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Production of a novel alkaline lipase by *Fusarium globulosum* using neem oil, and its applications*

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Abstract: A low-temperature, alkaline, and detergent-compatible lipase from *Fusarium globulosum* (FGL) has been produced using an inexpensive source, viz. neem oil. Maximum lipase production of 12 300 U/L was achieved in 96 h on optimizing various physicochemical parameters. The lipase exhibited remarkable stability in the presence of commercial detergents, bleaching agents, and proteases. The lipase showed a novel property of marked activation on prolonged incubation at alkaline pH. Wash performance analysis of the commercial detergent for removal of fatty stains improved upon addition of this lipase.

INTRODUCTION

Lipases (EC 3.1.1.3) catalyze the hydrolysis of triacylglycerides to diacylglycerides, monoacylglycerides, free fatty acids, and glycerol at the oil–water interface [1]. The biotechnological importance of lipases [2–6] has increased markedly in the last two decades owing to their versatile catalytic behavior [7]. They directly or indirectly form an integral part of the industries ranging from food [8], pharmaceuticals [9–11], and detergents [12–16] to oleo-chemicals, cosmetics [17], agriculture [18], leather, and tea industries [19,20]. However, the single biggest market for their use is in detergents where their functional importance lies in the removal of fatty residues in laundry, dishwashers, and for cleaning clogged drains [20,21]. Microbial lipases from *Humicola lanuginosa* (Lipolase), *Pseudomonas mendocina* (Lumafast), and *P. glumae* are being used commercially in detergent formulations, but most of them have a high-temperature optima [1]. The latest trend in detergent industry is toward lower wash temperatures that not only save energy, but also help maintain the texture and quality of fabrics [21]. Since fat stains are difficult to remove at low temperatures, understandably, lipase preparations are needed that are active at low to ambient temperatures and are compatible with the detergent formulations. Earlier, we reported the biocatalytic utility of *Fusarium globulosum* lipase (FGL) in different acetylation and deacetylation reactions for synthesis of bioactive compounds [22,23]. In this paper, we report studies on parametric optimization for its production and its evaluation as a potential detergent additive.

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Our results have shown that the FGL is active at ambient and low temperatures and alkaline pH. Besides being useful in detergent formulations, it also has many important properties, which are of significance in many industrial processes.

BIOCATALYTIC UTILITY OF *F. GLOBULOSUM* LIPASE

In recent years, chemoenzymatic synthetic strategies have become standard techniques for the preparation of a variety of chiral and nonchiral precursors and target molecules [24–45]. Among these methodologies, lipase-catalyzed acylation and deacylation reactions represent an important class of enzymatic transformations in organic synthesis as these enzymes can work in a variety of organic solvents. The capabilities of FGL were thus investigated (Fig. 1) for chemo- and enantioselective acetylation and deacetylation of hydroxymethylated phenols and hydroxyaryl alkyl ketones and their peracetylated derivatives.

Results have shown that FGL exhibits exclusive chemoselectivity for the acetylation of aliphatic hydroxyl group, monoacetates of 2-hydroxybenzyl alcohol and 3-hydroxy-1-(-4'-hydroxyphenyl)-2-methylpropanone were thus formed (Fig. 1). In addition, the acetylation of hydroxymethylaryl alkyl ketone led to its enantiomeric resolution. These reactions can thus be used for the synthesis of optically pure organic compounds.

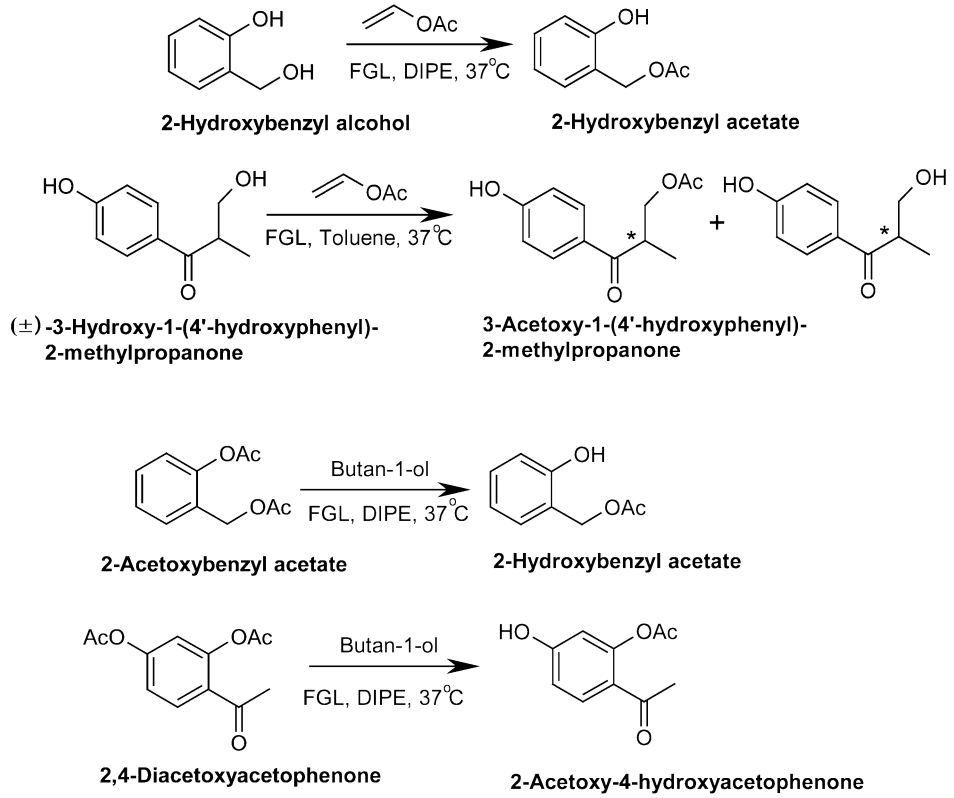

Fig. 1 Chemo-, regio-, and enatioselective reactions catalyzed by FGL.

Production medium for FGL and its activity

Fusarium globulosum was isolated by enrichment from a local oil factory effluent. The culture was maintained on potato dextrose agar slants and stored at 4 °C. Lipase production was carried out in the medium, which consisted of gl^{-1} distilled water: $NaNO_3$, 2; KCl, 0.52; $MgSO_4 \cdot 7H_2O$, 0.52; KH_2PO_4, 1.52; $Cu(NO_3)_2 \cdot H_2O$, 0.001; $FeSO_4 \cdot 7H_2O$, 0.001; $ZnSO_4 \cdot 7H_2O$, 0.001; glucose, 2.0; and olive oil, 15 ml. Initial pH of the medium was adjusted to 6.2. The medium was autoclaved at 121 °C (15 psi) for 15 min and inoculated with 5×10^7 spores/per 50 ml of the medium. The flasks were incubated at 25 ± 1 °C for 96 h.

The biomass estimation was done by filtering the culture broth through preweighed and predried Whatman filter paper no. I, and the culture filtrate was used as the source of extracellular lipase. The filter paper containing the biomass was dried at 80 °C to a constant weight and expressed as mg dry wt biomass/50 ml.

The lipase activity in the culture filtrate was determined by titrimetry employing *p*-nitrophenyl palmitate assay using olive oil as the substrate [46,47]. One unit of lipase activity is defined as the amount of enzyme required to release 1 µmol of fatty acid or *p*-nitrophenol at 37 °C under standard assay conditions (pH 7.0; reaction time 30 min). The total protein in the culture was estimated according to the method of Lowry et al. [48].

Parametric optimization for lipase production

Fusarium globulosum was selected as a potent lipase producer after screening of more than 200 bacteria and fungi.

Various physicochemical parameters were optimized in order to obtain maximum lipase production by *F. globulosum*. Sixteen different oils, namely, corn, coconut, groundnut, castor, soyabean, sunflower, walnut, mustard, vegetable, tallow, amla, jasmine, neem, rose, almond, and watermelon seeds were tested in addition to olive oil (used conventionally) for lipase production. Table 1 clearly describes the amount of lipase produced (U/L) along with the biomass and specific activities using different oils.

Table 1 FGL production using various oils at 25 °C, pH 6.2, and 250 rpm.

Oil	Lipase production (U/L)	Specific activity (U/mg protein)	Biomass (g dry wt/50 ml)
Olive	2080 ± 45	7.09 ± 1.01	0.37
Corn	3330 ± 54	5.43 ± 0.30	0.53
Sunflower	948 ± 65	2.97 ± 0.56	0.19
Groundnut	2650 ± 19	4.56 ± 0.33	0.30
Coconut	1080 ± 10	2.96 ± 0.90	0.20
Mustard	2765 ± 101	3.99 ± 0.49	0.33
Walnut	1840 ± 75	2.97 ± 0.66	0.20
Vegetable	2695 ± 45	1.01 ± 0.36	0.55
Tallow	1341 ± 26	4.57 ± 0.98	0.22
Amla	1690 ± 85	3.01 ± 0.11	0.43
Jasmine	2450 ± 45	4.56 ± 0.25	0.36
Neem	8976 ± 60	12.56 ± 0.31	0.52
Castor	3501 ± 10	5.99 ± 0.66	0.47
Rose	830 ± 75	1.97 ± 0.71	0.15
Almond	1050 ± 84	2.95 ± 0.36	0.20
Watermelon seeds	868 ± 95	2.11 ± 0.37	0.10

Neem oil was found to be the best suited oil for lipase production (Table 1). The composition of fatty acids in neem oil is: palmitic acid (16–34 %), stearic acid (6–24 %), oleic acid (25–58 %) and linoleic acid (6–17 %) [49]. It is the palmitic acid component of the neem oil that supports high lipase production. A similar trend has been observed for *Aspergillus terreus* [23] and *A. carneus* [50] where considerably higher palmitic acid content of the oil supported good lipase production.

Different concentrations (ranging from 0.5–3.5 %) of neem oil were studied. Neem oil at a concentration of 2.0 % v/v was found to be the best for lipase production (8976 U/L) in comparison to conventional olive oil (2080 U/L) initially used for screening at the same concentration (Table 1 and Fig. 2).

This is the first report of neem oil (which is a nonconventional oil) being used for lipase production. It is interesting to note that the cost of neem oil is 2.5 times lower than that of olive oil. Enzyme yields obtained are also comparable to those produced by a few other filamentous fungi, e.g., *A. niger* grown on olive oil [51], *Rhizopus japonicus* grown on rice bran oil [52], *Geotrichum candidum* grown on soyabean oil [53], etc.

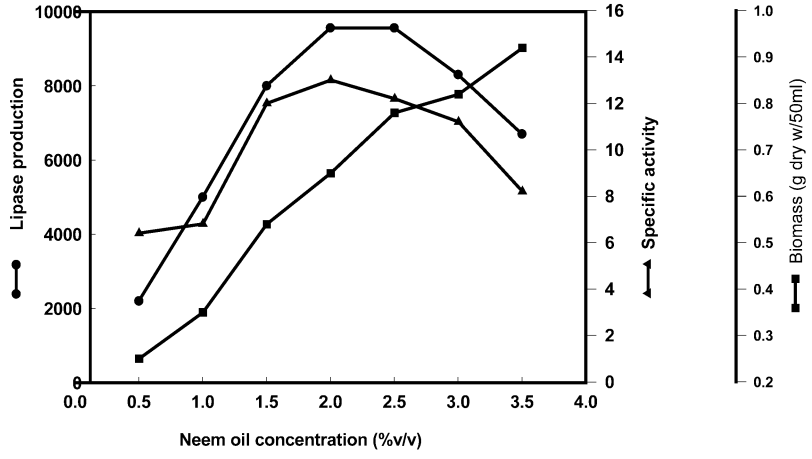

Fig. 2 Lipase production profile of *F. globulosum* at different concentrations of neem oil.

With the view to study the effect of pH on lipase production, pH of the minimal medium was varied in the range 3.0–10.0 (at an interval of one) using different buffer systems. The results showed that maximum lipase was produced at pH 7.0, though the organism was able to grow in the pH range 3.0 to 10.0 (Fig. 3). Hence, pH 7.0 was selected as the optimum pH for the production of lipase in all the subsequent experiments.

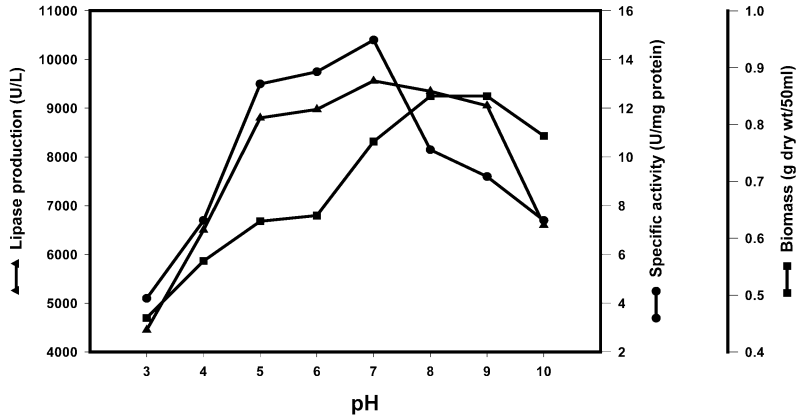

Fig. 3 Effect of pH on lipase production.

Lipase production was studied in the temperature range 20–45 °C (varied at an interval of 5 °C). Production was carried out in the minimal medium containing 2.0 % v/v neem oil (pH 7.0) and at 250 rpm for 96 h. The results (Fig. 4) showed that maximum lipase was produced in the temperature range 25–30 °C. The production declined at temperatures above 30 °C. Hence, 25 °C was selected as the optimal temperature for further studies.

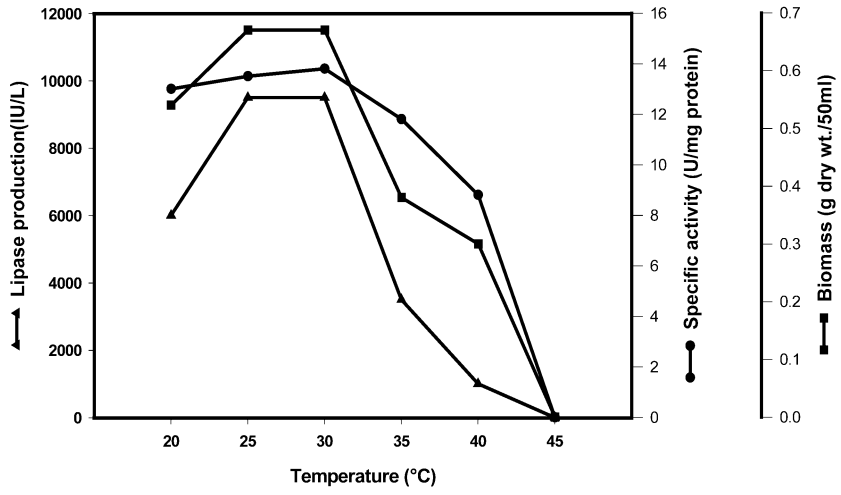

Fig. 4 Effect of temperature on lipase production.

The effect of nitrogenous additives on lipase production was also studied using various inorganic (ammonium chloride, ammonium nitrate, ammonium dihydrogen orthophosphate, ammonium sulfate) and organic (casein hydrolysate, peptone, beef extract, yeast extract, corn gluten meal, casein, tryptone, asparagine, and aspartic acid) nutrients. Among all, peptone further enhanced lipase production to 12330 U/L (Table 2). Peptone has also been shown to support lipase production in the case of *Rhizopus japonicus* [52] and *Streptomyces* sp. lipases [54].

Table 2 FGL production in the presence of different organic and inorganic nitrogenous additives at 25 °C, 250 rpm, and pH 7.0.

Nitrogenous additive	Lipase production (U/L)	Specific activity (U/mg protein)	Biomass (g dry wt/50ml)
(–) Control[a]	2080 ± 45	7.09 ± 1.01	0.37
(+) Control[b]	7850 ± 60	12.56 ± 0.31	0.52
Casein hydrolysate	9540 ± 51	10.56 ± 0.95	0.95
Peptone	12 330 ± 45	12.56 ± 0.56	1.02
Beef extract	8540 ± 101	8.99 ± 0.33	0.96
Yeast extract	7650 ± 96	7.56 ± 0.79	0.94
Corn gluten meal	12 310 ± 95	8.24 ± 0.96	1.25
Casein	10 660 ± 35	9.95 ± 0.72	0.80
Corn steeped liquor	9995 ± 105	11.24 ± 0.31	0.97
Tryptone	7665 ± 231	10.56 ± 0.29	0.83
Asparagine	6950 ± 43	7.54 ± 0.93	0.65
Aspartic acid	9995 ± 135	6.56 ± 0.76	0.67
Ammonium chloride	10 500 ± 101	10.33 ± 0.61	0.74
Ammonium nitrate	9693 ± 35	11.54 ± 0.21	0.75
Ammonium dihydrogen orthophosphate	8665 ± 43	12.39 ± 0.26	0.72

[a]Minimal medium* + olive oil
[b]Minimal medium* + neem oil
*Composition of minimal medium has been described under the heading "Production medium for FGL and its activity".

After optimizing the above physicochemical parameters for lipase production, incubation period was optimized. For this, *F. globulosum* was grown for different time periods, viz. 24–168 h in the optimized media. Lipase activity was estimated after every 24 h.

The results indicated that lipase production reached a peak in 96 h at the onset of the stationary phase (Fig. 5). A similar trend has also been reported in other filamentous fungi, e.g., *Humicola lanuginose* [55] and *Rhizopus delemar* [56].

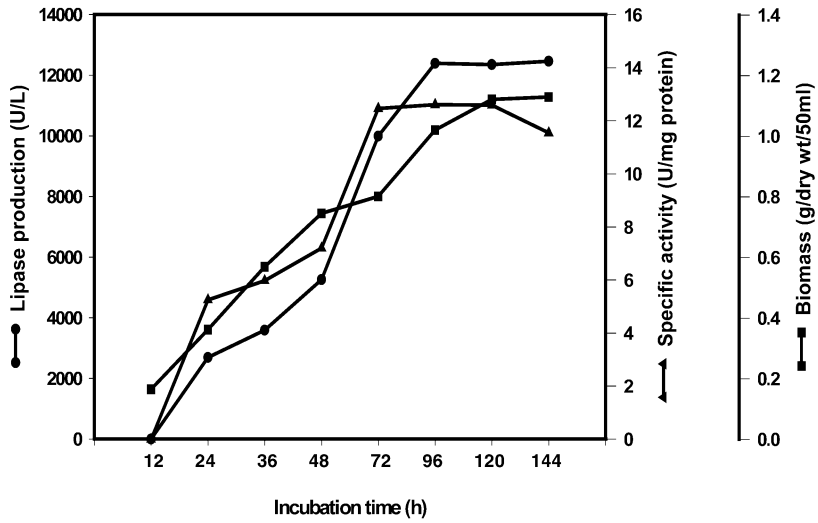

Fig. 5 Time course of lipase production by *F. globulosum.*

A total of 5.95-fold increase in lipase production from *F. globulosum* was observed on optimizing various physicochemical parameters against the (–) control viz. the first experiment where minimal medium + olive oil was used.

CHARACTERIZATION AND EVALUATION OF FGL AS POTENTIAL DETERGENT ADDITIVE

The lipase exhibited requisite pH and temperature kinetics suitable to detergent industry as the enzyme is active at neutral and alkaline pH (Fig. 6) and also in the *low* temperature range 10–30 °C (Table 3). Detailed characterization of the lipase was carried out to evaluate it as a potential detergent additive.

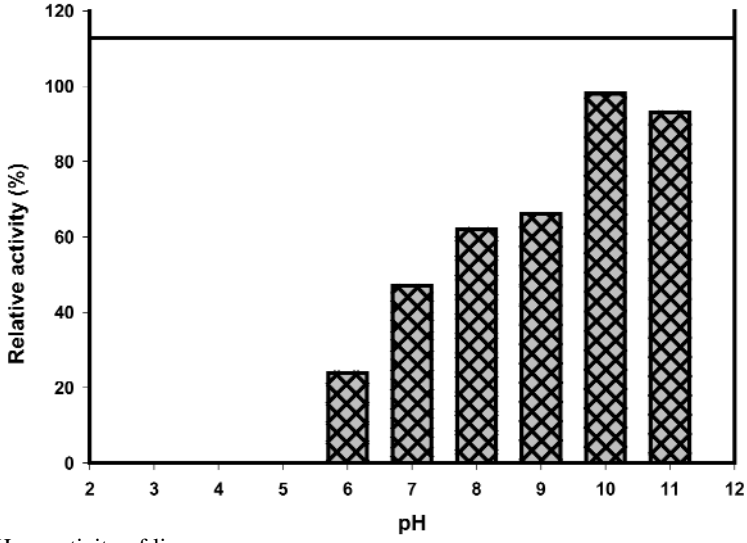

Fig. 6 Effect of pH on activity of lipase.

Table 3 Effect of temperature on activity of FGL at pH 10.0 after 30 min.

Temperature (°C)	Lipase activity (U/ml)	Relative activity (%)
10	2.85	71.1
20	3.24	80.8
30	3.85	96.0
37	4.01	100.0
50	3.80	94.8
60	2.25	56.1
70	1.32	32.9
80	1.29	32.1

The activity of FGL was assayed at different temperatures ranging from 10–80 °C at pH 10.0 (Table 3). The lipase showed more than 96, 81, and 71 % relative activities at 30, 20, and 10 °C, respectively. Thus, it was confirmed that the enzyme has the prerequisites of a detergent enzyme with respect to pH and temperature kinetics.

Culture filtrate containing FGL produced under optimized conditions was subjected to concentration using a 30 kDa ultra filtration membrane (Spin Trex, NBS, USA) and partial purification using

30 % acetone. The precipitate containing lipase was dissolved in 0.01 M phosphate buffer and evaluated for different biochemical properties.

FGL was incubated in different pH buffers ranging from 3.0–11.0 for varying time periods ranging from 0.5 to 24 h at 25 °C and the residual activities (%) were determined after the stipulated time period. The lipase showed marked stability in the pH range 6.0–11.0 (Table 4).

Table 4 pH stability of FGL.

pH	Residual activity (%) after				
	0.5 h	2 h	6 h	12 h	24 h
3	86.25	74.32	52.96	46.25	38.26
4	95.95	82.24	67.56	54.26	47.25
5	100.00	88.95	82.75	80.26	74.56
6	100.00	100.00	98.25	97.65	98.95
7	100.00	100.00	99.95	98.95	98.99
8	100.00	100.00	99.86	98.67	99.95
9	116.24	136.24	153.29	146.25	146.25
10	120.24	140.24	159.24	160.24	161.26
11	120.25	154.24	158.65	158.25	155.25

Interestingly, the lipase showed marked activation at alkaline pH of 9.0–11.0 (Table 4 and Fig. 7). More than 150 % residual activities were observed on incubation of lipase for more than 6 h at these pH values, thereby suggesting a more favored conformation for the enzyme at these pH values.

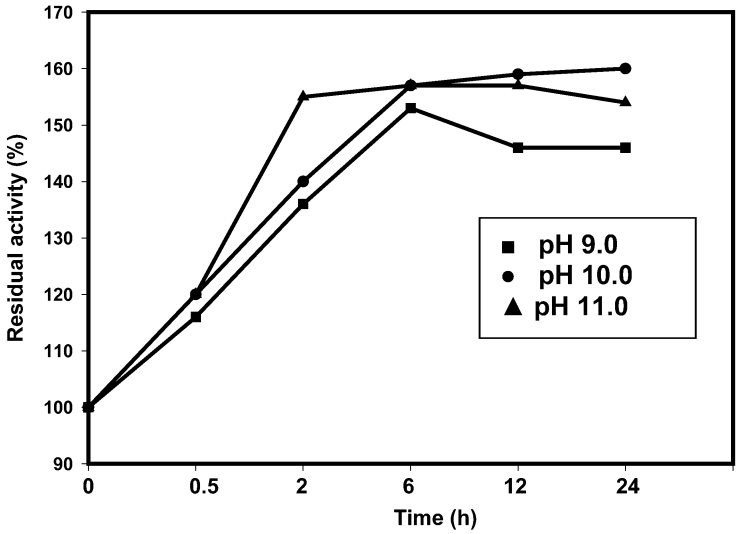

Fig. 7 Effect of alkaline pH on lipase stability.

With a view to see its potential use in detergent formulations, the lipase was incubated with different ionic and nonionic surfactants and commercial detergents at a concentration of 1 % w/v for 60 min at room temperature. The residual activities were assayed at optimal pH and temperature (pH 10.0 and 37 °C). For determining protease stability, FGL (5 U/ml) was incubated in the presence of 5 U/ml of protease from *Bacillus licheniformis* and *Aspergillus saitoi* (ICN Chemicals, USA) for 1 h and the residual activities were assayed at pH 10.0 and 37 °C (Table 5). It was found that the lipase ex-

hibited remarkable stability in the presence of *A. saitoi* (88 % residual activity) and *B. licheniformis* protease (100 % residual activity) even after 1 h of incubation.

Table 5 Stability of FGL in presence of surfactants, commercial detergents, bleaching agents, and proteases.

Detergent additive	Residual activity[a] (%)
Control	100.00
Surfactants	
Triton X-100	167.12
Tween-80	100.34
Tween-20	76.35
Saponin	115.24
Sodium cholate	82.25
Sodium taurocholate	88.67
Bleaching agents	
Hydrogen peroxide	91.34
Sodium perborate	90.56
International market detergents	
Surf	78.00
Ariel	73.24
Indian market detergents	
Nirma	73.00
Wheel	83.00
Fena	86.09
Protease	
A. saitoi	8.23
B. licheniformis	100.00

[a]100 % residual activity (control) corresponds to 7.5 U/ml assayed at pH 10.0 and 37 °C in the absence of any additive.

The results presented in Table 5 revealed that the lipase was stable in the presence of both ionic and nonionic surfactants with marked activation in presence of Triton X-100 and saponin and had retention of 100 % activities in presence of Tween 80. More than 70 % residual activities were observed in the presence of commercial detergents of international and Indian markets, viz. Surf, Ariel, Nirma, Wheel, and Fena.

To know the effect of oxidizing and bleaching agents, the lipase was incubated for 1 h in the presence of 0.1 M hydrogen peroxide and sodium perborate. The enzyme stability was expressed as residual activity (%) following lipolytic assay at pH 10.0 and 37 °C. The lipase exhibited remarkable stability in the presence of hydrogen peroxide and sodium perborate (>90 % residual activity), which are common detergent additives (Table 5).

Bott et al. [57] have advocated bleach stability as an important characteristic of enzymes to be used in detergents. In this respect, protein-engineering approaches have been employed to improve the oxidative stability of detergent enzymes. Here, the inherent capacity of FGL to be bleach-stable gives it an edge over engineered or tailor-made lipases from other sources.

In addition, FGL showed broad substrate specificity as it was able to hydrolyze a variety of kitchen oils and fats. The lipolytic activity of the enzyme was determined titrimetrically using the common kitchen oils and fats (Table 6).

Table 6 Hydrolysis of kitchen oils or fats by FGL at 37 °C, pH 10.0 for 30 min.

Oil/fat	Relative activity (%)
Olive	95.20
Corn	100.00
Groundnut	71.42
Sunflower	85.70
Soyabean	52.33
Coconut	71.43
Butter	52.33

The lipase showed more than 50 % relative activity for butter, a property rarely reported for lipases. Investigations by Pabai et al. [58] is thus far the only report of butter fat hydrolyzing lipase from *Pseudomonas fragi* CRDA 323.

The wash performance analysis of the lipase was examined for removal of oil stains from cloth pieces using three commercially used detergents in India viz. Surf, Nirma, and Wheel. Cotton swatches (8 × 8 cm) were stained with sudan red-dyed corn oil. The cloth pieces were dried overnight at 80 °C, 50 mg of FGL (equivalent to 500 lipase units) was added to 10 ml of the detergent solution (1.0 % w/v). The cloth pieces were soaked separately, in tap water (TW), tap water + lipase (TWL), detergent alone (D), and detergent + lipase (DL) for 20 min. The fatty acids released (mmol) were determined by titrating with 0.01 M NaOH.

The wash performance of FGL suggested that large amounts of fatty acids were released when FGL was used along with the detergent, leading to better stain removal (Table 7).

Table 7 Wash performance analysis of FGL in Indian market detergents.

	Amount of fatty acids released (mmol)			
Detergent	TW[a]	TWL[b]	D[c]	DL[d]
Surf	0.7	2.5	1.3	6.3
Nirma	0.5	2.2	1.6	5.5
Wheel	0.8	2.9	1.4	7.9

[a]Tap water.
[b]Tap water + lipase.
[c]Detergent only.
[d]Detergent + lipase.

OVERVIEW

Thus, it is clearly evident that the FGL has the requisite properties of alkaline pH optima, high activities at low temperatures, and stability in the presence of surfactants, commercial detergents, and bleaching agents, making it ideally suited for formulations in low-temperature wash detergents. Also, owing to its chemo- and regioselective properties, FGL can be used to carry out a variety of synthetic organic reactions, which can find applications in the pharmaceutical, oleo-chemical, food, and agrochemical industries.

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