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Identification of lipolytic enzymes isolated from bacteria indigenous to *Eucalyptus* wood species for application in the pulping industry

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ABSTRACT

This study highlights the importance of determining substrate specificity at variable experimental conditions. Lipases and esterases were isolated from microorganisms cultivated from *Eucalyptus* wood species and then concentrated (cellulases removed) and characterized. Phenol red agar plates supplemented with 1% olive oil or tributyrin was ascertained to be the most favourable method of screening for lipolytic activity. Lipolytic activity of the various enzymes were highest at 45–61 U/ml at the optimum temperature and pH of between at 30–35 °C and pH 4–5, respectively. Change in pH influenced the substrate specificity of the enzymes tested. The majority of enzymes tested displayed a propensity for longer aliphatic acyl chains such as dodecanoate (C_{12}), myristate (C_{14}), palmitate (C_{16}) and stearate (C_{18}) indicating that they could be characterised as potential lipases. Prospective esterases were also detected with specificity towards acetate (C_2), butyrate (C_4) and valerate (C_5). Enzymes maintained up to 95% activity at the optimal pH and temperature for 2–3 h. It is essential to test substrates at various pH and temperature when determining optimum activity of lipolytic enzymes, a method rarely employed. The stability of the enzymes at acidic pH and moderate temperatures makes them excellent candidates for application in the treatment of pitch during acid bi-sulphite pulping, which would greatly benefit the pulp and paper industry.

1. Introduction

Lipase and esterase are two major classes of hydrolase enzymes [1]. Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) catalyse the hydrolysis of long chain triacylglycerol substrates ($> C_8$), whereas esterases (EC 3.1.1.x) catalyse the hydrolysis of glycerolesters with short acyl chains ($< C_8$) [2]. The three-dimensional (3D) structures of both enzymes exhibit the characteristic α/β-hydrolase fold [3] a definite order of α-helices and β-sheets. The catalytic triad is comprised of Ser-Asp-His (Glu instead of Asp for some lipases) and typically also a consensus sequence (Gly-x-Ser-x-Gly) is found around the active site serine [4]. These lipolytic enzymes have been isolated from plants, animals, and microorganisms [1,5], however, microbial lipolytic enzymes are reported to be more robust in nature than plant or animal enzymes [6,7]. They are also appealing due to their low cost of production and they are simple to manipulate [1]. Some microbial species reported to produce these enzymes include *Bacillus* sp., *Pseudomonas* sp., *Burkholderia* sp., *Candida rugosa*, *Candida antarctica*, *Galactomyces geotricum*, *Saccharomyces cerevisiae*, *Yarrowia lipolytica*, *Trichosporon fermantans*, *Cryptococcus albidus*, *Aspergillus flavus*, *Thermomyces lanuginosus* and *Rhizopus oryzae* [8–13]. Due to the versatility of lipases and esterases, they have various applications in industries such as detergents, starch and fuels, food, baking, pulp and paper, fats and oils, organic synthesis, leather and environmental application [14,15].

In the pulp and paper industry, the presence of wood extractives plays a vital role. During pulping, pitch particles (composed of extractives such as triglycerides, fatty acid esters, glycosides, free and conjugated sterols) [16] tend to coalesce to form black pitch deposits in the pulp and on machinery which has a negative impact on the process and quality of pulp [17,18]. Sulphite pulps (acidic) in particular retain greater amounts of extractives in relation to kraft pulps (alkaline), as the alkaline method disbands and dissolves the wood resin [19]. The production of dissolving pulp, which is a high grade cellulose pulp, is generated using the acid bi-sulphite method.

Traditional methods for the control of pitch include seasoning and the addition of chemicals [20]. The biotechnological approach of using enzymes for pitch control is an alternative choice, especially for removal of glycerides. The treatment of pulp with lipases has been

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effective in reducing triglycerides (TG), however, steryl esters (SE) are frequently at the source of pitch formation [17]. Nonylphenol ethoxylates (NPEs) are the best chemicals for removing pitch components in chemical pulping. Unfortunately, their use is frowned upon due to their estrogen mimicking effects. Indeed, their use has been banned in North American and European chemical pulp mills as pulp handlers in European markets are reluctant to handle pulps treated with NPEs [21,22]. Also, the residual NPE in sulphite pulps are undesirable since the pulps are commonly used in pharmaceutical and food applications. Based on a mill study conducted by Sithole et al. (2010) it was suggested that the inclusion of an enzyme to target residual steryl esters could deliver a strategic solution to removing the extractives present in sulphite pulps [21].

Oxidative enzymes such as laccases have also been implemented in the degradation of various lipophilic extractives such as triglycerides, free and conjugated sterols, fatty acids and resin acids [23]. Laccases are typical for white-rot fungi and have been described as prime lignin degraders. Treatment of wood or pulp with these enzymes could offer a dual advantage in the company of redox mediators [24,25]. Redox mediators facilitate laccase removal of residual lignin, in conjunction with extensive degradation of extractives [26]. A decrease in kappa number and improved pulp brightness can also observed [19,26].

The enzymes characterized in this study are for application in the pulp and paper industry, for reduction or elimination of pitch deposit formation during pulping. Previous studies have reported the incomplete degradation of pitch by lipases [19,21], hence we are confident that the inclusion of esterases will assist in targeting the side groups that are theoretically present once the longer chain acyl chains (triacylglycerides) have been degraded by lipases. Lipases, esterases and laccases were included as part of this study and were selected based on their stability and activity at temperatures and pH levels employed during the acid bi-sulphite pulping of *Eucalyptus* wood species. To our knowledge, the lipolytic enzymes produced by microorganisms indigenous to *Eucalyptus* sp. wood have not been previously investigated. The results of the present study will provide more information on the characteristics of these enzymes and their potential for reduction of pitch components in pulps. For this study it was important to include different types of enzymes that could benefit the pulping process. Therefore purifications of the enzymes of interest were not necessary, as a cocktail of enzymes (excluding cellulases) is required and ideal in this study for the removal or degradation of all unwanted compounds (excluding cellulose). Combinations of hemicellulases, ligninases and other accessory enzymes are known to be essential for hydrolysis of plant biomass [27]. It was also important to test the effects of various conditions on substrate specificity as most researchers focus only on the pH and temperature optima of the enzyme and thereafter test substrate specificity at optimum conditions. Neglecting to investigate the effects of pH and temperature on substrate specificity of enzymes could have drastic implications for its efficiency and effectiveness. Therefore, the aim of this study was to screen indigenous microflora from *Eucalyptus* species for lipolytic activity and to determine the effects of pH and temperature on the hydrolysis of different substrates of these lipolytic enzymes (lipases, esterases and laccases).

2. Materials and methods

2.1. Isolation and identification of bacterial and fungal cultures

Five grams of wood chips from a commercial wood chip pile and individual *Eucalyptus* spp. were thoroughly washed by vortexing with 5 ml of phosphate buffer (pH 8.0) for 5 min. The washings were serially diluted and spread onto nutrient agar (NA) and potato dextrose agar (PDA) (Merck, South Africa) and incubated at 37 °C and 40 °C for 1 and 5 days, for the growth of bacteria and fungi, respectively. Colonies were selected based on morphological features; size, shape, pigmentation, margin, consistency and elevation and sub-cultured till pure isolates were obtained [28]. DNA was extracted from isolates and 16S rRNA and 18S rRNA for bacteria and fungi, respectively, were amplified according to Ramnath et al. [28]. Following PCR, the amplicons were sequenced (Inqaba Biotech, South Africa), and the sequences edited and entered in the Basic Alignment Search Tool (BLAST) algorithm [29] for identification of microorganisms.

2.2. Optimization of plate screening assays for lipolytic activity

There are a number of methods currently available for the screening of lipases and esterases. However, they vary with sensitivity, cost and ease of preparation. In this study a few methods were tested and evaluated.

All strains were pre-cultivated in Luria-Bertani (LB) medium and malt extract broth for bacteria and fungi, respectively. For detection of esterase activity a basal medium containing 0.5% (w/v) peptone, 0.3% (w/v) yeast extract and 2% bacteriological agar (pH 7) supplemented with 1%, 2% and 5% tributyrin was used. Five millimetre wells were bored into the agar plates and inoculated with 50 µl of pure bacterial cultures. Plates were incubated at 37 °C for 48 h. After incubation the isolates were observed for zones of hydrolysis (clear halos) around the colonies. Lipase activity was screened for on olive oil/rhodamine B agar plates. Rhodamine B (1 mg/ml; Sigma Chemical Co., Munich, Germany) was dissolved in distilled water and filter-sterilized. The agar plates contained 8 g nutrient broth, 4 g sodium chloride, 10 g agar (per litre) (pH 7). After autoclaving the medium was cooled to 60 °C, 31.25 ml olive oil and 10 ml of Rhodamine B solution (0.001% [wt/vol]) was added and stirred vigorously for 1 min. The medium was allowed to stand for 10 min to reduce foaming before pouring into sterile petri dishes. Lipase production was detected by irradiating plates with UV light at 350 nm [30]. Due to difficulty encountered with reading the screening plates using the above mentioned methods, two additional screening methods were tested, viz., assay with phenol red and tween agar plate screenings. Phenol red olive oil/tributyrin agar plates were prepared as follows (g/L); 0.01% (w/v) phenol red, 0.1% (w/v) $CaCl_2$, 1% (v/v) substrate, 2% (w/v) agar and pH adjusted to 7.3-7.4 with 0.1 N NaOH [31]. Organisms were inoculated onto the phenol red agar plates supplemented with 1% substrate and incubated at 37 °C for 2-4 days. The principle behind this assay is that a slight drop in pH from 7.3 (end point of the phenol red dye) to a more acidic pH will result in a change of colour from red to orange. The increase in acidity is due to the release of fatty acids following lipolysis [31]. A precipitation test using Tween 20 and Tween 80 agar plates was carried out to confirm lipolytic activity. Tween substrate plates were prepared as follows (g/L); 10 g peptone, 5 g $NaCl_2$, 0.1 g $CaCl_2{\cdot}2H_2O$, 20 g agar and 10 ml (v/v) Tween 20/80 [32]. This method is based on the principle of calcium salt precipitation. The hydrolysis of tween releases fatty acids which bind with the calcium in the medium to form insoluble crystals around the point of inoculation. Tween 80 is used for the detection of lipases as it contains esters of oleic acid, whilst Tween 20 is used for esterases as it contains esters of lower chain fatty acids [32]. The organisms were inoculated onto the plates and incubated at 37 °C for 2–4 days. A white precipitation around the boundary of the colony was indicative of lipase activity [31].

Fungal isolates were screened for laccase activity on PDA plates supplemented with and 0.2% bromophenol blue [33] (Merck, South Africa). Plates were incubated at 40 °C for 5 days, and then visually examined to evaluate the decolourizing ability of the fungal enzymes. To establish cellulase activity, substrates specific for the detection of exoglucanase (1% (w/v) avicel) and endoglucanase (1% (w/v) carboxymethyl cellulose (CMC)) were used to screen isolates on NA and PDA agar plates, for bacteria and fungi, respectively. All screening assays were performed in duplicate.

2.3. Enzyme assays

Lipolytic activity was determined spectrophotometrically by measuring the release of *p*-nitrophenol. *P*-nitrophenyl (*p*-NP) esters with various lengths of aliphatic acyl chains were used to determine esterase; *p*-NP acetate (C_2), *p*-NP butyrate (C_4), *p*-NP valerate (C_5) and lipase; *p*-NP octanoate (C_8), *p*-NP dodecanoate (C_{12}), *p*-NP myristate (C_{14}), *p*-NP palmitate (C_{16}), and *p*-NP stearate (C_{18}) activity. The substrate mixture consisted of 0.5 mM *p*-NP substrate in methanol, 50 mM tris-HCl buffer (pH 8) and 0.1% Triton X-100. The standard assay mixture contained 200 µl of substrate mixture and 20 µl of the crude supernatants, which were incubated at 37 °C for 1 h. The enzyme activity was determined by measuring the release of *p*-NP at an absorbance of 405 nm. One unit (U) of enzyme activity was defined as the amount of enzyme required to release 1 nM of *p*-NP per min under the assay conditions. Lipase/esterase and laccase activity was calculated from the formula derived from the Beer-Lambert Law: enzyme activity ($U\ ml^{-1}$) = $\Delta A.V/\varepsilon.t.v$. ΔA is the change in absorbance over time; V is the total volume of reaction mixture (ml); ε is the molar extinction coefficient in $nM^{-1}\ cm^{-1}$; t is the incubation time in minutes, and v is the volume of the enzyme in the assay mixture (ml) [34]. The appropriate extinction coefficient for each substrate under these assay conditions was used to calculate activity [35].

Laccase activity was determined based on the oxidation of syringaldazine substrate according to a protocol from Sigma-Aldrich (USA) [36]. The assay mixture (1 ml) contained 733 µl of acetate buffer (100 mM, pH 4/5) and 167 µl of laccase enzyme extract. The reaction vessels were equilibrated to 37 °C and absorbance monitored at 530 nm until constant. Thereafter 100 µl of 0.216 mM syringaldazine were added to the assay (to begin the reaction), followed by immediate mixing by inversion. The assays were incubated for 10 min and the increased absorbance was recorded using a UV-1800 Shimadzu UV Spectrophotometer (Japan). Production of the corresponding quinone was monitored at 530 nm ($\varepsilon 530 = 65\ 000\ M^{-1}\ cm^{-1}$). One enzyme unit is defined as the amount of enzyme that will oxidise 1 µmol of syringaldazine per min, under the assay conditions [37].

The dinitrosalicylic acid (DNS) assay was used to determine cellulase activity by detecting reducing sugars which are liberated by the hydrolytic action of endo- and exo-glucanase on different cellulose substrates (avicel and carboxymethylcellulose) [38].

2.4. Effects of temperature and pH on lipase/esterase activity and stability

The effect of temperature on enzyme activity was determined by conducting assays at incubation temperatures ranging from 25 to 50 °C (with 5 °C increments) and various *p*-NP esters as substrates [39]. Temperature stability of purified enzyme was determined by incubating the enzyme at various temperatures (25–50 °C) and estimating residual enzyme activities after incubation for 30 min, 1, 1.5, 2, 2.5, and 3 h. The effect of pH on enzyme activity was determined by assaying enzyme activity over a pH range of 3–12 using *p*-NP esters as substrates [39]. Citrate–phosphate buffer (pH 3 to 6), tris–HCl buffer (pH 7 and 8), carbonate–bicarbonate buffer (pH 9 and 10) and sodium-bicarbonate and sodium-phosphite buffer (pH 11 and 12) were used as buffer systems. Stability of the purified enzyme over a range of pH was also determined by measuring the residual activity after incubating 200 µl of the enzyme in 1800 µl of the above mentioned buffer systems (pH 3–12) for 3 h at the optimum temperature. Absorbance was read at 405 nm.

2.5. Production of crude enzyme extracts

The selected bacterial isolates were grown in basal medium containing 0.5% (w/v) peptone and 0.3% (w/v) yeast extract supplemented with 1% tributyrin. Flasks were incubated at 37 °C for 24 h at 180 rpm. Cells were harvested by centrifugation at 10 000 rpm for 10 min. The cell pellet was then resuspended in lysis buffer (20 mM Tris-HCl, 0.5 M NaCl, pH 8.0) and disrupted by ultrasonic treatment for 10 min in 10 s intervals. The cell lysate was centrifuged at 10 000 rpm for 10 min at 4 °C, and the supernatant was recovered to test intracellular activity. To test extracellular activity the cell free supernatant was collected and concentrated 10-fold by ultrafiltration with an Amicon system (Millipore, Massachusetts, USA) using first a 3 kDa cut-off membrane after which a 50 kDa cut-off membrane was used on the concentrated sample to remove proteins larger than 50 kDa.

2.6. Native & SDS-PAGE

Protein sizes were determined by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) as outlined by Judd (1996) [40]. Samples were electrophoresed by Native-PAGE (no SDS included) and SDS-PAGE in 12% polyacrylamide gels according to the method of Laemmli (1970) [41]. Protein concentration was determined using the Bradford assay (Bradford, 1976) [42].

Native SDS-PAGE was utilized to ensure removal of potential cellulases. To identify endo and exo-glucanases, 12% native-PAGE gels containing 1% avicel and carboxymethylcellulose, respectively, (prepared in 50 mM phosphate buffer pH 7) were prepared. Following electrophoresis at 100 V for approximately 90 min at room temperature, the gel slab was cut in two halves; one half was stained using 0.5% Coomassie Brilliant Blue R250 (Sigma-Aldrich, Germany) to determine the size of the proteins and the other portion was used to detect enzyme activity. The gel for activity staining was washed with 50 mM phosphate buffer (pH 7) for 5 min, followed by staining in Congo-Red solution (0.1%, [w/v]) for 15 min. The gel was then destained with 1 M NaCl to visualise the clearing zone of hydrolysis, and then fixed with 0.5% (v/v) acetic acid [43].

2.7. Statistical analysis

Results shown here are the means of three independent determinations. Standard deviations for each of the experimental results were calculated using Microsoft Excel software and represented as error bars.

3. Results and discussions

3.1. Identification of isolated bacteria and fungi

A total of ten different bacterial strains were isolated using the traditional culture and identification method using 16S rRNA sequencing: three *Bacillus* spp., three different *Pseudomonas aeruginosa* isolates, *Inquilinus* sp., *Micrococcus* sp., *Pantoea* sp., *Klebsiella*, *Streptomyces* sp. and *Cellulosimicrobium* sp. (Table 1) (all with a similarity index of more than 97%). *Bacillus* spp. were the predominant bacterial species (33%). Some of these genera such as *Bacillus*, *Pantoea*, *Klebsiella* and *Pseudomonas* have previously been identified in other woods [44,45], whilst others such as *Inquilinus* and *Mucilaginibacter* have not observed in woods. The two fungal isolates described in this study were identified as *Paecilomyces formosus* (F4) and *Phialophora alba* (X) using 18S rRNA sequencing. Both these fungal isolates have not been previously identified in *Eucalyptus* spp. woods.

3.2. Optimization of plate screening assays for lipase and esterase activity

One percent tributyrin (esterase activity) was optimal for bacteria isolated from the mixed wood sample (Table 1), however, 2% was optimal for bacteria from individual wood species (Table 2). Slight halos were observed for a few of the bacterial isolates in 5% tributyrin plates. Plate screening assays for lipase activity revealed minimum lipase activity for isolates from mixed wood species; however, for bacteria isolated from individual *Eucalyptus* species, 1% substrate concentration was optimal. Sixty-seven percent, 28% and 28% of the isolates displayed activity on 1%, 2% and 5% tributyrin plates,

Table 1
Lipase and esterase activity of bacteria isolated from a mixed *Eucalyptus* wood chip pile.

	Species	AccessionNumber	Esterase 1% Trb	Esterase 2% Trb	Esterase 5% Trb	Lipase 1% Oil	Lipase 2.5% Oil
B1	*Pseudomonas aeruginosa*	JX945659	+	+	−	−	−
B2	*Pseudomonas aeruginosa*	JX945660	+ +	−	−	−	−
B4	*Bacilllus firmus*	JX945657	+	+	−	+	+
B5	*Micrococcus luteus*	JX945661	+	+	−	+	−
B6	*Bacillus* sp.	JX945662	+ +	−	−	+	−
B7	*Inquilinus limosus*	JX945663	+ + +	−	+	−	−
B9	*Pantoea* sp.	JX945664	+ +	−	−	+	−
B10	*Klebsiella* sp.	JX945665	+	−	−	−	−
B12	*Bacillus ginsengihumi*	JX945658	+ +	+ +	+	+	−
B14	*Streptomyces costaricanus*	JX945666	−	−	−	−	−
B15	*Pseudomonas aeruginosa*	JX945667	−	−	+	−	−
B16	*Cellulosimicrobium cellulans*	JX945668	−	−	−	+	+

Key: + = slight halos (1–2 mm), + + = medium halos (2–5 mm), + + + = large halos (> 5 mm), Trb = tributyrin, Oil = olive oil, − = no halos.

respectively. *Bacillus firmus* was capable of hydrolysing all three concentrations of tributyrin, but largest halos were observed at 1% substrate concentration. *Micrococcus luteus*, *P. aeruginosa*, and *Cellulosimicrobium cellulans* were also identified as esterase producers. Eight percent, 63% and 22% of the isolates displayed activity on 1%, 2% and 5% tributyrin plates, respectively. *Curtobacterium flaccumfaciens*, *Bacillus thuringiensis*, *B. cereus*, *Pantoea agglomerans* and *P. vagans* produced the greatest zones of hydrolysis indicating esterase activity, with a halo zone of 2–5 mm (Fig. 1). Other studies have also had some degree of success with the use of tributyrin and olive oil/rhodamine B as substrates and methods for screening for lipolytic activity [32,46,47].

Due to difficulty encountered with visualization and of the clearing zones, additional assays such as phenol red and tween agar plate screenings were also performed to validate the results obtained. Both assays confirmed the results, however, the phenol red agar plate assay was more sensitive than the other assays. Distinct clearings for the phenol red plates and precipitation zones for the tween plates were observed (Fig. 2). The phenol red screening plates were used to quantify activity (Tables 1 and 2).

Lipases and esterases have been identified by screening microorganisms on various types of agar plates such as phenol red, rhodamine B, tween, Nile blue and so forth [48]. However varying degrees of success have been reported with the different methods of screening. An extracellular lipase isolated from a psychrotrophic *Pseudomonas* strain was discovered by screening on olive oil agar plates. Some researchers have found success with the rhodamine B dye method developed by Kouker and Jaeger (1987) [30,32,49,50]. However, others encountered difficulties in preparing the media, as well as visualizing activity of weaker lipases [51]. Based on the results from this study, the recommended method of screening for lipolytic activity would therefore be, phenol red agar plates supplemented with 1% olive oil or tributyrin.

In addition, isolates were also screened for cellulose activity. In the pulp and paper industry, the presence of cellulases has undesirable effects on the quality of pulp generated, particularly in the production of dissolving pulp (high grade cellulose pulp, > 98% cellulose content). Potential cellulases would hydrolyze the cellulose fibres resulting in a decrease in alpha cellulose, thus impacting yield [52]. Consequently, the detection and elimination of cellulase activity is important. Both the quantitative (screening plates) and qualitative (DNS assay) revealed negligible cellulase activity except for *C. flaccumfaciens* (Table 3). This was addressed by using spin columns with specific cut-off sizes to eliminate the larger proteins (> 50 kDa) which could be potential cellulases.

3.3. Native & SDS-PAGE

Native PAGE gels supplemented with carboxymethylcellulose and avicel were used to ensure that the minimal endoglucanase and

Table 2
Lipase and esterase activity of bacteria isolated from different *Eucalyptus* spp.

Species		GenBank Number	Esterase1% Trb	Esterase2% Trb	Esterase5% Trb	Lipase1% Oil	Lipase 2.5% Oil
	E. dunnii						
DF1	*Mucilaginibacter* sp.	JF999998.1	−	−	+	−	−
DF2	Unidentified	−	−	+ +	+	−	+
DF3	*Curtobacterium flaccumfaciens*	HE613377.1	−	+ +	+	−	−
DF5	*Pantoea vagans*	CP002206.1	−	−	+	+	−
DF6	Unidentified	−	−	+ +	+	+	−
DF7	*Bacillus thuringiensis*	FN667913.1	−	+ +	+	+	+
DF8	Unidentified	−	−	+	+	−	-
	E. grandis						
G1	*Pantoea agglomerans*	FJ11844.1	−	+ +	+	−	−
G2	*Curtobacterium flaccumfaciens*	JF706511.1	−	+ +	−	−	-
G3	*Pantoea vagans*	CP002206.1	−	+ +	+	+	−
G4	Unidentified	−	−	−	+	−	−
	E. nitens						
N1	*Bacillus cereus*	JF758862.1	+ +	+ +	+	−	-
N2	*Pantoea* sp.	JN853250.1	−	−	+	−	+
N3	*Curtobacterium* sp.	HQ219967.1	−	+ + +	+	−	−
N4	*Bacillus cereus*	JQ308572.1	−	−	+	−	+
N5	*Bacillus cereus*	EU621383.1	−	−	+	−	−
N6	*Bacillus* sp.	EU162013.1	−	+ +	+	−	+
N7	*Bacillus thuringiensis*	FN667913.1	−	+ +	+	−	−

Key: + = slight halos (1–2 mm), + + = medium halos (2–5 mm), + + + = large halos (> 5 mm), Trb = tributyrin, Oil = olive oil, − = no halos.

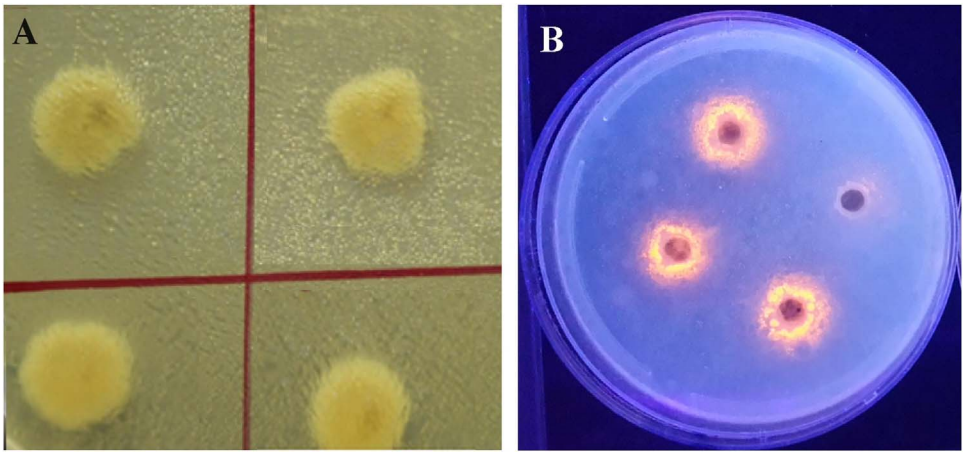

Fig. 1. A- 1% tributyrin plate screening assays for the detection of esterase activity of pure bacterial isolates from *Eucalyptus* wood species, B- 1% olive oil/rhodamine B plate screening assays for the detection of lipase activity of pure bacterial isolates from *Eucalyptus* wood species.

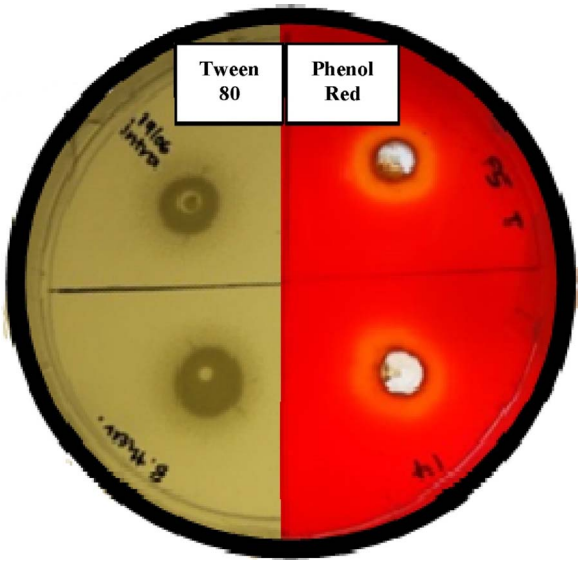

Fig. 2. 1% Tween 80 agar plates (left) and 1% tributyrin phenol red agar plates (right) for the detection of lipase and esterase activity, respectively, of pure bacterial isolates from *Eucalyptus* wood species.

exoglucanase activity observed was eliminated. Samples concentrated with the 3 kDa spin column were thereafter passed through a 50 kDa spin column to remove the larger proteins, presumably thought to be cellulases (Fig. 3). It is imperative that the enzyme extracts characterized here, contain no cellulase activity that may degrade the cellulose fibers. All other accessory enzymes such as xylanases, laccases, and ligninases that may be present will positively contribute to the production of high quality cellulose pulp. Bacterial lipases and esterases generally have an expected protein size of between 15 and 45 kDa [53]. Proteins larger than 50 kDa were regarded as potential cellulases. Cellulases have a negative impact on the final pulp by reducing cellulose chains. An esterase as small as 1.57 kDa from *Bacillus stearothermophilius* has been described by Simoes et al. (1997) [54]. *Bacillus thuringiensis* has been reported to produce a 38 kDa phospholipase [55].

3.4. Lipase and esterase activity

Upon evaluation of the preliminary screenings, the following isolates were selected for further study, DF3 – *C. flaccumfaciens*, DF7 – *B. thuringiensis*, B9–*Pantoea* sp. and BT – *B. thuringiensis*. In addition to the bacterial isolates selected, two fungal isolates F4–*P. formosus* and X – *P. alba* were chosen based on similar preliminary plate screenings (data not shown) as well as previous studies on laccase activity [56]. The effect of initial pH on the extracellular and intracellular lipase/esterase activity of the selected isolates was investigated at pH 8 and 37 °C with acetate and butyrate as substrates (generally selected for initial investigations). The results in Table 3 show a higher enzyme activity in the extracellular fractions of BT, DF7, and DF3, whilst B9 demonstrated higher activity in its intracellular fraction. Therefore, the appropriate fractions were used for further characterization of these enzymes. Fungi are known to produce extracellular enzymes to degrade polymers that cannot be absorbed [57], therefore it was not unexpected that the intracellular fraction yielded no enzyme activity.

3.5. Effects of temperature and pH on enzyme activity

Specificity of lipases are directed by a variety of properties such as type of substrate, position of esters fatty acids, stereospecificity and a combination of all four. These include factors that alter the binding of the enzyme to the substrate, the molecular properties of the enzyme, and structure of the substrate [58]. Therefore, in the work reported here, it was vital to institute an experimental design to test the effects of pH and temperature on a range of substrates. The majority of reported studies elect to determine pH and temperature optima and then test the

Table 3
Lipase/esterase and cellulase activity (endoglucanase and exoglucanase activity using the DNS assay) and protein concentrations of the intracellular and extracellular fractions from the different isolates.

		Acetate (U/ml)		Butyrate (U/ml)		Protein Conc. (μg/ml)		Endoglucanase Activity (U/ml)	Exoglucanase Activity (U/ml)
		Ext.	Int.	Ext.	Int.	Ext.	Int.		
BT	*Bacillus thuringiensis*	5.55	5.24	9.75	5.78	212.9	1.57	0.057	0.043
DF7	*Bacillus thuringiensis*	10.71	5.16	10.98	4.34	414.3	1.84	0.021	0.013
B9	*Pantoea* sp.	5.12	6.75	2.82	5.27	1.69	25	0.012	0.015
DF3	*Curtobacterium flaccumfaciens*	10.35	4.09	10.70	3.44	62.86	1.88	0.203	0.121
F4	*Paecilomyces formosus*	7.78	–	18.89	–	51.43	–	0.019	0.029
X	*Phialophora alba*	2.18	–	30.11	–	98.57	–	0.034	0.041

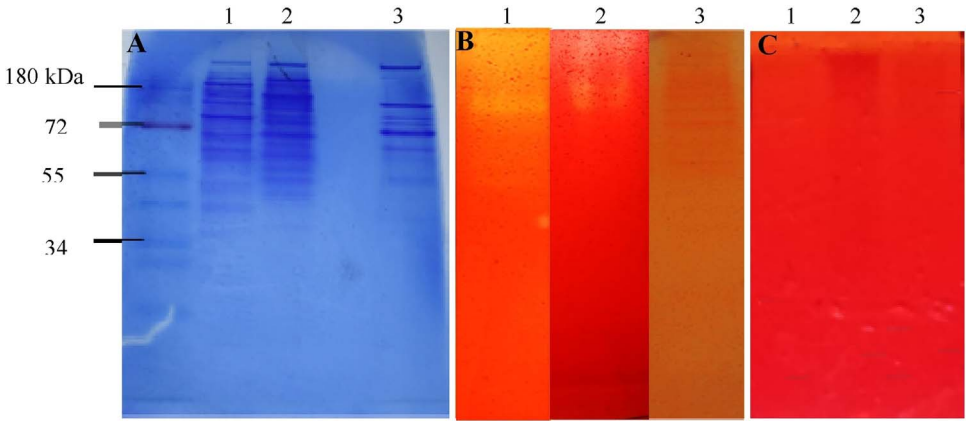

Fig. 3. Native PAGE gels supplemented with carboxylmethylcellulose (CMC) to confirm removal of any potential endoglucanases. A: SDS-PAGE of crude enzymes, 1- DF7, 2- DF3, 3-BT. B: Native PAGE of crude enzymes, 1- BT, 2-DF3, 3- DF7. C: Native page of crude enzymes after partial purification, 1- BT, 2-DF3, 3- DF7.

substrate specificity of the optimal expressed enzyme [59,60]; less detailed studies have demonstrated some effect of pH on substrate specificity of lipases and esterases [61]. Ertuğrul and colleagues found that at pH 6, lipases from a *Bacillus* strain demonstrated highest activity towards the long chain triglyceride trimyristin (C_{14}), however, at pH 9, the shorter chain triglycerides such as tributyrin (C4) and triacetin (C2) provided higher lipase activity compared to the longer chain triglycerides (C_8–C_{14}) [61]. This behaviour has also been reported for acetyl esterases from *Thermomyces lanuginosus* where, no activity was observed against *p*-NP-acetate at pH 9.0, however, activity at pH 4.0 was recorded [62]. This reveals the varying degrees of lipase and esterase activity depending on the pH of the medium, which may be attributed to the presence of isoenzymes. Results of our study indicate that substrate specificity is affected by changes in pH and temperature.

The enzymes in our study showed a preference for acidic conditions which is fairly uncommon amongst bacterial lipases. The majority of lipases are known to display their highest activities at a neutral or alkaline pH [63–65]. However, there are reports of the production of acidic lipases from bacteria although with varying amounts of activity. Ramani et al. (2010) described the production of an acidic lipase by *Pseudomonas gessardii* which had a maximum activity of 156 U/ml at a pH of 3.5 [66]. On the lower end of the scale, an acidic lipase produced by *Aeromonas* sp. demonstrated optimal activity of 0.7 U/ml at a pH of 6 [67].

The highest hydrolysis rates were obtained with potential lipases isolated from *B. thuringiensis* (BT and DF7) on *p*-NP-valerate (C_5) *p*-NP-octanoate (C_8), *p*-NP-dodecanoate (C_{12}), and *p*-NP-myristate (C_{14}), indicating the enzymes' propensity for longer acyl chain lengths (Fig. 5). The *p*-NP esters of palmitic and stearic acids were also good substrates, however the shorter acyl chain esters such as acetate, butyrate and valerate were hydrolysed at a lower rate but with relatively comparable activity to the longer chain acyl chain substrates. This suggests that the enzymes from both *B. thuringiensis* isolates could potentially produce both lipases and esterases. Lipases from *Bacillus* species such as *Bacillus stearothermophilus* have been reported to hydrolyse synthetic substrates with acyl group chain lengths between C_8 and C_{12} with optimal activity on C_{10} *p*-NP-caprate [68]. On the other hand, a lipase isolated from *B. stearothermophilus* had a wide substrate specificity towards triglycerides with C_4 to C_{18} [69].

Initially, when the enzymes were tested at pH 8, greater activity was observed with *p*-NP acetate and *p*-NP butyrate (data not shown). However, at the optimal pH of 4 and 5, greater activity towards dodecanoate, myristate and palmitate was noted (Table 4). This suggests that changes in pH have an influence on the substrate specificity of the enzyme. These findings may be explained by the phenomenon of induced fit model. This model claims that the substrate may cause substantial transformation in the three-dimensional link of the amino acids at the active site and these modifications in protein structure initiated by a substrate will bring the catalytic groups into a suitable orientation for reaction [70]. Post and Ray (1995) showed that conformational changes can enhance the specificity of an enzyme with suboptimal catalytic efficiency [71].

The enzymes isolated from the other microorganisms (DF3, F4, X) showed a preference for dodecanoate, palmitate, myristate, octanoate and stearate substrates. The enzymes' specificity in relation to lipids with fatty acid residues of C_8–C_{18} chain length compellingly suggests that the enzymes described in this study could be true lipases. Enzymes isolated from *Pantoea* sp. (B9) could potentially be classified as esterases due to their specificity towards butyrate and valerate. The criteria used to differentiate esterases from lipases, is that esterases do not hydrolyse esters containing an acyl chain length of longer than 10 carbon atoms [72]. It is unusual for isolate B9 to prefer pNP-butyrate over pNP-acetate, such specificity is uncommon in nature, however, novel esterases from *Lactobacillus casei* and *Escherichia coli* have previously demonstrated such catalytic preference [72,73]. *C. flaccumfaciens* (DF3) displayed highest activity of 60 U/ml at 30 °C with substrate specificity towards palmitate. *C. flaccumfaciens* is an endophytic bacteria associated with crops such as rice, potato, yam, tobacco, and cucumber and is capable of producing lipases [74]. This could be the first report of a characterized lipase from *C. flaccumfaciens* isolated from *Eucalyptus* wood.

Low activities were obtained for laccases (Fig. 4), and this is expected as extracellular laccases from basidiomycete fungi are known to be produced in low amounts [75]. It is recognized that when fungi are grown in a medium of pH 5, laccases will be produced in excess, however most studies show that a pH range of 3.6 to 5.2 is suitable for enzyme production [76]. Optimal temperatures for laccase activity can vary significantly amongst organisms. There are reports of activities in the range of 25 to 80 °C, with most enzymes having an optimum at 50 to 70 °C [77]. In this study the optimum temperatures of the lipases and esterases were 30 and 35 °C, respectively. Therefore, laccase activity and stability were tested at these temperatures as the final application of this study would be to create an enzyme cocktail to treat pulp for effective removal of lipophilic extractives. Nevertheless, there was minimal variation in activity from the optimal pH and temperature of isolates F4 and X. Isolate F4 displayed 6.8% and 9.7% more activity at the optimal conditions of 40 °C and pH 5.5, respectively. Isolate X showed 15.3% more activity at 50 °C, whilst the optimal pH remained the same. Our results are comparable to another study where the maximum production of laccase from *Trichoderma harzianum* was observed at 35 °C and pH 5 after 6 days [78].

In addition to demonstrating laccase activity (up to 3.1 U/ml) (Fig. 4), *P. formosus* (F4) and *P. alba* (X) also demonstrated high substrate specificity towards dodecanoate at 35 and 30 °C, respectively. Limited information has been published on the enzymes produced by *P. alba*, however, previous work indicate that xylanases from this microorganism were characterized with activity of up to 420 IU/ml [79]. The presence of enzymes from this microorganism could greatly assist in the

Table 4
Optimized pH, temperature and substrates for lipolytic enzymes from the different isolates.

Isolate	Optimum pH	Optimum Temperature	Substrate Specificity
BT	5	30 °C	Dodecanoate, Myristate, Octanoate, Acetate
DF7	4	35 °C	Dodecanoate, Octanoate, Valerate, Butyrate
B9	4	35 °C	Valerate, Dodecanoate, Butyrate, Octanoate
DF3	4	30 °C	Palmitate, Dodecanoate, Myristate, Octanoate
F4	4	35 °C	Dodecanoate, Palmitate, Octanoate, Myristate
X	5	30 °C	Dodecanoate, Stearate, Myristate, Octanoate

reduction of pitch formation as well as the breakdown of xylan which will reduce the amount of chemicals used in the downstream processing of pulp [80,81]. Laccases also have the ability to degrade both phenolic and non-phenolic compounds. Plant phenols released by hardwoods during pulping may have an inhibitory effect on enzyme activity [82], therefore the inclusion of fungal laccases in this study could mitigate the inhibitory effects of phenolic compounds.

3.6. Effects of temperature and pH on stability of enzymes

In the pulp and paper industry, the enzyme pre-treatment of pulp is a tricky affair. When considering the addition of enzymes to pulp, a number of variables such as dosage, incubation period, temperature, pH and combination of enzymes needs to be taken into account. Time is money, so minimal amount of time for enzyme pre-treatment would be optimal. Therefore, when determining enzyme stability, a shorter range for the incubation period was selected. Stability was however tested at 18 h to establish a broader range for incubation time, however, in industry pre-treatment times of up to 18 h are not feasible.

The enzymes from the various microorganisms appear to be relatively stable over a period of 18 h at their optimal temperature. Enzymes from DF3, DF7, and X maintained their lipolytic activity over a period of 3 h with minimal loss in activity and retained at least 60% activity after 18 h (Fig. 6). Enzymes isolated from BT, X, F4, DF3 and DF7 were fairly stable up to 2 h and thereafter a 30–40% decrease in activity was observed. More than 90% of the original activity was retained after 18 h for DF3 with dodecanoate and palmitate as substrates. Enzymes from DF7 and F4 retained more than 75% activity after 18 h with butyrate and valerate as substrates, respectively. B9 on the other hand, initially demonstrated high stability after 1 h of incubation followed by a drop in activity to 70% after 3 h of incubation. These results fare well in comparison to other studies under similar conditions. For example, in a study by Eggert et al. (2001) a variant of an esterase (LipB, EC 3.1.1.1) from *Bacillus subtilis* was found to be stable at pH 5 and 45 °C for 1 h [83].

Specificity of enzymes from DF3, DF7, F4 and X towards both the shorter and longer aliphatic acyl chains over the 18 h incubation period indicates the broad range of substrates these enzymes are able to act upon. The stability of these enzymes is a desirable characteristic and would offer an advantage in potential industrial applications. However, for the purpose of this study the addition of these enzymes to pulp as a pre-treatment step would be optimal up to 2–3 h. Similar results were reported by Massadeh and Sabra (2011) where a lipase isolated from *B. stearothermophilus* remained stable at a pH range of 7–9 after incubation for 1 h at 30 °C, with a residual activity remaining above 50% for pH 7–9 [84]. However, extremophilic organisms are capable of producing hardier lipases. A thermostable lipase from *Geobacillus thermodenitrificans* IBRL-nra was found to have an optimal temperature of 65 °C, at which it retained its initial activity for 3 h. Its highest lipase activity was reported at pH 7.0 and stable for 16 h at 65 °C [85]. Borkar et al. (2009) reported a lipase from a *P. aeruginosa* strain which was found to be completely stable at 55 °C after 2 h at pH 6.9 [86]. A lipase from a psychrotolerant *Pseudomonas fluorescens* strain was active at a temperature range of 15–65 °C, however, it exhibited maximum activity at 45 °C and pH 8.0. This enzyme demonstrated high stability, retaining 100% and 70% of its activity after an incubation period of 45 and 100 min, respectively, at 45 °C and pH 8.0. This particular lipase also showed a broad substrate specificity acting on *p*-nitrophenyl esters with C_8–C_{18} acyl groups as substrates [60].

Many researchers elect to clone genes coding for enzymes of interest in order to increase activity and improve production [87–89]. However, in industry this may not be a practical approach as screening of clone libraries involves conventional agar plate-based methods, which would require approximately 10,000 petri plates, each containing 10,000 clones. This is time-consuming and would greatly increase expenditure [90]. The enzyme activities observed in this study are comparable to, if not higher, than those of lipases and esterases which have not been modified or cloned (Table 5). The activities recorded in this study (up to 60 U/ml) could be invaluable in the reduction of pitch formation in the pulp and paper industry. In addition, the enzymes described here are indigenous to *Eucalyptus* wood species and have not been modified in any way, thus making them feasible and ideal for industrial applications. This is particularly the case for the acid-bisulphite pulping process used to produce dissolving pulp, as this process involves acidic pH

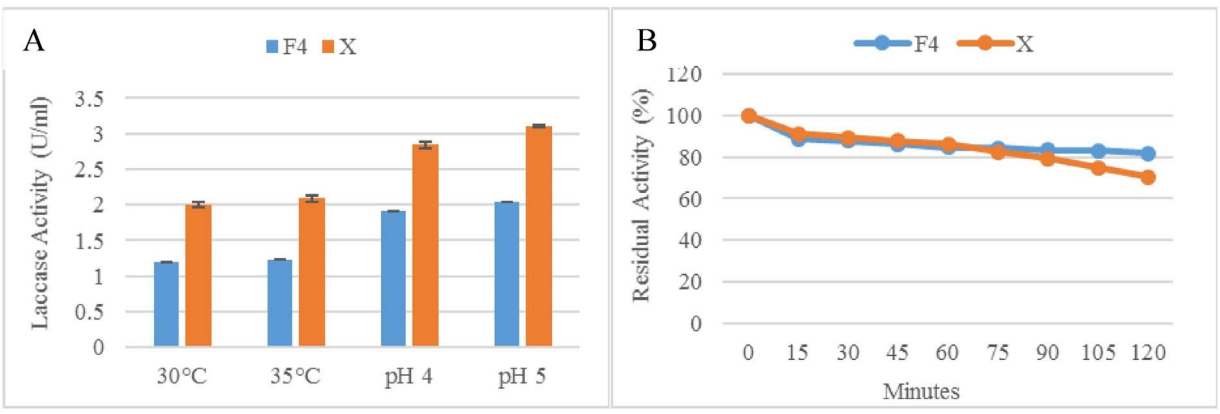

Fig. 4. Activity and stability of laccases from fungal isolates F4 and X. A: activity at 30 °C, 35 °C, pH 4 and pH 5; B: enzyme stability at 35 °C and pH 4 for F4 and 30 °C and pH 5 for X. .

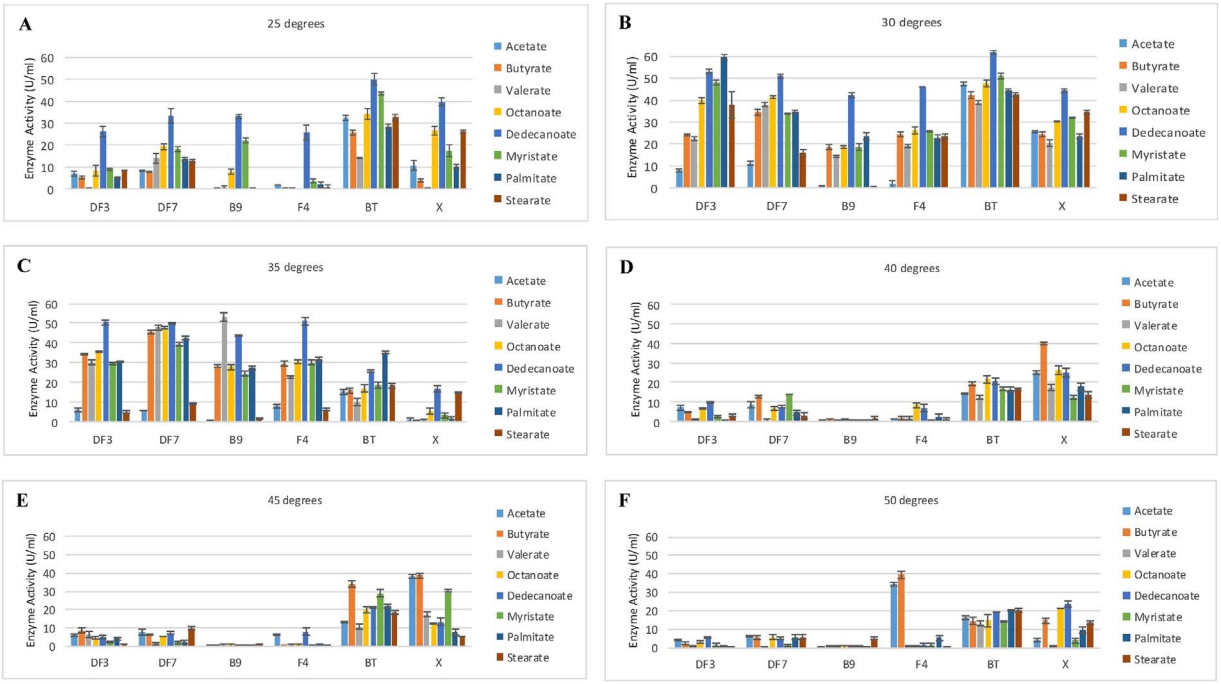

Fig. 5. Effect of temperature (at optimum pH 4 or 5) on the activity of esterases/lipases from isolates DF3 (pH 4), DF7 (pH 4), B9 (pH 4), F4 (pH 4), BT (pH 5) and X (pH 5) on *p*-NP esters (C_2–C_{18}).

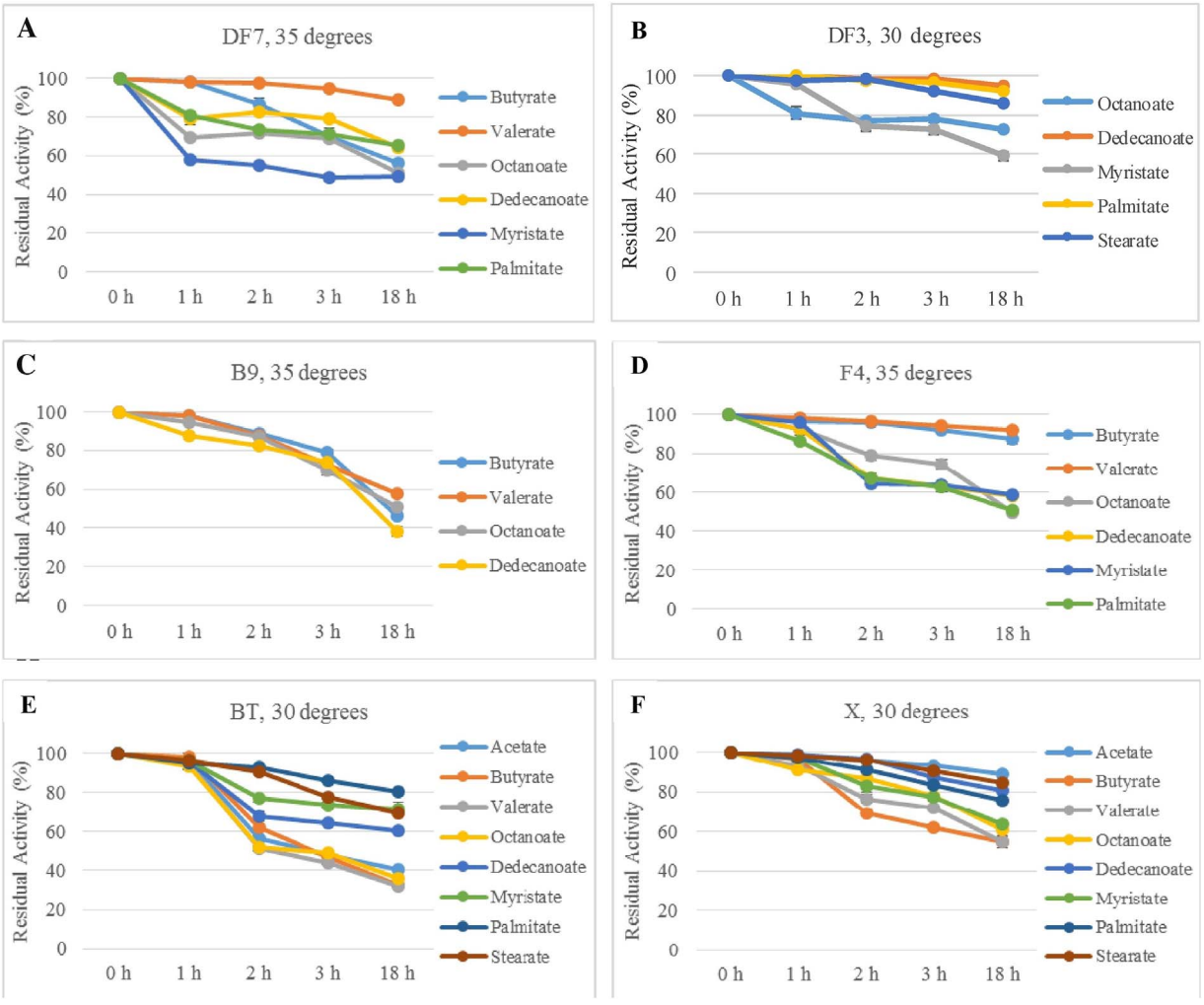

Fig. 6. Stability of esterases/lipases from DF3 (A); DF7 (B); B9 (C); F4 (D); BT (E) and X (F) at optimum temperature and pH.

Table 5
Comparison of optimal temperature and pH of some lipases and esterases isolated from different bacteria.

Isolate	Enzyme	pH	Temperature (°C)	Enzyme Activity (U/ml)	Refs.
Bacillus THL027	Lipase	7	70	8.3	Dharmsthiti and Luchai [91]
Bacillus coagulans BTS-3	Lipase	8.5	55	1.16	Kumar et al. [65]
Geobacillus zalihae sp.	Lipase	6.5	65	0.15	Rahman et al. [92]
Pseudomonas aeruginosa LP602	Lipase	8	55	3.5	Dharmsthiti and Kuhasuntisuk [93]
Pseudomonas gessardii	Lipase	3.5	30	156	Ramani et al. [66]
Burkholderia multivorans	Lipase	7	30	58	Gupta et al. [94]
Burkholderia multivorans V2	Lipase	8	37	14	Dandavate et al. [95]
Burkholderia sp. ZYB002	Lipase	8	65	22.8	Shu et al. [96]
Enterococcus durans NCIM5427	Lipase	4.6	30	207.6	Vrinda [97]
Streptomyces exfoliates LP10	Lipase	6	37	6.9	Aly et al. [98]
Salinivibrio sp. strain SA-2	Lipase	7.5	50	5.1	Amoozegar et al. [64]
Anoxybacillus gonensis A4	Esterase	5.5	60–80	0.8	Faiz et al. [99]
Bacillus sp. strain DVL2	Esterase	7	37	5.2	Kumar et al. [100]
Bacillus licheniformis	Esterase	8–8.5	45	12	Alvarez-Macarie et al. [101]
Geobacillus sp. DF20	Esterase	7	50	27.9	Özbek et al. [102]
Lactobacillus brevis NJ13	Esterase	8	50	48.12	Kim et al. [103]
Alcaligens faecalis	Esterase	8	30	0.27	Poornima and Kasthuri [104]
Burkholderia fungorum A216	Esterase	6.5	37	0.014	Jiao et al. [105]
Achromobacter denitrificans strain SP1	Esterase	8	50	89.5	Pradeep et al. [106]
Janthinobacterium lividum	Esterase	7	30	0.00568	Park et al. [107]
Pseudomonas sp. KWI-56	Esterase	7.5	22	51.6	Sugihara et al. [108]

process conditions which would be suitable for the enzymes described in this study.

4. Conclusions

In the present work, a cellulose-free cocktail of lipolytic and other enzymes was obtained from microorganisms indigenous to South African *Eucalyptus* wood chips. Lipases and esterases showed optimal activity at moderate temperatures (30 and 35 °C) and acidic pH range (pH 4 and 5). The enzymes' stability and activity on a broad range of lipophilic substrates could lead to potential biotechnological applications in the removal of lipophilic components that cause pitch problem in the manufacture of high purity chemical pulps such as dissolving wood pulp. The inclusion of laccases has the potential to assist in further degradation of these problematic lipophilic compounds. Future work will focus on applying these enzymes directly to the pulped wood chips and evaluating their potential to reduce the agglomeration of lipophilic compounds that cause pitch formation during pulping. The application of enzymes produced by indigenous microflora will aid in reducing cost and is a greener alternative to chemical treatments.

Conflict of interest

None.

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