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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.