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Biosynthesis optimization of STEAP1 protein in *Pichia pastoris* X33 MUT^{+}

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Resumo

O cancro da próstata é o tipo de cancro mais comum entre os homens com mais de 50 anos nos países ocidentais. As terapias atuais disponíveis são, em alguns casos, limitadas e ineficazes. Por isso, é necessário desenvolver terapias alternativas que possam combater de forma mais específica e eficaz as células tumorais. A proteína Six Transmembrane Epithelial Antigen of the Prostate 1 (STEAP1) é formada por seis domínios transmembranares, localizada nas junções célula-célula do epitélio prostático. Diversos estudos indicam que a STEAP1 se encontra sobre-expressa no cancro da próstata mas em tecidos normais, a sua expressão é mínima. A sua localização e estrutura celular sugere que esta proteína pode funcionar como um canal iónico. Logo, a expressão diferencial da STEAP1 entre tecidos, associada com a sua localização, sugerem o seu uso como possível alvo terapêutico do cancro. Para delinear novas formas de terapia usando como alvo a STEAP1, é necessário obter-se elevados níveis de proteína purificada. Portanto, o objetivo deste estudo é produzir a proteína STEAP1 em *Pichia pastoris* X33. A utilização de um sistema eucariótico irá permitir o enovelamento correto da proteína, visto a *Pichia pastoris* ser capaz de realizar reações pós-tradução. Os nossos resultados demonstraram que a proteína alvo é eficazmente produzida em *Pichia pastoris* e foi identificada na fração centrifugada a 16000g. Análise por Western blot revela a proteína com o peso molecular correto (~40 kDa). As melhores condições para a produção da proteína são um processo de fermentação de 6 horas em meio BMMH com 1,25% de metanol e 6% de DMSO a 30ºC, 250 rpm. O passo de solubilização deve ser efetuado utilizando SDS numa concentração de 1-3%.

Palavras-chave

STEAP1, Cancro da Próstata, Biossíntese, *Pichia pastoris*.

Resumo alargado

O cancro da próstata é o tipo de cancro mais comum entre os homens com mais de 50 anos nos países ocidentais e a quinta causa de morte no mundo. As terapias atuais disponíveis são, em alguns casos, limitadas e ineficazes. Por isso, é necessário desenvolver terapias alternativas que possam combater de forma mais específica e eficaz as células tumorais. A proteína Six Transmembrane Epithelial Antigen of the Prostate 1 (STEAP1) é formada por seis domínios transmembranares, localizada nas junções célula-célula do epitélio prostático. Diversos estudos indicam que a STEAP1 se encontra sobre-expressa no cancro da próstata mas em tecidos normais, a sua expressão é mínima. A sua localização na superfície da célula e estrutura sugere que pode funcionar como um canal iónico para pequenas moléculas tais como cálcio e potássio. Logo, a expressão diferencial da STEAP1 entre tecidos normais e cancerígenos, associada com a sua localização, sugerem o seu uso como possível alvo terapêutico do cancro. Para delinear novas formas de terapia usando como alvo a STEAP1, é necessário obter-se elevados níveis de proteína purificada. Portanto, o objetivo deste estudo é produzir a proteína STEAP1 em *Pichia* pastoris X33 Mut^+. A utilização de um sistema eucariótico irá permitir o enovelamento correto da proteína, visto a *Pichia pastoris* ser capaz de realizar reações pós tradução, como a glicosilação. O objetivo inicial foi a construção de um vetor de expressão, usando o plasmídeo pPICZαB. Neste foi colocado o gene da STEAP1 com uma cauda de 6 histidinas no fim para facilitar a purificação numa fase posterior. De seguida, o vetor foi inserido no genoma da *Pichia pastoris* e finalmente, foram testadas diferentes condições como temperatura, concentração de metanol e tempo de indução para determinar quais as melhores para a sua produção. Tendo em conta que a estirpe utilizada era Mut^+, esta consegue produzir a proteína alvo utilizando o promotor AOX, que é ativado pela presença de metanol no meio. Então, foi realizado inicialmente uma pré-fermentação com glicerol como fonte de carbono para promover o crescimento celular. De seguida, foi realizada uma fermentação com metanol para induzir a produção da proteína utilizando o promotor AOX. Os nossos resultados demonstraram que a proteína alvo é eficazmente produzida em *Pichia pastoris* no peso correto (~40 kDa) e foi identificada na fração do pellet, obtido após centrifugação a 16000g. Das três concentrações de metanol testadas (0,75%, 1% e 1,25%), a que levou à produção de uma maior quantidade de proteína foi a de 1,25%. O tempo de fermentação ideal foi de 6 horas, uma vez que os resultados demonstraram que a proteína só era produzida imediatamente após indução com metanol. Após este intervalo de tempo a proteína era degradada pelo microrganismo e só seria produzida após nova indução, mas em menor quantidade que na indução inicial. Também foram testadas duas Temperaturas de fermentação diferentes (25 e 30ºC), das quais a de 30ºC foi a mais eficaz, obtendo-se assim maior quantidade de proteína. A utilização do chaperone DMSO foi testado a duas concentrações (3 e 6%) e a que obteve melhores resultados foi a concentração de 6%, uma vez que a 3% ocorreu degradação da proteína.

Finalmente foram testados diferentes detergentes iónicos e não-iónicos para a solubilização, e a várias concentrações, sendo que a mais eficaz foi a utilização de SDS num intervalo de concentração de 1-3%. No entanto a otimização destes passos é vital e necessária, visto a solubilização não ser completa e ainda não foi testada nenhuma técnica de purificação da proteína alvo.

Abstract

Prostate cancer (PCa) is the most common type of cancer in men over the age of 50 in Western countries. The current therapies available are, in many cases, limited and ineffective. Therefore, it is necessary to develop alternative therapies, that can fight specifically and effectively cancer cells. The Six Transmembrane Epithelial Antigen of Prostate 1 (STEAP1) is a protein with six transmembrane domains located in the cell-cell junctions of the secretory epithelium of the prostate. Some studies have reported it as overexpressed in PCa but in normal tissues, its expression is minimal. The structure and cell location of STEAP1 suggest that it may function as a channel for ions. Moreover, it was demonstrated that STEAP1 overexpression induces proliferation of cancer cells. The differential expression of STEAP1 in tissues, associated with its localization, suggests its potential use as a target for cancer therapy. In order to delineate novel strategies for targeting STEAP1 protein, it is necessary to obtain high levels of purified protein. Therefore, the aim of this study is to produce STEAP1 protein in *Pichia pastoris* X33 Mut^{+}. The use of a eukaryotic system will allow the correct folding of the protein since *Pichia pastoris* is able to perform post-translation modifications. Our results showed that the target protein is being produced by *Pichia pastoris* strain and it was identified in the pellet fraction centrifuged at 16000g. Western blot analysis revealed the protein in the correct molecular weight (~ 40 kDa) The best conditions for protein production is a 6 hours fermentation process in BMMH medium with 1.25% methanol and 6% DMSO at 30ºC, 250rpm. The solubilization process should be done using SDS in a concentration of 1-3%.

Keywords

STEAP1, Prostate cancer, Biosynthesis, *Pichia pastoris*.

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List of Abbreviations

Ab	Antibodies
ACRATA	Apoptosis, cancer and redox associated transmembrane domain
AOX	Alcohol oxidase
AR	Androgen receptor
ARE	Androgen Response Elements
BMGH	Buffered Minimal Glycerol
BMMH	Buffered Minimal Methanol
BPH	Benign Nodular Hyperplasia
BSA	Bovine Serum Albumin
CAT	Catalase
DHAS	Dihydroxyacetone synthase
DHT	Dihydrotestosterone
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease I
E. coli	*Escherichia coli*
ES	Ewing's sarcoma
FSH	Follicle-stimulating hormone
gDNA	genomic Deoxyribonucleic acid
GnRH	Gonadotropin-releasing hormone
LAB	Lactic acid bacteria
LH	Luteinizing hormone
LSLB	Low Salt Luria-Bertani
MCT	Monocarboxylate Transporter
mRNA	messenger Ribonucleic acid
OD	Optical Density

PCa	Prostate cancer
PCR	Polymerase Chain Reaction
PIN	Prostatic intraepithelial neoplasia
PSA	Prostate specific antigen
PSCA	Prostate stem cell antigen
PSMA	Prostate-specific membrane antigen
SDS	Sodium Dodecyl Sulfate
STEAP	Six-Transmembrane Epithelial Antigen of Prostate
TAA	Tumor-associated antigens
YPD	Yeast Extract Peptone Dextrose

CHAPTER I- INTRODUCTION

1. Human prostate

1.1. Anatomy and Physiology

The prostate is a gland located in the pelvis, below the bladder, surrounding the upper part of the urethra. It is organized in lobules and surrounded by fibromuscular stroma (1, 2). Its dimensions are 3 cm high by 4 cm wide by 2 cm deep, about the size of a walnut (3). The prostate gland secretes an alkaline fluid that is used to suspend the ejaculated sperm, along with the fluid from the seminal vesicles and bulbourethral glands. This alkaline fluid helps to maintain sperm motility (3). During ejaculation, the smooth muscle of the prostate and other tissues contract, expelling the fluid into the urethra, forming the semen (3).

According to McNeal, the prostate gland can be divided into three main regions: a central zone, a peripheral zone, and a transition zone, as observed in Figure 1 (4–6). The central zone is a "vertical wedge of glandular tissue" which surrounds the ejaculatory ducts and comprises approximately 25% of the prostate and surrounds the ejaculatory ducts (4–7). The peripheral zone is the majority of the prostate's volume, about 65%. It is comprised of secretory epithelial cells and surrounded by smooth muscle that helps the release of prostatic secretions into the urethra at the time of ejaculation (3, 6). This zone is much more susceptible to prostatitis and adenocarcinomas, which can arise from peripheral ducts and acini (4, 5). The transition zone is comprised of two lobules located on the sides of the urethra and contains coarse and compact stroma cells. This zone comprises less than 5% of the prostate volume and has been associated with the appearance of glandular nodules, characteristics of Benign Prostatic Hyperplasia (BPH) (6, 7). Two more zones can be identified: the anterior fibromuscular stroma and the periurethral zone (8). The periurethral zone is comprised by the urethral glands, which are located close to the proximal urethra and the proximal urethra sphincter. The anterior fibromuscular stroma constitutes <5% of the mass of the prostate gland. It is comprised entirely of non-glandular tissue and its function is still unclear (9).

The epithelium of the prostate gland is composed of the secretory and basal cells. Secretory cells, also known as luminal cells, express, among others, prostate specific antigen (PSA) and the androgen receptor (AR). These cells do require androgens to differentiate (10, 11). Basal cells are multipotent and can generate all epithelial lineages of the prostate. A close observation of these cells can help to differentiate adenocarcinomas from benign conditions, since in the first case, these cells are absent. In addition, in normal conditions, basal cells do not express PSA. These two types of cells are interpolated by neuroendocrine cells in some

areas (5, 10, 11). Neuroendocrine cells are present in all zones, being more abundant in the periurethral and ductal regions. Although its function remains unclear, it has been suggested that they may influence growth or differentiation of surrounding epithelial cells. These cells express PSA but not AR (5, 10, 11).

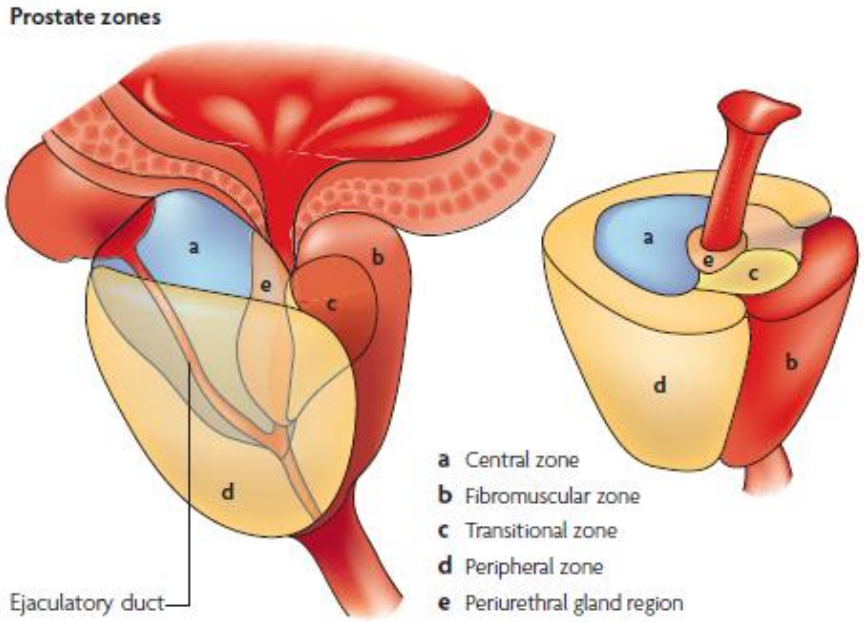

Figure 1: Prostate's anatomy with differentiated zones (Adapted from (8)).

The prostate is highly dependent on steroid hormones, such as androgens, for maintaining its secreting function and integrity (12). In normal conditions, the Gonadotropin-releasing hormone is secreted by the hypothalamus, which leads to the secretion of Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) by the Anterior pituitary (2, 13). FSH's primary function is to induce spermatogenesis and LH is to stimulate the production of testosterone in Leydig cells (2, 13). Testosterone acts on the prostate gland and can be converted into dihydrotestosterone (DHT) by the enzyme 5α-reductase type 2, present in some cells of the prostate. DTH can then activate the AR present in the secretory cells, as previously mentioned (2, 14, 15). Testosterone can also bind to AR, but with lower affinity when compared to DHT (12, 14, 15). This receptor is located in the cytoplasm, bound to Heat Shock Proteins (16–18). Once DHT binds to the receptor, there is a change in its conformation, allowing the AR to translocate into the nucleus. Then, AR-ligand binds to specific recognition DNA sequences, known as the androgen response elements (AREs) (16, 18). These AREs are localized in the promoter region of androgen-regulated genes, leading to regulation of genes responsible for DNA synthesis, cellular proliferation, differentiation, among others (14, 16, 17).

1.2. Prostate cancer

As it was previously mentioned, the peripheral zone of the prostate is the most common site for prostate adenocarcinoma, and the transitional zone for BPH (5). Prostate cancer (PCa) is regulated by androgens, such as BPH, although the latter can very rarely progress to carcinoma.

PCa can be developed due to the accumulation of genetic and epigenetic changes, leading to the inactivation of tumor-suppressor and caretaker genes and the activation of oncogenes. The triggering factor for its appearance is still unknown (8). However, several studies have suggested that infections, hormonal alterations, and physical traumas can create inflammation and proliferation of epithelial cells (8, 19–21). Changes in gene expression may further increase cell proliferation and morphological alterations leading to the appearance of prostatic intraepithelial neoplasia (PIN) lesions, as observed in Figure 2 (8). In fact, it has been suggested that PIN is a potential precursor of PCa. PIN lesions are characterized by basal cell disruption, the proliferation of the luminal epithelium, irregular cell spacing and nuclear and nucleolar enlargement (5, 22, 23). PIN is categorized into low grade and high grade, being the first less severe and the second the precursor of PCa (5, 22). The continuous tumor progression can lead to the disruption of the endothelial lining of blood vessels and cancer cells can enter the bloodstream and colonize other organs, leading to metastasis (8, 24).

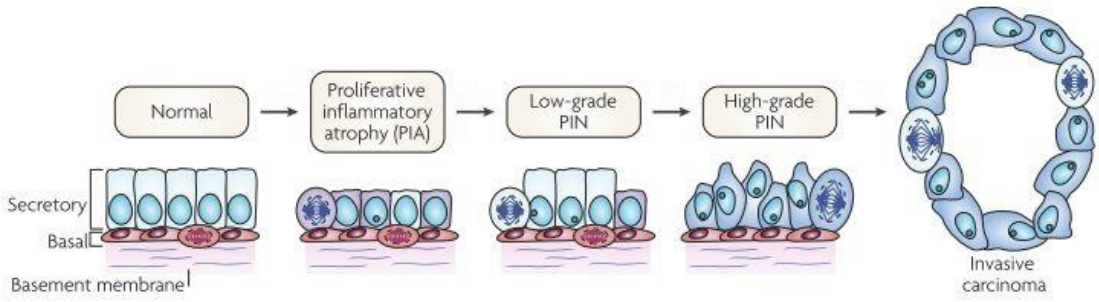

Figure 2: Stages of prostatic cancer (Adapted from (8)).

Since the prostate is a hormone-dependent tissue, the initial phases of PCa seem to be dependent of androgens (17). In several cases, the AR is overexpressed in PCa, leading to an increase in the expression of genes responsible for cell proliferation, and consequently the progression of PCa (17). In more advanced cases, especially when cancer becomes castration-resistant, several mutations and/or an increase of AR gene copy number have been identified (10). These molecular changes may turn the PCa cells more sensitive to androgens or increase the recruitment of co-activator proteins (10). In addition, it has been described that AR may be activated by other molecules, such as insulin growth factor, which can be found in high concentration in prostate cancer patients (24).

1.2.1 Epidemiology and Risk Factors

PCa is the most common type of cancer in men over the age of 50 in Western countries and the fifth leading cause of death in the world (25, 26). In Portugal, prostate cancer is the main cancer diagnosed in men and the third cause of death (25–27).

Risk factors for prostate cancer can be divided into two categories: endogenous and exogenous factors. Regarding endogenous factors, we can have family history, hormones, race, age, and oxidative stress. In exogenous factors, there is diet, environmental agents, and occupation (21).

Family history can be associated with high risk of PCa. Men who have a first-degree relative with prostate cancer will have a higher risk of having cancer. PCa can be classified as sporadic, familial or hereditary. Sporadic cases occur when no other case of cancer as appeared in the subject's family. Familial cases occur when one or more first-degree members are affected. Hereditary cases, being only 5-10% of cases, are considered when a pattern of cancer distribution is found within the subject's family. Some genes seem to be responsible for higher risk of prostate cancer when mutated, such as genes encoding proteins responsible for defending against inflammation and oxidative stress (21, 28, 29).

Androgens, such as testosterone and dihydrotestosterone, are responsible for the development, maturation, and maintenance of the prostate, as well as proliferation and differentiation in the luminal epithelium. In prostate cancer, some studies suggest that high levels of testosterone can increase the risk of developing it, although results have been inconsistent (19, 21, 29).

Prostate cancer has a very low chance of being diagnosed in men under 50 years and a higher chance in men over 65 years. The lowest incident rates of prostate cancer are found in Asia and the highest are among African-American men (19, 25). However, studies have shown that when people from Asian countries move to the USA, their risks of developing cancer increases, suggesting that other external factors may be involved, such as lifestyle and environmental factors (19, 29, 30).

A diet with high content of animal fat, such as red meat, has been linked to a higher risk of prostate cancer. The consumption of food with high-fat content seems to result in alteration of hormone pathways and increase of oxidative stress with potential carcinogenic effects. Vitamin D seems to have positive implications in prostate cancer. It has anti-proliferative and

pro-apoptotic effects and inhibits tumor growth. Its deficiency may be a risk for prostate cancer development (19, 21, 29, 31).

Some environmental agents, such as the endocrine disrupting chemicals and pesticides can alter hormone activity and affect reproductive organs and carcinogenesis (21).

An infection-associated inflammation and hyperproliferation can contribute to the development of prostate cancer. An infectious agent can induce an inflammatory process and lead to cellular alterations in the prostate. Also, sexually transmitted diseases and prostatitis are a risk factor for prostate cancer (8, 19, 29). Other factors such as obesity, smoking, alcohol ingestion, and certain professions can increase the risk of prostate cancer (19, 21, 25, 29, 30).

1.2.2. Diagnosis and Treatment

Diagnosis of prostate cancer can be carried out by measuring serum PSA, tissue biopsies and digital rectal examination (32).

The Gleason grading system is the preferred method to characterize prostate cancer. It was created by Dr. Donald F Gleason and is based on the histological pattern of carcinoma cells in the prostatic tissue. There are five basic grade patterns that can be used to obtain a histological score between 2 and 10. A primary and secondary pattern are characterized by the grade patterns and added to obtain the histological score. The primary pattern is the predominant one in the tissue and the secondary pattern is the second most common. The patterns differ in cell arrangement and shape, stromal invasion and gland size. Increasing Gleason grade is directly related to tumor size and invasion (33, 34). Other systems were created, such as the Tumor, Node and Metastasis system for the clinical stage with grading for tumor (T), node (N) and metastasis (M) (35).

As it was previously described, PSA is a serine protease secreted by the prostatic epithelium and the epithelial lining of the periurethral glands. It is involved in the liquefaction of seminal fluids. It is measured in the serum and detected by immunoassay (32, 36, 37). Its levels correlate with disease aggressiveness (37). However, PSA levels can be increased in BPH and prostatitis and lower in PCa. This leads to high chances of misdiagnosed and unnecessary biopsies. Some alternatives have arisen in Prostate cancer diagnosis (36, 37). PSA density takes into consideration the size of the prostate and can distinguish between BPH and prostate cancer. PSA velocity analyses the alterations in PSA levels over time. PSA velocity can correlate with cancer aggressiveness (37).

Regarding the treatment of prostate cancer, it depends on the age of the patient and stage of cancer (38, 39). In man with low-Grade Gleason score, the main action is simply active surveillance with measurements of serum PSA and prostate biopsies. In aggressive cancers, the most common treatments are androgen deprivation therapy, radiation, prostatectomy or a combination of both (40, 41). Many of these forms of treatment are aggressive and can diminish the quality of life of the patients. (42). Finding treatments with minimal toxicity is the goal and some can go through targeting tumor-associated antigens (TAA). These proteins are specific to cancer and that are either not expressed or minimally expressed in normal tissues and essential organs.

1.2.3. Putative Immunotherapeutic Target

As it was previously mentioned, current therapies for PCa are limited and induce unwanted side effects. Radiation therapy can destroy healthy cells and patients that undergo surgical castration can still develop cancer afterward, where previous treatments are ineffective. Therefore, it is necessary to develop new forms of treatment targeting specifically cancer cells. The overexpression of some membrane proteins in cancerous tissue may be a new way, as well as proteins involved in mechanisms that cancer cells use (42, 43).

Prostate-specific membrane antigen (PSMA) is a type II integral membrane glycoprotein, which is overexpressed in PCa cells. Some studies have shown a good correlation between PSMA level and Gleason score, and it has also been evaluated as a target for therapy with monoclonal antibodies (Ab) conjugated with toxins or radioisotopes (36, 44). A study with Ab against PSMA combined with an immunotoxin lead to its accumulation in LNCaP cells, leading to apoptosis of the cells overexpressing PSMA, but not in PSMA-negative PCa cells (45).

Prostate stem cell antigen (PSCA) is a glycoprotein expressed on the cell surface of prostate basal cells. Studies show that this protein increases with PCa stage and progression, and there is a good correlation between PSCA level and Gleason score (46, 47). At least two studies showed that targeting PSCA with specific Ab inhibits PCa cell growth (48, 49).

Six-transmembrane epithelial antigen of prostate 1 (STEAP1) is a transmembrane protein overexpressed in the epithelial cells of the prostate. It was demonstrated that STEAP1 silencing mediated by siRNA showed a decrease in proliferation of Ewing tumor cells (50). Other studies with Ab blockage of STEAP1 showed similar results in prostate and bladder tumor cells (51). This protein is considered a promising immunotherapeutic target since it meets the criteria for TAA, being overexpressed in cancer cells but not in normal tissues. In

addition, its location on the cell's surface facilitates recognition and can lead to a localized treatment, without damaging other tissues.

In cases of castration–resistant PCa, treatments to alter cancer cells' energy metabolism is a new approach that can be used. It is very well documented that cancer cells prefer glycolysis to obtain its energy instead of oxidative phosphorylation in Krebs cycle (52). Therefore, large amounts of lactic acid are produced, creating an acidic environment that facilitates tissue invasion and metastasis (53, 54). The Monocarboxylate Transporter (MCT) family is involved in the transport of lactic acid. In fact, MCT4 is overexpressed in multiple cancer types, and it is associated with poor prognosis of PCa (55, 56). The treatment of PCa cells with antisense oligonucleotides targeting MCT4 revealed a decrease in lactic acid secretion and inhibition of cell growth (55).

2. Human six transmembrane epithelial antigen of prostate proteins

The six-transmembrane epithelial antigen of prostate (STEAP) family is comprised of four proteins, STEAP 1 to 4. STEAPs are expressed in many different tissues and were found to be overexpressed in many cancer cell lines. All these proteins share a six transmembrane domain and intracellular N- and C- termini but differ in cellular functions and expression patterns. This family of proteins seems to contribute in maintaining metal homeostasis, oxidative stress response, cell-cell communication, proliferation, invasion, apoptosis, among others (57–59).
These proteins share similarities in the C terminus with the transmembrane domains of yeast FRE metalloreductases. The alignment of STEAP1-4 with *Saccharomyces cerevisiae* FRE1 domain shows similarities in a heme-binding domain, which was referred as the Apoptosis, Cancer and Redox Associated Transmembrane domain (ACRATA), and two conserved histidine residues. The FRE1 domain contains four histidine residues, creating two heme-binding domains. Since STEAP only has two, it only binds one heme domain. These residues are important because they allow the correct alignment of the heme group, facilitating electron transport through the domain (58–61).

The N-terminal domain also exhibits homology with the archaeal and bacterial $F_{420}H_2$: $NADP^+$ oxidoreductase (FNO) binding proteins in STEAP2-4 (59). This domain contains the Rossman-fold (GXGXXG/A motif), which allows proteins to bind to nucleotides such as NAD and a serine and arginine residues that are critical for binding to $NADP^+$. This FNO-like domain allows the binding of flavins as electron donors to reduce iron and copper, giving them oxidoreductase activity (57, 59). The specific domains presented in the N- and C-terminals of STEAP2-4

promote iron reduction, and possibly copper, as well as to stimulate their uptake into cells (59).

Although these proteins share many structural aspects, their expression and cellular localization are very diverse. STEAP1 mRNA and protein are mainly expressed in prostatic tissues, but also in liver, kidneys, and breast (59, 62). STEAP2 mRNA is expressed in normal prostate, pancreas, brain and fetal liver (59). STEAP3 mRNA is highly expressed in liver and it was also found at lower levels in pancreas and bone marrow (58). STEAP4 mRNA is found in bone marrow, placenta and fetal liver (58, 59, 63).

2.1. STEAP1

2.1.1. Structure, Function, and Expression

STEAP1 was firstly identified by Hubert in 1999, who has characterized it as a "cell-surface molecule with six transmembrane domains" (64). It contains 339 amino acids and a molecular mass of approximately 40 kDa (64). Its structure is comprised of six transmembrane domains, three extracellular loops, and two intracellular loops. The C- and N- terminal are both intracellular (64). It also contains a heme-binding domain, referred as the ACRATA domain, as previously described (51, 61, 62). Unlike all the other family members, STEAP1 neither contain the FNO-like domain nor the Rossman Fold, which implied the lacking of oxidoreductase activity (59). The lack of these structures may be the reason why it does not promote iron/copper uptake or reduction (59). Although STEAP1 does not contain these domains, it does seem to have a role in iron metabolism. STEAP1 is located in endosomal compartments containing the transferrin-transferrin receptor 1 (Tf-TfR1) complex. It was also found in other endosomal compartments that specialize in iron uptake, but do not contain this complex (58, 59, 62).

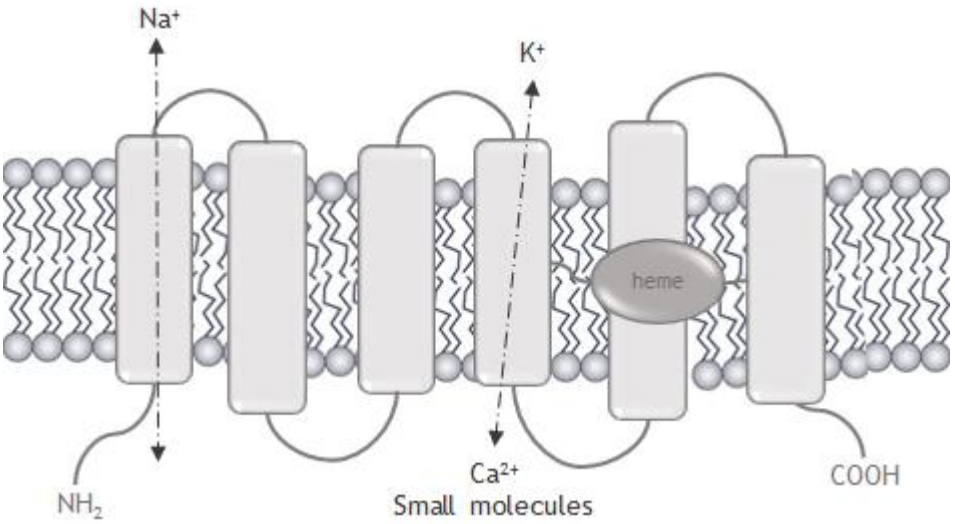

Figure 3: Schematic representation of the STEAP1 structure.

In normal tissues, the STEAP1 expression is restricted to prostate cells, but can also be found at low levels in other tissues such as liver and kidneys (59, 64). It is mainly localized in the cell-cell junctions of the plasma membrane, suggesting its role as an ion channel or transporter protein in tight junctions, gap junctions or cell adhesion (64). Some studies have shown that an increase of Na^+ channels facilitates cancer proliferation and invasiveness (65), supporting the hypothesis that STEAP1 may be involved in cancer proliferation through modulation of ion concentrations. In addition, it was demonstrated that STEAP1 is involved in intercellular communication, suggesting its implication in cancer proliferation (66). Also, studies of STEAP1 blockage with Ab showed that intercellular communication is compromised and inhibits cancer cell growth (51).

In cancer cells, STEAP1 protein is overexpressed in many cancer types. It is mainly overexpressed in PCa and at all stages (51, 67). It has also been found in lymph node and bone metastases and in the bladder, breast, lung, ovarian, colon and pancreatic cancer cell lines (51, 64, 68, 69). In Ewing's sarcoma (ES) STEAP1 is highly expressed and is associated with increased oxidative stress. STEAP1 increases cellular ROS levels and induced the expression of redox-sensitive and proinvasive genes (50, 70). STEAP1 is also overexpressed in breast cancer cells. This led to the suggestion that this protein may be regulated by estrogens. Effectively, STEAP1 is down-regulated by dihydrotestosterone and 17-ß estradiol, suggesting that STEAP1 may influence PCa progression through androgens and estrogens modulation (69).

2.1.2. STEAP1 as immunotherapeutic target

Although the function of STEAP1 remains to be determined, its over-expression in cancer cells, associated with its structure and cell location, support the idea of using this protein as a potential target for a variety of clinical applications that include antibody therapy, cancer-vaccine therapy, small-molecule therapy, and diagnostic imaging (51, 57, 62, 64, 71, 72). In fact, several studies have already demonstrated that STEAP1 can be used as a therapeutic target and/or biomarker. As it was previously mentioned, studies have shown that blocking STEAP1 using Ab against STEAP1 inhibited intercellular communication, resulting in tumor growth retardation (51).

Rodeberg and co-workers tested an epitope-based T-cell immunotherapy for cancer patients using a modified STEAP1 peptide. This study showed that the STEAP1 peptide was successfully capable of inducing naïve CD8+ precursors into CTLs that recognized STEAP-containing tumor cells in an MHC class I immune response (72).

In another study, it was evaluated an active immunization against either PSCA or STEAP1 using a Modified Vaccinia Ankara. The vaccination of mice against both antigens showed tumor reduction, the genitourinary tract was less pronounced and there was a reduction in the severity and extension of prostate lesions. In addition, the analysis of sections of the prostate tissue showed an increase of T-cell infiltration in the tumors of mice immunized against both antigens (73).

STEAP1 could also be used as a biomarker for prostate cancer early diagnosis, monitoring, screening, and treatment. Despite not being able to distinguish between PIN lesions and PCa, it can prevent misdiagnoses of BPH (67, 68). For these reasons, STEAP1 is a potential therapeutic target for PCa. However, in order to determine its structure and interactions with potential drugs it is vital to obtain high amounts of the purified protein.

3. Production and purification of transmembrane proteins

Proteins, in general, are synthesized in heterologous systems due to the impossibility to obtain satisfactory yields from natural sources. Some important steps in producing proteins are the selection of the ideal expression system and the appropriated growth conditions, as well as the characteristics of the target protein and downstream processes such as purification (74, 75).

3.1. Prokaryotic Systems

Prokaryotic organisms are the most used systems for protein production since they are easy to manipulate. However, they have several restrictions when it comes to heterologous protein production, such as post-translational modifications (74).

Escherichia coli is one of the most used prokaryotic organisms for recombinant protein production. In addition, it has been used to produce many pharmacological and food industry components because is easy to manipulate and to grow at a reduced cost, with high yield. However, when it comes to membrane proteins expression, *E. coli* has several restrictions. It is unable to do certain post-translational modifications and to properly fold foreign proteins. Also, sometimes proteins can form aggregates, becoming the purification process much more difficult or even impossible (74, 76).

Lactic acid bacteria (LAB), such as *Bacillus subtilis* and *Lactococcus lactis* are classified as Generally Recognized As Safe and widely used in the food industry. Its potential for heterologous protein expression has been explored in recent years, including membrane proteins. LAB are easy and inexpensive to grow, have well-established genetic methods and vector systems, moderate proteolytic activity and efficient targeting of MPs into the cytoplasmic membrane (74, 77). However, membrane protein production in these systems is limited since it can cause major stress to the cell and impair growth (78).

3.2. Eukaryotic Systems

In eukaryotic systems, yeast species are one of the most used organisms. Similar to prokaryotic organisms, they are easy to manipulate and to grow. Also, these systems are economical and can reach high cell density and express complex proteins (75). In opposition to prokaryotic systems, they are able to perform post-translational modifications.

Saccharomyces cerevisiae was the first eukaryotic organism having its genome sequenced (79), and its molecular and metabolic characteristics are already described (80). It has a high capacity to produce and consume ethanol, and presents a high tolerance to stress, such as low levels of oxygen. Although the *Saccharomyces cerevisiae* has a good capacity of glycosylation, the rate of protein secretion is low. Also, there has been cases of plasmid instability (81, 82), low protein production and hyperglycosylation of proteins (75, 83) that can alter its activity or even induce its degradation.

Pichia pastoris is a methylotrophic yeast, which can use methanol as its only carbon source to obtain energy. This organism does not need a complex growth medium and is easy to cultivate. Its genome contains the alcohol oxidase 1 (AOX1) promoter for methanol metabolism. This promoter is one of the strongest and most tightly regulated eukaryotic promoter. Since *Pichia pastoris* uses preferentially respiration to produce energy, low levels of ethanol and acetic acid accumulate in the medium and higher cell densities can be reached. Considering that *Pichia pastoris* is able to secrete proteins, along with the low levels of endogenous proteins in the medium, facilitates the purification process. Parameters such as pH, aeration, and carbon source feed rate can be controlled to improve protein production. However, the usage of methanol can be a fire hazard and its monitoring during the process is difficult (75, 84, 85).

Mammalian cell expression systems have the advantage of performing complex post-translational modifications and can imitate the original environment of a human protein (74, 77, 86). In order to introduce the recombinant DNA, two strategies can be applied: transient or stable gene expression (77, 86). In the transient expression, the DNA template is transferred into the cell by insertion of a plasmid containing the gene or infection with a recombinant virus. In the stable expression, the template is incorporated into the host cell genome. This process is very time-consuming and does not yield high amounts of proteins. Therefore, this system is usually used for structural studies, where large amounts of protein are not required (77, 86). Also, in some systems, the cell produces more protein than it can properly fold it, leading to protein accumulation in structures called aggresomes (74, 77). Nonetheless, some proteins have been successfully produced in mammalian cells such as the human ß2-adrenergic receptor in Chinese hamster ovary cells (87).

3.2.1. *Pichia pastoris* as a Bioreactor

There are many cases of protein production in *Pichia pastoris*, including several membrane proteins, such as human glucose transporter GLUT1 and GLUT4 (88), human mu-opioid receptor (89) and cytidine 5'-monophosphate-sialic acid transporter (90).

This organism is one of the most widely used systems for expression of heterologous proteins that require post-translational modifications. *Pichia pastoris* can reach high levels of expression and produce complex biomolecules that need to undergo post-translational modifications, such as proteolytic processing, disulfide bond formation, O- and N-linked glycosylation and processing of signal sequences (84, 91).

Another reason why this methylotrophic yeast is suited for heterologous protein expression is their easy ability to be genetically manipulated. Many techniques can be applied such as gene replacement, gene targeting, high-frequency DNA manipulation and cloning by function complementation. Since *Pichia pastoris* does not have native plasmids the expression of heterologous proteins can be achieved by inserting an expression vector into their chromosome. The integration can occur via gene insertion or gene replacement (84, 85, 92).

Regarding the type of metabolism to obtain energy, *Pichia pastoris* prefers respiration instead of fermentation (75, 91). This brings advantages since the cells during the fermentation process can accumulate ethanol to toxic levels, and consequently, to decrease the cell growth and production of recombinant proteins. Thus, a respiratory metabolism is preferable considering that the cultures may reach extremely high cell densities in a controlled environment (92).

As it was referred previously, *Pichia pastoris* can use methanol as its only carbon and energy source. This methanol utilization requires a metabolic pathway that involves several enzymes, such as AOX, catalase (CAT) and dihydroxyacetone synthase (DHAS) (76, 85, 93). AOX is responsible for the initial step, the oxidation of methanol to formaldehyde and hydrogen peroxide (Figure 4). The AOX is localized within the peroxisome along with CAT. This enzyme degrades hydrogen peroxide to oxygen and water. Part of the formaldehyde formed by the AOX leaves the peroxisome and is oxidized to formate and carbon dioxide by two cytoplasmic dehydrogenases. These reactions are a source of energy for cells growing on methanol. The rest of the formaldehyde is assimilated to form cellular constituents by a cyclic pathway that starts with the condensation of formaldehyde with xylulose 5-monophosphate, by DHAS, into glyceraldehyde 3-phosphate and dihydroxyacetone. These products leave the peroxisome and enter a cytoplasmic pathway that regenerates xylulose 5-monophosphate and, for every three cycles, one net molecule of glyceraldehyde 3-phosphate (76, 85, 92).

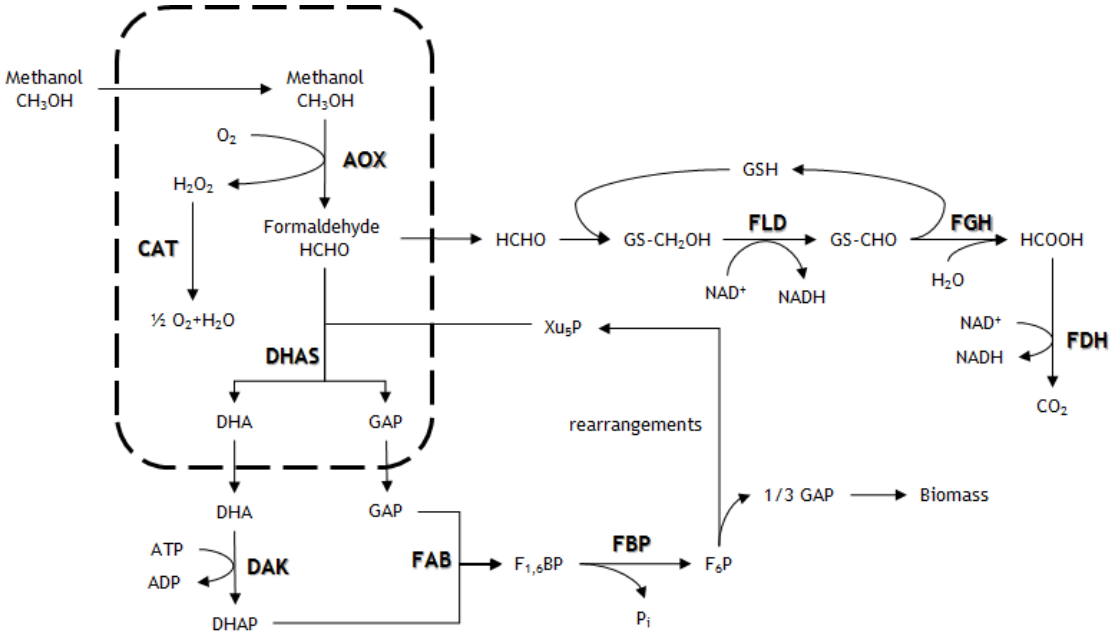

Figure 4: *Pichia pastoris*' methanol pathway. AOX: alcohol oxidase; CAT: catalase; DHAS: dihydroxyacetone synthase; DHA: dihydroxyacetone; GAP: Glyceraldehyde 3-phosphate; DAK: dihydroxyacetone kinase; DHAP: dihydroxyacetone phosphate; FAB: fructose 1,6-bisphosphate aldolase: fructose 1,6-bisphosphatase; FLD: formaldehyde dehydrogenase; FGH: S-formylglutathione hydrolase; FDH: formate dehydrogenase (Adapted from (92–94)).

The *Pichia pastoris* genome contains two genes for the alcohol oxidase enzyme, *AOX1* and *AOX2*. The *AOX1* gene is much strongly transcribed than *AOX2* and, consequently, is responsible for the majority of alcohol oxidase activity in the cell. A tight regulation and high levels of *AOX1* expression turns *AOX* into a strong promoter for expression of heterologous proteins (85).

There are three phenotypes for *Pichia pastoris*: Mut^+ (methanol utilization plus), Mut^S (methanol utilization slow) and Mut^- (methanol utilization minus). The Mut^+ phenotype has two functional *AOX* genes and can efficiently utilize methanol at the wild-type rate. The Mut^S phenotype has the *AOX1* gene deleted and relies on the *AOX2* for methanol metabolism. Strains with this phenotype grow more slowly and do not require large amounts of methanol like Mut^+. The Mut^- phenotype contains both genes deleted and is unable to utilize methanol. The carbon source used is usually glycerol (75, 76, 91, 92). All these strains have successfully produced heterologous proteins. Depending on the protein's characteristics, different strains can be more successful in its production. In general, Mut^+ strains are characterized by a higher growth rate than Mut^S and have higher production rates (95). However, the Mut^S strain requires less amount of methanol, which could be considered a fire hazard (96).

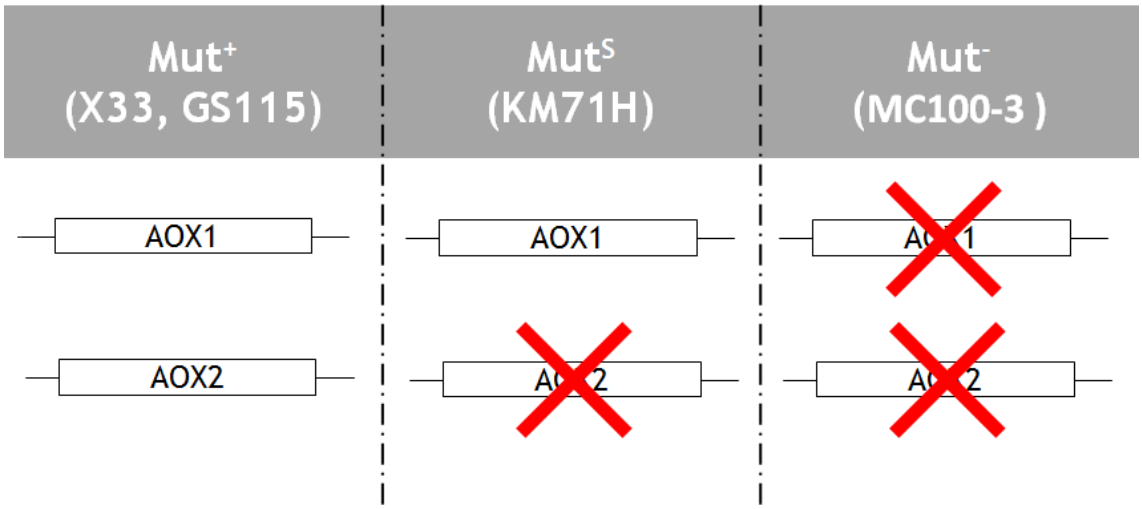

Figure 5: The different phenotypes of *Pichia pastoris* regarding its presence/absence of the two *AOX* genes (Adapted from (91, 97)).

Regarding the production of heterologous proteins, the fermentation process is usually achieved in a bioreactor with a fed-batch strategy with three or four stages (75). First, a batch stage is applied to a defined medium with glycerol as its carbon source to repress protein production and accumulate biomass (75, 85). Once glycerol is depleted in the medium, a fed-batch stage is applied with glycerol at a growth-limiting rate to further increase biomass. The final stage is a fed-batch with methanol at a slow rate, starting the induction of protein production (85, 92). Sometimes, an additional stage with a batch-methanol can be applied between the second and third stage to prepare the cells to fed-batch with a different carbon source (transition phase) (75, 85, 92).

CHAPTER II- AIMS

Due to STEAP1 over-expression pattern in cancer cells combined with its structure and cell location, several studies have pointed out STEAP1 as a potential therapeutic target for cancer. The clinical applications can include from treatment to management of cancer patients. In order to improve the use of STEAP1 as a therapeutic target, it should be determined its structure and interactions with potential drugs or other biological molecules. However, it is firstly required to obtain high levels of purified protein. Since STEAP1 is a protein that requires post-translational modifications, such as N-glycosylation, its production may be carried out in a eukaryotic system. *Pichia pastoris* is capable of performing post-translational modifications, making it an ideal system for protein production. Therefore, the present project aims to produce STEAP1 in *Pichia pastoris* X33. To achieve this goal, the following specific objectives were delineated:

- To construct the expression vector pPICZαB-STEAP1_6His;
- To insert the expression vector in *Pichia pastoris* X33's genome;
- To determine the optimal fermentation conditions and medium components to obtain high protein production;
- To determine the best detergent for protein solubilization.

CHAPTER III- MATERIALS AND METHODS

1. Materials

STEAP1 and AOX1 primers as well as Pst I restriction enzyme, NZYMiniprep kit, NZY First-Strand cDNA Synthesis kit, NZYDNA ladder VI marker and NZYColour protein marker II were obtained from Nzytech (Lisboa, Portugal). Not I and Sac I restriction enzymes were obtained from Takara Bio Inc. (Shiga, Japan). NucleoSpin Gel and PCR Clean-up kit were obtained from Macherey-Nagel (Düren, Germany). Wizard® Genomic DNA Purification Kit, T4 DNA Ligase and 1 µL Buffer 10x were obtained from Promega (Wisconsin, USA). BCA protein assay kit and Dream Taq Green PCR Master Mix (2x) were obtained from Thermo Fisher Scientific (Waltham, Massachusetts, EUA). Zeocin was obtained from InvivoGen (Toulouse, France). YNB, yeast extract, and Glycerol were obtained from HiMedia (Mumbai, India). Tris base, Glucose, Methanol, Dimethyl sulfoxide (DMSO), Triton X-100, Tween 20 and Tween 80 were obtained from Fisher Scientific UK (Loughborough, UK). DNAse and acid-washed glass beads were obtained from Sigma-Aldrich (St. Louis, MO, USA). SDS was obtained from AppliChem (Darmstadt, Germany). Biotin was obtained from Roche (Basileia, Swiss). European Bacteriological Agar was obtained from Laboratorios CONDA (Madrid, Spain). Peptone was obtained from Becton, Dickinson and Company (Sparks, MD).

2. Strains, plasmids, and media

The *Escherichia coli* TOP10 strain (Invitrogen, Carlsbad, USA) was used to produce the expression vector. The transformed cells were grown on Low Salt Luria-Bertani (LSLB) plates (1% peptone, 0.5% NaCl, 0.5% yeast extract, 1.5% European Bacteriological Agar, pH= 7.5) supplemented with 25 µg/mL zeocin at 37ºC. The *Pichia pastoris* X33 strain (Sigma-Aldrich, St. Louis, MO, USA) was used to produce the STEAP1 protein. The transformed cells were grown in YPD plates (1% yeast extract, 2% peptone, 2% glucose, 2% European Bacteriological Agar) supplemented with 100 µg/mL zeocin, YPDS plates (1% yeast extract, 2% peptone, 2% glucose,2% European Bacteriological Agar, 1 M sorbitol) supplemented with 100 µg/mL zeocin and YPD medium (1% yeast extract, 2% peptone, 2% glucose) supplemented with 100 µg/mL zeocin. The pre-fermentation process was performed BMGH medium (1 M potassium phosphate buffer pH 6.0, 1.34% yeast nitrogen base, $4x10^{-4}$ g/L biotin, 1% glycerol) and the fermentation in BMMH medium (1 M potassium phosphate buffer pH 6.0, 1.34% yeast nitrogen base, $4x10^{-4}$ g/L biotin, 0.5% methanol). The pPICZαB vector was obtained from Invitrogen (California, USA).

3. Construction of the pPICZαB-STEAP1_6His expression vector

Total RNA was previously extracted from LNCaP cell line as described in (98). From there, cDNA was synthesized with the NZY First-Strand cDNA Synthesis kit. The cDNA encoding STEAP1 protein was amplified by Polymerase Chain Reaction (PCR) using specific primers containing restriction sites for Not I and Pst I enzymes (STEAP1 FW: 5' AA GCT GCA GGA ATG GAA AGC AGA AAA GAC ATC 3'; STEAP1 RV: 5' AAG CGG CCG CTA ATG GTG ATG GTG ATG GTG CAA CTG GGA ACA TAT CTC AGT 3'). PCR conditions were conducted as followed: initial denaturation step at 95ºC for 5 minutes, 30 cycles of denaturation at 95ºC for 30 seconds, annealing at 60ºC for 30 seconds and extension at 72ºC for 1 minute and a final elongation step at 72ºC for 5 minutes.

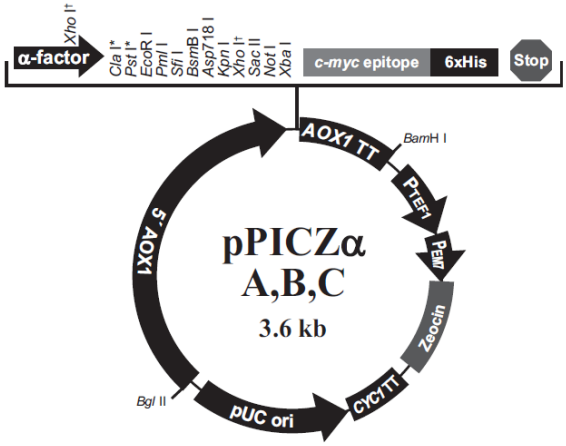

Figure 6: pPICZαB expression vector (Retrieved from Invitrogen, EasySelect™ *Pichia* Expression Kit no. 25, 2010).

PCR amplification was confirmed by 1% gel electrophoresis. PCR products were purified with the kit NucleoSpin Gel and PCR Clean-up following the manufacturer's instructions. The purified PCR products and vector pPICZαB (Figure 6) were digested with the restriction enzymes Not I and Pst I, and purified with kit NucleoSpin Gel and PCR Clean-up following the manufacturer's instructions. The digested products were incubated together with 0.5 µL T4 DNA Ligase and 1 µL Buffer 10x. Different conditions of temperature, time of incubation and ratio of vector:insert DNA were tested as suggested by the enzyme's manufacturer and presented in Table 1.

100 µL of competent *E. coli* TOP10 cells were transformed with the plasmid: 35 minutes on ice, followed by 2 minutes at 42ºC. The transformed cells were plated in LSLB medium with 25 µg/mL Zeocin. The plates were grown at 37ºC overnight. Several colonies were taken from

each plate, lysed (colony dissolved in 10 µL Mili-Q water and incubated for 10 minutes at 95ºC) and a screening by PCR was used to identify colonies with insert. Then, one colony was picked and grown in 2 mL of LSLB medium with 25 µg/mL Zeocin and incubated at 37ºC, 250 rpm, overnight. The plasmid was extracted and purified with the kit NZYMiniprep following the manufacturer's instructions. The purified vector was sequenced using the primers for STEAP1 as well as for the promoter AOX1 (AOX1 FW: 5' GACTGGTTCCAATTGACAAGC 3'; AOX1 RV: 5' CAAATGGCATTCTGACATCC) using the kit Dye Terminator Cycle Sequencing with Quick Start Kit from Beckman Coulter (California, USA). The program BlastX was used to analyze the DNA sequence in order to confirm the identity and frame of the insert.

4. *Pichia pastoris* X33 transformation

Using the sequenced vector, 10ng were digested with Sac I for 2 hours at 37ºC. The digested vector was purified with the kit NucleoSpin Gel and PCR Clean-up, and the purified linearized vector was stored at -80ºC.

Untransformed *Pichia pastoris* X33 cells were streaked on YPD plates, which were grown at 30ºC for 2-3 days. An isolated colony was picked and transferred to 5 mL of YPD medium in a 50 mL Falcon tube and grown overnight at 30ºC at 250 rpm. From there, 100 µL of the overnight culture was transferred to 250 mL YPD medium and the cells were grown at 30ºC until the OD_{600} reached 1.3-1.5. The culture was centrifuged in four separate 50 mL Falcon tubes at 4ºC for 5 minutes at 1500 g. The pellets were recovered and the supernatant discarded. Each pellet was dissolved in 20 mL of cold Sorbitol 1 M. All the pellets were gathered in one Falcon and centrifuged at 4ºC for 5 minutes at 1500 g. The supernatant was discarded and the pellet was dissolved in 1 mL of cold Sorbitol 1 M. The vector, previously stored at -80ºC, was added to 80 µL cells and the mixture was transferred to a cold 0.2 cm electroporation cuvette (Bio-Rad, California, USA) and incubated on ice for 5 minutes. The cells were pulsed once at 2500 V and, immediately after, 1 mL of cold Sorbitol 1 M was added. The mixture was transferred to a 15 mL Falcon tube and grown for 2 hours at 30ºC. Afterward, 12.5 µL, 25 µL, 50 µL and 100 µL of cells were streaked on four YPDS plates with 200 µg/mL Zeocin, and grown at 30ºC for 4 days.

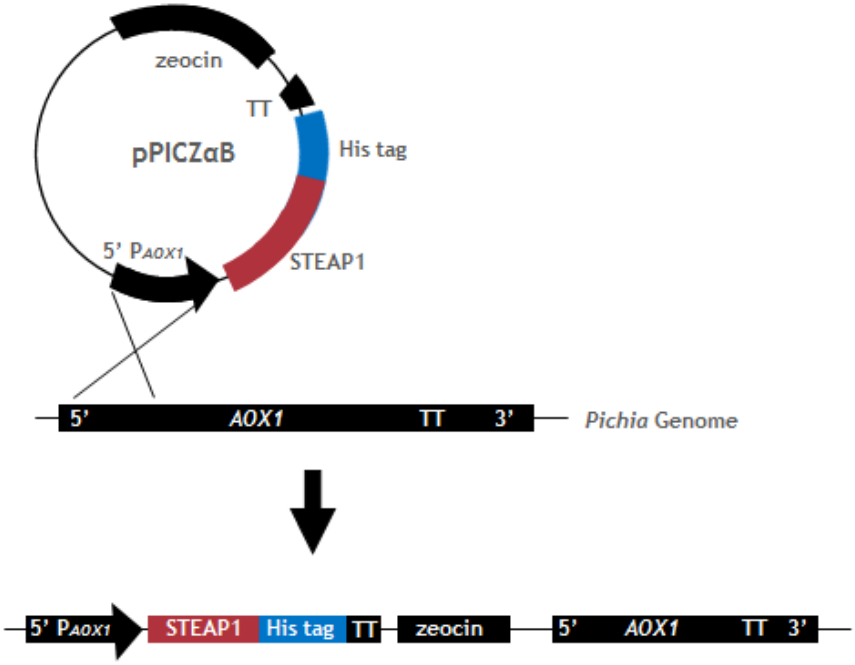

Figure 7: Schematics of gene insertion into *Pichia pastoris*' genome.

An isolated colony was picked from the ones originated from the 100 µL and transferred to 5 mL of YPD medium with 200 µg/mL of Zeocin in a 50 mL Falcon tube and grown overnight at 30ºC at 250 rpm. From there, 2 mL of the culture was added to 100 mL of YPD medium with 200 µg/mL of Zeocin and grown at 30ºC, 250 rpm, until the OD_{600} reached 1.2-1.3. To 3 mL of glycerol was added 7 mL of the culture and stored in aliquots with 500 µl at -80ºC.

To further confirm the presence of the vector, PCR was performed with *Pichia pastoris* genomic DNA (gDNA). From the fermentation process, 1 mL of medium was harvested and centrifuged at 14000 rpm for 2 minutes. The supernatant was discarded. The extraction and purification of gDNA were achieved with the Wizard® Genomic DNA Purification Kit. PCR reactions were carried out using STEAP1 and AOX1 primers.

5. Biosynthesis of STEAP1 in *Pichia pastoris*

An initial fermentation was carried out with the following conditions: an aliquot of transformed *Pichia pastoris* X33 with the vector pPICZαB-STEAP1_6His was streaked on YPD plates with 200 µg/mL Zeocin and grown at 30ºC for 2-3 days. An isolated colony was picked and transferred to 50 mL of BMGH medium in 250 mL shake flasks and grown at 30ºC, 250 rpm until OD_{600} reached 5-6. Then, an aliquot was collected and centrifuged at 15000 g, at room temperature for 5 minutes and added to 100 mL of BMMH medium in 500 mL shake flasks in

order to fix the initial OD_{600} at 1.0. To determine the volume of pre-fermentation to collect, the formula (1) was used:

$$OD_{pre-ferm} \times V_{pre-ferm} = \left(V_{pre-ferm} + V_{fermentation}\right) \times OD_{fermentation} \quad (1)$$

The fermentation was carried out during 120 h at 30ºC and 250 rpm and supplemented with 1% methanol every 24 hours. Finally, the cells were harvested by centrifugation at 1500g for 5 min at 4ºC. The supernatant/extracellular medium and pellet were stored at −20ºC until use. Afterward, the time of fermentation, methanol concentration, time of induction, temperature and use of chaperones were altered to optimize protein production.

6. Protein Recovery and Solubilization

The cell mass previously harvested was weighted and lysed in 2 volumes of lysis buffer (150 mM NaCl, 50 mM Tris, 1 mM $MgCl_2$, pH 7.8), 2 volumes of glass beads (500 µm, Sigma-Aldrich, St. Louis, MO, USA) and protease inhibitors cocktail (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's instructions. The mixture was vortexed 7 times for 1 min with an interval of 1 min on ice and centrifuged at 500g for 5 minutes at 4ºC. The supernatant was discarded and the pellet with the glass beads was resuspended in the same volume of lysis buffer. The resolubilized pellet was transferred to a lysis tube and added DNase (0.25 mg/mL), and centrifuged at 16000g for 30 min at 4ºC. The 16000g supernatant (S_{16000g}) was collected, and the 16000g pellet (P_{16000g}) was solubilized in the same volume of lysis buffer plus 1% Triton X-100 at 4ºC. For most samples, Triton X-100 was the detergent used. However, other detergents were tested, such as SDS, Tween 20 and Tween 80, as well as urea.

7. Agarose gel electrophoresis

The DNA electrophoresis was performed on a gel containing 1% agarose. The buffer used was a Tris-acetic acid buffer (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8.0). The run was realized at 120 V for 30 min and the bands were visualized under UV light using the program UVITEC FireReader (UVItec, Cambridge, UK).

8. Total protein quantification

The protein concentration in the samples obtained after solubilization were quantified with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), using bovine serum albumin (BSA) as the standard with concentrations from 25 to 2000 µg/mL according to manufacturer's instructions. The plate was read in xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad). The following curves were used to quantify the P_{16000g} fraction, according to the detergent used in the final step of solubilization.

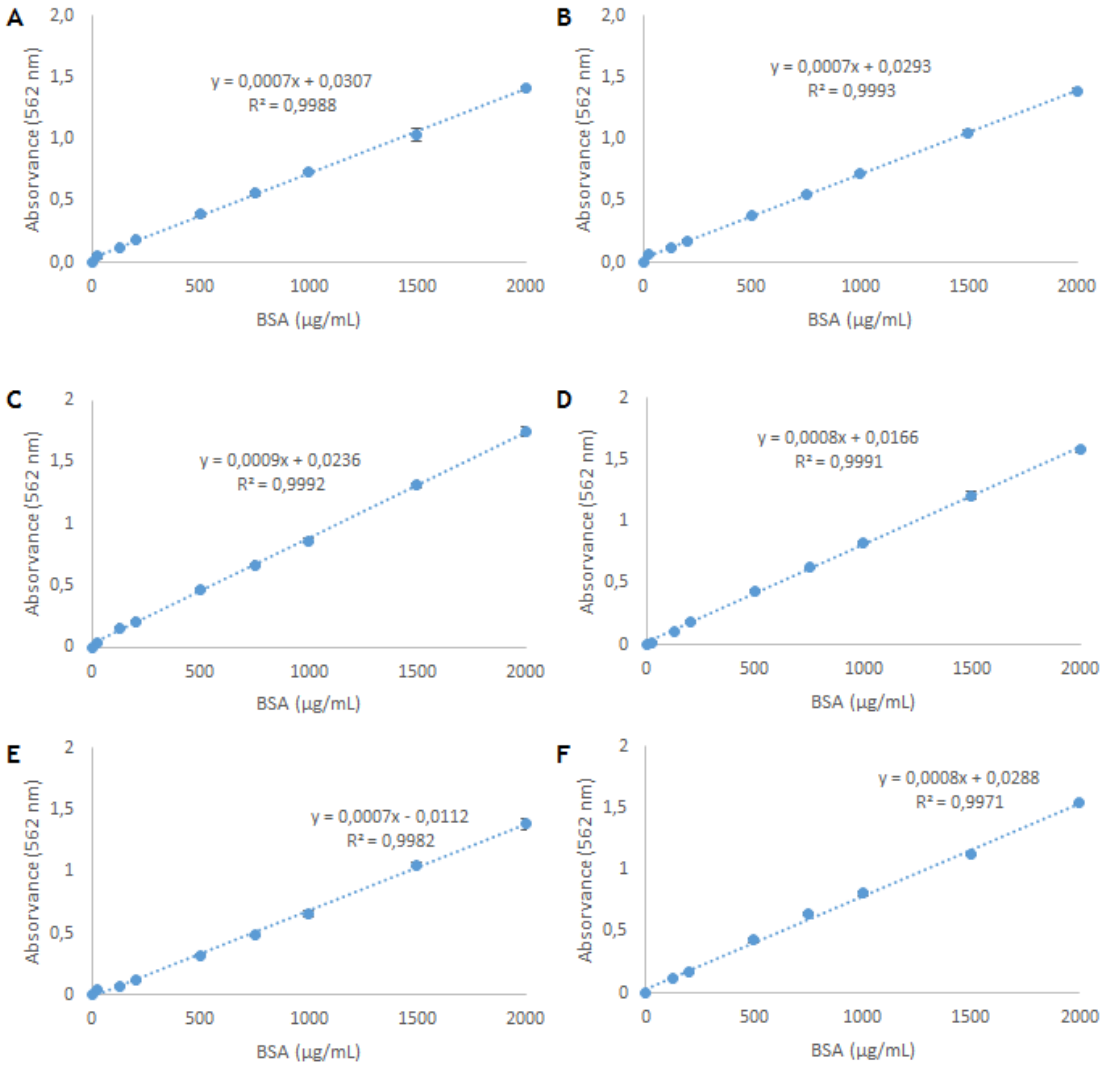

Figure 8: A- BSA calibration curve with Lysis Buffer; B- BSA calibration curve with Lysis Buffer (150 mM NaCl, 50 mM Tris, 1 mM MgCl2, pH 7.8) and Triton X-100 1%; C- BSA calibration curve with Lysis Buffer with and urea 6M; D- BSA calibration curve with Lysis buffer and Tween 20 1%; E- BSA calibration curve with Lysis buffer and SDS 1%; F- BSA calibration curve with Lysis buffer and Tween 80 1%.

9. Western Blot Analysis

Total protein was resolved by 12% SDS-PAGE gel at 120 V for 1h45min at room temperature with Running buffer, and then, electrotransferred to a Polyvinylidene difluoride membrane (GE Healthcare, Buckinghamshire, UK) at 0.75 A for 45 minutes in CAPS buffer. Membranes were blocked for 1 h in a 5% (w/v) milk solution and incubated overnight with a rabbit polyclonal antibody against human STEAP1 (diluted at 1:600, Santa Cruz Biotechnology, Dallas, Texas, U.S.A.) at 4ºC with constant stirring. The membranes were then washed with Washing buffer and incubated with a polyclonal antibody anti-rabbit (diluted 1:40000; Cosmo Bio Co. Ltd., Tokyo, Japan) for 1 h at room temperature with constant stirring. Finally, the membranes were once more washed with Washing buffer, exposed to ECL substrate (Advansta, CA, USA) for 5 minutes and visualized on the Molecular Imager FX (Biorad, Hercules, USA).

CHAPTER IV- RESULTS AND DISCUSSION

1. Construction of the expression vector pPICZαB-STEAP1_6His

The initial step was to amplify by PCR the *STEAP1* gene and insert it into the pPICZαB expression vector. The primers contained the restriction sites for two different restriction enzymes (Pst I and Not I, respectively). These restriction enzymes were chosen because they can be used to cut the expression vector pPICZαB in the multiple cloning site. Using two restriction enzymes instead of just one facilitates the directional insertion of the gene into the expression vector. It was also added a sequence coding a 6 Histidine tag in the primer reverse to facilitate the purification process. Since the primers had never been used before, in the first PCR done to amplify the *STEAP1* gene, three annealing temperatures were tested (60ºC, 62ºC, and 65ºC). All temperatures were effective, as it can be seen in Figure 9A. The temperature of annealing chosen was 60ºC since it created the band with the highest intensity.

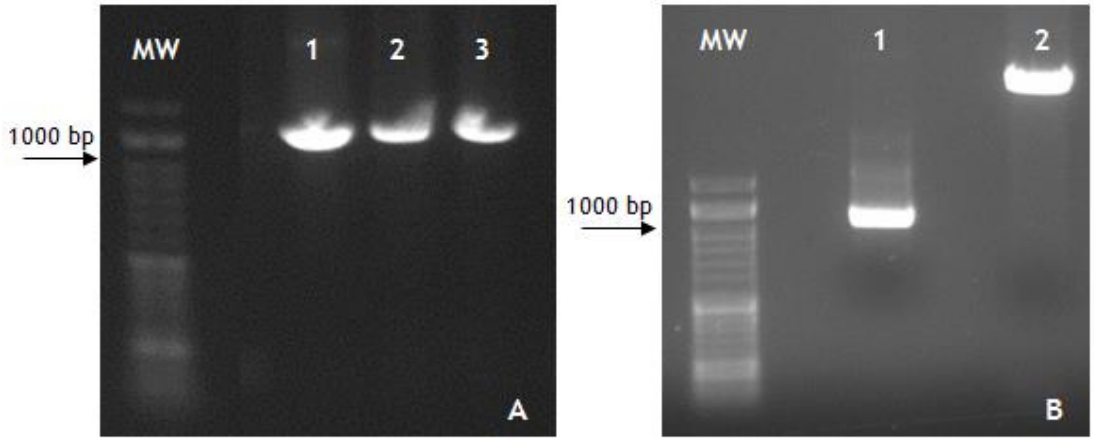

Figure 9: Agarose Gel 1%. A: M- NZYDNA ladder VI marker; 1- Annealing at 60ºC; 2- Annealing at 62ºC; 3- Annealing at 65ºC; B: MW- NZYDNA ladder VI marker; 1- Digested PCR products; 2- Digested pICZαB vector.

The PCR product and pPICZαB vector were digested with the restriction enzymes and purified. The digestion was complete as it can be seen in Figure 9B by the presence of a single band. In order to determine the best condition for DNA cloning, different quantities of digested PCR were added to 1 µL pPICZαB vector, supplemented by 1 µL Buffer, 0.5 µL T4 DNA Ligase and water Mili-Q until a total volume of 10 µL (Table 1).

Table 1: Different conditions tested for DNA cloning into a pPICZαB vector.

REACTION	PURIFIED PCR FRAGMENTS	TEMPERATURE	TIME
1	0.5 µL	Room T	3h
2	1 µL	Room T	3h
3	2 µL	Room T	3h
4	4 µL	Room T	3h
5	1 µL	4ºC	Overnight
6	2 µL	4ºC	Overnight
7	1 µL	Room T	4h
8	2 µL	Room T	4h

Of all the conditions tested, the best one was the Reaction 8, since it was the only reaction that was amplified by PCR with the STEAP1 primers (Figure 10, lane 5). The vector was purified and sequenced to confirm the correct placement of the gene. The PCR products were also sequenced and analyzed using the BlastX program.

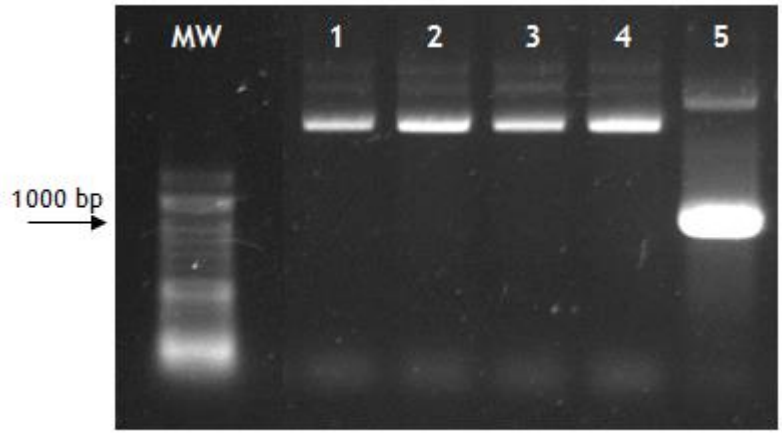

Figure 10: Agarose gel 1%; 1-4: reaction 1-4; 5- reaction 8.

2. *Pichia pastoris* X33 transformation

The sequenced vector was digested with the restriction enzyme Sac I, to linearize it and enable its insertion into the *Pichia pastoris'* genome. To confirm the correct insertion, the transformed cells were grown in YPD plates with zeocin. Since the resistance to this antibiotic is given by the vector, colonies that grew on the plate will have it. Several volumes of

transformed *Pichia pastoris* were plated: 12.5 µL, 50 µL, 100 µL and 200 µL. A colony from the 100 µL plate (Figure 11D) was selected and PCR amplification with STEAP1 and AOX1 primers confirmed the vector's correct insertion into *Pichia pastoris'* genome (Figure 12).

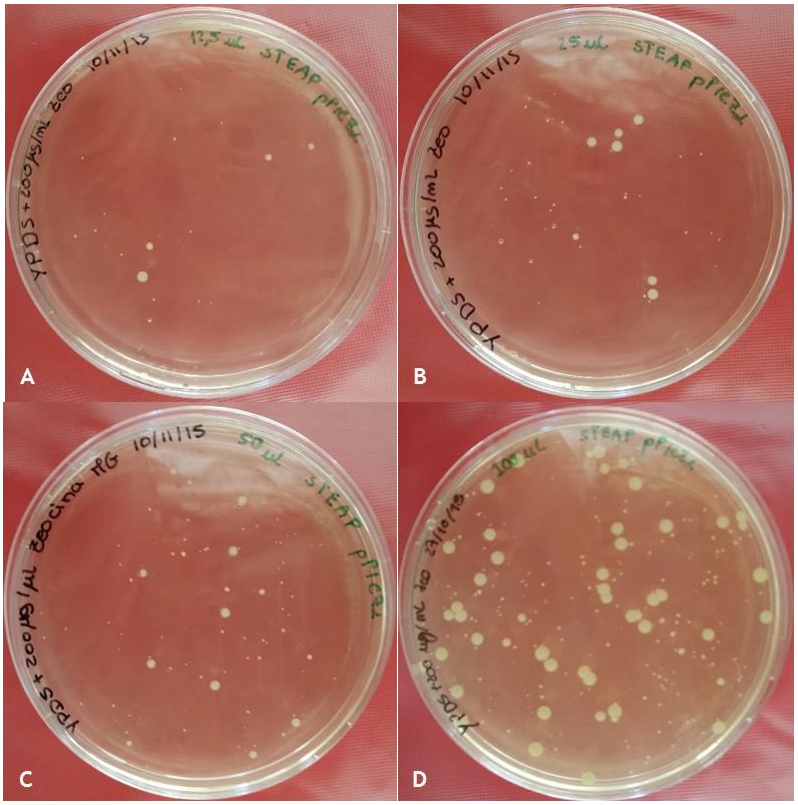

Figure 11: YPD plates with colonies with *Pichia pastoris* transformed with pPICZαB-STEAP1_6His expression vector; A- 12.5 µL of transformed cells; B- 25 µL of transformed cells; C- 50 µL of transformed cells; D- 100 µL of transformed cells.

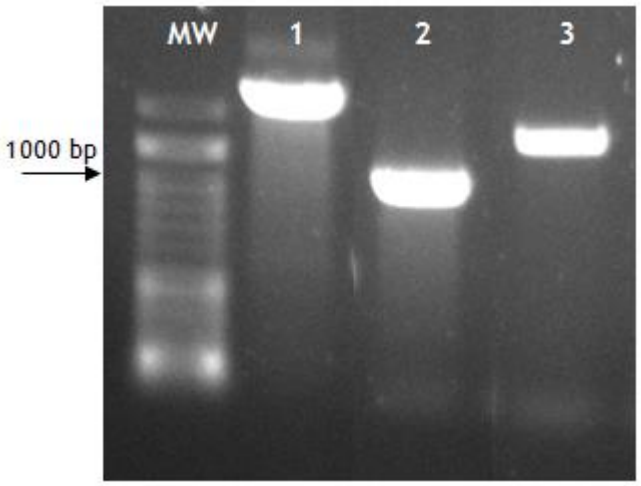

Figure 12: Agarose gel 1%; 1- PCR amplification with AOX1 primers; 2- PCR amplification with STEAP1 primers; 3- PCR amplification with AOX1 primers forward and STEAP1 primer reverse.

3. Biosynthesis of STEAP1 in *Pichia pastoris*

An initial fermentation was carried out with untransformed *Pichia pastoris* X33 to confirm that the strain did not produce STEAP1 protein. An aliquot of untransformed *Pichia pastoris* X33 was streaked on YPD plates and grown at 30ºC for 2-3 days. An isolated colony was picked and transferred to BMGH medium and grown at 30º C at 250 rpm until OD_{600} reached 5-6. Then, an aliquot was collected and centrifuged at 15000g, at room temperature for 5 minutes and added to BMMH medium in order to fix the initial OD_{600} at 1.0.

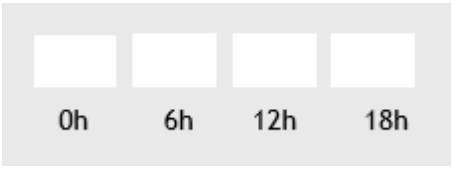

Figure 13: Western blot analysis of pellets obtained from untransformed *Pichia pastoris* X33.

Since no protein was detected (Figure 13), we can conclude that STEAP1 is not produced by the untransformed strain.

A fermentation with the transformed strain was done to determine its growth profile. The fermentation process was carried out in BMMH medium with the initial OD_{600} at 1.0 and for 120 hours. The medium was supplemented with methanol 1% every 24 hours and the OD_{600} was measured every 12 h.

The results in Figure 14 demonstrate that after 60 h of fermentation, *Pichia pastoris* seems to enter the stationary phase. Also, samples were taken every 12 hours for protein production analysis. The lysis process was carried out and Western blot analysis was performed to the various fractions obtained during the process. The S_{16000g}, P_{16000g} and extracellular medium were analyzed. An extra fermentation was carried out in parallel, but with no methanol supplementation, to determine if the protein was being produced without induction.

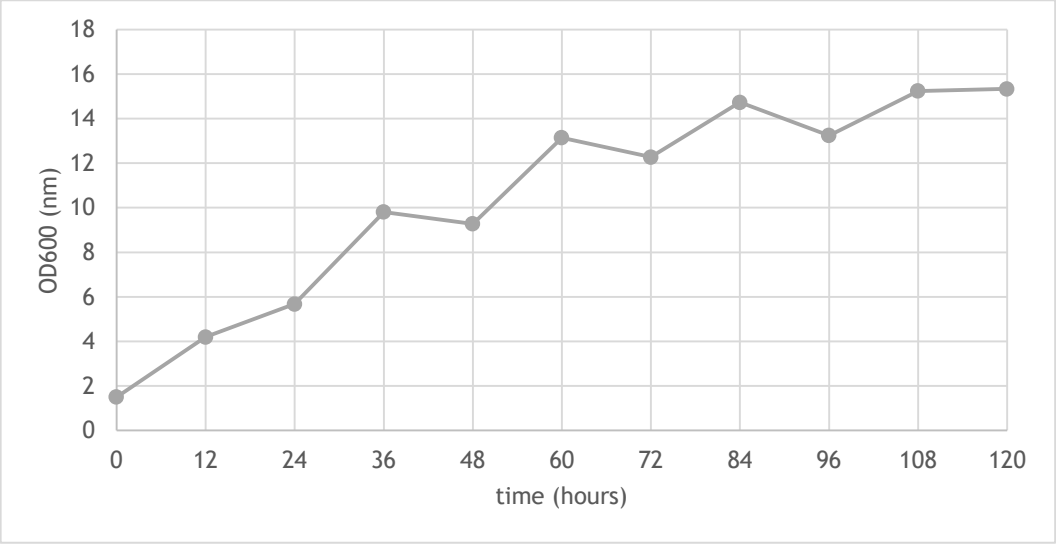

Figure 14: *Pichia pastoris* X33 with vector pPICZalphaB+STEAP1_6His growth profile.

Using a specific antibody against STEAP1, an immunoreactive band was found between the 35 and 48 kDa. Considering that STEAP1 molecular weight is approximately 40 kDa, our results suggest that the band detected corresponds to STEAP1 protein. The protein was found in the P_{16000g} fraction at 12h and in lesser amounts at 36h, 60h, and 84h (Figure 15A). No protein was found in the extracellular medium nor the S_{16000g} fractions (Figure 15B, C), indicating that the protein can only be found in the P_{16000g} fraction. In addition, no protein was found in any of the fractions taken from the fermentation process without induction. A closer look at the results of the P_{16000g} fraction, we can see that the protein does not appear in every sample taken over time. To further understand this process, another fermentation was carried out, taking samples every 6 hours. With the growth profile and the Western blot analysis, the time of fermentation was shortened from 120 to 60 hours since the protein that is being produced is a membrane protein and it is not being secreted. In addition, to better compare the STEAP1 production over time, the same amount of total protein (5 µg) was analyzed by Western blot.

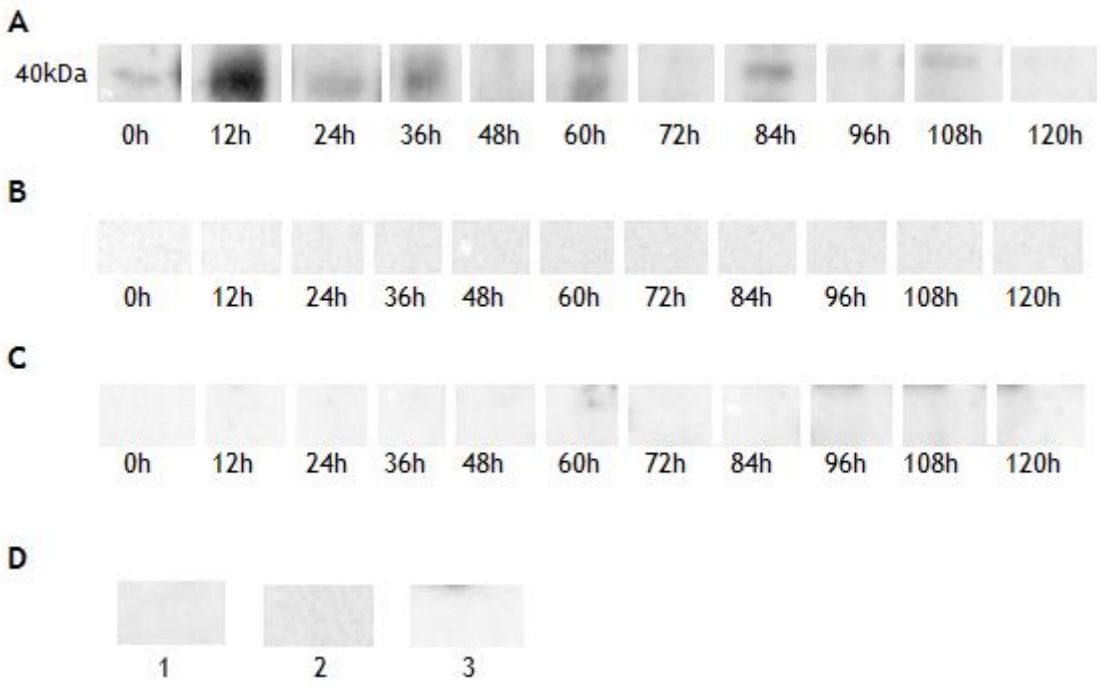

Figure 15: Western blot analysis of STEAP1 production in P_{16000g} (A), S_{16000g} (B) and extracellular medium (C) over 120h of fermentation with samples taken every 12h and methanol induction every 24h and fermentation without methanol induction (D) in P_{16000g} (1), S_{16000g} (2) and extracellular medium (3) at 120h.

Western blot analysis shows that STEAP1 protein is more produced at 12h, 30h, and 54h (Figure 16A). Curiously, the time of fermentation with high levels of STEAP1 occurs after methanol induction. The initial BMMH medium already contains methanol and the sample collected at 12h, presents high levels of STEAP1. At 18h of fermentation, its production diminishes considerably and at 24h no protein is detected. However, at the 24h of fermentation, 1% methanol was added to the medium, and the sample collected at 30h shows that STEAP1 is produced again at high levels. The production of STEAP1 decrease progressively until 48h of fermentation, a time-point that was added methanol, and consequently, the levels of STEAP1 increased at 54h of fermentation. These results suggest that protein production only occurs during a short period of time after methanol induction, or the induction requires a high concentration of methanol. Afterward, the protein is most likely being degraded by the microorganism due to its toxicity. Other cases have showed protein degradation during the fermentation process (99).

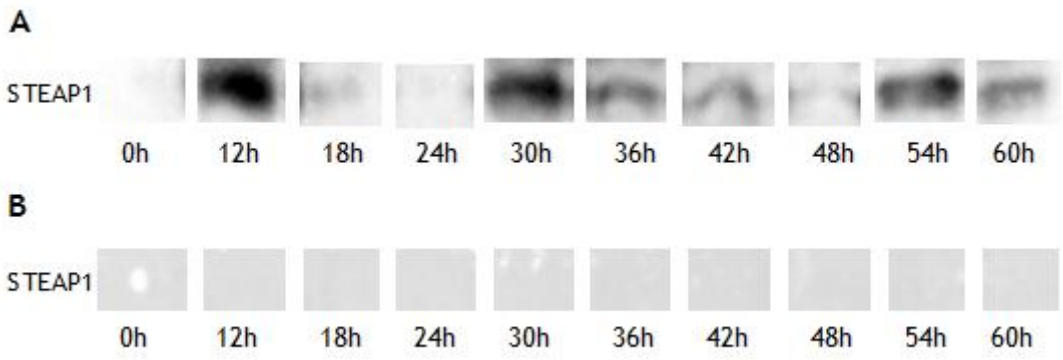

Figure 16: Western blot analysis of STEAP1 production in P_{16000g} (A) and S_{16000g} (B) over 60h of fermentation with samples taken every 6h and methanol induction every 24h.

Considering that STEAP1 production was apparently minor at 54h, the next fermentation has stopped at 36 hours, which is the sample taken after the second induction with methanol. Also, in an attempt to stabilize the protein, DMSO was added to the initial medium. DMSO is a chemical chaperone that can alter the expression of genes involved in the formation of membrane lipid components, leading to increased stabilization of proteins (100). The addition of DMSO has shown an increase of protein production in many cases (101–105). In this work, two concentrations of DMSO (3% and 6%) were tested, since these are the ones usually described in the literature.

This addition seemed to stabilize the protein since the bands obtained were very intense in both cases (Figure 17A and B). However, the production stopped after 6 hours and some protein degradation was found in the sample with 3% DMSO (Figure 17C lane 1). Then, an initial theory emerged, suggesting that *Pichia pastoris* may be consuming methanol at a higher rate than expected, and consequently, no more protein was produced. To test this hypothesis, the time of induction was evaluated, with 1% methanol added every 6 hours with 6% DMSO, since with this concentration there was not visible protein degradation (Figure 17C lane 2).

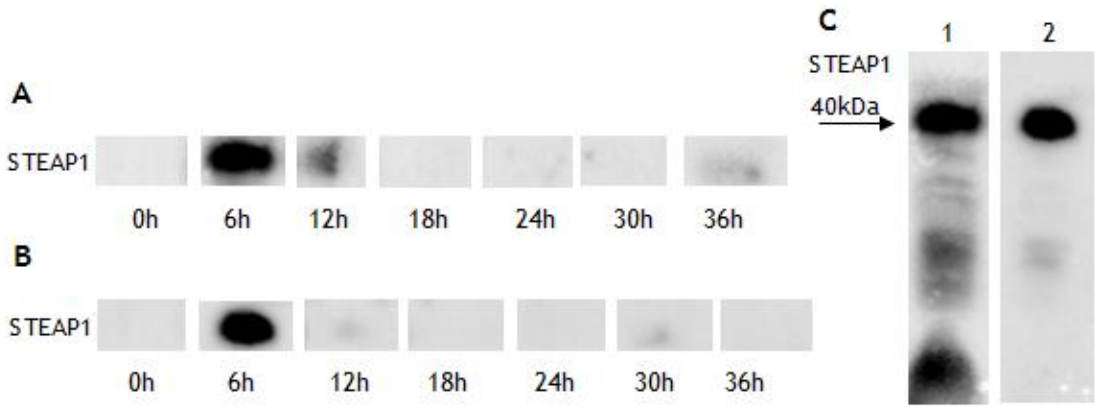

Figure 17: Western blot analysis of STEAP1 production in P_{16000g} over 36h of fermentation with samples taken every 6h, supplemented with methanol every 24h and 3% (A) and 6% (B) DMSO. C- Detail of the 6h sample band for 3% (A) and 6% (B) DMSO.

As seen in Figure 18, STEAP1 production was found at consecutive hours, with higher intensity at 12h, 18h, and 24h. The intensity of the bands seem to decrease with time, but the protein did not disappear, which is in accordance with previous fermentations. Interestingly, different immunoreactive bands were detected, suggesting that some interactions with other proteins from *Pichia pastoris* may occur. In addition, it is possible the formation of protein dimers (106), or even some alterations in the protein folding, which may lead to different molecular weights (99). In order to increase the production of STEAP1 protein, the concentration of methanol was varied. Thus, three fermentations were carried out with three different methanol concentrations: 0.75%, 1%, and 1.25%.

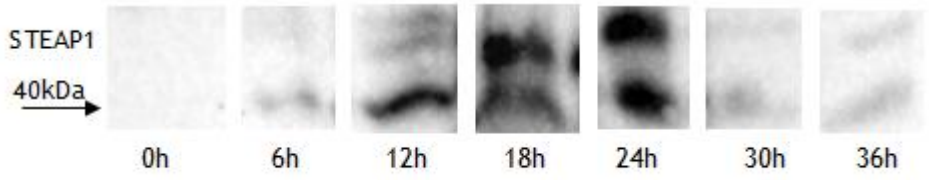

Figure 18: Western blot analysis of STEAP1 production in P_{16000g} over 36h of fermentation with samples taken every 6h supplemented with methanol every 6h.

Regarding 0.75% methanol induction, the protein production was very low, with an intense band only at 18h of fermentation (Figure 19A). It is possible that methanol concentration was too low to induce protein production after 6 hours, like in the previous results. With 1%, STEAP1 protein was found at a higher concentration at 6h (Figure 19B), but the protein appeared more degraded (Figure 19D lane 1). Concerning 1.25% methanol induction, the levels of STEAP1 protein seemed less than with 1% (Figure 19C), but presents less degradation (Figure 19D lane 2). These results allow to conclude that STEAP1 protein is not produced in sufficient amounts using 0.75% methanol, being then discarded. Considering that either 1% or

1.25% methanol allow to obtain good yield good results, it was decided to repeat the fermentations at 25ºC in an attempt to diminish protein degradation. In fact, other studies have been reported that lowering the temperature of fermentation may increase the protein stabilization (99, 103).

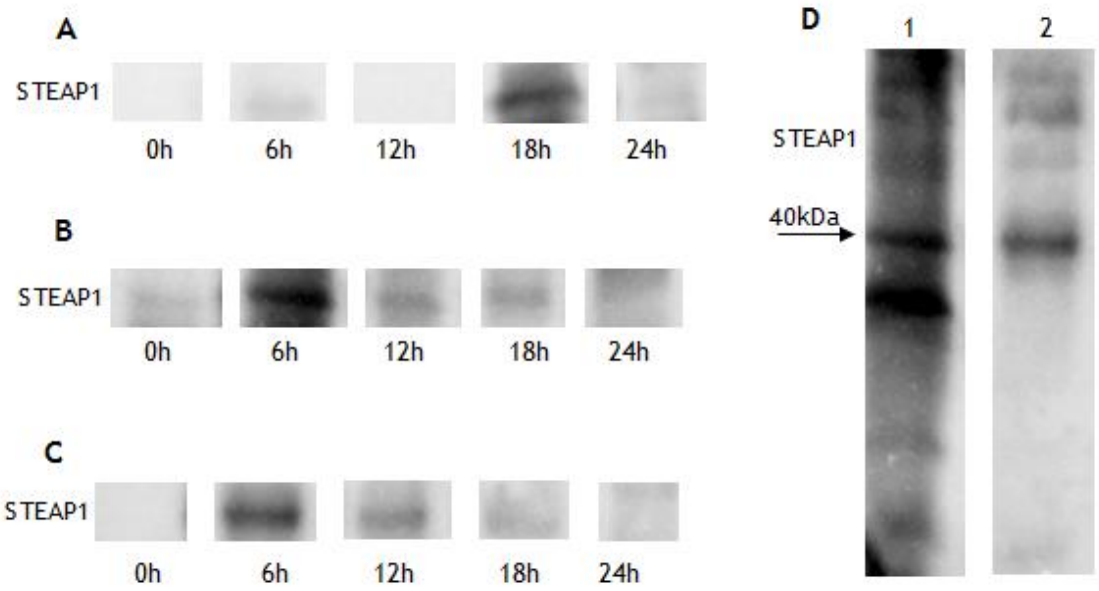

Figure 19: Western blot analysis of STEAP1 production in P_{16000g} over 24h of fermentation supplemented with methanol 0.75% (A), 1% (B) and 1.25% (C) every 6h; D- Detail of the 6h sample band for 1% (B) and 1.25% (C) methanol induction.

Protein production seemed to be higher at 6h in both situations, which is in accordance with previous results. The induction with 1.25% methanol seemed to obtain more protein than with 1% (Figure 20). However, it is unclear if the change in temperature improved protein production and stabilization. To confirm that temperature did not improve protein production, two fermentations were carried out with 1.25% methanol at 25ºC and 30ºC.

Figure 20: Western blot analysis of STEAP1 production in P_{16000g} over 18h of fermentation supplemented with methanol 1% (A) and 1.25% (B) every 6h at 25ºC.

Once more, the best time for protein production was at 6h of fermentation for both situations. As visualized in Figure 21, the best temperature for protein production was 30ºC and not 25ºC. In addition, the protein degradation was also lower at 30ºC than at 25ºC (data not shown). At 12h and 18h of fermentation, the protein seems to disappear in the 40 kDa

mark, but can still be visible at higher molecular weights. It is possible that protein may form aggregates. Other studies have shown that *Pichia pastoris* is capable of forming inclusion bodies (89). If these bands are the result of protein aggregation, their solubilization with urea may separate the proteins.

Figure 21: Western blot analysis of STEAP1 production in P_{16000g} over 18h of fermentation supplemented with methanol 1.25% every 6h at 25ºC (A) and 30ºC (B).

Of all these results, we can determine that the best medium for optimal protein production is a BMMH medium with 1.25% methanol and 6% DMSO. In addition, the best conditions for STEAP1 production are at 6 hours of fermentation at 30ºC and 250 rpm.

4. Protein Recovery and Solubilization

As previously mentioned, membrane proteins have the tendency to form aggregates. In addition, they are extremely hydrophobic, requiring detergents for solubilization. The choice of detergent may also affect the efficiency of downstream protein purification (107). Detergents are amphipathic molecules that contain both a hydrophobic and hydrophilic domain and form micelles in water. Micelles are a group of detergent molecules in which the hydrophilic head faces outward. Detergents can solubilize and stabilize the protein by binding to the hydrophobic part on one side and interacting with the aqueous part on the other (107, 108).

Different detergents were used to determine the best one to solubilize the protein. Triton X-100, Tween 20 and Tween 80 are non-ionic detergents. SDS is an anionic detergent. Urea was also tested to determine if the bands at higher molecular weights in Western blot analysis could result from protein aggregation.

Western blot analysis of the pellet solubilized with only the lysis buffer showed an intense band, suggesting that the protein could be solubilized without detergents (Figure 22). However, the band is not at the correct weight and detergents do seem to be necessary to stabilize the protein in a correct native folding. Urea did not seem to remove the protein

aggregation, as it was expected, with two bands at different weights. Tween 20 and Tween 80 both seem to be ineffective in solubilizing the protein. SDS is the only detergent that presented a band at the correct molecular weight. Other studies have shown the use of SDS to solubilize proteins and in conditions where it forms aggregation (89).

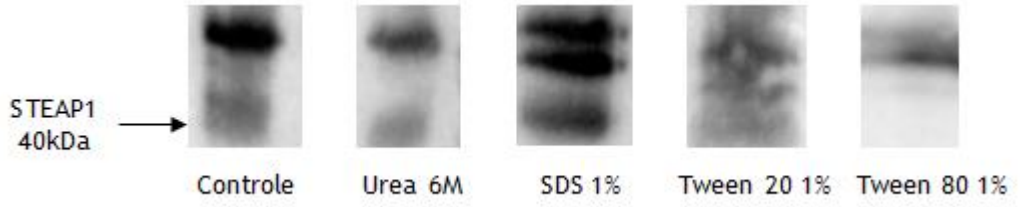

Figure 22: Western blot analysis of STEAP1 production in P_{16000g} over 6h of fermentation with methanol 1.25% at 30ºC and solubilized with different detergents.

Since SDS showed the capacity to solubilize the protein, different concentrations of this detergent were tested, as well as Triton X-100, since it was the one used in previous samples. The results showed that SDS has a higher capability of solubilizing STEAP1, and at higher amounts with the correct molecular weight (Figure 23). There are no significant differences between the different concentrations of SDS. Regarding the Triton, 3% concentration seem to be the best one but showed less protein when compared with SDS (Figure 23B). The bands at higher molecular weights are still visible in all samples, but they do stand out more in the bands solubilized with SDS. Interestingly, SDS was the only anionic detergent tested and the one with better results suggesting that other similar detergents could be tested in the future.

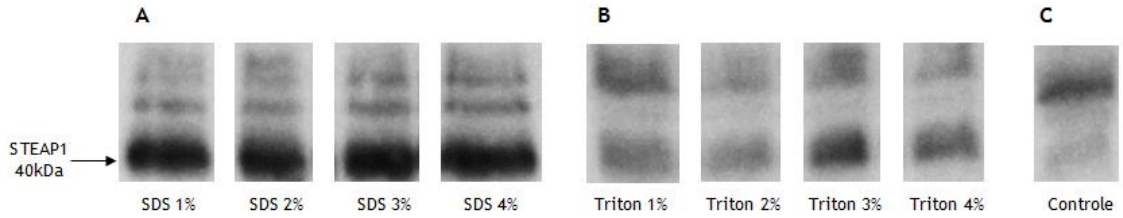

Figure 23: Western blot analysis of STEAP1 production in P_{16000g} over 6h of fermentation with methanol 1.25% at 30ºC and solubilized with different concentrations of SDS (A), Triton (B) and lysis buffer (C).

In conclusion, for protein solubilization, the best detergent seems to be SDS in a range between 1-3%, which is in accordance with other research groups (89, 90).

CHAPTER V- CONCLUSION AND FUTURE PERSPECTIVES

The expression vector pPICZαB+STEAP1_6His constructed was effective in the production of the recombinant protein STEAP1 in *Pichia pastoris* X33. The usage of a Mut^+ strain allowed using higher concentrations of methanol to induce protein expression, in conjunction with the AOX promoter. The best conditions for optimal protein production are a BMMH medium with 1.25% methanol and 6% DMSO and a fermentation process of 6 hours at 30ºC and 250 rpm. The protein was found entirely in the P_{16000g} fraction. The best solubilization conditions are a combination of the lysis buffer and SDS in the range between 1-3%.

For the first time, this expression system was used for STEAP1 production, and future works may involve further optimization of the production process. Since the highest methanol concentration tested was the one who gave better results, an even higher concentration may yield even more protein. In addition, other chaperones could be tested, such as histidine.

In addition, since protein production ceases after 6 hours of fermentation, a higher initial cellular mass could be a strategy to apply for higher protein production. Instead of a fermentation medium with methanol, glycerol would be added. Once it was reached a high OD and the glycerol was depleted, methanol would be added. Glycerol depletion could be monitored in a bioreactor through control of the oxygen levels.

The latest results regarding protein solubilization with SDS (Figure 23) showed different molecular weight for STEAP1 protein. It should be evaluated if the protein has a different folding process or if is aggregation with other host proteins. Nevertheless, the difference in the protein's molecular weight could influence its purification with IMAC, which will be tested.

REFERENCES

1. Longo DL, Fauci AS, Kasper DL, Hauser SL, Jameson JL, Loscalzo J. 2012. Harrison's Principle of Internal Medicine, 18th ed. McGraw-Hill, New York, NY.

2. Vander AJ, Luciano D, Sherman J. 2001. Human Physiology: The Mechanisms of Body Function, 8th ed. McGraw-Hill, New York, NY.

3. Scanlon VC, Sanders T. 2006. Essentials of Anatomy and Physiology, 5th ed. F. A. Davis Company, Philadelphia, PA.

4. McNeal JE. 1969. Origin and development of carcinoma in the prostate. Cancer 23:24–34.

5. Lalani el-N, Laniado ME, Abel PD. 1997. Molecular and cellular biology of prostate cancer. Cancer Metastasis Rev 16:29–66.

6. Kirby RS. 1995. Anatomy and Pathophysiology of the Prostate Gland, p. 3–12. *In* Epidemiology of Prostate Disease. Springer Berlin Heidelberg, Berlin, Heidelberg.

7. McNeal JE. 1980. Anatomy of the prostate: an historical survey of divergent views. Prostate 1:3–13.

8. De Marzo AM, Platz EA, Sutcliffe S, Xu J, Grönberg H, Drake CG, Nakai Y, Isaacs WB, Nelson WG. 2007. Inflammation in prostate carcinogenesis. Nat Rev Cancer 7:256–69.

9. McNeal JE. 1981. The zonal anatomy of the prostate. Prostate 2:35–49.

10. Shen MM, Abate-Shen C. 2010. Molecular genetics of prostate cancer: new prospects for old challenges. Genes Dev 24:1967–2000.

11. Peehl DM. 2005. Primary cell cultures as models of prostate cancer development. Endocr Relat Cancer 12:19–47.

12. Kumar VL, Majumder PK. 1995. Prostate gland: structure, functions and regulation. Int Urol Nephrol 27:231–43.

13. Mann T, Lutwak-Mann C. 1981. Male Reproductive Function and Semen. Springer London, London.

14. Hsing AW. 2001. Hormones and prostate cancer: what's next? Epidemiol Rev 23:42–58.

15. Marker PC, Donjacour AA, Dahiya R, Cunha GR. 2003. Hormonal, cellular, and molecular control of prostatic development. Dev Biol 253:165–74.

16. Patrão MTCC, Silva EJR, Avellar MCW. 2009. Androgens and the male reproductive tract: an overview of classical roles and current perspectives. Arq Bras Endocrinol Metabol 53:934–45.

17. Grosse A, Bartsch S, Baniahmad A. 2012. Androgen receptor-mediated gene repression. Mol Cell Endocrinol 352:46–56.

18. Gao W, Bohl CE, Dalton JT. 2005. Chemistry and structural biology of androgen receptor. Chem Rev 105:3352–70.

19. Hsing AW, Chokkalingam AP. 2006. Prostate cancer epidemiology. Front Biosci 11:1388–413.

20. Schmid M, Trinh Q-D, Graefen M, Fisch M, Chun FK, Hansen J. 2014. The role of biomarkers in the assessment of prostate cancer risk prior to prostate biopsy: which markers matter and how should they be used? World J Urol 32:871–80.

21. Bostwick DG, Burke HB, Djakiew D, Euling S, Ho S, Landolph J, Morrison H, Sonawane B, Shifflett T, Waters DJ, Timms B. 2004. Human prostate cancer risk factors. Cancer 101:2371–490.

22. Bostwick DG. 1996. Prospective origins of prostate carcinoma. Prostatic intraepithelial neoplasia and atypical adenomatous hyperplasia. Cancer 78:330–6.

23. Ayala AG, Ro JY. 2007. Prostatic intraepithelial neoplasia: recent advances. Arch Pathol Lab Med 131:1257–66.

24. Dasgupta S, Srinidhi S, Vishwanatha JK. 2012. Oncogenic activation in prostate cancer progression and metastasis: Molecular insights and future challenges. J Carcinog 11:4.

25. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. 2015. Global cancer statistics, 2012. CA Cancer J Clin 65:87–108.

26. Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, Rosso S, Coebergh JWW, Comber H, Forman D, Bray F. 2013. Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. Eur J Cancer 49:1374–403.

27. Bray F, Ren J-S, Masuyer E, Ferlay J. 2013. Global estimates of cancer prevalence for 27 sites in the adult population in 2008. Int J cancer 132:1133–45.

28. Carter BS, Bova GS, Beaty TH, Steinberg GD, Childs B, Isaacs WB, Walsh PC. 1993. Hereditary prostate cancer: epidemiologic and clinical features. J Urol 150:797–802.

29. Patel AR, Klein EA. 2009. Risk factors for prostate cancer. Nat Clin Pract Urol 6:87–95.

30. Gann PH. 2002. Risk factors for prostate cancer. Rev Urol 4 Suppl 5:S3–S10.

31. Loeb S, Schaeffer EM. 2009. Risk factors, prevention and early detection of prostate cancer. Prim Care 36:603–21.

32. Obort AS, Ajadi MB, Akinloye O. 2013. Prostate-specific antigen: any successor in sight? Rev Urol 15:97–107.

33. Humphrey PA. 2004. Gleason grading and prognostic factors in carcinoma of the prostate. Mod Pathol 17:292–306.

34. McNeal JE, Villers AA, Redwine EA, Freiha FS, Stamey TA. 1990. Histologic differentiation, cancer volume, and pelvic lymph node metastasis in adenocarcinoma of the prostate. Cancer 66:1225–33.

35. Bostwick DG, Myers RP, Oesterling JE. 2012. Staging of prostate cancer. Semin Surg Oncol 10:60–72.

36. Bradford TJ, Tomlins SA, Wang X, Chinnaiyan AM. 2006. Molecular markers of prostate cancer. Urol Oncol 24:538–51.

37. Leman ES, Getzenberg RH. 2009. Biomarkers for prostate cancer. J Cell Biochem 108:3–9.

38. DeSantis CE, Lin CC, Mariotto AB, Siegel RL, Stein KD, Kramer JL, Alteri R, Robbins AS, Jemal A. 2016. Cancer treatment and survivorship statistics, 2014. CA Cancer J Clin 64:252–71.

39. Attard G, Parker C, Eeles RA, Schröder F, Tomlins SA, Tannock I, Drake CG, de Bono JS. 2016. Prostate cancer. Lancet 387:70–82.

40. Prapotnich D, Cathelineau X, Rozet F, Barret E, Mombet A, Cathala N, Sanchez-Salas RE, Vallancien G. 2009. A 16-year clinical experience with intermittent androgen deprivation for prostate cancer: oncological results. World J Urol 27:627–35.

41. Warde P, Mason M, Ding K, Kirkbride P, Brundage M, Cowan R, Gospodarowicz M, Sanders K, Kostashuk E, Swanson G, Barber J, Hiltz A, Parmar MK, Sathya J, Anderson J, Hayter C, Hetherington J, Sydes MR, Parulekar W. 2011. Combined androgen deprivation therapy and radiation therapy for locally advanced prostate cancer: a randomised, phase 3 trial. Lancet 378:2104–2111.

42. McNeel DG. 2007. Prostate cancer immunotherapy. Curr Opin Urol 17:175–81.

43. Arlen PM, Mohebtash M, Madan RA, Gulley JL. 2009. Promising novel immunotherapies and combinations for prostate cancer. Future Oncol 5:187–96.

44. Gustavsson H, Jennbacken K, Welén K, Damber J-E. 2008. Altered expression of genes regulating angiogenesis in experimental androgen-independent prostate cancer. Prostate 68:161–70.

45. Zhang F, Shan L, Liu Y, Neville D, Woo J-H, Chen Y, Korotcov A, Lin S, Huang S, Sridhar R, Liang W, Wang PC. 2013. An anti-PSMA bivalent immunotoxin exhibits specificity and efficacy for prostate cancer imaging and therapy. Adv Healthc Mater 2:736–44.

46. Han K-R, Seligson DB, Liu X, Horvath S, Shintaku PI, Thomas G V, Said JW, Reiter RE. 2004. Prostate stem cell antigen expression is associated with gleason score, seminal vesicle invasion and capsular invasion in prostate cancer. J Urol 171:1117–21.

47. Reiter RE, Gu Z, Watabe T, Thomas G, Szigeti K, Davis E, Wahl M, Nisitani S, Yamashiro J, Le Beau MM, Loda M, Witte ON. 1998. Prostate stem cell antigen: a cell surface marker overexpressed in prostate cancer. Proc Natl Acad Sci U S A 95:1735–40.

48. Saffran DC, Raitano a B, Hubert RS, Witte ON, Reiter RE, Jakobovits A. 2001. Anti-PSCA mAbs inhibit tumor growth and metastasis formation and prolong the survival of mice bearing human prostate cancer xenografts. Proc Natl Acad Sci U S A 98:2658–63.

49. Gu Z, Yamashiro J, Kono E, Reiter RE. 2005. Anti-prostate stem cell antigen monoclonal antibody 1G8 induces cell death in vitro and inhibits tumor growth in vivo via a Fc-independent mechanism. Cancer Res 65:9495–500.

50. Grunewald TGP, Diebold I, Esposito I, Plehm S, Hauer K, Thiel U, da Silva-Buttkus P, Neff F, Unland R, Müller-Tidow C, Zobywalski C, Lohrig K, Lewandrowski U, Sickmann A, Prazeres da Costa O, Görlach A, Cossarizza A, Butt E, Richter GHS, Burdach S. 2012. STEAP1 is associated with the invasive and oxidative stress phenotype of Ewing tumors. Mol Cancer Res 10:52–65.

51. Challita-Eid PM, Morrison K, Etessami S, An Z, Morrison KJ, Perez-Villar JJ, Raitano AB, Jia X-C, Gudas JM, Kanner SB, Jakobovits A. 2007. Monoclonal antibodies to six-transmembrane epithelial antigen of the prostate-1 inhibit intercellular communication in vitro and growth of human tumor xenografts in vivo. Cancer Res 67:5798–805.

52. Warburg O. 1956. On the Origin of Cancer Cells. Science 123:309–314.

53. Gatenby RA, Gillies RJ. 2004. Why do cancers have high aerobic glycolysis? Nat Rev Cancer 4:891–9.

54. Raghunand N, Gatenby RA, Gillies RJ. 2003. Microenvironmental and cellular consequences of altered blood flow in tumours. Br J Radiol 76 Spec No:S11-22.

55. Choi SYC, Xue H, Wu R, Fazli L, Lin D, Collins CC, Gleave ME, Gout PW, Wang Y. 2016. The MCT4 Gene: A Novel, Potential Target for Therapy of Advanced Prostate Cancer. Clin Cancer Res 22:2721–33.

56. Pértega-Gomes N, Vizcaíno JR, Attig J, Jurmeister S, Lopes C, Baltazar F. 2014. A lactate shuttle system between tumour and stromal cells is associated with poor prognosis in prostate cancer. BMC Cancer 14:352.

57. Grunewald TGP, Bach H, Cossarizza A, Matsumoto I. 2012. The STEAP protein family: versatile oxidoreductases and targets for cancer immunotherapy with overlapping and distinct cellular functions. Biol Cell 104:641–57.

58. Ohgami RS, Campagna DR, Greer EL, Antiochos B, McDonald A, Chen J, Sharp JJ, Fujiwara Y, Barker JE, Fleming MD. 2005. Identification of a ferrireductase required for efficient transferrin-dependent iron uptake in erythroid cells. Nat Genet 37:1264–9.

59. Ohgami RS. 2006. The Steap proteins are metalloreductases. Blood 108:1388–1394.

60. Finegold AA, Shatwell KP, Segal AW, Klausner RD, Dancis A. 1996. Intramembrane bis-heme motif for transmembrane electron transport conserved in a yeast iron reductase and the human NADPH oxidase. J Biol Chem 271:31021–4.

61. Sanchez-Pulido L, Rojas AM, Valencia A, Martinez-A C, Andrade MA. 2004. ACRATA: a novel electron transfer domain associated to apoptosis and cancer. BMC Cancer 4:98.

62. Gomes IM, Maia CJ, Santos CR. 2012. STEAP proteins: from structure to applications in cancer therapy. Mol Cancer Res 10:573–87.

63. Sendamarai AK, Ohgami RS, Fleming MD, Lawrence CM. 2008. Structure of the membrane proximal oxidoreductase domain of human Steap3, the dominant ferrireductase of the erythroid transferrin cycle. Proc Natl Acad Sci U S A 105:7410–5.

64. Hubert RS, Vivanco I, Chen E, Rastegar S, Leong K, Mitchell SC, Madraswala R, Zhou Y, Kuo J, Raitano AB, Jakobovits A, Saffran DC, Afar DE. 1999. STEAP: a prostate-specific cell-surface antigen highly expressed in human prostate tumors. Proc Natl Acad Sci U S A 96:14523–8.

65. Smith P, Rhodes NP, Shortland AP, Fraser SP, Djamgoz MB, Ke Y, Foster CS. 1998. Sodium channel protein expression enhances the invasiveness of rat and human prostate cancer cells. FEBS Lett 423:19–24.

66. Yamamoto T, Tamura Y, Kobayashi J-I, Kamiguchi K, Hirohashi Y, Miyazaki A, Torigoe T, Asanuma H, Hiratsuka H, Sato N. 2013. Six-transmembrane epithelial antigen of the

prostate-1 plays a role for in vivo tumor growth via intercellular communication. Exp Cell Res 319:2617–26.

67. Ihlaseh-Catalano SM, Drigo SA, de Jesus CMN, Domingues MAC, Trindade Filho JCS, de Camargo JL V, Rogatto SR. 2013. STEAP1 protein overexpression is an independent marker for biochemical recurrence in prostate carcinoma. Histopathology 63:678–85.

68. Gomes IM, Arinto P, Lopes C, Santos CR, Maia CJ. 2014. STEAP1 is overexpressed in prostate cancer and prostatic intraepithelial neoplasia lesions, and it is positively associated with Gleason score. Urol Oncol Semin Orig Investig 32:53.e23-53.e29.

69. Maia CJB, Socorro S, Schmitt F, Santos CRA. 2008. STEAP1 is over-expressed in breast cancer and down-regulated by 17beta-estradiol in MCF-7 cells and in the rat mammary gland. Endocrine 34:108–16.

70. Moreaux J, Kassambara A, Hose D, Klein B. 2012. STEAP1 is overexpressed in cancers: A promising therapeutic target. Biochem Biophys Res Commun 429:148–155.

71. Azumi M, Kobayashi H, Aoki N, Sato K, Kimura S, Kakizaki H, Tateno M. 2010. Six-transmembrane epithelial antigen of the prostate as an immunotherapeutic target for renal cell and bladder cancer. J Urol 183:2036–44.

72. Rodeberg D a, Nuss R a, Elsawa SF, Celis E. 2005. Recognition of six-transmembrane epithelial antigen of the prostate-expressing tumor cells by peptide antigen-induced cytotoxic T lymphocytes. Clin Cancer Res 11:4545–52.

73. Krupa M, Canamero M, Gomez CE, Najera JL, Gil J, Esteban M. 2011. Immunization with recombinant DNA and modified vaccinia virus Ankara (MVA) vectors delivering PSCA and STEAP1 antigens inhibits prostate cancer progression. Vaccine 29:1504–13.

74. Ferrer-Miralles N, Saccardo P, Corchero JL, Xu Z, García-Fruitós E. 2015. General Introduction: Recombinant Protein Production and Purification of Insoluble Proteins, p. 1–24. *In* García-Fruitós, E (ed.), Insoluble Proteins Methods and Protocols. Springer New York, New York, NY.

75. Celik E, Calık P. 2012. Production of recombinant proteins by yeast cells. Biotechnol Adv 30:1108–18.

76. Daly R, Hearn MTW. 2005. Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production. J Mol Recognit 18:119–38.

77. Junge F, Schneider B, Reckel S, Schwarz D, Dötsch V, Bernhard F. 2008. Large-scale production of functional membrane proteins. Cell Mol Life Sci 65:1729–55.

78. Schlegel S, Hjelm A, Baumgarten T, Vikström D, de Gier J-W. 2014. Bacterial-based membrane protein production. Biochim Biophys Acta - Mol Cell Res 1843:1739–1749.

79. Goffeau A, Barrell BG, Bussey H, Davis RW, Dujon B, Feldmann H, Galibert F, Hoheisel JD, Jacq C, Johnston M, Louis EJ, Mewes HW, Murakami Y, Philippsen P, Tettelin H, Oliver SG. 1996. Life with 6000 genes. Science 274:546, 563–7.

80. Kayikci Ö, Nielsen J. 2015. Glucose repression in *Saccharomyces cerevisiae*. FEMS Yeast Res 15:1–8.

81. Madigan MT, Martinko JM, Stahl DA, Clark DP. 2011. Brock Biology of Microorganisms, 13th ed. Pearson Education, San Francisco.

82. Jensen NB, Strucko T, Kildegaard KR, David F, Maury J, Mortensen UH, Forster J, Nielsen J, Borodina I. 2014. EasyClone: method for iterative chromosomal integration of multiple genes in *Saccharomyces cerevisiae*. FEMS Yeast Res 14:238–48.

83. Emmerstorfer A, Wriessnegger T, Hirz M, Pichler H. 2014. Overexpression of membrane proteins from higher eukaryotes in yeasts. Appl Microbiol Biotechnol 98:7671–98.

84. Macauley-Patrick S, Fazenda ML, McNeil B, Harvey LM. 2005. Heterologous protein production using the *Pichia pastoris* expression system. Yeast 22:249–70.

85. Gonçalves AM, Pedro AQ, Maia C, Sousa F, Queiroz JA, Passarinha LA. 2013. *Pichia pastoris*: a recombinant microfactory for antibodies and human membrane proteins. J Microbiol Biotechnol 23:587–601.

86. Sarramegna V, Talmont F, Demange P, Milon A. 2003. Heterologous expression of G-protein-coupled receptors: comparison of expression systems from the standpoint of large-scale production and purification. Cell Mol Life Sci 60:1529–46.

87. Lohse MJ. 1992. Stable overexpression of human beta 2-adrenergic receptors in mammalian cells. Naunyn Schmiedebergs Arch Pharmacol 345:444–51.

88. Alisio A, Mueckler M. 2010. Purification and characterization of mammalian glucose transporters expressed in *Pichia pastoris*. Protein Expr Purif 70:81–7.

89. Sarramegna V, Muller I, Mousseau G, Froment C, Monsarrat B, Milon A, Talmont F. 2005. Solubilization, purification, and mass spectrometry analysis of the human mu-opioid receptor expressed in *Pichia pastoris*. Protein Expr Purif 43:85–93.

90. Maggioni A, Hadley B, von Itzstein M, Tiralongo J. 2014. Expression, solubilisation, and purification of a functional CMP-sialic acid transporter in *Pichia pastoris*. Protein Expr Purif 101:165–71.

91. Pedro AQ, Oppolzer D, Bonifácio MJ, Maia CJ, Queiroz J a., Passarinha L a. 2015. Evaluation of Mut^S and Mut^+ *Pichia pastoris* strains for membrane-bound catechol-O-methyltransferase biosynthesis. Appl Biochem Biotechnol 175:3840–55.

92. Cereghino JL, Cregg JM. 2000. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol Rev 24:45–66.

93. Hartner FS, Glieder A. 2006. Regulation of methanol utilisation pathway genes in yeasts. Microb Cell Fact 5:39.

94. Charoenrat T, Ketudat-Cairns M, Stendahl-Andersen H, Jahic M, Enfors S-O. 2005. Oxygen-limited fed-batch process: an alternative control for *Pichia pastoris* recombinant protein processes. Bioprocess Biosyst Eng 27:399–406.

95. Krainer FW, Dietzsch C, Hajek T, Herwig C, Spadiut O, Glieder A. 2012. Recombinant protein expression in *Pichia pastoris* strains with an engineered methanol utilization pathway. Microb Cell Fact 11:22.

96. Higgins DR, Cregg J. 1998. *Pichia* Protocols. Spatial vision. Humana Press, New Jersey.

97. Looser V, Brühlmann B, Bumbak F, Stenger C, Costa M, Camattari A, Fotiadis D, Kovar K. 2015. Cultivation strategies to enhance productivity of *Pichia pastoris*: A review. Biotechnol Adv.

98. Gomes IM, Santos CR, Socorro S, Maia CJ. 2013. Six transmembrane epithelial antigen of the prostate 1 is down-regulated by sex hormones in prostate cells. Prostate 73:605–613.

99. Jahic M, Gustavsson M, Jansen A-K, Martinelle M, Enfors S-O. 2003. Analysis and control of proteolysis of a fusion protein in *Pichia pastoris* fed-batch processes. J Biotechnol 102:45–53.

100. Murata Y, Watanabe T, Sato M, Momose Y, Nakahara T, Oka S, Iwahashi H. 2003. Dimethyl sulfoxide exposure facilitates phospholipid biosynthesis and cellular membrane proliferation in yeast cells. J Biol Chem 278:33185–93.

101. Ramón A, Marín M. 2011. Advances in the production of membrane proteins in *Pichia pastoris*. Biotechnol J 6:700–6.

102. Asada H, Uemura T, Yurugi-Kobayashi T, Shiroishi M, Shimamura T, Tsujimoto H, Ito K, Sugawara T, Nakane T, Nomura N, Murata T, Haga T, Iwata S, Kobayashi T. 2011. Evaluation of the *Pichia pastoris* expression system for the production of GPCRs for structural analysis. Microb Cell Fact 10:24.

103. André N, Cherouati N, Prual C, Steffan T, Zeder-Lutz G, Magnin T, Pattus F, Michel H, Wagner R, Reinhart C. 2006. Enhancing functional production of G protein-coupled receptors in *Pichia pastoris* to levels required for structural studies via a single expression screen. Protein Sci 15:1115–26.

104. Pedro AQ, Martins LM, Dias JML, Bonifácio MJ, Queiroz J a, Passarinha L a. 2015. An artificial neural network for membrane-bound catechol-O-methyltransferase biosynthesis with *Pichia pastoris* methanol-induced cultures. Microb Cell Fact 14:113.

105. Yurugi-Kobayashi T, Asada H, Shiroishi M, Shimamura T, Funamoto S, Katsuta N, Ito K, Sugawara T, Tokuda N, Tsujimoto H, Murata T, Nomura N, Haga K, Haga T, Iwata S, Kobayashi T. 2009. Comparison of functional non-glycosylated GPCRs expression in *Pichia pastoris*. Biochem Biophys Res Commun 380:271–6.

106. Abdulaev NG, Popp MP, Smith WC, Ridge KD. 1997. Functional Expression of Bovine Opsin in the Methylotrophic Yeast *Pichia pastoris*. Protein Expr Purif 10:61–69.

107. Smith SM. 2011. Strategies for the Purification of Membrane Proteins, p. 485–496. *In* Methods in Molecular Biology.

108. Hiruma-Shimizu K, Shimizu H, Thompson GS, Kalverda AP, Patching SG. 2015. Deuterated detergents for structural and functional studies of membrane proteins: Properties, chemical synthesis and applications. Mol Membr Biol 32:139–155.