Kinetic modeling, production and characterization of an acidic lipase produced by *Enterococcus durans* NCIM5427 from fish waste

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Abstract *Enterococcus durans* NCIM5427 (ED-27), capable of producing an intracellular acid stable lipase, was isolated from fish processing waste. Its growth and subsequent lipase production was optimized by Box Behnken design (optimized conditions: 5 % v/v fish waste oil (FWO), 0.10 mg/ml fish waste protein hydrolysates (FWPH) at 48 h of fermentation time). Under optimized conditions, ED-27 showed a 3.0 fold increase (207.6 U/ml to 612.53 U/ml) in lipase production, as compared to un-optimized conditions. Cell growth and lipase production was modeled using Logistic and Luedeking-Piret model, respectively; and lipase production by ED-27 was found to be growth-associated. Lipase produced by ED-27 showed stability at low pH ranges from 2 to 5 with its optimal activity at 30 °C, pH 4.6; showed metal ion dependent activity wherein its catalytic activity was activated by barium, sodium, lithium and potassium (10 mM); reduced by calcium and magnesium (10 mM). However, iron and mercury (5 mM) completely inactivated the enzyme. In addition, modifying agents like SDS, DTT, β -ME (1%v/v) increased activity of lipase of ED-27; while, PMSF, DEPC and ascorbic acid resulted in a marked decrease. ED-27 had maximum cell growth of 9.90309 log CFU/ml under optimized conditions as compared to 13 log CFU/ml in MRS. The lipase produced

has potential application in poultry and slaughterhouse waste management.

Keywords *Enterococcus durans* NCIM5427 · Acidic lipase · Kinetic modeling · Optimization · Fish waste

Introduction

Lipases (triacylglycerol acylhydrolases EC 3.1.1.3) belong to the class of esterase enzymes that catalyze hydrolysis and synthesis of esters formed from glycerol and long - chain fatty acids (Kumari et al. 2009). Lipase production is often dependent on initial pH, growth temperature and divalent cations (Gupta et al. 2004). Since lipases catalyze reactions like esterification, transesterification, acidolysis and alcoholysis reactions, they are used in chemical processing, oleochemical industries, dairy industries for improvement of flavour, paper industries, pharmaceuticals, synthesis of surfactants, detergent industries, leather industries and polymer synthesis (Hasan et al. 2006; Sharma et al. 2011).

Enterococcus durans is often regarded as an important microbe in the food industry as it is considered to be of non faecal origin (Franz et al. 1999) and is frequently associated with flavor and aroma improvement of cheeses (Tsakalidou et al. 1993). Even though extensive research has been carried out on bacterial lipases, reports on lactic acid bacterial lipases are limited. Lactic acid bacteria (LAB) are generally considered to be weakly lipolytic, as compared to other groups of microorganisms; and, among LAB, enterococci are found to possess the maximum lipolytic activity. Among them, *E. faecalis* is the most lipolytic strain followed by *E. faecium* and *E. durans* (Moreno et al. 2006). As per reports, *E. durans* was found to be the most prevalent in cheese curd, with levels of enterococci ranging from 10^4 to 10^6 CFU/g and from 10^5 to 10^7 CFU/g in the fully ripened cheese (Sarantinopoulos et al. 2001).

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Lipases are used as therapeutics and diagnostics and also in the food and flavoring industries. Microbial lipases constitute one of the main source of commercially available lipase enzymes; and attributed to *Bacillus*, *Pseudomonas* and *Burkholderia* (Lopes and Crespo 1999; Thapa et al. 2006). These organisms are opportunistic pathogens and their usual mode of pathogenicity is by lipolysis. They are capable of causing a variety of diseases ranging from minor to systemic infections in humans. In contrast, lipases produced by LAB will be of industrial significance in food and pharmaceutical industries as they are generally regarded as safe (GRAS) organisms. They do not cause any health hazards since they are the normal flora of the human body. As LAB are capable of growth in a wide pH range (4.4 to 9.6), the lipase produced by them are stable over a wide pH range. As mentioned earlier, though LAB have the ability to produce lipases, they are considered weakly lipolytic in comparison to other microorganisms. Hence, optimization of medium and cultural conditions for the enhancement of lipase production by LAB is very much essential. Kinetic models have been used to investigate the metabolism, which is very essential in defining the optimal fermentation conditions (Gombert and Nielson 2000). Kinetic modeling combined with optimization would certainly of help in increased production of desired enzyme by a microbe.

In the present study, a lipolytic strain of *E. durans* NCIM5427 (ED-27) (Vrinda et al. 2012) was subjected to enhanced biomass and lipase production by optimizing conditions using response surface methodology (RSM). Furthermore, the lipase produced was subjected to kinetic modeling to ascertain whether the lipase produced is growth dependent (Rajendran and Thangavelu 2007) apart from characterizing and studying the enzyme properties.

Materials and methods

Substrates and chemicals

Freshwater fish visceral waste devoid of air bladder was collected from local fish markets of Mysore, India. All microbiological media were procured from Hi-Media (M/s Hi-Media, Mumbai, India). Para-nitrophenyl acetate (*p*-NPA) and *p*-nitrophenol were obtained from SRL (SRL chemicals, Bangalore, India). All other chemicals, solvents and reagents used in the study were of analytical grade, unless otherwise mentioned.

Bacterial strains and inoculum preparation

E. durans NCIM5427 was isolated from fish processing waste and identified by 16S rDNA sequencing. The culture stands deposited in the National Collection of Industrial Microorganisms (NCIM), NCL, Pune (Vrinda et al. 2012). The strains

were maintained as MRS glycerol stocks at -20°C and subcultured periodically. Another deposited strain *E. faecium* NCIM5335 (EF-35) was used in the preparation of fish waste protein hydrolysates (FWPH) and separation of fish oil (FWO) from fish waste. Both FWPH and FWO were used in the study during optimization.

Screening of lipolytic activity on RBA plates

The culture was streaked onto rhodamine-B agar (RBA) plates supplemented with rhodamine (0.1%w/v) and tributyrin (1%v/v). The plates were incubated at 37°C for 3–7 days and observed under UV light for fluorescence. The fatty acids produced on degradation of a fat substrate, tributyrin, by lipase form a complex with rhodamine dye and this complex fluoresces in the presence of UV light (Kouker and Jaeger 1987).

Extraction of FWO and FWPH from fish waste

FWO was separated from the fish waste by fermentation using EF-35, as per the procedure of Amit et al. (2010) with minor modifications. Freshwater fish visceral waste devoid of air bladder was homogenized in a Waring blender, steam cooked at 85°C for 10 min and fermented for 72 h at 37°C using EF-35. The fermented mass was centrifuged at 6,000 rpm for 20 min to obtain three distinct layers. FWO separated out into the top layer with the protein rich FWPH forming middle aqueous layer followed by the collagenous residue settling at the bottom.

The FWPH was separated from the collagenous residue as per Bhaskar et al. (2008) with slight modifications. The FWPH separated from the collagenous residue was extracted thrice with distilled water in the ratio of 1:1 w/v. The extracts were then lyophilized to get the FWPH in solid form for further use in the studies. The concentration of protein present in the FWPH was measured by Biuret method (Gornall et al. 1949).

Optimization experiments

Media

The medium used for the optimization experiments was of the same composition as that of commercial deMan Rogosa Sharpe (MRS) medium that is used commonly for the growth and metabolite production by LAB, except for the carbon and nitrogen sources. The carbon (dextrose) and nitrogen (peptone, yeast extract and beef extract) sources were replaced by FWO and FWPH respectively, in concentrations as shown in the optimization experiments. The composition of the formulated fish waste medium consisted of 0.01 % Magnesium Sulphate, 0.005 % Manganese Sulphate, 0.2 %

Dipotassium Hydrogen Phosphate, 0.5 % sodium acetate, 0.2 % ammonium citrate and 0.1 % (v/v) Tween-80. FWPH (mg/ml) and FWO (% v/v) were added as protein and carbon replacement sources as per the concentrations indicated in Table 1. The protein concentration of FWPH was estimated to be 33.53 mg/ml estimated by Biuret method.

Box Behneken design

The effect of FWO levels (X1, %v/v), FWPH levels (X2, mg/ml) and time (X3, h) (Table 1) was studied on the growth and lipase production by ED-27 using the Box Behneken design. The design, comprising of 15 runs, (Table 2) was employed for optimization of growth and lipase production by ED-27. Growth (Y_1) and lipase activity (Y_2) were determined as the responses (dependent) variables.

Lipase assay

The optimization experiments were carried out in 250 ml Erlenmeyer flasks containing 100 ml media, as per the pre-determined levels of independent variables outlined in Table 2. All the runs were performed in triplicates. Sample aliquots were collected at time intervals indicated and centrifuged at 10,000 rpm for 10 min. Cell pellet was separated and sonicated in phosphate buffer (pH 7.0) for complete lysis. The lysed cells were centrifuged to obtain the lysed cell free extract (LCFE) and lipase assay was performed for the LCFE.

Lipase activity was determined spectrophotometrically as described by Wang et al. (2010) using *p*-NPA as the substrate with slight modifications. LCFE (300 μ l) was mixed with 900 μ l solvent mixture (acetonitrile: ethanol: phosphate buffer (pH 6.8) in the ratio of 1:4:95 v/v/v) followed by the addition of 800 μ l of 100 mM *p*-NPA in acetonitrile and incubated at 37 °C for 15 min. The liberated *p*-nitrophenol was measured at 408 nm. One unit of lipase activity was defined as the amount of enzyme required to liberate one μ mol of *p*-nitrophenol per minute under the standard assay conditions.

Statistical analysis

The optimization experiments were designed by STATISTICA software (Statsoft 1999) using the experimental design module.

Table 1 Maximum and minimum levels of variables used in Box Behneken design

	Factors	Levels		
		−1	0	1
X1: FWO concentration (%v/v); X2 FWPH concentration (mg/ml); X3 Time (h)	X1	1	3	5
	X2	0.1	0.15	0.2
	X3	24	48	72

Table 2 Actual levels of independent variables with the observed values of the response variables, cell count (log CFU/ml; Y_1) and Lipase activity (U/ml; Y_2)

Run #	X1	X2	X3	Y_1	Y_2
1	1	0.1	48	10	298.9
2	5	0.1	48	10	592.34
3	1	0.2	48	9.954243	316.219
4	5	0.2	48	9.69897	913.25
5	1	0.15	24	9.69897	148.216
6	5	0.15	24	6	625.547
7	1	0.15	72	9.778151	138.247
8	5	0.15	72	9.30103	247.324
9	3	0.1	24	9.69897	403.946
10	3	0.2	24	9.60206	598.301
11	3	0.1	72	9.60206	137.857
12	3	0.2	72	8.477121	387.953
13	3	0.15	48	9.69897	607.174
14	3	0.15	48	9.69897	612.423
15	3	0.15	48	9.69897	616.798

X1 : FWO concentration (%v/v); X2 : FWPH concentration (mg/ml); X3 : Time (h)

Y_1 : Cell count (CFU/ml); Y_2 : Lipase activity (U/ml)

The data generated from the experiments were analyzed by the same software to obtain the optimized conditions, as well as for validation runs.

Validation of the second order model

Validation of the model was done by carrying out experiments using randomly selected values for independent variables. These were independent of the experimental runs mentioned in the previous section. The observed values of growth and lipase production were then compared to the respective values predicted by the second order polynomial equation obtained from Box Behneken design, to evaluate the goodness of fit of the model.

Characterization of lipase

ED-27 was grown under the optimized conditions to obtain lipase, which was further characterized. As detailed earlier, lipase assay for LCFE using *p*-NPA as the substrate was also carried out throughout the characterization experiments.

Determination of optimum pH for the enzyme activity

Five hundred μ l of LCFE was added to 500 μ l of buffers of pH varying from 2.6 to 12.0 and incubated at 37 °C (assay temperature) for 30 min. The buffers used were citric acid

buffer (pH 2.6–7.0), phosphate buffer (pH 8.0), carbonate-bicarbonate buffer (pH 9.2), bicarbonate-NaOH buffer (pH 10.0–11.0) and phosphate-NaOH buffer (pH 12.0). Lipase assay was then performed for the incubated samples.

Determination of optimum temperature for the enzyme activity

Equal volumes of LCFE at the optimized pH level were incubated at different temperatures viz., 30, 40, 50, 60, 70, 80, 90 and 100 °C for 30 min. Lipase assay was then performed for the incubated samples and the temperature at which maximum activity expressed, was taken as the optimum temperature.

Effect of metal ions on enzyme activity

Chloride salts of metal ions i.e. Fe^{3+} , Mg^{2+} , Hg^{2+} , Ba^{2+} , Ca^{2+} , Li^{+} , K^{+} and Na^{+} were dissolved at a concentration of 5 mM and 10 mM in buffer of optimum pH for enzyme activity. The effect of metal ions on the enzyme activity was studied by incubating equal volumes of these solutions and LCFE for 30 min at the optimum temperature. Lipase assay was performed for the incubated samples and the effect of metal ions on enzyme activity was studied.

Effect of organic solvents on enzyme activity

The effect of 12 organic solvents i.e., xylene, propanol, ethanol, butanol, methanol, pyridine, acetone, acetonitrile, DMSO, ethyl acetate, diethyl ether and hexane on the activity of lipase was checked by incubating 1 ml of LCFE with 1% v/v of the organic solvents at optimum temperature for 30 min. Lipase assay was performed for the incubated samples and the effect of organic solvents on enzyme activity was studied.

Effect of modifying agents on enzyme activity

The effect of different modifying agents viz., ethylene diamine tetra acetic acid (EDTA; chelating agent), potassium iodide (KI, oxidizing agent), ascorbic acid (reducing agent), β -mercaptoethanol and di-thio thieritol (DTT; denaturant of disulphide bonds), diethyl-pyrocabonate (DEPC, histidine modifier), PMSF (serine modifier) and sodium do-decyl sulfate (SDS; anionic surfactant) on the activity of lipase produced by ED-27 at optimum pH and temperature was also evaluated. LCFE (1 ml) was incubated with 1 % v/v of the respective modifying agents at optimum pH and temperature for 30 min. Lipase assay was performed to ascertain the effect of the modifying agents on enzyme activity.

Comparison of growth of ED-27 in the optimized fish waste medium and MRS medium

ED-27 (10^7 CFU/ml) was inoculated into separate Erlenmeyer flasks (250 ml) containing 100 ml of optimized fish waste medium and MRS medium respectively. The fish waste medium was formulated by eliminating the protein supplements in MRS (yeast extract, beef extract and proteose peptone) with 0.1 mg/ml of FWPH and the carbon source (Dextrose) was replaced with 5 % (v/v) of FWO. The other components like salts and Tween80 were added in the same composition as MRS. The experiments were performed in triplicates. Sample aliquots were collected at regular intervals and serially diluted. 50 μ l of appropriate dilutions (10^{-4} to 10^{-6}) were spread plated onto MRS agar plates. The plates were incubated at 37 °C for 24 h. The colonies were counted manually and represented in terms of CFU/ml.

Growth and product modelling by unstructured models

Aliquots of optimised medium, in which cells were grown, were collected at an interval of 2 h for determination of cell mass and lipase activity. The optical density of the broth was measured at 600 nm and cell mass was obtained by using a calibration curve based on the relationship between optical density at 600 nm and dry cell weight. Lipase activity was measured spectrophotometrically using *p*-NPA as the substrate. For bacterial growth most of the times, an unstructured growth model (usually the logistic model) has been employed to describe the bacterial growth processes (Rajendran and Thangavelu 2007). The logistic model used in our case was

$$dX/dt = \mu_o(1-X/X_{max})X \quad (1)$$

where μ_o is the initial specific growth rate (h^{-1}) and X_{max} is the maximum cell mass concentration (g L^{-1}). Equation 1 on integration with limits $X_o = X$ gives a sigmoidal variation $X(t)$ that represents both the exponential and stationary phase; and, is mentioned in Eq. 2 below.

$$X(t) = X_o e^{\mu_o t} / (1 - X_o/X_{max})(1 - e^{\mu_o t}) \quad (2)$$

Upon rearrangement of Eq. 2, we can obtain logistic equation (Eq. 3) which can be explained in terms of a straight line. Equation 3 is as below -

$$\mu_{max} t = \ln(X_{max}/X_o) + \ln(\bar{A}/1-\bar{A}) \quad (3)$$

where $\bar{A} = X/X_{max}$, if the data fits the logistic equation then, it can be explained in terms of straight line equation. The plot of $\ln(\bar{A}/1-\bar{A})$ v/s time (t) should give a straight line of slope

Table 3 ANOVA table for cell count of ED-27 as affected by FWO concentration (X1), FWPH concentration (X2) and time (X3)

	SS	df	MS	F	p*
Independent variables					
X1 (L)	2.11218E+19	1	2.11218E+19	9.82926	0.02581
X1 (Q)	1.08299E+19	1	1.08299E+19	5.0398	0.07475
X2 (L)	1.43113E+19	1	1.43113E+19	6.65991	0.04939
X2 (Q)	1.17959E+19	1	1.17959E+19	5.48934	0.06614
X3 (L)	3.61675E+17	1	3.61675E+17	0.16831	0.69861
X3 (Q)	4.42635E+19	1	4.42635E+19	20.5986	0.00618
Interactions					
X1* X2	4E+18	1	4E+18	1.86145	0.23066
X1* X3	2.495E+17	1	2.495E+17	0.11611	0.74715
X2* X3	1.8225E+18	1	1.8225E+18	0.84812	0.39934
Error	1.07443E+19	5	2.14887E+18		
Total SS	1.25074E+20	14			

* $p < 0.05$ indicates significance at 95 % confidence level

μ_{max} and intercept - $\ln(X_{max}/X_0)$ (Rajendran and Thangavelu 2007). These were used for growth modeling.

For the purpose of product (i.e., lipase) modeling Luedeking-Piret model was employed. Luedeking and Piret (1959) formulated that the rate of product formation is directly proportional to the cell mass concentration i.e., $X(t)$ and growth rate dX/dt . Hence the rate of product formation could be represented as -

$$dP/dt = \alpha dX/dt + \beta \quad (4)$$

where α (gP gX^{-1}) and β ($\text{gP gX}^{-1} \text{h}^{-1}$) are the growth dependent and growth independent rate constants respectively.

Table 4 ANOVA table for lipase activity of ED-27 as affected by FWO concentration (X1), FWPH concentration (X2) and time (X3)

	SS	df	MS	F	p*
Independent variables					
X1 (L)	272646.3762	1	272646.3762	97.464	0.00018
X1 (Q)	27990.57731	1	27990.57731	10.0059	0.025
X2 (L)	76573.4978	1	76573.4978	27.373	0.00338
X2 (Q)	96.53151725	1	96.53151725	0.03451	0.85993
X3 (L)	93447.87165	1	93447.87165	33.4052	0.00218
X3 (Q)	204308.2118	1	204308.2118	73.0349	0.00036
Interactions					
X1* X2	23041.87382	1	23041.87382	8.23687	0.03499
X1* X3	33902.71652	1	33902.71652	12.1193	0.01763
X2* X3	776.7647702	1	776.7647702	0.27767	0.62076
Error	13987.03044	5	2797.406087		
Total SS	739309.3332	14			

* $p < 0.05$ indicates significance at 95 % confidence level

Upon integration of Eq. 4 and substituting Eq. 1 at the appropriate place, a straight line equation as represented in Eq. 5 can be obtained.

$$(P_t - P_0)/B(t) = \alpha A(t)/B(t) + \beta \quad (5)$$

Wherein,

$$A(t) = X_0 \{ e^{\mu_{max} t} / [1.0 - (X_0/X_{max})(1 - e^{\mu_{max} t})] - 1.0 \} \quad (6)$$

and

$$B(t) = X_{max} / \mu_{max} \ln[1.0 - X_0/X_{max}(1 - e^{\mu_{max} t})] \quad (7)$$

while, α and β in Eq. 5 can be determined by plotting $P_t - P_0/B(t)$ against $A(t)/B(t)$ (Rajendran and Thangavelu 2007).

Results and discussion

Screening of lipolytic activity on RBA plates

Orange halos were observed around the colonies of ED-27 grown in the presence of tributyrin on exposure to UV light after 5 days of incubation (data not shown). This test is specific and sensitive for bacterial lipases (Hou and Johnston 1992). The results clearly indicated that the enzyme produced by ED-27 was a true lipase.

Optimization of parameters for growth and lipase production by Box Behneken design

The experimental results of growth and lipase production were obtained by the Box Behneken design (Table 2). Regression

analysis was performed on the results obtained from the design experiments leading to mathematical models represented by the

following second order equations for both growth (Y_1) and lipase activity (Y_2).

$$Y_1 = -683.97 + 201.12 * X_1 - 21.76 * X_1^2 - 14.91 * X_2 + 0.2045 * X_2^2 + 38.71 * X_3 - 0.4083 * X_3^2 + 7.58 * X_1 * X_2 - 1.917 * X_1 * X_3 + 0.116 * X_2 * X_3$$

$$Y_2 = 10E9 - 2.1E9 * X_1 + 4.28E8 * X_1^2 - 1.84E9 * X_2 + 7.14E7 * X_2^2 + 6.36E8 * X_3 - 6E7 * X_3^2 - 1E8 * X_1 * X_2 + 5.2E6 * X_1 * X_3 - 5.6E6 * X_2 * X_3$$

The coefficient of variation (R^2) for growth and lipase production was 0.9141 and 0.9801, respectively indicating that the models were able to explain 91.41 % and 98.01 % of variation in the growth and lipase production by ED-27. The R^2 value lies between 0 and 1, and a value ≥ 0.75 indicates the goodness of the model (Kaur and Satyanarayana 2005). The significance of independent variables, their interactions was determined by t -test and p -values (Tables 3 and 4). The linear coefficients of FWO, FWPH and quadratic coefficients of FWPH and time had significant effects on the growth of ED-27 (Table 3), whereas, the linear coefficients of FWO,

FWPH and time ($p < 0.05$), quadratic coefficients of FWO and time ($p < 0.05$) and interaction effects of FWO*time and FWO*FWPH ($p < 0.05$) had remarkable effects on the lipase production by ED-27 (Table 4). The quadratic coefficient of time ($p = 0.00618$) and linear coefficient of FWO ($p = 0.00018$) were more significant than the other factors for cell count of ED-27 and its lipase activity, respectively. Previous reports have depicted that the linear effects of sesame oil and olive oil were highly significant on production of lipase by *Bacillus sphaericus* and *Burkholderia multivorans* (Gupta et al. 2007; Rajendran and Thangavelu 2007).

Fig. 1 Three dimensional plot showing the effect of **a** FWPH concentration, FWO concentration; **b** FWO concentration, time; on cell count of ED-27 and **c** FWPH concentration, FWO concentration; **d** FWO concentration, time; on lipase production by *Enterococcus durans* NCIM5427 (ED-27)

Fig. 2 Desirability profiles for cell count (CFU/ml; Y_1) and lipase activity (U/ml; Y_2) along with the desirability levels for FWO concentration, FWPH concentration and incubation time for optimum lipase production and growth of ED 27

The response surface plots showed in Fig. 1 shows the interaction between two factors keeping the third at the centre of its level. A simultaneous increase in FWO concentration with FWPH concentration resulted in a decrease in the cell count of ED-27, whereas change in FWPH had a moderate effect on the cell count (Fig. 1a). Oh et al. (1995) have reported a similar effect of tryptone on the cell count of *Lactobacillus casei* YIT 9018. An increase in incubation time led to an increase in cell count up to a certain extent (48 h), but further rise in time led to its decrease (Fig. 1b). This may probably be due to the reason that the cells attain stationary phase or reach death phase. A similar decrease in growth after 48 h of incubation was observed during growth optimization of *Bifidobacterium pseudocatenulatum* G4 (Shuhaimi et al. 2009). A continuous increase in lipase activity of ED-27 was observed with an increase in both FWO and FWPH concentration (Fig. 1c). Fish waste and visceral peptones have been used as a nitrogen source for enhanced lipase production from *Staphylococcus* species (Rebah et al. 2008; Souissi et al. 2009). Additions of a triglyceride or fatty acid to the medium were reported to induce the production of both extra- and intra-cellular lipases (Teng and Xu 2008). Lipase activity increased with increase in time but a decrease was observed after 64 h of incubation (Fig. 1d). The main reason for the decrease in lipase activity could be complete hydrolysis of FWO and FWPH provided in the medium after 64 h. Decrease in lipase activity after 67 h of incubation was also observed in case of *Burkholderia* sp. C20 (Liu et al. 2006). The optimized conditions for enhanced biomass and lipase production by ED-27 were obtained from the desirability profile (Fig. 2), as predicted by STATISTICA software. Based on the desirability

profile, the optimum conditions for biomass and lipase production by ED-27 were at 5%v/v FWO, 0.1 mg/ml of FWPH at 48 h of incubation time.

Validation of the second order model

The second order model was validated by a random set of experiments other than the experimental runs. The observed values of cell count and lipase activity were compared with the values as predicted by the second order models (Table 5). The observed values were very close to the predicted values, which indicated that the model was highly significant. The lipase activity obtained in MRS medium was 207.6 U/ml after 48 h of fermentation by ED-27. After optimization of the medium composition and the fermentation conditions, the activity of

Table 5 The observed values and values of validation runs as predicted by the second order model for the dependent responses

X1	X2	X3	Y_1^a	Y_1^b	Y_2^a	Y_2^b
2.5	0.175	48	9.72	9.70	610.77	603.44
4.0	0.10	72	9.61	9.48	246.64	248.97
5.0	0.15	48	9.71	9.70	709.67	701.00
1.0	0.15	48	9.92	9.90	340.45	345.60
1.5	0.10	24	9.85	9.85	204.83	213.50

X1=FWO concentration (%v/v), X2=FWPH concentration (mg/ml), X3=Time (h)

Y_1 = Cell count (log CFU/ml), Y_2 =Lipase activity (U/ml)

^a Predicted values

^b Observed values

Fig. 3 **a** Effect of pH on lipase activity of ED-27 ($n=6$). **b** Effect of temperature on lipase activity of ED-27 ($n=6$). **c** Effect of organic solvents on lipase activity of ED-27

lipase was approximately 3.0 fold higher (614.53 U/ ml). Cell count of 9.90309 log CFU/ml was obtained on growing ED-27 in the optimized medium and fermentation conditions.

Table 6 Effect of metal ions on lipase enzyme activity

Metal ions	Concentration (mM)	Relative activity (%)
Control	—	100
FeCl ₃	5	0
FeCl ₃	10	0
HgCl ₂	5	0
HgCl ₂	10	0
BaCl ₂	5	116.33
BaCl ₂	10	143.49
MgCl ₂	5	79.02
MgCl ₂	10	67.85
CaCl ₂	5	84.69
CaCl ₂	10	72.6
NaCl	5	102.50
NaCl	10	107.32
LiCl	5	118.78
LiCl	10	150.63
KCl	5	105.41
KCl	10	109.86

Characterization of lipase produced at optimized conditions

Determination of optimum pH and temperature for enzyme activity

Lipase from ED-27 showed optimal activity at pH 4.6 and at temperature 30 °C (Fig. 3a and b). Similar acidic pH activity

Table 7 Effect of additives on lipase enzyme activity

Additives	Relative Activity (%)
Control	100
SDS	107.57
EDTA	63.76
Ascorbic acid	0
Potassium iodide	94.06
β-mercaptoethanol	120.08
DTT	120.73
DEPC	34.5
PMSF	0

Fig. 4 Time course profile of growth of ED-27 in MRS medium and optimized fish waste medium

Table 8 Unstructured model parameters evaluated for cell mass and lipase production by ED-27

Kinetic model parameters	
$\mu_{\max}$ (h^{-1})	0.152
X_0 (g/L)	0.132
$X_{\max}$ (g/L)	2.651
α (Ug/X)	404.1
β (Ug/Xh)	-4.954

Ca^{2+} and Mg^{2+} significantly reduced the activity of the lipase, and Fe^{3+} and Hg^{2+} lead to strong reduction in the lipase activity. The strong inhibition by Hg^{2+} suggests the presence of key cysteine residues in the enzyme since Hg^{2+} binds to the thiol group (Dheeman et al. 2010).

has been observed for a lipase from *Pseudomonas gessardii* isolated from slaughterhouse waste (Ramani et al. 2010). The optimum pH for most of the lipases is in the range 8.0–9.0 with few exceptions wherein the optimum pH lies between 5.0 and 6.0 (Neves-Petersen et al. 2001).

Effect of metal ions on enzyme activity

The effect of metal ions was studied by incubating the enzyme with 5 mM and 10 mM of metal ions at its optimum pH and temperature for 30 min. Table 6 indicated that both 5 mM and 10 mM of monovalent ions i.e. Na^+ , Li^+ , Mg^+ and Ba^{2+} significantly stimulated the lipase; especially Ba^{2+} , Li^+ and increased 43 % and 50 % of its activity, respectively. However,

Effect of organic solvents on lipase activity

The effect of organic solvents was studied by incubating the enzyme with 1%v/v of organic solvents at its optimum pH and temperature for 30 min. Lipase activity remained stable in the presence of ethyl acetate, pyridine, xylene and butanol, whereas the other organic solvents reduced the activity of lipase with acetone causing the maximum reduction (Fig. 3c). Lipase activity of *Pseudomonas aeruginosa* MTCC 2488 was drastically reduced in the presence of 2-propanol, hexane and butanol (Borkar et al. 2009). Also, the lipase activity of *Pseudomonas aeruginosa* SRT 9 was drastically reduced in the presence of ethanol, butanol and isopropanol (Chouhan and Dawande 2010).

Fig. 5 **A** Comparative evolution of lipase activity and cell biomass ED-27 with respect to time. **B** Experimental and model predictions of (a) cell mass by logistic model and (b) lipase activity by Luedeking-Piret model of ED-27. **C** Comparison of observed and predicted values of (a) cell mass by Logistic model and (b) lipase activity by Luedeking-Piret Model of ED-27

Effect of modifying agents on enzyme activity

The effect of modifying agents was studied by incubating the lipase with 1%v/v or 1%w/v of additives at its optimum pH and temperature for 30 min (Table 7). Most lipases had a catalytic triad consisting of Ser- His-Asp/Glu similar to that in serine proteases (Gupta et al. 2004). The enzyme was completely inactivated in the presence of PMSF (serine modifier), and DEPC (histidine modifier) inhibited the lipase activity by 55.6 % indicating the presence of histidine and serine in its catalytic site. Lipase activity was inhibited by 36.24 % in the presence of EDTA (divalent metal chelating agent), which indicated that the activity is dependent on metal ions implying the enzyme is of metalloprotein in nature. The activity was slightly reduced (around 6 %) in the presence of potassium iodide (oxidising agent), and slightly increased (7.57 %) in the presence of SDS (anionic detergent). Ascorbic acid (reducing agent) completely inhibited the lipase activity. β -mercaptoethanol and DTT increased the lipase activity by about 20 %. Since these compounds act by denaturing the disulphide bonds, this suggests that the intracellular lipase produced by ED-27 probably does not have any disulphide bonds near its catalytic site.

Comparison of growth of ED-27 in the optimized fish waste medium and MRS medium

Figure 4 shows the time course profile of growth of ED-27 (represented by log CFU/ml) in the optimized fish waste medium and MRS medium for a period of 55 h. The MRS medium produced more number of viable cells in comparison to the fish waste medium at all time intervals. However, MRS is an expensive medium which contains 22 g/l of complex nitrogen source and 20 g/l of carbon source, which restricts its use in commercial scale (Horn et al. 2005). Even if the number of viable cells in case of optimised fish waste medium is lesser than the MRS medium, a cell count of approximately 9.90309 log CFU/ml was obtained at 48 h and this medium was found to be more economical than the MRS medium. Horn et al. (2005) reported that the replacement of the 22 g/l complex nitrogen source in standard MRS medium with only 5 g/l of fish peptone reduced the biomass yield of *Lactobacillus plantarum* only by 10 %.

Growth and product modeling by unstructured models

The unstructured mathematical models were evaluated for growth and lipase production kinetics by ED-27. Figure 5A shows the time course profile of cell mass and lipase production in the optimized fish waste medium. It has been observed that the lipase production increases linearly with cell mass. Figure 5B shows the experimental and model predicted values of cell mass and lipase activity. Table 8 presents the kinetic

model parameters of cell growth and lipase production by ED-27. The values of $\mu_{\max}$, α and β were found to be 0.152 h⁻¹, 404.1 Ug/X and -4.954 Ug/Xh, respectively. Since the non-growth associated parameter β was found to be negative, the lipase production by ED-27 was proven to be growth dependent. The experimental cell mass and lipase activity were compared with the values as predicted by the logistic model and Luedeking- Piret model (Fig. 5C). The logistic model and the Luedeking- Piret model were successful in describing the cell growth and lipase production by ED-27 with a high R² value of 0.947 and 0.966, respectively.

Conclusions

The present study indicated that *Enterococcus durans* NCIM5427 (ED-27) was capable of producing an acidic lipase enzyme, which showed optimal activity at 30 °C and pH 4.6. Moreover, a cost effective medium for maximal lipase production by ED-27 was designed. The kinetics of cell growth and lipase production was studied using unstructured models and the lipase production was found to be growth dependent. The significance of the study lies in the fact that this enzyme has application in poultry and slaughterhouse waste management.

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