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1 **Optimized expression of the *Starmerella bombicola* lactone esterase in *Pichia pastoris* through temperature**
2 **adaptation, codon-optimization and co-expression with *HAC1***

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20 **Abstract**

21 The *Starmerella bombicola* lactone esterase (SBLE) is a novel enzyme that, *in vivo*, catalyzes the intramolecular
22 esterification (lactonization) of acidic sophorolipids in an aqueous environment. In fact, this is an unusual reaction
23 given the unfavorable conditions for dehydration. This characteristic strongly contributes to the potential of SBLE to
24 become a 'green' tool in industrial applications. Indeed, lactonization occurs normally in organic solvents, an
25 application for which microbial lipases are increasingly used as biocatalysts. Previously, we described the production
26 of recombinant SBLE (rSBLE) in *Pichia pastoris* (syn. *Komagataella phaffii*). However, expression was not optimal
27 to delve deeper into the enzyme's potential for industrial application. In the current study, we explored codon-
28 optimization of the *SBLE* gene and we optimized the rSBLE expression protocol. Temperature reduction had the
29 biggest impact followed by codon-optimization and co-expression of the *HAC1* transcription factor. Combining these
30 approaches, we achieved a 32-fold improvement of the yield during rSBLE production (from 0.75 mg/L to 24 mg/L
31 culture) accompanied with a strong reduction of contaminants after affinity purification.
32

33 **Keywords**

34 Lipase; lactonase; *Starmerella bombicola*; *Pichia pastoris*; protein purification; Green chemistry.

35 **Highlights**

36 - Production of *Starmerella bombicola* lactone esterase (SBLE) in *P. pastoris* is evaluated
37 - Expressing rSBLE at 16°C decreased the amount of contaminants during purification.
38 - Codon-optimization and expressing at 16°C increased rSBLE yield seventeenfold.
39 - Co-expression of rSBLE with *HAC1* increased the yield approximately twofold.

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43

44 **Abbreviations**

45 rSBLE : Recombinant *Starmerella bombicola* lactone esterase; rSBLEopt : Recombinant Starmerella bombicola
46 lactone esterase obtained after codon-optimization; EndoH: Endoglycosidase H; SEC: Size-exclusion
47 chromatography; IMAC: immobilized metal-ion affinity chromatography; MALDI-TOF : Matrix-Assisted Laser
48 Desorption/Ionization Time-Of-Flight; UPR : unfolded protein response

49

50 **Introduction**

51 Microbial lipases are widely used as biocatalysts in a variety of biotechnological applications[1]. Although the typical
52 natural activity of lipases in aqueous media is the hydrolysis of triglycerides or phospholipids, these enzymes can also
53 catalyze inter- and intra-esterifications in organic media. This is used for the synthesis of intermediates for the
54 production of chiral compounds such as many pharmaceuticals[1]. Intra-esterifications result in the formation of
55 lactones, which occur widely in nature as hormones (spironolactones and mevalonolactones), antibiotics (erythromycin
56 and amphotericin B) or neurotransmitters (butyrolactones and avermectins). Intermolecular esterification occurs also
57 in the biosynthesis of sophorolipids (SLs), a family of fungal biosurfactants that naturally exist in two forms: a closed
58 lactone and an open acidic form[2]. Each form has distinct properties: acidic SLs have better foaming properties, while
59 lactonic SLs are better in surface tension reduction and have antimicrobial activity[3].

60 We have previously described that ring closure to form lactonic SLs in *S. bombicola* is catalyzed by a novel member
61 of the *Pseudozyma (Candida) antarctica* – A (CAL-A) lipase family[4], which was designated as the *Starmerella*
62 *bombicola* lactone esterase (SBLE)[5]. This enzyme is rather unique as it lactonizes sophorolipids in the overabundance
63 of water[5, 6], whereas normally lipases perform esterifications in non-aqueous media[7]. Indeed, only a few enzymes
64 are described to be capable to perform esterification reactions in an aqueous environment[8, 9]. At present,
65 lactonization reactions are mostly performed using a different lipase of *Pseudozyma* (*Candida*) *antarctica*, the
66 *Pseudozyma* (*Candida*) *antarctica* lipase B (CAL-B), commercialized as Novozyme® 435 by Novozymes. CAL-B has
67 been used for the synthetic preparation of lactonized SLs, but hazardous solvents (dry tetrahydrofuran (THF) containing
68 vinyl acrylate and vinyl acetate) are required [10]. In addition, CAL-B links the fatty acid moiety to the C-6″ glycosyl
69 hydroxyl group whereas the natural reaction, as performed by SBLE, involves the C-4″ hydroxyl group[10].

70 The unique properties of SBLE could provide an alternative for the catalysis of lactonization reactions in green
71 chemistry applications[6, 11]. However, in order to valorize SBLE for industrial use, a better production system is
72 required. We have previously reported a *Pichia pastoris* production system for the production of rSBLE (Figure 1,
73 Table 1).

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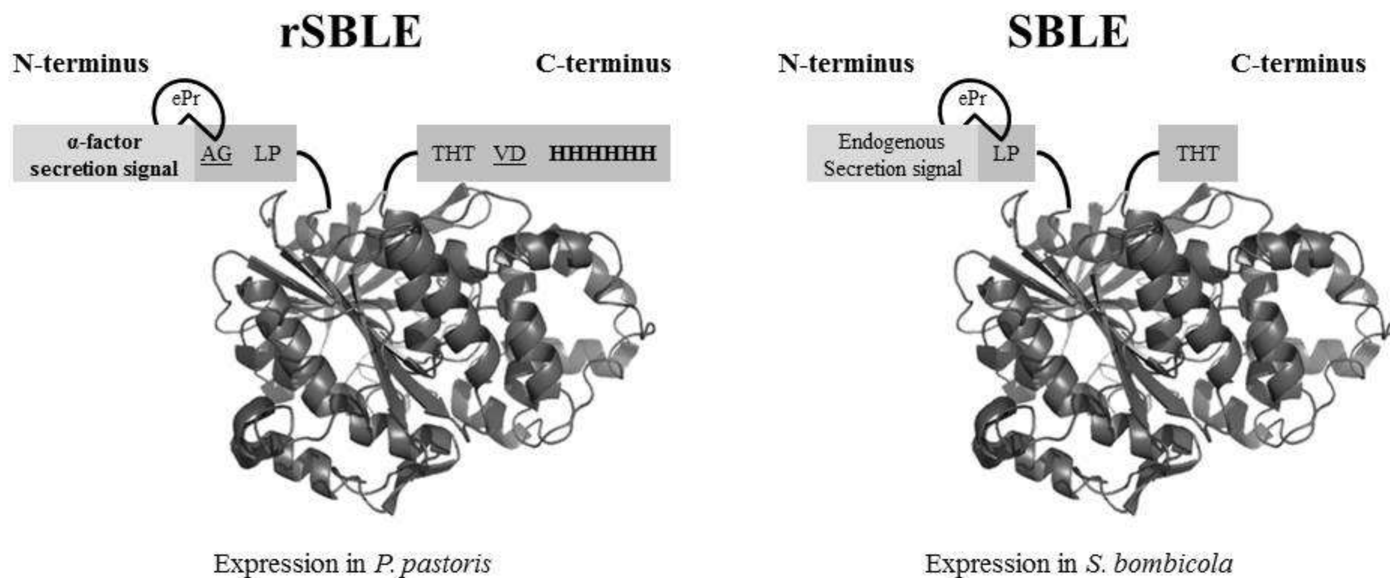


75

76 **Figure 1: Comparison of rSBLE (left) and SBLE (right).** We replaced the endogenous secretion signal used in *S. bombicola* with *Saccharomyces*
77 *cerevisiae's* α factor secretion signal. This leaves two additional amino acids (AG) at the N-terminus of the protein. At the C-terminal we added a His_6 tag. In
78 addition two amino acids are inserted (underlined) to allow restriction site compatibility. The structure displayed is a model based on the structure of CAL-A
79 (PDB id: 2VEO) [12].

80

81 **Table 1: Characteristics of the *Starmerella bombicola* lactone esterase.**

	rSBLE (SBLE-His_6)	**SBLE**
Molecular weight (kDa) *	45.122	44.085
Subunits/isoforms	None	None
pI (theoretical)	4.88	4.63
pH optimum	3.5 - 6.0	Not determined
Temperature optimum	40°C	Not determined

82 Temperature and pH optima have been retrieved from [6], while pI and molecular weight have been obtained from ExPASY [13]. *: the influence of
83 glycosylation on the molecular weight has been excluded due to the uncertainty of the actual amount of N-glycosylation sites.

84

85 Unfortunately, this system was so far unsatisfactory for two main reasons: first, only a low yield of rSBLE (0.75
86 mg/liter culture) was obtained. Second, during His Tag purification, several contaminants were co-purified. Therefore
87 this study foremost aimed to improve the rSBLE production system in view of further exploitation of this enzyme. To
88 improve the production of rSBLE we followed a dual approach. On the one hand, we optimized the expression
89 conditions using the strain expressing the non-codon-optimized SBLE (containing the plasmid pPICZαB_rSBLE)[6]
90 and on the other hand, we generated a novel, codon-optimized rSBLE production strain. In brief, we investigated the

91 influence of aeration, pH, induction-time, methanol feeding and temperature on rSBLE accumulation in the culture
92 supernatant. Afterwards, these optimized expression conditions were implemented on the codon-optimized construct
93 to reach large-scale expressions. Additionally, we verified whether co-expression of the transcriptional activator gene
94 *HAC1* improved production yields. Indeed, during expression of recombinant protein, proteins can become unfolded
95 leading to the unfolded protein response (UPR). The transcriptional activator Hac1p is produced during this unfolded
96 protein response, stimulating the transcription of several genes related to translocation, glycosylation and protein
97 folding[14, 15]. Co-expression of Hac1p has been used successfully to increase the yield of the protein of interest, due
98 to the recovery of unfolded protein[15]. Overall, our efforts led to a significant improvement of production yields for
99 SBLE production.
100

101 <u>Materials and methods</u>

102 Strains and media:

103 The strains used in this study are shown in Table 2. *Escherichia coli* DH5α (New-England-Biolabs (NEB)) was used
104 for cloning and plasmid amplification. Bacteria were propagated in low-salt lysogeny broth (LS-LB) medium,
105 consisting of 0.5% (w/v) sodium chloride (Merck), 0.5% (w/v) yeast extract (Lab M) and 1.0% (w/v) tryptone (Lab M)
106 with or without 1.5% agar (BD) and with the required antibiotics. The *Pichia pastoris* (syn. *Komagataella phaffii*)
107 NRRL-Y-11430 strain (kindly obtained from Prof. Nico Callewaert (VIB, Ghent University)) was used for recombinant
108 protein production. Yeast strains were plated on yeast extract peptone dextrose (YPD) plates (1% (w/v) yeast extract,
109 2% (w/v) peptone (BD), 2% (w/v) dextrose (Merck), 1.5% agar (Difco)) with the required antibiotics. All antibiotics
110 were purchased from Thermo Fischer Scientific with the exception of carbenicillin, which was obtained from Gold
111 Biotechnology. For recombinant protein expression, strains were grown in buffered glycerol-complex medium
112 (BMGY) and induction was performed in buffered methanol-complex medium (BMMY). Both BMGY and BMMY
113 consist of 1% (w/v) yeast extract, 2% (w/v) peptone (BD), 100 mM phosphate buffer (Sigma-Aldrich) at pH 6.0 and
114 1.34% (w/v) yeast nitrogen base (YNB, Formedium) with 1% glycerol (v/v Sigma) or 1% methanol (v/v, VWR) as
115 sole carbon source respectively.

Cloning of the codon optimized rSBLE

All enzymes required for cloning were obtained from NEB. The original DNA construct for rSBLE has been described in [6]. In brief, the coding sequence of mature SBLE (GenBank accession no. **JB750219**), lacking the secretion signal and the stop codon, was cloned in-frame with *Saccharomyces cerevisiae*'s prepro α-mating factor secretion signal and a C-terminal His-tag. To determine the impact of possible codon bias in *P. pastoris*, the SBLE ORF was codon-optimized by Genscript®'s proprietary algorithm and ordered synthetically. The codon-optimized SBLE construct was cloned to the pPICZαB plasmid (Invitrogen®, Carlsbad, USA) (Table 2; sequence in supplementary Figure 1). Therefore, the SBLE construct was digested with PstI-HF® and SalI-HF® and ligated to the similarly opened pPICZαB plasmid using T4 DNA ligase. The ligation reaction mixture was used to transform electrocompetent DH5α cells and positive transformants were selected on LS-LB plates containing Zeocin^{TM} (50 µg/ml medium). Twelve positive clones were selected and screened by colony PCR using primers FW_rSBLE and Rev_rSBLE (5'-ATTGCTGCAGGACTCCCTTTGGGTTAT-3' and 5'-ATTAGTCGACTGTGTGGGTAGAATTAACTGG-3', respectively. The underlined sequence indicates the PstI- and SalI-sites). A single clone was selected, the resulting plasmid (pPICZαB_rSBLEopt) was isolated and sequence-verified by GATC biotech (Konstanz, Germany) using the primers mentioned above.

Table 2: Plasmids and strains used in this study.

Plasmid	Characteristics	Size (bp)	Source
pPICZαB	Zeo^R, α-factor secretion signal, His_6 Tag, C-myc Epitope tag, AOX1 promoter, 3' AOX TT	3597	Life Technologies®
pPICZαB_rSBLE	Zeo^R, α-factor secretion signal, His_6 Tag, AOX1 promoter, 3' AOX TT, linearized with SacI, coding original rSBLE	4678	[5]
pPICZαB_rSBLEopt	Zeo^R, α-factor secretion signal, His_6 Tag, AOX1 promoter, 3' AOX TT, linearized with SacI, coding codon-optimized rSBLE	4678	This study

pPICHyg_HAC1spliced	Hyg^R, Amp^R, AOX1 promoter, 3' AOX TT , linearized with PmeI, coding spliced *HAC1*	6426	[16]
Strains	**Characteristics**		**Source**
NRRL-Y-11430	*Pichia pastoris* (*syn. Komagataella phaffii*) strain, used for expressing protein of interest		Prof. Nico Callewaert (VIB, Ghent University)
DH5α	F-, Φ80lacZΔM15 ΔlacZYA-argF) U169 recA1 endA1 hsdR17 (rK-, mK+) phoA supE44 λ- thi-1 gyrA96 relA1, used for producing the plasmids of interest and cloning purposes		NEB®

Amp, ampicillin; AOX, alcohol oxidase; bp, basepairs; His, histidine; Hyg, hygromycin; opt, optimized; R, resistant to; rSBLE, recombinant *Starmerella bombicola* lactone esterase; TT, transcription terminator; Zeo, Zeocin^{TM}.

Transformation of codon-optimized rSBLE and co-expression vectors

The plasmid of interest (pPICZαB_rSBLEopt) was linearized in the AOX1 promoter region by SacI and 100 ng of the plasmid was transformed to *P. pastoris* NRRL-Y-11430 cells using electroporation, as in the protocol of Wu *et al*[17]. Transformants were selected on YPD medium containing Zeocin^{TM} (500 µg/ml) and the best producing clone was chosen based on Western blot analysis.

To study the influence of Hac1p co-expression, the best producing rSBLE clone was made to be electrocompetent using the protocol of Wu *et al*[17]. Subsequently, 100 ng of pPIC9K_HAC1spliced, linearized by PmeI in the AOX1 promotor region, was added to these electrocompetent cells. Transformants were then selected on YPD medium supplemented with Hygromycin B (300 µg/ml) and Zeocin^{TM} (500 µg/ml).

Evaluation of the effect of aeration using baffled flasks

Cells, containing the plasmid of interest, were grown in a preculture of 10 ml BMGY overnight at 250 rpm, while maintaining Zeocin^{TM} (500 µg/ml) selection. This preculture was divided between two types of shake flasks, both containing 500 ml BMGY, and grown for 48 hours to evaluate the effect of aeration. The first type (Neubert-Glas,

152 0735-10-3000) did not have any baffles, while the latter shake flask did contain 4 baffles (Neubert-Glas, 0749-10-
153 3000). Both flasks types had a volume of 3000 ml. After 48 hours of induction the cells were centrifuged and the
154 supernatant was studied regarding rSBLE production.
155
156 **Screening for optimal conditions for expression of rSBLE.**
157 Cells were grown in a 24-well plate sealed with AreaSeal film (Sigma-Aldrich). Initially, each well contained 2 ml
158 BMGY. Cells were allowed to grow for 48 hours (28°C, 250 rpm) to accumulate biomass. Cells were centrifuged, and
159 washed. Afterwards, the cells resuspended in BMMY for induction under different expression conditions to screen for
160 the effect of pH, methanol-feeding and induction-time. The following parameters were tested: pH (0.1 M citrate buffer,
161 pH 2.0, 3.0, 4.0, 5.0, 6.0; 0.1 M phosphate buffer, pH 6.0), percentage of methanol for induction (0.5%, 0.75%, 1.0%
162 and 1.5%), induction-time (48h, 60h, 72h, 84h, 96h, 102h and 120h) and induction-temperature (16°C and 28°C). The
163 results were always compared with the reference condition as used in [6] where expression conditions were : pH 6.0
164 (0.1 M phosphate buffer), temperature 28°C, duration 48h and methanol concentration 1%. After the appropriate
165 induction time, the cultures were centrifuged (6000 *g*) and the supernatant was collected for analysis. The cleared
166 supernatant was precipitated using DOC-TCA precipitation. In brief, 0.05% (w/v, final concentration) sodium
167 deoxycholate (DOC, Sigma-Aldrich) was added to 1 ml of supernatant. After incubation, 20% (w/v, final
168 concentration) of trichloroacetic acid (TCA, Sigma-Aldrich) was added and the sample was incubated on ice.
169 Subsequently, the sample was centrifuged and the obtained pellet was washed twice with ice-cold acetone. After
170 washing with 70% (v/v) ethanol, the pellet was resuspended in 50 µl phosphate buffered saline (PBS). Prior to SDS-
171 PAGE analysis, 5 µl of sample was treated with Endoglycosidase H (Endo H, 500 U, NEB) in order to remove
172 heterogeneity due to N-glycosylation and to allow a more accurate molecular weight estimation on SDS-PAGE.
173
174 **Protein analysis: SDS-PAGE and Western blotting**.

175 Protein samples were analyzed by SDS-PAGE on 12.5% gels (Tris-HCl). The Precision Plus Protein™ Unstained
176 Standard (Bio-Rad) was used as a molecular weight marker. Gels were stained with Coomassie brilliant blue G,
177 unless Western blotting was used.
178 For Western blot analysis of rSBLE, rSBLE was detected using an Anti-His Tag horseradish peroxidase-coupled
179 antibody (Anti-His (C-Term)-HRP, 46-0707, Invitrogen, Carlsbad, USA). Blots were revealed using luminol and
180 H_2O_2 (Pierce ECL Western blotting substrate, both obtained from Thermo Scientific) and using the Precision Plus
181 Protein™ Dual Color Standard (Bio-Rad). The gels were scanned using a GS-800 calibrated densitometer (Bio-Rad)
182 and visualized using the Quantity One software package (Bio-Rad).
183
184 **Protein purification**
185 For purification of rSBLE, a pre-culture was prepared in 10 ml BMGY and grown overnight (at 28°C, 250 rpm) under
186 antibiotic selection. The next day, the pre-culture was used to inoculate 500 ml BMGY in 3L baffled flasks (at 28°C,
187 250 rpm). At an optical density at 600 nm (OD_{600}) of approximately 41, cells were harvested by centrifugation (10
188 minutes at 4,000 *g)*. The cells were resuspended with BMMY containing 1% methanol (v/v) to induce expression. To
189 maintain the induction, 1% methanol (final concentration) was added to the cells every 10 to 12 hours. After 48 hours
190 of induction, the cultures were centrifuged and the supernatant was harvested.
191 All purifications were done on an ÄKTA Purifier system (GE Healthcare). Prior to loading the sample, 100 mg/l
192 reduced glutathione (Sigma-Aldrich) and 2 mM (final concentration) of magnesium sulfate (Sigma-Aldrich) were
193 added and the pH was adjusted to 7.5. Any precipitation was removed by filtering the sample over a 0.22 µm bottle top
194 filter (Sarstedt). The cleared supernatant was loaded overnight on a 5 ml HisTrap™ FF column (GE Healthcare)
195 previously equilibrated with washing buffer (50 mM Na_2HPO_4 and 500 mM NaCl (pH 7.5)). The column was washed
196 with washing buffer until the UV(280nm) absorption reached baseline. The column was step-wise eluted with 20-, 200
197 and 400 mM imidazole in washing buffer. rSBLE eluted at 20 mM imidazole already, but a significant amount was
198 recovered at 200mM as well. These two fractions were concentrated by ultrafiltration to a volume of 1.0 ml using 10

199 kDa molecular weight cut-off Vivaspin® columns (EMD Millipore). Ten µl of these concentrated fractions was
200 analyzed by SDS-PAGE as described above.
201 To further purify rSBLE, the pooled and concentrated IMAC fraction was injected onto a HiLoad® 16/600 Superdex®
202 200pg column (GE Healthcare) equilibrated with 150 mM NaCl in 50 mM Tris (pH 7.5) and eluted with the same
203 buffer. The fractions containing rSBLE were concentrated to 1.0 ml by ultrafiltration using Vivaspin® (10 kDa
204 molecular weight cut-off, Merck millipore) from which 10 µl was taken for SDS-PAGE analysis. The size-exclusion
205 chromatogram was represented as a graph made by GraphPad prism 6.0. Every purification was performed with at least
206 2 technical replicates and the average ± standard deviation of the total yield was used to compare with other conditions.
207 The obtained concentrated SEC fraction was stored at -80°C until further analysis.
208

209 **Determination of protein concentration**

210 The rSBLE concentration was determined by UV spectrometry with a NanoDrop® 2000 (Thermo Scientific) using
211 parameters: ε, 73.355 M^{-1} cm^{-1} and a molecular weight of 45.5 kDa as calculated using ProtParam (ExPASY[13]).
212

213 **Activity assays of rSBLE**

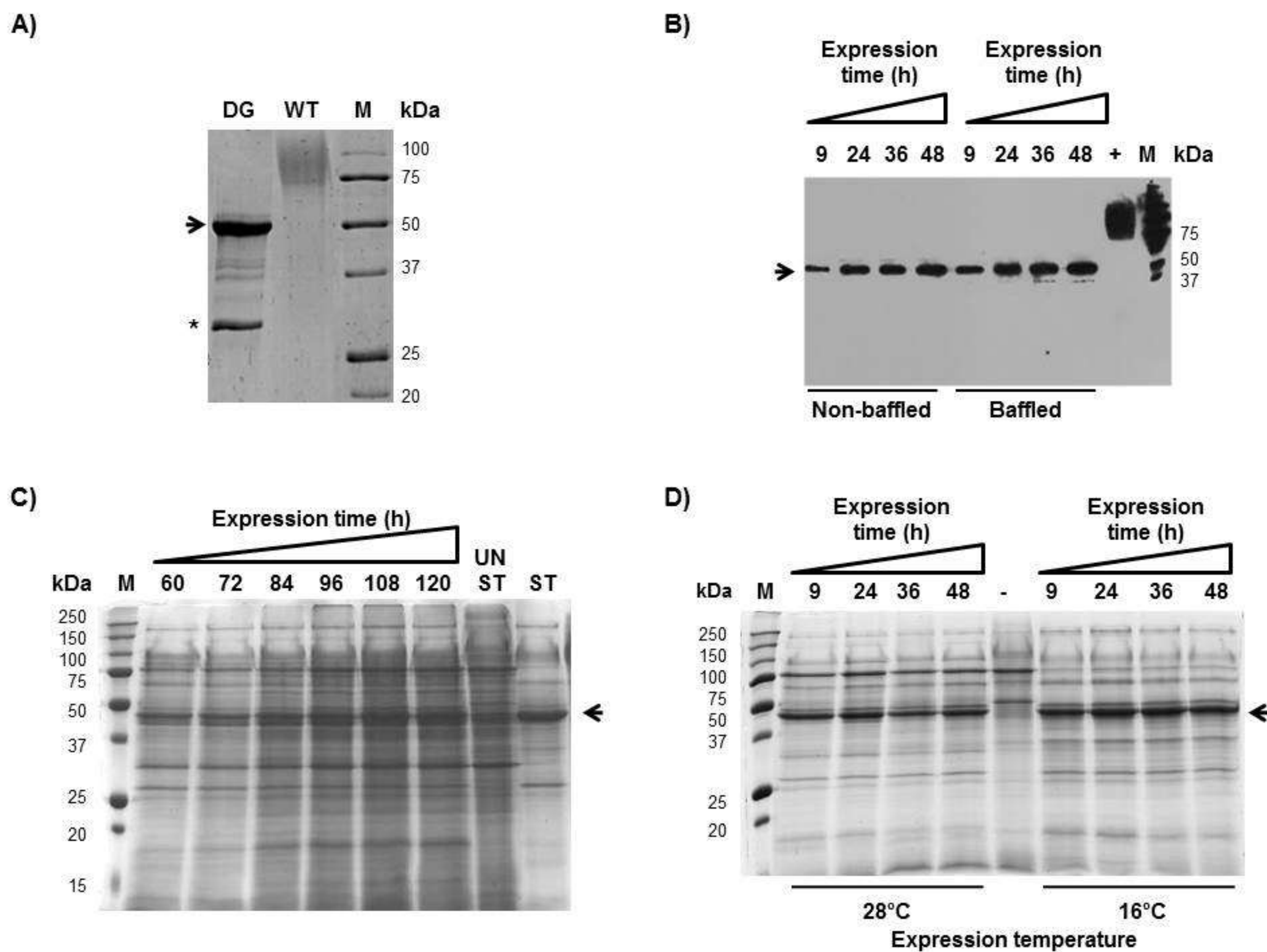
214 The activity of rSBLE, isolated from different productions was analyzed using an HPLC-based activity assay following
215 the protocol described in Ciesielska *et al* [6]. In brief, 2 µg of purified rSBLE was added to 500 µl of reaction buffer,
216 containing 5 mM of acidic diacetylated sophorolipids and 50 mM sodium citrate. The mixture was incubated for 1 hour
217 at 1400 rpm after which reaction was stopped using 500 µl 100% (v/v) EtOH. After concentrating the sample using a
218 SpeedVac vacuum centrifuge (Thermo Savant, Holbrook, NY) to 120 µl, the samples were analyzed using HPLC.
219

220 Results

221 When rSBLE is produced in *P. pastoris* as described previously [6] it displays a heterogeneous pattern on SDS-PAGE
222 analysis (Figure 2A) and on Western blotting (Figure 2B, right lane). In order to more accurately estimate rSBLE
223 quantity, samples were deglycosylated prior to SDS-PAGE analysis. rSBLE appears, after deglycosylation, as a

224 predominant band with an apparent molecular weight of approximately 50 kDa and a minor band was observed around
225 37 kDa (Figure 2AB).
226
227 **Influence of aeration, pH, percentage of methanol, temperature and expression duration on rSBLE**
228 **production.**
229 First, we evaluated whether aeration of the cultures has an impact on rSBLE expression. To this end we compared the
230 production yields obtained using Erlenmeyers with and without baffles. From these experiments we found that using
231 baffled flasks (Neubert-Glas, 0749-10-3000) led to a modest increase in rSBLE expression (Figure 2B).
232 Next, we tested whether altering the pH of the medium during induction would influence rSBLE expression compared
233 to a standard phosphate-buffered medium at pH 6.0. When inducing the cultures in a citrate buffer pH 3.0, we observed
234 a somewhat increased accumulation but this coincided with an increased degradation (Supplementary Figure 2). No
235 effect on rSBLE accumulation was observed when the induction was performed pH of 2.0, 4.0 and 5.0 (results not
236 shown). Based on these results, the standard phosphate buffer (pH 6.0) was maintained.
237 We also determined whether varying the methanol feed during induction could improve rSBLE induction compared to
238 the use of 1.0% methanol. However, altering methanol feed did not have any impact rSBLE yields (results not shown).
239 To determine the optimal induction time and to decipher whether rSBLE continues to accumulate in the culture
240 medium, we compared various extended induction times (60h, 72h, 84h, 96h, 108h and 120h). The gel patterns of
241 proteins obtained after DOC-TCA precipitation of the medium were compared to a previously obtained sample
242 collected after 48h induction time. Although over time, there is a small increase in intensity of the band corresponding
243 to rSBLE, we also observed this was also the case for several other proteins in the sample. Therefore, we concluded
244 that increasing induction time would probably also compromise protein purification. (Figure 2C). Consequently, the
245 48 hours induction time was maintained in future experiments.
246 Finally, we determined the effect of the temperature used during expression. To this end, we first grew the cells at 28°C
247 but then lowered the temperature to 16°C for induction. We also maintained a reference culture at 28°C during
248 induction. After SDS-PAGE analysis, we observed that performing the induction at 16°C resulted in a considerable

249 increase of rSBLE, compared to induction at 28°C (Figure 2D). Curiously, based on the band intensity it seems that
250 the rSBLE yield at the 9 hour timepoint is almost as good as after 48 hours of induction. Nevertheless, Western blotting
251 does display a difference (Figure 2B). This conflicting result could be explained by assuming a limited
252 sensitivity/dynamic range of Coomassie Brilliant Blue staining compared to immunoblotting.
253 Based on these experiments, we further performed protein productions in baffled shake flasks and the induction was
254 performed by feeding cultures with 1% methanol at pH 6.0 using a standard phosphate buffer at 16°C for 48 hours.



255
256
257
258 **Figure 2: Optimization of rSBLE production.** A) Coomassie Brilliant Blue stained SDS-PAGE of purified wild type rSBLE (WT) and deglycosylated
259 rSBLE (DG). The star indicates Endo H® B) Western blot analysis showing the influence of aeration on rSBLE-accumulation. rSBLE production in non-
260 baffled flask was compared to a production in baffled flask at different time points, indicated in hours after induction. Samples were deglycosylated prior to
261 analysis. '+' represents non deglycosylated rSBLE. C) Coomassie Brilliant Blue stained SDS-PAGE analysis to evaluate rSBLE production at prolonged
262 induction times (60-120 hours). The reference (ST) is rSBLE obtained after 48 h induction time, a sample obtained from an independent experiment and its
263 untreated, non-deglycosylated control (UN). D) Coomassie Brilliant Blue stained SDS-PAGE analysis to compare rSBLE production at different temperatures
264 (16°C and 28°C). Cells were harvested after 48 hours post-induction. "-" represents a control sample where rSBLE production was not induced by methanol
265 addition. In all pictures, 'M' stands for marker, molecular weight is shown in kDa. The black arrow indicates deglycosylated rSBLE.

266

Influence of expression temperature on rSBLE yield and retained contaminants during purification

We reported that during protein purification of rSBLE, two dominant contaminants co-eluted in during IMAC purification (phosphotyrosine phosphatase and phosphatidylinositol-4-kinase) [6]. These proteins were presented in a low molecular weight smear on an SDS-PAGE gel, together with degradation products of rSBLE. We investigated whether these contaminants were present after purification of samples evolved from our improved induction protocol (Figure 3). When comparing the SDS-PAGE patterns of the rSBLE containing fractions after IMAC purification (Figure 3A), we noticed that the samples obtained from cultures induced at 16°C contained much less low molecular weight contaminants or rSBLE degradation (the ‘smearing’ around 25 kDa) than the cultures induced at 28°C. The dramatic reduction of protein degradation or copurification of contaminants is also observed when comparing the size-exclusion chromatograms of these IMAC elution fractions (Figure 3B). Not only did we observe less contaminants, we also observed an approximate sixfold increase of rSBLE yield after purification, from 0.75 mg/L rSBLE to 4.7 mg/L as determined by UV spectroscopy.

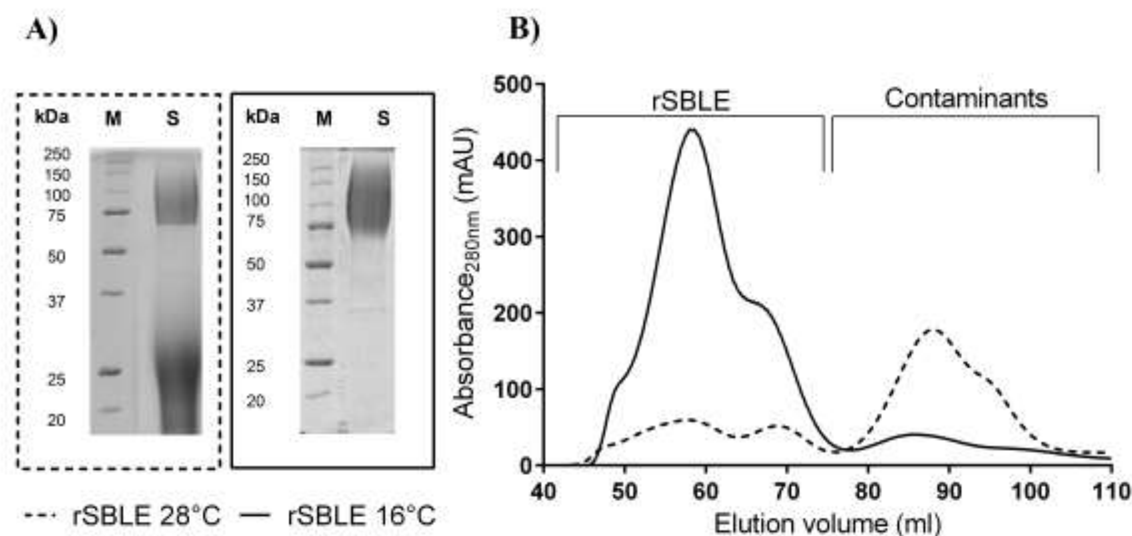
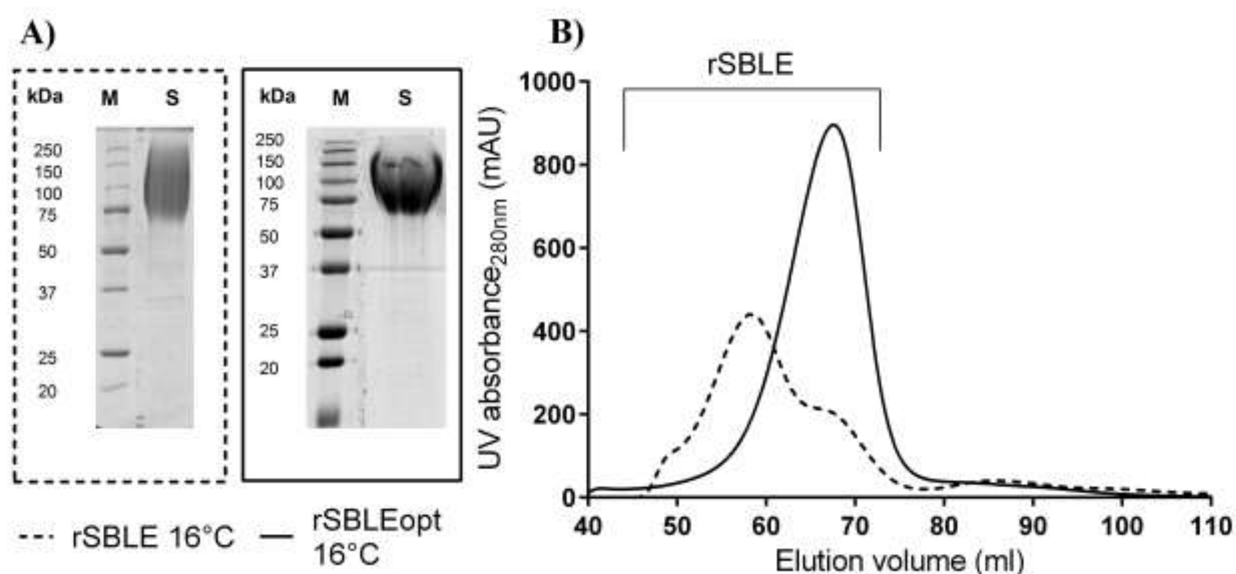


Figure 3: Comparison of rSBLE production expressed by the non-codon-optimized construct at 28°C and 16°C. A) Coomassie® Brilliant Blue stained SDS-PAGE patterns comparing the concentrated protein fractions (S) eluted from IMAC after imidazole elution. B) Size-exclusion chromatograms after loading the IMAC fraction from the original construct expressed at 16°C (solid line) and at 28°C (dashed line). The void volume, until 40 ml elution volume, is not shown. ‘Contaminants’ represent the co-eluting low molecular weight contaminants and possible rSBLE degradation products. M, molecular weight marker in kDa; mAU, milli absorbance units.

Evaluation of the codon-optimized rSBLE construct.

287 In parallel with the evaluation of the expression conditions described above, we produced a strain expressing a codon-
288 optimized rSBLE construct. We isolated the rSBLE produced by this strain (rSBLEopt) using the same purification
289 scheme, and compared the results with the product obtained using the previously described construct (Figure 4). We
290 achieved a 3-fold increase in yield for the rSBLEopt sample, as yields went from 4.7±0.5 mg/l to 13.7±1.5 mg/l (Figure
291 4A). In addition, size-exclusion chromatography displayed a single peak with the codon-optimized constructs. The
292 chromatogram of the non-codon-optimized construct showed a more heterogeneous pattern that we attributed to
293 difference in glycosylation. (Figure 4B, [6]). We tested whether this led to a difference in activity using a HPLC assay,
294 but this was not the case (Supplementary Figure 3).



295

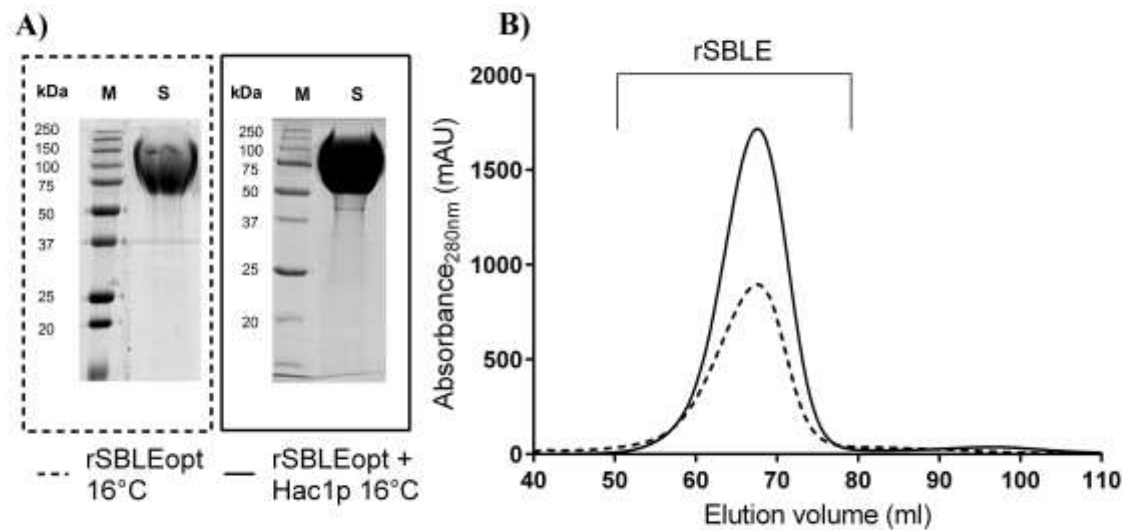
296 **Figure 4: Comparison of the expression of the original rSBLE construct (dashed line) and the codon-optimized rSBLE construct (solid line).** A)
297 Coomassie Brilliant Blue stained SDS-PAGE analysis of the fractions eluted from IMAC using imidazole (S, concentrated sample eluted from IMAC with
298 imidazole). B) Size-exclusion chromatography analysis of both concentrated IMAC fractions. The void volume, with the exception of 40 to 45 ml, was not
299 shown. The dashed line chromatogram represents the SEC profile of the expressed rSBLE from the original construct while the solid line chromatogram shows
300 expressed rSBLE from the codon-optimized construct. M, molecular weight marker in kDa; mAU, milli absorbance units; S, concentrated IMAC sample.

301

302 **The influence of co-expression of *HAC1* on codon-optimized rSBLE's yield.**

303 We finally tested the effect of co-expression with the transcriptional activator *HAC1* on rSBLEopt yield. Activity of
304 HAC1 in this strain was confirmed by verifying increase of production of Kar2p and Pdip, two proteins known to be
305 affected by HAC1 activity (Supplementary file and Supplementary Figure 4) Effectively, this led to a dramatic
306 improvement, i.e. the yield raised from 13.7±1.5 mg/l to 24.1±1.0 mg/l (Figure 5A). On the size-exclusion

307 chromatogram, the *HAC1* co-expressed rSBLEopt eluted again as a single peak, at the same retention volume (Figure 308 5B). rSBLE, whether it was co-expressed with *HAC1* or not, was tested for activity as was described in [6] but no 309 difference was observed (Supplementary Figure 3).



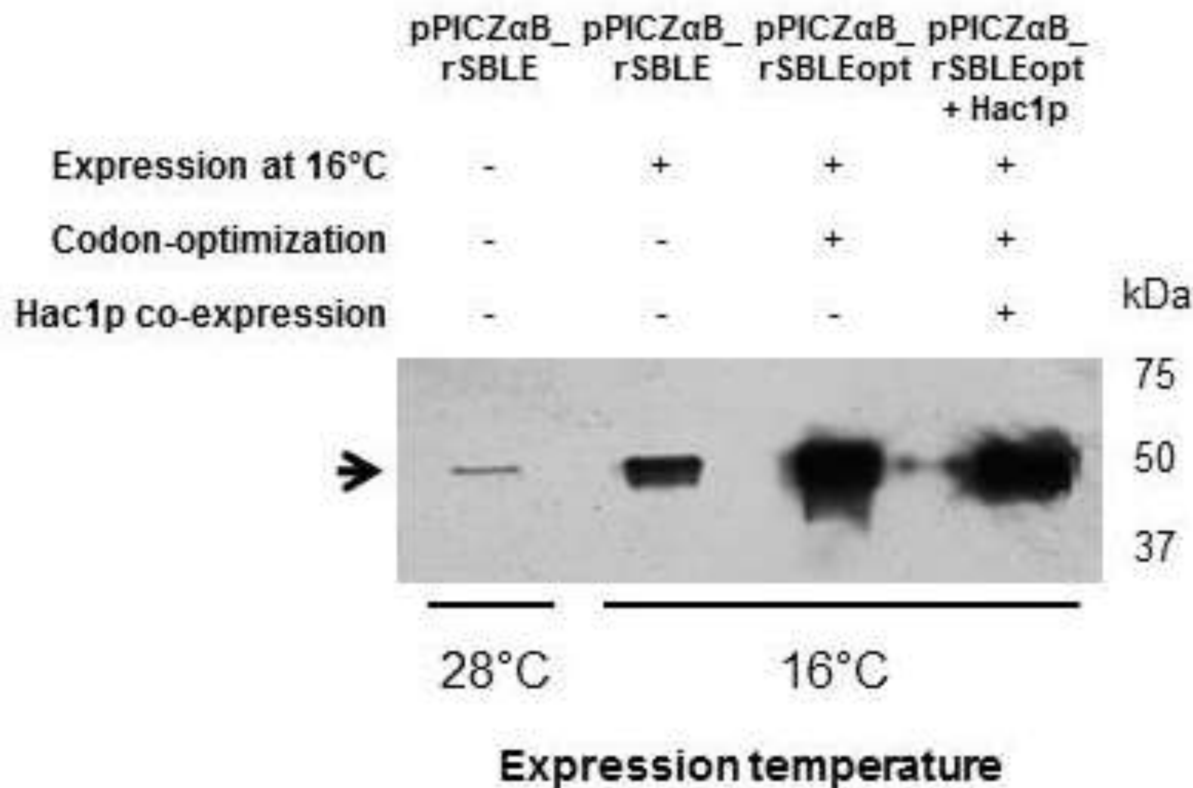
310

311 **Figure 5: Comparison of the expression of the codon-optimized rSBLE (dashed line) and the codon-optimized rSBLE (solid line) co-expressing *HAC1*.** 312 A) Coomassie Brilliant Blue stained SDS-PAGE analysis of both concentrated IMAC fractions (S, concentrated IMAC sample). B) The void volume, with 313 the exception of 40 to 45 ml, was not shown. The dashed line chromatogram was the SEC profile of the expressed rSBLE from the codon-optimized construct 314 while the solid line chromatogram represents codon-optimized rSBLE co-expressed with *HAC1*. M, molecular weight marker in kDa; mAU, milli absorbance 315 units; S, concentrated IMAC sample.

316

317 While comparing the different obtained yields, one could argue that a lower temperature and codon-optimization led 318 to a protein that can be more easily purified, and that improvement of yield is not necessarily caused by an increase of 319 expression. To test this possibility, the 3 different strains producing rSBLE, rSBLEopt and rSBLEopt co-expressed 320 with Hac1, were compared. After 48 hours induction, the obtained secreted proteins were collected using DOC-TCA 321 precipitation. Protein yield was verified by Western Blotting. The results of this analysis proved that improvement of 322 yield after purification is effectively the result of an increased protein production in the medium (Figure 6).

323



324

325 **Figure 6: Western blot analysis of different production strains.** Samples (1 ml of culture each) were precipitated using DOC-TCA precipitation. Bradford
326 analysis was used to normalize the protein concentrations after which an equal amount of total protein was loaded. The black arrow represents deglycosylated
327 rSBLE.

328

329 **Discussion**

330 In this study we aimed to optimize the expression conditions for rSBLE production in *P. pastoris* [5, 6]. It has been
331 previously reported that the flask design is a crucial parameter in the fermentation design[18]. Therefore we first studied
332 the effect of using a baffled shake flask on the expression of rSBLE. Western blotting showed an increase in band
333 intensity of rSBLE. Indeed, baffled shake flasks typically result in a higher amount of cells and improved aeration,
334 which can in turn lead to a higher protein production. It should be mentioned that the actual design of the baffled flasks
335 can also have an effect on the protein production[18], but we only tested the four-baffled shake flask.

336 Another approach to increase the expression of target proteins is to optimize the expression conditions. More precisely,
337 optimization of the cultivation temperature, methanol concentration, pH and the expression time can greatly increase
338 protein production[19]. In the case of rSBLE, neither the increase of the expression time nor a variation of the methanol
339 concentration influenced rSBLE expression, in contrast to different examples described in the literature[20]. While

340 expressing rSBLE at a pH of 3 led to an increase of band intensity, this coincided with an increase in degradation. This
341 could be explained by a decrease in stability of the protein when exposed to low pH. However, during sophorolipid
342 fermentation, pH typically drops to 3.5 which did not hinder SBLE's activity [6]. Therefore, it seems more likely that
343 the observed degradation is caused by proteolytic activity. *Pichia pastoris* is known to produce intracellular proteases
344 and unwanted proteolysis can be largely solved by protein production in *pep4* (SMD1168) mutant cells, devoid of the
345 major intracellular protease[21]. As a last parameter, we performed induction at 16°C instead of 28°C. By reducing the
346 induction temperature, we decrease the rate of protein synthesis, and give time for newly translated recombinant protein
347 to fold properly. According to other literature examples, lowering induction temperature does not only lead to a higher
348 expression, it can also lead to a more active protein [22] and an increase in cell viability[23, 24]. Indeed,
349 transcriptomic[25] and proteomic[26] analyses of *Pichia pastoris* recombinant protein expression at a lower
350 temperature showed a strong decrease in folding stress, noticed by the decrease of chaperones and other folding-related
351 proteins. Changing the expression temperature had the largest impact on production yield, raising it to 4.7±0.5 mg/l
352 culture.
353 Besides its low yield, the rSBLE production as described so far was also compromised by an overabundance of
354 contaminants which exhibit high affinity for the IMAC column. These contaminants were identified as intracellular
355 proteins with an apparent molecular weight of 15 and 30 kDa according to SDS-PAGE, and were identified as a
356 phosphotyrosine phosphatase (UniProt code: C4QXK8) and a fragment of phosphatidylinositol-4-kinase (UniProt
357 code: C4QV87). Additionally, tryptic peptides from rSBLE were also identified from SDS-PAGE bands cut at lower
358 molecular weight, indicating rSBLE degradation[6]. Interestingly, the identified contaminants were not yet described
359 in the literature[27]. We observed that expressing rSBLE at 16°C not only increases the protein yield but also reduces
360 the amount of such contaminants avoiding the need for additional clean-up steps.
361 Codon bias is another major barrier to obtain high protein yields. This codon bias occurs when foreign coding DNA is
362 significantly different from that of the host. As a consequence, during the synthesis of the recombinant protein,
363 depletion of low-abundance tRNAs can arise. This can lead to amino acid disincorporation or truncation of the
364 polypeptide, thus affecting the heterologous protein production levels[28]. When we compared the codon usage of *P.*

365 *pastoris* with the *SBLE* coding sequence using the online Graphical Codon Usage Analyzer tool
366 (http://gcua.schoedl.de)[29], we indeed noticed several rare codon usages in the original SBLE gene (Supplementary
367 files: Figure 5). These differences were most striking for arginine (CGC, CGG), glycine (GGC), leucine (CTT) and
368 valine (GTG). Therefore we cloned the codon-optimized construct into the vector of interest and observed a threefold
369 increase in yield compared to the original construct at an induction temperature of 16°C. The protein product from this
370 construct showed a more uniform peak in size-exclusion chromatography. Indeed, under non-optimized conditions, we
371 observed a double peak, and we assumed that the peak eluting first represented a dimeric species. Several lipases are
372 reported to form dimers [30-32]. Our current findings indicated that the first peak is probably the result of a partially
373 unfolded or more extensively glycosylated rSBLE.
374
375 Regarding the possible influence of biomass, variation in final optical density (OD_{600nm}) of the different producing
376 strains did not change drastically (Supplementary Figure 6). More precisely, the small variation in cell mass could
377 therefore not be responsible for the observed increases in yield. Indeed, Zhong et al. also observed that, during methanol
378 induction, there was no statistical influence of a lower temperature on the growth curve of recombinant *Pichia*
379 *pastoris*[23]. Although their experiment used an induction temperature of 20°C, it is remarkable that these results hold
380 true for 16°C as well.
381 As a last effort to increase rSBLE expression yield we also investigated the effect of *HAC1* on the expression of the
382 codon-optimized construct. Indeed, *HAC1* leads to transcriptional activation of endoplasmic-reticulum resident
383 chaperones or foldases, which are normally activated during the UPR pathway [14, 15, 33]. Unfolded protein is
384 typically produced during the high level expression of heterologous proteins, such as rSBLE, and therefore these
385 foldases will recover (partially) unfolded rSBLE, that would normally be degraded [15, 33]. This co-expression led to
386 an even higher increase of yield (Table 3). As of now, we have not yet reached expression levels such as 60 mg/liter
387 for the *Geotrichum candidum* lipases[34] or, an even higher yield, *Rhizomucor miehei* lipase (220 mg/liter)[35]. The
388 current lab-scale yield is acceptable for the coming crystallization trials and valorization of this enzyme. In future
389 experiments we will move our expression system to a bioreactor, in order to obtain an even greater increase in yield[36].

390

391 **Table 3: Total overview of the different yields during this study.**

Expression of:	**pPICZαB_rSBLE at 28°C**	**pPICZαB_rSBLE at 16°C**	**pPICZαB_rSBLEopt at 16°C**	**pPICZαB_rSBLEopt + pPICHyg_HAC1spliced at 16°C**
Yield (mg/l):	0.75±0.05	4.7±0.5	13.7±1.5	24±1.0
Improvement	/	Compared to 28°C: approximately sixfold	Compared to non-codon-optimized: approximately threefold	Compared to no co-expression: approximately twofold

392
393 This table represents the total yield ± standard deviation (average of at least 2 technical repeats each). Equal production volumes were compared, as the
394 optical density was not significantly different. After IMAC and size-exclusion purification, the corresponding peak(s) were concentrated to a volume of 1.0
395 ml and the concentration of the corresponding faction was measured using NanoDrop®.

396

397 A remaining bottleneck for crystallization and batch-to-batch reproducibility in protein production is the inherent
398 heterogeneous glycosylation of rSBLE. In previous studies we were able to fully deglycosylate rSBLE *in vitro* using
399 Endo H® without denaturation. Therefore to pave the way for crystallization, the easiest approach to solve this problem
400 would be to perform *in vitro* deglycosylation with Endo H® on the native protein. To counter the batch-to-batch
401 reproducibility in protein production, we also explored co-expression with Endo-N-acetyl-beta-D-glucosaminidase T
402 (EndoT)[37] but this decreased rSBLE expression (results not shown). Although we did not pursue any optimization,
403 a possible explanation could be that complete deglycosylation favors proteolytic degradation or aggregation[38] of
404 rSBLE, therefore, further experiments are needed to assess this. Alternatively, other glyco-engineered strains could be
405 used to express rSBLE[39], for example, using a *P. pastoris* strain, deficient in OCH1p. Although, this strain delivers
406 a more homogeneous N-glycosylation pattern, consisting mainly of $Man_8GlcNAc_2$, the yield from such a strain is
407 compromised [40, 41]. Another approach could be to co-express rSBLE with a different Endo-N-acetyl-beta-D-
408 glucosaminidase, such as Endo H.

409

To conclude, our study solved the two main bottlenecks of rSBLE production in *P. pastoris* and cleared the path for structural and functional studies to understand rSBLE's illustrious mechanism and, eventually, rSBLE's valorization.

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Compliance with ethical standards

All authors have approved the final version of this article

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Conflict of interest

IVB, KC, BD, and WS have been granted a patent (US9394559 B2) on the enzyme and its use. The other authors declare that they have no conflict of interest.

Ethical approval

This article did not require any studies with human participants or animals by any of the authors.

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