Secretion of *Pseudomonas aeruginosa* Lipoxygenase by *Pichia pastoris* upon Glycerol Feed

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**Pseudomonas aeruginosa** lipoxygenase (PaLOX) catalyzes the peroxidation of unsaturated fatty acids. Not only linoleic acid, but also linolenic acid and oleic acid are oxidized. The natural host secretes PaLOX into the periplasmic space. Herein, the aim is to secrete PaLOX to the culture supernatant of *Pichia pastoris*. Since protein background in the culture supernatant is typically rather low, this strategy allows for almost pure production of PaLOX applicable for the valorization of renewable fatty acids, for example for the production of green leaf volatiles. Using the CAT1 promoter system and the well-established α-factor signal sequence for secretion, methanol- and glycerol-induced secretion are compared and the latter shows four times more LOX activity in the culture supernatant under methanol-free conditions. In addition, secreted PaLOX is purified and the specific activity with enzyme in culture supernatant is compared. Notably, the predominant specific activity is achieved for enzyme in culture supernatant - 11.6 U mg⁻¹ - reaching five times higher specific activity than purified PaLOX.

1. Introduction

Lipoxygenases (LOXs; EC 1.13.11.X) are non-heme iron or manganese containing enzymes, which are widely abundant in animals, plants, fungi as well as bacteria.[1,2] LOXs catalyze the peroxidation of (poly)unsaturated fatty acids (PUFA), both regio- and stereospecifically, forming hydroperoxides (HPOs), that is, in case of 13-LOX, 13-hydroperoxy-9,11-octadecadienoic acid (13-HPOD), and 13-hydroperoxy-9,11,15-octadecatrienoic acid (13-HPOT) from linoleic and linolenic acid, respectively.

Among the most extensively studied LOXs are the mammalian enzymes. These are responsible for the biosynthesis of lipid mediators, which are involved in a multitude of metabolic responses, for example, in inflammation or cell development.[3-5] Plant LOXs are crucial for various defense mechanisms, like wound healing, plant–plant communication, response against microbial pathogens, and also protection against herbivorous animals and insects. Due to the production of secondary metabolites like jasmonates, green leaf volatiles, or phyto-oxylipins, plants are able to counteract when distressed.[6-11] In contrast to LOXs originating from animals and plants, research on microbial LOXs have been somewhat neglected in the past. In many fungal species like oomycetes, basidiomycetes, and yeasts, different types of LOXs have been described. Furthermore, some fungal LOXs, like *Fusarium oxysporum* LOX (FoxLOX), utilize manganese in their catalytic site, in contrast to the major class of iron-dependent LOXs (Wennman et al. 2015; Stolterfoht et al. 2019).[12,13] One of the most recently studied group of LOXs are those derived from bacteria. Since most bacteria do not possess the requirements for generating PUFA's, their biological relevance has yet to be clarified.[14] LOXs expressed in *Escherichia coli* highlight the importance of this enzyme class for the application in the functionalization of fatty acids. In 2018, An et al. demonstrated the conversion of PUFA's into eight types of hepxyoxipins and trioxylins, metabolites for mammalian enzymes, which might aid in the development of drugs based on lipid mediators.[15] Another study also used microbial LOX to convert PUFA's into plant oxylipins, which are involved in plant pathogen specific defense mechanisms against fungal infections.[16]

The LOX from *Pseudomonas aeruginosa* (PaLOX)[17,18] is a well-studied bacterial lipoxygenase. Numerous biological roles were proposed in *P. aeruginosa*, among others PaLOX may contribute to i) adjustment of lipid signaling among pathogen and host via biofilm production, ii) the assembly of anti-inflammatory lipoxins to aid with the down regulation of the host's immune response, and iii) oxidation of membrane lipids, thus acting as virulence factors while infecting the host.[19-23]
PaLOX is located in the periplasm of the gram-negative bacterium P. aeruginosa and has a molecular mass of 45 kDa in its monomeric form. The enzyme has considerable activity at 55 °C, belonging to a rather small group of resilient enzymes. Notably, its most captivating property is perhaps its substrate spectrum. In addition to linoleic- and linolenic acid, PaLOX also peroxidises oleic acid and arachidonic acid. PaLOX has been successfully expressed in the prokaryotic host E. coli. Interestingly, and in contrast to its native host, heterologous expression in E. coli resulted in a protein with significantly higher molecular mass (70 kDa vs 45 kDa). Despite this fact, both variants showed similar catalytic properties. The lower molecular mass in the native host compared to the recombinant protein suggests proteolytic truncation that is prevalent in P. aeruginosa but not in E. coli.\(^{[24]}\)

Further studies on PaLOX expressed in E. coli focused on the characterization of the HPO products. By forming relatively high amounts of 13(S)-HPOD, recombinant PaLOX was categorized as a 13(S)-LOX, when using linoleic acid as the substrate. Specific activity of heterologous PaLOX in crude extract was 2.1 U mg\(^{-1}\). After extensive precipitation, purification, and filtration steps a specific activity of 28.3 U mg\(^{-1}\) was determined.\(^{[25]}\)

The eukaryotic expression host Pichia pastoris, aka Koma-gataella phaffii,\(^{[26]}\) has the potential to implement complex post-translational modifications. In addition, this host system is rather easy to manipulate and cultivation is both, simple and fast. Furthermore, secretion of the recombinant protein into the culture supernatant, facilitating down-stream processing tremendously.\(^{[27]}\) On top of that, P. pastoris can rapidly grow to very high cell densities, allowing large-scale production for industrial applications.\(^{[30,31]}\)

The most common promoter recommended for P. pastoris is the methanol-inducible alcohol oxidase 1 promoter (P\(_{\text{AOX}}\)). However, in regard to safety the use of methanol in large-scale applications is considered difficult.\(^{[32–34]}\) Consequently, methanol-free alternative inducers of protein expression are topping the wishlists in industry. Fischer et al. (2019) discovered a novel promoter system, that is, the promoter of the P. pastoris CAT1 gene.\(^{[35]}\) Using this system, a methanol-induced, but also a methanol-free expression approach can be established, providing flexibility in terms of application. The CAT1 promoter is repressed in the presence of glucose or glycerol, and de-repressed upon depletion of these carbon sources.\(^{[35]}\) Here, we report PaLOX production in P. pastoris for the first time. As secreted protein, down-stream processes and analysis of the enzyme are tremendously simplified.

2. Results

2.1. Activity Assay of αPaLOX

P. pastoris CBS7435 Mut\(^5\) was transformed with 3 μg of linearized plasmid DNA harboring the PaLOX expression cassette. To analyze expression levels, eight clones were picked and cultivated in small (0.5 mL) and large scale (50 mL, data not shown) using glycerol or methanol for induction. Assays were carried out with P. pastoris culture supernatant containing αPaLOX, that is, PaLOX secreted upon a-mating factor signal sequence fusion. The transformant which showed the highest activity on the substrate linoleic acid was investigated in more detail.

Surprisingly, αPaLOX containing supernatants from methanol-induced samples showed significantly less activity than glycerol-induced samples. While samples induced with methanol reached 0.003 AU min\(^{-1}\), samples induced with glycerol reached a fourfold higher value of 0.012 AU min\(^{-1}\) (Figure 1).

After confirming LOX activity, SDS-PAGE analysis of methanol-chloroform precipitated culture supernatants was performed. Samples were concentrated \(\approx\)30 times. For comparison, we produced and purified PaLOX without the α-factor secretion signal (methanol induction) (Figure 2, lane 3) and also secreted αFoxLOX (Figure 2, lane 2) in P. pastoris. SDS-PAGE analysis revealed a 70 kDa secreted αPaLOX (glycerol) band (Figure 2, lane 4) showing a marginally higher molecular weight.

Figure 1. LOX activity of αPaLOX for peroxidation of linoleic acid. Formation of hydroperoxide with enzyme in culture supernatant is observed after 72 h of cultivation, with either glycerol or methanol induction. In addition, methanol-induced samples were concentrated 5X via Vivaspin ultrafiltration columns. EVC, empty vector control—negative control.
controls, EVC as negative control. The secreted enzyme was furthermore visualized by SDS-PAGE (Figure 2) which revealed a band at \( \approx 70 \text{ kDa} \) for the methanol but not for the glycerol-induced samples or the empty vector control. In comparison, methanol-induced cell-free extract showed an \( \alpha \text{PasLOX} \) signal at the same height as secreted \( \alpha \text{PasLOX} \) (glycerol), supporting our hypothesis of a partially unprocessed signal sequence of \( \alpha \text{PasLOX} \) (glycerol).

### 2.2. Determination of the Enzyme Concentration/Specific Activity

In order to determine the specific activity of \( \alpha \text{PasLOX} \). 3 L of culture supernatant were concentrated via crossflow (CF, 10 kDa MWCO) to \( \approx 240 \text{ mL} \), resulting in 12.5-fold concentration. All of the concentrated culture supernatant (cSN 12.5xconc) was then purified by immobilized metal-affinity chromatography (on Ni-Sepharose). After desalting of the protein (3.5 mL), activities of all samples were measured spectrophotometrically (Figure 3). Surprisingly, the flow through of the IMAC-purification column (FT IMAC) showed almost the same activity as the 12.5-fold concentrated culture supernatant prior to loading it onto the purification column, indicating a low binding affinity of His-tagged \( \alpha \text{PasLOX} \). Nevertheless, a small portion of purified \( \alpha \text{PasLOX} \) was obtained and activity for linoleic acid was confirmed. However, the purified enzyme only showed a 2.5-fold increase in activity, compared to the concentrated culture supernatant (\( \alpha \text{PasLOX}_\text{cSN} 12.5\text{xconc} \)). Considering the effort of purification, working with concentrated culture supernatants is unambiguously favored here.

To calculate yield, titre and specific activity of the recombinant enzyme in the culture supernatant, total protein concentrations were determined for secreted \( \alpha \text{PasLOX} \) and the respective EVC. Total protein concentrations ranged from 0.293 to 0.460 \( \mu \text{g mL}^{-1} \) for culture supernatant of \( \alpha \text{PasLOX} \) secretion strains, induced with glycerol and methanol, respectively. A lower value of 0.167 to 0.255 \( \mu \text{g mL}^{-1} \) was determined for the empty vector control (glycerol/methanol-induced) samples (Table 1). In contrast, the methanol-induced samples showed much more total protein than glycerol-induced samples, which is again in agreement to the results in Figure 3.

Being aware of a relatively high endogenous protein background in the precipitated culture supernatants, probably due to suboptimal process conditions and consequently higher cell lysis, (Figure 2 and high protein content of EVC), the \( \alpha \text{PasLOX} \) (glycerol) content in culture supernatant was estimated by SDS-PAGE in comparison to bovine serum albumin (BSA) (Figure 4). Approximately 1–1.5 \( \mu \text{g} \) of protein per milliliter were estimated for secreted \( \alpha \text{PasLOX} \) (glycerol). Protein concentration of IMAC-purified enzyme was determined by NanoDrop and BCA-assay. Calculation of the protein concentration resulted in a concentration of 0.37 mg mL\(^{-1}\).

The secreted enzyme was furthermore visualized by SDS-PAGE and Western Blot at \( \approx 70 \text{ kDa} \) for both, the IMAC-purified and methanol-chloroform precipitated samples. Again, \( P. \text{pastoris} \) secreted \( \alpha \text{FoxLOX} \) was used as a positive control (Figure 5). Western Blot analysis was performed in order to check the presence and integrity of the His-tag on \( \alpha \text{PasLOX} \). Interestingly, purified \( \alpha \text{PasLOX} \) showed two bands, of which the higher, fainter band matched the size of the highly active, unpurified secreted

![Figure 2. Expression analysis of \( \alpha \text{PasLOX} \) culture supernatant (cSN) and cell-free extracts (lysate), induced with either glycerol or methanol. LOX expression was analyzed after 72 h cultivation and methanol/chloroform precipitation or Y-PER treatment of culture supernatant and cell pellet, respectively. Arrows indicate \( \alpha \text{PasLOX} \) and \( \alpha \text{PasLOX} \) bands. Secreted \( F. \text{oxypropum} \) \( (\alpha \text{FoxLOX}) \) culture supernatant and \( \alpha \text{PasLOX} \) were applied as positive controls, EVC as a negative control.](image-url)

![Figure 3. Determination of the enzyme concentration/Specific activity](image-url)
Figure 3. Volumetric activity of \(\alpha PaLOX\) with linoleic acid. After 72 h of cultivation and induction with glycerol, different fractions were analyzed at 234 nm for 30 min to detect formation of hydroperoxide. cSN, culture supernatant; cSN 12.5x conc, 12.5-fold concentrated culture supernatant; FT IMAC, flow through of IMAC-purification; total volumes of the respective fractions are indicated on top of bars in the diagram.

Table 1. Total protein concentrations of \(\alpha PaLOX\) and EVC (glycerol/methanol) in culture supernatant (cSN).

| Induction | Total protein concentration [\(\mu g \mu L^{-1}\)] |
|-----------|-----------------------------------------------|
| Glycerol  | \(\alpha PaLOX\) cSN 0.293 EVC 0.167          |
| Methanol  | \(\alpha PaLOX\) cSN 0.460 EVC 0.255          |

\(\alpha PaLOX\) (glycerol). However, the lower size second band of the purified sample, did give a distinct Western Blot signal. This might be the same fraction, with lower or no activity, as shown for the methanol-induced secreted enzyme or intracellularly expressed \(PaLOX\) (Figure 2). Apparently, this lower size/activity fraction is also present in the culture supernatant of glycerol-induced samples, but much less abundant than the higher activity fraction (Figure 4, lane 3).

Finally, a yield and titre of 0.019 mg g\(^{-1}\) and 1.25 mg L\(^{-1}\), respectively, were calculated for the enzyme in culture supernatant. This corresponds to a high specific activity of 11.6 U mg\(^{-1}\). In contrast, yield and titre calculated for IMAC-purified \(PaLOX\) were much lower (specific activity of 2 U mg\(^{-1}\), Table 2) and in the same range as reported for \(PaLOX\) expressed in \(E. coli\).[25] This might be due to the fact that only a smaller portion of the high size/activity fraction of \(\alpha PaLOX\), with parts of the \(\alpha\)-factor signal peptide attached, was purified. The smaller size/low activity fraction, without \(\alpha\)-factor signal peptide, was enriched after IMAC. The unprocessed parts of the \(\alpha\)-factor signal peptide might have caused decreased accessibility of the His-tag, explaining the high level of total activity remaining in the IMAC flow through. Compared to the IMAC-purified enzyme, the secreted \(\alpha PaLOX\) in culture supernatant seems to have more than five times higher specific activity, making purification obsolete for this enzyme. In summary, we showcased the benefits of using untreated \(\alpha PaLOX\)-containing culture supernatant for HPOD formation.
Figure 5. Detection of IMAC-purified \( \alpha \text{PaLOX} \) and \( \alpha \text{PaLOX} \) in culture supernatant (cSN). A) SDS-PAGE of IMAC-purified \( \alpha \text{PaLOX} \) and enzyme in culture supernatant (cSN). Arrows indicate \( \alpha \text{PaLOX} \) (partially unprocessed secretion signal) and PaLOX bands. B) Western Blot detection after 72 h of cultivations. \( \alpha \text{PaLOX} \) was detected at \( \approx 70 \) kDa. Secreted \( F. \text{oxy}sporum \) (\( \alpha \text{FoxLOX} \)) culture supernatant was used as positive control; EVC, empty vector control.

Table 2. Yield, titre, and specific activity of \( \alpha \text{PaLOX} \) from culture supernatant (cSN) and IMAC-purification.

|                      | \( \alpha \text{PaLOX} \) (cSN) | IMAC \( \alpha \text{PaLOX} \) |
|----------------------|---------------------------------|-------------------------------|
| Yield [mg protein g\(^{-1}\) biomass] | 0.019                           | 0.001                         |
| Titre [mg L\(^{-1}\) medium]            | 1.25                             | 0.43                          |
| Specific activity [U mg\(^{-1}\)]       | 11.6                             | 2.08                          |

3. Discussion

In this study, we present for the first time secretion of \( P. \text{aerugino} \)osa lipoygenase in \( P. \text{pastoris} \). To date, heterologous expression of PaLOX was only shown for \( E. \text{coli} \) for which some difficulties are reported in the expression of PaLOX, for example, extensive formation of inclusion bodies and exhaustive enzyme filtration and purification steps.\(^{[25]}\) Secretion of \( \alpha \text{PaLOX} \) into the culture supernatant could alleviate these obstacles.

Considering the difference in apparent mass, it seems possible that \( \alpha \text{PaLOX} \) (glycerol) polypeptides still possess some parts of the \( \alpha \)-factor secretion signal, while samples induced with methanol do not show any indication of an unprocessed signal sequence. The \( \alpha \)-factor signal consists of a 19 amino acid pre-peptide which is followed by a 66 amino acid pro-sequence.\(^{[16]}\) While the \( \alpha \)-factor signal peptide is processed in the endoplasmic reticulum, the pro-sequence might still be associated with the secreted protein. This pro-region has been described to act as a molecular chaperone for secreted proteins by promoting folding, probably slowing down rapid folding and therefore, stabilizing the enzyme.\(^{[37,38]}\) This might also be the case for our secretory \( \alpha \text{PaLOX} \) (glycerol), which shows higher molecular weight and significantly higher activity compared to the methanol-induced samples or the intracellular reference. While processing of the complete secretion factor may not have occurred, it might influence the stability and activity of the enzyme in the culture supernatant. Notably, protein purification of glycerol induced culture supernatant via the attached His-tag did lead to a decrease in enzyme activity as compared to the crude culture supernatant. We attribute this observation to the presence of \( \alpha \)-mating factor signal sequence-linked \( \alpha \text{PaLOX} \) in the culture supernatant and a selective depletion thereof when purifying \( \alpha \text{PaLOX} \). This theory is backed up by the detected signals of proteins with different sizes in the Western Blot, the SDS-PAGE of retained \( \alpha \text{PaLOX} \) from cell-free extracts of methanol induced samples and furthermore, by the remaining activity in the flow through of IMAC, showing almost the same total activity as unpurified enzyme in culture supernatant.
By using *P. pastoris* we provided excellent groundwork for the use of a PaLOX in industrial biotechnology. The *P. pastoris* system in secretion mode facilitates simple downstream processing and suggests the use of a PaLOX in the culture supernatant matrix. Moreover, specific activity of a PaLOX in *P. pastoris* culture supernatant was shown to be five times higher than crude extract of PaLOX expressed in *E. coli*. This was facilitated by the CAT1 promoter system, which allows methanol-free and methanol-induced expression. In contrast, other well-established methanol based promoter systems require a two-step cultivation protocol, which demands higher process times and more laborious handling. The use of methanol is considered hazardous (toxic and flammable) and in addition, removal of methanol contaminated cells can be problematic for a lot of branches. This is one rare example in which glycerol as a sole carbon source (an inexpensive and safe compound) clearly outcompetes methanol in terms of activity yields. Methanol-free cultivation of *P. pastoris* might be more than an alternative, here. Based on these results and further research in this field, a PaLOX secreted by *P. pastoris* seems a valuable catalyst for future applications in industrial biocatalysis.

### 4. Experimental Section

**Vector and Strain Construction:** For PaLOX expression the vector pBSY3S1Z (bisy GmbH) was used. A codon-harmonized gene variant (MT039000) of PaLOX (NP_249860.1; umodified gene) was chosen, designed manually by applying the *P. pastoris* codon usage (https://www.kazusa.or.jp/codon/), and cloned behind the coding sequence of the α-factor signal sequence to secrete the enzyme into the culture supernatant of *P. pastoris*. Strain deposition numbers are #8009 and #8010 of IMBT, Graz University of Technology, strain collection, for secreted and intracellular PaLOX, respectively. Antibiotic selection was based on a Zeocin resistance gene (ZeoR) and the N-terminal His-tag was used for detection and purification.

*P. pastoris* strain CBS7435 WT Mut5 was used as host strain. This strain has a dysfunctional alcohol oxidase 1 (AOX1) gene and shows a Mut5, methanol utilizing slow, phenotype. Expression vectors were linearized with SmI for integration of the expression cassette into the genome of *P. pastoris*. Routinely, electrocompetent *P. pastoris* cells were transformed with linearized plasmid and colonies were selected and cultivated according to Cregg et al. (2009).

**Cultivation:** Cultivation of recombinant *P. pastoris* CBS7435 Mut5 was done in both, 50 mL of BMGY and BMMY according to Fischer et al. (2019). Main cultures were induced for 72 h by addition of 0.25% glycerol every 24 h for BMGY and 1% methanol every 12 h for BMMY cultivations. Cells were cultivated at 28 °C and 130 rpm in 300 mL wide-necked, baffled shake flasks covered with two layers of cotton cloth. Culture supernatant was harvested by centrifugation at 4000 × g and 4 °C for 10 min. For intracellular PaLOX samples, 100 mg of wet cell pellets were treated according to the Y-PER yeast protein extraction reagent manual.

**Sample Preparation:** In order to obtain higher enzyme concentrations, culture supernatants and cell-free extracts were concentrated using Viivapin ultrafiltration columns (Sartorius) or crossflow filtration (Pall Corporation), with a 10 kDa molecular weight cut-off. Proteins were purified by nickel affinity chromatography using the gravity flow protocol for Ni-Sepharose 6 Fast Flow columns by GE Healthcare. The column was equilibrated with 10 column volumes (CV) of binding buffer (50 mM NaH2PO4, pH 7.0, 300 mM NaCl, and 10 mM imidazole). Approximately 240 mL of concentrated aPaLOX culture supernatant from glycerol-induced cultivation, were added to the column. The column was washed with binding buffer and washing buffer containing 30 mM imidazol and eluted with elution buffer containing 300 mM imidazole. Fractions that contained protein were pooled and concentrated to a volume of n=2.5 mL. Buffer exchange was performed via size exclusion chromatography using PD-10 columns (GE Healthcare). This column had been equilibrated with 100 mM of sodium-phosphate buffer, pH 7.4. Fractions were collected and stored on ice before further analysis.

aPaLOX was concentrated by methanol-chloroform precipitation. Approximately 840 μL of aPaLOX culture supernatant were combined with 480 μL of methanol and 160 μL of chloroform. Tubes were centrifuged at 12 200 rpm for 5 min and the aqueous phase on top was carefully removed. To the precipitated protein on the interphase, 300 μL of methanol were added, and the samples were vortexed and centrifuged at 12 200 rpm for 30 min and 4 °C. The supernatant was fully removed, and the pellet was dried for ≤45 min at room temperature.

To determine protein concentration of purified sample and culture supernatants, NanoDrop 2000C Spectrophotometer (Thermo Fisher Scientific) and Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) were used according to the manufacturer’s manuals.

Proteins were visualised by SDS-PAGE and Western blotting. Pellets of precipitated protein or purified protein samples were denatured by addition of SDS sample buffer (NuPage LDS Sample Buffer (4x) and NuPage Sample Reducing Agent). After incubation at 70 °C for 10 min, 4 μL of PageRuler Prestained Protein Standard and 30 μL of sample were loaded onto NuPage 4–12% Bis-Tris Protein Gels (125 V and 30 mA for ≥50 min). Gels were stained with Coomassie-Blue.

Proteins were transferred from SDS-PAGE gels to nitrocellulose membranes (Amersham Hybond ECL) using transfer buffer (25 mm Tris, 190 mm glycine, 19% methanol) for 1 h and 15 min at 230 mA. The membrane was blocked with a TBST milk solution (5% skim milk powder) overnight at 4 °C. On the next day, the membrane was incubated with the primary antibody—a monoclonal anti-poly-Histidine antibody produced in mouse (H1029 – Sigma-Aldrich, 1:2000 diluted in TBST milk)—for 1 h. Subsequently, the membrane was washed and incubated with the secondary antibody—a polyclonal anti-mouse IgG-peroxidase antibody (A4416 – Sigma-Aldrich, 1:5000 diluted in TBST milk)—overnight at 4 °C. On the next day, the membrane was incubated with the secondary antibody—a polyclonal anti-mouse IgG-peroxidase antibody (A4416 – Sigma-Aldrich, 1:5000 diluted in TBST milk), linked to horseradish peroxidase enzyme (HRP)—for 1 h.

For detection, SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific), and luminol-based enhanced chemiluminescent (ECL) horseradish peroxidase (HRP) substrate were used.

**Activity Assay:** Lipoxygenase activity was determined spectrophotometrically by monitoring the increase in the absorbance at 234 nm based on the transformation of linoleic acid to the respective conjugated hydroperoxidey[46–48]. The assays were performed in 96-well UV-Star microtiter plates containing 175 μL McIlvaine citrate-phosphate buffer pH 7.0 (0.1 M citric acid and 0.2 M Na2HPO4) and 5 μL enzyme solution per well. The reaction was initiated by adding 40 μL of freshly prepared 2 mM linoleic acid solution. This substrate solution was prepared by mixing 20 μL (18 mg) linoleic acid with 30 μL (33 mg) Tween 20, 100 μL of 1 M NaOH and distilled water, first in 2 mL until the solution was clarified, and then diluted with water to 32 mL. Blank reactions without substrate and without enzyme were carried out in parallel. Absorbance at 234 nm was recorded every 30 s and monitored for 30 min using a plate reader (Synergy Mx, BioTek) at 30 °C. All experiments were performed in quadruplicates.

**Acknowledgements**

acb: Next Generation Bioproduction is funded by BMVIT, BMDW, SFG, Standortagentur Tirol, Government of Lower Austria, and Vienna Business Agency in the framework of COMET (Competence Centers for Excellent Technologies). The COMET Funding Program is managed by the Austrian Research Promotion Agency FFG. This work was also supported by the Austrian Science Fund (FWF), no. 860262.

**Conflict of Interest**

The authors declare no conflict of interest.
Author Contributions

H.P. developed the research idea. H.S. and C.R. conceived and designed expression constructs. S.S. generated constructs. C.H. conducted experiments and analyzed data. M.W. acquired funding and managed the project. M.W. and H.P. supervised the project. C.H. wrote the manuscript. All authors discussed the results and contributed to the final manuscript.

Keywords

lipoygenase, methanol-free protein expression, Pichia pastoris, protein secretion, Pseudomonas aeruginosa

Received: April 30, 2020
Revised: July 7, 2020
Published online: October 7, 2020

[1] Y.-C. Joo, D.-K. Oh, Biotechnol. Adv. 2012, 30, 1524.
[2] R. Heshof, L. H. de Graaff, J. J. Villaverde, A. J. D. Silvestre, T. Haarman, T. K. Dalsgaard, J. Buchert, Crit. Rev. Biotechnol. 2016, 36, 665.
[3] J. Z. Haeggström, C. D. Funk, Chem. Rev. 2011, 111, 5866.
[4] A. R. Brash, J. Biol. Chem. 1999, 274, 23679.
[5] H. Kuhn, S. Banthiya, K. Van Leyen, Biochim. Biophys. Acta – Mol. Cell Biol. Lipids 2015, 1851, 308.
[6] M. N. ul Hassan, Z. Zainal, I. Ismail, Plant Biotechnol. J. 2015, 13, 727.
[7] I. T. Baldwin, R. Halitschke, A. Paschold, C. C. Von Dahl, C. A. Preston, Science 2006, 311, 812.
[8] C. Westernack, B. Hause, Ann. Bot. 2013, 111, 1021.
[9] E. Blee, Trends Plant Sci. 2002, 7, 315.
[10] C. Westernack, Ann. Bot. 2007, 100, 681.
[11] N. J. Bate, S. J. Rothstein, Plant J. 1998, 16, 561.
[12] A. Wennman, A. Magnuson, M. Hamberg, E. H. Oliw, J. Lipid Res. 2015, 56, 1606.
[13] H. Stolterfoht, C. Rinnofer, M. Winkler, H. Pichler, J. Agric. Food Chem. 2019, 67, 13367.
[14] A. Garreta, S. P. Val-Moraes, Q. García-Fernández, M. Busquets, C. Juan, A. Oliver, A. Ortiz, B. J. Gaffney, I. Fita, A. Manresa, X. Carpena, FASEBJ. 2013, 27, 4811.
[15] J.-U. An, Y. S. Song, K. R. Kim, Y. J. Ko, D.-Y. Yoon, D.-K. Oh, Nat. Commun. 2018, 9, 4281.
[16] J. U. An, I. G. Lee, Y. J. Ko, D. K. Oh, J. Agric. Food Chem. 2019, 67, 3209.
[17] M. Busquets, V. Deroncelé, J. Vidal-Mas, E. Rodríguez, A. Guerrero, A. Manresa, Antonie van Leeuwenhoek 2004, 85, 129.
[18] R. E. Vance, S. H. Hong, K. Gronert, C. N. Serhan, J. M. Mekalanos, Proc. Natl. Acad. Sci. U. S. A. 2004, 101, 2135.
[19] J.-H. Bae, C. T. Hoo, H.-R. Kim, Biotechnol. Bioprocess Eng. 2010, 15, 1022.
[20] M. Aldrovandi, S. Banthiya, S. Meckelmann, Y. Zhou, D. Heydeck, V. B. O’Donnell, H. Kuhn, Biochim. Biophys. Acta – Mol. Cell Biol. Lipids 2018, 1863, 152.
[21] S. Banthiya, J. Kalms, E. Galemou Yoga, I. Ivanov, X. Carpena, M. Hamberg, H. Kuhn, P. Scheerer, Biochim. Biophys. Acta – Mol. Cell Biol. Lipids 2016, 1861, 1681.
[22] S. Banthiya, M. Pekárová, H. Kuhn, D. Heydeck, Arch. Biochem. Biophys. 2015, 584, 116.
[23] J. D. Deschamps, A. F. Ogunsola, J. B. Jameson, A. Yasgar, B. A. Flitter, C. J. Freedman, J. A. Melvin, J. V. M. H. Nguyen, D. J. Maloney, A. Jadhav, A. Simeonov, J. M. Bomberger, T. R. Holman, Biochemistry 2016, 55, 3329.
[24] J. Vidal-Mas, M. Busquets, A. Manresa, Antonie Van Leeuwenhoek 2005, 87, 245.
[25] X. Lu, J. Zhang, S. Liu, D. Zhang, Z. Xu, J. Wu, J. Li, G. Du, J. Chen, Appl. Microbiol. Biotechnol. 2013, 97, 5793.
[26] C. P. Kurtzman, J. Ind. Microbiol. Biotechnol. 2009, 36, 1435.
[27] S. Macaulay-Patrick, M. L. Fazenda, B. McNiel, L. M. Harvey, Yeast 2005, 22, 249.
[28] E. Paifer, E. Margolles, J. Cremata, R. Montesino, L. Herrera, J. M. Delgado, Yeast 1994, 10, 1415.
[29] J. Burgard, C. Grünwald-Gruber, F. Altmann, J. Zanghellini, M. Valli, D. Mattanovich, B. Gasser, Microb. Biotechnol. 2020, 13, 479.
[30] J. L. Cereghino, J. M. Cregg, FEMS Microbiol. Rev. 2000, 24, 45.
[31] L. M. Damasceno, C. J. Huang, C. A. Batt, Appl. Microbiol. Biotechnol. 2012, 93, 31.
[32] R. M. Bill, Front. Microbiol. 2014, 5, 85.
[33] J. M. Cregg, J. L. Cereghino, J. Shi, D. R. Higgins, Mol. Biotechnol. 2000, 16, 23.
[34] K. Weinhandl, M. Winkler, A. Glieder, A. Cammattari, Microb. Cell Fact. 2014, 13, 5.
[35] J. E. Fischer, A.-M. Hatzl, A. Weninger, C. Schmid, A. Glieder, J. Vis. Exp. 2019, 143, e58589.
[36] J. Kurjan, I. Herskowitz, Cell 1982, 30, 933.
[37] B. Chaudhuri, K. Steube, C. Stephan, Eur. J. Biochem. 1992, 206, 793.
[38] E. Fabre, J. M. Nicaud, C. Gaillardin, J. Biol. Chem. 1991, 266, 3782.
[39] T. Vogl, L. Sturmbberger, P. C. Fauland, P. Hyden, J. E. Fischer, C. Schmid, G. G. Thallinger, M. Geier, A. Glieder, Biotechnol. Bioeng. 2018, 115, 1037.
[40] T. Vogl, A. Glieder, New Biotechnol. 2013, 30, 385.
[41] O. Cos, A. Serrano, J. L. Montesinos, P. Ferrer, J. M. Cregg, F. Valero, J. Biotechnol. 2005, 116, 321.
[42] A. Kübler, J. Schneider, G. G. Thallinger, I. Anderl, D. Wibberg, T. Hajek, S. Jaenicke, K. Brinkrolf, A. Goessmann, R. Szczepański, A. Pühler, H. Schwab, A. Glieder, H. Pichler, J. Biotechnol. 2011, 154, 312.
[43] L. Sturmbberger, T. Chappell, M. Geier, F. Krainer, K. J. Day, U. Vide, S. Trstenjak, A. Schiefer, T. Richardson, L. Soriaga, B. Darnohor, R. Birner-Gruenberger, B. S. Click, I. Tolsturov, J. Cregg, K. Madden, A. Glieder, J. Biotechnol. 2016, 235, 121.
[44] J. Lin-Cereghino, W. W. Wong, S. Xiong, W. Jiang, L. T. Luong, J. V. S. Johnsson, G. P. Lin-Cereghino, BioTechniques 2005, 38, 44.
[45] J. M. Cregg, I. Tolsturov, A. Kusari, J. Sunga, K. Madden, A. Glieder, J. Mol. Catal. B Enzym. 2013, 87, 99.