Elevating the expression level of biologically active recombinant human alpha 1-antitrypsin in *Pichia pastoris*

Sareh Arjmand[1,2,3] · Abbas Sahebghadam Lotfi [2,4] ✉ · Mehdi Shamsara[2] · Seyed Javad Mowla[1]

[1]Tarbiat Modares University, Faculty of Biological Sciences, Department of Molecular Genetics, Tehran, Iran
[2]National Institute of Genetic Engineering and Biotechnology, Shahrak-e Pajoohesh, Tehran, Iran
[3]Zanjan University of Medical Sciences, School of Pharmacy, Ayatollah Mousavi Hospital, Zanjan, Iran
[4]Tarbiat Modares University, Faculty of Medical Sciences, Department of Clinical Biochemistry, Tehran, Iran

✉ Corresponding author: lotfi-ab@nigeb.ac.ir

Abstract

Background: Human alpha 1-antitrypsin (AAT) is a potent inhibitor of multiple serine proteases, and protects tissues against their harmful effects. Individuals with reduced or abnormal production of this inhibitor need intravenous administration of exogenous protein. In this study, we employed the methylotrophic (methanol utilizing) yeast *Pichia pastoris* (*P. pastoris*) as a preferential host for efficient production and secretion of recombinant AAT. Furthermore, we examined different strategies to maximize the yield of the secreted protein.
Results: Our findings revealed that optimizing the codon usage of AAT gene for *P. pastoris* had positive effects on the level of secreted AAT under the control of inducible alcohol oxidase 1 (AOX1) and constitutive glycerol aldehyde phosphate dehydrogenase (GAP) promoters. Compared to AOX1, the GAP promoter increased the yield of AAT by more than two fold. It was also demonstrated that the human AAT native signal sequence was more effective than the well-known yeast signal sequence, alpha mating factor (α-MF). Doubling gene dosage nearly doubled the production of AAT, though dosages exceeding this limit had negative effects on the yield.
Conclusion: *P. pastoris* is shown to be an efficient expression system for production of recombinant and biologically active AAT. Also different strategies could be used to elevate the amount of this secretable protein.

Keywords: human alpha 1-antitrypsin, *Pichia pastoris*, recombinant proteins.

INTRODUCTION

Human alpha 1-antitrypsin (AAT), a serum glycoprotein, is one of the best-known prototypes of serine protease inhibitors (serpin) superfamily (Heutinck et al. 2010). AAT inhibits a range of proteases including one powerful protease known as neutrophil elastase (NE). The released protease from white blood cells plays a pivotal role in inflammation, and can be deleterious if not properly controlled. Much of AAT is produced by hepatic cells, and then transported to the lungs via the blood flow (Greene and McElvaney, 2009). Defects in the blood levels of AAT (below 15% of normal level) or its deposition in liver cells is responsible for early onset of chronic liver disease, emphysema, aneurysm (Rice-McDonald, 2005; Kok et al. 2007; Fairbanks and Tavill, 2008) and possibly other disorders such as bronchiectasis, asthma, vasculitis and Wegener's granulomatosis (Lonardo et al. 2002; Takii et al. 2003; Shoemark et al. 2007). Currently, the only available treatment for patients with AAT deficiency is intravenous augmentation therapy with human serum derived AAT. Although the results suggest the effectiveness of this therapy in slowing the disease progression (Chapman et al. 2009), it has some disadvantages including source limitation and safety concerns (Stockley, 2010). The latest generation

of treatment for AAT deficiency focuses on the recombinant AAT and new approaches in drug delivery (Brantly et al. 2006; Martin et al. 2006; Pemberton et al. 2006; Chulay et al. 2011).

In a previous study, we used methylotrophic yeast *P. pastoris* as a suitable host for expression and secretion of human AAT gene, while replacing its signal peptide with that of *Saccharomyces cerevisiae* (*S. cerevisiae*) α-MF. The results showed that this eukaryotic host can produce considerable quantities of functionally active recombinant AAT in its glycosylated form (Arjmand et al. 2011). We hoped to increase this amount using various environmental culture conditions and also genetic strategies to construct a productive yeast clone which expresses heterologous AAT in high quantities. This improved fabricated host strain would be economically profitable later, in large scale production.

Regardless of the host type, there are various strategies to improve the expression or secretion of recombinant proteins. In the simplest method, one can optimize conditions of the culture such as culture medium, cell density, inducer concentration, temperature, pH, aeration, etc. (Maldonado et al. 2007; Maldonado et al. 2008; Zhao et al. 2008; Fredericks et al. 2010; Varghese et al. 2010). However, most of the time, improving external factors is not sufficient, whereas changing some genetic factors can significantly impact the production of recombinant protein (Wang et al. 2006; Li et al. 2008). Based on the previous studies, the kind of genetic alterations depend mainly on the kind of recombinant protein. The same strategy may have contradictory effects on different recombinant proteins, even in identical hosts, which makes prediction almost impossible. Thus, for every single individual protein, there should be different strategies to elevate its production. Some of the most common methods for adjusting and increasing recombinant protein expression are: 1) optimizing the sequence of heterogonous gene according to the host, which subsequently affects the RNA secondary structure and its G+C composition, 2) switching from an inducible promoter to a constitutive one or vice versa, 3) trying for different signal peptides in cases of secretary proteins, and 4) increasing the copy number of transfected gene in the host genome. The results vary from several times increase in protein production to no effects or even negative effects on the level of protein products.

The aim of the present study was to improve the amount of secreted AAT in *P. pastoris* by codon optimization, replacing promoter and signal peptide, and increasing gene dosage.

MATERIALS AND METHODS

P. pastoris strain and vectors

Wild-type *P. pastoris* X-33 (Invitrogen, USA) was applied as a host cell to express recombinant AAT protein. The expression cassettes including inducible and constitutive promoters were compared for protein expression. The plasmids pPICZαB and pPICZA were used to construct the methanol-inducible AAT expression vectors. In all cases the methanol utilization plus (Mut[+]) phenotype clones were used for analysis and comparison. Constitutive expression was constructed using pGAPZαA and pGAPZA plasmids. Both plasmid contained GAP promoter instead of AOX1. *XhoI* and *XbaI* restriction sites were added to sense and antisense primers, respectively, for cloning of AAT fragment in the he pPICZαB and pGAPZαA plasmids. *EcoRI* and *NotI* restriction sites were used for cloning in the pPICZA and pGAPZA plasmids in the same way. Structure of all constructs is depicted in Figure 1. Cloning steps were performed in *E. coli* DH5-α.

Media and culture conditions

YPD liquid medium or agar plates (2% peptone, 1% yeast extract, 2% dextrose, and 20 g/l agar) were used routinely for culture of *P. pastoris*. Other culture media used in this study are as follows: buffered glycerol complex (BMGY) medium (1% yeast extract, 2% peptone in 100 mM potassium phosphate buffer pH 6.0, 1.34% yeast nitrogen base (YNB), 4 x 10^{-5}% biotin containing 2% glycerol) used for *P. pastoris* cell growth, buffered methanol complex (BMMY) medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer pH 6.0, 1.34% YNB, 4 x 10^{-5}% biotin, and 0.5% methanol was used for AAT inducible expression). MM and MD agar plates (1.34% YNB, 4 x 10^{-5}% biotin, 2% dextrose, 2% agar), for MM, 0.5% filter-sterilized methanol was added instead of 2% dextrose used for screening of methanol utilizing phenotype. In all experiments, the cultivation was held in 250 ml baffled flasks (working volume of 50 ml) at 30ºC and at the agitation rate of 200 rpm.

Isolation of genomic DNA

Genomic DNA from *P. pastoris* was isolated using DNeasy® blood and tissue kit (Qiagen, Germany) according to the supplier's supplementary protocol for the purification of chromosomal DNA from yeast. DNA concentration was measured at OD_{260} with spectrophotometer (Jenway Genova).
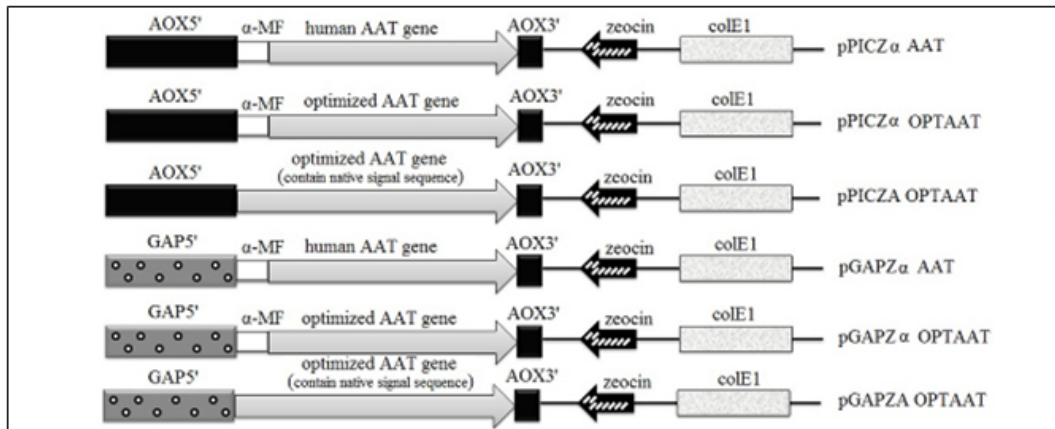

Fig. 1 Six expression cassettes constructed for this study. AOX5': AOX promoter; GAP5': GAP promoter; AOX3': AOX transcription terminator sequence; α-MF: secretion signal peptide; zeocin: Streptoalloteichus hindustanus (*Sh ble*) gene which confer zeocin resistance; colE1: bacterial replication sequence.

P. pastoris transformation

Fresh competent X-33 *P. pastoris* cells were generated according to the Easyselect™ *Pichia* expression kit manual (Invitrogen). 100 µl of the cells plus 10 µg of purified linearized plasmid DNA pulsed in 0.1 cm electroporation cuvettes at 1.5 kV, 25 F, 400 Ω and 8 millisec (Bio-Rad gene-pulser). Transformants were selected on YPD zeocin plates, and then screened for the insert at AOX1 and GAP locus, by PCR on yeast genomic DNA using 5' and 3'AOX1 (5'-GACTGGTTCCAATTGACAAGC-3' and 5'-GTCCCTATTTCAATCAATTGAA-3') and pGAP forward (5'-GTCCCTATTTCAATCAATTGAA-3') primers (Invitrogen). Positive clones were used in subsequent analysis.

Determination of methanol utilizing phenotype

Transformation of X-33 cells with linearized DNA generated two different methanol utilization (Mut) phenotypes in host cells. Since single crossover recombination at the AOX1 promoter region is favoured, most of the transformants had Mut[+] phenotype, in which the AOX1 gene is intact. However, due to the presence of the AOX1 terminator sequences in the plasmid, there is a chance for second recombination at this region which results in the replacement of AOX1 gene and creating Mut[S] phenotype. The latter is weak in metabolizing methanol. The Mut phenotype for the transformed X-33 cells was determined by growing 100 clones on minimal media with methanol (MM) and minimal media with dextrose (MD) plates. The results were confirmed by PCR amplification, using AOX primers obtained in previous step.

Construction of multi-copy clones

Clones with possible harbouring of two or three copies of the inductive or constitutive AAT expression cassette were selected using the zeocin screening method as described elsewhere (Vassileva et al. 2001). For this purpose the transformants were streaked on YPD plates containing 100, 500 and 1000 µg/ml zeocin. The precise number of AAT copies in the transformants was determined by real-time PCR (see below).

Quantification of AAT gene dosage by real-time PCR amplification

Expression cassette copy numbers were determined by real-time PCR using SYBR Green I dye technique. The $2^{-\Delta\Delta Ct}$ method of relative quantification was used to estimate integrated AAT copy number (Ferreira et al. 2006). The *P. pastoris* homoserine-O-transacetylase (MET2) was used as a house-keeping gene (reference gene) of constant copy number in all samples. Genomic DNA extracted from a *P. pastoris* clone which had been proven by southern blot (Inan et al. 2006) to harbour a single copy of integrated AAT (the data was not shown) was used as a calibrator. The $\Delta\Delta C_t$ calculation for the relative quantification of target was used as follows $\Delta\Delta C_t$ = (C_t, AAT gene - C_t, MET2) $_X$ - (C_t, AAT gene - C_t, MET2) $_Y$, where C_t is threshold cycle, X = sample with unknown copy of AAT and Y = sample with known one copy of AAT. Results for each sample were normalized to MET2 relative to the copy number of the target gene in known sample, according to the following equation; amount of target = $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).
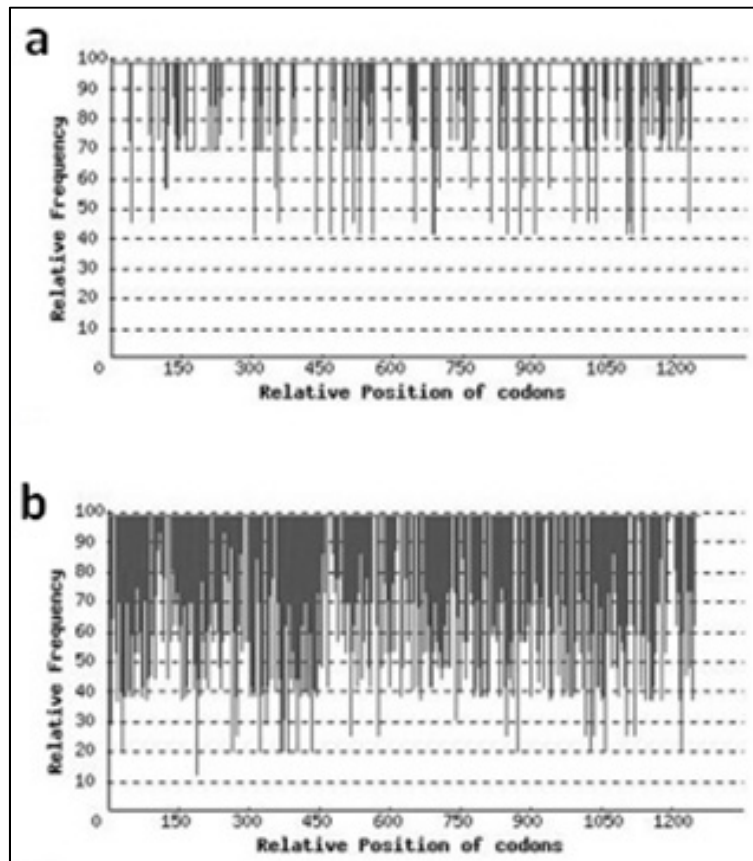

Fig. 2 Diagram of relative frequency (%) of AAT gene codons for *P. pastoris* according their positions. (a) codon optimized sequences; (b) human native sequences.

Real-time PCR reactions were carried out in a StepOne™ (48 well) real-time PCR system (ABI) using 10 µl of power SYBR Green master mix (ABI), 1 ng recombinant *P. pastoris* genomic DNA and 0.4 µM of each primer in a final volume of 20 µl. The amplification profile included one cycle of 95ºC for 5 min followed by 40 cycles of initial denaturation at 95ºC for 10 sec and 60ºC for 30 sec. Out of the four primers used, AATF2 (5'- ATAAGGCTGTGCTGACCATCGAC-3') and AATR2 (5'- TTGGGTGGGATTCACCACTTTTC-3') primers amplified a sequence of AAT gene that was 179 bp in length, while MET2F1 (5'- GCCCGAAAACCCATTCTCACTTC -3') and MET2R1 (5'- CACTGGAACCCGTTAAAGCAGG -3') amplified a sequence of host MET2 gene of the same length. The quantity of each target was expressed as the difference in threshold cycle (C_t) for each reaction. All experiments were performed in duplicate in each run and repeated at least two times in different runs.

Synthetic AAT cDNA with optimized codon usage

Human AAT gene coding sequence was recreated synthetically by GenScript corp. to optimize codon usage for expression in *P. pastoris*. The rare codons were replaced with more frequent ones for this host, and the GC content and stability of possible secondary RNA structures were reduced. Evaluation and comparison of the secondary structures and their free energy (ΔG) in original type sequence and the newly synthesized one was done using the web free software Centroidfold (http://www.ncrna.org/centroidfold) (Sato et al. 2009a). This tool predicts RNA secondary structures with the γ-centroid estimator which is a kind of posterior decoding method based on statistical decision theory. Relative adaptiveness of the newly synthesized gene towards the codon usage of highly expressed genes in *P. pastoris* was measured by codon adaptation index (CAI), using rare codon analysis tool (http://www.genscript.com/cgi-bin/tools/rare_codon_analysis). This numerical value is the ratio of the usage of each codon to that of the most abundant codon for the same amino acid and ranges from 0 to 1. A high value of relative adaptiveness indicates a high proportion of the most abundant codons, while a low value of this measurement points to a high chance that the desired gene will be expressed poorly (Sharp and Li, 1987).

Quantification of AAT by enzyme-linked immunosorbent assay (ELISA)

Culture media at different time points (0, 24, 48 and 72 hrs) were subjected to ELISA assay to quantify the amounts of recombinant AAT. The experiments were done using human alpha 1-antitrypsin ELISA Quantitation Kit (GenWay Biotech, Inc), in accordance with the manufacturer's instructions. All samples were diluted equally in sample/conjugate diluents buffer (50 mM Tris, 0.14 M NaCl, 1% (w/v) BSA, 0.05% (v/v) Tween 20, pH 8.0) and assayed in triplicate. Commercial human AAT (Sigma) and the medium of non-recombinant *P. pastoris* were applied as positive and negative controls, respectively.
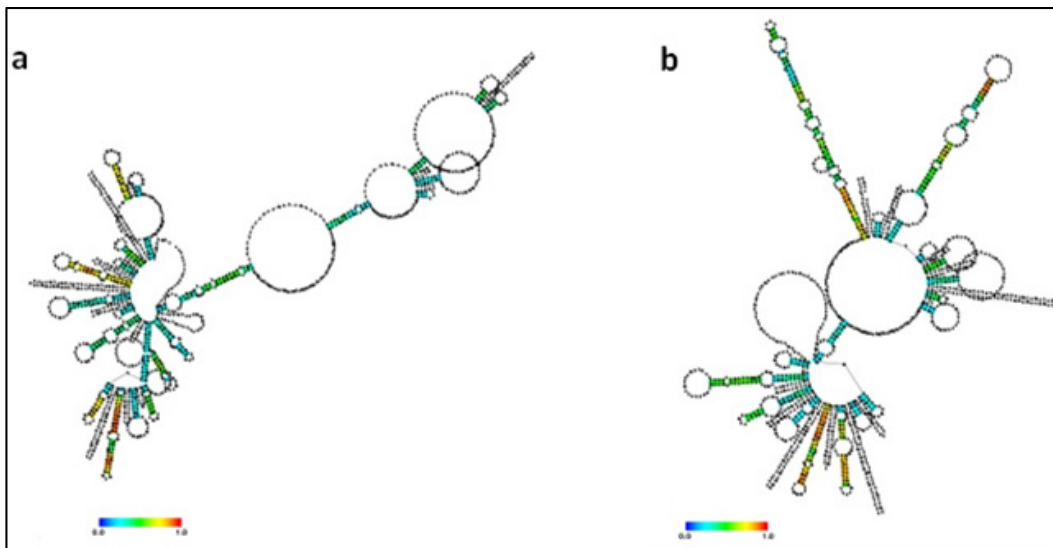

Fig. 3 Secondary structure of AAT RNA. (a) for native human AAT gene (ΔG = -244.69); (b) for codon optimized AAT gene (ΔG = -149.99).

Elastase Inhibition Capacity (EIC)

Biological inhibitory activity of recombinant AAT against its main substrate, elastase, was detected using EnzChek® Elastase Assay Kit (Molecular Probes, Inc.), in accordance with the manufacturer's recommendations. The alteration in fluorescence level was monitored using a standard fluorometer (Hitachi F-3010) with a maximum absorption at 505 nm and a maximum fluorescence emission at 515 nm. Standard curve was plotted using the known concentrations of commercial human AAT. Non-recombinant X-33 medium was used as a negative control.

RESULTS

Codon optimization and following analysis

The structural AAT gene is composed of 1254 nucleotides that encodes for a protein of 418 amino acids. According to the codon bias of *P. pastoris*, 312 nucleotides were replaced in new synthetic sequence. There was 75.2% similarity between the human and synthetic sequence of AAT. Furthermore, the CAI value is increased in *P. pastoris* from 0.66 for human AAT gene sequence to 0.96, amount of rare codons (< 30%) was decreased from 5% to 0 (Figure 2) and GC content was shifted from 52% to 36%. Moreover, the optimization caused substantial changes in mRNA folding, and, subsequently, in its total free energy (ΔG) from -244.69 to -149.99 (Figure 3).

Quantification of gene dosage

Real-time PCR results indicated that clones grown on the same concentration of zeocin (and not grown on the higher concentrations) showed the same C_t values for AAT gene amplification. The observed C_t difference between clones grown on 100 and 500 µg/ml zeocin was almost one, while the difference in C_t for 100 and 1000 µg/ml zeocin was observed to be almost one and a half cycles. A constant C_t was obtained for all clones of reference gene (15.03 ± 0.044) and calibrator (26.4 ± 0.02). Integrated AAT copy number was calculated according to equations mentioned in materials and methods (Table 1). No significance difference was observed between expected and calculated copy numbers (P value > 0.95).
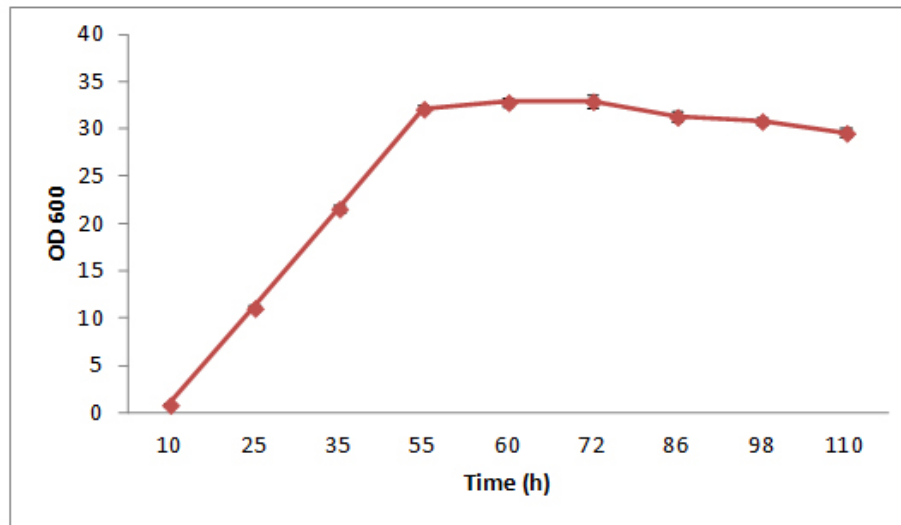

Fig. 4 The growth curve of *P. pastoris* cells in inductive medium BMMY. Logarithmic phase continues until 55 hours and death phase starts after 3 days. The samples were diluted to enable photometric measurement in the linear range between 0.1-0.5 for high OD_{600} reading. Error bars represents standard errors.

Quantification of secreted AAT from different constructs

In all experiments, the quantity of measured recombinant AAT in media indicated a constant increase over three consecutive days, with almost a twofold elevation in each day. This doubling showed a good compatibility with the *P. pastoris* growth kinetic in BMMY media (Figure 4).

The effect of gene dosage on the production and secretion of AAT, both extra- and intracellulary, was shown in Figure 5. The graph represents the obtained data on the third day. Doubling the gene dosage increased the amount of AAT produced almost proportionally, while with three copies of the gene, an overall reduction in the amount of AAT was observed.

Comparison of the amount of AAT production under AOX1 and GAP promoters for human and yeast-optimized AAT sequence is represented in Figure 6a. For this protein, GAP promoter and expression in constant mode seems to be considerably more efficient. Regardless of promoter type, optimizing the AAT sequence in favour of host improved expression rate more than 40%.

The data on the influence of signal sequence on the secreted AAT with codon optimized sequence are shown in Figure 6b. The amount of AAT protein in the media was more than twice when native human signal sequence was used compared to that of yeast.

Biological activity of secreted AAT from different constructs

The protease inhibitor activity of constructed AATs was measured for pPICZα AAT, pGAPZα AAT and pPICZα OPTAAT. The activities of the produced AATs are in agreement with their quantity (Figure 7).

DISCUSSION

The methylotrophic yeast *P. pastoris* expression system is similar to the well-known *S. cerevisiae* system without having some of the difficulties often encountered in *S. cerevisiae* production systems, such as mitotic instability of recombinant strains, undesirable hyperglycosylation and low production yield (Kim et al. 2005). This single cell organism is capable of many of the functions carried out in higher eukaryotic cells such as glycosylation, folding and disulphide bond formation, and yet its molecular manipulation needs simple and established techniques used for prokaryotes (Bollok et al. 2009). This system is less expensive than insect and mammalian tissue culture cell systems and has been widely used, both intracellulary and extracellulary, for the production of negligible to high quantities of foreign heterologous proteins (Macauley-Patrick et al. 2005; Vad et al. 2005).
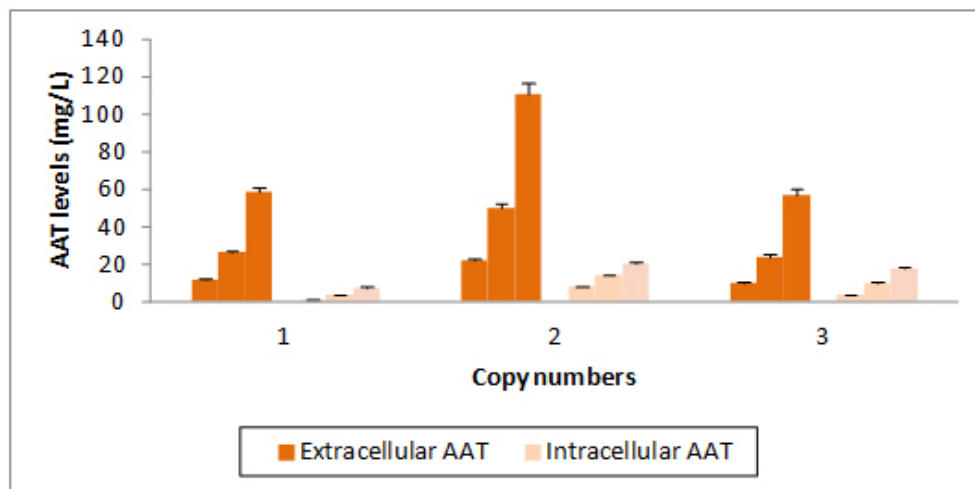

Fig. 5 Graphs related to comparison of secreted and intracellular recombinant AAT protein in clones with 1, 2 and 3 copies of integrated AAT gene, in three consecutive days. Error bars represent standard errors.

Despite long time experience and great advances in recombinant protein production, finding appropriate hosts for the expression of desired protein or improving the protein yield following a successful expression is a matter of trial and error (Stevens, 2000; Yokoyama, 2003). Moreover, there are potential bottlenecks for the optimum expression of every heterologous protein including promoter strength (Kim et al. 2009), the gene copy number (Vassileva et al. 2001), the codon usage of recombinant gene (Xu et al. 2010), and finally processing in ER and Golgi and secretion out of cell in case of secretary protein (Inan et al. 2006). Based on previous experiments, each of these factors can be considered for elevating the heterologous protein expression, though there are no reliable means of predicting the precise effect of each factor (Strausberg and Strausberg, 2001; Luo et al. 2006;

Geymonat et al. 2007; Ramachandran et al. 2008; Reisinger et al. 2008; Pérez-García et al. 2010). AAT protein was expressed and partially characterized in the previous work (Arjmand et al. 2011). In the present study, we selected some known parameters and evaluated their effects on the expression of heterologous AAT in *P. pastoris* to create a clone with maximal expression. Each of these parameters displayed characteristically distinct contribution in the AAT expression level.

Changing the AAT gene codons in favour of *P. pastoris* was the first strategy considered for increasing the AAT expression levels. The observation that preferred codons are consistent across the genes of specific species and their correlation with the most abundant aminoacyltRNA results in the codon bias theory and has been investigated (Grantham et al. 1980; Sinclair and Choy, 2002). Currently, frequencies of codons for different investigated organisms have been calculated and are available as tables of codon usage. Different studies have been done to examine the effect of varying codon bias in numerous expression systems from simple prokaryotes to higher eukaryotic hosts and led to different results from slight change to 100 fold increase in heterologous protein production (Wu et al. 2009; Tiwari et al. 2010). Besides codon bias, RNA secondary structure is another important factor restructured by codon change and represents significant effects on heterologous gene expression and hence should be placed under great observation during codon optimization. Lower folding energies (higher stability) slow down the ribosome and decrease translation efficiency (Lammertyn et al. 1996; Tuller et al. 2010). On the other hand, it appears that codon bias in lower eukaryotes tends towards A+T (Sinclair and Choy, 2002). In present study, the sequence of AAT was optimized for the expression in *P. pastoris* in a way that decreased G+C content and increased the RNA free energy. The synthetic construct was then cloned under both AOX 1 and GAP promoters. Regardless of promoter, this optimization caused about 40% increase in medium detected AAT. Although in comparison with other studies this percentage is, by number, not significant, given the relatively high amount of protein obtained in baffled flask culture from natural human AAT sequence (at least ~ 60 mg/L for pPICZα AAT), it is considerable (at least ~24 mg/L).
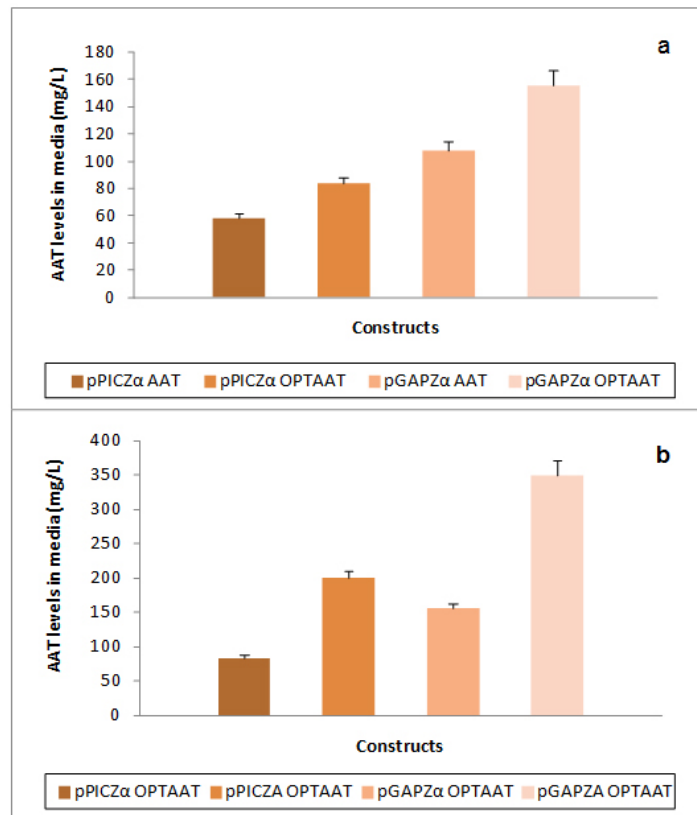

Fig. 6 (a) Graphs related to comparison of secreted AAT from constructs containing native and optimized AAT gene sequence under AOX1 and GAP promoter. Error bar represents standard errors. (b) Graphs related to comparison of secreted AAT from constructs containing optimized AAT gene sequence with yeast α-MF signal sequence and native AAT signal sequence under AOX1 and GAP promoter. Error bars represent standard errors.

One reason for the popularity of *P. pastoris* is availability of strongly regulated AOX1 promoter which is especially useful for the expression of cytotoxic protein. This promoter is the most widely utilized, and is induced when cells are grown on methanol (Macauley-Patrick et al. 2005). However, other promoter options are available for the production of foreign protein in *P. pastoris*. Constitutive GAP, for instance, which promote the expression of heterologous protein in different carbon sources like glycerol and glucose and avoids some of the problems encountered in using AOX1 such as fire hazard of methanol (Cereghino and Cregg, 2000; Daly and Hearn, 2005). According to our results, human AAT was expressed more efficiently by the GAP promoter. Although the GAP promoter seems to be a suitable choice on this particular scale, the results of our study may not be applicable to larger scale situations, where other determining factors arising from the choice of promoter including carbon sources, their availability and cost, productivity and etc., would be involved; the effects of these on large-scale production requires further research. Despite indication of good performance for GAP promoter, AOX1 has been used considerably more in fermentation studies (Koganesawa et al. 2002; Damasceno et al. 2004; Ning et al. 2005; Naested et al. 2006; Jacobs et al. 2010; Vester-Christensen et al. 2010).
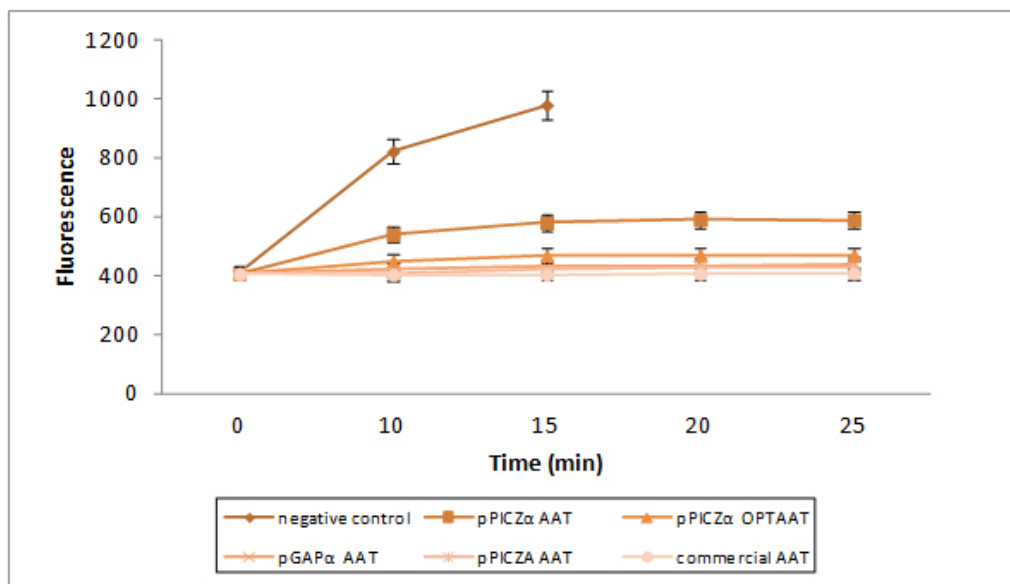

Fig. 7 Graph related to comparison of elastase inhibition activity of recombinant AAT. 100 µl of X-33 media containing pPICZα AAT, pGAPZα AAT and pPICZα OPTAAT and pPICZA OPTAAT used for each assay. It is expected that these values contain about 5.8, 10, 8.4 and 20 µg of AAT protein respectively. 100 µg of commercial AAT and 100 µl of non-recombinant X-33 used as positive and negative controls respectively. Error bars represent standard errors.

In the present research, we employed the *P. pastoris* secretion machinery system. Due to secretion of few proteins, expression of heterologous protein in secretary manner in this host is considered as the first step in purification. An important parameter involved in the secretion is signal peptide which guides protein trafficking through the secretary route. The most common signal peptides which are incorporated in commercial *P. pastoris* plasmids include PHO1 obtained from *P. pastoris* acid phosphatase and *S. cerevisiae* α-MF. As yet these signal peptides have been used successfully for the secretion of a variety of heterologous proteins in *P. pastoris* (Ha et al. 2001; Kommoju et al. 2007). Numerous experiments demonstrated the ability of this yeast to identify and properly cleave signal peptides belong to prokaryotic or eukaryotic cells, including human (Colao et al. 2006; Huang et al. 2008; Liu et al. 2008; Oliveira et al. 2008; Sato et al. 2009b). However, conflicting results have been achieved in some studies (Ghosalkar et al. 2008). Our study shows that α-MF secretion signal is suitable for direct secretion of AAT; however, the native codon optimized AAT signal sequence worked better since the rate of secreted AAT was increased more than two times and led to a remarkably higher yield of AAT in culture media which should be separated from a few yeast secreted proteins.

The amount of recombinant protein is affected by the number of integrated copies of the expression cassette. In *P. pastoris* transformation is the result of recombination events which occur through the

homologous regions in vector and *P. pastoris* genome mostly by a single crossing over event. Hence most of cassettes incorporated into the specific locus by insertion (rather than replacement). In 1-10% of transformants repeated recombination events result in tandem multiple integrations (Daly and Hearn, 2005). Several studies have shown that there is a positive correlation between integrated gene copy number and productivity in *P. pastoris*(Vassileva et al. 2001; Mansur et al. 2005; Luo et al. 2006; Li et al. 2009). By contrast, there are studies that show negative effects of increasing copy number on saturation of secretary pathway and therefore, decrease the amount of secreted proteins (Inan et al. 2007). Whereas *sh ble* gene product inactivates zeocin in a dose-dependent manner, resistance to high concentration of zeocin is a sensitive way to screen for multiple integration events. Vassileva et al. (2001) reported that transformants resistant to 100 μg/ml of zeocin generally contained one copy of new cassette, those resistant to 500, 1000 and 2000 μg/ml had two, three and four copies respectively. This is a simple method to make multi-copy clones, but not quietly accurate and needs to be confirmed. In this study, we have shown that by increasing the concentration of antibiotic, yeasts with multi-copy integrated AAT can be achieved. Integrated gene copy number is a key issue to study the effectiveness of protein expressed and data interpretation. Typical southern blot has been applied as a gold standard method to determine the copy number of newly integrated gene (Liu et al. 2005; Abad et al. 2010; Ohta et al. 2010). Despite the high accuracy, southern blot is a tedious and time consuming technique which requires considerable amounts of DNA. The quantitative real time PCR is increasingly adopted as a fast, sensitive and effective technique for copy number analysis, especially in transgenic animal and plants. Two types of chemistries are developed for this technique: the double-stranded DNA-intercalating agent SYBR Green I dye and the sequence-specific TaqMan probes (Chiang et al. 1999; Bustin et al. 2009). SYBR Green dye makes the assay more cost-effective, simplifies the experimental design and generates results comparable to those obtained with TaqMan chemistry (De Preter et al. 2002; Bel et al. 2011). In this work, we used real-time PCR with SYBR Green I dye detection and analysis of obtained C_t for each sample to quantify the gene copy number. It is assumed that a two-fold difference in gene copy number should have one C_t difference no matter where the threshold is set within the exponential phase of amplification, provided the efficiency is close to 100% (Dorak, 2006). The results indicated that extracellular AAT level increased 1.8 folds by increasing the gene copy number from one to two. With an opposite trend, a slight loss in the AAT level was obtained when the gene copy number increased from one to three. On the other hand, in comparison to single copy clones, intracellular detected ATT levels were higher in multi-copy clones than the single copy clones. In conclusion, it seems that increasing the AAT copy number to more than two is a bottleneck in both intracellular and extracellular expressions in *P. pastoris*. More accurate interpretation can be achieved using RNA transcription analysis.

Table 1. Obtained C_t values and AAT copy numbers related to extracted yeast genomes from different clones grown in different concentration of zeocin.

Target clones	C_t value	Expected AAT copy no.	Calculated AAT copy no.
Resistant to 100 (but not to 500) μg/ml zeocin	26.39 ± 0.022	1	1.00
Resistant to 500 (but not to 1000) μg/ml zeocin	25.43 ± 0.031	2	1.95
Resistant to 1000 (but not to 2000) μg/ml zeocin	24.84 ± 0.028	3	2.94

As demonstrated in this study, changing of gene dosage could be an effective tool for increasing the yield of a heterologous protein, and it seems that the amount of this effectiveness for different parameters is cumulative. Checking the environmental parameters along with these internal changes could be concerned as a complementary study for improving the expression of heterologous proteins which can be helpful for future efforts.

CONCLUDING REMARKS

We found *P. pastoris* a valuable and efficient host for overproduction of recombinant AAT. Using different genetic strategies, the appropriate ways were investigated for increasing the expression of AAT in *P. pastoris* and yeast clones where high level AAT production were created which were promising for large scale production of AAT after optimizing environmental conditions. The results also

indicated increase in the biological activity alongside increase in AAT expression which was our desired outcome.

ACKNOWLEDGMENTS

The authors thank Mrs. Mandana Bank, for her help with editing the paper.

REFERENCES

ABAD, S.; KITZ, K.; HÖRMANN, A.; SCHREINER, U.; HARTNER, F.S. and GLIEDER, A. (2010). Real-time PCR-based determination of gene copy numbers in *Pichia pastoris*. *Biotechnology Journal*, vol. 5, no. 4, p. 413-420. **[CrossRef]**

ARJMAND, S.; BIDRAM, E.; LOTFI, A.S.; SHAMSARA, M. and MOWLA, S.J. (2011). Expression and purification of functionally active recombinant human alpha 1-antitrypsin in methylotrophic yeast *Pichia pastoris*. *Avicenna Journal Medical Biotechnology*, vol. 3, no. 3, p. 127-134.

BEL, Y.; FERRÉ, J. and ESCRICHE, B. (2011). Quantitative real-time PCR with SYBR Green detection to assess gene duplication in insects: Study of gene dosage in *Drosophila melanogaster* (Diptera) and in *Ostrinia nubilalis* (Lepidoptera). *BMC Research Notes*, vol. 4, no. 84. **[CrossRef]**

BOLLOK, M.; RESINA, D.; VALERO, F. and FERRER, P. (2009). Recent patents on the *Pichia pastoris* expression system: Expanding the toolbox for recombinant protein production. *Recent Patents on Biotechnology*, vol. 3, no. 3, p. 192-201. **[CrossRef]**

BRANTLY, M.L.; SPENCER, L.T.; HUMPHRIES, M.; CONLON, T.J.; SPENCER, C.T.; POIRIER, A.; GARLINGTON, W.; BAKER, D.; SONG, S.; BERNS, K.I.; MUZYCZKA, N.; SNYDER, R.O.; BYRNE, B.J. and FLOTTE, T.R. (2006). Phase I trial of intramuscular injection of a recombinant adeno-associated virus serotype 2 *α*1-antitrypsin (AAT) vector in AAT-deficient adults. *Human Gene Therapy*, vol. 17, no. 12, p. 1177-1186. **[CrossRef]**

BUSTIN, S.A.; BENES, V.; GARSON, J.A.; HELLEMANS, J.; HUGGETT, J.; KUBISTA, M.; MUELLER, R.; NOLAN, T.; PFAFFL, M.W.; SHIPLEY, G.L.; VANDESOMPELE, J. and WITTWER, C.T. (2009). The MIQE guidelines: Minimum Information for Publication of Quantitative real-time PCR Experiments. *Clinical Chemistry*, vol. 55, no. 4, p. 611-622. **[CrossRef]**

CEREGHINO, J.L. and CREGG, J.M. (2000). Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. *FEMS Microbiology Reviews*, vol. 24, no. 1, p. 45-66. **[CrossRef]**

CHAPMAN, K.R.; STOCKLEY, R.A.; DAWKINS, C.; WILKES, M.M. and NAVICKIS, R.J. (2009). Augmentation therapy for α1 antitrypsin deficiency: A meta-analysis. *COPD: Journal of Chronic Obstructive Pulmonary Disease*, vol. 6, no. 3, p. 177-184. **[CrossRef]**

CHIANG, P.W.; WEI, W.L.; GIBSON, K.; BODMER, R. and KURNIT, D.M. (1999). A fluorescent quantitative PCR approach to map gene deletions in the Drosophila genome. *Genetics*, vol. 153, no. 3, p. 1313-1316.

CHULAY, J.D.; YE, G.J.; THOMAS, D.L.; KNOP, D.R.; BENSON, J.M.; HUTT, J.A.; WANG, G.; HUMPHRIES, M. and FLOTTE, T.R. (2011). Preclinical evaluation of a recombinant adeno-associated virus vector expressing human alpha-1 antitrypsin made using a recombinant herpes simplex virus production method. *Human Gene Therapy*, vol. 22, no. 2, p. 155-165. **[CrossRef]**

COLAO, M.C.; LUPINO, S.; GARZILLO, A.M.; BUONOCORE, V. and RUZZI, M. (2006). Heterologous expression of *lcc1* gene from *Trametes trogii* in *Pichia pastoris* and characterization of the recombinant enzyme. *Microbial Cell Factories*, vol. 5, no. 31. **[CrossRef]**

DALY, R. and HEARN, M.T.W. (2005). Expression of heterologous proteins in *Pichia pastoris*: A useful experimental tool in protein engineering and production. *Journal of Molecular Recognition*, vol. 18, no. 2, p. 119-138. **[CrossRef]**

DAMASCENO, L.M.; PLA, I.; CHANG, H.J.; COHEN, L.; RITTER, G.; OLD, L.J. and BATT, C.A. (2004). An optimized fermentation process for high-level production of a single-chain Fv antibody fragment in *Pichia pastoris*. *Protein Expression and Purification*, vol. 37, no. 1, p. 18-26. **[CrossRef]**

DE PRETER, K.; SPELEMAN, F.; COMBARET, V.; LUNEC, J.; LAUREYS, G.; EUSSEN, B.H.; FRANCOTTE, N.; BOARD, J.; PEARSON, A.D.; DE PAEPE, A.; VAN ROY, N. and VANDESOMPELE, J. (2002). Quantification of *MYCN*, *DDX1*, and *NAG* gene copy number in neuroblastoma using a real-time quantitative PCR assay. *Methods in Pathology*, vol. 15, no. 2, p. 159-166. **[CrossRef]**

DORAK, M.T. (2006). *Real-time PCR*. Abingdon: Taylor & Francis Group. 333 p. ISBN 9780415377348.

FAIRBANKS, K.D. and TAVILL, A.S. (2008). Liver disease in alpha 1-antitrypsin deficiency: A review. *The American Journal of Gastroenterology*, vol. 103, no. 8, p. 2136-2141. **[CrossRef]**

FERREIRA, I.D.; ROSARIO, V.E. and CRAVO, P.V.L. (2006). Real-time quantitative PCR with SYBR Green I detection for estimating copy numbers of nine drug resistance candidate genes in *Plasmodium falciparum*. *Malaria Journal*, vol. 5, no. 1. **[CrossRef]**

FREDERICKS, D.; CLAY, R.; WARNER, T.; O'CONNOR, A.; DE KRETSER, D.M. and HEARN, M.T. (2010). Optimization of the expression of recombinant human activin A in the yeast *Pichia pastoris*. *Biotechnology Progress*, vol. 26, no. 2, p. 372-383. **[CrossRef]**

GEYMONAT, M.; SPANOS, A. and SEDGWICK, S.G. (2007). A *Saccharomyces cerevisiae* autoselection system for optimised recombinant protein expression. *Gene*, vol. 399, no. 2, p. 120-128. **[CrossRef]**

GHOSALKAR, A.; SAHAI, V. and SRIVASTAVA, A. (2008). Secretory expression of interferon-alpha 2b in recombinant *Pichia pastoris* using three different secretion signals. *Protein Expression and Purification*, vol. 60, no. 2, p. 103-109. **[CrossRef]**

GRANTHAM, R.; GAUTIER, C. and GOUY, M. (1980). Codon frequencies in 119 individual genes confirm consistent choices of degenerate bases according to genome type. *Nucleic Acids Research*, vol. 8, no. 9, p. 1893-1912. **[CrossRef]**

GREENE, C.M. and MCELVANEY, N.G. (2009). Proteases and antiproteases in chronic neutrophilic lung disease-relevance to drug discovery. *British Journal of Pharmacology*, vol. 158, no. 4, p. 1048-1058. **[CrossRef]**

HA, S.H.; PARK, J.J.; KIM, J.W.; JEONG, J.W.; NOH, K.S.; JEON, Y.J.; KIM, H.S. and KIM, H.B. (2001). Molecular cloning and high-level expression of G2 protein of hantaan (HTN) virus 76-118 strain in the yeast *Pichia pastoris* KM71. *Virus Genes*, vol. 22, no. 2, p. 167-173. **[CrossRef]**

HEUTINCK, K.M.; TEN BERGE, I.J.; HACK, C.E.; HAMANN, J. and ROWSHANI, A.T. (2010). Serine proteases of the human immune system in health and disease. *Molecular Immunology*, vol. 47, no. 11-12, p. 1943-1955. **[CrossRef]**

HUANG, Y.S.; CHEN, Z.; CHEN, Y.Q.; MA, G.C.; SHAN, J.F.; LIU, W. and ZHOU, L.F. (2008). Preparation and characterization of a novel exendin-4 human serum albumin fusion protein expressed in *Pichia pastoris*. *Journal of Peptide Science*, vol. 14, no. 5, p. 588-595. **[CrossRef]**

INAN, M.; ARYASOMAYAJULA, D.; SINHA, J. and MEAGHER, M.M. (2006). Enhancement of protein secretion in *Pichia pastoris* by overexpression of protein disulfide isomerase. *Biotechnology and Bioengineering*, vol. 93, no. 4, p. 771-778. **[CrossRef]**

INAN, M.; FANDERS, S.A.; ZHANG, W.; HOTEZ, P.J.; ZHAN, B. and MEAGHER, M.M. (2007). Saturation of the secretory pathway by overexpression of a hookworm (*Necator americanus*) Protein (Na-ASP1). *Methods in Molecular Biology*, vol. 389, p. 65-76. **[CrossRef]**

JACOBS, P.P.; INAN, M.; FESTJENS, N.; HAUSTRAETE, J.; VAN HECKE, A.; CONTRERAS, R.; MEAGHER, M.M. and CALLEWAERT, N. (2010). Fed-batch fermentation of GM-CSF-producing glycoengineered *Pichia pastoris* under controlled specific growth rate. *Microbial Cell Factories*, vol. 9, no. 93. **[CrossRef]**

KIM, Y.J.; OH, Y.K.; KANG, W.; LEE, E.Y. and PARK, S. (2005). Production of human caseinomacropeptide in recombinant *Saccharomyces cerevisiae* and *Pichia pastoris*. *Journal of Industrial Microbiology & Biotechnology*, vol. 32, no. 9, p. 402-408. **[CrossRef]**

KIM, S.J.; LEE, J.A.; KIM, Y.H. and SONG, B.K. (2009). Optimization of the functional expression of *Coprinus cinereus* peroxidase in *Pichia pastoris* by varying the host and promoter. *Journal of Microbiology and Biotechnology*, vol. 19, no. 9, p. 966-971.

KOGANESAWA, N.; AIZAWA, T.; SHIMOJO, H.; MIURA, K.; OHNISHI, A.; DEMURA, M.; HAYAKAWA, Y.; NITTA, K. and KAWANO, K. (2002). Expression and purification of a small cytokine growth-blocking peptide from armyworm *Pseudaletia separata* by an optimized fermentation method using the methylotrophic yeast *Pichia pastoris*. *Protein Expression and Purification*, vol. 25, no. 3, p. 416-425. **[CrossRef]**

KOK, K.F.; WAHAB, P.J.; HOUWEN, R.H.; DRENTH, J.P.; DE MAN, R.A.; VAN HOEK, B.; MEIJER, J.W.; WILLEKENS, F.L. and DE VRIES, R.A. (2007). Heterozygous alpha-I antitrypsin deficiency as a co-factor in the development of chronic liver disease: A review. *Netherlands the Journal of Medicine*, vol. 65, no. 5, p. 160-166.

KOMMOJU, P.R.; MACHEROUX, P. and GHISLA, S. (2007). Molecular cloning, expression and purification of L-amino acid oxidase from the Malayan pit viper *Calloselasma rhodostoma*. *Protein Expression and Purification*, vol. 52, no. 1, p. 89-95. **[CrossRef]**

LAMMERTYN, E.; VAN MELLAERT, L.; BIJNENS, A.P.; JORIS, B. and ANNÉ, J. (1996). Codon adjustment to maximise heterologous gene expression in *Streptomyces lividans* can lead to decreased mRNA stability and protein yield. *Molecular and General Genetics MGG*, vol. 250, no. 2, p. 223-229. **[CrossRef]**

LI, Z.; HONG, G.; WU, Z.; HU, B.; XU, J. and LI, L. (2008). Optimization of the expression of hepatitis B virus e gene in *Pichia pastoris* and immunological characterization of the product. *Journal of Biotechnology*, vol. 138, no. 1-2, p. 1-8. **[CrossRef]**

LI, Y.T.; LI, M.T.; FU, C.H.; ZHOU, P.P.; LIU, J.M. and YU, L.J. (2009). Improvement of arachidonic acid and eicosapentaenoic acid production by increasing the copy number of the genes encoding fatty acid desaturase and elongase into *Pichia pastoris*. *Biotechnology Letters*, vol. 31, no. 7, p. 1011-1017. **[CrossRef]**

LIU, S.H.; CHOU, W.I.; SHEU, C.C. and CHANG, M.D. (2005). Improved secretory production of glucoamylase in *Pichia pastoris* by combination of genetic manipulations. *Biochemical and Biophysical Research Communications*, vol. 326, no. 4, p. 817-824. **[CrossRef]**

LIU, F.C.; CHEN, H.L.; CHONG, K.Y.; HSU, A.L. and CHEN, C.M. (2008). Production of recombinant porcine colipase secreted by *Pichia pastoris* and its application to improve dietary fat digestion and growth of postweaning piglets. *Biotechnology Progress*, vol. 24, no. 6, p. 1333-1341. **[CrossRef]**

LIVAK, K.J. and SCHMITTGEN, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods*, vol. 25, no. 4, p. 402-408. **[CrossRef]**

LONARDO, A.; MEDICINA, D.; LEONELLI, M.; BAGNI, A. and CALLEA, F. (2002). Intestinal Wegener's granulomatosis in a patient with severe alpha-1-antitrypsin deficiency resulting from a unique combination of two deficiency alleles (PiZ and PiMProcida). *European Journal of Gastroenterology & Hepatology*, vol. 14, no. 12, p. 1389-1392. **[CrossRef]**

LUO, H.Y.; HUANG, H.Q.; BAI, Y.G.; WANG, Y.R.; YANG, P.L.; MENG, K.; YUAN, T.Z. and YAO, B. (2006). Improving phytase expression by increasing the gene copy number of *appA-m* in *Pichia pastoris*. *Chinese Journal of Biotechnology*, vol. 22, no. 4, p. 528-533. **[CrossRef]**

MACAULEY-PATRICK, S.; FAZENDA, M.L.; MCNEIL, B. and HARVEY, L.M. (2005). Heterologous protein production using the *Pichia pastoris* expression system. *Yeast*, vol. 22, no. 4, p. 249-270. **[CrossRef]**

MALDONADO, L.M.; HERNÁNDEZ, V.E.B.; RIVERO, E.M.; BARBA DE LA ROSA, A.P.; FLORES, J.L.F.; ACEVEDO, L.G.O. and DE LEON RODRÍGUEZ, A. (2007). Optimization of culture conditions for a synthetic gene expression in *Escherichia coli* using response surface methodology: The case of human interferon beta. *Biomolecular Engineering*, vol. 24, no. 2, p. 217-222. **[CrossRef]**

MALDONADO, M.C.; BELFIORE, C. and NAVARRO, A.R. (2008). Temperature, soluble solids and pH effect on *Alicyclobacillus acidoterrestris* viability in lemon juice concentrate. *Journal of Industrial Microbiology & Biotechnology*, vol. 35, no. 2, p. 141-144. **[CrossRef]**

MANSUR, M.; CABELLO, C.; HERNANDEZ, L.; PAÍS, J.; VARAS, L.; VALDÉS, J.; TERRERO, Y.; HIDALGO, A.; PLANA, L.; BESADA, V.; GARCIA, L.; LAMAZARES, E.; CASTELLANOS, L. and MARTINEZ, E. (2005). Multiple gene copy number enhances insulin precursor secretion in the yeast *Pichia pastori*s. *Biotechnology Letters*, vol. 27, no. 5, p. 339-345. **[CrossRef]**

MARTIN, S.L.; DOWNEY, D.; BILTON, D.; KEOGAN, M.T.; EDGAR, J. and ELBORN, J.S. (2006). Safety and efficacy of recombinant alpha(1)-antitrypsin therapy in cystic fibrosis. *Pediatric Pulmonology*, vol. 41, no. 2, p. 177-183. **[CrossRef]**

NAESTED, H.; KRAMHØFT, B.; LOK, F.; BOJSEN, K.; YU, S. and SVENSSON, B. (2006). Production of enzymatically active recombinant full-length barley high p*I* α-glucosidase of glycoside family 31 by high cell-density fermentation of *Pichia pastoris* and affinity purification. *Protein Expression and Purification*, vol. 46, no. 1, p. 56-63. **[CrossRef]**

NING, D.; JUNJIAN, X.; QING, Z.; SHENG, X.; WENYIN, C.; GUIRONG, R. and XUNZHANG, W. (2005). Production of recombinant humanized anti-HBsAg Fab fragment from *Pichia pastoris* by fermentation. *Journal of Biochemistry and Molecular Biology*, vol. 38, no. 3, p. 294-299.

OHTA, K.; FUJIMOTO, H.; FUJII, S. and WAKIYAMA, M. (2010). Cell-associated β-xylosidase from *Aureobasidium pullulans* ATCC 20524: Purification, properties, and characterization of the encoding gene. *Journal of Bioscience and Bioengineering*, vol. 110, no. 2, p. 152-157. **[CrossRef]**

OLIVEIRA, C.; FELIX, W.; MOREIRA, R.A.; TEIXEIRA, J.Á. and DOMINGUES, L. (2008). Expression of frutalin, an α-D-galactose-binding jacalin-related lectin, in the yeast *Pichia pastoris*. *Protein Expression and Purification*, vol. 60, no. 2, p. 188-193. **[CrossRef]**

PEMBERTON, P.A.; KOBAYASHI, D.; WILK, B.J.; HENSTRAND, J.M.; SHAPIRO, S.D. and BARR, P.J. (2006). Inhaled recombinant alpha 1-antitrypsin ameliorates cigarette smoke-induced emphysema in the mouse. *COPD: Journal of Chronic Obstructive Pulmonary Disease*, vol. 3, no. 2, p. 101-108. **[CrossRef]**

PERÉZ-GARCÍA, G.; BASURTO-RÍOS, R. and IBARRA, J.E. (2010). Potential effect of a putative σ^H-driven promoter on the over expression of the Cry1Ac toxin of *Bacillus thuringiensis*. *Journal of Invertebrate Pathology*, vol. 104, no. 2, p. 140-146. **[CrossRef]**

RAMACHANDRAN, S.; KNICKELBEIN, J.E.; FERKO, C.; HENDRICKS, R.L. and KINCHINGTON, P.R. (2008). Development and pathogenic evaluation of recombinant herpes simplex virus type 1 expressing two fluorescent reporter genes from different lytic promoters. *Virology*, vol. 378, no. 2, p. 254-264. **[CrossRef]**

REISINGER, H.; STEINFELLNER, W.; STERN, B.; KATINGER, H. and KUNERT, R. (2008). The absence of effect of gene copy number and mRNA level on the amount of mAb secretion from mammalian cells. *Applied Microbiology and Biotechnology*, vol. 81, no. 4, p. 701-710. **[CrossRef]**

RICE-MCDONALD, G. (2005). Alpha-1 antitrypsin deficiency, emphysema and chronic obstructive pulmonary disease. *Internal Medicine Journal*, vol. 35, no. 12, p. 732-733. **[CrossRef]**

SATO, K.; HAMADA, M.; ASAI, K. and MITUYAMA, T. (2009a). CENTROIDFOLD: a web server for RNA secondary structure prediction. *Nucleic Acids Research*, vol. 37, no. S2, p. W277-W280. **[CrossRef]**

SATO, R.; MATSUMOTO, T.; HIDAKA, N.; IMAI, Y.; ABE, K.; TAKAHASHI, S.; YAMADA, R.H. and KERA, Y. (2009b). Cloning and expression of carp acetylcholinesterase gene in *Pichia pastoris* and characterization of the recombinant enzyme. *Protein Expression and Purification*, vol. 64, no. 2, p. 205-212. **[CrossRef]**

SHARP, P.M. and LI, W.H. (1987). The codon adaptation index-a measure of directional synonymous codon usage bias, and its potential applications. *Nucleic Acids Research*, vol. 15, no. 3, p. 1281-1295. **[CrossRef]**

SHOEMARK, A.; OZEROVITCH, L. and WILSON, R. 2007. Aetiology in adult patients with bronchiectasis. *Respiratory Medicine*, vol. 101, no. 6, p. 1163-1170. **[CrossRef]**

SINCLAIR, G. and CHOY, F.Y. (2002). Synonymous codon usage bias and the expression of human glucocerebrosidase in the methylotrophic yeast, *Pichia pastoris*. *Protein Expression and Purification*, vol. 26, no. 1, p. 96-105. **[CrossRef]**

STEVENS, R.C. (2000). Design of high-throughput methods of protein production for structural biology. *Structure*, vol. 8, no. 9, p. R177-R185. **[CrossRef]**

STOCKLEY, R.A. (2010). Emerging drugs for alpha-1-antitrypsin deficiency. *Expert Opinion on Emerging Drugs*, vol. 15, no. 4, p. 685-694. **[CrossRef]**

STRAUSBERG, R.L. and STRAUSBERG, S.L. (2001). Overview of protein expression in Saccharomyces cerevisiae. *Current Protocols in Protein Science*, Chapter 5, Unit 5-6. **[CrossRef]**

TAKII, Y.; INDUE, H.; KARASHIMA, E.; AKAHOSHI, M.; FURUGO, I.; HATTORI, S.; YAMANAKA, M.; KINOSHITA, S.; HAMASAKI, N.; SHIMODA, K.; NAKASHIMA, H.; TSUKAMOTO, H.; HORIUCHI, T.; OTSUKA, T. and HARADA, M. (2003). Systemic vasculitis associated with α1-antitrypsin deficiency. *Internal Medicine*, vol. 42, no. 7, p. 619-623. **[CrossRef]**

TIWARI, A.; SANKHYAN, A.; KHANNA, N. and SINHA, S. (2010). Enhanced periplasmic expression of high affinity humanized scFv against Hepatitis B surface antigen by codon optimization. *Protein Expression and Purification*, vol. 74, no. 2, p. 272-279. **[CrossRef]**

TULLER, T.; WALDMAN, Y.Y.; KUPIEC, M. and RUPPIN, E. (2010). Translation efficiency is determined by both codon bias and folding energy. *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 8, p. 3645-3650. **[CrossRef]**

VAD, R.; NAFSTAD, E.; DAHL, L.A. and GABRIELSEN, O.S. (2005). Engineering of a *Pichia pastoris* expression system for secretion of high amounts of intact human parathyroid hormone. *Journal of Biotechnology*, vol. 116, no. 3, p. 251-260. **[CrossRef]**

VARGHESE, V.M.; PRASAD, T. and KUMARY, T.V. (2010). Optimization of culture conditions for an efficient xeno-feeder free limbal cell culture system towards ocular surface regeneration. *Microscopy Research and Technique*, vol. 73, no. 11, p. 1045-1052. **[CrossRef]**

VASSILEVA, A.; CHUGH, D.A.; SWAMINATHAN, S. and KHANNA, N. (2001). Effect of copy number on the expression levels of hepatitis B surface antigen in the methylotrophic yeast *Pichia pastoris*. *Protein Expression and Purification*, vol. 21, no. 1, p. 71-80. **[CrossRef]**

VESTER-CHRISTENSEN, M.B.; HACHEM, M.A.; NAESTED, H. and SVENSSON, B. (2010). Secretory expression of functional barley limit dextrinase by *Pichia pastoris* using high cell-density fermentation. *Protein Expression and Purification*, vol. 69, no. 1, p. 112-119. **[CrossRef]**

WANG, S.; FARFAN-ARRIBAS, D.J.; SHEN, S.; CHOU, T.H.; HIRSCH, A.; HE, F. and LU, S. (2006). Relative contributions of codon usage, promoter efficiency and leader sequence to the antigen expression and immunogenicity of HIV-1 Env DNA vaccine. *Vaccine*, vol. 24, no. 21, p. 4531-4540. **[CrossRef]**

WU, Z.L.; QIAO, J.; ZHANG, Z.G.; GUENGERICH, F.P.; LIU, Y. and PEI, X.Q. (2009). Enhanced bacterial expression of several mammalian cytochrome P450s by codon optimization and chaperone coexpression. *Biotechnology Letters*, vol. 31, no. 10, p. 1589-1593. **[CrossRef]**

XU, L.; JIANG, X.; YANG, J.; LIU, Y. and YAN, Y. (2010). Cloning of a novel lipase gene, *lipJ08*, from *Candida rugosa* and expression in *Pichia pastoris* by codon optimization. *Biotechnology Letters*, vol. 32, no. 2, p. 269-276. **[CrossRef]**

YOKOYAMA, S. (2003). Protein expression systems for structural genomics and proteomics. *Current Opinion in Chemical Biology*, vol. 7, no. 1, p. 39-43. **[CrossRef]**

ZHAO, H.L.; XUE, C.; WANG, Y.; YAO, X.Q. and LIU, Z.M. (2008). Increasing the cell viability and heterologous protein expression of *Pichia pastoris* mutant deficient in *PMR1* gene by culture condition optimization. *Applied Microbiology and Biotechnology*, vol. 81, no. 2, p. 235-241. **[CrossRef]**

How to reference this article:

ARJMAND, S.; LOTFI, A.S.; SHAMSARA, M. and MOWLA, S.J. (2012). Elevating the expression level of biologically active recombinant human alpha 1-antitrypsin in *Pichia pastoris*. *Electronic Journal of Biotechnology*, vol. 16, no. 1. http://dx.doi.org/10.2225/vol16-issue1-fulltext-4