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Production and properties of an alkaline, thermophilic lipase from *Pseudomonas fluorescens* NS2W

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Eighteen bacterial strains were isolated from soil samples and screened for alkaline, thermophilic lipase production. *Pseudomonas fluorescens* NS2W was selected and its production of lipase was optimized in shake flasks using a statistical experimental design. Cell growth and lipase production were studied in shake flasks and in a 1-l fermenter in the optimized medium. Maximum lipase yields were 69.7 and 68.7 U ml^{-1}, respectively. The optimized medium resulted in about a five-fold increase in the enzyme production, compared to that obtained in the basal medium. The lipase had an optimal activity at pH 9.0 and was stable over a wide pH range of 3–11 with more than 70% activity retention. The lipase had an optimal activity at 55°C and was stable up to 60°C with more than 70% activity retention for at least 2 h.
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Introduction

Lipases (EC 3.1.1.3) are glycerol ester hydrolases that hydrolyze tri-, di- and monoglycerides at an oil–water interface. Lipases show different substrate and positional specificities, and as a consequence, they find uses in various industries like food, chemical, pharmaceutical, cosmetic, leather processing and in the detergent industry [14,17]. Triglycerides in stains on fabrics are difficult to remove because they are hardly saponified compared to fatty acids. Therefore, lipases functioning at alkaline pH values are required.

The aim of the present work was to determine culture conditions for maximum lipase production by a newly isolated *Pseudomonas fluorescens* NS2W and to characterize the enzyme. Medium composition, medium pH and the incubation temperature were examined for optimization of the lipase production. Medium optimization for production of the lipase was achieved using a two-level full factorial design with four variables. The lipase was characterized with respect to optimal pH and temperature as well as stability.

Materials and methods

Materials

Refined, edible vegetable oils were purchased locally. Tributyrin was from Aldrich. Poly vinyl alcohol (PVA; average molecular weight 30,000–70,000), butyric acid and caproic acid were from Sigma. All other chemicals of analytical grade were from SD Fine Chemicals (Boisar, India). Media components were from HiMedia (Mumbai, India), while Proflo and Pharmamedia were gifts from Traders Protein (Memphis, TN, USA).

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Enrichment of lipase-producing microorganisms in liquid medium

Soil samples were inoculated in 50 ml of enrichment medium containing (g l^{-1}): castor oil 20, K_2HPO_4 2.5, $(NH_4)_2SO_4$ 1.3, $MgSO_4 \cdot 7H_2O$ 0.5, yeast extract 0.05, filter-sterilized urea solution 0.65 ml (200 g l^{-1} stock), at pH 7.4. The flasks were incubated at 30°C on a rotary shaker at 220 rpm, for 24 h. After incubation, the culture fluid was used for inoculation of another set of enrichment flasks and screening medium plates.

Isolation and screening of lipase-producing microorganisms

The solid medium used for screening and maintenance of the isolates contained (g l^{-1}): castor oil 2.0, $(NH_4)_2SO_4$ 5.0, $MgSO_4 \cdot 7H_2O$ 1.0, NaCl 1.0, yeast extract 0.5, filter-sterilized urea solution 1 ml (200 g l^{-1} stock), agar 20, at pH 7.4. The above mixture was sonicated and then autoclaved. Appropriate dilutions of enriched cultures were spread on agar plates and incubated up to 5 days, at 30°C. Material from colonies having a clearance zone due to oil hydrolysis, was selected and transferred to agar slants.

Screening for lipase activity in liquid medium

The screening medium contained (g l^{-1}): peptone 10, castor oil 10, yeast extract 5, NaCl 1, Na_2HPO_4 8.63, NaH_2PO_4 6.08, at pH 7.4. One milliliter of the sterile $MgSO_4 \cdot 7H_2O$ stock solution (500 g l^{-1}) was added after autoclaving. Inoculum was prepared in the screening medium, devoid of oil. The flasks were inoculated from a slant culture and incubated at 30°C, for 24 h.

The screening medium flasks were inoculated with 10% inoculum and incubated on a rotary shaker at 220 rpm for 48 h. Samples were withdrawn and the cells were removed by centrifugation at 20,000×*g* for 10 min. The lipase activity in the supernatant was estimated by titrimetry, as described below, and gas chromatography [8]. The bacterial isolate that produced maximum lipase was selected for further work.

Lipase assay

Titrimetric assay of lipase: Twenty milliliters of olive oil was added to 80 ml of 20 g l^{-1} polyvinyl alcohol solution and sonicated using Branson sonifier. The reaction mixture, composed of 5 ml of olive oil emulsion, 4 ml of glycine–NaOH buffer (0.1 M, pH 9.0) and 1 ml of the enzyme sample, was incubated at 30°C in a shaking water bath at 180 rpm, for 1 h. At the end of the incubation, the emulsion was broken by addition of 20 ml of acetone:ethanol mixture (1:1) and the liberated fatty acids were titrated with 0.05 N NaOH. One unit of lipase was defined as the amount of enzyme that liberated 1 μmol of fatty acid per minute.

Gas chromatographic assay of lipase: Tributyrin was used as the substrate for enzyme reaction during the gas chromatography assay as described earlier [8]. The reaction mixture, composed of 0.25 ml of tributyrin emulsion, 0.25 ml of glycine–NaOH buffer (0.1 M, pH 9.0) and 0.4 ml of enzyme, was incubated at 30°C in a shaking water bath at 180 rpm, for 30 min. The reaction was terminated by addition of 0.1 ml of *ortho*-phosphoric acid (14.5 M) and tubes were centrifuged at 10,000×*g* for 10 min. After centrifugation, a 0.5-ml aliquot of the aqueous phase was removed and the liberated butyric acid was quantified by gas chromatography, using caproic acid as an internal standard. One unit of lipase was defined as the amount of enzyme that produced 1 μmol of butyric acid per minute, under the assay conditions.

Taxonomic study

The bacterial isolate was identified according to Palleroni [13] and "Bergey's Manual of Systematic Bacteriology" [7].

Medium optimization

A 5% inoculum was added to a 500-ml Erlenmeyer flask containing 50 ml of basal medium containing (g l^{-1}): peptone 5, yeast extract 2.5, K_2HPO_4 1, $MgSO_4 \cdot 7H_2O$ 0.2 and castor oil 5 (as an emulsion with gum *Acacia*), at initial pH 7.4. The flasks were incubated on a rotary shaker at 220 rpm, at 30°C. Samples were removed at intervals for growth and lipase activity measurement. A 1-ml aliquot was centrifuged at 20,000×*g* for 10 min and the lipase activity in the supernatant was determined. Lipase activity was estimated using the gas chromatography method [8]. For estimation of cell growth, the pellet was suspended in 1 ml of saline (8.5 g l^{-1} NaCl solution), diluted appropriately, and absorbance was measured at 600 nm.

For all experiments, the inoculum was grown in the basal medium, devoid of oil, for 10 h at 30°C. To test the effect of different carbon sources on lipase production, castor oil was replaced by the respective carbon source, at an equal carbon content. Replacing peptone with various nitrogen sources, on an equal nitrogen content basis, assessed the effect of nitrogen sources. The effects of $MgSO_4 \cdot 7H_2O$, NaCl and $CaCl_2 \cdot 2H_2O$ on lipase production were studied individually, as well in combination with each other.

A factorial experiment was designed using the approach given in standard texts on design of experiments [1,3]. The variability in lipase production was initially determined using five flasks of identical medium (at the center of the first factorial design). Effects of concentrations of the four variables, namely ammonium dihydrogen phosphate, groundnut oil, calcium chloride and magnesium sulfate, on lipase production were studied using a two-level factorial design. The data obtained from the factorial experiments were fitted to the following polynomial:

$$\text{Activity} = \alpha_0 + \alpha_1 x_1 + \alpha_2 x_2 + \alpha_3 x_3 + \alpha_4 x_4 + \alpha_{12} x_1 x_2 + \alpha_{13} x_1 x_3 + \alpha_{14} x_1 x_4 + \alpha_{23} x_2 x_3 + \alpha_{24} x_2 x_4 + \alpha_{34} x_3 x_4$$

where the α values are fitted constants and X_1, X_2, X_3 and X_4 are coded variables for ammonium dihydrogen phosphate, groundnut oil, calcium chloride and magnesium sulfate, respectively. The α values were calculated from main effects and interactions, as described by Box *et al* [1]. Alternatively, these coefficients can also be obtained by simple linear regression.

Coefficients smaller than "two times the standard error" were neglected [12]. The direction of maximum increase in lipase production is given by the gradient of the above polynomial and the next set of experiments was conducted along this direction. This amounted to one-dimensional optimization along the line of steepest increase.

Time course of lipase production

The time course of lipase production was studied in the optimized medium in shake flasks incubated for 60 h. A 5% inoculum was added to 50 ml of medium, in 500-ml Erlenmeyer flasks and incubated at 220 rpm on a rotary shaker, at 30°C, for 60 h. Samples were removed periodically and bacterial growth as well as lipase activity in the culture supernatant were determined. The profile of lipase production was also studied in a 1-l fermenter (Gallenkamp, UK). Forty milliliters of inoculum was added to 360 ml of optimized medium in the fermenter. The fermenter was operated at 30°C with 0.7 l min^{-1} airflow rate and 400 rpm agitator speed. Samples were removed periodically and analyzed for growth and the lipase activity. The effects of temperature and initial pH of the medium on growth and lipase production were also studied in a 1-l fermenter, using optimized medium.

Enzyme characterization

P. fluorescens NS2W was grown in shake flasks in the optimized medium for 52 h and the cells were removed by centrifugation at 20,000×*g* for 10 min, at 4°C. Proteins in the culture broth were precipitated using cold acetone (1:2 vol/vol). The precipitate was recovered by centrifugation at 0°C, at 20,000×*g* for 10 min, dissolved in appropriate buffer and used for study of enzyme properties. The activity of the crude enzyme preparation was studied in the substrate pH range 3–11. Enzyme stability was determined by incubating the enzyme preparation in buffers of different pH in the range of 3–11 for 2 h, at 30°C, followed by activity determination at pH 9.0. The temperature optimum was determined in the range 30–70°C. Enzyme stability at different temperatures was studied by incubating the enzyme in the range 30–70°C, for 2 h each, followed by activity estimation at 30°C.

Results and discussion

Screening of lipase-producing microorganisms

The lipase production by 18 bacterial strains isolated from four soil samples was examined. Only three strains showed more than 2 U ml^{-1} activity as determined by the gas chromatography method

[8]. Among them, NS2W was selected as the highest lipase producer. It produced 10 U ml^{-1} lipase in the screening medium. The lipase exhibited 2 U ml^{-1} activity as determined by titrimetry with olive oil as substrate.

Identification of NS2W

From growth, morphology and nutritional characteristics, the organism was identified as *P. fluorescens* and was designated strain NS2W. The isolate is deposited in the National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India, as NCIM 5145.

Effect of carbon source on lipase production

The study of lipase production with various carbon sources in the medium showed that enzyme production was maximal when groundnut oil was used as the carbon source (Table 1). *P. fluorescens* NS2W utilized several vegetable oils as well as hexoses and pentoses, but disaccharides and polysaccharides resulted in poor growth. The isolate secreted more than 12 U ml^{-1} lipase with all vegetable oils examined as carbon source. With tributyrin as sole carbon source, the secreted enzyme activity in the supernatant was only 2.6 U ml^{-1}. Among the sugars used as carbon source, glucose resulted in maximal lipase production. Sztajer and Maliszewska [15] reported that starch was the best carbon source for lipase production by *P. fluorescens*. In the present study, groundnut oil was the best carbon source.

Effect of nitrogen source on lipase production

P. fluorescens NS2W grew well with all the nitrogen sources tested and the optical density reached above 10. Ammonium dihydrogen phosphate was the best among all nitrogen sources tested, yielding 56.9 U ml^{-1} lipase, which was almost threefold more compared to the basal medium (Table 2). Lipase production was very low with soybean meal and corn steep liquor, whereas the enzyme productions observed with other organic and inorganic nitrogen sources were similar. Makhzoum *et al* [10] reported that arginine, threonine and lysine as well as some ammonium salts of mineral acids supported good growth and lipase production by *P. fluorescens* 2D. Tryptone and casamino acids in combination with ammonium were reported to be the best nitrogen sources for lipase production by *Acinetobacter calcoaceticus* strains [2].

Table 1 Effect of carbon source on lipase production

Carbon source	Cell growth (OD 600 nm)	Lipase yield (U ml^{-1})
Arabinose	8.8	2.6
Coconut oil	13.5	17.2
Fructose	9.1	4.7
Glucose	11.7	8.0
Groundnut oil	15.0	21.3
Lactose	3.7	4.5
Maltose	4.2	6.5
Mustard oil	17.1	15.2
Olive oil	14.8	14.7
Sesame oil	15.7	13.9
Soluble starch	4.4	1.9
Sucrose	3.4	4.0
Sunflower oil	16.8	18.8
Tributyrin	7.7	2.6
Xylose	12.7	3.0
Basal medium	16.7	14.7

The medium contained (g l^{-1}): peptone 5, yeast extract 2.5, K_2HPO_4 1, $MgSO_4 \cdot 7H_2O$ 0.2, to which different carbon sources were added, with carbon content equivalent to 5 g l^{-1} triolein incubated at 30°C.
Samples were diluted suitably for OD and enzyme activity measurement.

Table 2 Effect of nitrogen source on lipase production

Nitrogen source	Cell growth (OD 600 nm)	Lipase yield (U ml^{-1})
Ammonium nitrate	10.8	17.7
Ammonium dihydrogen phosphate	15.6	56.9
Ammonium sulfate	12.5	21.9
Beef extract	15.9	23.4
Casein acid hydrolysate	13.6	17.4
Corn steep liquor	15.3	2.9
Peptone	19.3	19.5
Pharmamedia	N.A.	11.8
Potassium nitrate	12.7	17.7
Proflo	N.A.	12.8
Sodium nitrate	14.8	18.6
Soyapeptone	15.8	18.5
Soybean meal	N.A.	3.2
Tryptone	15.5	24.5
Urea	11.6	27.4
Yeast extract	21.6	19.8

The medium contained (g l^{-1}): yeast extract 2.5, K_2HPO_4 1, $MgSO_4 \cdot 7H_2O$ 0.2, 5 g l^{-1} groundnut oil as gum *Acacia* emulsion. Different nitrogen sources with nitrogen content equivalent to 5 g l^{-1} of peptone were added. N.A. — not attempted due to particulate nitrogen source. Samples were diluted suitably for OD and lipase activity measurement.

Effect of minerals on lipase production

$MgSO_4 \cdot 7H_2O$ and $CaCl_2 \cdot 2H_2O$, individually or in combination, increased lipase production by 26% while NaCl decreased it (Table 3). Several authors reported stimulatory effects of sodium, magnesium and calcium ions on lipase production by different organisms. Addition of 0.01% NaCl to the fermentation medium stimulated lipase production by *Candida utilis* and *Debaryomyces hansenii* [4]. Among several metal ions, calcium stimulated lipase production by *P. fluorescens* 2D while magnesium did not have any stimulatory effect [10]. In the case of *P. fluorescens* B52, a lower yield of lipase was observed in the absence of calcium when the culture was grown in minimal medium PMS_7 [11]. Secretion of extracellular lipase by *Aspergillus terreus* was also increased by addition of Ca^{2+} and Mg^{2+} ions to the growth medium [5].

Table 3 Effect of mineral salts on lipase production

Salt (0.02% individually)	Cell growth (OD 600 nm)	Lipase yield (U ml^{-1})	Change (%)
None	11.6	47.2	0
$MgSO_4 \cdot 7H_2O$	11.4	57.8	22
$CaCl_2 \cdot 2H_2O$	10.9	50.4	7
NaCl	13.8	32.2	−32
$MgSO_4 \cdot 7H_2O + CaCl_2 \cdot 2H_2O$	13.1	59.3	26
$MgSO_4 \cdot 7H_2O$ + NaCl	11.9	54.6	16
$CaCl_2 \cdot 2H_2O$ + NaCl	11.3	48.8	3
$MgSO_4 \cdot 7H_2O + CaCl_2 \cdot 2H_2O$ + NaCl	12.0	52.3	11

Cultivated in a medium containing (g l^{-1}): groundnut oil 5, $NH_4H_2PO_4$ 5.134, K_2HPO_4 1, yeast extract 2.5, each mineral salt 0.2 g l^{-1}. Samples diluted, if necessary.

Table 4 Medium optimization by factorial design[a,b]

Flask number	$NH_4H_2PO_4$ (X_1)	Groundnut oil (X_2)	$CaCl_2 \cdot 2H_2O$ (X_3)	$MgSO_4 \cdot 7H_2O$ (X_4)	Lipase yield ($U ml^{-1}$)
1	+	+	+	+	61.7
2	+	+	+	–	60.8
3	+	+	–	+	60.6
4	+	+	–	–	64.2
5	+	–	+	+	67.4
6	+	–	+	–	66.7
7	+	–	–	+	58.1
8	+	–	–	–	54.3
9	–	+	+	+	12.4
10	–	+	+	–	17.6
11	–	+	–	+	26.4
12	–	+	–	–	27.3
13	–	–	+	+	16.0
14	–	–	+	–	12.9
15	–	–	–	+	53.7
16	–	–	–	–	73.7

[a]Levels ($g l^{-1}$): $NH_4H_2PO_4$, (+) 10.26 and (–) 5.134; groundnut oil, (+) 10 and (–) 5; $CaCl_2 \cdot 2H_2O$, (+) 0.4 and (–) 0.2; $MgSO_4 \cdot 7H_2O$, (+) 0.4 and (–) 0.2.
[b]Effects α_1: 30.94; α_2: –8.52; α_3: –13.47; α_4: –1.86; α_{12}: 8.74; α_{13}: 18.13; α_{14}: –2.32; α_{23}: 5.57; α_{24}: 0.8775; α_{34}: 2.11.

Factorial design

The effect of concentrations of ammonium dihydrogen phosphate, groundnut oil, calcium chloride and magnesium sulfate on lipase production was studied using a two-level full factorial design. Using five flasks containing identical medium, the standard error in enzyme production was calculated to be 2.57.

The experimental results obtained from the factorial design showed that all the variables, except $MgSO_4 \cdot 7H_2O$, had a significant effect on lipase production. The effect of $MgSO_4 \cdot 7H_2O$ was negligible at the selected concentrations, $NH_4H_2PO_4$ had a positive effect while groundnut oil and $CaCl_2 \cdot 2H_2O$ had negative effects (Table 4).

An experiment was then conducted in the linear direction of steepest ascent; however, further increase in lipase production was not observed. The pH of the medium decreased to 5.8 or lower when the culture was grown in shake flasks. As the concentration of $NH_4H_2PO_4$ increased, the pH decreased rapidly and this might have inhibited growth and lipase production. Thus, the optimum medium was found to be ($g l^{-1}$) groundnut oil 7.5, ammonium dihydrogen orthophosphate 7.7, yeast extract 2.5, calcium chloride 0.3 and magnesium sulfate 0.3.

The medium optimization resulted in a fivefold increase in lipase production compared to the basal medium. The maximum lipase yield achieved was 69.7 U ml^{-1}.

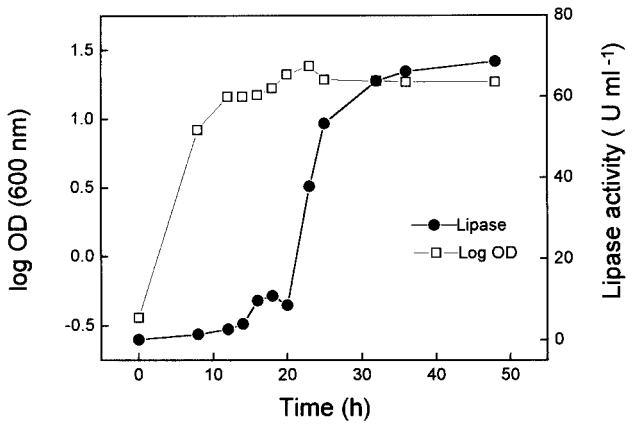

Figure 1 Growth and lipase activity of *P. fluorescens* NS2W in 1-l Gallenkamp fermenter.

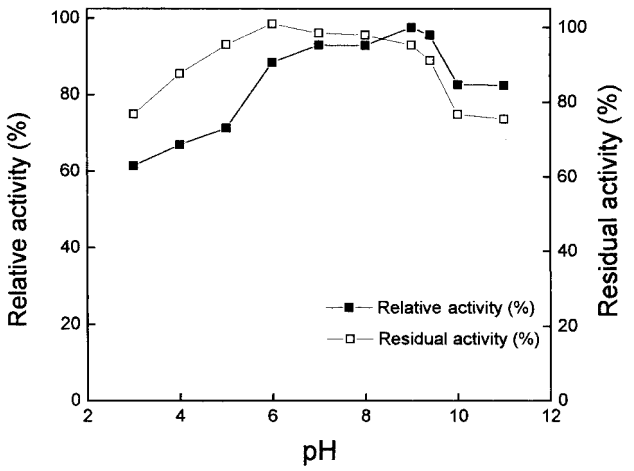

Figure 2 Effect of pH on lipase activity and stability. The buffers used for activity determination were: pH 3–7, sodium citrate–phosphate; pH 8, sodium phosphate; pH 9–10, glycine–NaOH; pH 11, sodium phosphate–NaOH. The activity was assayed as described in Materials and methods. The initial activity (158.8 U ml^{-1}) was considered as 100% relative activity and 100% residual activity.

Growth and lipase production

Lipase production was studied in shake flasks and in a 1-l fermenter with the optimized medium (Figure 1). Lipase was secreted in the late logarithmic phase. The lipase activity obtained with optimized medium in shake flasks was 69.7 U ml^{-1} and that in a 1-l fermenter was 68.7 U ml^{-1}.

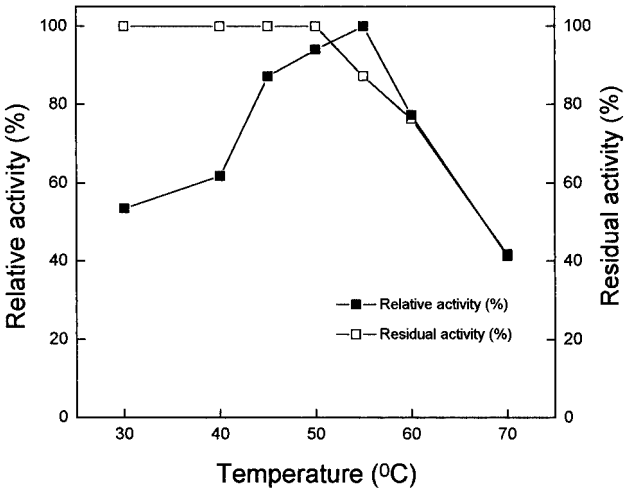

Figure 3 Effect of temperature on lipase activity and stability. Activity was assayed at pH 9.4 as described in Materials and methods. The maximum activity obtained (158.8 U ml^{-1}) was considered as 100% relative activity and 100% residual activity.

Effect of the medium pH and incubation temperature

The effect of pH and incubation temperature on lipase production was studied in a 1-l fermenter. The pH was maintained by acid or base addition during the batch fermentation, using a pH controller (New Brunswick Scientific, USA). Growth and enzyme production were highest when pH was controlled at 7.2 and temperature was maintained at 30°C.

Optimal pH and stability

The lipase activity estimated in different pH buffers showed that the optimal activity was at pH 9.0 (Figure 2). The enzyme was stable over a wide pH range of 3–11 and retained more than 70% activity, for at least 2 h, at 30°C in this pH range. *Pseudomonas* lipases are optimally active at alkaline pH values. A lipase from *P. fluorescens* had optimum activity at pH 8, with 80% activity retention in the range 7–11.5 [16]. Kojima *et al* [6] also reported optimal pH in the range 8–10 with more than 80% activity retention in the pH range 4–10 for a *P. fluorescens* lipase. We reported an enzyme with pH optimum of 9.6 and more than 70% activity retention in the pH range 5–9 for another *Pseudomonas* sp. isolate [9]. The present lipase has a substantially wider pH range.

Effect of temperature on lipase activity and stability

Optimal activity of the present lipase was found at 55°C (Figure 3). It retained more than 70% activity between 30°C and 60°C for at least 2 h. A lipase from *P. fluorescens* has a similar temperature optimum and stability [6].

Conclusions

The optimized medium resulted in fivefold higher production of lipase as compared to that in the basal medium. One can take a convenient and efficient two-step approach consisting of medium optimization in shake flasks followed by translation of the results to fermenters. The use of optimized medium in the shake flask and fermenter resulted in the same degree of improvement in enzyme production.

The present lipase is stable over a wide range of pH and had optimal activity in an alkaline environment. The lipase had optimal activity at 55°C and was stable until 60°C. These properties suggest that this enzyme would be suitable for use in detergent formulations and also can find applications in reactions performed at higher temperature and in alkaline environments.

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