CONSTITUTIVE EXPRESSION OF THE ACTIVE FRAGMENT OF HUMAN VASOSTATIN Vs30 IN *Pichia pastoris* SMD1168

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Abstract

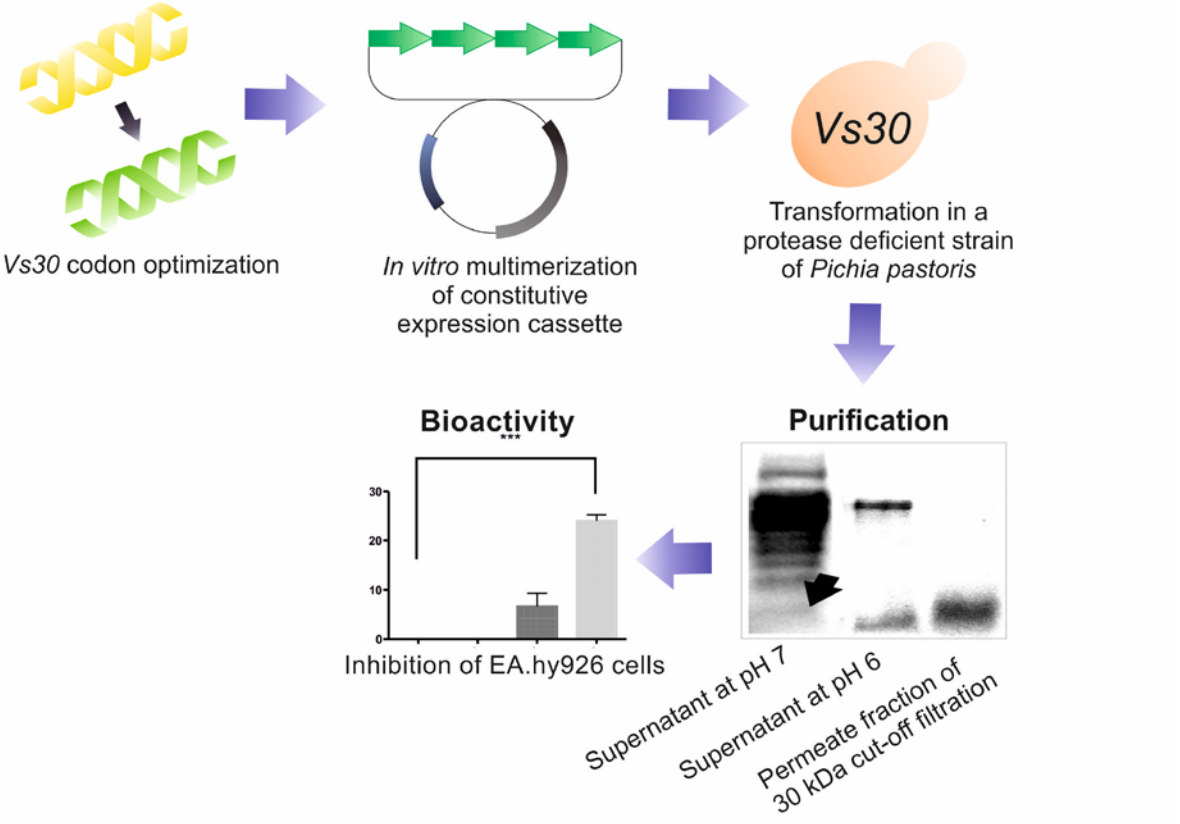
Vasostatin 30 (Vs30) is an active fragment derived from the N-terminal region (135-164aa) of human calreticulin (hCALR) and has the ability to inhibit angiogenesis. In this work, the expression and secretion of Vs30 was performed using a protease-deficient strain of the methylotrophic yeast *Pichia pastoris*. The *vs30* gene was optimized for *P. pastoris* preferential codon usage and inserted into pGAPZαA, a constitutive expression vector. In addition, a multi-copy strain was obtained using an *in vitro* multimerization approach. The flask fermentation conditions were: a culture volume of 25 mL in 250 mL baffled flasks at a 28°C, pH 6 and a harvest time of 48 h. Up to 21.07 mg/L Vs30 were attained. Vs30 was purified by ultrafiltration with a 30-kDa cut-off membrane. Thus, 10.47 mg/L of purified and dialyzed Vs30 was obtained. Bioctivity of Vs30 was confirmed by the inhibition of EA.hy926 cell proliferation *in vitro.* This work constitutes the first report on the expression of Vs30 in *Pichia pastoris* using a constitutive promoter and multimerization approach such as strategies to improve the recombinant protein expression.

Keywords: Angiogenesis; constitutive expression; multi-copy strain; spheroids; vasostatin.

Highlights:

- Constitutive expression of Human Vs30 was achieved in *Pichia pastoris*.
-The synthetic *vs30* gene was optimized based on *Pichia pastoris* codon preference.
- The increase in the number of expression cassette copies had a positive influence on the amount of Vs30.
-Purification of Vs30 from supernatants was performed by ultrafiltration.
-The purified Vs30 was functionally active.

Graphical Abstract



1. Introduction

Angiogenesis consists in capillaries formation from preexisting blood vessels, and it is essential for the development of primary and metastatic tumors [1]. Inhibition of angiogenesis represents a promising approach for treatment of solid tumors and other pathologies as diabetic retinopathy and rheumatoid arthritis [2, 3]. Consequently, several peptides have been developed to inhibit one or more angiogenesis steps such as endothelial cells proliferation, migration and tubule formation [4]. Vasostatin peptide is an angiogenesis inhibitor derived from the N-terminal region of human calreticulin, and has the ability to inhibit endothelial proliferation and tubulogenesis *in vitro* [5, 6]. Additionally, vasostatin has an anti-inflammatory activity and does not affect the wound-healing process, and *in vivo* experiments have shown angiogenesis inhibition and tumor growth reduction [7, 8].

The active fragment of human vasostatin (Vs30), a small and potent anti-proliferative peptide, is comprised of 30 aminoacids (135-164aa) [9]. Previously, we expressed Vs30 in *E. coli* BL21-SI cotransformed with a T7 promoter-based expression vector and pLysSRARE, a vector that contains unusual tRNAs for *E. coli*. However, the yield of soluble Vs30 after purification was only 2.98 mg/L [10]. Therefore, in order to increase the yield of Vs30, we decided to employ a yeast expression system.

The methylotrophic yeast *Pichia pastoris* represents a robust system of production and secretion of recombinant proteins. To date, several proteins with antiangionegic activity have been expressed in *P. pastoris* such as endostatin [11], angiostatin [12], tumstatin [13], canstatin [14], calreticulin-N58 [15] and vasostatin 120-180 [16]. In the present study, we expressed constitutively Vs30 in a secreting clone of *P. pastoris* SMD1168, a protease deficient strain. The

expression strategy was comprised of optimizing the *vs30* sequence in accordance to the preferential codon usage of *P. pastoris* as well as multimerizing the expression cassette.

2. Materials and methods

2.1 Strains and plasmids

Escherichia coli TOP10F´ strain (Invitrogen) was used as the host for plasmid amplification and maintenance. Zeocine, *Pichia pastoris* SMD1168H (*pepA*) a protease deficient strain, and the constitutive expression vector pGAPZαA were purchased from Invitrogen. Restriction enzymes were obtained from New England Biolabs Company. The kits for DNA recovery from agarose and plasmid extraction were bought from ZYMO RESEARCH. The routine chemicals were analytical grade (Sigma).

2.2 Construction of multi-copy expression vector

The *vs30* sequence was optimized in accordance to preferential codon usage for *P. pastoris* using the OptimumGene™ software and synthesized by Genscript™. The synthetic *vs30* (111 bp gene) was cloned in frame, downstream of the α-mating factor signal peptide into *Eco*RI site of the expression vector pGAPZαA to obtain pGAP-Vs30-1x. The expression cassette of pGAP-Vs30-1x was amplified and sequenced using the 5´α-Factor and 3´ AOX1 primers to confirm the correct reading frame between the signal peptide and *vs30.* To generate pGAP-Vs30-4x, a vector with four copies of expression cassette in tandem configuration, a *Bam*HI-*Bgl*II *in vitro* multimerization strategy was used as previously described [17].

2.3 Transformation of *Pichia pastoris* SMD1168

The expression vector pGAP-Vs30-4x was linearized with *Bam*HI and transformed into competent *P. pastoris* SMD1168H cells by electroporation (Electroporator 2510, Eppendorf) at 1500 V using 2 mm width cuvettes. The transformants clones were incubated on YPDS (YPD medium with 1M Sorbitol) plates containing 100 µg/mL of Zeocine antibiotic for 48-72 hours at 28°C and the positive ones were screened by colony PCR using the 5´α-Factor and 3´ AOX1 primers.

2.4 Yeastern blot

To select Vs30 secreting clones, several colonies were picked and patched on YPD plates. After incubation at 28°C for 72 h, a nitrocellulose filter (0.2 µm) was placed on the colonies. The filter was removed and washed with TBS (50 mM Tris, 150 mM NaCl, pH 7.5) after incubation at 28°C for 24 h. The membrane was treated with a polyclonal mouse anti-His tag primary antibody (Millipore) 1: 5,000 dilution followed by an alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma) 1: 2,000 dilution. In accordance with the intensity of the signals, Vs30 secreting clones were selected.

2.5 Expression and purification of Vs30

The selected clone was cultured in 250 ml baffled flask with 25 ml YPD medium with an initial optical density of 0.1 at 600 nm. The culture medium was incubated at 28 °C and shaking at 180 rpm for 120 h. Cell supernatants were collected every 24 h by centrifugation at 4°C and stored at -20°C.

To purify Vs30, the supernatant was collected by centrifugation at 16,000 x*g* for 30 min and filtered through a stirred ultrafiltration cell 8050 (Amicon) with a 30-kDa cut-off membrane (Millipore). The supernatant was pretreated with a protease inhibitor cocktail (cOmplete^{TM}, Sigma) and adjusted pH to 6 with NH_4OH. Then, 50 mL of the supernatant were added to stirred cell at 20°C and concentrated 10 times. To filter the supernatant, a 30 kDa cut-off polyethersulfone membrane with a diameter of 44.5 mm and an effective membrane area of 13.4 cm^2 was used. Compressed air was used to pressurize the system at 10 psi. Samples of retentate and permeate were collected for analysis and stored at -20°C. The purity of the permeate fraction was determinated by densitometric analisys of proteins on SDS-PAGE using ImageJ software.

To concentrate the permeate fraction, ammonium sulfate was added to reach 80 % salt saturation and incubated for 12 h at 4°C. Precipitated proteins were collected by centrifugation at 16, 000 x*g* for 30 min and resuspended in 0.1% acetic acid. To remove remnant ammonium sulfate, the sample was dialyzed by ultracentrifugation using 4 kDa filter units (Millipore). Vs30 yield was measured at 280 nm using the nanodrop 1000 spectrophotometer (Thermo). Vs30 bioactivity was performed by the inhibition of cell proliferation assays as described below.

2.6 Cell culture

Immortalized EA.hy926 endothelial cells derived from fusion of human umbilical vein endothelial cells (HUVECs) with A549 lung carcinoma cells [18] were obtained from ATCC. Cells were grown in Iscove´s Modified Dulbecco´s Medium (IMDM, Sigma) supplemented with 10% fetal bovine serum (FBS, Gibco), glutamine and 1x Antibiotic Antimycotic Solution (100x) Stabilized (Sigma). Cultures were maintained at 37°C and 5% CO_2 in a humidified atmosphere and routinely harvested by 0.025% trypsine-EDTA Ix (Gibco) treatment.

138

139 **2.7 Proliferation assay**

140 The EA.hy926 cells ($2x10^4$/mL) were seeded in 96-well flat-bottom culture plates (Corning, USA)
141 with 100 µL culture medium (2% SFB), and 350, 500 and 750 µg/mL of Vs30 were added in
142 presence or absence of 35 ng/mL of FGF factor (Sigma), the cultures were maintained for 72 h at
143 37°C. To determine the cell viability 20 µL of a mixture of 3-(4,5-dimethylthiazol-2-yl)-5-(3-
144 carboximethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS
145 Promega) were added to each well and the culture was incubated for 2 h at 37°C, the absorbance
146 at 490 nm was recorded using ELISA plate reader (iMark ™, BIO-RAD).

147

148 **2.8 *In vitro* angiogenesis assay**

149 A modification of a previously reported protocol for spheroids of endothelial cells [19] was done in
150 order to get spheroids of the EA.hy926 cells. Thus, $4x10^3$ cells/mL were suspended in IMDM
151 culture medium containing 20% (v/v) methocel seeded into non-adherent round-bottom 96-well
152 plates and cultured overnight at 37°C and 5% CO_2. The spheroids were harvested by gently
153 pipetting and centrifuged at 300 x*g* for 15 min and embedded into collagen gels and 1.2%
154 methylcellulose, they were transferred into 96-well plates and incubated for 30 min until the
155 collagen gel solidified, following 50 µL IMDM medium supplemented with 5% FBS and PBS, Native
156 proteins from supernatant of untransformed *Pichia pastoris* or Vs30 were aggregated and
157 incubated for 72 h at 37°C. The images were captured at 0 and 72 h after treatments by using a
158 camera that was linked to an inverted microscope using the 10X objective (Leica).

159

160 **2.9 Statistical analysis**

Statistical analysis by an unpaired *t*-test was carried out using the GraphPad PRISM software Version 6.0c.

3. Results and discussions

3.1 Optimization of the *vs30* gene and construction of multi-copy expression vector

The sequence of *vs30* was adapted to the preferential codon usage of *Pichia pastoris* without changing the amino acid sequence. In addition, other aspects were considered for the design of synthetic *vs30*, such as GC content, RNA stability and cryptic splice sites. In this way, 18 of 30 codons were optimized, the GC content was adjusted from 50% to 38.8% and the codon adaptation index (CAI) was increased from 0.7 to 0.84. Consequently, 60% of *vs30* codons were increased at the highest frequency and the rare codons were removed (Figure 1).

A strategy that has been used to increase efficiently the amount of recombinant protein in *P. pastoris* is to increase the gene dose by multiple insertions of the expression cassette into the genome [20, 21]. Consequently, plasmids pGAP-Vs30-1x, pGAP-Vs30-2x and pGAP-Vs30-4x were obtained with 1, 2 and 4 copies of the expression cassette, which has a length of 1.35 Kb and consists of the constitutive promoter pGAP, *Saccharomyces cerevisiae* secretion signal alpha-factor, *vs30* gene and alcohol oxidase 1 terminator. Thus, the expression cassette was duplicated in each scaling, *i. e.* 2.7 Kb (2 copies) and 5.4 kb (4 copies), while the skeleton vector that was containing the zeocine resistance gene remained constant at 1.9 kb (Figure 2).

3.2 Selection of secreting strain of *Pichia pastoris* SMD1168

P. pastoris clones transformed with pGAP-Vs30-1x and pGAP-Vs30-4x were screened by yeastern colony blot. This method enables the identification of secreting transformants by signal developed on the nitrocellulose membrane, after direct contact and incubation with colonies. The selected strain, transformed with pGAP-Vs30-4x, produced more intense signals in contrast to the strain that was containing one copy of Vs30 expression cassette and untransformed strain (Figure 3a). The supernatants were precipitated with methanol, and analyzed by an SDS-PAGE and western blot (Figure 3b and 3c, respectively). Thus, a band below 10 kDa was visualized, which coincides with the Vs30 theoretical molecular weight. This band was observed only in the multi-copy strain supernatants. Most published studies have shown that the production of protein is proportional to the number of expression vector copies integrated in genome of *P. pastoris* [22,23]. However, sometimes the use of multi-copy strains had negative effects on protein production [24]. Our results demonstrate that 4 copies of the expression cassette have a positive influence on the amount of Vs30 produced by *Pichia pastoris* SMD1168.

3.3 Expression and purification of Vs30

It has been observed, that pH is an important parameter for expression in *P. pastoris*. Indeed, the increase in cultivation time and pH are related with protease secretion in culture medium [25]. Thus, *P. pastoris* cells were incubated at 6 and 7 pH conditions, and the supernatants were harvested and analyzed. As shown in tris-tricine polyacrylamide gel (Figure 4a), the number of proteins found in supernatant was reduced drastically at pH 6, which simplified the purification procedure. The effect of the dissolved oxygen on Vs30 production was also tested by incubation of culture medium in baffled flasks, which were used to increase the dissolved oxygen concentration in solution. With this, Vs30 production was higher when baffled flasks were used in comparison to

standard flask (Figure 4b). The optimal supernatant harvest time was 48 h. After 72 h cultivation, the yield of Vs30 had decreased probably by a proteolytic action (Figure 4c).

Supernatants were ultrafiltered using a stirred cell with a 30-kDa cut-off membrane. Thus, retentate and permeate fractions were collected and analyzed by SDS-PAGE. Vs30 was visualized as a unique band below 10 kDa only in the permeate fraction (Figure 5). Thus, SDS-PAGE analysis showed that the purity of Vs30 was up to 98%. In our laboratory, we were able to obtain 2.98 mg/L of soluble Vs30 by using the *E. coli* BL21-SI as an expression host [10]. In this work, we successfully expressed Vs30 in *P. pastoris*, and increased the post-purification amount to 10.47 mg/L. Then, we simplified the purification step reducing the time and cost of production. Furthermore, antiangiogenic peptides produced in bacterial expression systems require additional controls for effects of endotoxins [26, 27]. Previously, VAS (120-180aa), a longer version of Vs30 (135-164aa), was expressed in *P. pastoris* KM17 under a methanol inducible promoter [16]. On the other hand, we used the vector pGAPZαA based on the constitutive glycerlaldehyde-3-phosphate dehydrogenase (GAP) promoter. The constitutive expression simplifies the production process; it avoids the use of potentially hazardous methanol, and maintains continuous transcription of the gene of interest with different carbon sources as glucose, glycerol and sorbitol.

3.4 Vs30 decrease the proliferation of EA.hy926 cells

In order to assay the biological activity of Vs30 derived from *P. pastoris,* the proliferation of EA.hy926 cells was measured in presence of several concentrations of Vs30 (250, 500 and 750 µg/mL) by MTS reactive as described in material and methods. The results showed that the concentration of Vs30 (750 µg/mL) was responsible of 25% inhibition of proliferation of EA.hy926 cells compared to a vehicle, no effect of Vs30 was observed at 250 and 500 µg/mL (Figure 6).

These results proved that Vs30 is capable to affect cell proliferation from endothelial origin such as previously reported [9]. The efficiency of fusion albumin to increase the half live of recombinant proteins and peptides and increase the bioavailability has been determined before [28–31] so in spite to extend the half-life of Vs30 peptide and improve its bioavailability we suggest taking advantage of this strategy.

3.5 Vs30 inhibits the capillary-like structures *in vitro.*

Angiogenesis process involves proliferation, migration and extracellular matrix degradation by endothelial cells and it requires a three-dimensional endothelial organization thus an *in vitro* angiogenesis model was implemented in our lab using spheroids of EA.hy926 cells instead of HUVEC cells as commonly reported [19, 32], to avoid the technical limitations such as the number of passes from primary cultures. To analyze the biological effect of Vs30 over the capillary formation, spheroids of EA.hy926 cells embedded into collagen gels were treated by 72 h with IMDM plus %5 FBS, 50 µg of Vs30 or untransformed strain supernatants from *P. pastoris* or PBS as vehicle as previously described. Vs30 inhibited sprouting, spheroids were disaggregated and the contact cell to cell was lost while PBS and NC had no effect over basal sprouting give it by FBS (Figure 7). Before Vs30 from *Pichia pastoris* showed inhibition of angiogenesis *in vitro* and the mechanism would be resides in the disruption of junction of endothelial cell adhesion proteins such as platelet endothelial cell adhesion molecule (PECAM), Nectin or Endothelial cell selective adhesion molecule (ESAM) because all of them contribute to endothelial cell to cell adhesion [33]. On these assays with 5% FBS was enough to induce sprouting, however a grow factor such as VEGF of FGF recombinant could be necessary if we decide to describe the specific pathway involved in this response [32].

4. Conclusions

The expression, secretion and purification of Vs30 were achieved in a protease deficient strain of *P. pastoris*. In this work, the production of Vs30 was improved using a multicopy strain of *P. pastoris*. The expression of Vs30 at pH 6 decreased the amount of secreted yeast proteins into the culture medium simplifying the Vs30 recovery. Consequently, Vs30 was purified by ultrafiltration with a 30-kDa cut-off membranes. Thus, this one-step purification method provided an efficient way to obtain soluble Vs30 with purity up to 98%. Bioctivity of Vs30 was confirmed by the inhibition of EA.hy926 cell proliferation *in vitro.*

Authors´ contribution

Sergio Calderon-Salais, Prisiliana Velazquez-Bernardino and Antonio De Leon-Rodriguez: conceptualized and designed the experiments.

Sergio Calderon-Salais: clonation, transformation, expression and purification experiments.

Prisiliana Velazquez-Bernardino: *In vitro* angiogenesis assays.

Victor E. Balderas-Hernandez and Ana P. Barba-de la Rosa: Technical advice and support, they revised the manuscript and improved the discussion.

Conflicts of interest: none

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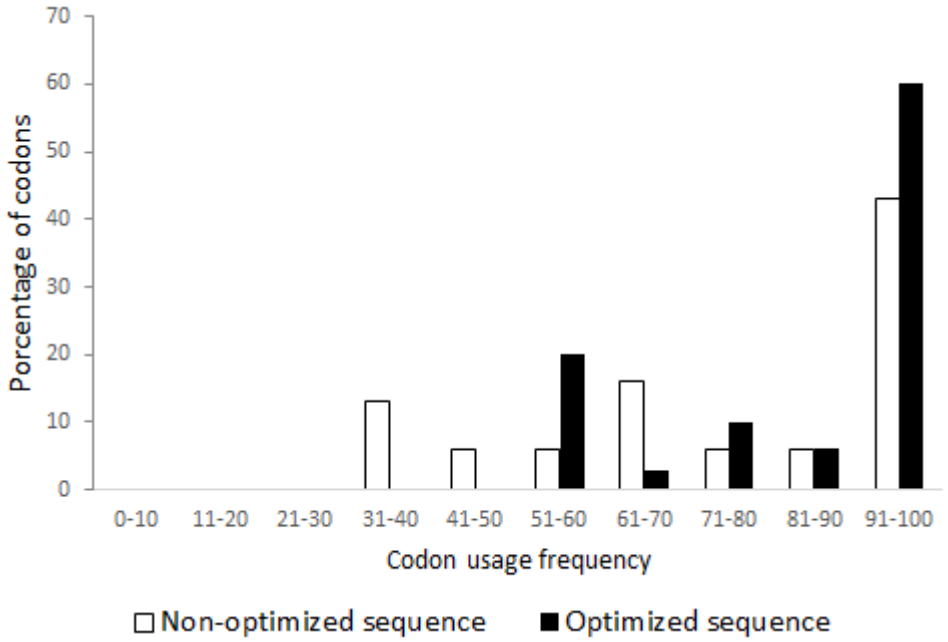

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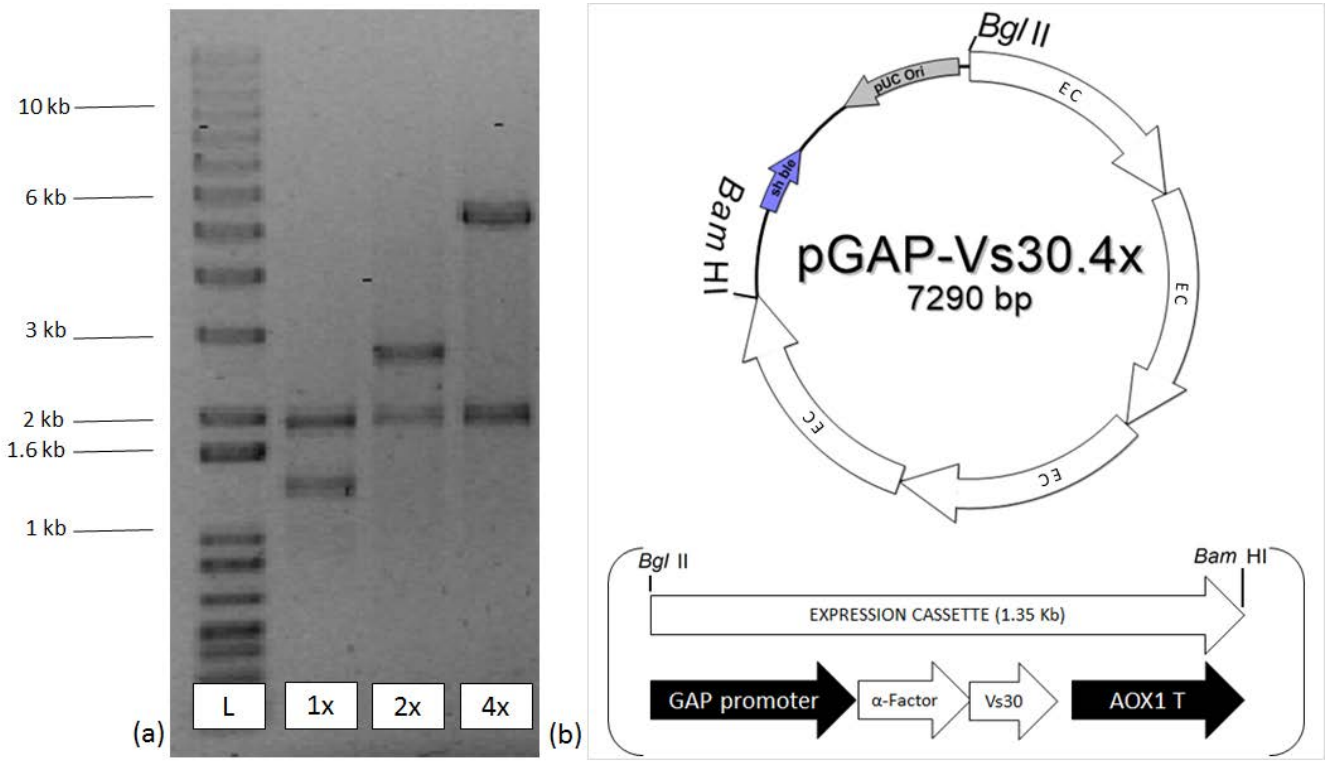
366 Figure 1: The percentage distribution of Vs30 codons. The value of 100 is set for the codon with

367 the highest usage frequency for a given amino acid in *Pichia pastoris*.



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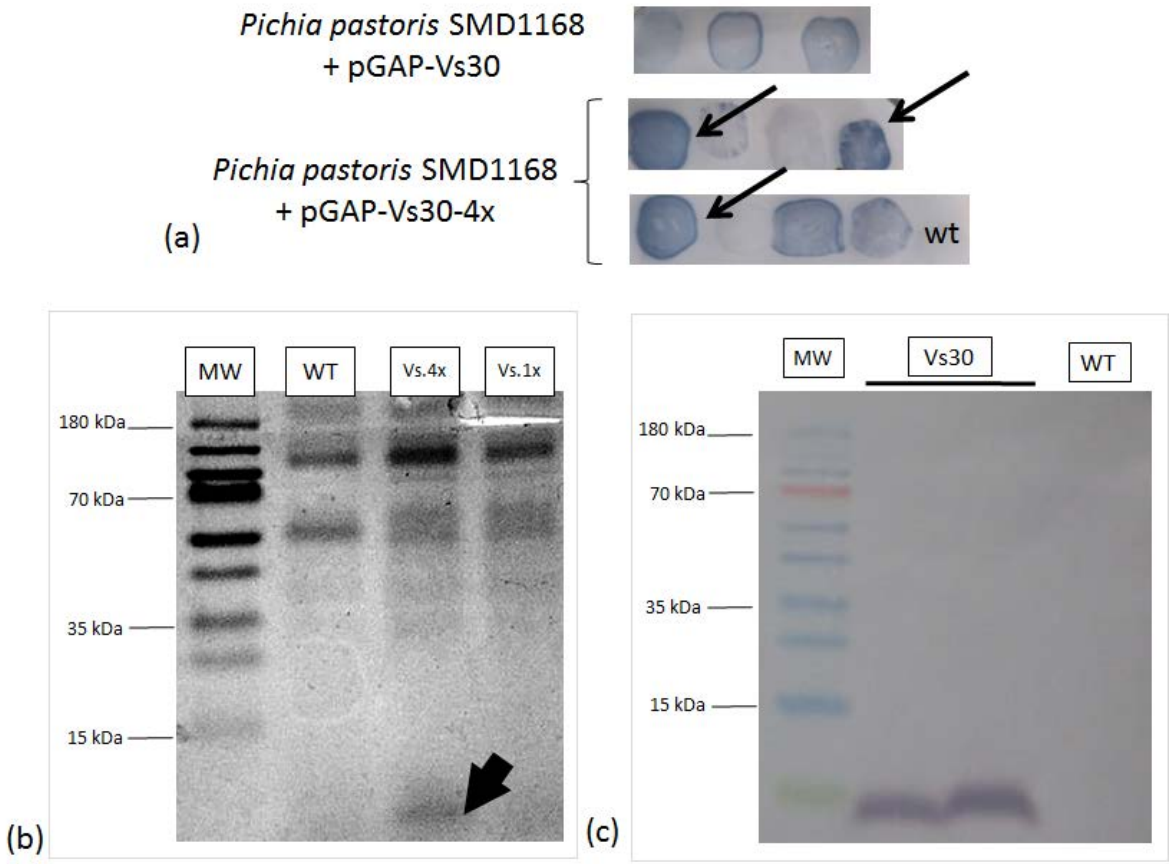
369 Figure 2: Construction of multi-copy expression vectors. (a) BamHI / BglII restriction analysis of the
370 vectors with 1, 2, and 4 copies of the expression cassette vs30 (L = 1 kb plus ladder, 1x = 1 copy of
371 the expression cassette, 2x = 2 copies, and 4x = 4 copies). (b) Schematic representation of vector
372 with 4 copies of the expression cassette (EC). The expression cassette has a length of 1.35 Kb and
373 is represented by a white arrow that is equivalent to the constitutive promoter pGAP, the
374 secretion signal alpha factor, the *vs30* gene and the Alcohol Oxidase 1 terminator.



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376 Figure 3: Selection of *Pichia pastoris* strains and electrophoretic analysis of supernatant proteins.
377 (a) Yeastern blot revealed with the alkaline phosphatase system. The arrows indicate the most
378 intense signals obtained from *Pichia pastoris* SMD1168 transformed with pGAP-Vs30-4x (b) SDS-
379 PAGE revealed with Coomasie blue. The arrow indicates the presence of a band in the lane (Vs.4x),
380 which is not visualized in the supernatants of the untransformed SMD1168 (WT) or the strain with
381 one copy of *vs30* (Vs.1x). (c) Western Blot revealed with the alkaline phosphatase system. The
382 presence of the band was observed in the strains transformed with pGAP-Vs30 and not in
383 untransformed SMD1168.

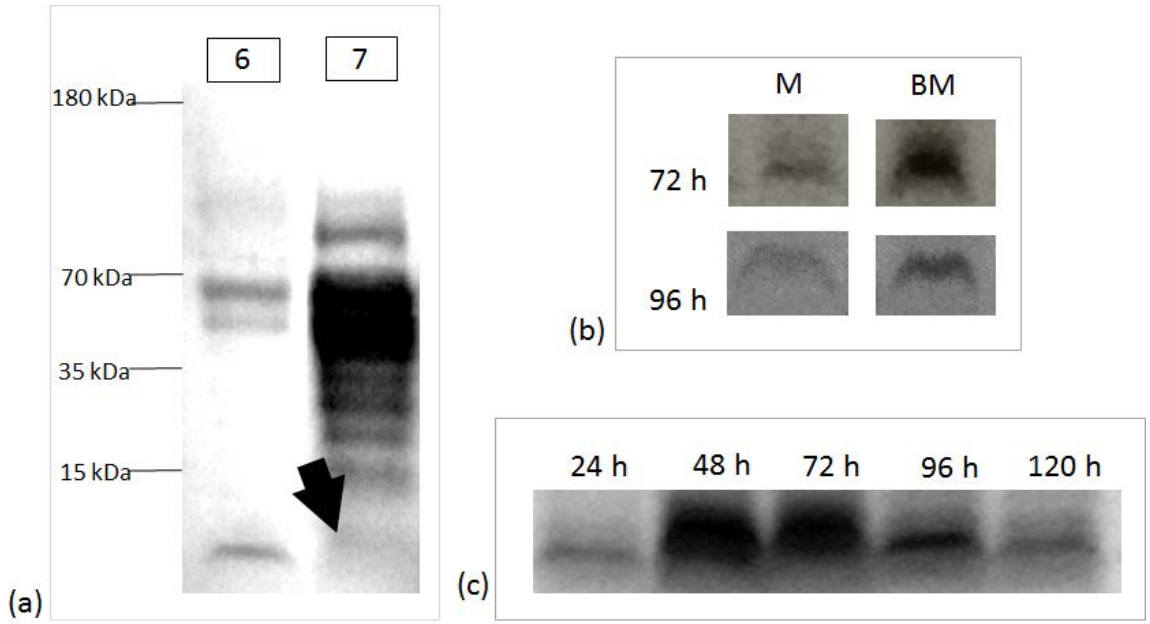
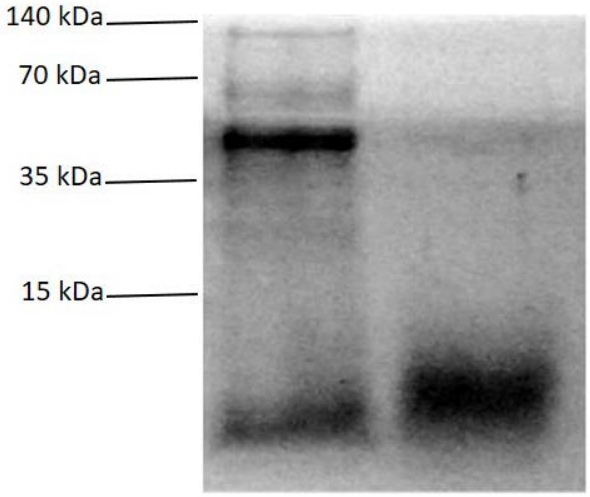


Figure 4: Effect of pH, flask type and time on proteins found in the culture medium. (a) SDS-PAGE of the supernatant expressed under different pH (6 = pH 6, 7 = pH 7, Arrow = Vs30). (b) SDS-PAGE of supernatants taken at 72 and 96 hours. M = 25 mL of culture medium incubated in 250 mL flask. BM = 25 mL of culture medium incubated in 250 mL baffled flask. (c) SDS-PAGE to samples taken at 24, 48, 72, 96 and 120 hours.



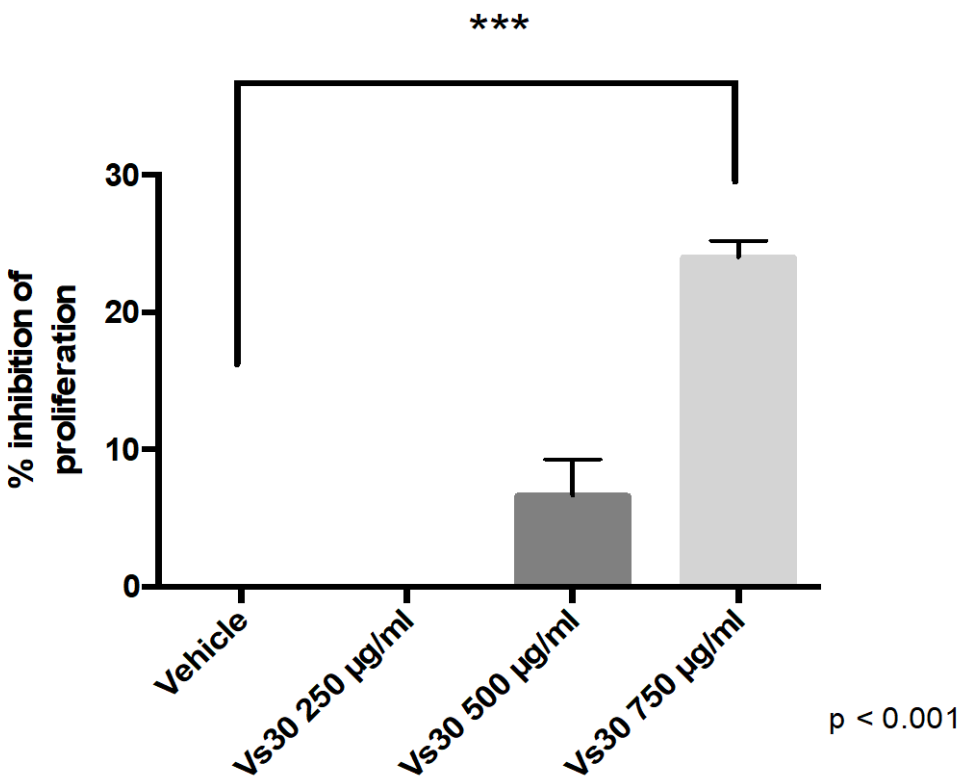
393

394 Figure 5: Purification of Vs30. SDS-PAGE of 30 kDa cut-off ultratilfration fractions. Left lane =

395 Retentate fraction. Right lane = Permeate fraction - Purified Vs30.

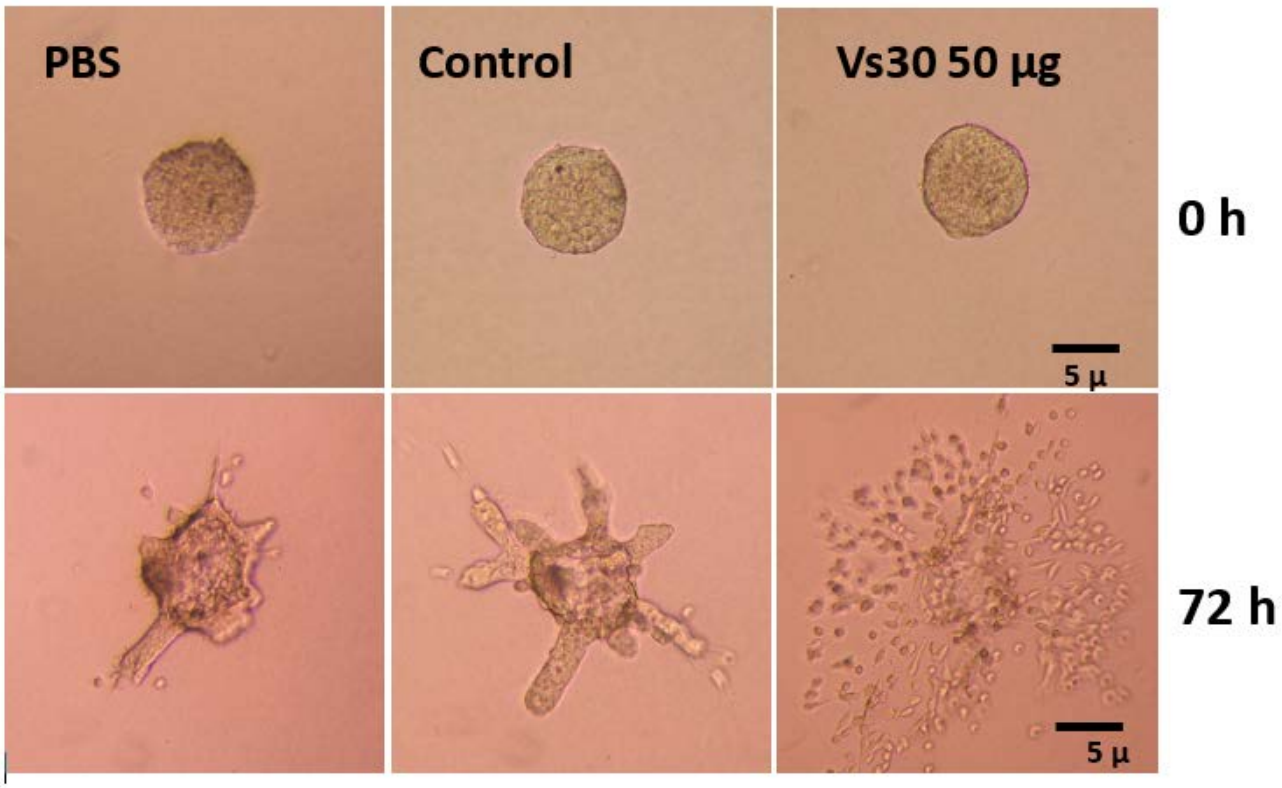
396

397



398

399 Figure 6: Inhibition of EA.hy926 proliferation by Vs30. EA.hy926 cells were incubated with 250, 500
400 and 750 µg/mL of Vs30 derived from *Pichia pastoris* for 72 h. 750 µg/mL of Vs30 increased the
401 percentage of inhibition of proliferation in EA.hy926 cells compared to vehicle. The values belong
402 to the mean of three determinations, $p < 0.001$.



403

404 Figure 7: Vs30 inhibit the basal sprouting of spheroids from EA.hy926 cells. Spheroids of EA.hy926
405 cells were imbibed into collagen gels, after, the stimulation of sprouting with IMDM supplemented
406 with 5% SFB plus vehicle (PBS), 50 µg of Vs30 or 50 µg of native proteins from supernatant of
407 *Pichia Pastoris* culture (Negative Control, NC) was assayed for up to72 h, while the treatment with
408 PBS and NC showed basal sprouting by the formation of capillary-like structures the spheroids that
409 were stimulated with Vs30 were segregated avoiding the initiation and extension of capillary.