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Characterization of 1,3-regiospecific lipases from new *Pseudomonas* and *Bacillus* isolates

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Abstract

Lipase producing ability of 120 bacterial isolates was examined qualitatively, resulting in 32 lipase producers, which were further screened for 1,3-regiospecificity. Three *Bacillus* (GK-8, GK-31 and GK-42) and one *Pseudomonas* (GK-80) were found to produce 1,3-regiospecific lipases. These lipases were alkaline in nature as they showed pH optima of 9.0 and high stability in the alkaline pH range of 8.0–11.0. The lipases from three *Bacillus* isolates, viz. GK-8, GK-31 and GK-42 showed temperature optima of 37 °C, whereas the *Pseudomonas* (GK-80) lipase showed optimum activity at 50 °C. The lipase of GK-8 was highly stable and showed enhanced activity in different organic solvents like petroleum ether (172%), diethyl ether (143%) and acetone (135%).

Keywords: 1,3-Regiospecificity; Lipase; *Bacillus* spp.; *Pseudomonas* spp.; Triolein

1. Introduction

Microbial lipases (triacylglycerol ester hydrolases EC 3.1.1.3) are amongst the most versatile biocatalysts, which can carry out a multitude of bioconversion reactions such as hydrolysis, esterification, alcoholysis, acidolysis and amonolysis [1–3]. Their importance lies in the fact that they have clearly defined specificities for reactions catalyzed [4–6]. In this context, regiospecificity is one of the most important properties exhibited by lipases. Regiospecific lipases are of two types: *sn*-1,3 specific (1,3-regiospecific) and *sn*-2 specific (2-regiospecific) [4]. However, amongst them 1,3-regiospecific lipases are very important due to their multifarious application in different industries such as oil modification, food processing and dairy, pharmaceutical, organic synthesis, polymer synthesis and agrochemicals [7–14].

Mostly 1,3-regiospecific lipases have been reported from fungal spp. which include *Rhizopus arrhizus*, *R. delemar*, *R. japonicus*, *Mucor miehei*, *Aspergillus niger*, *A. oryzae*, *A. carneus*, *A. terreus*, *Penicillium cyclopium* and *Fusarium heterosporum* [15–22], while very few reports are available on bacterial 1,3-regiospecific lipases [23,24]. The present investigation is therefore an attempt to identify newer 1,3-regiospecific lipases from *Bacillus* and *Pseudomonas* groups and their subsequent characterization.

2. Experimental

A total of 120 bacterial isolates (55 *Pseudomonas* and 65 *Bacillus* strains) were procured from laboratory stock culture collection. These isolates were grown on nutrient agar slants (composition g/l: peptone 5.0, beef extract 1.5, yeast extract 1.5, NaCl 5.0, pH 7.4 ± 0.2) at 30 °C for 24 h and maintained at 10 °C in a BOD incubator (Yorco Sales Pvt. Ltd., New Delhi). Subculturing was carried out after every 15 days.

2.1. Screening and selection of lipase producers

Qualitatively, the lipase producers were screened using tributyrin agar (TBA) plates [composition g/l: peptone 5.0, beef extract 3. 0, tributyrin 15 ml (v/v), agar-agar 15.0, pH 7.0 ± 0.2]. The isolates were streaked on TBA plates and incubated at 30 °C for 48 h. Lipase activity was observed as a zone of hydrolysis around the bacterial colonies. Lipase activity was further confirmed by streaking the positive isolates on Rhodamine B plates [composition g/l: nutrient broth 8.0, NaCl 4.0, gum acacia 1.0, sodium taurocholate 1.0, olive oil 30.0 (v/v), agar-agar 15.0, pH 7.0 ± 0.2] and incubated at 30 °C for 48 h. The lipolytic activ-

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ity was observed under UV light as an orange fluorescent zone around the bacterial colonies.

The isolates found to be lipase producers by qualitative methods were grown in a production medium [composition g/l: glucose 2.0, peptone 0.5, yeast extract 5.0, Na_2SO_4 2.0, KH_2PO_4 1.0, K_2HPO_4 3.0, $MgSO_4 \cdot 7H_2O$ 0.10, olive oil 10.0 ml (v/v), pH 7.0 ± 0.2] for 48 h at 30 °C and 200 rpm. After the desired incubation period, the cultures were harvested by centrifugation at 10,000 rpm for 10 min. The supernatant was examined for lipase activity using *p*-nitrophenyl palmitate (*p*-NPP) assay method.

2.2. Assay procedure

To 2.4 ml of freshly prepared *p*-NPP solution, 0.1 ml of enzyme sample was added and the reaction mixture was incubated at 37 °C in a water bath shaker for 30 min. The reaction was terminated by the addition of 0.2 ml of 100 mM $CaCl_2$ solution to the reaction mixture and keeping it on ice. The reaction mixture was centrifuged to clarify the solution and the absorbance of the yellow colour of supernatant was read at 410 nm. For control, same reaction was carried out with heat-inactivated enzymes. Lipase activity was calculated from standard curve of *p*-nitrophenol prepared in the range of 10–100 μg/ml.

2.3. Enzyme unit

One international unit of lipase is defined as the amount of enzyme required to release 1 μmol of free phenol from the substrate per ml per minute under the standard assay conditions.

2.4. Evaluation of lipases for 1,3-regiospecificity

The 1,3-regiospecific nature of the lipases produced from various isolates was determined by incubating 1 ml of triolein with 5 ml of enzyme samples at 30 °C and 100 rpm for 24 h. Aliquots of 200 μl were withdrawn after 24 h and mixed with 5 ml of diethyl ether for termination of the reaction. The products formed as a result of hydrolysis of triolein by lipases were evaluated on thin-layer chromatography (TLC) plates (Silica gel 60 F, Merck, Germany) using a solvent system comprising of petroleum ether, diethyl ether and acetic acid in the ratio of 80:30:1. Spots were visualized by incubating the TLC plates in a saturated iodine chamber. The 1,3-regioepecific lipase of *Rhizomucor miehei* obtained from Sigma (St. Louis, USA) was used as control.

2.5. Characterization of the selected lipases

The selected four 1,3-regiospecific lipases were characterized with respect to the following properties.

2.5.1. pH-tolerance

pH-tolerance of the selected bacterial lipases were examined in the range of 4.0–12.0 using the standard *p*-NPP method. Buffers (0.05 M) of different pH (citrate phosphate for pH 4.0 and 5.0, phosphate buffer for pH 6.0 and 7.0, Tris–HCl buffer for pH 8.0, glycine–NaOH buffer for pH 9.0 and 10.0 and hydroxide–chloride buffer for pH 11.0 and 12.0) were used for the preparation of *p*-NPP solution.

2.5.2. pH stability

pH stability of the selected bacterial lipases were examined in the range of 4.0–12.0 by incubating the enzyme samples for 24 h with different buffers. Residual activity was estimated using *p*-NPP method under standard assay conditions and expressed as percentage of the initial activity.

2.5.3. Temperature tolerance

Temperature tolerance of the bacterial lipases was examined at different temperature from 30 to 70 °C (30, 40, 50, 60 and 70 °C) by assaying their activity at the respective temperatures.

2.5.4. Temperature stability

Temperature stability of the lipases were examined by incubating them at different temperatures viz. 30, 40, 50, 60 and 70 °C for 60 min and is expressed as its percentage (%) residual activity.

2.5.5. Organic solvent stability

Twenty milligrams of each of the four partially purified lyophilized lipase samples were mixed with 1 ml of different organic solvents (butanol, carbinol, hexane, toluene, acetone, diethyl ether, di-isopropyl ether and petroleum ether) and incubated for 24 h. The samples were centrifuged and the organic solvents were decanted and evaporated at room temperature. Dried samples were then dissolved in 1 ml of glycine–NaOH buffer (0.05 M, pH 9.0) and the residual activity was determined.

Appropriate controls were kept for all the experiments.

3. Results and discussion

On the basis of screening of 120 isolates (55 *Pseudomonas* and 65 *Bacillus*) on TBA and Rhodamine B plates, 32 were found to be lipase producers (Table 1). Amongst these, three *Bacillus* isolates (GK-8, GK-31 and GK-42) and one *Pseudomonas* isolate (GK-80) were found to produce 1,3-regiospecific lipase as determined by TLC analysis (triolein hydrolysis products from these lipases showed spots corresponding to standard monoolein, diolein, and oleic acids as well as to the triolein hydrolysis products of standard 1,3-regiospecific lipase from *Rhizomucor miehei* on the TLC plate) (Fig. 1).

Lipases from all the four 1,3-regiospecific lipase producing isolates showed optimum activity at pH 9.0 and more than 55% activity in the alkaline pH range of 8–11 (Fig. 2). These results are in accordance with the earlier reports of alkaline lipases of *Bacillus thermocatenulatus*, *Bacillus subtilis*, *Staphylococcus hyicus* and *Pseudomonas fragi* which showed optimal activity in the pH range of 8–10 [23,25–27]. Although, all the four lipases were stable in the pH range of 4.0–12.0 after 24 h of incubation, however, only GK-80 lipase showed maximum stability of 98% between pH 6 and 12 after 24 h of incubation (Fig. 3). Similar pH stability profiles have been reported for *Pseudomonas* sp. [28] and *P. fluorescens* NS2W [27] lipases but these experiments were performed for very short durations of 1–2 h only.

Table 1
Qualitative and quantitative analysis of lipase positive isolates

S. no.	Isolates	Qualitative estimation, zone of hydrolysis (mm) on TBA plates 48 h	Quantitative estimation (*p*-NPP method), lipase activity (IU/ml) 48 h
1	*Bacillus* GK-1	4.0	1.71
2	*Bacillus* GK-2	3.0	1.20
3	*Bacillus* GK-3	5.0	1.31
4	*Bacillus* GK-8	8.0	3.45
5	*Pseudomonas* GK-9	5.0	1.57
6	*Bacillus* GK-11	6.0	1.42
7	*Bacillus* GK-31	8.0	3.52
8	*Pseudomonas* GK-33	4.0	1.28
9	*Pseudomonas* GK-39	3.0	1.63
10	*Bacillus* GK-42	6.0	2.30
11	*Pseudomonas* GK-45	5.0	1.46
12	*Bacillus* GK-46	5.0	1.50
13	*Pseudomonas* GK-48	4.0	1.61
14	*Bacillus* GK-49	4.0	1.73
15	*Bacillus* GK-51	4.0	1.80
16	*Bacillus* GK-52	5.0	1.52
17	*Pseudomonas* GK-80	8.0	30.0
18	*Bacillus* GK-81	5.0	2.10
19	*Bacillus* GK-83	6.0	2.16
20	*Bacillus* GK-84	4.0	2.23
21	*Bacillus* GK-86	3.0	1.40
22	*Bacillus* GK-92	4.0	1.54
23	*Pseudomonas* GK-93	5.0	1.37
24	*Pseudomonas* GK-94	4.0	1.28
25	*Bacillus* GK-101	6.0	1.23
26	*Pseudomonas* GK-105	4.0	2.01
27	*Bacillus* GK-107	5.0	1.89
28	*Pseudomonas* GK-110	4.0	0.96
29	*Pseudomonas* GK-111	6.0	1.23
30	*Bacillus* GK-114	4.0	1.40
31	*Pseudomonas* GK-117	5.0	0.95
32	*Pseudomonas* GK-119	3.0	1.00

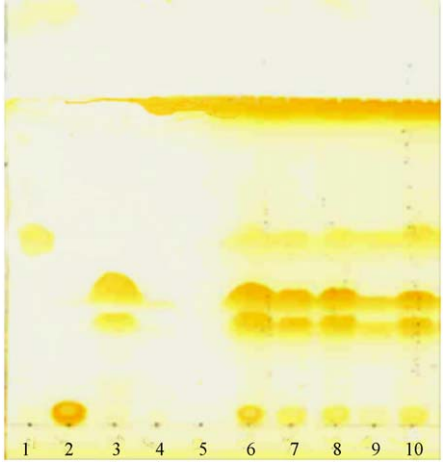

Fig. 1. TLC analysis of products of triolein hydrolysis by selected lipases: 1, oleic acid; 2, monoolein; 3, diolein; 4, triolein; 5, glycerol; 6, *Rhizomucor miehei* lipase; 7, GK-8 lipase; 8, GK-31; 9, GK-42; 10, GK-80.

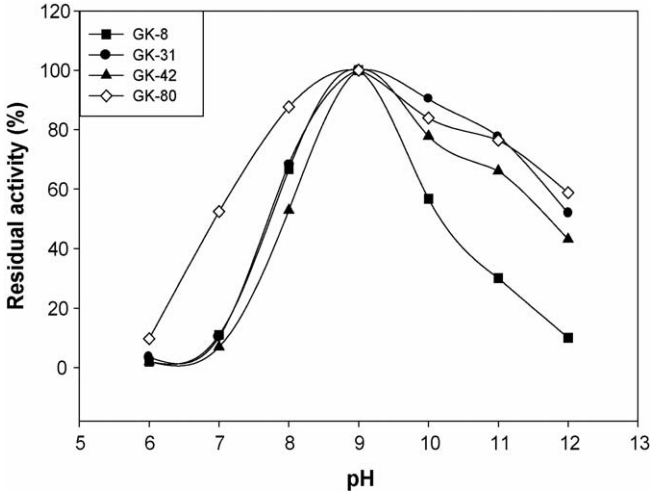

Fig. 2. pH tolerance profile of the selected lipases.

The lipases from GK-8, GK-31 and GK-80 showed maximum stability [82%, 94%, and 98%, respectively] at pH 9.0 whereas the lipase from GK-42 was maximally stable (almost 80%) at pH 8.0 after 24 h of incubation (Fig. 3).

Temperature tolerance profiles of the four lipases showed that GK-8, GK-31 and GK-42 lipases have temperature optima at 37 °C, whereas GK-80 lipase exhibited optimum activity at 50 °C (Fig. 4). These results are in accordance with previous works where a temperature optimum of 37 °C was reported for *B. subtilis* and *Pseudomonas* sp. strain KB 700A [22,29] lipases, and 50 °C was reported for *Bacillus coagulans* lipase [30].

GK-80 lipase exhibited 95.19%, 93.83% and 77.08% residual activities at 50, 60 and 70 °C, respectively after 1 h of incubation indicating its thermostability (Fig. 5). On the other hand, lipase from *Bacillus thermolevorans* ID-1, exhibited only 50% residual activity at 60 and 70 °C after 60 and 30 min, respectively [31]. The lipases from GK-8, GK-31 and GK-42 exhibited 85.95%, 87.47% and 71.59% residual activities, respectively at 50 °C. Even at 60 °C more than 50% activity was exhibited by GK-8 and GK-31 lipases after 1 h of incubation. These results are supported by those reported for *B. thermocatenulatus* lipase which

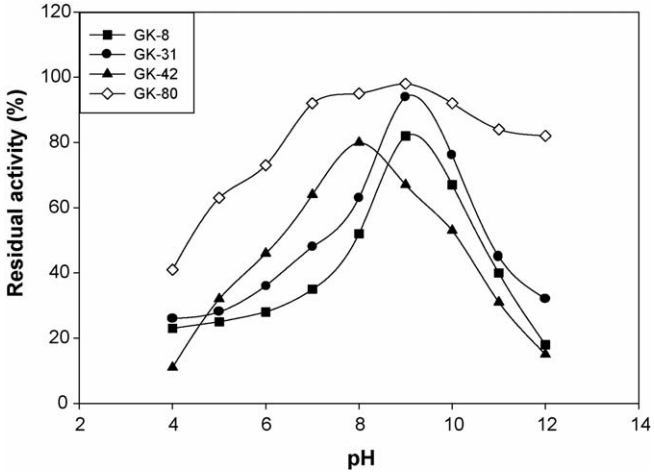

Fig. 3. pH stability profile of the selected lipases.

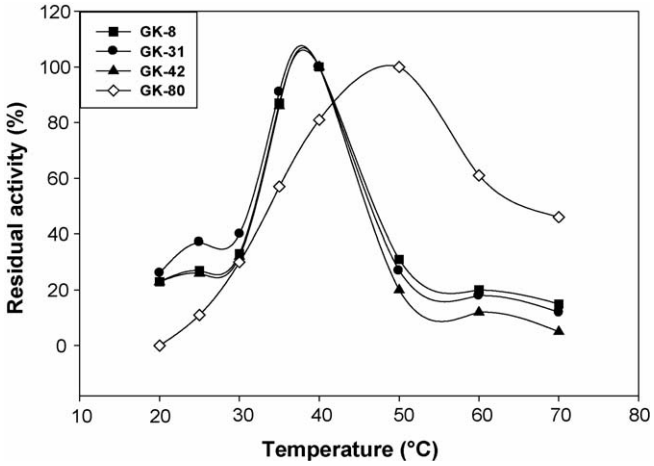

Fig. 4. Temperature tolerance profile of the selected lipases.

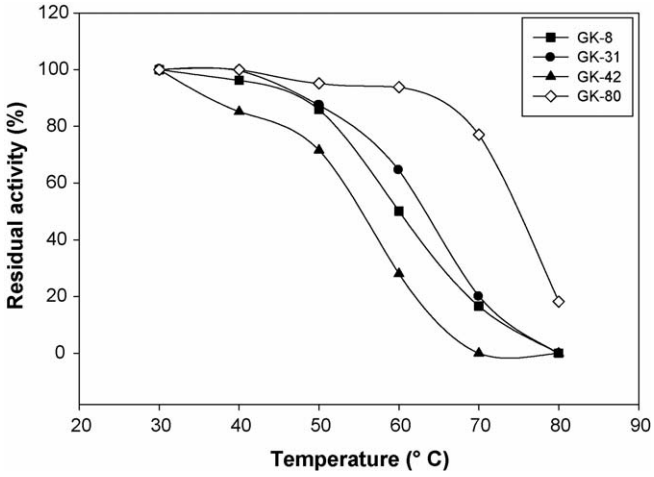

Fig. 5. Temperature stability profile of the selected lipases.

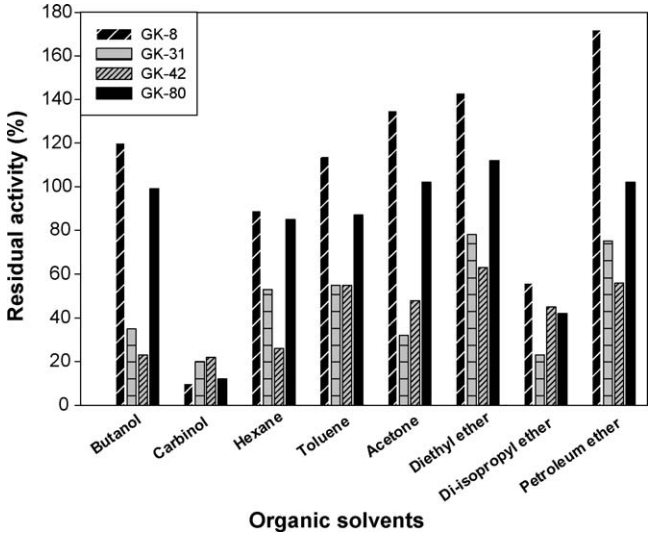

Fig. 6. Organic solvent stability profile of the selected lipases.

showed 48.5% activity at 60 °C after 30 min of incubation [32] and *Bacillus* sp. RS-12 lipase which was stable at 50 °C (99% stability after 60 min) and 55 °C (97% activity after 30 min) of incubation [33].

An enhanced activity of lipase from GK-8 was observed on incubation with organic solvents like petroleum ether (172%), diethyl ether (143%), and acetone (135%). Similarly, GK-80 lipase showed slightly higher activity in diethyl ether (112%). However, lipases from GK-31 and GK-42 showed moderate stability in different organic solvents (Fig. 6). These results are supported by earlier reports, where stimulation of lipase activity by organic solvents has been observed [25].

4. Conclusions

Till date in literature, fungal 1,3-regiospecific lipases have been mostly reported except for the few reports available on bacterial 1,3-regiospecific lipases. The present investigation adds new candidates to the known bacterial 1,3-regiospecific lipases. Furthermore, characterization of these lipases has yielded important information about their optimal catalytic conditions as well as their temperature, pH and organic solvent stability, which gives an additional information to design experiments or reaction conditions, to achieve 100% output from these lipases.

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