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, $NaCl_2$, $MgSO_4$, and $Fe_2(SO4)_3$. 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.

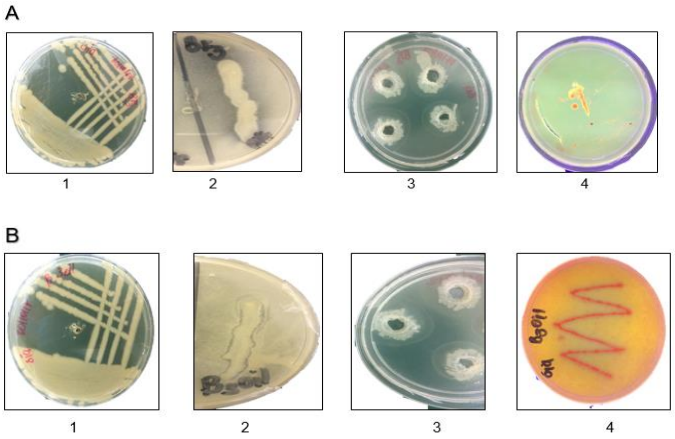

Fig. 1: (a) The screening for lipolytic activity of *BF3* on nutrient agar (1); Tributyrin agar plate (2); Tween 80 agar plate (3), Olive oil rhodamine b dye (4). (b) The screening for *B30* on nutrient agar (1); Tributyrin agar plate (2); Tween 80 agar plate (3), Olive oil Rhodamine B dye (4).

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 *B3O*, and while *BF3* was coccus. Identification methods using 16S rRNA sequencing confirmed the isolates to be *Bacillus cereus*, with Genbank accession numbers CP023179.1 (*B3O*) 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* (B3O) 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.

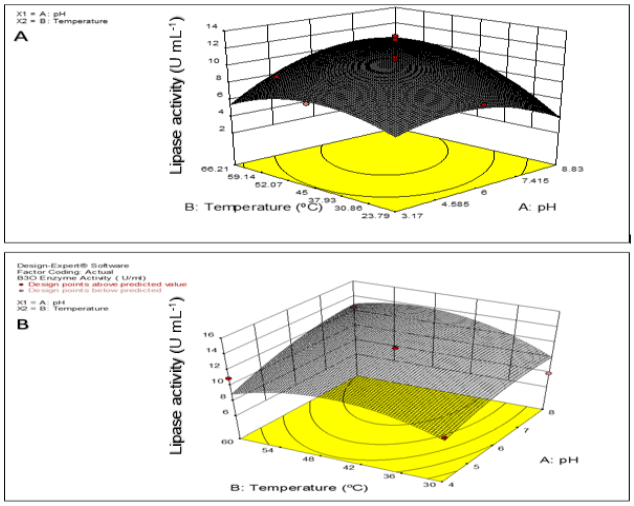

Fig. 2: The interactive role of pH and temperature on lipase activity of strain *BF3* (a) and *B30* (b).

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].

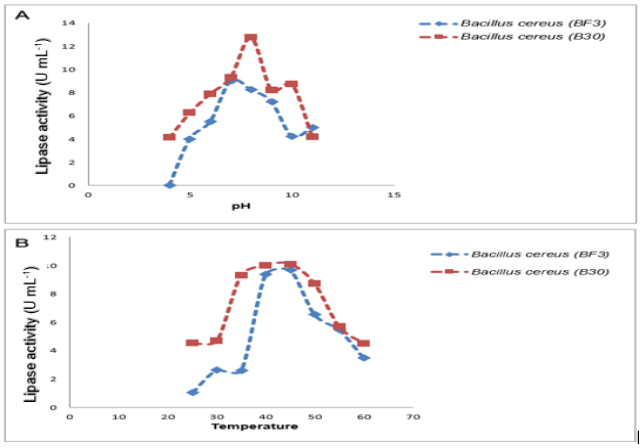

Fig. 3: The impact of pH (A) and temperature (B) on enzyme activity for *Bacillus cereus AB1* (*BF3*) and *Bacillus cereus CC-1* (*B30*) strains.

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 Mg^{2+} (1 mM) whereas more than 90 % activity was lost in the 1 mM EDTA, Na^{+}, Fe^{2+}, and K reaction mixtures. It was not suprising 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* (*B3O*)

Metal ion and detergent (A)	**Concentration (mM)**	**Relative activity (%) *BF3***	**Relative activity (%) *B3O***
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 (%) *BF3***	**Relative activity (%) *B3O***
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 toulene, methanol, isopropanol, hexane, ethanol and chlorofom. 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 acivity and stability. Future works should focus on the applications of these lipolytic microorganisms in bio-delipidation systems.

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