

## Title

Identification and kinetics characterization of a wax ester hydrolase from a feather-degrading actinomycete

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## Abstract

*Streptomyces fradiae* var. k11 is a Gram-positive soil microorganism capable of degrading chicken feathers. Apart from being mostly protein, chicken feathers have a considerable level of lipids, with wax esters being the largest lipid class. The waxes may pose a challenge while rendering the feathers into coproducts, such as feather meal, and so the identification of a wax-ester hydrolase is warranted. A draft genome sequence of *S. fradiae* var. k11 was used to identify 14 gene sequences of potential lipid-degrading enzymes. The genes were expressed in *E. coli* BL21(DE3) cells on a pET vector and screened for activity. Four of the 14 enzymes had detectable activity, with two of the enzymes, SFK3309 and SFK3087, active against p-nitrophenyl palmitate, a representative water-insoluble substrate. A modified enzymatic assay was designed to measure activity against three model wax substrates: jojoba oil, beeswax, and cetyl-palmitate. SFK3309 was characterized to hydrolyze all three wax substrates. Kinetic experiments for SFK3309 were performed with cetyl-palmitate at 37°C, pH 8.0. The  $K_m$  was determined to be 850  $\mu\text{M}$  and the  $K_{cat}$  was 11.63  $\text{s}^{-1}$ . Through the characterization of SFK3309 as a wax-ester hydrolase, biotechnological implications of wax ester hydrolases in the rendering of many industrial wastes can be substantiated for further studies.

## Keywords

Wax, lipase, hydrolase, esterase, kinetics

## Introduction

Chicken feathers are well known for being predominately protein (90%), mostly comprised of recalcitrant  $\beta$ -keratin (Gupta et al. 2012). However fat content has been known to vary inversely with protein content in feather meal, anywhere from 2-12% (Dale 1992). Some groups have estimated chicken feather fat to be near 11% (Kondamudi et al. 2009). Chickens secrete lipids from the uropygial gland onto their plumage for protection from the elements in an act known as preening. Wax (di)esters account for 49.3% of total preen oil composition and 38.2% of total feather lipids (Wertz et al. 1986).

Feathers represent approximately 3-6% of a chicken's whole body weight (Leeson and Walsh 2004) and with the annual slaughter of 8.9 billion chickens in the USA (USDA (U.S. Department of Agriculture) 2017), effective rendering of chicken feathers is necessary to combat pollution. The protein from chicken feathers has considerable economic value, but the significant presence of fat in chicken feathers may pose a challenge for adequate degradation of the  $\beta$ -keratin, and thus lower the economic potential.

Over half a century ago, *Streptomyces fradiae* was identified for the biodegradation of wool and feather keratin at enriched waste sites (Noval and Nickerson 1959) and our lab has recently been investigating enzymes from *S. fradiae* var. k11 for industrial relevance. Already the research community has identified many keratinases and reductases in *S. fradiae* (Gupta and Ramnani 2006), but we wanted to determine if lipolytic enzymes capable of hydrolyzing the waxy feather lipids were also present. Multiple strains of *Streptomyces* have been previously characterized to utilize beeswax found on ancient artwork as a sole carbon source (Sakr et al. 2013). Given that *S. fradiae* var. k11 is of the same genus, it may be reasonable to suspect *S. fradiae* var. k11 also produces enzymes capable of catalyzing the hydrolysis of wax esters found on chicken feathers.

A wax ester is a fatty acid esterified to a fatty alcohol and is critical for multiple physiological events. Wax esters are used by some plants and marine organisms as an energy storage (Leray 2006). Often on the epicuticular or epidermal layers of plants and animals, wax esters provide defense against pathogens and desiccation (Leray 2006). Wax esters are also a buoyancy regulator in fish (Phleger 1998). The unique properties of wax esters have significant industrial potential for use in cosmetics (Schlossman and Shao 2014), pharmaceuticals (Coffey 2012; El Mogy 2004), art, food products (Hepburn et al. 2000), fuel (Al-Widyan and Al-Muhtaseb 2010), and as a lubricants (Bhatia et al. 1990; Kalscheuer and Steinbuchel 2003). There have been multiple groups that have focused on the

biosynthesis of wax esters (Heilmann et al. 2012; Rodriguez et al. 2014; Santala et al. 2014; Trani et al. 1991; Tsujita et al. 1999), but studies focused on the hydrolysis of wax esters have been few. The literature contains a few examples of wax ester hydrolases (EC 3.1.1.50) from plant (Huang et al. 1978; Kalinowska and Wojciechowski 1985; Moreau and Huang 1981), fungal (Brahimihorn et al. 1989), marine (Benson et al. 1975; Kayama et al. 1979; Mankura et al. 1984; Patton et al. 1975), and bacterial (Kalscheuer and Steinbuchel 2003) origins have previously been reported, however kinetic information is scant (Huang et al. 1978; Kalinowska and Wojciechowski 1985).

The identification and characterization of wax ester hydrolases has utility in the development of biotechnology to process agricultural byproducts with waxy surface lipids, such as wool (Brahimihorn et al. 1991) and feathers. Moreover, the similar physical characteristics of waxes and plastics (solid, insoluble) has facilitated in the discovery of a wax worm digesting plastic bags (Bombelli et al. 2017) or cutinase enzymes (EC: 3.1.1.74) hydrolyzing polyethylene terephthalate (PET) (Guebitz and Cavaco-Paulo 2008). Our report identified, and characterized, one wax ester hydrolase from a feather-degrading actinomycete with wax ester hydrolase activity.

## **Materials and Methods**

### **Strains and chemicals**

Chemicals were purchased from Sigma Aldrich (St. Louis, Missouri, USA) unless otherwise noted. Bacteria strain *Streptomyces fradiae* var. k11 was gifted to the lab from collaborators (Meng et al. 2007).

### **Cloning of wax esterase genes**

*S. fradiae* var. k11 was cultured in brain heart infusion broth and genomic DNA was isolated as described previously by Nicodinovic, et al (Nikodinovic et al. 2003). Previous work in the lab has yielded a draft genome for *S. fradiae* var. k11. The genome was annotated on the Rapid Annotation using Subsystem Technology (RAST) server using the RASTClassic annotation scheme to identify potential lipolytic enzymes (Aziz et al. 2008).

Additional enzymes were identified with patterns PDOC00110, PDOC00842, and PDOC00903 from the PROSITE database (Hulo et al. 2006). Some of the partial sequences were completed using sequence information from Ju et al.

(Ju et al. 2015) and the previously reported *lips221* sequence (Zhang et al. 2008) was downloaded from GenBank (ID: EF429087.1) (Benson et al. 2013). Genomic DNA served as template for a polymerase chain reaction (PCR) to amplify out the genes. Pred-TAT (Bagos et al. 2010), an online signal peptide recognition tool, was used to predict the presence of any signal sequence. Oligonucleotides were designed to pull out the different genes without any native signal peptide or stop codon, while incorporating NcoI and XhoI restriction sites at the 5' and 3' ends, respectively.

The PCR reaction was performed using Platinum pfx polymerase purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). The placement of restriction sites allowed for simplified cloning of PCR products into the pET22b(+) and pET28a vectors via double digestion. Ligated constructs were cloned into *E. coli* BL21(DE3) cells and selected over LB amp<sup>+</sup> plates. Plasmid DNA was isolated using a QIAGEN (Venlo, Netherlands) Qiaprep Spin miniprep kit and the construct was verified at the Cornell Biotechnology Resource Center DNA Sequencing facility using T7 oligonucleotides. Sequencing results were analyzed against a vector map using ApE - A Plasmid Editor (Wayne Davis, Salt Lake City, Utah, USA; version 2.0.49.10) (Davis 2008).

### **Gene expression and protein purification**

Starter cultures in Luria broth (LB) were grown overnight and used to inoculate expression cultures. Expression cultures used either LB, terrific broth (TB), or autoinduction media (Teknova, Hollister, California, USA) and were grown at 37°C to OD<sub>600</sub> of 0.6-0.8, then placed into a 16°C, shaking incubator for 22 hours. Cultures in LB or TB were induced with 0.1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG).

Protein was harvested by first collecting the cells via centrifugation (5000 × *g* for 5 minutes). Cells were resuspended in lysis buffer (20 mM HEPES, 500 mM NaCl, 10 mM imidazole, pH 7.6). Cells were then sonicated for 8 minutes using 4 second pulses at 45% amplitude on a Sonics (Newtown, Connecticut, USA) Vibracell sonicator (model: VC 130). The disrupted cells were then centrifuged at 15,000 × *g* for 20 minutes and the total soluble protein was mixed with Ni-NTA agarose (McLab, South San Francisco, California, USA) for 1 hour on a rolling shaker at 4°C. The slurry was then loaded into a gravity flow column and allowed for the agarose to settle. Upon the agarose settling, the column was uncapped and the flow-through was collected. The Ni-NTA agarose was then washed and collected three times with wash buffer (20 mM HEPES, 500 mM NaCl, and 20 mM imidazole, pH

7.6). Protein bound to the Ni-NTA agarose was then eluted with elution buffer (20 mM HEPES, 500 mM NaCl, and 250 mM imidazole, pH 7.6).

The eluted protein was then concentrated in an Amicon ultra-15 centrifuge tube with a molecular weight cut-off of 10 kDa. Concentrated protein then further purified using an ÄKTA Explorer FPLC system (GE Healthcare, USA). Size exclusion chromatography (SEC) was performed on all NiNTA purified proteins. The column employed for purification was Superdex 200 10/300 GL. Standards used to calibrate the SEC column were a lyophilized mix of thyroglobulin, bovine  $\gamma$ -globulin, chicken ovalbumin, equine myoglobin, and vitamin B12 (Molecular weight range: 1,3500-670,000 Da, pI range: 4.5-6.9) (Bio-Rad, Hercules, California, USA). Proteins were stored at 4°C in SEC buffer (20 mM HEPES, 500 mM NaCl, 5 mM CaCl<sub>2</sub>, pH 7.6) until further analyzed for activity and purity. Protein purity was determined by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) using Coomassie blue staining (Simpson 2007).

### **Enzyme activity assays**

Protein concentration was determined as previously described (Walker 2009), with the exception of the incubation taking place at 37°C instead of 60°C. Lipase assays were conducted using p-nitrophenyl palmitate as substrate. Assay conditions have been previously described (Zhang et al. 2008). Briefly, p-nitrophenyl palmitate (pNPP) was dissolved in 2-propanol to make a 10 mM solution. A reaction buffer was prepared (50 mM sodium phosphate, pH 8.0, 0.1% gum arabic, 0.2% sodium deoxycholate) and mixed with the pNPP solution in a 9 to 1 ratio. Enzyme sample (10  $\mu$ L) was loaded into a 96-well microtiter plate and 240  $\mu$ L of the substrate solution was added immediately before taking a kinetic read for 2 minutes with 15 second intervals using a wavelength of 410 nm on a SpectraMax M2e spectrophotometer (Molecular Devices, Sunnyvale, California, USA). One enzyme unit was defined as the hydrolysis of 1.0  $\mu$ mole of p-nitrophenyl palmitate per minute at pH 8.0 at room temperature. Other p-nitrophenyl ester substrates (butyrate, decanoate, and stearate) were used interchangeable with pNPP for the assay.

Wax ester hydrolase activity was determined using three different wax substrates: beeswax, cetyl palmitate, and jojoba oil. Wax substrate was solubilized in 2-propanol, using a heat block when necessary and mixed with the same reaction buffer from the pNPP assay and at the same ratio. The substrate solution was then added to a microcentrifuge tube that contained the enzyme sample and was incubated in a 37°C, shaking incubator for up to 1

hour. Released fatty acid products from wax ester hydrolysis were then detected using reagents from the NEFA-HR(2) kit from Wako Chemicals (Richmond, Virginia, USA). Briefly, in a 96-well microtiter plate an 8  $\mu\text{L}$  sample from the enzyme reaction was mixed with 144  $\mu\text{L}$  color reagent A, which contained coenzyme A (CoA), acyl-CoA synthetase, and adenosine triphosphate and resulted in the formation of acyl-CoA. After a 5-minute incubation at 37°C, the microtiter plate was read at  $A_{550}$ . Color reagent B (acyl-CoA oxidase, peroxidase, 3-methyl-ethyl-N-( $\beta$ -hydroxyethyl)-aniline(MEHA), and 4-aminoantipyrine) was then added (48  $\mu\text{L}$ ), which oxidized the acyl-CoA into hydrogen peroxide, thus allowing MEHA to undergo oxidative condensation with 4-aminoantipyrine and create a purple colored product. After a 5-minute incubation at 37°C, a second  $A_{550}$  reading was made. A standard curve was created using varying concentrations of oleate and an enzyme unit was defined as the release of 1 millimole-equivalent free fatty acid per minute per milligram of enzyme.

Thin layer chromatography (TLC) was performed as described previously (Yamada et al. 2016). Briefly, an enzyme reaction with a wax substrate was set up as described earlier. The reactions were then incubated in a 37°C shaking incubator for 1 hour. Methanol (100  $\mu\text{L}$ ) was added to the sample and vortexed. Chloroform (200  $\mu\text{L}$ ) was then added and the sample was again vortexed. A sample collected from the chloroform layer was used to spot a HPTLC silica gel 60 TLC plate and double-developed with eluent hexane: diisopropyl ether: acetic acid (50:50:1). The plate was dried and then sprayed with coloring agent (27.4 mM 12-molybdo(VI) phosphoric acid n-hydrate and 50 mM sodium perchlorate dissolved in ethanol). The plate was then heated in an oven for 2 minutes until the bands were revealed. A separate reaction was set up with a heat-deactivated enzyme and was performed concurrently with the reaction containing the active enzyme. The enzyme was heated to 65°C for 10 minutes to fully deactivate it.

Data analysis was carried out using the Pandas module in python (McKinney 2011). Plots were generated using Matplotlib (Hunter 2007).

## Results

### Identification of lipolytic enzymes from *Streptomyces* genome

From a draft genome sequence of feather-degrading actinomycete *S. fradiae* var. k11, fourteen genes were identified as potential lipases from annotations and ProSite patterns. Four genes, out of the pool of 14, had detectable

enzymatic activity against p-nitrophenyl acetate or pNPP (Table 1). Lipase activity was observed only in two of the four identified enzymes (SFK3087 and SFK3309), as demonstrated by their ability to hydrolyze pNPP. Both SFK3309 and SFK3087 were tested against the wax ester substrate jojoba oil, but only SFK3309 retained activity and further characterization of SFK3309 was warranted. **Sequence characterization of SFK3309**

The cloned construct of SFK3309 in the pET22b(+) was verified via Sanger sequencing. Gene sequences of SFK3309 and lips221 which served as the gene template for cloning were aligned (Fig. 1). The translated sequence codes for four alternative residues from lips221: P86L, L88P, N223S, and L237P.

### **Expression optimization and purification**

Initially, culturing pET22b SFK3309 in *E. coli* BL21(DE3) cells appeared to have some toxicity as indicated by a reduced OD<sub>600</sub> at harvest time compared to that of the time of IPTG induction. Cultures expressing SFK3309 were tested using different growth media for optimization: Luria broth (LB), terrific broth (TB), and Studier's ZYM-5052 autoinduction media (AT). Not only did the cell density of cultures grown in AT increase compared to LB and TB (OD<sub>600</sub> ~6.0), the enzyme activity in the lysate was ~3X higher, as seen in Figure 2.

The C-terminal His tag on the pET vector was used for the first purification step of SFK3309. After eluting SFK3309 from the Ni-NTA agarose column, the protein was run on an SDS-PAGE gel and stained using Coomassie blue (Fig. 3). A subsequent gel filtration step was used and separated the multimeric protein forms.

### **Enzyme activity analysis**

Substrate chain-length preference of SFK3309 was determined by the release of nitrophenol from synthetic substrates with varying length carbon-chains esterified to nitrophenyl. Substrates included: para nitrophenyl-butyrate (C4), para nitrophenyl-decanoate (C10), para nitrophenyl-palmitate (C16), and para nitrophenyl-stearate (C18). Preference of SFK3309 for the shorter carbon chain of para nitrophenyl-butyrate was evident (Fig. 4).

Kinetic studies of SFK3309 with cetyl-palmitate as substrate were performed. A Lineweaver-Burk plot was used for the determination of the  $K_m$  and  $V_{max}$  values (Fig. 5). The  $K_m$  was worked out to be  $8.5 \times 10^{-3}$  M and the  $K_{cat}$

was  $11.55 \text{ s}^{-1}$ , yielding a  $K_{cat}/K_m$  value of  $1.35 \cdot \text{mMs}^{-1}$  (Table 2). Visual hydrolysis of cetyl-palmitate, alongside two more substrates, jojoba oil and beeswax, was carried out using thin layer chromatography (Fig. 6).

## Discussion

An initial set of 14 gene candidates were identified in feather-degrading bacterial strain *S. fradiae* var. k11 to encode for lipolytic activity. From this set, four enzymes expressed successfully in *E. coli* BL21 (DE3) and had detectable activity towards pNPA or pNPP (Table 1). The simplicity of the pNPP and pNPA assays allowed for a rapid identification of active enzymes and permitted the differentiation of enzymes capable of hydrolyzing long-chain, water-insoluble substrates (pNPP) or only active towards short-chained water-soluble substrates (pNPA). As the physical properties of wax suggest, it was necessary to identify the enzymes capable of acting on the water-insoluble substrate. Two enzymes were capable of hydrolyzing pNPP: SFK3087 and SFK3309.

The Lipase Engineering Database (LED) (Fischer and Pleiss 2003) classified SFK3087 as part of the alpha-beta hydrolase superfamily 25, while the ESTHER database (Lenfant et al. 2013) classified the enzyme as a member of the lipase class 1 and polyesterase-lipase-cutinase family. SFK3309 was classified by LED to the alpha-beta hydrolase 16 superfamily and the ESTHER database suggested the enzyme belongs to the lipase class 2 family. Given SFK3087 was classified as a member of the polyesterase-lipase-cutinase family, it seemed likely the enzyme would perform well against longer-chained substrates, such as plant cutin. However, only SFK3309 was active against wax substrates, and not SFK3087. Therefore, SFK3309 was selected as the central enzyme of the study.

DNA sequence analysis of SFK3309 cloned in the pET22b vector indicated four missense mutations (P85L, L87P, N223S, and L237P) from the lips221 sequence, as shown in the MAFFT (Katoh and Standley 2013) alignment (Fig. 1). Secondary structure prediction of lips221 by JPRED4 (Drozdetskiy et al. 2015) suggested all SFK3309 mutations fell in loop regions and are less probable to severely disrupt overall protein structure.

A pelB signal sequence on the pET22b vector directed SFK3309 to the periplasm to provide an ideal environment for secretory protein expression (Boock et al. 2015). Expression of SFK3309 was carried out in *E. coli* BL21 (DE3) cells. Lipolytic activity in the supernatant as well as a decrease in final OD<sub>600</sub> suggested some level of toxicity from expressing SFK3309 in the cells, a familiar issue in heterologous lipase expression (Drouault et al. 2000). Three different growth media were evaluated for improved SFK3309 expression: LB, TB, and Studier's



ZYM-5052 media. LB is standard media found in labs, while TB provides added glycerol and uses a phosphate buffer (Boock et al. 2015), an understandable advantage considering free fatty acid products from lipolysis can affect pH. Studier's ZYM-5052 media is also buffered, but was able to further improve protein titers through using a blend of carbon sources and bypassing the need for IPTG induction for stable expression (Studier 2005). Expression experiments demonstrated LB and TB activity in the cell lysate was comparable, while Studier's ZYM-5052 medium improved expression levels about 3-fold (Fig. 2).

By using the C-terminal 6X-histidine tag in the pET22b vector SFK3309 was purified from the cell lysate and able to reach approximately 90% purity from one round of Ni-NTA purification as demonstrated from SDS-Page (Fig. 3). Yields of purified protein for 250 mL cultures were about 2 mg. A subsequent size-exclusion chromatography step was used to further separated SFK3309 from different oligomeric states. While Zhang et al. struggled with *E. coli* BL21 (DE3) as a heterologous host for extracellular expression of lipS221 (Zhang et al. 2008), our experience suggests reasonable protein expression levels can be achieved in *E. coli* BL21 (DE3) for SFK3309 by coupling periplasmic expression with autoinduction media. The reported  $K_m$  of 0.139 mM of lipS221 expressed in *Pichia pastoris* (Zhang et al. 2008) was comparable to the  $K_m$  of 0.078 mM that our group produced with SFK3309 against pNPP. The  $K_{cat}$  of SFK3309 was determined to be  $1668 \text{ s}^{-1}$ , which was within reason of values reported by similar enzymes (Dheeman et al. 2011).

SFK3309 was evaluated for wax ester hydrolase activity using three different substrates: jojoba oil, beeswax, and cetyl palmitate. Jojoba oil is derived from the seeds of *Simmondsia chinensis*, a plant native to the southwestern United States. The major lipid form in jojoba oil are wax esters, with the dominant carbon chain length being  $C_{40}$  and  $C_{42}$  (Bussonbreyse et al. 1994). Jojoba oil was an ideal substrate to work with initially due to the low melting point ( $10^\circ\text{C}$ ) allowing for easy handling and the negligent levels of free fatty acids made the detection of reaction products unambiguous. Beeswax is produced by honey bees and is 35% composed of wax monoesters with mostly  $C_{40} - C_{48}$  chain lengths (Tulloch 1971) and myricyl-palmitate as the main component (Riemenschneider and Bolt 2005). The higher melting temperature ( $63^\circ\text{C}$ ) of beeswax required heating in order to dissolve into 2-propanol, and the raw substrate further tested enzyme utility. Cetyl-palmitate was the third wax substrate used for analysis of wax-ester hydrolase activity. Cetyl-palmitate, like beeswax is a solid at room temperature (melting point =  $54^\circ\text{C}$ ) and thus required similar handling. However, given cetyl-palmitate is easily obtained as a pure substrate, wax-ester hydrolase kinetic experiments were possible.

Previous groups have reported wax ester hydrolase assays that rely on radioactively labelled substrates (Bogevik et al. 2008; Brahimihorn et al. 1989; Tsujita et al. 1999), which require resources not accessible to many labs. Others have also reported colorimetrically detecting free fatty acid products by first converting the fatty acids to copper salts and measured with sodium dithiocarbamate (Heinen and De Vries 1966; Huang et al. 1978) but these assays are time consuming and often rely on using strong organic solvents such as chloroform, thus limiting the use of plastic ware. The wax ester hydrolase assay used for this study first incubates an enzyme with a standard wax substrate, then utilizes the Wako NEFA HR(2) commercial kit to detect released free fatty acids. The entire assay can be performed in a basic lab equipped with a spectrophotometer using standard microtiter plates and microcentrifuge tubes and does not require an extraction step. The linear detection range of free fatty acids using the Wako NEFA HR(2) kit is 0.01-4.0 mEq/L. Since the standard curve is constructed using oleic acid, the millimole-equivalent unit is used to denote any free fatty acid released. Jojoba oil was used initially as a substrate to confirm the reliability of the assay.

SFK3309 exhibited enzyme activity against all three of the wax ester substrates using the described colorimetric assay. TLC was used to confirm the observed enzyme activity. From Figure 6, the active enzymes produced thicker lower bands, corresponding to the hydrolysis of the substrate, though beeswax was an anomaly, which may be due to a high level of background. Enzyme kinetics were determined using cetyl-palmitate as substrate. Two groups have previously reported on the kinetics of wax-ester hydrolases and their results are available for comparison in Table 2. In Huang et al.'s study the synthetic substrate N-methylindoxyl myristate was used for kinetic experiments of an enzyme from jojoba cotyledons (Huang et al. 1978). Their work found the enzyme's  $K_m$  value to be  $9.3 \times 10^{-5}$  M, within close proximity to the work of Kalinowska and Wojciechowski, who used cetyl [ $1-^{14}\text{C}$ ]palmitate as substrate and reported a  $K_m$  of  $2.8 \times 10^{-5}$  M for an enzyme from the roots of white mustard (Kalinowska and Wojciechowski 1985). The constructed Lineweaver-Burk plot from our study suggests a good linear fit for our data (Fig. 5). The  $K_m$  of SFK3309 was determined to be  $8.5 \times 10^{-4}$ , which is close to the other groups' values. We also found the  $K_{cat}$  of SFK3309 to be  $11.63 \text{ s}^{-1}$

In conclusion, we identified two lipases from the feather-degrading bacteria *S. fradiae* var. k11 and characterize one of the enzymes as a wax-ester hydrolase. The described wax-ester hydrolase assay from our study allowed for the evaluation of enzyme activity against various wax substrates and permitted a kinetics experiment against cetyl-palmitate. Given the presence of waxy lipids on the surface of chicken feathers, the existence of wax-

ester hydrolase activity from SFK3309 in *S. fradiae* var. k11 suggests a potential application in industrial feather hydrolysis.

Current literature on the enzymatic hydrolysis of wax esters is limited, with kinetic studies even more scarce. The adaptation of the wax ester hydrolase assay as reported here allows for a more accessible approach to evaluate wax ester hydrolase activity and yield results comparable with traditional techniques. Waxes are an important lipid class and have a presence across multiple industries. A more comprehensive investigation of enzymes capable of hydrolyzing wax esters may contribute to the development of existing and novel industrial processes.

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### **Compliance with Ethical Standards**

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Conflict of Interest: M.B. declares that he has no conflict of interest. D.M. declares that he has no conflict of interest. X.G.L. declares that he has no conflict of interest.

Ethical Approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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**Fig. 1** SFK3309 alignment with lips221. Using MAFFT (Katoh and Standley 2013) the top performing lipase from this study (SFK3309) was aligned to lips221 (GenBank ID: EF429087.1), a highly similar sequence initially reported by Zhang, et al (Zhang et al. 2008). Four missense mutations were present between the two sequences: P86L, L88P, N223S, and L237P. Alignment was visualized in Chimera (Pettersen et al. 2004)

**Fig. 2** Optimal growth media for SFK3309. Luria broth (LB), Terrific broth (TB), and Studier's ZYM-5052 autoinduction media (AT) were tested for impact on protein expression. Cultures were grown as described in *Materials in Methods*. Assays were performed in duplicate and the standard deviation is represented by the error bars. Both LB and TB had comparable activity in the lysate ~22 hours post IPTG induction while AT had 2.9X the activity in the lysate at the same time period

**Fig. 3** Coomassie blue stained SDS-PAGE gel of his-tag purified SFK3309. The left lane is the Bio-Rad broad range protein marker and its bands are labelled with their respective molecular weights. The right lane is the purified SFK3309 (29.6 kDa) eluted from the Ni-NTA agarose (see Materials and Methods)

**Fig. 4** Substrate chain-length preference of SFK3309. Purified SFK3309 was evaluated for enzyme hydrolysis activity against the chromophore p-nitrophenol (pNP) esterified to different carbon-chain length carboxylates: butyrate (C4), decanoate (C10), palmitate (C16), and stearate (C18). Reaction conditions were carried out at room temperature buffered with 0.05 M sodium phosphate, pH 8.0 and performed in duplicate. The average activity of SFK3309 against pNP-Butyrate was considered absolute (100%) and the standard deviation was represented by the error bars. The data suggests SFK3309 has preference for the shorter-chained substrate, yet retains broad specificity, allowing the enzyme to retain activity against all the substrates tested

**Fig. 5** Lineweaver-Burk plot for kinetics calculations of cetyl-palmitate hydrolysis by SFK3309. Cetyl-palmitate concentrations ranged from 0.1 to 5 mM. Reactions were carried out at 37°C for 5 minutes. The concentration of SFK3309 was 0.206 mg/mL. All reactions were performed in triplicate and the average values were plotted. The  $K_{cat}$  was determined to be  $11.63 \text{ s}^{-1}$  and the  $k_{cat}/k_m$  was  $13.74/\text{mM}\cdot\text{s}^{-1}$ . The plot was made in python using Matplotlib (Hunter 2007)

**Fig. 6** Thin layer chromatography of SFK3309 wax hydrolysis products. Wax-ester hydrolase assays were set up with substrates jojoba oil, cetyl-palmitate, and beeswax and treated with active SFK3309 (A) or SFK3309 that had been heat-deactivated (B). The samples with active SFK3309 appeared to create more products than the heat-deactivated SFK3309 against jojoba oil and cetyl-palmitate as represented in the two thicker lower bands, though the differences in beeswax were negligible

Table 1 Lipolytic activity screen for genes from *Streptomyces fradiae* var. k11

ID	Annotation	Vector	Activity
SFK0710	esterase/lipase/thioesterase	pET22b	-
SFK0767	putative ATP-dependent RNA helicase	pET28a	-
SFK1518	putative lipase/esterase	pET28a	Esterase
SFK2174	lipase/acylhydrolase	pET22b	Esterase
SFK2553	thioesterase	pET28a	-
SFK3087	triacylglycerol lipase precursor	pET22b	Lipase
SFK3128	hypothetical protein	pET28a	-
SFK3309	putative secreted lipase	pET22b	Lipase
SFK3682	lysophospholipase L1	pET22b	-
SFK4182	Endo-1,4-beta-xylanase A precursor	pET22b	-
SFK4461	putative hydrolase	pET28a	-
SFK4667	hypothetical protein	pET28a	-
SFK5000	cholesterol esterase	pET22b	-
SFK5309	lysophospholipase L1	pET22b	-

Genes were amplified from genomic DNA in a PCR reaction with gene-specific primers, then cloned into pET22b and pET28a vectors and expressed in *E. coli* BL21(DE3) cells. Activity in the supernatant and cell lysate was tested for hydrolysis of p-nitrophenyl palmitate to represent lipase activity and p-nitrophenyl acetate hydrolysis to represent esterase activity. Four of the fourteen constructs resulted in gene expression with detectable activity. If no activity was detected then samples were marked with a minus sign

Table 2 – Comparison of reported wax-ester hydrolysis kinetics values.

$K_m$ (M)	$K_{cat}$ (1/s)	$K_{cat}/K_m$ (1/mMs <sup>-1</sup> )	Organism	Reference
$2.8 \times 10^{-5a}$	--	--	<i>Sinapis alba</i> L.	(Kalinowska and Wojciechowski 1985)
$9.3 \times 10^{-5b}$	--	--	<i>Simmondsia chinensis</i>	(Huang et al. 1978)
$8.5 \times 10^{-4c}$	11.63	13.7	<i>S. fradiae</i> var. k11	Current study

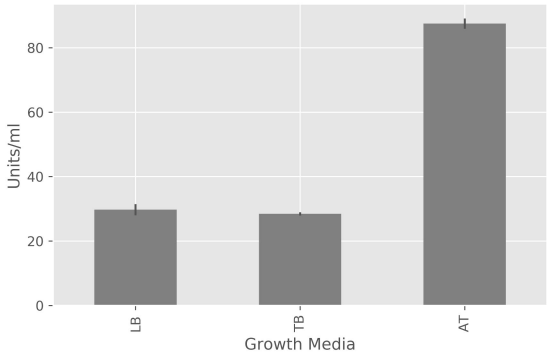
<sup>a</sup> Radiolabeled cetyl-palmitate was used as substrate in this study.

<sup>b</sup> N-methylindoxyl myristate was used as substrate.

<sup>c</sup> Unlabeled cetyl-palmitate was used as substrate.



ATPPAATDR	11 TAATAATAGA	21 SGAGWNDYAC	31 KPSAAHPRPV	41 VLVHGT
ATPPAATDR	TAATAATAGA	SGAGWNDYAC	KPSAAHPRPV	VLVHGT
ATPPAATDR	TAATAATAGA	SGAGWNDYAC	KPSAAHPRPV	VLVHGT
DNWLGLAPY	61 LVRRGYCVFS	71 LDYGQLPGVP	81 FFHG p p VAE	91 SAGQLD
DNWLGLAPY	LVRRGYCVFS	LDYGQLPGVP	FFHGPGLVAE	SAGQLD
DNWLGLAPY	LVRRGYCVFS	LDYGQLPGVP	FFHGLGPVAE	SAGQLD
VLGATGARE	111 VDIVGHSQGG	121 MMPRHYLKFL	131 GGAEKVNALV	141 GIAPDN
VLGATGARE	VDIVGHSQGG	MMPRHYLKFL	GGAEKVNALV	GIAPDN
VLGATGARE	VDIVGHSQGG	MMPRHYLKFL	GGAEKVNALV	GIAPDN
HGLTRLLPH	161 FPGAADLISA	171 KTPALADQIA	181 GSPFLQRLNE	191 GGDTV
HGLTRLLPH	FPGAADLISA	KTPALADQIA	GSPFLQRLNE	GGDTV
HGLTRLLPH	FPGAADLISA	KTPALADQIA	GSPFLQRLNE	GGDTV
VIATRYDQV	211 VTPYRSSFLD	221 GP s VRNVLLQ	231 DLCPLD p SEH	241 LAIGLV
VIATRYDQV	VTPYRSSFLD	GPNVVRNVLLQ	DLCPLDLSSEH	LAIGLV
VIATRYDQV	VTPYRSSFLD	GPSVRNVLLQ	DLCPLDPSSEH	LAIGLV
HEVANALDP	261 ARATPTTCAS	271 VLG - - - - -	281 -	
HEVANALDP	ARATPTTCAS	VLG - - - - -	-	
HEVANALDP	ARATPTTCAS	VLGLEHHHHH	H	



202 kDa

114 kDa

83 kDa

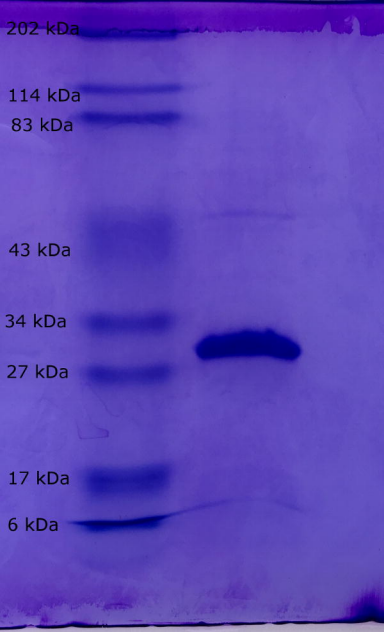
43 kDa

34 kDa

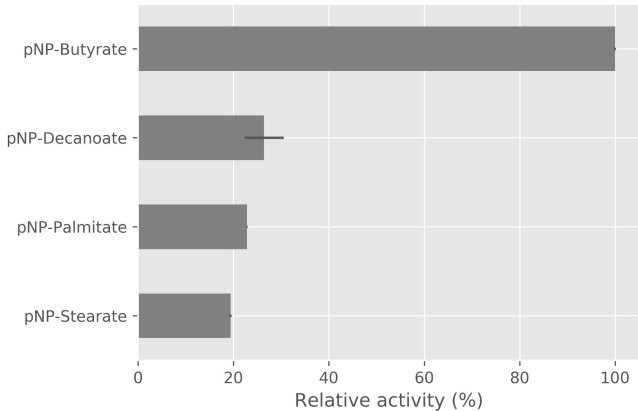
27 kDa

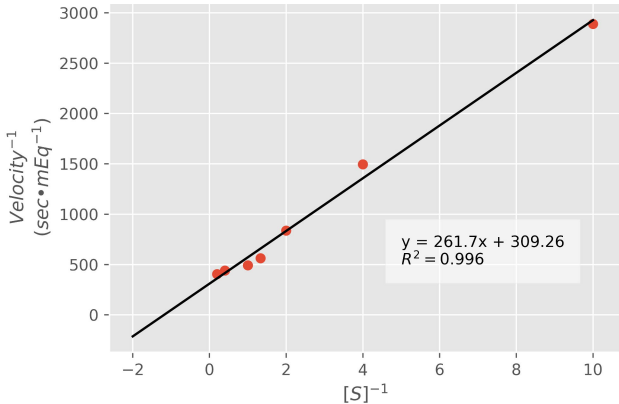
17 kDa

6 kDa









**Jajoba Oil**    **Cetyl-Palmitate**    **Beeswax**  
A            B            A            B            A            B

