



Molecular cloning and recombinant protein expression of 9-LOX gene from *Solanum tuberosum* in *Pichia pastoris* yeast cells

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Abstract

Synthetic fungicides have effectively controlled crop pathogenic fungi, but their continued use has been detrimental to natural biological systems, and sometimes resulted in development of fungal resistance. They also have undesirable effects on non-target organisms and foster environmental and human health concerns, thus, new biodegradable natural alternatives have to be investigated. Lipxygenases (LOX) are ubiquitous non-heme iron containing dioxygenases that catalyze the addition of molecular oxygen to polyunsaturated fatty acids (PUFAs) such as linoleic acid to form oxylipins that possess anti-microbial activity. The aim of this study was to generate a recombinant 9-Lipoxygenase protein for the chemoenzymatic synthesis of oxylipin-based biodegradable fungicides. Golden gate assembly, a molecular cloning method that allows assembly of various DNA fragments into a complete piece using Type II restriction enzymes, and T4 DNA ligase was used to clone the complete 9-LOX gene into the expression plasmid vector pPICZαB. Protein expression in *Pichia pastoris* yeast cells was induced by addition of absolute methanol after every 24 h for up to four days. Analysis of protein expression from cell lysates was achieved by SDS-PAGE and Western blotting probed with anti-histidine antibodies, which showed putative protein bands of 97 kDa representing recombinant 9-LOX protein. In the future, optimization studies on the yeast *kex2* convertase and the α - secretion factor need to be performed in order to enable the secretion of recombinant *Solanum tuberosum* 9-LOX protein, since the protein in this study was recovered from cell lysates.

Keywords 9-Lipoxygenase · Golden gate cloning · *Pichia pastoris* · Oxylipins · *Solanum tuberosum*

Introduction

The use of synthetic plant protection products, such as fungicides, insecticides, and herbicides, is paramount for controlling diseases and pests in agriculture, but their safety,

costs, and availability are a growing concern (Carvalho 2017; Jacquet et al. 2022; Lykogianni et al. 2021; Toffolatti et al. 2023). In the past four decades, disease management has contributed massively to plant health and food production but global harvests are still reduced by 10–16% due

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to plant diseases caused by infectious microorganisms like bacteria, viruses, nematodes and fungi (Adigun et al. 2023). This has prompted research institutions worldwide to focus on the discovery of naturally derived and produced biocides (Dean et al. 2012). Oxylipins such as trihydroxy fatty acids (TriOHs) occupy an important role in natural plant defense, possessing certain bioactive properties, including antifungal, bactericidal and insecticidal activity (Andreou et al. 2009; Feussner and Wasternack 2002; Göbel et al. 2002). Oxylipins also act as modulators of reactive oxygen species (ROS)-production and as constituents of the cuticle, a physical barrier to the pathogen attack (Righetti et al. 2023). This study describes the cloning and expression of 9-LOX protein which is an early phase research leading towards application of oxylipins as biological fungicides and preservatives in the industry to improve health protection and food safety. In comparison, conventional synthetic fungicides have a problem of bioaccumulation and non-biodegradability, thus, there is need to deploy techniques for production of natural fungicides such as oxylipins that confer antifungal properties to field crops with no bioaccumulation. This scientific work focuses on providing a recombinant plant 9-lipoxygenase that, at later stages of the research, will be used for the chemo-enzymatic synthesis of oxygenated derivatives of linoleic acid, especially 9,10,13- and 9,12,13-trihydroxy-11/10E-octadecenoic acid isomers (THOEs). There are 16 stereoisomers of THOEs, resulting from the two regioisomers and the three existing stereogenic centers (Hamberg 1991a, b), all of them most likely having different bioactive properties. The further application of racemic and isomer-pure TriOHs as natural fungicides onto mold-infected plants and as preservatives in food products will also be an essential part of the later stages of the research project.

In plants, THOEs are most likely formed enzymatically via three main formation steps: 1) first a lipoxygenase-catalyzed oxygenation or autooxidation of linoleic acid; 2) prior to an epoxidation of the formed polyunsaturated hydroperoxy derivatives into epoxyols, catalyzed by epoxy alcohol synthase; 3) and subsequent hydrolysis catalyzed by epoxide hydrolase (Garbe et al. 2005). Lipoxygenases (LOXs) belong to a family of monomeric proteins that catalyze oxidation of polyunsaturated fatty acids (PUFA) (linoleic, linolenic, and arachidonic acid) to form hydroperoxides (Lončarić et al. 2021). The LOXs are ubiquitous in animal, plant, and fungi kingdoms, as well as in cyanobacteria (Newcomer and Brash 2015). During insertion of molecular oxygen into the polyunsaturated fatty acids, the oxygen can be added to both sides of the pentadiene system, a term

known as regio specificity (Conconi et al. 1996). According to Brash (1999), the addition of molecular oxygen to an PUFA can either occur at carbon position 9 or 13, yielding two products, the 9- or the 13- hydroperoxy fatty acids, respectively. The unsaturated fatty acid hydroperoxides produced as a result of LOX activity are further metabolized into oxylipins. These oxylipins act as mediators. For example, in plants, oxylipin-based lipid mediators yielded include volatile aldehydes and jasmonates (Feussner and Wasternack 2002). These mediators have been shown in several studies to confer antimicrobial action to fungal and bacterial attack, for example, in tobacco following infection with *Phytophthora parasitica* var. *nicotianae* and in potato infected by *Phytophthora infestans* (Polkowska-Kowalczyk et al. 2008). Righetti et al. (2023), investigating metabolic plant response toward Deoxynivalenol accumulation in four winter cereals found that during the process of infection, initial plant defenses are activated by the detection of Reactive Oxygen Species (ROS) that promote lipid peroxidation. As membrane lipids, PUFAs may be released in response to a pathogen attack, triggering the formation of oxygenated fatty acids and oxylipins, which act as key signaling compounds. The oxylipins in plants with the best characterization are called jasmonates that include jasmonic acid (JA), 12-oxo-phytodienoic acid (12- OPDA), and JA derivatives such as methyl jasmonate and JA-isoleucine (Parveen et al. 2023). Pathogen infection and pest wounding are the best-studied environmental triggers in this context. JA and its derivatives act as defence regulators, influencing the reproductive and pathogenetic processes during the interaction with both beneficial and pathogenic microorganisms (Parveen et al. 2023).

More so, the accumulation of the 9-LOX-derived oxylipins in *Solanum tuberosum* has been shown to be rapid and intense in stimulation of plant defence gene expression, modulation of cell death and/or showing antimicrobial activity; hence, they are considered to be potential biodegradable defence compounds (Polkowska-Kowalczyk et al. 2008). Figure 1 shows the planned synthesis strategy for the chemical, chemo-enzymatic or enzymatic synthesis of THOEs with the depicted 1,2,5-trihydroxy-3(E)-pentene structure. This study reports on the molecular cloning and recombinant protein expression of the 9-LOX gene from *S. tuberosum* in *Pichia pastoris* yeast cells. The 9-LOX enzyme will then be used for reaction step one (oxygenation) in the synthesis of THOEs (Fig. 1).

In this study, the golden gate cloning strategy was used to clone the complete 9-LOX gene into the pPICZaB expression vector. After cloning, the *P. pastoris* X-33 expression

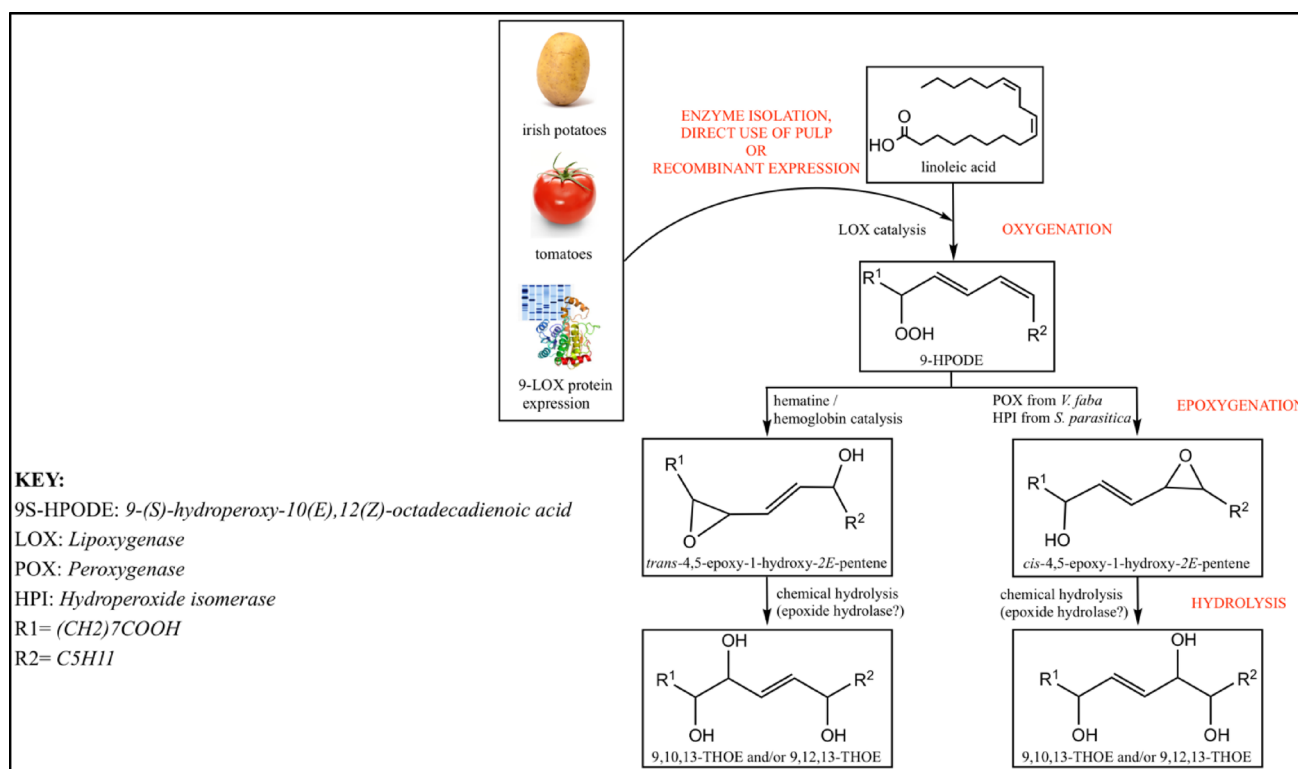


Fig. 1 Synthesis strategy for the chemo-enzymatic or enzymatic synthesis of THOEs

cell system was utilized to express the recombinant protein of interest. Golden Gate cloning is a method used to assemble multiple DNA fragments in a defined linear order into a recipient vector using a one-pot assembly procedure. The principle of golden gate cloning is based on the use of a Type II restriction enzyme for digestion of the DNA fragments and vector (Marillonnet and Grützner 2020). *P. pastoris* is a methylotrophic yeast, implying that it can grow with only methanol as a source of energy. The gene that codes for the desired protein is mainly achieved by inducing AOX1 promoter through addition of absolute methanol (Koutz et al. 1989). The study objectives, therefore, were 1) to successfully clone the 9-LOX gene into plasmid vector pPICZαB, 2) express the recombinant 9-LOX protein using *P. pastoris* X-33 strain.

Materials and methods

Sources of materials used in the study

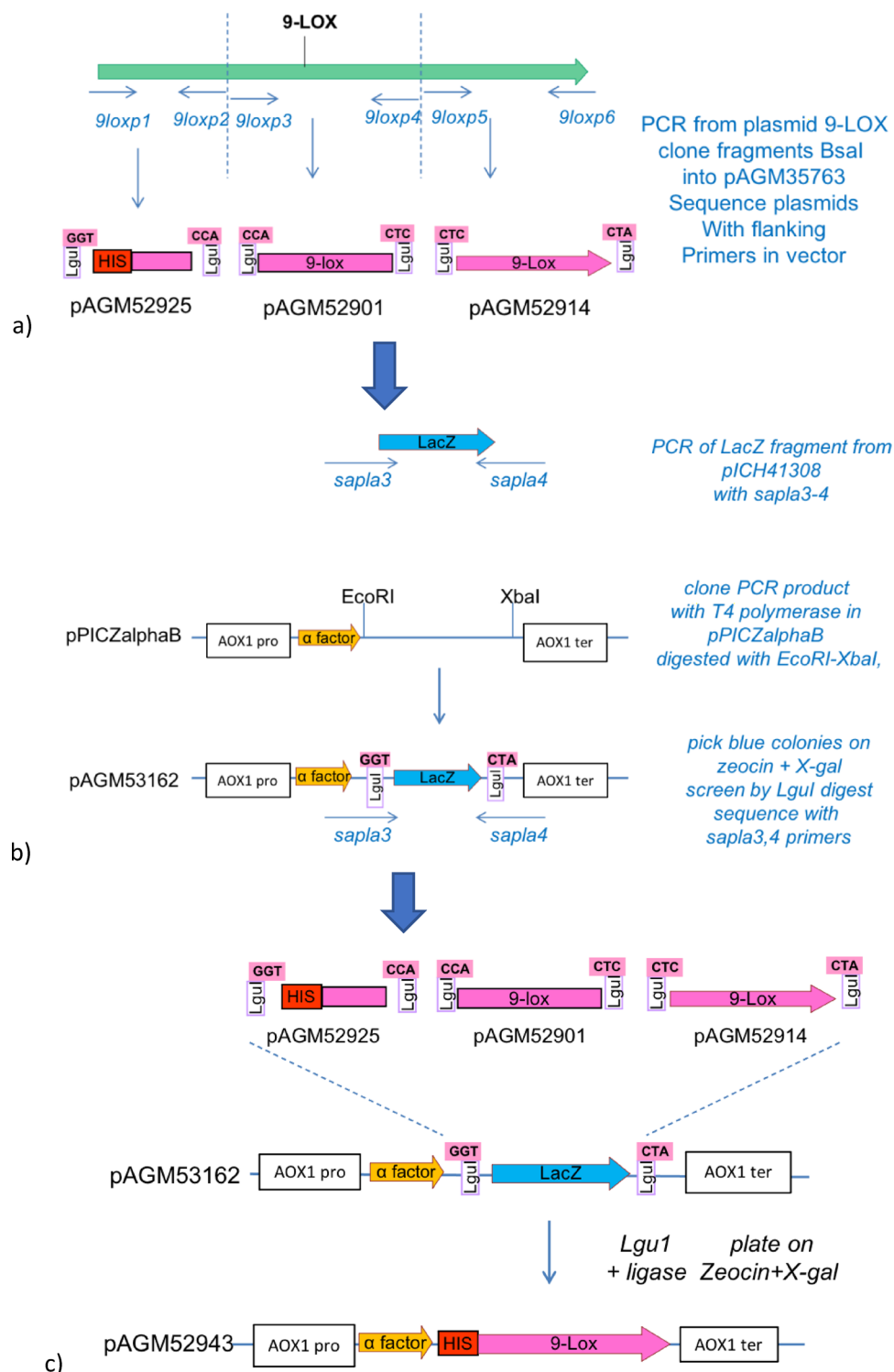
The recombinant PET-28a-9-LOX plasmid vector (Royo et al. 1996) used as a template for 9-LOX amplification in this study was donated by Professor Sabine Rosahl of the

Leibniz Institute of Plant Biochemistry (Halle, Germany). Cloning vectors pAGM35763 and plasmid pICH41308 were provided by Dr. Sylvestre Marillonnet of the Leibniz Institute of Plant Biochemistry, Germany. Primers were designed using Vector NTI Version 11.5.5 (Thermo Fisher Scientific) and purchased from MWG (now Eurofins), Germany. The PCR Kit, NucleoSpin Gel and PCR Clean-up used in the study were purchased from Macherey–Nagel (Düren, Germany). The expression kit containing pPICZαB plasmid was purchased from Thermo Fisher Scientific, Germany. Primary and secondary antibodies used in the Western blot analysis were purchased from Abcam, England. The restriction enzymes (*Bsa*I, *Lgu*I, and *Pme*I) were purchased from New England Biolabs, whereas *Eco*RI and *Xba*I were purchased from Thermo Fisher Scientific. Ligase enzyme and buffer were purchased from Promega.

Cloning of 9-LOX gene into pPICZαB plasmid vector

Since the 9-LOX coding sequence is 2.9 kb, it was easier to sub-clone it in three smaller fragments. Three 9-LOX gene fragment regions selected by the Vector NTI software were amplified by PCR using the 3 pairs of primers (Table 1) which added *Bsa* I sites flanking at both ends of each of

Fig. 2 Schematic representation of the cloning steps. **a** Amplification of 9-LOX regions and sub-cloning into pAGM35763 Vector. **b** Schematic representation of T4 cloning of *LacZ* amplicon into pPICZαB plasmid vector. **c** Schematic representation of restriction-ligation assembly of 9-LOX fragments into pPICZαB expression vector



the three 9-LOX amplicons for restriction-ligation cloning into intermediary vector *pAGM35763* using *BsaI* Type II restriction enzyme (Fig. 2a). The PCR was performed using KOD Hot start DNA polymerase since it has a low error rate, unlike Taq polymerase (Engler and Marillonnet 2013). After, the intermediary vector *pAGM35763* with compatible

BsaI sites was used to sub-clone the three 9-LOX amplicons in a restriction-ligation reaction to generate intermediary recombinant vectors *pAGM52925* containing a 548 bp fragment, *pAGM52901* containing a 993 bp fragment and *pAGM52914* containing a 1144 bp fragment (Fig. 2a). In preparation for the final cloning step, the destination

plasmid vector pPICZαB was digested with EcoRI and XbaI restriction enzymes (Fig. 2b). Using the plasmid vector *pICH41308* as a template for *LacZ* gene amplification, sapla 3 forward primer (5'gaggctgaagctgcaggaattggttgaagagcgcagctggcagcagagtttc3') and sapla 4 reverse primer (5'gatcctctctgagatgagttttgttctagtgaagagcgtcacagctt-gtctgtaagcgg3') were used to introduce *LguI* recognition sites (bold and underlined) compatible with the *LguI* sites in the intermediary vector used in sub-cloning of 9-LOX fragments. The *LacZ* amplicon was then cloned into the digested destination plasmid vector pPICZαB using T-4 cloning protocol adopted from Thieme et al. (2011). The intermediary recombinant vectors *pAGM52925* containing the 548 bp fragment, *pAGM52901* containing the 993 bp fragment, *pAGM52914* containing the 1144 bp fragment and destination plasmid vector pPICZαB containing the *LacZ* fragment (649 bp) were used for the golden gate assembly (Fig. 2c) using reaction conditions adopted from Terfrüchte et al. (2014). The restriction-ligation product was then used to transform competent *E. coli* DH10B cells using the heat shock method. Plasmid DNA extraction was done using Nucleospin plasmid Easy Pure Kit following manufacturer's instructions. Restriction analysis was done on the recombinant plasmid DNA (*pAGM52943*) using *BsaI*, *BstXI*, and *NheI* restriction enzymes (New England Biolabs) to confirm the expected size of the insert followed by sequencing. From the extracted plasmid DNA, one sample was used for recombinant protein expression analysis.

Linearization of 9-LOX recombinant plasmid DNA using *Pme I* restriction enzyme

Linearization was performed by restriction analysis using *Pme I* enzyme basing on a protocol adopted from Guo et al. (2016). *P. pastoris* X-33 strain was transformed with linearized 9-LOX plasmid DNA using electroporation method. Mixture was then incubated at 30 °C without shaking for 2 h to allow expression of zeocin resistance. From the transformed cell mixture, 250 µl was spread on an YPDS (Yeast Extract Peptone Dextrose) agar plate containing 25 µg/ml of zeocin using sterile glass beads and left to air dry for 30 min then incubated at 30 °C for up to 10 days. Five colonies were picked using a sterile wire loop and streaked individually on five fresh YPDS agar plates containing 25 µg/ml of zeocin and incubated for 10 days at 30 °C to obtain pure colonies for expression.

Expression and analysis of recombinant 9-LOX protein

Protein expression was achieved by growing recombinant *P. pastoris* X-33 strain in Buffered Glycerol Complex Medium (BMGY) for 18 h and thereafter grown in Buffered Methanol Complex Medium (BMMY) to induce protein expression. At 24 h intervals, absolute methanol was added to a final concentration of 0.5% to maintain expression of

Table 1 Primer pairs used for amplification of three 9-LOX gene fragments from *S. tuberosum*

Primer	Sequence (5' to 3')	Binding site	Expected fragment size (bp)
9-LOXp1(F)	tt ggtctc a ttca ggttctcatcatcatcatcatcggtattgtgggtggactaattggtggc	11.82	548
9-LOXp2(R)	tt ggtctc a ttcg tgggtttcacttgggagatatggctg	527.553	
9-LOXp3 (F)	tt ggtctc a ttca ccagagcttttgcgcaataacagag	549.575	993
9-LOXp4(R)	tt ggtctc a ttcg gagtatacactttactaataacaccaaattg	1487.1518	
9-LOXp5(F)	tt ggtctc a ttca ctccaagtcatcaaggtgttgagagc	1514.1540	1144
9-LOXp6(R)	tt ggtctc a ttcg tagctatattgacacactgttggaattcc	2603.2629	

KEY

-  Any nucleotide of choice
-  Linkers
-  BsaI recognition sequence
-  6x Polyhistidine tag
-  Fusion site
-  9-LOX Complementary sequence

recombinant 9-LOX protein. Briefly, five single colonies from the transformed *P. pastoris* X-33 cells were selected and each grown in 3 ml of YPDS media in 50 ml falcon tubes at 30 °C with shaking at 250 rpm. From each of the overnight cultures, 500 µl was aliquoted and inoculated into five 250 ml baffled Erlenmeyer flasks containing 30 ml of BMGY for cell growth at 30 °C with shaking at 250 rpm for 18 h before inducing protein expression. The cultures were then spun down at 3000xg for 5 min and the pellets resuspended in 30 ml BMMY and centrifuged at 3000xg for 5 min to remove excess glycerol. The resultant pellets were resuspended in 30 ml of BMMY to induce protein expression and these were allowed to grow at 30 °C with shaking at 250 rpm for 96 h. To maintain expression, 100% methanol was added to the first 4 flasks at a final concentration of 0.5% at 24 h intervals for the time points 24 h, 48 h, 72 h, and harvested at 96 h. The 5th flask was used as a control for uninduced expression since absolute methanol was not added to it. After expression, 30 ml of the resultant culture from each of the five baffled flasks were spun at 3000xg for 5 min and 1 ml of supernatants pipetted off into separate Eppendorf tubes. The resultant pellet samples and the supernatants were stored at –80 °C freezer until future analysis. The pellet samples were analyzed for protein expression

using SDS-PAGE and Western blotting using PVDF membrane probed with anti-histidine (Abcam) coupled with a pre-stained protein marker-180 kDa (Thermo-Fisher Scientific).

Dot blot analysis of recombinant 9-LOX protein expression in *P. pastoris*

In the dot blot analysis, 5 µl of the supernatant from each of the viva flow® 200 concentrated expressed proteins from *pAGM52943* at 24 h, 48 h, 72 h, and 96 h of expression were spotted on a nitrocellulose membrane in the concentrate order 1,2,3,4. Blots 5 and 6 are from the viva flow filtrate samples. Blots 7–10 represent supernatant samples from *pAGM52943* at 24 h, 48 h, 72 h, and 96 h of expression. Blot 11 was the uninduced sample at 96 h.

After spotting, the nitrocellulose membrane was left to dry for 10 min then blocked with 5% skimmed milk in 1×PBS at 4 °C overnight to prevent nonspecific binding of antibodies. The membrane was then washed 3 times with 1×PBS containing 0.05% Tween-20. Each wash was 10 min at room temperature in a glass dish while rocking gently. The membrane was then incubated with primary antibody (anti-histidine produced in mouse), at dilution of 1:2000 in 1×PBS containing 0.5% skimmed milk at room temperature for one hour. The membrane was then washed 3 times with PBS containing 0.05% Tween-20, each wash for 10 min. The membrane was then incubated with secondary antibody (anti-mouse conjugated to alkaline phosphatase) at 1:2000 dilution in 1×PBS containing 0.5% skimmed milk. This was incubated at room Temperature for 1 h. The membrane was then washed 3 times with 1×PBS containing 0.05% Tween-20. Each wash was 10 min at room Temperature.

After, the membrane was washed 2 times in Alkaline Phosphate (AP) solution which contained 0.1 M Tris HCl, 0.1 M NaCl, 5 mM MgCl₂ and made up to pH 9.5. The membrane was stained with 15 ml AP containing 20 µl BCIP (Bromochloroindolyl phosphate) and 0.5% skimmed milk. Color development was monitored for 20 min and the reaction stopped by adding 200 µl of 1 mM EDTA, pH 8.0 in 50 ml of PBS.

Peroxide test strip measurement of recombinant 9-LOX activity

Enzyme activity testing was achieved by using 500 mg of pure linoleic (Sigma-Aldrich, purity 64–74%, impurities 18–32% oleic acid) acid as substrate that was dissolved in 3.0 mL Ethanol (99%). The linoleic acid was converted into

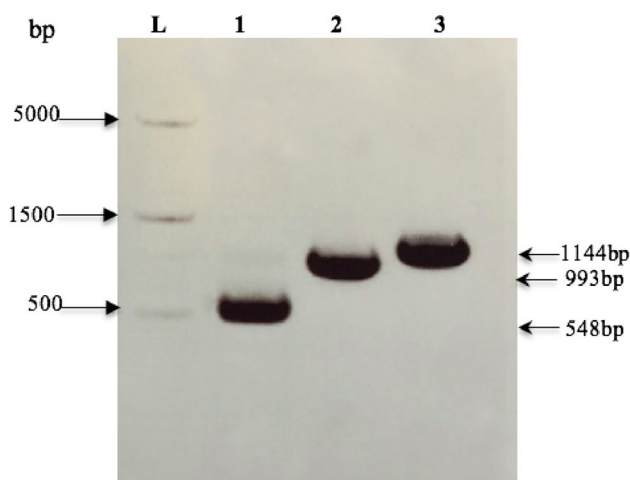


Fig. 3 A representative 1% agarose gel showing 9-LOX amplicons. Lane L: Gene ruler 1 kb plus-RTU (Thermo-Fisher Scientific); Lanes 1, 2, and 3 represent the 9-LOX amplified fragments of band sizes 548 bp, 993 bp, and 1144 bp respectively. After PCR amplification, 5 µl of each of the three 9-LOX amplicons were mixed with 6X gel loading dye (Thermo-Fisher Scientific), loaded on a 1% agarose gel and run alongside a 1 kb DNA ladder (RTU-Thermo Scientific) at 125 V in 1X Tris–Acetic acid-EDTA (TAE) buffer containing 0.5 µg/ml ethidium bromide for 18 min. The ethidium bromide-stained gel was visualized using the UV gel documentation system- Fusion FX (Vilber, Lourmat)

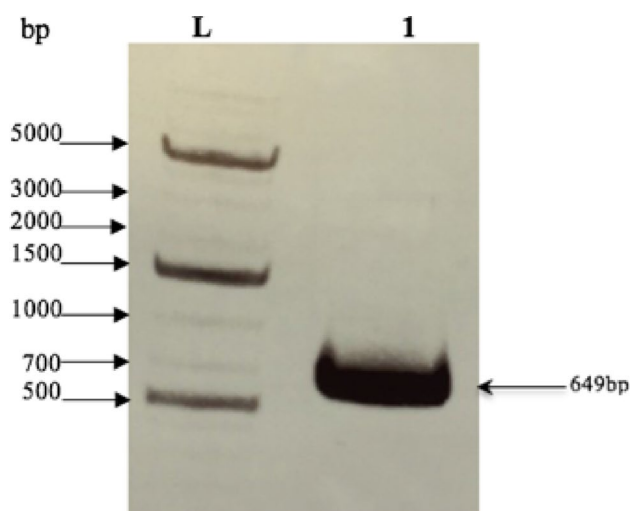


Fig. 4 A representative 1% agarose gel showing *LacZ* amplicon. Lane L: Gene ruler 1 kb plus DNA ladder RTU (Thermo-Fisher Scientific); lane 1: PCR amplicon of *LacZ* fragment size (649bp) from *pICH41308* plasmid. After PCR amplification, 5 µl of reaction product was mixed with 6X gel loading dye (Thermo-Fisher Scientific), loaded on a 1% agarose gel and run alongside a 1 kb DNA ladder (RTU-Thermo Scientific) at 125V in 1X Tris–Acetic acid-EDTA (TAE) buffer containing 0.5 µg/ml ethidium bromide for 18 min. The ethidium bromide-stained gel was visualized using the UV gel documentation system- Fusion FX (Vilber, Lourmat)

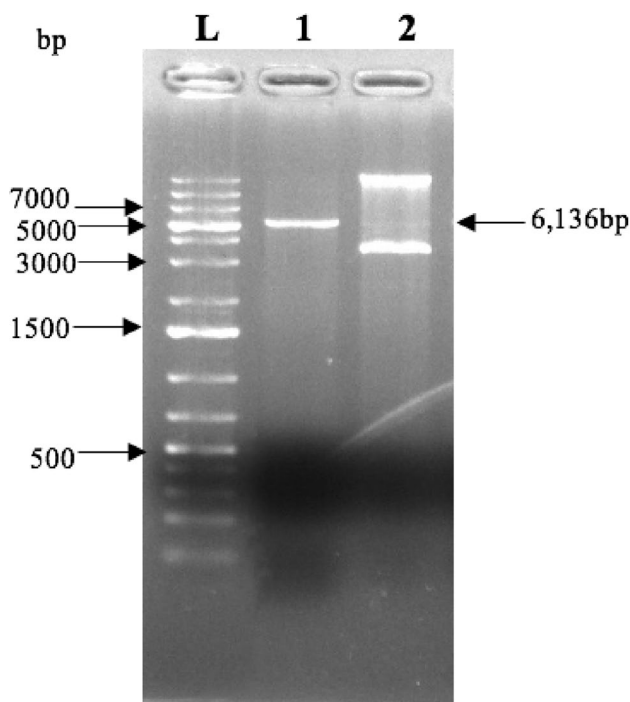


Fig. 5 A representative 1% agarose gel with linearized and non-linearized recombinant plasmid DNA. Lane L: Gene ruler 1 kb plus DNA ladder RTU (Thermo-Fisher Scientific); lanes 1 and 2 represent linearized and non-linearized *pAGM52943* plasmid vectors, respectively

the linoleate by the addition of 120 µl NH₄OH, diluted with 100 ml distilled water and adjusted to pH 9.0 with 0.1 M HCl, while the mixture was stirred. 300 ml 100 mM sodium borate buffer (pH 9.1) and 50 µl of Tween-20 were added while stirring and the reaction vessel set under a steady oxygen stream. Incubation was started by addition of 5 ml cell lysate containing crude recombinant 9-LOX protein (dissolved in sodium borate buffer pH 9.1) at a temperature of 25 °C for 15 min and hydroperoxide production was tested after 5/10/15 min via Quantofix® peroxide test stripes (Roth, Germany, Peroxide 100). At the end of the reaction, the mixture was acidified to pH ≈ 4.0 with 2 M HCl.

Results

Three amplified 9-LOX DNA fragments of *S. tuberosum*

The three 9-LOX DNA fragments were successfully amplified by PCR using the primer pairs, which added *Bsa*I sites flanking each of the three DNA fragments generated (Fig. 3). The three fragments were individually sub-cloned into the intermediary plasmid vector *pAGM35763*.

Amplified *LacZ* fragment from *pICH41308* plasmid vector

The *pICH41308* plasmid vector was used as a template for *LacZ* gene amplification using sapla 3 forward and sapla 4 reverse primers. A 649 bp amplicon was generated (Fig. 4).

*Pme*I linearized 9-LOX recombinant plasmid *pAGM52943*

Linearization of recombinant plasmid *pAGM52943* was achieved by using *pme*I restriction enzyme that gave a single linear band of approximately 6136 bp (Fig. 5).

A 12% SDS-PAGE and western blot of recombinant 9-LOX expressed in *P. pastoris* X-33 strain

The recombinant 9-LOX protein has a molecular weight of approximately 97 kDa. The expression of the recombinant 9-LOX protein in *P. pastoris* X-33 cells was analyzed by SDS-PAGE and Western blotting and showed bands from cell lysate samples (Fig. 6).

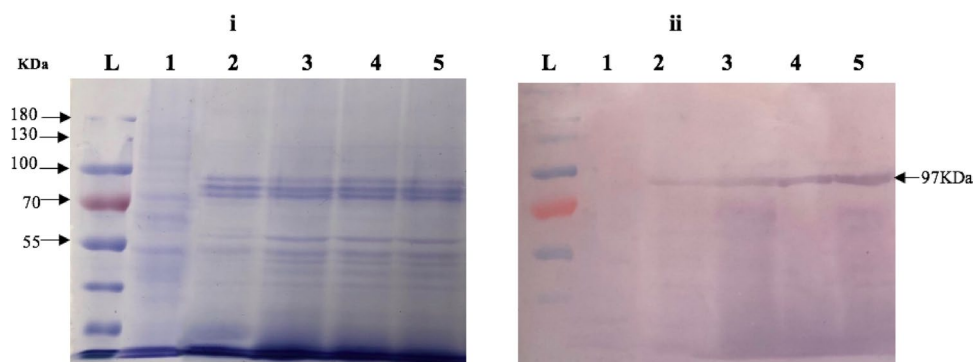
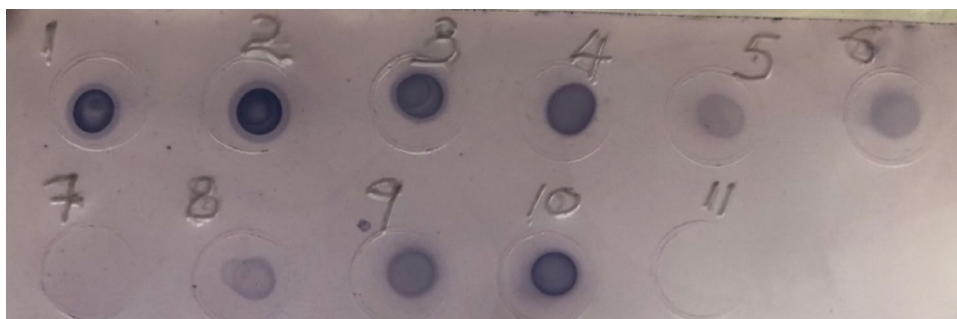


Fig. 6 A 12% SDS-PAGE and Western blot of the recombinant 9-LOX expressed in *P. pastoris*. (i) Coomassie blue staining of polyacrylamide gel; Lane L: Prestained protein marker-180 kDa (Thermo-Fisher Scientific); lane 1: uninduced recombinant *P. pastoris* cells at 96 h post expression; lanes 2, 3, 4, and 5 represent proteins from induced *P. pastoris* cells integrated with *pAGM52943* plasmid at 96 h post induction with 100% methanol to a final concentration of 0.5%. All the four induced samples showed expected band size of 97KDa. (ii) Western blot analysis of recombinant 9-LOX protein gel; Lane L: Prestained

protein marker-180 kDa (Thermo-Fisher Scientific); lane 1: PVDF (Thermo-Fisher Scientific) transferred proteins from uninduced *P. pastoris* cells integrated with *pAGM52943* plasmid at 96 h of expression; lanes 2, 3, 4 and 5 represent proteins from induced cells containing *pAGM52943* plasmid at 96 h post induction with 100% methanol to a final concentration of 0.5%. Western transfer probed with anti-His monoclonal antibodies resulted in bands of 97KDa that correspond to the recombinant 9-LOX protein. Non induced sample showed no signal

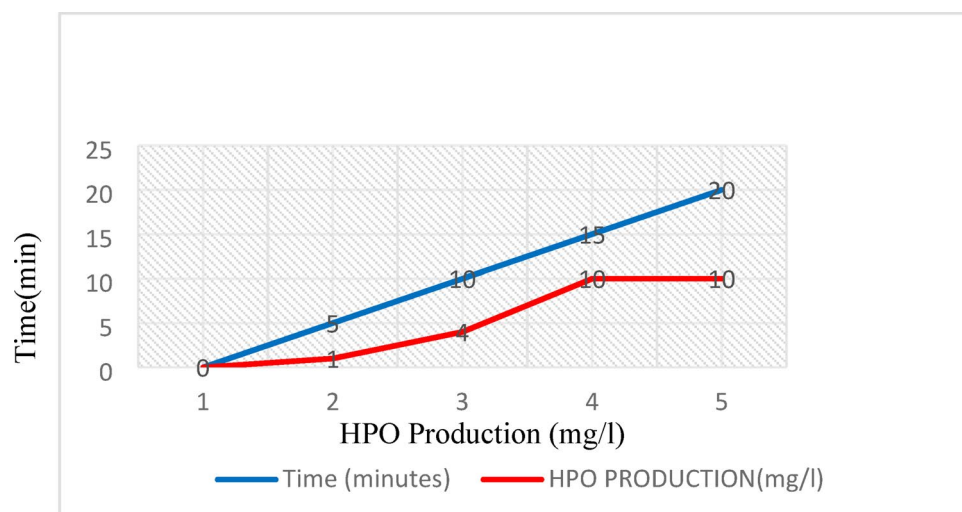
Fig. 7 Nitrocellulose membrane showing dot blots from supernatant samples developed using BCIP



Dot blot analysis of recombinant 9-LOX protein expression in *P. pastoris*

Dot blots 1–4 shows are viva flow® 200 concentrates of 9-LOX proteins indicated by the colour intensity. However, some proteins were also detected in the viva flow filtrate (blots 5 and 6 for protein samples from *pAGM52943* and yet the viva flow filter has a cut off of 50KDa. This can be attributed to the retention capacity of the filter used or other proteins present in the supernatant. Blot 7–10 show aliquots of induced expression from *pAGM52943* at different time frames as above. Blot 11 is uninduced sample containing *pAGM52943* at 96 h (Fig. 7).

Blots 1,2,3, and 4 are viva flow® 200 concentrates from *pAGM52943* at different time points (24 h, 48 h, 72 h, 96 h) post induction with 100% methanol to a final concentration of 0.5%. Blots 5 and 6 are viva flow filtrates from *pAGM52943*. Blot 7–10 show aliquots of induced expression from *pAGM52943* at different time frames as above. Blot 11 is uninduced sample containing *pAGM52943* at 96 h.



Graph 1 Hydroperoxide (HPO) production against time

There was a steady increase of linoleic HPO formed up to 15 min after which it is constant possibly due to other reactions in the solution that consume the HPO formed.

Discussion

This study aimed at generating recombinant *S. tuberosum* 9-LOX protein using *P. pastoris* as an expression system. For many years, plant pathogenic fungi have caused devastating losses worth millions of dollars in crops worldwide (Yoon et al. 2013). Synthetic fungicides have, for many years, controlled plant pathogenic fungi. However, their repeated use over decades has disrupted natural biological systems, and sometimes resulted in the development of fungal resistance (Yoon et al. 2013). They have undesirable effects on non-target organisms and foster environmental and human health concerns and thus alternative approaches that yield biodegradable fungicides have to be investigated. One of these alternative approaches would be the application of plant-derived (directly or indirectly) oxylipins, especially THOEs. This study has successfully proven that *P. pastoris* can generate recombinant 9-LOX protein and recombinant cell lines can be prepared for production of large quantities of recombinant 9-LOX protein, which would then be available for large scale selective biotechnological production processes of diverse THOE isomers.

The reason why we chose recombinant expression of 9-LOX is that it is difficult to attain pure 9-LOX protein in high amounts besides dealing with the impurities which come along with the extraction. Such impurities like phenolic compounds interfere with the downstream experiments. Recombinant 9-LOX can be an equally efficient alternative as compared to commercial reagent alpha linoleic acid and

linolenic acid in the production of 9R-hydroxy-10E,12Z-octadecadienoic acid (9R-HODE) and 9R-hydroxy-10E,12Z,15Z-octadecatrienoic acid (9R-HOTE) (Kim et al. 2015). Though achievable through plant extraction or chemical synthesis, LOX extraction is costly and inefficient due to low concentrations. Consumer preference for natural additives further discourages synthetic methods (Huang and Schwab 2011). To illustrate, the natural green notes market stands at 5–10 tons per year and 3000 US dollars per kilogram (Muller et al. 1995). Also, the occurrence of different isoenzymes in plant-based sources, which differ in their biochemical properties prompted us to use recombinant 9-LOX protein. As far as we are aware, Golden Gate cloning has not been previously used to insert a 9-LOX gene from *S. tuberosum* into the expression plasmid vector pPICZαB. This study also presents the first time 9-LOX protein from *S. tuberosum* is being expressed in X-33 strain of *P.pastoris*.

In this study, *P. pastoris* expression system was chosen because of its significant similarities with higher eukaryotic systems, such as Chinese Hamster Ovary (CHO) cell lines, while being more affordable and offering quicker expression and processing, coupled with posttranslational modification (Karbalaie et al. 2020). Large-scale protein production is achievable from small culture volumes through industrial bioreactors. Notably, studies indicate that the *Pichia* expression system is particularly well-suited for membrane proteins, including potassium and calcium channels, phosphate and nitrate transporters, among others (Byrne 2015). Beyond that, *P. pastoris* effectively secretes recombinant proteins into the culture medium and its limited endogenous protein secretion aids purification (Tachioka et al. 2016).

In addition, the system's prior success in expressing numerous LOX proteins, such as secreted *Pseudomonas aeruginosa* LOX proteins, expression and molecular

characterization of LOX from tomato, screening of single domain antibodies, expression of polyproteins, and activity analysis of lipoproteins justifies its use in the current study (Barone et al. 2023; Han et al. 2012; Hu et al. 2011; Kelle et al. 2014; Rahbarizadeh et al. 2006; Reddy et al. 1994; Zhang et al. 2022). In conclusion, the *P. pastoris* expression system provides appropriate folding and secretion of recombinant proteins into the extracellular environment alongside simplified purification (Karbalaee et al. 2020).

As for cloning, the Golden Gate technique was used due to the large size of the 9-LOX insert (2629 bp) that codes for a protein of approximately 97 kDa. The vector NTI Bioinformatics tool Version 11.5.5 was used to select the three 9-LOX fragments that were sub-cloned into intermediary level 0 vector as described by Engler and Marillonnet (2013). Direct assembly of 9-LOX PCR products into the destination vector is possible; however, column purification is recommended to remove DNA polymerase and primer dimers formed during PCR. Primer dimers containing fusion sites (incorporated into the primers) are prone to miscloning, generating incorrect constructs (Werner et al. 2012). This study was, therefore, able to successfully clone a 2629 bp 9-LOX gene into pPICZαB plasmid vector using a restriction-ligation reaction. The golden gate reaction conditions used in this study are similar to those described by Marillonnet and Grützner (2020) and Terfrüchte et al. (2014).

The level 0 intermediary vector *pAGM35763* was cloned with individual 9-LOX fragments of 548 bp size attached to a 6xHistag, 9-LOX fragment of 993 bp, and 9-LOX fragment of 1144 bp. All the 3 fragments sub-cloned into level 0 intermediary vectors carried *BsaI* restriction sites introduced during PCR amplification. The level 0 intermediary vector carrying each of the three 9-LOX fragments was then used in a final restriction-ligation step in which the fragments were assembled into a level 1 destination vector (pPICZαB) which generated recombinant plasmid *pAGM52943*. All level 0 intermediary vectors met the following criteria as described by Engler and Marillonnet (2013): (1) They were flanked with *BsaI* sites in the opposing direction. The enzyme *BsaI* (isoschizomer: *Eco31I*) with the non-palindromic recognition/hydrolysis site is active at both 37 °C and 50 °C, has a recognition site of 6 bp separated by one random nucleotide from the site of hydrolysis and generates a 4 bases overhang (5'-GGTCTC(N)1-3'/3'-CCAGAG(N)5-5', (2) all DNA parts of the same type were flanked by same pair of fusion sites, (3) recognition sequences of the restriction endonuclease *BsaI* were absent in all internal sequences of the selected 9-LOX fragments, (4) the pPICZαB vector backbone contained a *LacZ* resistance gene for negative selection introduced by T4 polymerase and ligase buffer without using ligase. These guiding cloning principles were

also similar to those described by Marillonnet and Grützner (2020). According to Jeong et al. (2012), T4 cloning was used to undertake a one-step sequence and ligation independent cloning similar to this study since ligase enzyme was not used in the cloning reaction of the *Lac Z* gene into the digested pPICZαB plasmid vector. This is attributed to the fact that T4 DNA polymerase has a 3' to 5' exonuclease activity (Jeong et al. 2012). Sequencing of the recombinant destination plasmid vector *pAGM52943* showed no mutations in the shuffled 9-LOX gene fragments. This was expected because the modules in the intermediary vector constructs were made using PCR, but then sequenced before being used for restriction-ligation assembly and correct orientation was noted.

After, the recombinant *pAGM52943* plasmid was used for protein expression using the methylotrophic *P. pastoris* X-33 strain as a host system. The recombinant 9-LOX protein was expected to be secreted in the supernatant since the 9-LOX gene was cloned upstream of the alpha secretion factor using the golden gate protocol as described by Engler and Marillonnet (2013). Attempts to recover recombinant 9-LOX protein from supernatant were futile which could be attributed to vacuolar proteases such as carboxypeptidase Y and proteinase B. These are significant factors in protein degradation especially in fermenter cultures owing to the high cell density environment in combination with lysis of small percentage of cells (Koutz et al. 1989). An example of a strategy to counter proteolytic degradation of secreted recombinant proteins is to add casamino acids (Sinha et al. 2004), use mutant strains deleted in the vacuolar proteases saccharopepsin (Protease A) and/or cerevisin (Protease B). These proteases are mainly present in the extracellular medium as a result of cell lysis during protein expression observed by frothing in the baffled flasks (Werten et al. 2019). Methanol can also induce cell lysis by oxidative stress and heat-shock responses, eliciting a proteolytic response when the cells are growing exponentially, which results in high cell-density fermentation (Vieira et al. 2017). In this regard, proteins damaged by oxidative stress are a probability, since methanol metabolism demands high oxygen and a by-product is hydrogen peroxide. Excess production of the vacuolar proteases can result in a secretion to cytosol and eventually to the culture medium. (Stevens et al. 1986).

Since recombinant proteins can either accumulate inside the cells or be secreted to the outside, it will make a huge difference in the purification process (Ruh 2023). When working with intracellular proteins, cells must first be broken by homogenization. Established techniques include continuous-fill French press, sonication, or enzymatic digest (Ruh 2023). Naturally, the cell lysate will have a lot more impurities such as nucleic acids and cell debris that must

be removed before chromatography steps. Either way, centrifugation is an essential technique that must be performed whether the protein is intracellular or secreted. It helps to remove unbroken cells, cell debris and ribosomal material and other particulates. If necessary, phase partitioning or membrane filtration can be performed subsequently (Wingfield 2002).

There are numerous chromatographic techniques available for protein purification though affinity chromatography is regarded as the most specific and effective. This technique exploits the ability of molecules to form reversible bonds with affinity ligands (Roque et al. 2007). For the purification of recombinant proteins, immobilized metal affinity chromatography (IMAC) is commonly utilized (Falke and Corbin 2004). The technique involves 4 steps. Firstly, metal ions are immobilized onto the matrix. Subsequently, the sample will be loaded onto the column. The targeted protein should interact with the metal ions and be retained. The third step consists of washing the column to remove unwanted components. Lastly, the targeted protein will be eluted (Corona et al. 1993; El Hajj et al. 2021). A popular example of the IMAC is purification with His6-tag. Six consecutive histidines are introduced in a cloning vector to the N- or C-terminus of a recombinant protein (Ruh 2023). Additional purification steps for recombinant proteins include Ion-exchange chromatography, Gel filtration, Affinity chromatography, Chromatofocusing, Isoelectric focusing, Immunoprecipitation, Solubilization (membrane proteins) lectin affinity chromatography and are discussed thoroughly in Guide to Protein Purification (Deutscher 1990).

The absence of recombinant 9-LOX protein in the culture media in the present study can also be attributed to very low level of secretory protein expression. Putting these into account, attempts were made to recover 9-LOX recombinant protein from the cell lysates. SDS-PAGE analysis showed an expected band size of approximately 97KDa that was confirmed by immunoblotting of PVDF membrane probed using anti-histidine antibody which is very good for lowly expressed proteins although it gives a high background (Xiang et al. 2021). The expression results from this study are similar to those from a study by Rodrigo et al. (2013), where expressed recombinant protein was recovered from the cell lysates as was revealed by SDS-PAGE and Western blot analysis after NTI-affinity chromatography purification. During decades of using *P. pastoris* as an eukaryotic protein expression system, there has been a problem of inconsistent secretory productivity among different recombinant proteins as some proteins could reach extremely high yields while some others had little or no expression at all. This has always been a major obstacle for routine application in both research and industry (Yang et

al. 2013). The same study by Yang et al. (2013) suggested a new strategy to increase *P. pastoris* secretory productivity by optimizing the yeast convertase Kex2 cleavage, which increased secretory protein in comparison with yeast vector with no Kex2 modification since this was not possible in our study. Also, artificial addition of the Kex2 protease gene into the host genomic DNA expressing Venus fluorescence protein doubles the productivity of the recombinant protein according to a study by Juturu and Wu (2018).

Therefore, in the future studies, to enable secretion of recombinant 9-LOX protein into the culture media, optimization of the α -secretion factor and yeast kex2 convertase in comparison to yeast vector with no kex2 modification can be investigated. Artificial addition of the Kex2 protease gene into the host genomic DNA should be investigated during expression to enhance recombinant protein secretion into the culture media and protease inhibitors should be included during expression to avoid loss of protein due to proteolytic activity of proteases in the culture media. The 9-LOX Protein yield, purification and potential testing of the recombinant 9-LOX protein for its chemoenzymatic activity should also be investigated. Also, other strains within the *P. pastoris* kit such as GS115 and KM71H can be used since we only used the X-33 strain.

Conclusion

In this study, expression of the recombinant *S. tuberosum* 9-LOX protein from cell lysates was achieved using the *P. pastoris* X-33 strain. This study supports further research on the chemo-enzymatic synthesis of oxygenated derivatives of linoleic acid, especially 9,10,13- and 9,12,13-trihydroxy-11/10E-octadecenoic acid isomers (THOEs), development of potential application as natural fungicides via in situ experiments onto mold-infected plants and as preservatives in food products.

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Author contributions Conceptualization, funding, and project administration were done by L.A.G., F.S. and M.K. Methodology and investigation were done by A.O., A.N., E.H.B.P., S.M., and R.G. Cloning constructs were provided by S.M., R.G. and E.H.B.P. Project supervision was done by A.N. and L.A.G. M.S.K. provided mentorship during the protein expression work. The manuscript was written by A.O., A.N. and F.S. Reviewing and editing was performed by A.O., A.N., M.K., S.M., R.G. and F.S.

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Data availability All supporting data and supplementary information regarding this study are available upon request.

Declarations

Conflict of interest The Authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

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