

Microbial Screening for Bio-delipidation System of Pre-treated Poultry Slaughterhouse Wastewater: Impact of Solvents, Metal Ions and Detergents on Lipase Activity

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Abstract— This study reports on the microbial screening for a bio-delipidation system of lipid-rich slaughterhouse wastewater, and on the optimal conditions for lipase production and activity. In this study, swaps were collected from the poultry slaughterhouse discharge point for screening, isolation and characterisation of lipolytic microorganisms using molecular techniques. *Bacillus cereus* strains *AB1* (*BF3*) and *CC-1* (*B30*) were identified using 16S rRNA techniques. Maximal lipase production for both strains was observed between pH 6–8 and 45–60 °C. Optimal lipase activity for *BF3* and *B30* was achieved at pH 8 and 60 °C, and at pH 8.83 and 45°C, respectively. After partial purification, increased activity was observed for *BF3* and *B30* strains. Solvents, metal ions and detergents (triclosan and trichlorocarbonilide) affected lipase activity. It was concluded that *BF3* and *B30* strains were suitable candidates for bio-delipidation systems.

Keywords— Bio-delipidation, Poultry slaughterhouse wastewater, Lipase activity

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I. INTRODUCTION

Compared to plants and animals, bacterial lipases are the most significant biocatalytic agents for numerous industrial applications [1]. In addition, microbial sourced lipases have also been considered important for biotechnological industrial applications due to their ability to catalyse reactions both in aqueous and non-aqueous systems. Generally, various microorganisms including those from microbial genera *Pseudomonas*, *Bacillus*, *Staphylococcus*, *Streptomyces*, *Penicillium*, *Aspergillus* and *Rhizopus* [2], commercially produce lipase. These enzymes can be produced by submerged fermentation (SMF) and solid-state fermentation (SSF) on an industrial scale whereby solid substrates such as agro-waste and oils are used as inducers or substrates for lipase production.

Like many other microbially produced extracellular protein, lipase production is also influenced by factors such as, media composition and physicochemical factors, i.e. carbon source, pH and temperature [3]. In most instances, the lipase production is induced by the presence of lipids, oils and triacylglycerols [4]. However, optimisation of the production conditions is also essential for process performance and production costs [5]. Furthermore, the applications of thermo- and alkaline-stable lipases in the bio-delipidation systems is critical due to the extreme/harsh nature of the process conditions [6]. As a result, the optimisation of fermentation conditions and enzyme activity is prioritised to assess the effect of these conditions on enzyme functionality in an environment in which the enzymes will be applied.

Some of the pollutants in poultry slaughterhouse wastewater (PSW) are heavy metals, solvents, and pharmaceuticals including antibiotics and detergents [7]. Their presence in PSW has been established, and they are known to be detrimental to human and environmental health [7]. Hence, the effect of these pollutants on the functionality and stability of the enzymes was investigated in the current study. The aim of this study was therefore, to isolate and characterize potential microorganisms from PSW, optimise lipase production and investigate the impact of environmental factors on the lipase activity and stability.

II. MATERIALS AND METHODS

A. Isolation and Identification of Lipolytic Bacteria

Sterile swabs were used to collect lipolytic bacteria from PSW and a local poultry slaughterhouse wastewater discharge point in Cape Town, South Africa. Nutrient broth with olive oil was used as an inducer and the cultures were maintained on nutrient agar by a streaking technique subsequent to incubation at 37 °C for 48 h, with a regular sub-culturing strategy being implemented to obtain pure colonies. Pure cultures with high lipase activity were subjected to identification by morphologically studying each species under a microscope using a gram staining method, followed by further identification using 16S rRNA sequencing techniques. For 16S rRNA analyses, samples were sent to Inqaba Biotechnical Industries (Pretoria, South Africa) for sequencing, with the DNA from the isolates being obtained using a ZR Fungal/Bacterial DNA Kit™ (Zymo Research, USA). The 16S rRNA target region was amplified by DreamTaq™ DNA polymerase (Thermo Scientific™, SA) and the primers used were: (forward) 16S-27F 5'-AGAGTTTGATCMTGGCTC-3' and (reverse) 16S-1492R 5'-CGGTTACCTTACGACTT-3'. PCR products were gel extracted using a Zymoclean™ Gel DNA Recovery kit (Zymo Research, USA). This was followed by sequencing in the forward and reverse directions on an ABI PRISM™ 3500xl Genetic Analyser. Purified sequences were analysed using a CLC Main Workbench 7 and subjected to a Basic Alignment Search Tool (BLAST) to compare the sequence with those available on the NCBI-NIH database (National Centre for Biotechnical Information, n.d.) for identification of the microorganisms.

B. Screening of Lipase Production/Activity on Agar Plates

Lipase activity of the isolated strains was determined using agar plate screening methods adapted from [8], using tributyrin medium containing (per litre): 2.5 g peptone from casein, 3 g yeast extract, and 12 g nutrient agar. After autoclaving and cooling of the medium to 60 °C, 10 mL of tributyrin was added. A homogeneous mixture was maintained prior to pouring the media into Petri plates. A loopful of each culture was inoculated on the agar plates and incubated at 37 °C for 48 h. Presence of lipase activity/production was monitored by a clear zone around the colonies. Microorganisms identified to be lipolytic, using the tributyrin medium screening method, were further screened using a Rhodamine B agar plate method. Rhodamine agar plates consisted of the following (per litre): 8 g peptone, 4 g yeast extract, 3 g NaCl, and 20 g nutrient agar. Rhodamine B dye (1 mg mL⁻¹) was prepared and filter-sterilised to make a 10 mL solution. After autoclaving and cooling of the agar medium to 60 °C, 30 mL of olive oil and 10 mL of the filter sterilised Rhodamine B solution were added. A loopful of each strain was inoculated on the agar plates and incubated at 37 °C for 48 h. UV irradiation (350 nm) was used to assess clearing zones.

C. Optimisation of Lipase Production using Response Surface Methodology (RSM)

Response surface methodology was employed to optimise the production of lipases. Design-Expert® software v.6.0.8 (Stat-Ease, Inc, USA) was used for RSM analyses and for

reactor parameter optimisation. A set of 13 experiments was used to ascertain optimum conditions for high lipase production by the isolated strains, *B3O* and *BF3*. Two parameters (pH and temperature) were considered for the RSM design to optimize lipase production. pH (ranging from 3.17 - 8.83) and temperature (ranging from 23.79 - 66.21 °C) conditions were set according to the RSM experimental design, and tested for lipase production and activity. All the experiments were conducted in triplicates.

D. Lipase Production

Positive lipase-producing isolates were inoculated in a fermentation medium (pH 7), incubated at 37 °C for 72 h, and agitated at 121 rpm. The fermentation medium comprised (g/L): 5.0 g of peptone, 10 g yeast extract and 5.0 g NaCl supplemented with 10mL of filter-sterilized olive oil [8] to induce lipase production. Quantification of bacterial growth was performed by measuring absorbance at 660nm using a UV/V is Jenway spectrophotometer, Cole Parmer, USA.

E. Partial Purification

Culture broth was centrifuged at 7000 rpm at 4 °C for 25 min. All supernatants were separated from the biomass pellet with the pellet being resuspended in 50mMTris-HCl buffer (pH 8.0) by vortexing. In order to harvest intracellular lipases, a sonicator was used for the disruption of the cells constituting the pellet for 10 min at 10 s intervals to minimise overheating, with sonicated samples being put on ice (4 °C) between sonication cycles. The mixture from the sonicator was centrifuged at 15000 rpm and 4 °C for 25 min. Crude enzyme extracts in the form of supernatants from the centrifugation and sonication procedures were recovered and stored at 4 °C prior to enzyme activity quantification in triplicates. The crude enzyme extracts with high enzyme activity were concentrated by loading on to Bio-Rad/Bio-Gel® P-60 size exclusion chromatography columns equilibrated with potassium phosphate buffer (50 mM pH 7.0) with the fractions (5 mL) collected and stored at 4 °C for further use in this study.

F. Determination of Protein Content and Lipase Activity

Bradford's assay was used to determine the total protein concentrations [9]. Enzyme activity was determined using the lipase assay methods previously used by [10], [11], [12], [13].

G. Effect of pH and Temperature on Enzyme Stability and Activity

Optimum pH for lipase (semi purified) stability was determined spectrophotometrically using an adapted method from [8]. 50mM concentrations of citrate buffer (pH 3.0-5.0), Tris-HCl buffer (pH 6.0-7.0), K₂HPO₄-KH₂PO₄ (pH 8.0-9.0) and glycine-KOH buffer (pH10.0) were used for reaction mixtures. A typical assay reaction mixture contained 200µL of the semi-purified enzyme and 1800µL of the buffer and incubation at 37 °C for 15 min. Optimum temperature for stability was studied at a pre-determined optimum pH by incubating the assay mixtures at different temperatures, i.e. 25, 45, 55, 65 and 75 °C in a 50 mM phosphate buffer for 30 min, after which the samples were allowed to cool subsequent to

enzyme activity determination. In both cases, the enzyme activity was measured as described above.

H. Effect of Solvents, Metal ions and Detergents on Enzyme Stability and Activity

The effect of solvents on enzyme stability was investigated using isopropanol, 2-mercaptoethanol, acetone, methanol, ethanol, hexane, chloroform and toluene. The impact of metal ions on the lipase activity was also studied by similarly incubating the enzymes in the presence of metal salts, i.e. CaCl_2 , KCl , NaCl , MgSO_4 , and $\text{Fe}_2(\text{SO}_4)_3$. Triclosan (TCS) (5-chloro-2-(2,4-dichlorophenoxy)-phenol), EDTA, and trichlorocarbanilide (3,4,4-trichlorocarbanilide) (TCC) were used for assessing the role of these detergents prior enzyme activity determination. Prior to enzyme activity determination, the reaction mixture of each solvents / metal ions / detergents (1.8 mL) and crude enzyme (0.2 mL) were incubated at 37 °C for 30 min. For comparative analysis, a reference experiment without the solvents, metal ions, detergents was also used.

III. RESULTS AND DISCUSSION

A. Plate Assays: Microbial Screening and Lipase Production

Pure culture samples obtained from poultry slaughterhouse wastewater (PSW), and from a discharge point at a local poultry slaughterhouse, were screened to isolate lipolytic strains using qualitative assay methods. Isolates ($n = 20$) were maintained on nutrient agar, following lipase activity screening procedures on agar plates supplemented with tributyrin. A number ($n = 2$) of isolates produced distinct zones of hydrolysis (clearance) as shown in Figure 1-a2 for strain *BF3* and Figure 1-b2 for strain *B30*. The clearing of zones on tributyrin agar plate was attributed to tributyrin hydrolysis and esterases enzyme activity [14].

The lipase activity was observed in Tween 80 agar plates for *BF3* (Fig. 1-a3) and *B30* strain (Fig. 1-b3), thus confirming the potential applicability of the isolates in bio-delipidation systems. Additionally, the esterification of free fatty acids and long chain alcohols was confirmed when *BF3* (Fig. 1-a4) and *B30* strains (Fig. 1-b4) were tested using the Rhodamine B dye assay method. Although, the current study successfully screened for lipase producing isolates using plate assays, very few other studies managed to achieve similar results [15], [8]. The low success rate is a resultant of the insensitivity of the quantitative methods and low enzyme doses on plates, especially when Rhodamine B dye assay is used under UV light.

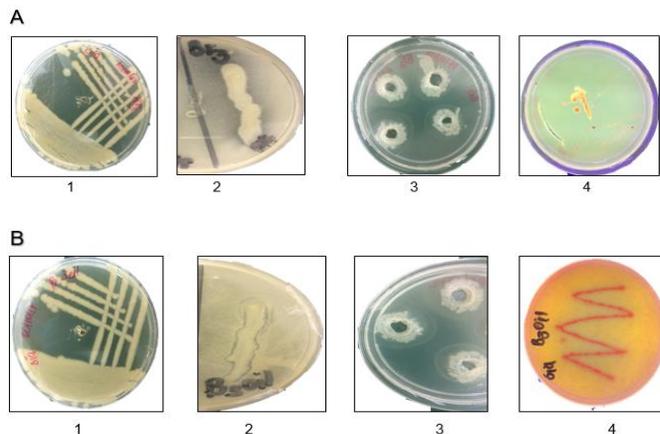


Fig. 1: (a) The screening for lipolytic activity of *BF3* on nutrient agar (1); Tributyrin agar plate (2); Tween 80 agar plate (3), Olive oil rhodamine b dye (4). (b) The screening for *B30* on nutrient agar (1); Tributyrin agar plate (2); Tween 80 agar plate (3), Olive oil Rhodamine B dye (4).

B. Isolation and Identification

Microbial identification of the isolates that showed a high lipolytic potential was achieved by morphological identification and 16S rRNA sequencing. The microorganisms were subjected to gram staining for morphological characterisation, with both being gram positive, with a rod shape and a yellow color for *B30*, and while *BF3* was coccus. Identification methods using 16S rRNA sequencing confirmed the isolates to be *Bacillus cereus*, with Genbank accession numbers CP023179.1 (*B30*) and MF800922.1 (*BF3*).

C. Optimisation of Lipase Production

Graphical illustrations were generated to study the interactive influence of pH and temperature on lipase production, while maintaining the production time (72 h) as an independent parameter. It was crucial to understand these interactions on the semi-purified enzymes because fermentation conditions, environmental, as well as physiological factors are known to impact on lipase production [2]. In different studies, maximum production of lipases by *Pseudomonas sp.* was achieved between 48 and 96 hours [16], [17], [18]. Maximal lipase production for both strains was achieved between pH 6 and 8, and between at 45 and 60°C. Maximum lipase activity for *Bacillus cereus* strain *AB1* (*BF3*) (11.25 U mL^{-1}) (Fig. 2a) and *Bacillus cereus* *CC-1* (*B30*) (15.50 U mL^{-1}) (Fig. 2b) was obtained under the conditions of pH 8 and 60°C, and pH 8.83 and 45 °C, respectively. These observations clearly showed the importance of strain variability and the direct role of environmental factors on lipase production. Interestingly, post purification activity for *Bacillus cereus* strains *AB1* (*BF3*) and *CC-1* (*B30*) was quantified as 19.47 and 28.36 U mL^{-1} respectively (data not shown). This improvement in lipase activity highlighted the importance of enzyme purifications for bio-delipidation systems.

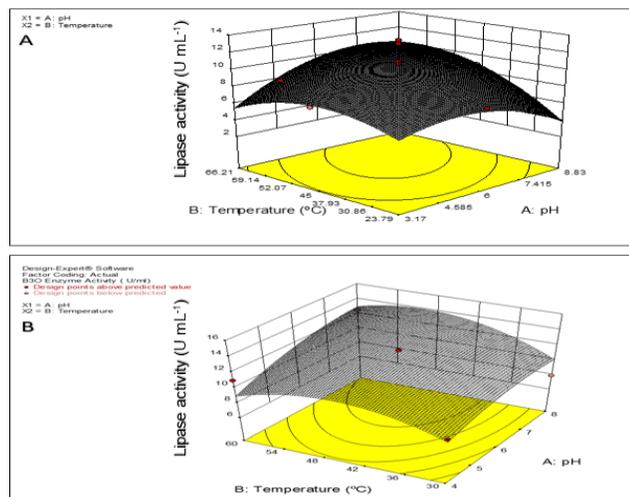


Fig. 2: The interactive role of pH and temperature on lipase activity of strain *BF3* (a) and *B30* (b).

D. Effect of pH and Temperature on Enzyme Stability and Activity

As indicated in Fig 3a, the optimal lipase activity for *Bacillus cereus* (*BF3*) and *Bacillus cereus* (*B30*) was obtained at pH 7 (8.25 U mL⁻¹) and at pH 8 (12.72 U mL⁻¹), respectively. This profile made these enzymes more suitable for bio-delipidation in near neutral pH conditions. Hence, the pH of the dissolved air floatation (DAF) pre-treated PSW are usually higher than pH 6 [23]. A strain dependent characteristic was noted when the lipase enzymes produced by *Bacillus cereus* (*B30*) retained a higher activity over a broad range of pH conditions (pH 4 - 11). However, many lipases from *Bacillus* sp. were reported stable at alkaline conditions (pH 7 - 9) [19]. Generally, an optimum temperature facilitates enzyme-substrate binding, leading to high substrate conversion rates. For both isolates (Fig 3b), a decrease in activity was observed when the temperature was increased beyond 45 °C, as opposed to other thermostable *Bacillus* sp., that can retain their activity at temperatures beyond 60°C [20].

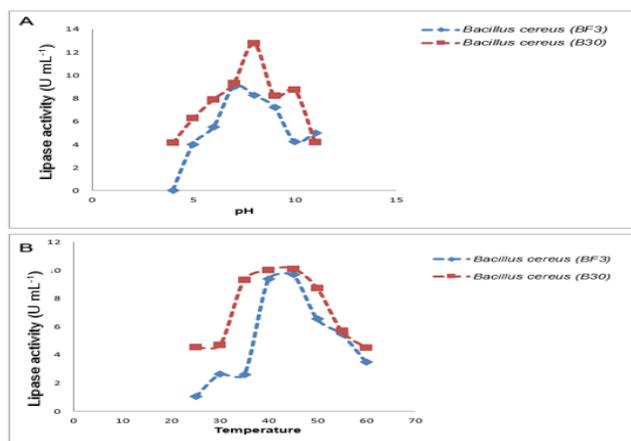


Fig. 3: The impact of pH (A) and temperature (B) on enzyme activity for *Bacillus cereus* AB1 (*BF3*) and *Bacillus cereus* CC-1 (*B30*) strains.

E. Effect of Solvents, Metal ions and Detergents on Enzyme Stability and Activity

Table 1a shows the relative influence of metal ions and detergents on the enzyme activity of the isolates. 95.01 % (*BF3*) and 89.94 % (*B30*) lipases activity was retained in the presence of Mg²⁺ (1 mM) whereas more than 90 % activity was lost in the 1 mM EDTA, Na⁺, Fe²⁺, and K reaction mixtures. It was not surprising since most metal ions and detergents are known to negatively impact on activity and stability of lipases [21]. Detergents and metal ions are known to have an influence on the biological function of enzymes, by enhancing or inhibiting their activity via various mechanisms, such as acting as an electron donor or acceptor and forming complexes with limited reactivity [22]. Like any other enzyme, inhibition of lipase activity by these metal ions is caused by inhibition of the catalytic site [19].

TABLE I: EFFECT OF METAL IONS, DETERGENTS (A) AND ORGANIC SOLVENTS (B) ON LIPASE ACTIVITY OF *BACILLUS CEREUS* STRAINS AB1 (*BF3*) AND CC-1 (*B30*)

Metal ion and detergent (A)	Concentration (mM)	Relative activity (%) <i>BF3</i>	Relative activity (%) <i>B30</i>
Control	0.0	100	100
CaCl ₂	1.0	9.41	11.95
NaCl	1.0	3.70	8.83
KCl	1.0	7.33	12.03
MgSO ₄	1.0	95.01	89.94
FeSO ₄	1.0	2.65	4.48
EDTA	5.0	4.85	8.83
Triclosan	1.0	81.36	73.91
Trichlorocarbonilide	1.0	91.43	85.32
Organic solvent (B)	Concentration (% v/v)	Relative activity (%) <i>BF3</i>	Relative activity (%) <i>B30</i>
Control	0.0	100	100
2-Mercaptoethanol	30	25.4	23.49
Acetone	30	15.56	26.72
Chloroform	30	61.77	61.63
Ethanol	30	71.20	71.05
Hexane	30	79.90	77.68
Isopropanol	30	85.14	85.97
Methanol	30	84.47	83.78
Toulene	30	88.70	90.00

Inconsistently, activity of lipases from both strains was also considerably inhibited by Ca⁺, unlike lipases from numerous microorganisms, including those from *Bacillus* sp. that are Ca⁺ dependent. Considerable lipases activity was retained in reaction mixtures separately containing TCS (81.36 % and 73.91 % for *BF3* and *B30*, respectively) and TCC (91.43 % and 85.32 % for *BF3* and *B30*, respectively), which are often used as

antimicrobial agents and disinfectants in slaughterhouses. In agreement, [23] observed more than 90 % lipase retainment for *C. aquatica* BF-3 and *Bacillus* sp. BF-2 when TCS and TCC were also tested.

The presence of acetone and 2-Mercaptoethanol inhibited the lipase activity of both isolates by over 70 % (Table 1b). In contrast, the enzymes from both isolates retained an activity of more than 60 % in the presence of toluene, methanol, isopropanol, hexane, ethanol and chloroform. It was interesting to realise that most organic solvents can support and maintain enzyme conformity that stimulates activity [24], [25].

IV. CONCLUSION

Lipolytic microorganisms, *Bacillus cereus* AB1 (BF3) and CC-1 (B3O) were isolated from the PSW, identified using 16S rRNA sequencing techniques and successfully screened for lipase activity. The interactive impact of environmental factors (pH and temperature) on lipase production was revealed by RSM. Temperature, pH, metal ions, detergents and organic solvents affected significantly on the lipase activity and stability. Future works should focus on the applications of these lipolytic microorganisms in bio-delipidation systems.

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