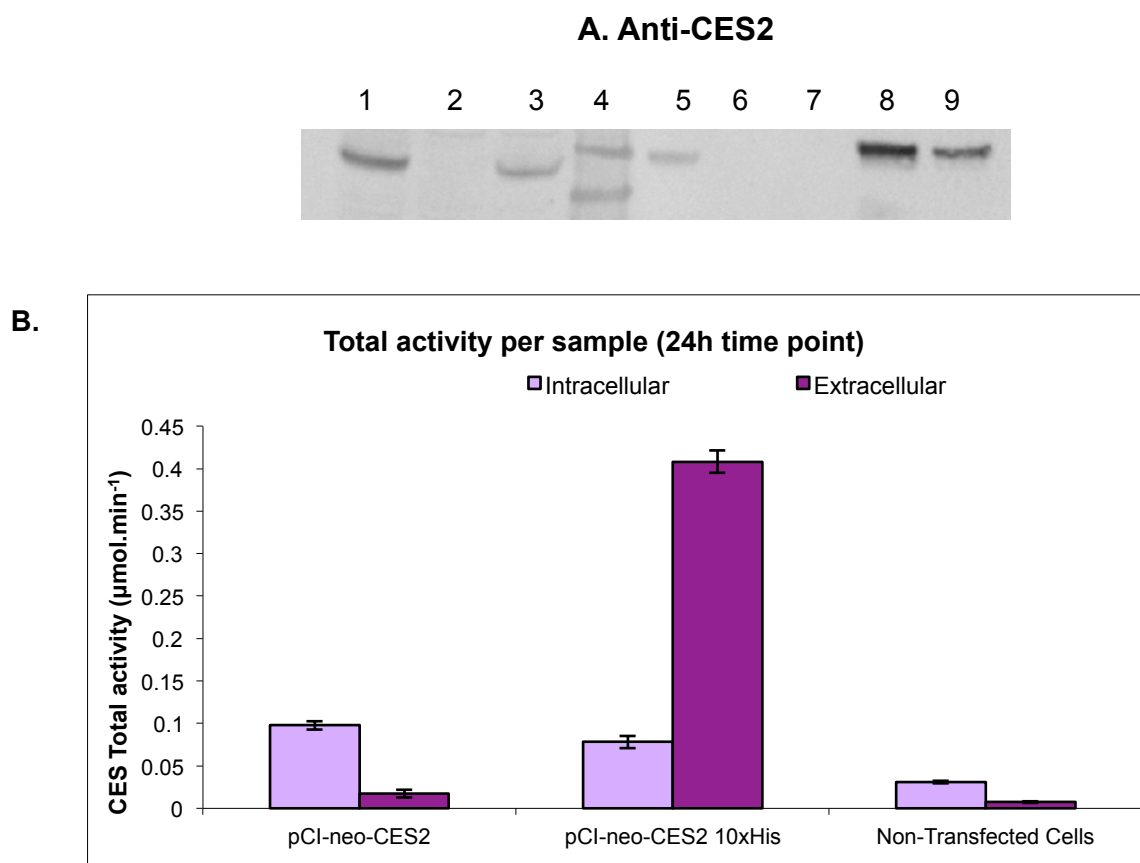


**Figure 4.4.7:** Matrix effect of the recombinant CES2 production in supernatants and cellular extracts from HEK-293T cells in suspension. Samples were obtained from transfection with 5  $\mu\text{g}/\text{mL}$  of pCI-neo-CES2-10xHis, at 24h. Incubation was made with anti-CES2 antibody. Samples from lanes 2 to 4 have the same percentage of sample (1.5% (v/v) relative to the initial amount) Lanes: **1.** SeeBlue® Plus2 Ladder; **2.** Supernatant from transfected cells; **3.** Cell extract from transfected cells; **4.** Cell extract and supernatant (1:1) from transfected cells.

As it can be observed (Figure 4.4.7), the differences in the apparent protein weight of the different samples (cells extracts vs supernatant) were confirmed to be due to a matrix effect of the samples, as only one band was observed when both samples (cells extracts and supernatant) were mixed. This effect may be due to the presence, intracellularly, of a high amount of proteins that may be masking the CES2 protein migration, dragging it. Anti-CES2 (1:200) and anti-rabbit alkaline phosphatase conjugated secondary antibody (1:2000) were used and detection was performed by addition of NBT/BCIP reagent.

#### 4.5 CES2 secretion from transfected HEK-293T cells in suspension

To evaluate if the histidine tag, located at the carboxy-terminal amino acid sequence HTEL, was the responsible for the protein secretion, a western blot was performed (Figure 4.5.1 A) with supernatant samples from transfection with pCI-neo-CES2 and pCI-neo-CES2-10xHis at 24 h (in this culture time, the protein is already being secreted) using with 5  $\mu\text{g}/\text{mL}$  of DNA.



**Figure 4.5.1:** Recombinant CES2 production in supernatants and cellular extracts from HEK-293T cells in suspension. **A.** Western Blot (range from 62 to 49 kDa, incubation with anti-CES2) with samples obtained after transfection (with pCI-neo-CES2 and pCI-neo-CES2-10xHis) with 5 µg/mL of DNA for both cellular extracts (50 µg of total protein) and supernatant samples (10 µL) (24 h time point). **Lanes:** 1. Cell extract from transfected cells with 5 µg/mL of pCI-neo-CES2-10xHis; 2 Cell extract from non-transfected cells; 3. Cell extract from transfected cells with 5 µg/mL of pCI-neo-CES2; 4. SeeBlue® Plus2 Ladder; 5. Supernatant from transfected cells with 5µg/mL of pCI-neo-CES2-10xHis; 6. Supernatant from non-transfected cells; 7. Supernatant from transfected cells with 5 µg/mL of pCI-neo-CES2; 8. Commercial CES2 (R&D Systems, Minneapolis, U.S.A.) (100 ng); 9. Commercial CES2 (50ng); **B.** CES activity towards 4-MUBA was evaluated spectrophotometrically with both the cellular extracts (intracellular) and with supernatant (extracellular), as described in Materials and Methods. Error bars represent the standard deviation of three independent analytical assays.

It is possible to see that CES2 is being expressed in both cell extracts from both transfections, as expected (lanes 1 to 3). However, only the extracellular sample from the transfection with pCI-neo-CES2-10xHis is positive for the presence of CES2 protein, which supports the hypothesis that it is the histidine tag that influences the secretion of the protein (secretion is not observed for the protein without the histidine tag). The data (Figure 4.5.1 B) shows that CES2 is being secreted as soon as 24 h after transfection with pCI-neo-CES2-10xHis but not pCI-neo-CES2. Considering the total activity of each sample, both intra and extracellularly, it becomes clear that more than 80% of the activity is detected in the supernatant of HEK-293T cells transiently transfected with pCI-neo-CES2-10xHis expression vector (Table 4.4).

**Table 4.4:** CES2 activity in transiently HEK-293T transfected cells, 24 h post-transfection, represented as the relative percentage of the total activity (intra and extracellular) of pCI-neo-CES2-10xHis sample.

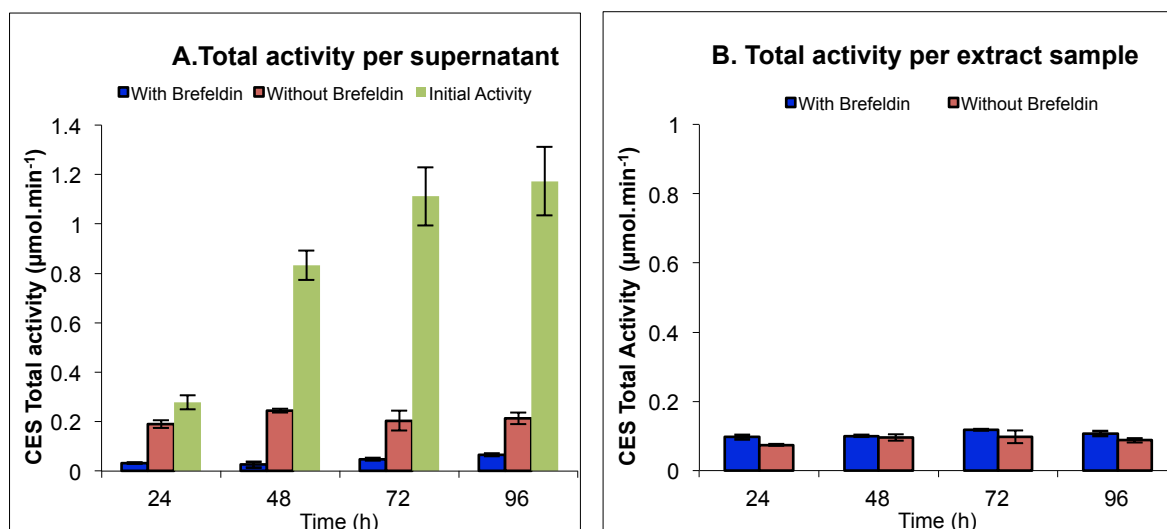
	Activity (%)	
	Intracellular	Extracellular
<b>pCI-neo-CES2-10xHis</b>	16.07	83.93
<b>pCI-neo-CES2</b>	20.11	3.56
<b>Non-Transfected Cells</b>	6.32	1.50

This secretion is not due to cell lysis as no decrease in the cell viability (Figure 4.4.1) was detected at this time point (initial viability:  $95 \pm 3.3\%$ ; 24 h post-transfection:  $90 \pm 8.5\%$ ; average  $\pm$  standard deviation of three independent assays). This five-fold increase is only possible due to the secretion of the protein. The production of human recombinant CES2-10xHis, in a secreted form, is increased four times, when compared to the production with pCI-neo-CES2. This is a very important issue as one of the main drawbacks of recombinant protein expression in mammalian cells is the low yield usually obtained (68, 87, 88).

To further assess if the secretion was due to the interference with the carboxy-terminal amino acid sequence signal for permanent retention of proteins in the endoplasmic reticulum, an experiment was conducted with Brefeldin A, an antibiotic that rapidly causes disassembly of the stack of Golgi cisternae and blocks the formation of vesicles involved in protein transport from the endoplasmic reticulum to the *cis*-Golgi cisterna (29). Proteins destined to be secreted undergo three crucial sorting and translocation steps. The first segregation step is into the lumen of the ER, the second step from the ER to the Golgi complex and finally the third step from the Golgi complex to the cell surface. By using this antibiotic, protein retention inside the cell is promoted by inhibition of the secretion mechanisms in the Golgi complex (11, 29) and also by the destabilization of the ER itself.

It has been previously reported that the expression of mutated human alveolar macrophage CES and rabbit liver CES, missing the HTIEL C-terminal motif, in COS-7 monkey fibroblasts, originates the secretion of these enzymes through the classical ER/Golgi-dependent exocytosis (81).

The incubation of pCI-neo-CES2-10xHis transiently transfected HEK-293T suspension cells with Brefeldin A inhibits the secretion of the protein, as the CES activity detected in the supernatant significantly drops (Figure 4.5.2 A).



**Figure 4.5.2:** CES activity in the supernatant of pCI-neo-CES2-10xHis transiently HEK-293T transfected cells in suspension upon the treatment with brefeldin A. CES activity towards 4-MUBA was evaluated, spectrophotometrically as described in Materials and Methods, 24, 48, 72 and 96 h post-transfection, with or without a 4 h treatment with 10 µg/mL of brefeldin A. The initial activity corresponds to the activity in the culture time before the 4 h incubation period. **A.** 5 µg/mL of pCI-neo-CES2-10xHis expression vector was used and 50 µL of each supernatant sample was used per assay. CES activity is shown as total activity, in µmol.min<sup>-1</sup> in 10 mL of supernatant, the total volume of sample in each time point. Each result represents the average of three independent assays, and error bars represent the standard deviation; **B.** 5 µg/mL of pCI-neo-CES2-10xHis expression vector was used and 10 µg of total protein in cellular extracts was used per assay. CES activity is shown as total activity, in µmol.min<sup>-1</sup> in 200 µL of sample volume in each time point. Each result represents the average of three independent assays, and error bars represent the standard deviation.

This decrease in extracellular CES activity is accompanied by a small increase in the detected intracellular CES activity in the sample treated with brefeldin A (Figure 4.5.2 B). As previously, the data also suggests that pCI-neo-CES2-10xHis transiently HEK-293T transfected cells keep their ability to produce and excrete CES2 at a fairly constant productive rate throughout time, even 96 h after transfection (Figure 4.5.2). The incubation of transiently HEK-293T transfected cells with brefeldin A did not cause any decrease in cell viability in comparison to the non-incubated cells (data not shown).

These results clearly demonstrate that CES2-10xHis is being exported to the supernatant through the classical secretory pathway. Thus, it is possible to assume that CES2-10xHis is undergoing its normal processing pathway, entering the ER where it can be properly folded and post-translationally modified (81). The addition of a 10xHis tag in the C-terminus of CES2 may be masking the HTEL motif, hampering its binding and retention inside the ER. Secretion of CES2-10xHis is not due to its overexpression and inability of the cell to cope with large amounts of the protein, since the transfection of HEK-293T cells with a similar expression vector lacking only the 10xHis tag does not lead to the secretion of the protein (Figure 4.5.1). Additionally, secretion of the protein is not due to cell lysis as, as soon as 24 h after transfection CES2 activity can be detected in the supernatant of pCI-neo-CES2-10xHis transiently transfected HEK-293T cells but not in the one from pCI-neo-CES2 transiently transfected cells (Figure 4.5.1). Moreover, the addition of brefeldin A completely abolishes the secretion of CES2-10xHis to the culture media (Figure 4.5.2). These results fully support the hypothesis that the deletion of the KDEL anchoring motif is not necessary to promote the secretion.

The in frame His tag located immediately after this sequence is sufficient to lead to the secretion of the protein without the need to mutate or remove the retention sequence or to add any extra signal peptides to the N-terminal region. Also, one can assume that CES2-10xHis, despite its secretion, is being properly folded and post-translationally modified as it enters the ER.

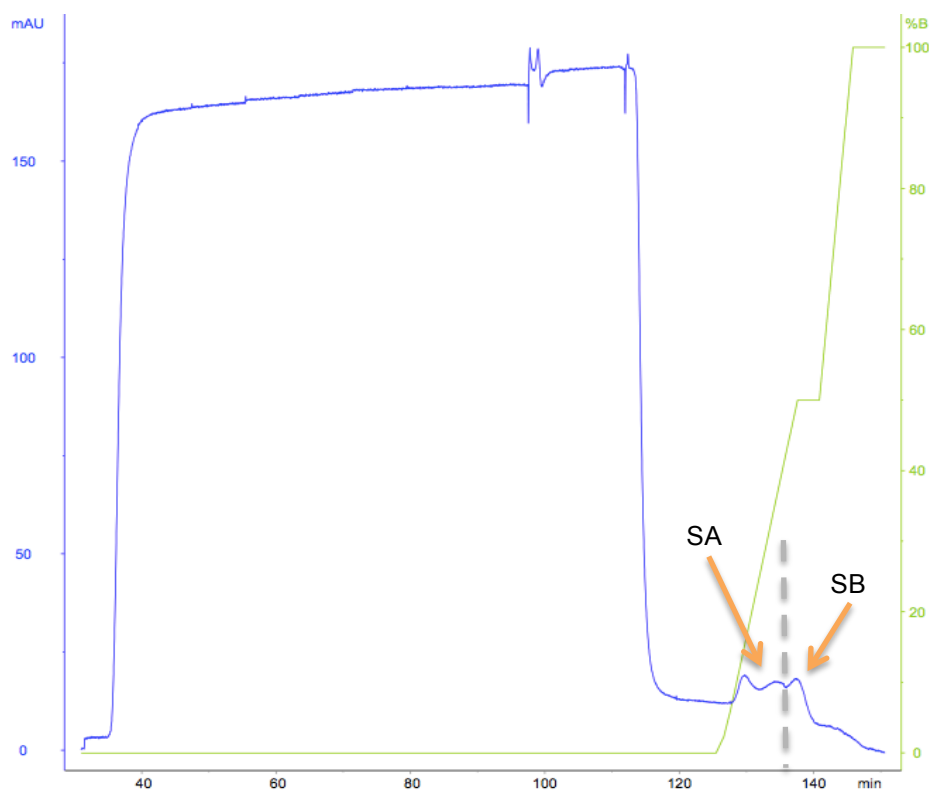
### **4.6 CES2 purification from supernatant samples of transfected HEK-293T cells in suspension**

Human recombinant CES2-10xHis enzyme was purified in the ÄKTAexplorer™ 10S System through a nickel affinity chromatography process, as described in the Materials and Methods section. The main goal of this purification process performed over the Erlenmeyer production was to determine the best purification conditions to later purify recombinant CES2 produced in bioreactor. Thus, a total of 210 mL of supernatant samples recovered throughout 96 h after HEK-293T cells transfection from 5, 10 and 20 µg/mL, were pooled together in order to analyse the purification profile and obtain purified CES2-10xHis enzyme.

A two-step imidazole gradient was used for separation of CES2-10xHis from the remaining proteins present in the supernatant. Three main fractions could be detected in the chromatogram of the purification (Figure 4.6.1): the first one (the larger one), eluted with the addition of the wash buffer, the second one eluted in the beginning of the gradient with Elution buffer (with 65 mM of imidazole), and the third one eluted in the end of the first step of the gradient with Elution buffer (with 250 mM of imidazole).

Two samples were collected during the purification process (marked by the orange arrows in figure 4.6.1):

- The second fraction, eluted in the beginning of the gradient with Elution buffer (with 65 mM of imidazole) - two first peaks (SA);
- The third fraction, eluted in the end of the first step of the gradient with Elution buffer (with 250 mM of imidazole) - last peak (SB);



**Figure 4.6.1:** Purification chromatogram of recombinant CES2 purification from supernatant samples of transfected HEK-293T cells in suspension with pCI-neo-CES2-10xHis. Imidazole gradient (v/v) (%) is represented in green while in blue is the absorbance at 280nm (mAU). The collected samples are indicated with orange arrows.

Both fractions were immediately processed for buffer exchange (in Vivaflow cassettes) in Storage Buffer, in order to remove imidazole from the samples, as previously mentioned. All samples were filtered in sterile conditions and stored at  $-80\text{ }^{\circ}\text{C}$  with 20% glycerol (v/v) to preserve the protein stability, as described in the Materials and Methods section.

The total protein concentration was determined for both samples using the Micro BCA™ Kit. The results are shown in Table 4.5.

**Table 4.5:** Total protein concentration of the collected samples during protein the purification process and their quantification with the Micro BCA™ Kit

Sample ID	Sample Description	Sample Concentration ( $\mu\text{g}/\mu\text{L}$ )
<b>SA</b>	Fraction eluted with 65 mM of imidazole (23.5 mL)	$0.026 \pm 0.001$
<b>SB</b>	Fraction eluted with 250 mM of imidazole (18 mL)	$0.0093 \pm 0.0005$

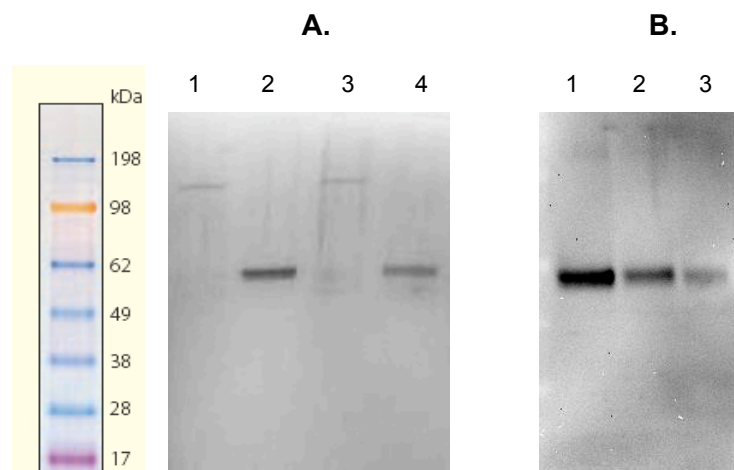
These quantifications already take into account the 20% dilution made by the addition of glycerol. Protein quantification revealed that eluted SA had higher protein content (611  $\mu\text{g}$ ) than SB (167.4  $\mu\text{g}$ ).

The enzymatic kinetic profile was determined for both samples, as described before, and both eluted fractions showed CES activity. However, the specific enzymatic activity of SB ( $22.8 \pm 2.1 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ ; average  $\pm$  standard deviation) was five times higher than the one obtained in the eluted SA ( $4.3 \pm 0.3 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ ; average  $\pm$  standard deviation). Table 4.6 summarizes these data.

**Table 4.6:** Characterization of the purification samples (SA and SB)

	SA	SB
<b>Imidazole (mM)</b>	65	250
<b>Protein (<math>\mu\text{g}/\text{mL}^{-1}</math>)</b>	26.4	9.3
<b>Volume (mL)</b>	23.5	18
<b>Specific enz. activity (<math>\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}</math>)</b>	$4.3 \pm 0.3$	$22.8 \pm 2.1$

Also, all samples were analyzed by SDS-PAGE, using NuPAGE Bis-Tris 4-12% gels, followed by Silver Staining to evaluate protein purity (Figure 4.6.2 A).

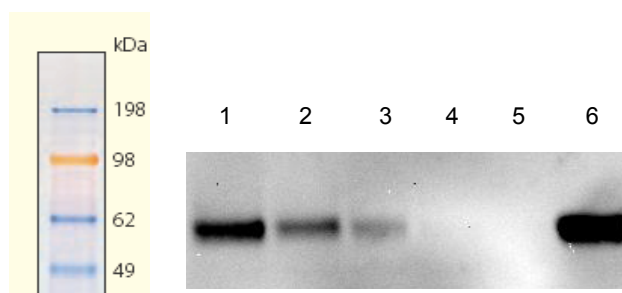


**Figure 4.6.2:** Purified Recombinant CES2 protein. **A.** Silver staining of SDS-PAGE electrophoresis, for protein purity evaluation, performed as described in Materials and Methods. **Lanes:** 1. 85 ng of SA; 3. 84.4 ng of SB; 4. 42.6 ng of SA; 5. 42.2 ng of SB; **B.** Western blot of the SB from the purification process. 5, 2.5 and 1.25 ng (lanes 1, 2 and 3, respectively) of the sample were loaded. Rabbit anti-CES2 and HRP-conjugated antibodies were used.

SB shows a clear and defined band corresponding to the described 60 kDa CES2 weight. Moreover, no major contaminants are seen with only one extra faint band visible in the lane. To further confirm that this band corresponds to CES2, a western blot was performed (Figure 4.6.2 B) with anti-CES2 primary antibody (1:200) and anti-rabbit ECL HRP conjugated secondary antibody (1:20000).

Detection was performed with Amersham ECL Plus. The results indicate that CES2-10xHis protein is present in SB with a high purity level.

With this purified CES2 protein and having a commercially available anti-CES2 antibody, it was possible to perform a Western Blot (Figure 4.6.3) with different amounts of this protein (5, 2.5 and 1.25 ng) and also supernatants from the production in Erlenmeyer and bioreactor with 5  $\mu\text{g}/\text{mL}$  of pCI-neo-CES2-10xHis at 96 h, in order to quantify the protein amount in these samples and also to estimate the yield of the process. Note that it is not possible to quantify directly the supernatants due to interference in the BCA assay.



**Figure 4.6.3:** Purified Recombinant CES2 protein. Western Blot picture (range from 98 to 49 kDa) with 5, 2.5 and 1.25 ng (lanes 1, 2 and 3, respectively) of the purified protein and 0.5  $\mu\text{L}$  of supernatant from transfected cells with 5  $\mu\text{g}/\text{mL}$  of pCI-neo-CES2-10xHis (recovered at 96h; lane 6). Rabbit anti-CES2 and HRP-conjugated antibodies were used.

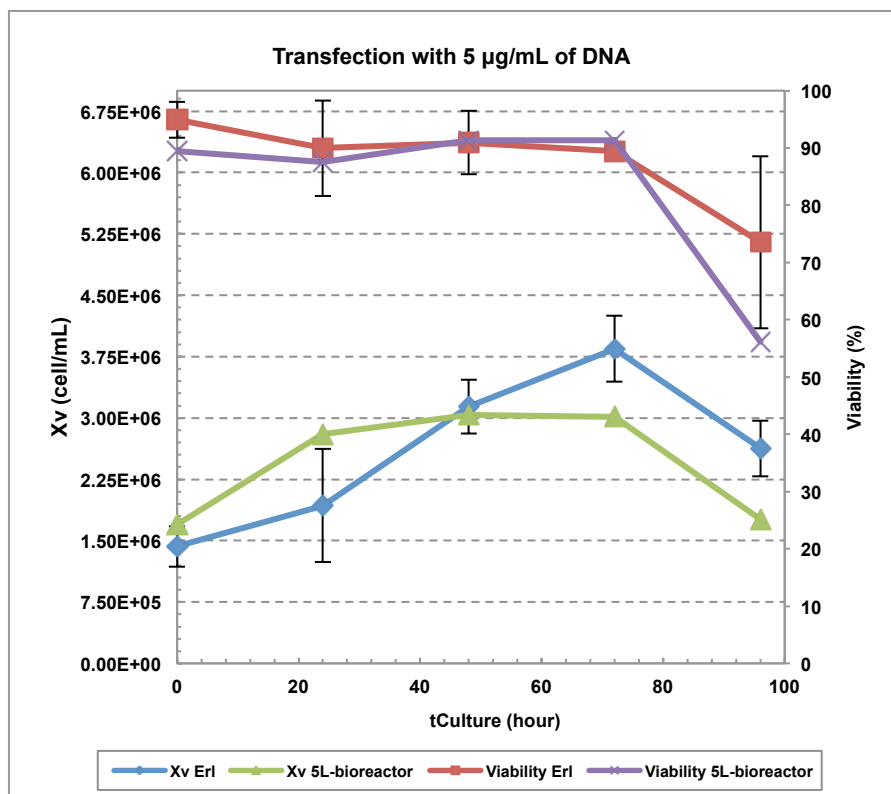
Thus, plotting a standard curve with the integrated density vs the loaded protein amount, it was estimate that the supernatant of HEK-293T cells transiently transfected with 5  $\mu\text{g}/\text{mL}$  of pCI-neo-CES2-10xHis contains  $44.1 \pm 15.5 \mu\text{g}/\text{mL}$  (average  $\pm$  standard deviation) of CES2 human recombinant protein (in the 200 mL sample). Considering only the CES2-10xHis protein present at a highly pure rate in SB, a 2% yield is estimated in this purification process (0.8 mg/L of highly pure protein). This low obtained yield may be explained by the possible formation of very large aggregates, or multimers. These aggregates with more than 500 kDa (68) were previously mentioned for CES, but no information concerning those aggregates and their activities are available. CES2 may be aggregating and thus masking the C-terminal His-tag, and this aggregation will lead to the formation of inactive CES2, thereby reducing the obtained yields.

#### 4.7 CES2 production in bioreactor with transfected HEK-293T cells in suspension

CES2-10xHis production was scaled to a five-litre bioreactor process with the previously optimized conditions in Erlenmeyer. Cell transfection was performed with pCI-neo-CES2-10xHis purified with QIAprep® Gigaprep, as described in Materials and Methods section. The yield obtained in this purification process was significantly higher than the predicted by the manufacturer. This was due to

the fact that this purification was supposed to be done in bacteria cultures in LB media. However, in this particular case, the bacteria culture was performed in TB media, which allows a higher growth of the bacteria.

After cell transfection with pCI-neo-CES2-10xHis, cell viability (Figure 4.7.1) and CES2 activity (Figure 4.7.2 A) were followed throughout time in order to control the process.



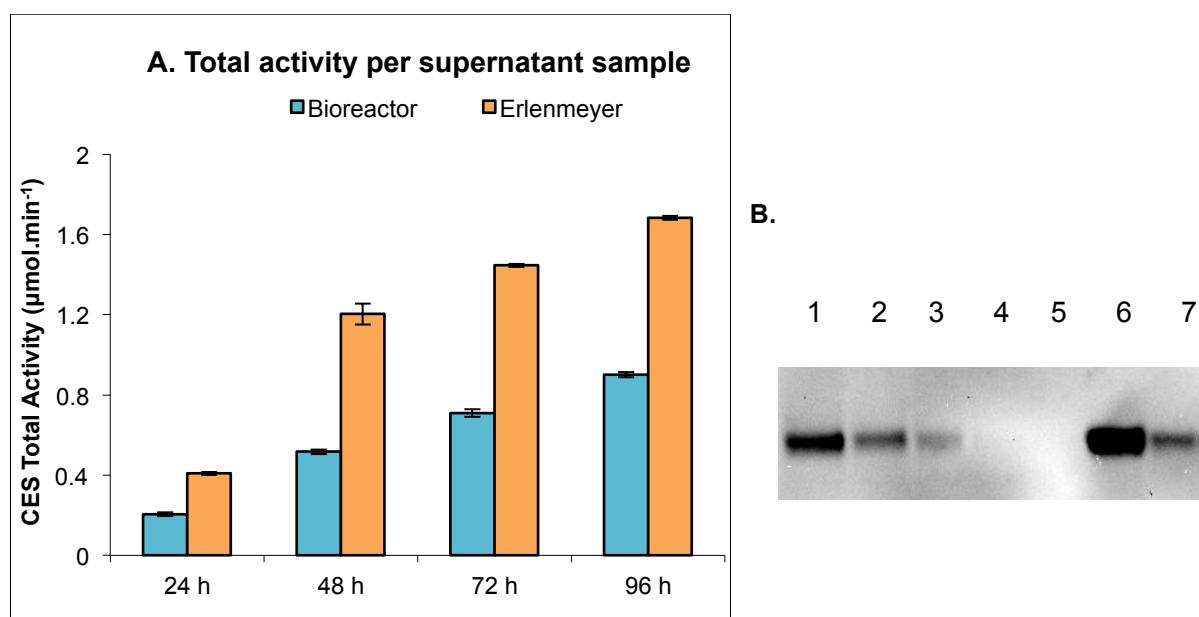
**Figure 4.7.1:** Characterization of CES2 production in bioreactor. Cellular growth of transfected HEK-293T in suspension with 5 µg/mL of DNA in the 5L bioreactor (green) and Erlenmeyer (blue). Xv represents the viable cell concentration. In red and purple are represented the cellular viability for the erlenmeyer and bioreactor, respectively. The presented error bars represent the standard deviation of three independent transfection assays.

Cell density and viability profiles in the bioreactor were similar to the ones obtained before in the Erlenmeyer platform (Figure 4.7.1). The metabolites formation due to cellular growth was also quantified for the bioreactor production, as described in the Materials and Methods Section (Table 4.6). The cellular growth is accompanied by a consumption of glucose in the media and with an increase of the production of lactate. There is a high glucose consumption as well as lactate formation during the first 24 h, corresponding to a higher cellular growth (Figure 4.7.1). This consumption and production stabilized in the following time points, indicating a lower growth rate.

**Table 4.7:** Metabolite formation by the suspension cells in the bioreactor

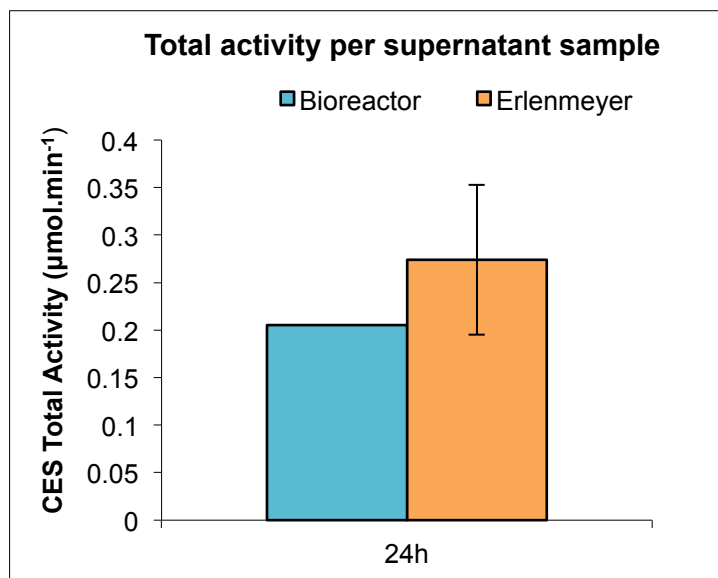
Sample	Glucose (mmol/L)	Lactate (mmol/L)
0 h	20.7	0.19
24 h	9.25	17.3
48 h	6.90	15.8
72 h	6.31	13.8
96 h	5.22	11.9

CES2 activity in the bioreactor production was in agreement with the obtained values in Erlenmeyer, since it increases over time (Figure 4.7.2 A). However, a decrease in CES2 activity ( $0.2 \pm 0.02 \mu\text{mol}\cdot\text{min}^{-1}$ ; average  $\pm$  standard deviation) (Figure 4.7.2 A), as well as in CES2 amount, estimated by western blot (Figure 4.7.2 B), were noticed in comparison to the transient transfection experiments performed in Erlenmeyer.



**Figure 4.7.2:** Characterization of CES2 production in the 5L-bioreactor. **A.** CES activity in supernatant of pCI-neo-CES2-10xHis transiently transfected HEK-293T cells in erlenmeyer and bioreactor. CES activity towards 4-MUBA, evaluated spectrophotometrically. Each result represents the average of three independent analytical assays, and error bars represent the standard deviation; **B.** Western Blot (range from 62 to 49 kDa) with 5, 2.5 and 1.25 ng (lanes 1, 2 and 3, respectively) of the previously purified protein and 0.5  $\mu\text{L}$  of supernatant sample from transfected cells with 5  $\mu\text{g}/\text{mL}$  of pCI-neo-CES2-10xHis (recovered at 96h) in Erlenmeyer (lane 6) and bioreactor (lane 7). Anti-CES2 and anti-rabbit HRP-conjugated antibodies were used.

However, analysing three independent transfection assays for the erlenmeyer platform (in Figure 4.7.3) for one time point, it was possible to determine the experimental variation associated with these assays in that same time point (in this case, 24 h post-transfection).



**Figure 4.7.3:** Evaluation of the experimental variability. CES activity towards 4-MUBA in supernatant of pCI-neo-CES2-10xHis transiently transfected HEK-293T cells in erlenmeyer and bioreactor was evaluated spectrophotometrically. Each result represents the average of three independent analytical assays. The presented error bars represent the standard deviation of three independent transfection assays.

The analysis of the variability between three independent transient transfection experiments performed in Erlenmeyer revealed that these results are explained by the experimental variability ( $0.27 \pm 0.08 \mu\text{mol}\cdot\text{min}^{-1}$ ; average  $\pm$  standard error of the mean), i.e., the obtained bioreactor result ( $0.21 \mu\text{mol}\cdot\text{min}^{-1}$ ) actually is comprised by the experimental variability determined in erlenmeyer ( $0.19$  to  $0.35 \mu\text{mol}\cdot\text{min}^{-1}$ ).

Taken together, these results show that CES2-10xHis production obtained through chemical transient transfection of adapted HEK-293T cells in suspension is possible to be scaled-up.

## 5. Final Remarks

The main goal of this thesis project was to establish a process for the expression and production of the human carboxylesterase enzyme in mammalian cells.

Adherent HEK-293T cells were a good platform for the process development, but since high amounts of purified enzyme were required to proceed for crystallographic studies in order to unravel the structure of this protein, scale-up of the process was required.

The optimization of CES2-10xHis production conditions in suspension culture was performed in Erlenmeyer. The DNA amount used in the transfection process and the time of harvest were analysed, and transfection with 5 µg/mL of DNA and harvesting 96 hours post-transfection proved to be the best condition, where higher activity was obtained, and thus chosen to proceed to the CES2 production in the bioreactor.

The addition of an in frame C-terminal Histidine tag to CES2 protein showed to influence the protein localization, leading to its secretion without the need to mutate or delete the C-terminus motif of the protein (that enables its binding and retention inside ER) or the addition of N-terminal signalling sequences. Experiments conducted with samples with CES2 with and without the histidine tag and also with brefeldin A confirmed this hypothesis.

At the selected condition, the levels of CES2 activity are fifteen times higher comparing to 24 h after transfection (with the histidine tag), since no evident decrease in CES2 activity occurs from 48 to 96 h. These results indicate that the cells are producing and excreting CES2-10xHis at a fairly constant rate and the protein is being accumulated extracellularly, in an active form.

A simple and fast affinity chromatography purification process was applied to obtain CES2-10xHis protein at a high purity grade. A 2% yield was estimated in this purification process, although, further optimization is still required. Later studies, outside the scope of this thesis, pointed to aggregation as the main factor responsible for this low yield.

CES2-10xHis production was successfully scaled to a five-litre bioreactor and all the optimized conditions in Erlenmeyer were kept. A decrease in CES activity as well as in CES2 amount were observed in comparison to the transient transfection experiments performed in Erlenmeyer. However, these results are explained by the experimental variability inherent to the transfection experiments.

Overall, this thesis reflects the successful implementation of production and purification strategies of CES2 protein in HEK-293T cells. Despite being an ER anchored protein, secretion of CES2 to serum free media was achieved due to the presence of the in frame C-terminal 10xHistidine tag, which contributed to a good production yield.



## 6. Future Work

The next step in the follow up of this work will consist in purify the CES2 obtained in the 5L-bioreactor, in order to have enough protein to perform a stability assay (ThermoFluor®) and also crystallographic studies.

To produce sufficient protein for structural studies, an increase of CES2 purification yields is needed without compromising protein purity and activity. This low yield obtained will be a target of attention - the possible formation of very large aggregates, or multimers, with more than 500 kDa (68) was mentioned for CES, but no information concerning those aggregates and their activities are available. CES2 may be aggregating and thus masking the C-terminal His-tag, and this aggregation will lead to the formation of inactive CES2, thereby reducing the obtained yields.

Beside the importance of the crystallographic studies, the purified protein can also be used for its further characterization and to study its ability to form these aggregates.

Also, if this low yield prevail in further purifications, and if this system doesn't allow to produce the CES2 amounts required to proceed to crystallographic studies, different CES2 purification strategies and/or different production platforms, such as *Pichia pastoris* or Sf9 insect cells, may have to be explored.

A validation of a high quality *in silico* homology model of CES2, using BuChE (butyrylcholinesterase) as a template, will be performed (with directed mutagenesis of key amino acid residues predicted from the model to account for differences to BuChE). This model has a number of features expected to be present in CES2 from kinetic analysis, for example, a side-door allowing product exit from the active site and it shows overall very high structural similarity with BuChE around the catalytic site. Thus, this model has good potential for understanding the catalytic mechanism of CES2 and the origins of its specificity, which could be used for predicting drug interactions with the enzyme, and for designing inhibitors for potential use in cancer adjunct therapy. Although this work was predicted in the scope of the present thesis, it was delayed and replaced by the scale up of the production process due to the necessity of increasing the amount of protein produced.



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