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Pichia pastoris engineering for the production of a modified phospholipase C

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ABSTRACT

Crude vegetable oils are refined to remove impurities that adversely impact in their stability, color and flavor. In recent years, enzymatic degumming methods using phospholipase C (PLC) enzymes provide an environmentally friendly process for phospholipids removal with improved oil recovery yields.

In this study, *Pichia pastoris* was used as the expression system for the production of PLC-Y, a modified PLC enzyme derived from *Bacillus cereus*. Production of secreted PLC-Y driven by the methanol inducible *AOX1* promoter was optimized by genetic strain engineering which included gene codon optimization, generation of multi-copy chromosomal integrations and the co-expression of helper factors supporting protein folding, processing and secretion processes. In addition, tunable promoters directing helper factor expression were tested.

In batch cultures, a strain harboring seven integrated copies of the PLC-Y expression cassette and co-expressing the HAC1 transcription factor under an attenuated *AOX1* promoter showed a 6.2 fold increase in the production titers compared to the strain harboring a single gene copy. A fed-batch fermentation process developed using this engineered strain produced 4.5 g/l of this enzyme. The results presented in this work show the viability of using PLC-Y for oil degumming and provide a manufacturing process for its cost effective production.

1. Introduction

Type C Phospholipases (PLC) are enzymes that cleave phospholipids to yield diacylglycerol (DAG) and phosphate monoesters. This class of enzymes, produced by many microorganisms including *Bacillus*, *Pseudomonas and Clostridium* species [1–4], have lately received considerable attention because of their use for the enzymatic degumming of edible oils at industrial scale. This environmentally friendly process removes phospholipids and increases the recovery of oil. The extra yield is obtained as a result of: (i) the generated DAGs, miscible with triacylglycerols (TAGs) and (ii) the smaller quantity of TAGs trapped due to the reduction of the emulsion produced by the gums [5].

The PLC from *Bacillus cereus* (BC-PLC) is a secreted monomeric protein encoded by an 852 nucleotide chromosomal gene. This enzyme preferentially catalyzes the hydrolysis of phosphatidylcholine (PC) but it also hydrolyzes phosphatidylethanolamine (PE) with a lower catalytic efficiency [6]. As PC and PE are two of the most abundant phospholipids present in vegetable oils [1,7], its specificity makes BC-PLC an interesting candidate to be used in oil degumming processes [5,8]. Recently, we and others have engineered PLCs to create mutants with improved properties [7,9,10]. Our modified BC-PLC was designated PLC-Y and possesses a point mutation, replacing the natural phenylalanine at position 66 by tyrosine. According to the crystallographic structure, this residue is part of the choline binding pocket and its exchange for a tyrosine residue makes the protein more selective for PE than the wild-type BC-PLC enzyme [1]. This increased activity towards PE makes this mutant more suitable for enzymatic degumming of soybean oil which contains an average of 24% of PE in its phospholipids composition. Thus, a cost-effective robust manufacturing process for the production of PLC-Y is required to bring this enzyme to a commercial stage.

B. cereus is a pathogenic microorganism and therefore expression of the modified *plc-y* gene in a heterologous host is needed to establish a safe large scale industrial production process. The yeast *Pichia pastoris* is considered one of the most efficient expression systems for the production of recombinant proteins [11–14],

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because of its extraordinary capacity to secrete the target proteins, the available genetic tools including promoters with variable strength [15–17], and its remarkable ability to grow in fermenters to very high cell densities using inexpensive substrates [18,19].

In some cases, cost-effective production of recombinant proteins in *P. pastoris* is hampered by limitations in folding and secretion [20]. Recently, many helper proteins participating in these pathways have been identified and it has been shown that over-expression of some of their encoding genes results in higher productivities [21,22]. However, the current knowledge is not sufficient to anticipate which helper protein may contribute to boost the production of a particular target.

Here we describe a systematic work for *P. pastoris* strain engineering for the production of PLC-Y, a novel enzyme for oil degumming. The approach includes the use of a codon optimized gene, establishing the optimal gene dose per cell, identifying and modulating the expression of an accessory gene, and fed-batch fermentation development to obtain a high-producing strain displaying robust growth in high-cell density fermentations.

2. Materials and methods

2.1. Strains, plasmid and media

Escherichia coli TOP10 (Invitrogen, USA) was used for DNA manipulation and gene cloning. The recombinant strains were screened using Luria-Bertani (LB) medium plates (5 g/l yeast extract, 10 g/ltryptone, 10 g/lNaCl and 2% agar) containing 50 μg/ml kanamycin or LB low salt (5 g/l yeast extract, 10 g/l tryptone, 5 g/l NaCl and 2% agar) containing 25 μg/ml Zeocin. *P. pastoris* GS115 (Mut$^+$) strain was transformed with pBLHis vector [23] to complement histidine auxotrophy (GS115H). GS115H was grown on YP medium (10 g/l yeast extract, 20 g/l peptone) supplemented with 2% dextrose (YPD). pJ912 and pJ915 yeast expression vectors were purchased from DNA 2.0. pJ912 K and pJ915 K were constructed by replacing the Zeocin resistance gene in pJ912 or pJ915 vector with the kanamycin/G418 resistance marker from pFA6a-kanMX6 plasmid using *Nco*I and *Eco*RI restriction sites. Derived plasmids and oligonucleotides used in this study are listed in Table 1 and Supplementary Table 1, respectively.

2.2. Construction of expression vectors and transformation of P. pastoris

A synthetic version of PLC gene derived from *Bacillus cereus* (PLC-Y) was synthesized and codon optimized by Genscript (GenBank accession no. KU641164). *plc-y* gene was digested with *Xho*I and *Not*I and ligated into pJ912 vector for secretory expression.

For the construction of pJ912:PLC-Y_NTS, a PCR product for rDNA fragment was obtained using genomic *P. pastoris* DNA as template and primers NTSup and NTSdo (Supplementary Table 1). This region located in the rDNA repeat locus was designed as NTS (non-transcribed intergenic spacer) and cloned upstream the *AOX1* promoter (P_{AOX1}) in the pJ912-PLC-Y vector.

pJ912-PLC-Y and pJ912_NTS plasmids were linearized by *Sac*I and *Spe*I, respectively, and transformed into *P. pastoris* GS115H by electroporation with a Gene Pulser apparatus (Bio-Rad, USA) according to manufacturerís protocol. Transformants were selected on YPD-Zeocin plates. By using P_{AOX1} driven integration, gene insertion through a single crossover can lead to multi-copy being integrated in approximately 1–10% of all transformants [24,25]. In the case of NTS_PLC-Y, clones were stepwise transferred to YPD agar plates with increasing Zeocin concentration from100 μg/ml, and then restreaked onto 500, 1000, 2000 and 5000 μg/ml, as previously described [26]. In both cases, gene copy number integration was determined by real time PCR.

AOX1 promoter variants were made by introducing single point mutations using primers 737GCfw and 737GCrv or by deleting two base pairs using primers d737-38fwand d737-38rv as described by Hartner and co-workers [27], to construct *AOX1-30* and *AOX1-5* (Supplementary Table 1). These modified sequence promoters retain 30% and 5% of *AOX1* wild type promoter activity, respectively.

2.3. Expression tests based on PLC-Y activity

For monitoring *P. pastoris* transformants, single colonies were inoculated in 2 ml of YPD and grown at 30 °C in a deep 24-well microplate in a shaking incubator for 16–18 h at 250 rpm. These cultures were diluted to an OD_{600} of 1 in 2 ml of YPM (YP, 100 mM phosphate buffer pH6 and 0.5% methanol) to induce gene expression and incubated in the same conditions as previously. Methanol was added every 24 h in order to maintain0.5% methanol concentration. After 72 h of induction, cells were harvested by centrifugation and the supernatant analyzed for protein expression by SDS-PAGE.

2.4. PC-PLC activity determination

PC-PLC expression was monitored using 4-Nitrophenyl phosphorylcholine (NPPC, Toronto Research Chemicals) as a substrate. The assay was carried out at 50 °C in a 96 microwell plates by incubating 10 μl of culture supernatant, 10 μl 100 mM NPPC and 80 μl of buffer HEPES20 mM at pH 7.0 and sorbitol 60%. The rate of NPPC hydrolysis by phospholipase C was monitored at various time intervals during 30 min by measuring the p-nitrophenol released at 405 nm in a Synergy HT microplate reader (Biotek).

2.5. Isolation of genomic DNA from P. pastoris and quantitative real time PCR (qRT-PCR)

Genomic DNA isolation from *P. pastoris* was performed as previously described [28]. The copy number of target genes was determined by qRT-PCR of the integrated *AOX1* promoter copy number using GS115H as the control strain. Data were normalized using the housekeeping gene *ARG4* gene as the endogenous control. The primers AoxUp, AoxDo, Arg4Up and Arg4Do were used for qRT-PCR (Supplementary Table 1) and purchased from Genbiotech (Argentina). According to manufacturerís instructions, PCR was performed in a 20 μl reaction mixture containing 10 μl of 2× MezclaReal (Biodynamics, Argentina), 2.5 μl of each 5 μM primer and 5 μl of 1/10 dilution of genomic DNA sample. The amplification reaction was initiated with a 2 min step at 98 °C, followed by 40 cycles of 10 s at 95 °C, 20 s at 55 °C and 30 s at 72 °C. The data collection of the fluorescence signal was performed at the end of the elongation step by using a StepOne Real-Time PCR System (Applied Biosystems, Argentina). The amplification period was followed by a melting curve to exclude amplification of unspecific products. A ten-fold serial dilution (10^{-2}-10^{-7}) of target and reference genes was utilized as templates for setting the standard curves and the PCR efficiency was calculated from the slope of each curve (data not shown).

2.6. PCR amplification and co-expression of chaperones and helper factors

The gene sequence data from all chaperones and helper factors were obtained from Genbank database and previous publications [21,22,29]. The respective homologs of genes encoding for Pdi, Sso2, Hac1, Bmh2 and Ssa4 were amplified from *Sacharomyces cerevisae* genomic DNA while for Kar2, Kex2, YDJ1, Ssa1 and Sec63 genes were amplified from *P. pastoris* DNA. These genes were amplified

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Table 1
Plasmids used in this study.

Plasmid	Characteristics	Source/reference
pBLHis	rep *colE1*, *bla*, HIS4	Lin Cereghino et al. [23]
pJ912	pUC origin, zeo, AOX1 promoter, alpha-factor signal sequence	DNA 2.0
pJ915	pUC origin, zeo, GAP promoter, alpha-factor signal sequence	DNA 2.0
pUC57	rep *PMB1*, *bla*	Genscript
pFA6a-kanMX6	pBR322_origin, bla, KanR2 (variant)	Bahler et al. [67]
pJ912K	pJ912-G418	This work
pJ915K	pJ915-G418	This work
pKCN173	pJ912:PLC_Y	This work
pKCN174	pJ915:PLC_Y	This work
pKCN183	pJ912(Kan):HAC1	This work
pKCN188	pJ912:PLC_Y_NTS	This work
pKCN202	pBLHIS:PLC_Y	This work
pKCN251	pJ915:HAC1	This work
pKCN254	pJ912K:AOX30:HAC1	This work
pKCN351	pJ912K:AOX5:HAC1	This work
pKCN255	pJ912K:Ssa1	This work
pKCN258	pJ912K:Sec63	This work
pKCN262	pJ912K:YDJ	This work
pKCN267	pJ912K:Kar2	This work
pKCN181	pJ912K:Bmh2	This work
pKCN184	pJ912K:Pdi	This work
pKCN185	pJ912K:Ssa4	This work
pKCN186	pJ912K:Sso2	This work
pKCN 212	pJ912K:Kex-2	This work

using specific primers with 5′oligonucleotide extension that creates *Eco*RI or *Mfe*I and *Not*I restriction enzyme sites (Table 1 and Supplementary material, Table S1). The amplified products were digested, purified and ligated into pJ912 K or pJ915 K vectors for cytoplasmic expression. A low amount of DNA (~500 ng/80 μl cells) was used to guarantee the prevalence of single-copy integration [30]. The recombinant plasmids were linearized with *Bgl*I and introduced by electroporation in PLC-Y_7strain. Positive recombinant transformants were identified using YPD plates supplemented with G418 (400 μg/ml).

2.7. High cell density cultures of P. pastoris

The fermentation inoculum of *P. pastoris* was prepared by cultivating the cells at 30 °C for 20 h in a shaking flask containing YPD medium. This culture was diluted 1/10 in a 3-L fermenter (Infors Labfors 4, Switzerland) with 900 ml of the BSM medium (Invitrogen), 40 g of glycerol and 4.35 ml of PTM1 trace salts solution per liter. PMT1 solution is composed of 6 g/l $CuSO_4{\cdot}5H_2O$, 0.08 g/l NaI, 3 g/l $MnSO_4{\cdot}H_2O$, 0.2 g/l $Na_2MoO_4{\cdot}2H_2O$, 0.02 g/l H_3BO_3, 0.5 g/l $CoCl_2$, 20 g/l $ZnCl_2$, 65 g/l $FeSO_4{\cdot}7H_2O$, 0.2 g/l biotin and 0.5% v/v H_2SO_4.

Throughout the entire cultivation process, temperature was maintained at 30 °C, pH was adjusted to 5 using NH_4OH 28%, dissolved oxygen was maintained over 20% of air saturation by controlling the agitation speed and the air flow rate (or pure oxygen when required) was kept at 0.5 VVM.

The fermentation process comprised three different phases: the glycerol batch, the glycerol fed batch phase and the methanol fed batch phase [31–33]. Briefly, once the initial glycerol from the batch culture was consumed and the OD_{600} reached approximately 150, a fed batch was started with a solution containing glycerol 50% w/v and 1.2% of PTM1. After 4 h of feeding with a constant flow rate of 18.1 ml/l.h the induction phase was started with a solution containing methanol 100% and 1.2% of PTM1. The feed rate was started at 3.6 ml/l.h during 4 h, then the flow rate was doubled to 7.2 ml/l.h for the next 2 h (adaptation phase). Once the culture was fully adapted to methanol utilization, a constant flow rate of 11 ml/l.h was maintained until the end of the process. Samples were taken regularly to monitor biomass and protein production by phospholipase activity tests and quantified by SDS-PAGE analysis using BSA (Sigma Aldrich) as standard. PLC-Y recovered from *P. pastoris* fermentation broth after cell mass separation by centrifugation, was diafiltrated against 20 mM sodium acetate pH 6.0 treatment.

2.8. Enzymatic soybean oil degumming using PLC-Y

Soybean oil was supplied by Molinos Rio de la Plata (Argentina) and Bunge (Argentina). Oil degumming experiments were performed using 1 kg of crude soybean oil (Phospholipids = 2.5%, FFA = 1%; Phosphorous = 1000 ppm). For this purpose, crude oil was preincubated in a beaker immersed in a thermostatic bath equipped with an agitation unit which consisted of a 3-bladed propeller-type impeller (70 mm diameter) to maintain continuous stirring during the reaction. 5 mg of PLC-Y enzyme in 30 ml of 50 mM citrate buffer pH 6.0 was added per 1 kg of crude soybean oil. The mixture was emulsified using an Ultra-Turrax T-50 Homogenizer (IKA) and continuous stirring (500 rpm) was maintained at 50 °C for 2 h. Finally, heat inactivation of the enzyme was carried out by incubation at 85 °C for 10 min to end the reaction.

2.9. NMR analysis of phospholipids composition in oil

Oil degumming experiments were performed as described above. Treated oil was emulsified using an Ultra-TurraxT-50 Homogenizer (IKA) for 1 min before taking 300 mg samples for further analysis. Oil samples were extracted with 900 μl of NMR solution (100 mM Tris-HCl pH 10.5, 50 mM EDTA, 2.5% sodium deoxycholate) during 1 h at 37 °C with constant agitation. Finally, the resulting aqueous phase was extracted with 600 μl hexane. For ^{31}P NMR phospholipids analysis, 50 μl of D_2O was added to the aqueous phase and spectra were acquired using a Bruker 300 Ultrashield equipment. Samples of phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid and phosphatidylinositol (Avanti Polar Lipids) were used as standards (data not shown).

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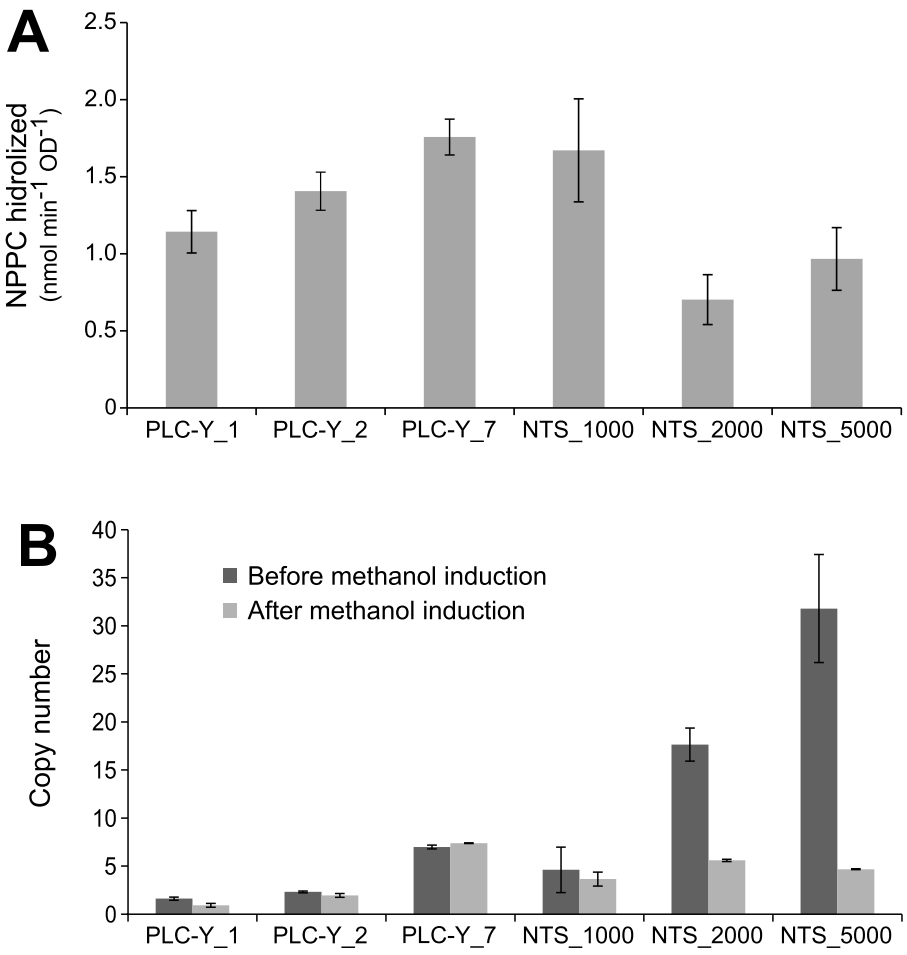

Fig. 1. A-Expression screening test based on PLC activity. Samples from culture supernatants of PLC-Y_1, PLC-Y_2 and PLC-Y_7 (P_{AOX1} driven integration of the PLC-Y expression cassette) or NTS-1000, NTS-2000 and NTS-5000 (NTS-derived clones) were tested for PLC activity after 72 h of methanol induction. PLC activity directly correlates with PLC production levels. **B-Gene copy number integration was assessed by qRT-PCR.** Samples were taken before and after methanol induction, under non antibiotic selective condition. The data represent the mean ± SD values from three independent measurements done in triplicate.

3. Results

3.1. Gene design, strain construction and effect of gene copy number on PLC production

PLC-Y is an engineered *B. cereus* phospholipase showing superior properties for oil degumming [9]. PLC-Y sequence was first codon optimized for its expression in *P. pastoris* using a codon randomization method which consisted on randomly assigning a triplet for each amino acid using a preference table (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=4922), with a probability based on the weight of each codon within the set encoding a given amino acid [34]. The synthetic gene was cloned under the control of P_{AOX1} for extracellular secretion in *P. pastoris* [35,36] in such a way that after Kex-2 cleavage, the mature enzyme conserved the initial N-terminal tryptophan residue which is essential for enzyme activity.

In order to evaluate PLC-Y production in *P. pastoris* transformants, we first sought to assess the impact of gene copy number. Two different alternatives were used to obtain transformants with variable gene doses: (i) P_{AOX1} driven integration or (ii) NTS-integration into rDNA locus of PLC-Y expression cassette. Fig. 1A shows the *P. pastoris* GS115H strain containing one, two or seven integrations obtained by P_{AOX1} driven integration, grown in YPD medium in deep well microplates and induced with methanol for 72 h. The enzyme was recovered from culture supernatants, and expression tests based on PLC-Y activity [37] clearly demonstrate that production of PLC-Y increases with the number of gene copies integrated.

Since multi-copy clones show higher PLC-Y titers, we surmised that gene dose may still be a bottleneck for productivity. To test this hypothesis, NTS-driven integration was used as an alternative to direct gene copy number amplification into the ribosomal DNA locus, seeking to obtain clones with more than seven copies per cell. For this, PLC-Y was expressed from the *AOX1* promoter integrated into the NTS region of the rRNA gene locus. A recombinant colony was initially isolated on YPD plates containing 100 μg/ml of Zeocin and re-streaked on YPD plates supplemented with increasing Zeocin concentrations of 500, 1000, 2000 and 5000 μg/ml as described previously [26]. The resulting new clones were named according to the selection condition used as NTS_1000, NTS_2000 and NTS_5000 (data not shown for NTS_500). Clones harboring 5,16 and 31 copies of the PLC-Y expression cassette were obtained for clones NTS_1000, NTS_2000 and NTS_5000 respectively, as determined by qRT-PCR analysis (Fig. 1B).

For heterologous protein expression, maintaining the stability of a producing strain under non-selective conditions is a critical issue. For this reason, all the strains were restreaked on YPD plates without antibiotics and then assayed in liquid medium for PLC-Y expression after 72 h of methanol induction. NTS clones did not show sustainable PLC-Y secretion levels, contrary to what was observed for clones obtained by P_{AOX1} homologous integration

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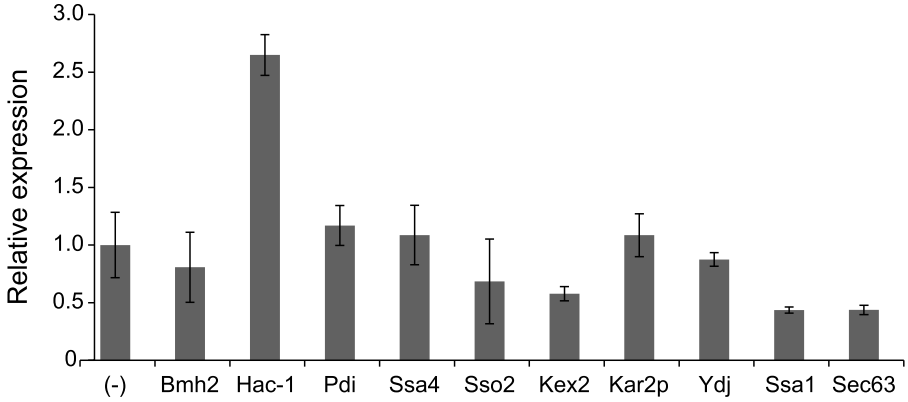

Fig. 2. PLC relative expression levels obtained by co-expression of helper proteins compared to PLC-Y-7 strain used as control. The data represent the mean ± SD values from three independent measurements done in triplicate.

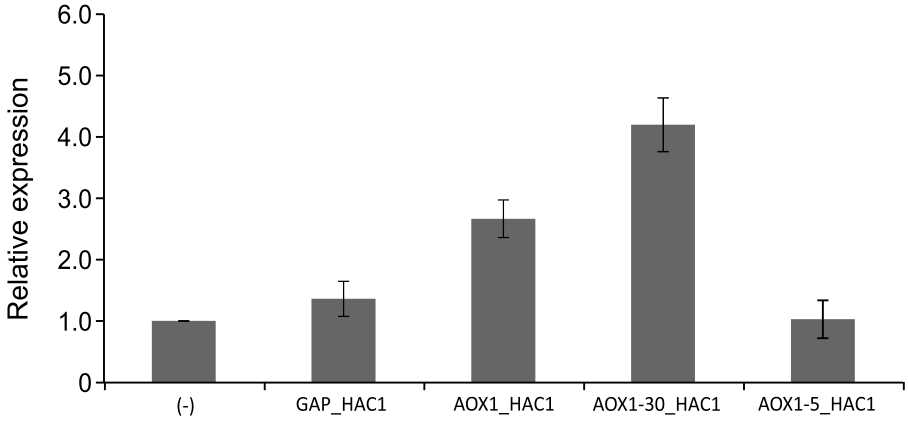

Fig. 3. Tuning Hac1 expression level by using different promoters. The constitutive *GAP* promoter (P_{GAP}_Hac1), the methanol induced *AOX1* promoter (P_{AOX1}_Hac) or derived P_{AOX1} mutants retaining 30% or 5% of WT activity (AOX1-30_Hac1 or AOX1-5_Hac1, respectively) were used to control Hac1 expression in PLC-Y-7 strain. The data represent the mean ± SD values from three independent measurements done in triplicate.

(Fig. 1A). In keeping with this observation, when we checked for gene copy number in clones obtained by P_{AOX1} or NTS driven integration before and after 72 h of methanol induction, NTS derived clones were not stable as determined by the decrease in gene copy number analyzed by qRT-PCR (Fig. 1B).

Taking together, these results suggest that the P_{AOX1} integration strategy provides strains with higher stability and the strain harboring seven copies of the expression cassette is the optimal for PLC-Y production under the tested conditions. We therefore decided to continue the strain engineering process using the PLC-Y-7 strain.

3.2. Co-expression of helper proteins to enhance PLC-Y production

When using *P. pastoris* as an expression host, a linear correlation between protein titers and gene copy number integration was widely reported for intracellular expression [26,38]. In the case of proteins targeted to the secretory pathway, there is often evidence of saturation causing a plateau in protein production titers as the gene dose increases [39,40]. It has been previously described that several factors involved in the unfolded protein response (Hac1), protein folding (Kar2, YDJ, Ssa1, Sec63, SSa4), disulfide bridge formation (Pdi), vesicle transport (Bmh2, Sso2), and release (Kex-2) from the cell can affect recombinant protein production [21,22,41]. We therefore explored whether the co-expression of these helper factors can contribute to increase the production of PLC-Y (Fig. 2). For this, PLC-Y-7 strain was transformed with the plasmids containing the expression cassette for the helper proteins under the control of *AOX1* promoter (Table 1). The resulting clones were grown in triplicates in deep well microplates in YPD medium and induced for 72 h with methanol. Fig. 2 shows that co-expression of Hac1, provided a 2.5 fold increase in PLC-Y titers compared to those obtained with the parental PLC-Y-7 strain, while no significant titer improvements were detected for the co-expression of the remaining helper proteins.

Hac1 has been extensively described as the key regulator of the unfolding protein response (UPR), a signaling pathway that leads to restore ER homeostasis which in turn affects the productivity of recombinant proteins [42–44]. Thus, we decided to evaluate if different Hac1 levels may further impact on PLC-Y production. For this purpose, *Hac1* gene was cloned under the control of different promoters varying from constitutive expression with the P_{GAP} promoter to methanol induced mutant promoters with different strength: *AOX1-5* (5% of expression compared to wild type), *AOX1-30* (30% of expression compared to wild type) and *AOX1* wild type [27]. In all cases, clones containing a single inserted copy of the Hac1 expression cassette were selected. Fig. 3 shows that the PLC-Y-7_AOX1-30_Hac strain, where the expression of Hac1 is driven by the 30% mutant *AOX1* promoter produced ~30% more PLC-Y enzyme than the strain carrying Hac1 under the control of the wild type *AOX1* promoter and more than 6.2 fold more recombinant enzyme than the first generation PLC-Y-1 strain. This strain was therefore chosen for the development of a fed batch fermentation process for the production of PLC-Y.

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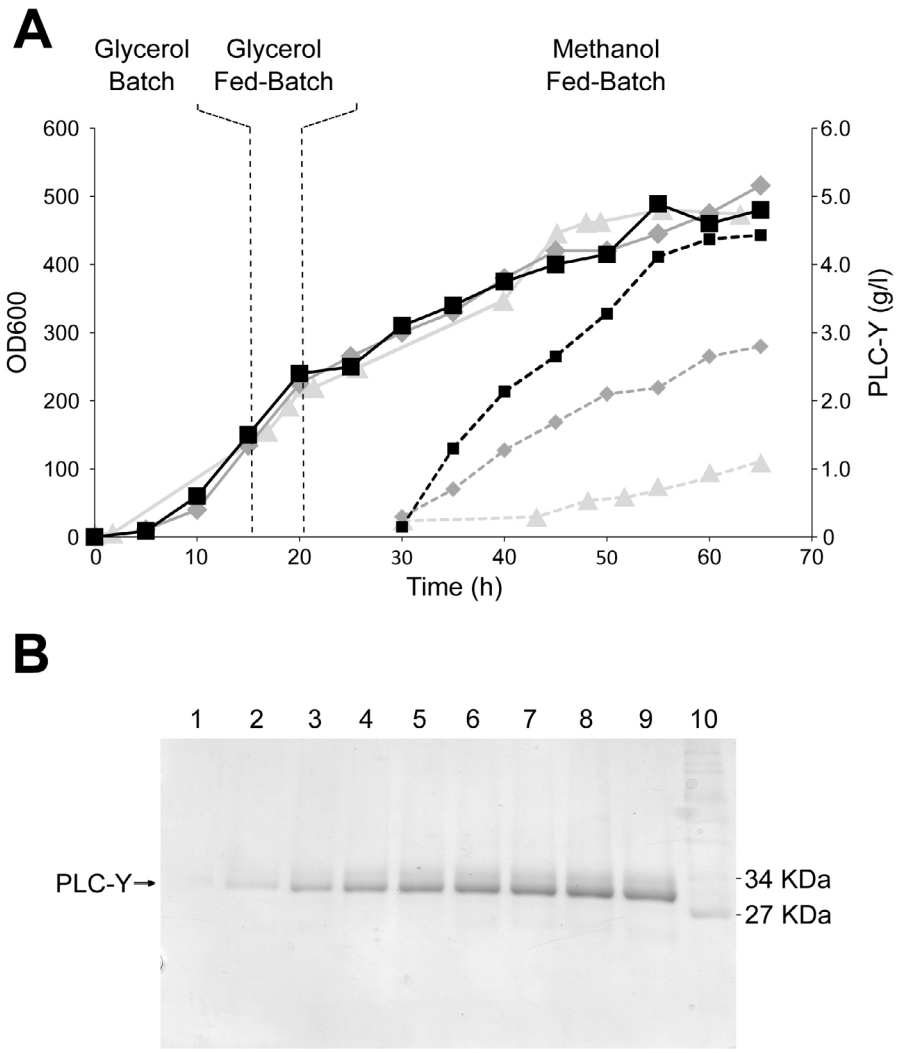

Fig. 4. High cell density culture for PLC-Y production. (A) Time course comparison of biomass (solid line) and total protein concentration (dashed line) during fed-batch fermentation in the 3-L fermenter using methanol as the sole carbon source and inductor. PLC-Y-1: grey triangle; PLC-Y_7: grey rhombus; PLC-Y_7-AOX1-30_Hac1: black square. Values shown are means of three independent experiments. The standard deviations were in all the cases less than 10% of the corresponding means. (B) Time course of SDS-PAGE analysis of culture supernatants during methanol fed-batch cultivation in a 3L fermenter. Lanes 1–9, 20 μl of 1/20 dilution of culture supernatant of PLC-Y_7-AOX1-30_Hac1 strain in a 3-L fermenter after 30, 35, 40, 45, 50, 55, 60 and 65 h of cultivation. Lane 10, molecular weight marker (NEB).

3.3. PLC-Y production in fed batch fermentation

For the recombinant production of PLC-Y in *P. pastoris*, we developed a fed batch fermentation process using glycerol as a carbon soured during the growth phase and methanol as the inducer and sole carbon source for the induction phase. Using this process, we compared the performance of the wild type GS115H strain transformed with an empty vector, PLC-Y-1, PLC-Y-7 and PLC-Y-7_AOX1-30_HAC strains to assess the toxicity of PLC-Y, the effect of the *plc-y* gene dose and the impact HAC 1 over PLC-Y expression under high cell density culture growth conditions.

The growth profile of all the recombinant strains (Fig. 4A), as well as the wild type strain transformed with the empty vector (data not shown) were similar, indicating that neither PLC-Y expression nor the overexpression of Hac1 have any impact in cell viability or growth rate. A final OD_{600} of ~500 units, equivalent to 125 g/l DCW (dry cell weight) was obtained for all strains. Cell mass yield of 0.83 OD_{600}/g of methanol remained constant during the entire fermentation. The production of PLC-Y was detectable 7 h after the beginning of the methanol induction phase for all the strains, and its production continuously increased until a maximum titer of 4.5 g/l for the PLC-Y-7_AOX1-30_Hac, 2.8 g/l for PLC-Y-7, and 1.1 g/L for PLC-Y-1 strain, achieved in all cases at around 38 h after the beginning of the induction phase (Fig. 4A, B and supplementary Fig. 1). The concentration of both glycerol during the fed batch phase, and methanol throughout the induction phase were undetectable, indicating that the carbon source added was completely depleted (data not shown). Three fermentation runs were carried out for each strain, where the PLC-Y productivity and growth profile were very similar, indicating the robustness of the fermentation process developed.

3.4. Enzymatic oil degumming with PLC-Y

To assess the performance of the recombinant PLC-Y produced in the engineered *P. pastoris* PLC-Y-7_AOX1-30_HAC strain for edible oil treatment, laboratory scale degumming reactions were set up simulating the conditions used for enzymatic oil degumming on industrial scale as described before. In order to confirm the specificity of the reaction catalyzed by PLC-Y, the phospholipid fraction remaining after the enzymatic treatment was separated and analyzed by NMR. The NMR spectra for treated oil and a control where

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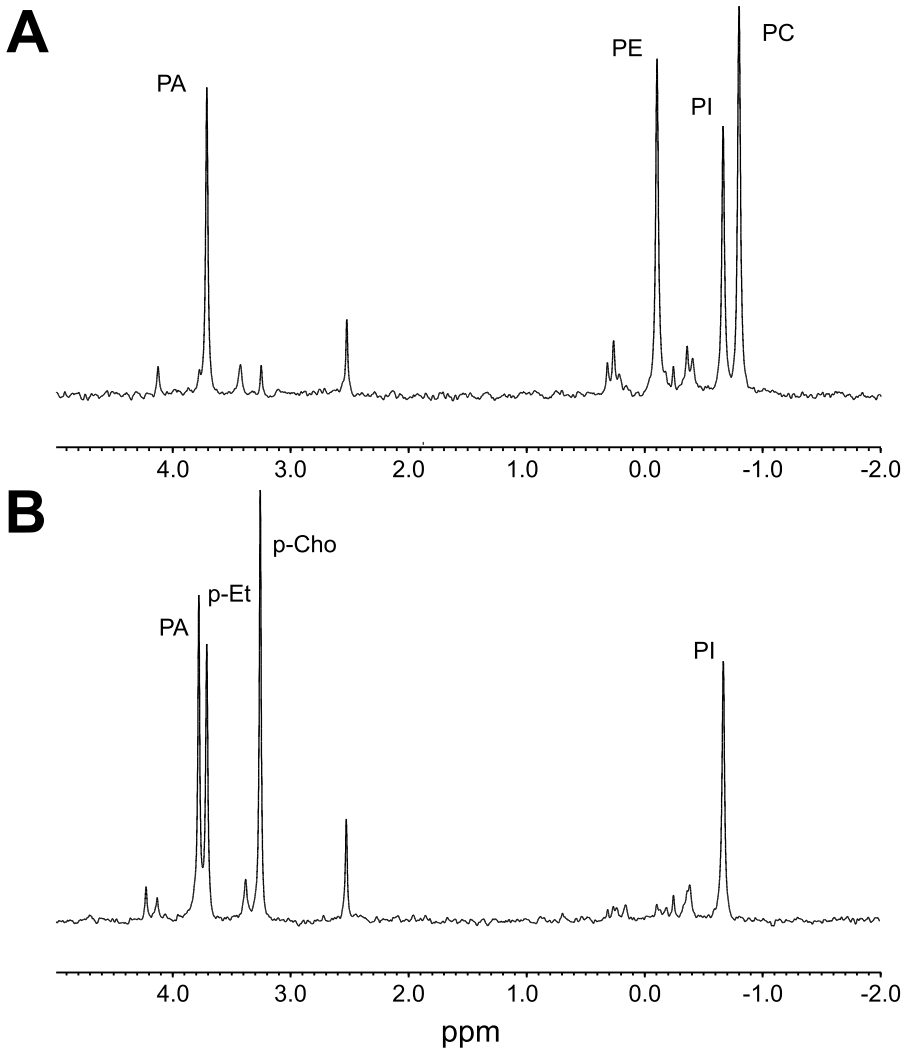

Fig. 5. Analysis of phospholipid hydrolysis in soybean oil by PLC-Y.^{31}P-NMR spectra for a water treated soybean oil (A) or PLC-Y treated oil (B). PLC-Y produced was able to completely hydrolyze PC and PE in oil treated samples, generating phosphocoline (pCho) and phosphoethanolamime (pEt) as determined by ^{31}P RMN peak profile. PI and PA indicates phospatidylinositol and phosphatidc acid respectively.

no enzyme was added are shown in Fig. 5. It can be observed upon the treatment with PLC-Y that the peaks corresponding to PC and PE are no longer detectable while peaks for phosphocholine (pCho) and phosphoethanolamime (pEt), the hydrolysis products, are generated. Taken together, these results clearly indicate that PC and PE are completely hydrolyzed by the recombinant PLC-Y produced with the fed batch fermentation.

4. Discussion

Novel PLC enzymes that can efficiently catalyze the hydrolysis of phospholipids to yield 1,2-DAGs may contribute to establish enzymatic oil degumming, an environmentally friendly process that is quickly replacing traditional aqueous degumming in oil refining processes [5]. However, like for most of industrial enzymes, manufacturing cost is a critical factor that needs to be addressed [34,45].

B. cereus PLC is an attractive enzyme to be used for oil degumming naturally produced by a pathogenic microorganism. Recombinant production of BC-PLC in *P. pastoris* and *B. subtillis* has been reported under laboratory conditions [46,47], although obtained yields were far below to those required to establish a cost-effective process. Expression attempts were also made in *E. coli*, where the recombinant PLC formed inclusion bodies, making its recovery in an active form expensive since it required multiple steps including a large volume refolding process [48]. We recently reported a production of 5 g/L of this enzyme in *C. glutamicum*, but this microorganism was unable to remove the signal peptide and therefore the obtained protein needs to be treated with an additional protease to obtain a functional enzyme [49]. Thus, there is a need for efficient strains and fermentation processes capable of providing large quantities of this enzyme at affordable prices.

PLC-Y variant is an engineered BC-PLC enzyme with superior properties for oil degumming [9]. We therefore designed and synthesized a codon optimized gene for PLC-Y expression using the *P. pastoris* GS115H strain as a host. Initially, the effect of gene dose on PLC-Y titers obtained was investigated. It was reported by different authors that increasing gene dose has a significant impact on recombinant protein production [50–52], although the genetic stability of the resulting recombinant strains has not been evaluated in most of the cases. Previous studies demonstrated that for P_{AOX1} driven integration, clones with high copy number could be stable in the absence of methanol, but these strains may show a dramatic decrease in their stability after induction [53]. However, this type of analysis has not been systematically carried out in recombinant

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strains with NTS driven integration under non-selective conditions [26,54]. In our study, strains with a copy number higher than seven were only obtained by using NTS driven integration, which turned out to be unstable. In this case we are not able to distinguish if the instability is only associated with the number of integrated copies rather than the integration method used. The best results under the tested conditions were obtained when inserting seven copies of *plc-y* gene into the GS115H strain by P_{AOX1} driven integration, and thus decided to use this strain for further optimization.

Recent results show that the over-expression of a variety of helper proteins increase the production of several heterologous proteins in *P. pastoris* [21,22]. For this reason, we explored the impact of nine of these proteins involved in protein folding, fusion of vesicles and secretion. In our hands, only the overexpression of the transcription factor Hac1 provided an increase in the production of PLC-Y.

When comparing clones co-expressing a single inserted copy of Hac1, production of PLC-Y was higher when Hac1 expression was inducible rather than constitutive, and the increase in heterologous protein expression levels was even greater when using the attenuated variant for *AOX1* promoter, *pAOX1-30*. While Hac1 expression is regulated by a unique stress-regulated splicing mechanism in *S. cerevisiae* [55], splicing in *P. pastoris* was shown to occur constitutively, suggesting primarily an UPR regulation by the Hac1 transcript levels in *P. pastoris* [44]. The activation of the UPR affects the transcription of nearly 400 genes in yeasts [56,57] and the aim of this pathway is to maintain cellular protein homeostasis and alleviate endoplasmic reticulum stress [58]. Under prolonged signaling, the ER-associated degradation (ERAD) pathway is activated [56,59], resulting in the shuttle of misfolded protein to the proteasome for degradation, which impacts negatively on protein production and reduces the overall yield [60]. This may explain why both the constitutive expression and expression under the strong *AOX1* promoter resulted in lower titers of PLC-Y than those obtained with the *pAOX1-30*, which, among the tested promoters, may be providing the optimal amount of Hac1 required to achieve the highest expression of PLC-Y under our experimental conditions.

Previous studies using protein fusions to well-known secreted proteins [61] and the co-expression of possible combination of helper factors [22,62,63] have been made as different attempts to alleviate cellular stress. In this sense, the use of promoters with varied strength regulating Hac1 levels could be useful tools in increasing heterologous protein production by controlling cell burden and activation of UPR pathway. Previous works showing the transcriptome analyses of Hac1 overexpressing strains allowed for the identification of cellular targets leading to improved protein production [64]. It would be interesting to perform a similar analysis for the expression of PLC-Y in Hac1 overexpressing strains, as it could provide useful insights for rational strain engineering for further improvements in protein production.

Finally, in order to develop a high yield fermentation process for production of PLC-Y, a classical fed batch strategy using glycerol and methanol as carbon sources was tested. Using a mineral salt medium, a final titer of 4.5 g/l was obtained in 60 h, which correspond to a volumetric productivity of 0.075 g/l.h. The growing, and therefore the physiological conditions of *P. pastoris* in fed batch fermentations are different to those of batch cultures grown on YPD medium. However, the over-expression of Hac1 in fed batch cultures provided the highest titer for PLC-Y production, similar to the results obtained for the batch cultures, suggesting that the conditions used for the screening served well to predict the behavior of the engineered strains in high cell density fermentations. The production of PLC-Y did not affect the growth of the host, indicating that a continuous production process might be designed to further aid to reduce the production cost. To the best of our knowledge, this is the highest yield reached for a PLC type enzyme. A further increase in the productivity may be obtained by optimizing the post-induction feeding profile [65,66]. Since this substrate is completely depleted in all the fermentation runs described in this work, it is possible that the strain may grow and produce more PLC at higher feeding rates.

In summary, by combining strain engineering and fed batch fermentation development, we have designed a lab-scale efficient process for the production of PLC-Y. The results described here provide a path for the large scale production of this novel enzyme which can contribute to the adoption of enzymatic oil degumming, a more efficient and environmentally friendly process for oil refining. Next challenges include the scaling up of the fermentation process to manufacture PLC-Y and establishing the optimal conditions for oil degumming at industrial scale.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.procbio.2016.08.022.

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