ELSEVIER

Lipase from solvent tolerant *Pseudomonas aeruginosa* strain: Production optimization by response surface methodology and application

Gaur Ruchi, Gupta Anshu, S.K. Khare *

Chemistry Department, Indian Institute of Technology, Delhi, Hauz Khas, New Delhi 110 016, India

Abstract

Solvent tolerant *Pseudomonas aeruginosa* strain PseA has been studied for lipase activity. This strain has earlier been reported to be secreting alkaline and solvent stable protease. It produced an extra cellular lipase with suitable properties for detergent applications viz. (i) alkaline in nature, (ii) stability and compatibility towards bleach oxidants, surfactants and detergent formulations and (iii) resistant to proteolysis. Since the culture supernatant contains both protease and lipase which are together required in detergent formulations, enzymes from *P. aeruginosa* seem ideal for use as detergent additive. *P. aeruginosa* lipase exhibited remarkable stability in wide range of organic solvents at 25% (v/v) concentration. This property can be useful for solvent bioremediation and biotransformations in non-aqueous media. Media optimization for cost effective production of lipase was carried out by response surface methodology which led to 5.58-fold increase in lipase production (4580 IU/ml) over un-optimized media.
© 2007 Elsevier Ltd. All rights reserved.

Keywords: *Pseudomonas aeruginosa*; Solvent tolerant; Lipase; Detergents; Response surface methodology

1. Introduction

Detergent industries account for largest share of world enzyme market (Maurer, 2004). Protease and lipases are the key enzymatic constituents in detergent formulations. Such application necessitates enzyme stability in alkaline pH and compatibility with surfactants and other detergent ingredients. Protease and lipase, in general, lack these requisite properties. Currently used detergent protease mainly originate from *Bacillus* sp. viz. Alcalase®, Esperase®, Everlase® and Savinase® (Novozymes, Biotech, Inc., Denmark) and PurafectOxP™ and Properase™ (Genencor, Int., USA) (Joo and Chang, 2006), whereas lipase come from *Pseudomonads*, such as *Pseudomonas stutzeri* ATCC 19.154 lipase (British Patent 1,372,034) and Lipase P from *Pseudomonas*

fluorescens IAM 1057 (Amano Pharmaceutical Co. Ltd., Nagoya, Japan). Ten percent growth in volume of detergent enzyme sales is predicted by major enzyme manufacturers (Detergent enzyme, 2006). Cost effective production of detergent compatible enzymes is anticipated as major demands from enzyme researches. For this reason, intensive research has been directed to obtain protease and lipase, suitable for detergent applications, by enzyme engineering, chemical modifications and screening of new microbial strains (Beselin et al., 2006; Freire et al., 1999).

Lipases improve the washing capacity of the protease-containing detergents and removes fatty food stains and sebum from fabrics which are difficult to remove under normal washing condition (Rathi et al., 2001). Most of the commercial detergents are formulated by mixing lipase and protease both derived from different microbial sources. Single microbial strain producing protease and lipase having detergent properties and compatible with each other are rarely reported. This is because lipase as a protein is

---

* Corresponding author. Tel.: +91 11 26596533; fax: +91 11 26581073.
E-mail address: khare_sk@hotmail.com (S.K. Khare).

likely to be hydrolyzed by protease. Detergent lipases, thus all the more need to be protease resistant as well.

We have previously reported solvent tolerant strain of *Pseudomonas aeruginosa* PseA and its extra cellular alkaline protease exhibiting remarkable stability towards range of solvents and surfactants (Gupta and Khare, 2006). The same strain is observed to secrete alkaline lipase which is not acted upon by its protease. Thus cell free supernatant from this strain exhibit both lipase and protease activities attractive for detergent formulations.

Present work describes the distinct features of this lipase viz. (i) alkaline nature of lipase and stability in surfactants, (ii) simultaneous production with protease, (iii) stability towards its own protease and other commercial proteases, (iv) stability in organic solvents and (v) high level of production. Optimization of lipase production using response surface methodology (RSM) is also described.

2. Methods

2.1. Chemicals

The *p*-nitrophenyl palmitate (pNPP) substrate for lipase was purchased from Sigma Chemical Co., USA. Cellulose acetate membrane filters (0.22 μm) were supplied by Millipore India Pvt. Ltd., Mumbai, India. Media components were purchased from Hi-Media Laboratories Pvt. Ltd., Mumbai, India. Proteases: Palkotase™ and Palkodent™ were purchased from Maps (India) Ltd., Ahmedabad. All the chemicals used were of analytical grade.

2.2. P. aeruginosa PseA

The solvent tolerant strain of *P. aeruginosa* was used. Its isolation and characterization have been reported by us previously (Gupta and Khare, 2006).

2.3. Simultaneous protease and lipase production from P. aeruginosa

Pseudomonas aeruginosa PseA was maintained at 4 °C on nutrient agar slants and subcultured at monthly interval. Inoculum was prepared by transferring loopful of this stock culture to the nutrient medium containing (g l^{-1}): peptone, 5.0; yeast extract, 3.0; NaCl, 0.5; pH 7.0 .The cultivation was performed at 30 °C with shaking at 120 rpm until the A_{660} reached to ∼1.0.

One hundred milliliters of optimized media containing (g l^{-1}): tryptone, 10.1; yeast extract, 0.2; gum arabic, 0.2; NaNO$_3$, 0.2; MgSO$_4$, 1.0 and glucose, 0.3 (pH adjusted to 6.5 with 0.1 M NaOH) in 500 ml Erlenmeyer flask was seeded with 1 ml of inoculum. The incubation was carried out at 100 rpm in an orbital shaker maintained at 25 °C. Cell growth was monitored by recording A_{660}. For estimating lipase and protease production, periodically withdrawn samples were centrifuged at 10,000g for 10 min and enzyme activity was assayed in the supernatant.

2.4. Lipase assay

Lipase activity was determined by following the method of Kilcawley et al., 2002. 1.8 ml of solution containing 0.15 M NaCl and 0.5% Triton X-100 in 0.1 M Tris–HCl buffer (pH 8.0) was preincubated at 40 °C with 200 μl of suitable dilution of cell-free culture supernatant (crude lipase). Twenty microliters of substrate (50 mM pNPP in acetonitrile) was added to the reaction mixture and incubated at 40 °C for 30 min. The amount of liberated *p*-nitrophenol (pNP) was recorded at 400 nm. One unit is defined as the amount of enzyme liberating 1 nmol of pNP under standard assay conditions.

2.5. Protease assay

Protease activity was determined as described by Shimogaki et al. (1991) using casein as substrate. Enzyme solution (0.5 ml) was added to 3.0 ml of substrate solution (0.6% casein in 0.1 M, pH 8.0) and the mixture was incubated at 37 °C for 20 min. The reaction was stopped by addition of 3.2 ml of TCA mixture (containing 0.11 M trichloroacetic acid, 0.22 M sodium acetate and 0.33 M acetic acid) and kept at room temperature for 30 min followed by filtration through Whatman filter paper No 1. The absorbance of filtrate was measured at 280 nm. One unit of protease activity is defined as the amount of enzyme required to produce 1 μg of tyrosine per minute under the conditions described above.

2.6. Stability of crude P. aeruginosa lipase towards additives and proteases

To investigate the stability of lipase in presence of additives, it was incubated in the presence of various surfactants, oxidizing agents [at 2% and 5% (w/v) prepared in assay buffer (0.1 M Tris–HCl, pH 8.0)] at 30 °C for 1 h. Cetyl trimethyl ammonium bromide (CTAB) and sodium dodecyl sulfate (SDS) were used at 0.1% and 1% (w/v) concentrations. Control was run under identical conditions without additives. The residual lipase activity in each sample was calculated with respect to control as 100%.

To check the stability of *P. aeruginosa* lipase against native protease, the cell-free culture supernatant was incubated at 4 and 30 °C. Lipase activity was determined as a function of time up to 72 h. Lipase activity at 0 h was taken as 100%.

2.7. Stability of P. aeruginosa lipase in organic solvents

Three milliliters of cell-free culture supernatant was mixed with 1 ml of organic solvents (25%, v/v) of different log *P* values (logarithm of the partition coefficient of a particular solvent between *n*-octanol and water) in screw-capped tubes. The mixture was incubated at 30 °C with constant shaking at 150 rpm. Samples were withdrawn periodically from the aqueous phase and residual lipase

activity was assayed under standard conditions. The filtrate incubated without solvent was treated as control. Stability is expressed as the residual lipolytic activity relative to the control (100%).

2.8. Optimization of lipase production media

Media optimization for maximum lipase production was carried out following the statistical approach. Plackett–Burman design (PB) was employed for initial screening of the factors, potentially influencing the response. These selected factors were subjected to response surface methodology (RSM) for obtaining the optimum concentration of individual factors.

2.9. Plackett–Burman design

Set of 12 experiments was constructed using the Design expert (version 5.0.9) software (Stat-Ease Corporation, USA) for 11 components: glucose, glycerol, xylose, gum arabic as carbon sources; peptone, tryptone, NaNO$_3$, NH$_4$Cl as nitrogen sources; MgSO$_4$, NaCl and yeast extract as vitamin source which are considered to be important for lipase production. Each component was tested at two concentration levels, low and high. The concentration range taken for each component was (%, w/v): peptone (0.02–1.00); tryptone (0.02–1.00); NH$_4$Cl (0.02–0.20); NaNO$_3$ (0.02–0.20); yeast extract (0.02–2.00); glucose (0.10–0.50); glycerol (0.02–0.10); xylose (0.03–0.30); gum arabic (0.02–0.10); MgSO$_4$ (0.02–0.10) and NaCl (0.02–0.10). Concentration levels were decided on the basis of literature reports on lipase production from other *P. aeruginosa* strains.

The experiments were carried out in 150 ml Erlenmeyer flasks containing 30 ml media at 100 rpm and 25 °C. Response was measured as lipase activity and growth in the periodically withdrawn samples. These responses from individual 12 experiments were subjected to compatible analysis, which yielded regression coefficient values. The variables yeast extract, tryptone, MgSO$_4$ and gum arabic gave highest positive regression coefficient values, hence considered to significantly influence lipase production.

2.10. Response surface methodology

A 2^n factorial Central Composite Design (CCD) developed by the Design Expert software was used to optimize the concentration of above four significant factors yielding a set of 30 experiments. The remaining factors were maintained at their low values throughout. The factors (NH$_4$Cl, glycerol and NaCl) showing negative regression coefficient values for lipase production or both lipase and biomass were omitted from the medium. Experiments were conducted in 150 ml Erlenmeyer flasks containing 30 ml media (pH 6.5) prepared according to the design. Inoculum size of 1% (v/v) was used for each experiment. The incubation was done at 25 °C and 100 rpm. Lipase activity and growth were recorded as response at the end of 72 h. Response data were fed and analyzed by the software which generated 3D contours plots indicating the optimum concentrations and interaction among these factors.

2.11. Validation of the model

To check the validity of chosen quadratic model, experiments, as predicted by the point prediction feature of the Design Expert software were conducted in triplicates. Lipase activity and growth were estimated and compared with the predicted values.

All the experiments were done in triplicate and the variation was within ±5%.

3. Results and discussion

3.1. Lipase from P. aeruginosa PseA

Enzymes from solvent tolerant bacteria are novel for various applications. Isolation of a solvent tolerant *P. aeruginosa* strain and detailed studies of an extra cellular protease secreted by it have previously reported (Gupta et al., 2005; Gupta and Khare, 2006). We further observed lipase production from the same stain, as indicated by zone of hydrolysis on tributyrin agar plates (picture not shown).

Fig. 1 shows growth curve and time course of lipase and protease production. Substantial lipase production started at 24 h and reached to maximum in late log phase after 48 h. Protease production also commenced at about 24 h but remained constant thereafter. Simultaneous production of lipase and protease by single strain is cited only in few cases (Henriette et al., 1993; Jensen et al., 2002; Baselin et al., 2006). In all such cases, lipase is resistant to proteolytic attack. Although influence of nutritional factors on simultaneous production in *Serratia marcescens* (Henriette et al., 1993) and differential regulation of lipase and protease production by proximal and distal locations of the
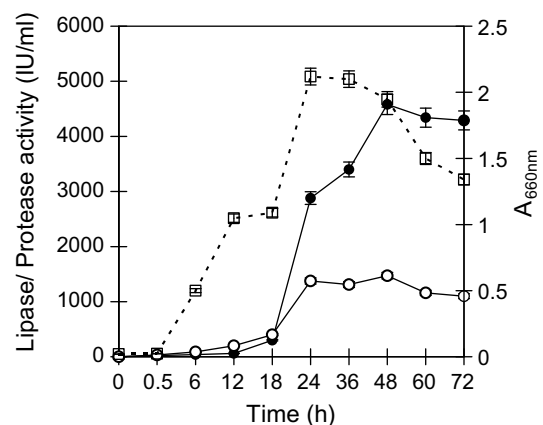

Fig. 1. Time course of enzyme production by *P. aeruginosa* PseA. Lipase production medium was seeded with 1% (v/v) inoculum and incubated at 25 °C for 72 h at 100 rpm. Growth (–□–) was monitored by recording absorbance at 660 nm. Lipase activity (-●-) and protease activity (-○-) were estimated in the cell-free supernatant as described in Section 2.

respective genes lipA and aprX in *P. fluorescens* (McCarthy et al., 2004) are pointed out but the structural features in lipase imparting the resistance towards native protease are not studied. The possible reason which may be attributed in present case is that *P. aeruginosa* protease is preferential for Z-Phe-X-Ala wherein the X- should be bulky and hydrophobic (Morihara and Tsuzuki, 1975). This combination is not exposed for attack in its lipase (lipA and lipB gene, Strover et al., 2000).

In order to confirm lipase stability towards native protease, cell-free supernatant (contains both lipase and protease) was incubated at 4 and 30 °C. The residual lipase activity was estimated in the aliquots withdrawn at various time intervals. It was found that lipase retained good activity even after 24 h at both the temperatures (data not shown). This confirms that lipase of *P. aeruginosa* PseA is not very susceptible to its own protease and commercial proteases viz. Palkobate™ and Palkogent™ at 30 °C at least up to 3 h (data not shown).

3.2. Properties for detergent applications

Systems containing both lipase and protease are more useful especially for laundry applications where a cocktail of enzymes is added to the detergent for improving its quality. Detergent formulations are fortified with both proteases (Maurer, 2004; Joo and Chang, 2006; Paridans and Lee, 1989) and lipases (Andree et al., 1980; Lenting et al., 1993) individually. A major problem of using both enzymes together is proteolysis of lipase by protease. Therefore, the preparations having both activities together and lipase not being prone to protease will have better applicability. Inspired by the stability of *P. aeruginosa* lipase in presence of native protease, lipase was further investigated for stability in surfactants, bleach oxidants and other commercial proteases. It retained 90–100% activity in presence of high concentrations of all the non-ionic surfactants (5%, v/v) and oxidizing agents (2%, v/v) at 30 °C (Table 1). Bleach

Table 1
Stability of *P. aeruginosa* lipase in surfactants and oxidizing agents

Additives	Residual activity (%)	
	Concentration % (w/v or v/v)	
Surfactants	2	5
1. Triton X-100	113	95
2. Triton X-114	100	100
3. CHAPS	95	90
4. Span-20	115	130
5. Span-80	120	110
6. Tween 20	100	90
7. Tween 80	100	93
8. Brij-35	100	90
9. CTAB	5.3	0
10. SDS	100	56
Oxidizing agents		
1. H_2O_2	102	60
2. Sodium hypochlorite	100	98

Residual activity was calculated after 1 h of incubation at 30 °C.

stability is an important property for detergent compatible proteases and lipase (Rathi et al., 2001), that has been achieved by site-directed mutagenesis (Outtrup et al., 1995) and protein engineering (Wolff et al., 1996; Gupta et al., 1992). Interestingly, the present lipase retained its full activity in presence of 5% oxidizing agents, as compared to Lipolase® which is reported to retain only 33% and 43% activity in presence of these additives at 1% concentration (Rathi et al., 2001). The stability in surfactant and bleach oxidants is desirable for lipases to remain active in detergent formulations wherein surfactants and oxidants are present as ingredients (Hemachander and Puvanakrishnan, 2000).

3.3. Production of P. aeruginosa lipase

Media optimization was carried out to enhance lipase production level for cost effectiveness. Eleven factors namely glucose, glycerol, xylose, gum arabic, peptone, tryptone, $NaNO_3$, NH_4Cl $MgSO_4$, NaCl and yeast extract were selected for initial screening by Plackett–Burman design prior to optimization. PB design serves as a very good tool for initial screening of large number of seemingly important factors in a very small number of experiments for reliable short-listing of relevant factors for further optimization (Kalil et al., 2000). Each factor was tested at two concentration levels, high and low. A design of 12 experiments was generated using the software. Table 2 shows distribution of factors according to the Design Expert software and the response in the study. Based on response values, the software further generated regression coefficient values for the effect of factor i.e. medium components on biomass and lipase production. It is clear from Table 3, that all other factors had a positive effect on lipase production except NH_4Cl, glycerol, NaCl and xylose. Therefore, these factors having no effect, were omitted from the media during further optimization.

Response surface methodology is a successive, exploratory approach to establish the relation between positive variables obtained in responses. Four factors viz. tryptone, yeast extract, gum arabic and $MgSO_4$ showing positive influence on lipase production as evident by the regression coefficient values obtained after Plackett–Burman optimization, were selected and Central Composite Design (CCD) was used to determine their optimum concentration. Peptone and tryptone both are organic sources of nitrogen and showed equal values of regression coefficients. Out of the two, tryptone was chosen for further optimization due to relatively lower cost of tryptone than peptone. RSM allows establishing the relationship between multiple variables with the obtained responses more efficiently than traditional design. A total of 30 experiments (Table 4) with different combinations of tryptone (A), yeast extract (B), gum arabic (C) and $MgSO_4$ (D) were generated by RSM. These experiments were performed and the responses obtained were fed to Design Expert Software. Following cubic regression equations were obtained:

Table 2
Plackett–Burman experimental design and results

Run no.	Peptone (%, w/v)	Tryptone (%, w/v)	NH$_4$Cl (%, w/v)	NaNO$_3$ (%, w/v)	Yeast extract (%, w/v)	Glucose (%, w/v)	Glycerol (%, w/v)	Xylose (%, w/v)	Gum arabic (%, w/v)	MgSO$_4$ (%, w/v)	NaCl (% w/v)	Growth (A$_{660nm}$)	Lipase activity (IU/ml)
1	1.00	0.02	0.20	0.20	0.02	0.50	0.02	0.03	0.02	0.10	0.10	4.99	1600
2	1.00	0.02	0.20	0.02	0.02	0.10	0.10	0.30	0.10	0.02	0.10	3.38	1150
3	1.00	1.00+	0.02	0.20	0.02	0.10	0.02	0.30	0.10	0.10	0.02	2.21	2480
4	0.02	1.00	0.20	0.20	0.02	0.50	0.10	0.03	0.10	0.02	0.02	0.33	330
5	1.00	0.02	0.02	0.02	2.00	0.50	0.10	0.03	0.10	0.10	0.02	1.69	1250
6	0.02	0.02	0.02	0.02	0.20	2.00	0.50	0.02	0.30	0.02	0.10	3.64	1340
7	0.02	0.02	0.20	0.20	2.00	0.10	0.10	0.30	0.02	0.10	0.10	2.73	520
8	0.02	0.02	0.02	0.02	0.02	0.10	0.02	0.03	0.02	0.02	0.02	1.30	440
9	1.00	1.00	0.02	0.20	2.00	0.10	0.10	0.03	0.02	0.02	0.10	6.89	2000
10	0.02	1.00	0.20	0.02	2.00	0.10	0.02	0.03	0.10	0.10	0.10	4.94	630
11	1.00	1.00	0.20	0.20	0.02	2.00	0.50	0.02	0.30	0.02	0.02	2.86	1010
12	0.02	1.00	0.02	0.02	0.02	0.50	0.10	0.30	0.02	0.10	0.10	2.99	670

Table 3
Regression coefficient values obtained after Plackett–Burman data analysis: effect of medium components on growth and lipase production

Factors	Name	Lipase (IU/ml)	Growth (A$_{660nm}$)
1	Peptone	185.00	0.67
2	Tryptone	185.00	0.15
3	NH$_4$Cl	−43.33	−0.22
4	NaNO$_3$	80.00	−0.093
5	Yeast extract	170.00	1.23
6	Glucose	56.67	0.47
7	Glycerol	−120.00	0.38
8	Xylose	−81.67	−0.31
9	Gum arabic	301.67	−0.006
10	MgSO$_4$	241.67	0.54
11	NaCl	−350.00	−0.028

Table 4
Experimental design for response surface methodology and observed responses

Run no.	Gum arabic A (%, w/v)	MgSO$_4$ B (%, w/v)	Tryptone C (%, w/v)	Yeast extract D (%, w/v)	Growth (A$_{660nm}$)	Lipase activity (IU/ml)
1	0.02	0.02	1.00	0.02	0.85	3030
2	2.99	0.06	0.51	1.01	2.125	668
3	0.02	0.10	0.02	2.00	1.275	1460
4	1.01	0.14	0.51	1.01	1.275	1095
5	0.02	0.10	1.00	0.02	0.2	4790
6	2.00	0.10	1.00	0.02	1.7	1480
7	1.01	0.06	0.51	1.01	2	885
8	2.00	0.02	0.02	2.00	2.85	350
9	2.00	0.10	1.00	2.00	2.55	120
10	1.01	0.06	0.51	−0.97	0.65	1570
11	2.00	0.10	0.02	2.00	1.825	115
12	1.01	0.06	1.49	1.01	3	874
13	0.02	0.10	1.00	2.00	15	540
14	1.01	0.06	0.51	1.01	2.125	890
15	0.02	0.10	0.02	0.02	0.625	38
16	2.00	0.02	0.02	0.02	0.625	410
17	0.02	0.02	1.00	2.00	0.65	690
18	−0.97	0.06	0.51	1.01	1.275	2410
19	2.00	0.02	1.00	2.00	3.2	450
20	1.01	0.06	0.51	1.01	1.925	880
21	1.01	0.06	0.51	1.01	2.05	888
22	0.02	0.02	0.02	0.02	0	0 90
23	2.00	0.02	1.00	0.02	1.075	3890
24	1.01	0.06	0.51	2.99	5.15	129
25	1.01	0.06	0.51	1.01	1.875	904
26	2.00	0.10	0.02	0.02	0	109
27	0.02	0.02	0.02	2.00	2.125	1180
28	1.01	0.06	0.51	1.01	1.825	904
29	1.01	0.06	−0.47	1.01	1.825	904
30	1.01	−0.02	0.51	1.01	0.08	2130

Lipase activity $= 915.99 - 262.67A - 33.58B + 1015.33C - 624.25D + 133.85A^2 + 152.23B^2 + 85.85C^2 - 38.52D^2 - 319.62AB - 82.87AC - 48.50AD - 51.37BC + 35.50BD - 865.50CD - 43.21A^3 - 56.29B^3 - 312.96C^3 + 66D^3 - 224.12ABC + 232.75ABD + 272.25ACD - 14.25BCD.$

Growth $= 0.75 + 0.26A + 0.36B + 0.45C + 0.66D + 4.16 \times 10^{-4}A^2 - 0.096B^2 + 0.013C^2 + 0.12D^2 - 0.38AB - 0.24AC - 0.26AD + 0.39BC + 0.30BD + 0.27CD + 0.086A^3 - 0.063B^3 - 0.054C^3 - 0.053D^3 - 0.31ABC - 0.38ABD - 0.32ACD + 0.39BCD.$

In addition, 3-D contour plots were also generated which delineates predicted response over a range in the design surface (Figs. 2a and b). In 3D contours, the responses were studied taking two factors at a time while keeping other two at a fixed level. Fig. 2a shows that increasing concentration of tryptone and gum arabic increases the growth. Fig. 2b shows the prominent effect of tryptone on lipase activity. Lipase activity increased continuously with increasing concentration of tryptone and gum arabic. The optimum concentrations of four factors for maximum lipase production found out by studying the responses in detail for all possible combinations keeping two factors constant at a time using the point prediction feature of the software. Applying point prediction feature, maximum lipase yield is obtained when tryptone and gum arabic were at a concentration of 1.01% and at 0.02%, respectively, keeping MgSO$_4$ at 0.10% and yeast extract at 0.02%. Under these conditions, the predicted lipase yield was 4540 IU/ml and growth of 1.21 (A$_{660}$). However, the experimental yield of lipase under the optimized medium was found to be 4580 IU/ml after 48 h of
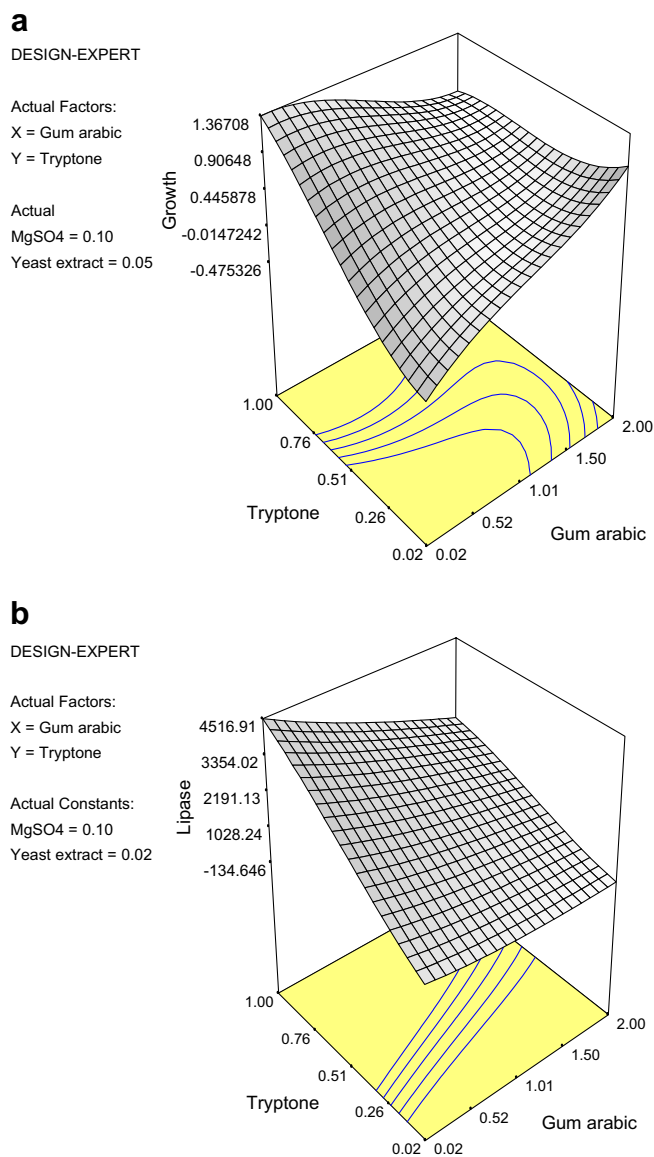
Fig. 2. Response surface 3-D contour plot for growth and lipase activity as response variables for *P. aeruginosa*. The contour plots represent effect of tryptone, gum arabic and their mutual interaction on (a) growth and (b) lipase activity (IU/ml).

Table 5
Stability of *P. aeruginosa* lipase in organic solvents

Solvent	log P	Residual lipase activity (%)
None	–	100
Isopropanol	-0.28	30
Acetone	-0.23	20
Acetonitrile	-0.15	26
n-Butanol	0.8	19
Toluene	2.5	99
p-Xylene	3.1	125
Cyclohexane	3.2	168
Hexane	3.5	183
n-Heptane	4.0	175
Isooctane	4.5	97
Dodecane	6.6	116

Residual lipase activity was determined after 72 h.

2007). Only report of RSM in lipase production optimization by *P. aeruginosa* is by Gao et al. (2000) where 87.5 IU/ml lipase activity was achieved. Thus the level of lipase production by our strain is significantly high and can be cost effective for its applications.

We also observed a simultaneous increase in the level of protease production, which was enhanced by 2.2-fold (3275 IU/ml) as compared to 1472 IU/ml in the unoptimized media. Reddy et al. (2007) have reported similar enhancement in protease level using *Bacillus* sp. RKY3 following optimization by RSM.

3.4. Enzymatic properties

The kinetic characteristics revealed *P. aeruginosa* lipase to be alkaline in nature having pH optima at 8.0. The enzyme was moderately thermostable showing a half-life time of 6.12 h at 45 °C. It has K_m value 0.11 mM and V_{max} value 43.10 μmol/mg protein/min towards pNPP as substrate.

Stability of *P. aeruginosa* lipase towards organic solvents was explored. It exhibited remarkable stability in most of the hydrophobic solvents having log P above 2.5 and did not loose any activity up to 72 h (Table 5). Enzyme activity decreased drastically in the presence of hydrophilic solvents of log P values ranging from -0.28 to 0.8. The reason for better stability in presence of hydrophobic solvents may be the surface–solvent interaction leading to interfacial activation. The results are in agreement with those reported by Ogino et al. (1999).

4. Conclusions

The stability of crude lipase in presence of high concentration of surfactants, oxidizing agents, commercial detergents, range of organic solvents and resistant to protease make it potential candidate for detergent formulation and biotransformations in non-aqueous media. Using statistical approach, the lipase production could be enhanced

incubation. Thus the lipase production time could also be reduced from 72 h to 48 h after optimization. Hasanuzzaman et al. (2004) have reported a lipase yield of 0.5 IU/ml from *P. aeruginosa* using 1% salad oil as inducer. Other strains have been reported to produce lipase activity of 2.3 IU/ml and 3.5 IU/ml (Ito et al., 2001; Sharon et al., 1998). However in the mentioned cases, optimization has been done by one factor at a time approach. There have been a few reports on lipase production optimization by *Pseudomonas* sp. Whereas Liu et al. (2006) have reported 5-fold increase in lipase yield after optimization, production of an alkaline lipase from *Burkholderia multivorans* increased by 12-fold by a combination of RSM and scale-up attempt using 14 L bioreactor (Gupta et al.,

from 820 IU/ml in unoptimized media to 4580 IU/ml giving 5.58-fold increase in lipase production.

The media optimization by RSM, and simultaneous production of solvent stable and detergent compatible lipase with protease are the highlights of the present work.

Acknowledgements

References

Andree, H., Muller, W.R., Schmid, R., 1980. Lipases as detergent components. J. Appl. Biochem. 2, 218–219.

Beselin, A., Rosenau, F., Jaeger, K.E., Breuer, M., Hauer, B., 2006. Protease and protease cDNA and their use in enhancement of microbial lipase production. PCT Int. Appl. 55.

Detergent enzyme sales in a slow spin despite new products. Focus on Surfactants 2006. 2006, 3. Available from: <http://www.chemicalmarketreporter.com>.

Freire, D.M.G., Sant'Anna Jr., G.L., Alves, T.L.M., 1999. Mathematical modeling of lipase and protease production by Penicillium restrictum in a batch fermenter. Appl. Biochem. Biotechnol. 79, 845–856.

Gao, X.G., Cao, S.G., Zhang, K.C., 2000. Production, properties and application to nonaqueous enzymatic catalysis of lipase from a newly isolated Pseudomonas strain. Enzyme Microb. Technol. 27, 74–82.

Gupta, N., Sahai, V., Gupta, R., 2007. Alkaline lipase from a novel strain Burkholderia multivorans: statistical media optimization and production in a bioreactor. Process Biochem. 42, 516–518.

Gupta, A., Roy, I., Khare, S.K., Gupta, M.N., 2005. Purification and characterization of a solvent stable protease from Pseudomonas aeruginosa PseA. J. Chromatogr. A 1069, 155–161.

Gupta, A., Khare, S.K., 2006. A protease stable in organic solvent from solvent tolerant strain of Pseudomonas aeruginosa. Bioresour. Technol. 97, 1788–1793.

Gupta, R., Gupta, K., Saxena, R.K., Khan, S., 1992. Bleach stable, alkaline protease from Bacillus sp.. Biotechnol. Lett. 21, 135–138.

Hasanuzzaman, M., Umadhay-Briones, K.M., Zsiros, S.M., Morita, N., Nodasaka, Y., Okuyama, H., 2004. Isolation, identification and characterization of a novel oil degrading bacterium, Pseudomonas aeruginosa T1. Curr. Microbiol. 49, 108–114.

Hemachander, C., Puvanakrishnan, R., 2000. Lipase from Ralstonia picketti as an additive in laundry detergent formulations. Process Biochem. 35, 809–814.

Henriette, C., Zinebi, S., Aumaitre, M.F., Petitdemange, E., Petitdemange, H., 1993. Protease and lipase production by a strain of Serratia marcescens (532 S). J. Ind. Microbiol. 12, 129–135.

Ito, T., Kikuta, H., Nagamori, E., Honda, H., Ogino, H., Ishikawa, H., Kobayashi, T., 2001. Lipase production in two-step fed-batch culture of organic solvent tolerant Pseudomonas aeruginosa LST-03. J. Biosci. Bioeng. 91, 245–250.

Jensen, B., Nebelong, P., Olsen, J., Reeslev, M., 2002. Enzyme production in continuous cultivation by the thermophilic fungus, Thermomyces lanuginosus. Biotechnol. Lett. 24, 41–45.

Joo, H.S., Chang, C.S., 2006. Production of an oxidant and SDS-stable alkaline protease from an alkalophilic Bacillus clausii I-52 by submerged fermentation: feasibility as a laundry detergent additive. Enzyme Microb. Technol. 38, 176–183.

Kalil, S.J., Maugeri, F., Rodrigues, M.I., 2000. Response surface analysis and simulation as a tool for bioprocess design and optimization. Process Biochem. 35, 539–550.

Kilcawley, K.N., Wilkinson, M.G., Fox, P.F., 2002. Determination of key enzyme activities in commercial peptidase and lipase preparations from microbial or animal sources. Enzyme Microb. Technol. 31, 310–320.

Lenting, H.B.M., Misset, O., Labout, J.J.M., Bolle, R., Oomen, A.J.A.M., Mulleners, L.J.S.M., 1993. Identification of lipase for use in household detergents. In: International Meeting: Lipases Structure, Function and Protein Engineering (Abstract). Elsinore, Denmark.

Liu, C.H., Lu, W.B., Chang, J.S., 2006. Optimizing lipase production of Burkholderia sp. by response surface methodology. Process Biochem. 41, 1940–1944.

Maurer, K.H., 2004. Detergent proteases. Curr. Opin. Biotechnol. 15, 330–334.

McCarthy, C.N., Woods, R.G., Beacham, I.R., 2004. Regulation of aprX-lipA operon of Pseudomonas fluorescens B52: differential regulation of proximal and distal genes encoding protease and lipase, by ompR-envZ. FEMS Microbiol. Lett. 241, 243–248.

Morihara, K., Tsuzuki, H., 1975. Pseudomonas aeruginosa elastase: affinity chromatography and some properties as some metallo-neutral proteinase. Agr. Biol. Chem. 39, 1123–1128.

Ogino, H., Miyamoto, K., Yasuda, M., Ishimi, K., Ishikawa, H., 1999. Growth of organic solvent-tolerant Pseudomonas aeruginosa LST-03 in the presence of various organic solvents and production of lipolytic enzyme in the presence of cyclohexane. Biochem. Eng. J. 4, 1–6.

Outtrup, H., Dambmann, C., Christiansen, M., Aaslyng, D.A., 1995. Patent No US 5:, 466–594.

Paridans, L., Lee, T.N., 1989. Lipase and protease produced by Pseudomonas strain and their use in detergents. Eur. Pat. Appl., 8.

Rathi, P., Saxena, R.K., Gupta, R.A., 2001. Novel lipase from Burkholderia cepacia for detergent formulation. Process Biochem. 37, 187–192.

Reddy, L.V.A. et al., 2007. Optimization of alkaline protease production by batch culture of Bacillus sp. RKY3 through Plackett–Burman and response surface methodological approaches. Bioresour. Technol. 99, 2242–2249.

Sharon, C., Furugoh, S., Yamakido, T., Ogawa, H., Kato, Y., 1998. Purification and characterization of a lipase from Pseudomonas aeruginosa KKA-5 and its role in castor oil hydrolysis. J. Ind. Microbiol. 20, 304–307.

Shimogaki, H., Takeuchi, K., Nishino, T., Ohdera, M., Kudo, T., Ohba, K., Iwama, M., Irie, M., 1991. Purification and properties of a novel surface active agent and alkaline-resistant protease from Bacillus sp. Y. Agric. Biol. Chem. 55, 2251–2258.

Strover, C.K., Pham, X.Q.T., Ervin, A.L., Mizoguchi, S.D., Warrener, P., Hickey, M.J., Brinkman, F.S.L., Hufnagle, W.O., Kowalik, D.J., Logrou, M., Garber, R.L., Goltry, L., Tolentino, E., Westbrook-Wadman, S., Yuan, Y., Brody, L.L., Coulter, S.N., Folger, K.R., Kas, A., Larbig, K., Lim, R.M., Smith, K.A., Spencer, D.H., Wong, G.K.S., Wu, Z., Paulsen, I.T., Rezer, J., Saier, M.H., Hancock, R.E.W., Lory, S., Olson, M.V., 2000. Complete genome sequence of Pseudomonas aeruginosa PA01, an opportunistic pathogen. Nature 406, 959–964.

Wolff, A.M., Showell, M.S., Venegas, M.G., Barnett, B.L., Wertz, W.C., 1996. Laundry performance of subtilisin protease. In: Bott, R.C. (Ed.), Subtilisin Enzymes Practical Protein Engineering. Plenum Press, New York, pp. 113–120.