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Screening and characterization of lipase-producing, wax-degrading bacteria isolated from cotton mealybugs

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ABSTRACT

Lipases (EC 3.1.1.3) are triacylglycerol acylhydrolases that hydrolyze triglycerides to glycerol and fatty acids. The numerous applications of lipases in chemical, food, pharmaceutical, biotechnological, detergents and surfactants, and agricultural industries and in waste treatment plants proved their importance. In this study, 23 wax-degrading bacterial strains isolated from four cotton mealybug species were screened for lipase production. On the basis of the 16S rRNA gene sequences, lipase-positive strains were classified into six genera, namely, *Pseudoxanthomonas*, *Acinetobacter*, *Klebsiella*, *Providencia*, *Enterobacter*, and *Serratia*. *Acinetobacter lwoffii* PSAD2 and *A. beijerinckii* PSAD7 isolates showed the maximum lipase production of 2.3 and 1.7 U/mL, respectively in the quantification process. The analysis of fatty acid methyl esters showed that *A. lwoffii* PSAD2 cells produced monounsaturated and hydroxyl fatty acids, whereas *A. beijerinckii* PSAD7 produced iso- and anteiso-methyl branched fatty acids. In addition, the growth curve analysis confirmed that lipase production is associated with the growth phase of the isolates. The results of this study will be helpful in developing lipase-based microbial insecticides for the management of waxy cuticle-protected insect pests.

Key words: Lipase, wax, bacteria, mealybug, 16S rRNA, fatty acid methyl esters.

INTRODUCTION

Wax (derived from Anglo-Saxon word *weax*), a type of lipid, is technically an ester of long-chain fatty acids with long-chain fatty alcohols (Jetter et al., 2006). The wax esters, sterol esters, fatty alcohols, diols, ketones, aliphatic aldehydes, beta-diketones, triacylglycerol, and other basic biochemical constituents of wax vary with their origin. Essentially, plant and insect waxes are made of wax esters, alkyl esters, fatty acids, long-chain alcohols, aldehydes, ketones, beta-diketones, hydroxy-beta-diketones, and triacylglycerols (Hansen et al., 1997; Gołębiowski et al., 2011); however, the combination and composition of wax vary greatly between plants and insects (Nguyen et al., 2014; Arunkumar et al., 2018). After serving their intended purpose mainly as a protective layer, wax residues from living organisms are degraded by a group of opportunistic wax-degrading microorganisms present in the environment. Waxes are hydrolyzed by these microorganisms by pseudosolubilization, microbial surfactant production, or the secretion of extracellular microbial lipases (Roper, 2004).

Lipases are glycerol ester hydrolases (EC 3.1.1.3) produced by plants, animals, and microorganisms to hydrolyze lipids by acting on the carboxyl ester bonds of triglycerides that results in fatty acids and glycerol (Madeira et al., 2017). The production of extracellular microbial lipases using agricultural residues such as rice bran, sugarcane bagasse, and wheat bran, which makes them more economical, is relatively cheaper than that of animal or plant lipase (Babu and Rao, 2007). Microbial lipases are widely used in the biodegradation of crude oil (Benelli and Rajasekar, 2017), detergents and surfactants industry, pharmaceutical and food industry, agricultural

industry, and pulp and paper industry, as they are eco-friendly and zero toxic and leave no harmful residues after action (Rajan et al., 2011).

In the agricultural industry, apart from residue management, microbial lipases are used in insect pest control along with chitinase, protease, chitin deacetylase, beta-1,3-glucanase, and chitosanase. Mealybugs contain powdery waxy coating all over the surface to protect them from desiccation and penetration of toxic chemicals (Watson and Kubiriba, 2005), which also make their cadavers a suitable habitat for the isolation of complex hydrocarbon-degrading microorganisms. From the cadavers of *Maconellicoccus hirsutus*, the pink mealybug, three novel wax-degrading isolates identified as *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Serratia marcescens* were isolated by Salunkhe et al. (2013), with focus on eco-friendly mealybug management. Wax-protected insect cuticle is made easily accessible for degradation by chitinases and proteases after the action of lipase enzyme, which was confirmed by the laboratory bioassay of *Ceratovacuna lanigera* (sugarcane woolly aphid) with fungal and bacterial lipases (Chavan, 2009). In addition, the modern day DNA-based bacterial identification and GC analysis of microbial fatty acid methyl esters (FAMEs) will be helpful in the identification and differentiation of closely related bacterial communities (Cook et al., 2003). Hence, this study aimed to screen and characterize lipase-producing, wax-degrading bacteria (WDB) isolated from mealybugs for their possible applications in future.

MATERIALS AND METHODS

Location and chemicals

The experiments were performed at the Indian Council of Agricultural Research-Central Institute for Cotton Research, Regional Station, Coimbatore, Tamil Nadu, India during the year 2017 (11.014327 °N latitude, 76.929456 °E longitude). The analytical reagent grade chemicals were used for media preparation and biochemical studies (HiMedia, Mumbai, India).

Isolation of wax-degrading bacteria from mealybug

A total of 23 WDB isolates designated as PSAD (1 to 23) were previously isolated in the laboratory (Arunkumar et al., 2017) using modified Davis minimal agar medium containing mealybug wax (2 g/L), ammonium sulfate (1.0 g/L), dipotassium phosphate (7 g/L), magnesium sulfate (0.1 g/L), and agar (15 g/L) at pH 7. Mealybug cadavers of four mealybug species, *Ferrisia virgata* Cockerell, *Phenacoccus solenopsis* Tinsley, *Drosicha mangiferae* Green and *Paracoccus marginatus* Williams and Granara de Willink, were separately immersed in the sterile saline solution (0.8%) in 50 mL centrifuge tubes, followed by vortex for 5 min. Furthermore, a suspension of 1 ml was plated on modified Davis minimal agar incubated at 32°C for 72 h, and the microbial colonies producing clear haloes of more than 10 mm (antibiotic zone scale-C PW297, HiMedia, India) after incubation were further screened for lipase production.

Screening for lipase production

Starter culture

The starter culture for the screening of all qualitative and quantitative lipases was prepared by inoculating a single colony of the isolated WDB isolates into 50-mL Erlenmeyer flasks containing 10 ml of Davis minimal broth supplemented with olive oil 2.0% (v/v) grown overnight at 28 ±2C and 120 rpm.

Rhodamine B fluorescence-based lipase assay

The enzyme production was screened by streaking a loopful of the starter culture on rhodamine B olive oil agar medium (pH 6.5) containing trypticase peptone (8 g/L), yeast extract (4 g/L), NaCl (3 g/L), agar (20 g/L), olive oil 2.0% (v/v), and rhodamine B 0.001% (w/v) for the detection of enzyme activity with three replicates for each WDB. The assay plates were incubated at 37°C, and after 36 h, each Petri dish was exposed to ultraviolet (UV) irradiation (UV-A, 350 nm) for visualizing orange haloes around the colonies.

Tributyrin agar well method for lipid hydrolysis

Lipase production by the WDB isolates was further screened using tributyrin agar (TBA) plates containing 5.0 g/L of peptone and 3.0 g/L of beef extract, 20.0 g/L of agar autoclaved and cooled to approximately 60°C, and l% tributyrin (v/v) was filter sterilized and added to the base medium. For the lipid hydrolysis assay, 40 µl of supernatant from the overnight grown starter culture was filter sterilized and inoculated into 6-mm diameter wells cut into TBA plates using a cork borer. The plates were incubated at 28 ±2°C and the zone of clearance (hydrolysis) around the colonies was observed and documented up to seven days.

Enzyme production and cell growth

The 1% (v/v) starter culture containing 10^9 cfu/ml was inoculated into 100-ml Erlenmeyer flask containing 25 ml of medium supplemented with peptone 0.2% (w/v), $NH_4H_2PO_4$ 0.1% (w/v), NaCl 0.25% (w/v), $MgSO_4$ $7H_2O$

0.04% (w/v), $CaCl_2.2H_2O$ 0.04% (w/v), olive oil 2.0% (v/v) at pH 7.0, and a drop of Tween 80 as emulsifier with rotary shaker at 150 rpm at 28 ±2°C. After incubation, for every 12 h, each bacterial culture was centrifuged at 10,000 rpm for 20 min at 4°C, and the cell-free culture supernatant was used for the estimation of extracellular enzyme until 96 h. Simultaneously, the cell density was evaluated by measuring the optical density at 600 nm against the cell-free control.

Simplified p-nitrophenyl laurate assay for lipases

The lipase activity of the WDB isolates was assayed every 12 h using p-nitrophenyl laurate (pNPL; Sigma-Aldrich, USA) as the substrate. The samples (0.1 mL) of WDB culture supernatants were mixed with 0.9 ml of the substrate solution (containing 3 mg of pNPL dissolved in 1 mL propanol-2-ol, which was diluted in 9 ml of 50 mM Tris-HCl at pH 8.0 with 40 mg of Triton X–100 and 10 mg of gum arabic). After 30 min of incubation at 38°C, the reaction was then terminated by adding 1 ml of ethanol, and the absorbance was measured spectrophotometrically (SmartSpecTM3000 UV spectrophotometer, Bio-Rad) at 410 nm against an enzyme-free control. In addition, one lipase unit was defined as the amount of enzyme that liberates 1 µmol p-nitrophenol/min under the assay conditions described above. All enzyme assays were performed in triplicate, and the average values were calculated.

Molecular identification using 16S rDNA sequencing and phylogenetic classification

Total genomic DNA was extracted from the WDB isolates using a genomic DNA preparation kit and purified using the bacterial genomic DNA purification kit (HiMedia, India). The 16S rRNA gene from the bacterial isolates was amplified using universal eubacterial primers, 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT; Weisburg et al., 1991). The amplified products were analyzed by electrophoresis in 1.5% agarose gels. After the separation of polymerase chain reaction products in the agarose gel, the products were observed and documented using Alpha imager TM1200 gel documentation and analysis system. The band of the expected size was gel purified using spin columns and eluted using sterile Milli-Q water. Sanger sequencing was performed at Chromus Biotech Pvt. Ltd., Bangalore, Karnataka, India. The identity of 16S rRNA sequence was established by performing a similarity search against the NCBI (National Center for Biotechnology Information), EZtaxon, and DDBJ (DNA Data Bank of Japan) nucleotide sequence databases using the Basic Local Alignment Search Tool (BLASTn) program. The phylogenetic tree of WDB isolates was constructed using the 16S rRNA gene sequence of the type strains of the species obtained from the NCBI, and ClustalX program was used for multiple sequence alignment. The phylogenetic tree was constructed using the neighbor-joining method using MEGA software package version 4.0, and bootstrapping was used to estimate the reliability of the phylogenetic reconstructions (1,000 replicates).

Fatty acid analysis

The WDB isolates were grown overnight in 10 ml of Davis minimal broth at 28 ±2°C; bacterial cells were harvested by centrifugation (6000 g, 10 min, 4°C) and washed with 0.9% NaCl to remove the residue of the culture medium from the pellets. The analysis of FAME was performed by Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India. FAMEs were separated on a gas chromatograph (Hewlett-Packard 6890) equipped with an HP Ultra 2 capillary column (25 m, 0.22 mm ID) and hydrogen as the carrier gas. By using a flame ionization detector, the FAMEs were detected and identified using MIDI Microbial Identification System software (Sherlock TSBA 6.1 method and TSBA6 library; MIDI Inc., Newark, DE, USA). For easier interpretation, the results were classified as saturated, unsaturated, hydroxyl, and branched fatty acids.

RESULTS AND DISCUSSION

A vast array of wax-degrading microorganisms exists in nature (Elisa et al., 2006); however, only a few are documented and used in commercial applications. To use these elite microorganisms in various fields, their process of wax degradation has gain attention. The WDB from mealybugs have proved their effectiveness in the microbial surfactant production, a key process involved in complex hydrocarbon degradation (Arunkumar et al., 2017b), and this study aimed to visualize the lipase-producing ability of these novel WDB strains. This study has originated from a previous study in which lipolytic microorganisms belonging to different genera, including *Bacillus*, *Brevibacterium*, *Corynebacterium*, *Staphylococcus*, *Klebsiella*, and *Stenotrophomonas*, were isolated from the silkworm *Bombyx mori* L. (Feng et al., 2011).

Screening of wax-degrading bacteria for lipase production

The fluorescence-based interaction of rhodamine B with fatty acids released during the enzymatic hydrolysis of triglycerides is an effective qualitative assay for lipase (Jette and Ziomek, 1994). The primary screening of the 23 WDB

Table 1: Screening of the wax degrading bacterial (WDB) isolates for lipase production on rhodamine B and tributyrin agar well.

WDB isolate	Rhodomine B Assay*	Maximum hydrolysis reached (days)	Activity**
PSAD 1	D	7	+++
PSAD 2	D	4	+++
PSAD 3	D	5	++
PSAD 4	ND	7	---
PSAD 5	D	7	++
PSAD 6	D	6	+++
PSAD 7	D	5	+++
PSAD 8	D	6	+++
PSAD 9	D	6	+++
PSAD 10	ND	7	---
PSAD 11	ND	7	---
PSAD 12	ND	7	---
PSAD 13	ND	7	---
PSAD 14	ND	7	---
PSAD 15	ND	7	---
PSAD 16	ND	7	---
PSAD 17	ND	7	---
PSAD 18	ND	7	---
PSAD 19	ND	7	---
PSAD 20	ND	7	---
PSAD 21	D	6	+
PSAD22	ND	7	---
PSAD23	ND	7	---

*D– an orange halo zone detected; ND– no orange halo zone detected.
** + – weak activity (zone greater than 15 mm); ++ – medium activity (zone greater than 20mm); +++ – strong activity (zone greater than 25 mm); --- – no activity (no zone).

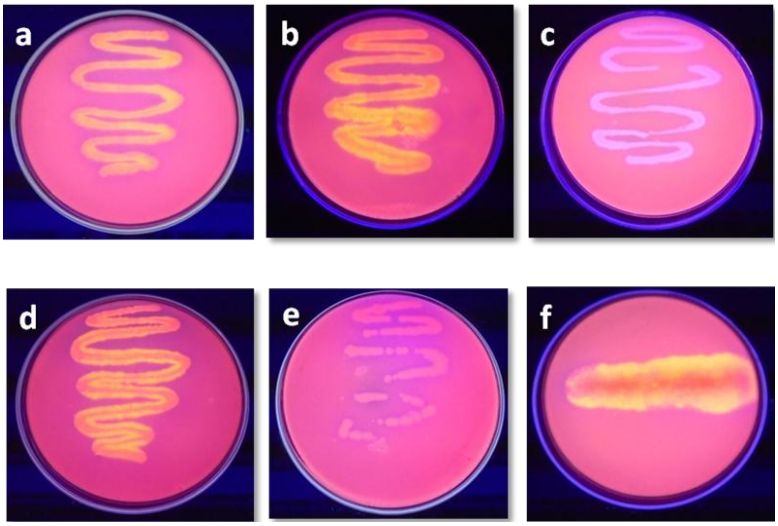

Figure 1: Screening for lipase activity on rhodamine B agar plate (**a**) PSAD1, (**b**) PSAD2, (**c**) PSAD4, (**d**) PSAD7, (**e**) PSAD16, (**f**) PSAD 21.

isolates by rhodamine B assay resulted in 9 lipase-positive bacterial isolates (PSAD1, PSAD2, PSAD3, PSAD5, PSAD6, PSAD7, PSAD8, PSAD9, and PSAD21) from *P. solenopsis* and *F. virgata* (Table 1 and Figure 1). Other 14 isolates from *D. mangiferae* and *P. marginatus* did not produce any orange halo zone, which initially proved their ineffectiveness in

lipase production. The complex formation between uranyl fatty acid ion and cationic rhodamine B during the lipid hydrolysis by lipase and further exposure to longer wavelengths such as UV radiation result in the excited dimmers of the rhodamine B complex-liberating fluorescence (Boonmahome, 2013).

Further screening using TBA well method showed that PSAD1, PSAD2, PSAD6, PSAD7, PSAD8, and PSAD9 (zone greater than 25 mm) had the highest activity, followed by PSAD3 and PSAD5 (zone greater than 20 mm), whereas PSAD21 showed the least activity (zone less than 20 mm). Lipase-producing strains can be conventionally screened on tributyrylglycerol or TBA plates, and the zone of tributyrin hydrolysis is a clear indicative of lipase activity (Gupta et al., 2003). The effectiveness of tributyrin hydrolysis was shown in the previous studies on lipase-producing microorganisms, such as *P. fragi*, *Staphylococcus aureus*, *Burkholderia glumae*, *Clostridium tetanomorphum, Lactobacillus plantarum*, *L. delbrueckii* subsp. *bulgaricus*, *L. sakei*, *L. reuteri*, *Enterococcus faecium*, and *Leuconostoc citreum* (Petersen and Daniel, 2006; Dincer and Kivanc, 2018). As PSAD4, PSAD10, PSAD11, PSAD12, PSAD13, PSAD14, PSAD15, PSAD16, PSAD17, PSAD18, PSAD20, PSAD22, and PSAD23 showed negative results in screening, and PSAD21 with least tributyrin hydrolyzing efficiency was rejected, the other eight potential strains were evaluated for lipase quantification and cell growth studies.

Comparison of cell growth and lipase production in wax-degrading bacteria

Initially, the starter cultures of the potential eight WDB isolates were grown on olive oil as a carbon source, and the lipase quantification and growth analysis were performed on a defined medium containing peptone and olive oil for accurate results, as the media supplemented with olive oil is prevalently used for the screening and quantification of lipase-positive microorganisms (Martinez and Soberon-Chavez, 2001). Further quantification of lipase production by pNPL assay showed that the isolate PSAD2 had higher lipase production of 2.3 U/mL, followed by PSAD7 (1.7 U/mL), whereas PSAD3 and PSAD5 showed least lipase production of 0.032 and 0.024 U/mL, respectively between 48 and 60 h of incubation in the given medium (Figure 2) and thereafter started declining. The finding is similar to the results obtained by Gowland et al. (1987) for *Bacillus* species (4 U/mL) and Hamid et al. (2003) for *Bacillus* species (4.58 U/mL) and *Ralstonia paucula* (3.51 U/mL). The growth of the eight screened positive WDB isolates PSAD1, PSAD2, PSAD3, PSAD5, PSAD6, PSAD7, PSAD8, and PSAD9 reached the maximum cell density at 60 h after incubation and decreased after completing the stationary phase in 72 h. The growth curve analysis of the eight positive WDB isolates confirmed that lipase production is associated with the growth phase of the isolates, and after reaching the stationary phase, the lipase production and cell growth decreased, which may be because of the depletion of nutrients, as confirmed by Biswas et al. (2016).

Molecular-based phylogeny of the wax-degrading bacterial isolates

The genomic DNA of the WDB isolates was amplified, and a phylogenetic tree constructed using NJ method with 16S rRNA amplified gene sequence, as shown in Figure 3. The phylogenetic tree inferred from 16S rRNA gene sequences confirmed that the bacterial isolates were *Pseudoxanthomonas suwonensis* PSAD1 (GenBank accession number KY780940), *Acinetobacter lwoffii* PSAD2 (GenBank accession number KY780941), *Klebsiella aerogenes* PSAD3 (GenBank accession number KY780942), *Providencia rettgeri* PSAD5 (GenBank accession number KY780943), *Enterobacter cloacae* PSAD6 (GenBank accession number KY780944), *A. beijerinckii* PSAD7 (GenBank accession number KY780945), *K. aerogenes* PSAD8 (GenBank accession number MF373207), and *S. marcescens* PSAD9 (GenBank accession number MF373208); the sequences are presently available in the NCBI GenBank database. In a previous study by Syihab et al. (2017), a thermostable and alcohol-tolerant lipase from *Pseudoxanthomonas* species was isolated from domestic compost. On the basis of 16S rRNA gene sequence analysis, 12 strains of *Bacillus, Klebsiella, Pseudomonas*, and *Enterobacter* were collected from soil and water samples and optimized for lipase production (Lin et al., 2012). Extracellular novel lipase-producing *Acinetobacter* species have been isolated from olive oil-enriched soil (Wang et al., 2011) and the subalpine region of western Himalaya, India (Kasana et al., 2008).

Cellular fatty acid profiles of the lipase-producing, wax-degrading bacterial isolates

In this study, the lipase production by *A. lwoffii* PSAD2 and *A. beijerinckii* PSAD7 was approximately ten times higher than that by other WDB isolates. The lipase production by *Acinetobacter* species and their potential applications in pharmaceutical, agricultural, biotechnology, and food industries have elaborately been reviewed by Snellman and Colwell (2004). The cellular fatty acid composition of the lipase-producing WDB isolates identified through gas chromatograph is shown in Figure 4. The cellular fatty acid composition of the *A. lwoffii* PSAD2 strain (Figure 4A) showed that the only fatty acids in the straight monounsaturated group were $C_{18:1}$w9c (33.69%) and $C_{17:1}$w8c (2.33%) and the remaining were straight saturated fatty acids $C_{16:0}$ (22.79%), $C_{17:0}$ (3.63%), and $C_{12:0}$ (3.50%) and straight hydroxyl fatty acids $C_{12:0}$3OH (3.62%) and $C_{12:0}$2OH (2.77%), and it was completely devoid of iso- and anteiso-branched fatty acids. The fatty acid profiles of

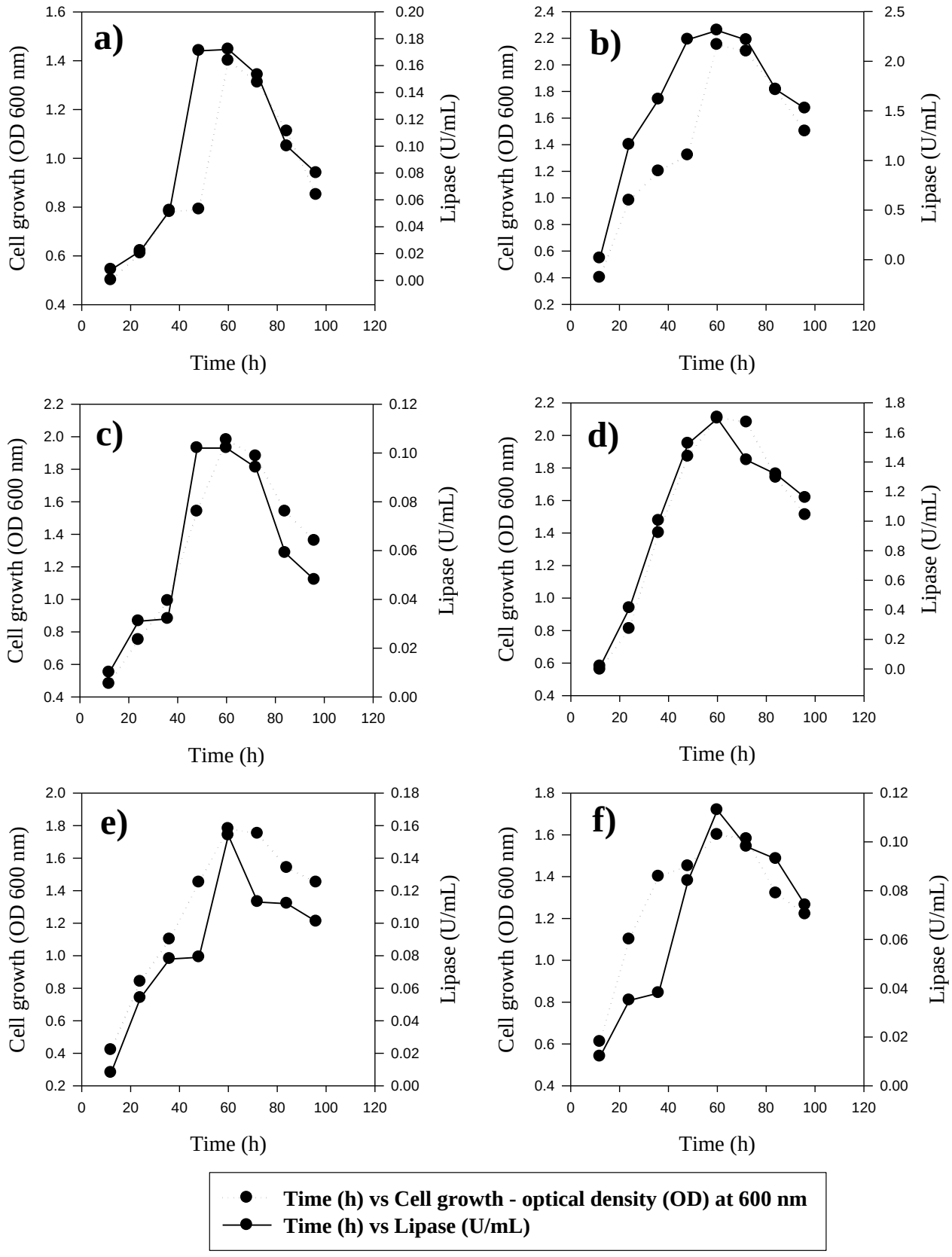

Figure 2: Comparison of cell growth and lipase production in wax degrading bacteria (WDB); **a**) PSAD1, **b**) PSAD2, **c**) PSAD6, **d**) PSAD7, **e**) PSAD8, **f**) PSAD 9.

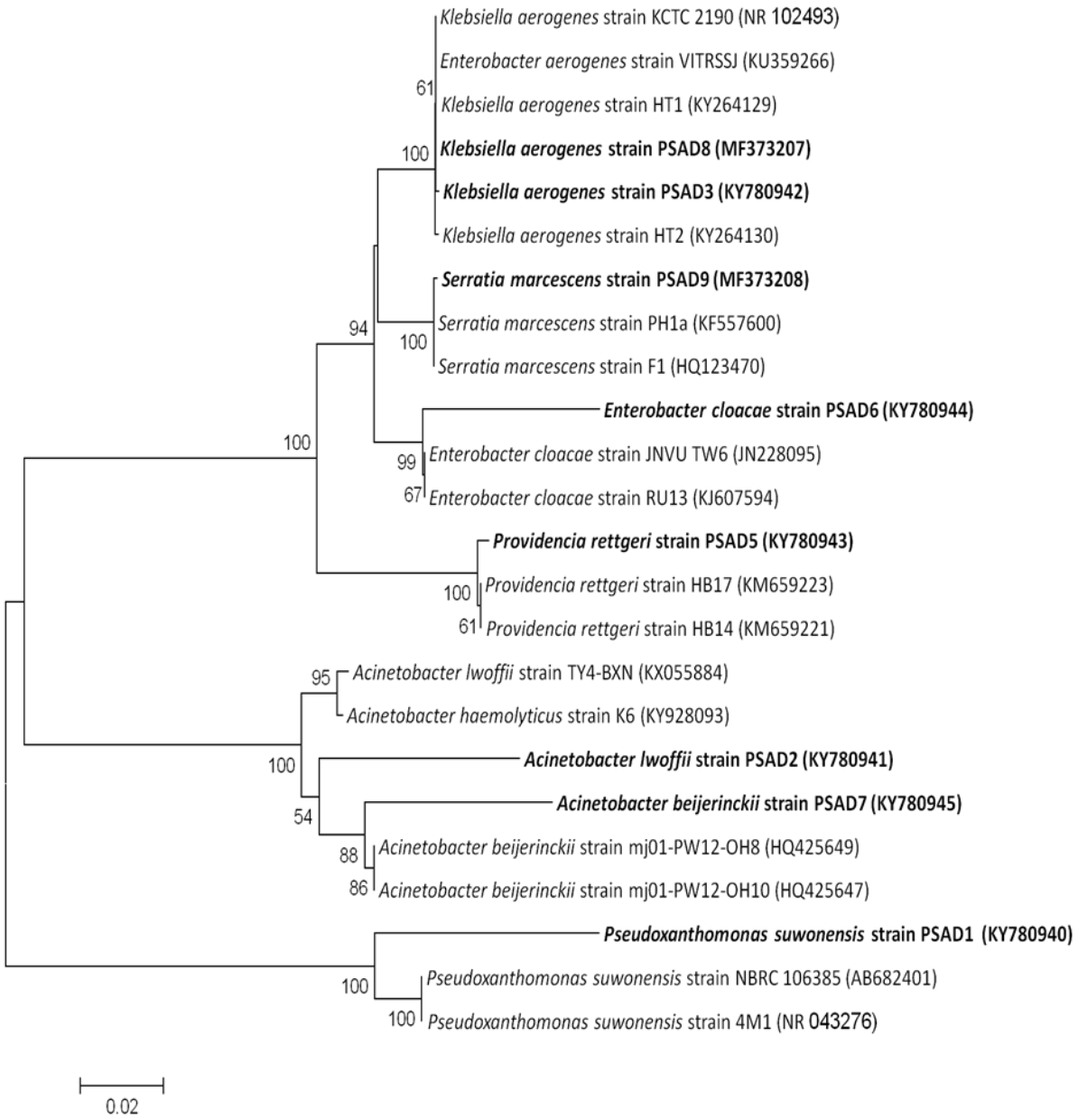

Figure 3: Phylogenetic classification of wax degrading bacteria isolated from cotton mealybugs by Neighbor-joining phylogenetic tree based on complete 16S rRNA sequences. Bar, 0.02 nucleotide changes per positions. The wax degrading bacterial strains obtained in this study are shown in bold. A bootstrap value ≥ 50 is shown.

A. beijerinckii PSAD7 cells indicated the presence of branched fatty acids such as anteiso-$C_{15:0}$ (55.56%), iso-$C_{15:0}$ (8.55%), iso-$C_{16:0}$ (8.33%), and anteiso-$C_{17:0}$ (7.15%) with few straight saturated fatty acids $C_{16:0}$ (11.93%) and $C_{14:0}$ (11.93%; Figure 4B).

The FAME results suggest that the major difference was the presence of monounsaturated and hydroxyl fatty acids in *A. lwoffii* PSAD2 strain and iso- and anteiso-methyl branched fatty acids in *A. beijerinckii* PSAD7 by which the two strains can be well differentiated on the basis of the cellular fatty acid composition profile. This finding is consistent with the results of various studies on *Acinetobacter* species for the occurrence of saturated, unsaturated, hydroxyl, and branched fatty acids (Malhotra et al., 2012). The results obtained by Santala et al. (2011) on the fatty acid composition of six different strains, namely, *A. radioresistens* DSM 6976, *A. venetianus* ATCC 31012, *A. baumannii* LMG 1041, *A. parvus* LMG 21765, *A.* LMG 998 and *A. soli* JCM 15062, confirmed the variations in fatty acids at species level.

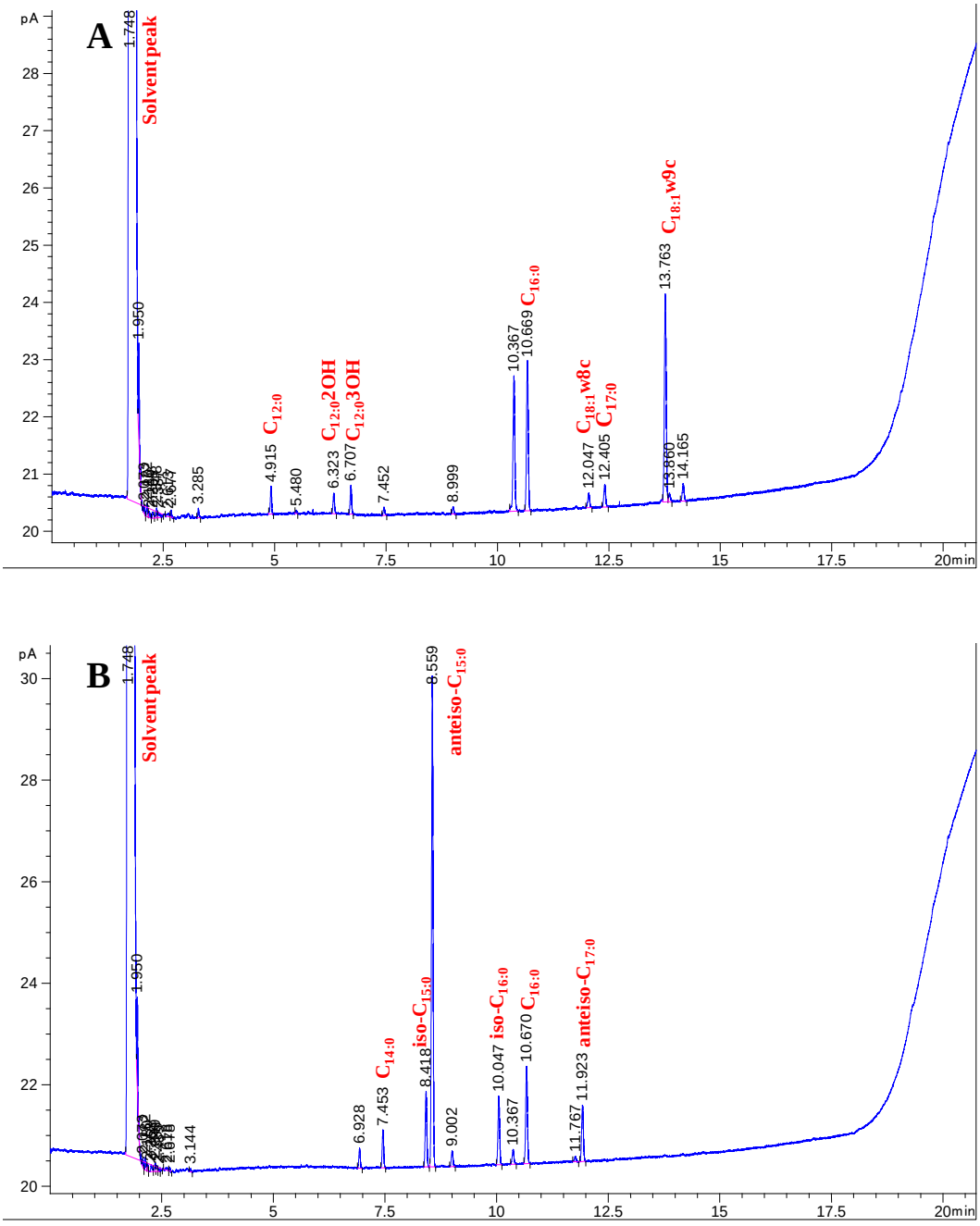

Figure 4: Cellular fatty acid profiles of (**A**) *A. lwoffii* PSAD2 and (**B**) *A. beijerinckii* PSAD7. The gas chromatogram shows the overall composition of fatty acids: saturated fatty acids (C12:0, C14:0, C16:0, C17:0, C12:02OH, C12:03OH, unsaturated fatty acids ($C_{18:1}$w9c and $C_{17:1}$w8c) and branched fatty acids (anteiso-$C_{15:0}$, iso-$C_{15:0}$, iso-$C_{16:0}$ and anteiso-$C_{17:0}$).

CONCLUSIONS

Microbial lipases are widely used in various industries, as they are economical and eco-friendly. Previously, lipases from *Bacillus*, *Pseudomonas*, *Alcaligenes*, *Arthrobacter*, lactic acid bacteria, and *Chromobacterium* were used commercially. However, the interest in lipase-producing *Acinetobacter* is recently gaining importance for their multiple applications with little implications. This study is the first to report on lipase-producing WDB from cotton mealybugs, highly confirmed with screening and identification studies. Furthermore, this study explored the potential of these lipase-producing WDB isolates in developing a potential microbial insecticide against cotton

mealybug.

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