J Ind Microbiol Biotechnol (2010) 37:531–535
DOI 10.1007/s10295-010-0700-2

SHORT COMMUNICATION

Production of a novel extracellular acidic lipase from *Pseudomonas gessardii* using slaughterhouse waste as a substrate

K. Ramani · Evvie Chockalingam · G. Sekaran

Received: 19 December 2009 / Accepted: 10 February 2010 / Published online: 4 March 2010

Abstract An isolate exhibiting high extracellular lipolytic activity was identified as *Pseudomonas gessardii* by 16S rDNA gene sequence analysis. The slaughterhouse waste, goat tallow, was used as a lipid substrate for the production of acidic lipase by *P. gessardii.* The maximum lipase activity of 156 U/ml was observed at an acidic pH of 3.5 and at 0.31 g substrate concentration. The purification steps resulted in the isolation of acidic lipase with a specific activity of 1,473 U/mg and a molecular weight of 94 kDa. One interesting feature of this purified lipase is its stability at highly acidic pH ranging from 2.0 to 5.5 with a high molecular weight. The amino acid composition was determined using HPLC. This acidic lipase has potential applications in the medicinal field as a substitute for pancreatic lipases for enzyme therapy, oleochemical and in biotechnological industries.

Keywords *Pseudomonas gessardii* · Acidic lipase · Lipase production · Slaughterhouse waste

Introduction

Lipid-rich solid waste is generated in considerable quantity each year from slaughterhouses, the food processing industry, edible oil processing industry, dairy products industry and olive oil mills [1]. These solid wastes have been used as raw material for the production of low value products like soap and detergents. Recovery of high value-added products from solid wastes has been a largely neglected field. Enzyme production using lipid solid waste as the substrate has been demonstrated as a viable technique for by-product recovery. Lipases are able to catalyze hydrolysis of long-chain triglycerides at the water-oil interface system [2]. Microbial lipases from yeast, fungi and bacteria are the main sources of current commercial lipases. New sources for the production of lipases are still a greater scope in applications such as stability towards wide pH range and high thermal stability. Hence, screening of extremophiles has become an important criterion for the production of highly active and stable lipases towards extreme environmental conditions.

Despite of a number of lipase-producing bacterial strains reported, the literature available on the acidic lipase-producing bacterial strain is nil. *Aspergillus niger* NCIM 1207 is the only reported fungal strain for the production of highly acidic lipases using olive oil as a substrate [3–5]. The acidic lipases have been used considerably in food, flavor industries (where aroma esters such as isoamyl acetate are formed under an acidic environment), acid bating of fur and wool, and digestive aids for medical treatment [6]. The acidic lipases (highly active at less than pH 4.0) are used as a substitute of pancreatic lipases for enzyme therapy [7]. Therefore, the production of acidic lipase appears to command a huge market potential.

In the present investigation, *Pseudomonas gessardii* was isolated from tallow acclimatized soil for the production of acidic lipase by the aerobic fermentation of the slaughterhouse waste, goat tallow. Goat tallow is a fat-rich solid substance, obtained from slaughterhouses; it was used as a lipid substrate for the production of acidic lipase. Papanikolaou et al. [8] have used an industrial derivative of beef tallow for the production of lipase using *Yarrowia lipolytica* with a maximum activity of 2.5 U/ml. However,

K. Ramani · E. Chockalingam · G. Sekaran (✉)
Environmental Technology Division, Central Leather Research Institute (CSIR), Adyar, Chennai 600 020, Tamilnadu, India
e-mail: ganesansekaran@hotmail.com;
microramana@yahoo.co.in

lipases with higher activity are of great interest for use in industrial and catalytic applications. There are no reports on the utilization of goat tallow for the production of acidic lipase. Therefore, considering the applications of acidic lipase, this study was focused on the production of acidic lipase from *P. gessardii* using goat tallow as a substrate, followed by purification and characterization.

Materials and methods

Goat tallow and its characterization

The goat tallow used in this study is a whitish solid substance rich in fat with a mild odor; it was obtained from a slaughterhouse in Chennai. The percentage composition of carbon, hydrogen and nitrogen content was determined using a CHNS 1108 Carlo-Erba analyzer. The mineral composition was determined using a Perkin Elmer 3110 atomic absorption spectrophotometer. Acetylene and air were used to maintain the temperature at 2300°C to atomize the samples.

Isolation of *P. gessardii*

The acidic lipase producing bacterial strain, *P. gessardii*, was isolated from the animal tallow acclimatized black soil with a nutrient broth (NB) composition containing peptone (5.0 g/l), yeast extract (1.5 g/l), beef extract (1.5 g/l) and NaCl (0.5 g/l), and was kept for 48 h; this served as the broth. Further, the isolation process was performed by serial dilution of tallow-acclimatized culture onto tributyrin and rhodamine agar plates. The colonies showing clear zones were picked out from the plate and inoculated into the basal medium containing the goat tallow for the maximum yield of lipase. Among 15 isolated strains, 1 showed a better lipolytic activity than others, and it was selected for the production of lipase. The strain was identified by 16S ribosomal DNA (16S rDNA) sequencing and phylogenetical analysis.

Identification of *P. gessardii* by 16S rDNA gene sequencing and phylogenetical analysis

The genomic DNA was isolated in accordance with the procedure of Marmur [9]. The small subunit rRNA gene was amplified using two primers, 16S1 (5-GAT CCT GGC TCA GGA TGA AC-3) and 16S2 (5-GGA CTA CCA GGG TAT CTA ATC-3). The purified DNA product of approximately 1.5 kb was sequenced using five forward and one reverse and an internal primer as described by Reddy et al. [10]. The deduced sequence was subjected to basic local alignment search tool (BLAST) for the closest match in the database. Phylogenetic analysis was performed by subjecting the deduced sequence to the 16S rDNA database to obtain the closely related sequences.

Substrate preparation

The solid goat tallow (10 g) was melted to get the lipid source alone from the tissues, and this was used as a substrate for the production of lipase. The specific gravity of the goat tallow was found to be 1.05 g/ml, which was determined using a specific gravity bottle.

Determination of culture conditions for lipase production

Lipase production was optimized by varying the time (0–96 h), pH (1.0–7.5), temperature (10–70°C), substrate concentration (0.11, 0.21, 0.31, 0.42 and 0.52 g/100 ml of the basal medium), metal ions (K_2HPO_4, KH_2PO_4, $MgCl_2$, NaCl, $CaCl_2$ and $FeSO_4$) and different concentration of Ca^{2+} ions (0.05, 0.1, 0.15, 0.2 and 0.25 g/100 ml of the basal medium).

Production and purification of acidic lipase

P. gessardii was grown in the basal medium containing KH_2PO_4 (1.0 g/l); $NH_4(SO_4)_2$ (0.5 g/l); $CaCl_2$ (1.0 g/l); 0.31% (w/v) goat tallow and 1% (v/v) gum arabic at pH 3.5. Biomass in the broth was removed by centrifugation at 6,500×*g* for 20 min at 4°C, and the supernatant was subjected to lipase activity determination according to the method followed by Deuerlu and Akpinard [11] using olive oil emulsion as a substrate. One activity unit of lipase was defined as the amount of enzyme that released 1 μM of fatty acid per min under assay conditions. For enzyme purification, the crude supernatant was precipitated by 80% ammonium sulphate precipitation, and proteins recovered from the ammonium sulphate precipitation were resuspended in 100 mM acetate buffer (pH 3.5) and dialyzed. The dialyzed enzyme was loaded onto a DEAE-cellulose column. The elution of the adsorbed proteins was performed with a linear increase of NaCl concentration from 0 to 1.0 M NaCl. The most active fractions were pooled and subjected to Sephadex G-100 column chromatography. The fractions containing lipase were pooled and lyophilized, and the resulting material was used for the determination of homogeneity and molecular weight by SDS-PAGE [12].

Amino acid composition of acidic lipase, analysis by HPLC

The acidic lipase was hydrolyzed at 100°C for 20 h in 6 N HCl and neutralized with NaOH. The amino acid

composition was analyzed using Agilent 1100 HPLC amino acid analyzer.

Effect of pH on purified acidic lipase activity and stability

The optimal pH of the enzyme was determined by measuring the lipase activity at 37°C at various pH levels in the following buffers: 100 mM acetate buffer (pH 2.0 to 5.0) and 100 mM phosphate buffer (6.0 to 7.5). The pH stability was studied by incubating the purified enzyme in various buffers with pH ranging from 2.0 to 7.5 for 24 h at 37°C. The residual activity was then assayed under standard assay conditions.

Determination of kinetic parameters

Olive oil emulsion substrate at different concentrations and at pH 3.5 were employed to determine the kinetic parameters, maximum reaction rate (V_{max}) and Michaelis-Menten constant (K_m) for the purified lipase. The kinetic parameters were estimated from the Lineweaver-Burk equation plot (1).

$$\frac{1}{v} = \frac{k_m}{V_{max}} \times \frac{1}{[s]} + \frac{1}{V_{max}} \quad (1)$$

where [S] is the substrate concentration (mM) and V is the initial reaction rate of the enzyme (mM/min).

Results and discussion

Characterization of goat tallow

The physicochemical characteristics and mineral composition of the melted goat tallow are as follows: free fatty acid 57 ± 0.15%, carbon, hydrogen and nitrogen content 10.44 ± 0.1, 2.33 ± 0.13 and 0%, respectively. Among the various elements studied, Ca^{2+} ions were in relatively higher concentration (10.16 ± 0.32 ppm) than the other elements determined. Goat tallow has 2% water solubility. The water-soluble compound of the goat tallow was free fatty acid content 12%. The elemental composition of water-soluble goat tallow is as follows: carbon 1.26%, hydrogen 0.42%, Zn 0.08 ppm, Ca 3.6 ppm, Mg 0.08 ppm, Fe 0.11 ppm, Mn 0.03 ppm, Na 0.25 ppm and K 0.12 ppm.

Isolation and identification of microorganisms

Of the 15 strains tried, 1 showed higher lipolytic activity in the presence of tallow (52 U/ml) and clear zone formation in the tributyrin and rhodamine agar plates, indicating the production of an extracellular lipase. The control sample (without tallow) also was kept to determine the lipolytic activity in the absence of tallow, in which the lipase activity was found to be 6 U/ml. Based on nucleotide homology (16S rDNA sequencing) and phylogenetical analysis, the organism was identified as *Pseudomonas gessardii.* The 1,466-bp sequence was submitted to GenBank (NCBI) and obtained the accession number "FJ943496." The BLAST algorithm was used to search for homologous sequences in GenBank. The strain *Pseudomonas gessardii* had 99% homology with the strain *Pseudomonas fluorescens* FLM05-2 (accession no. DQ084460).

Evaluation of culture conditions for the production of lipase

P. gessardii showed maximum acidic lipase production (52 U/ml) at the stationary growth phase (48 h) with final cell density of 4.52 (OD_{600}).

The pH of the culture is one of the most important environmental parameters affecting microbial cell growth and enzyme production. The effect of initial pH on the lipase production from *P. gessardii* was investigated in the pH values from 1.0 to 7.5 as shown in Fig. 1a. The values presented in Fig 1a were compared with the basic statistical analysis, namely Duncan's multiple range test applied as one-way ANOVA revealed, significant differences ($P \leq 0.05$). All statistical analyses were performed with SPSS 10.0 version (SPSS Inc., Chicago, IL). Although lipase production was favored between pH 1.0 to 6.0, the maximum lipase activity was obtained at initial pH of the growth medium, i.e., pH 3.5. However, lipase production declined sharply between pH 6.0 to 7.5. Hence, the lipase produced by the strain *P. gessardii* was acidic lipase. The acidic lipases (less than pH 4.0) are used in the medicinal field as a substitute of pancreatic lipases for enzyme therapy [7]. The temperature dependence profile showed that the acidic lipase produced in the goat tallow fermentation is a mesophilic enzyme, and it displayed high-hydrolytic efficiency at a temperature of 37°C.

Lipase production is a constitutive aspect of microbes using lipid as a carbon source. Some substrates, typically carbohydrates and acids not related to fats, support good growth, but very low lipase production was obtained. Lipid substrates induced lipase production, the fatty acids being the most effective inducers. The lipase activity gradually increased from 137 U/ml to 156 U/ml for the goat tallow concentration of 0.11 to 0.31 g/100 ml of the basal medium, and it decreased with increase in the concentration of goat tallow beyond 0.31 g. Thus, the optimum concentration of substrate was 0.31 g/100 ml (w/v) of the basal medium.

The addition of 0.1% (w/v) metal ions such as K^+, Mg^{2+}, Na^+ and Fe^{2+} displayed a slight inhibition with

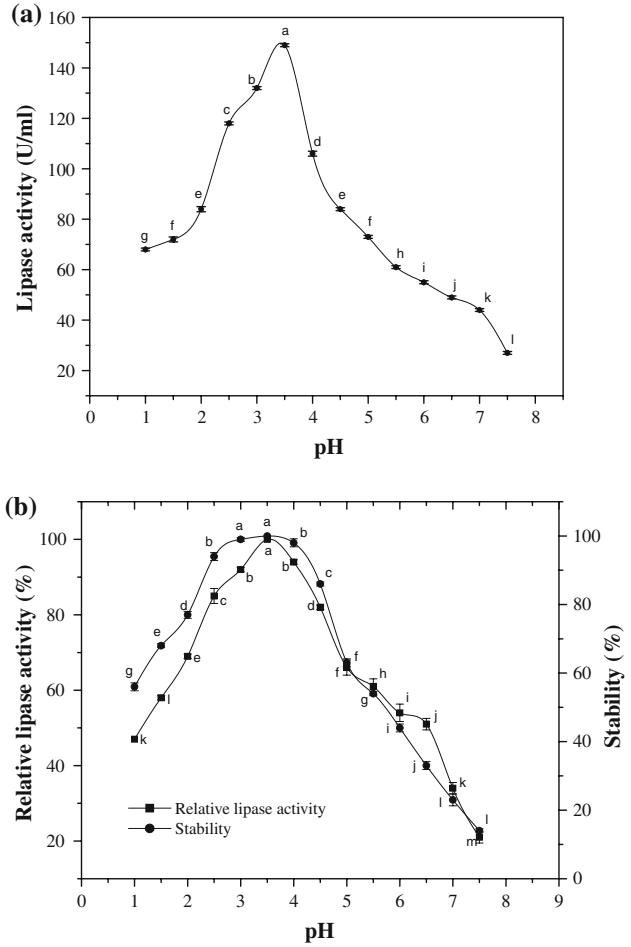

Fig. 1 **a** Effect of pH conditions: fermentation time, 48 h; temperature 37°C) on lipase production by *P. gessardii*. **b** Relative lipase activity and stability of purified extremely acidic lipase at different pHs (100% relative activity and stability represent an enzyme activity of 286 U/0.2 mg/min). Note: [a]Means within a column followed by the same letter are not significantly different at $P \leq 0.05$ using Duncan's multiple range test

lipase productivity of 159, 82, 59 and 68 U/ml, respectively, except for Ca^{2+} with a stimulatory effect on lipase productivity of 162 U/ml. We also investigated the effect of lipase activity at varying concentration of Ca^{2+} ions (0.05. 0.1, 0.15 and 0.2%). The data show that lipase activities were 158, 162, 166 and 166 U/ml at Ca^{2+} ion concentrations of 0.05, 0.1, 0.15 and 0.2%, respectively. Thus, the optimum concentration of Ca^{2+} was 0.15%. The results confirmed that the acidic lipase activity was induced by the presence of calcium.

Purification and characterization of acidic lipase

P. gessardii acidic lipase was purified to homogeneity by a three-step purification process that included ammonium sulphate precipitation, ion exchange and gel filtration

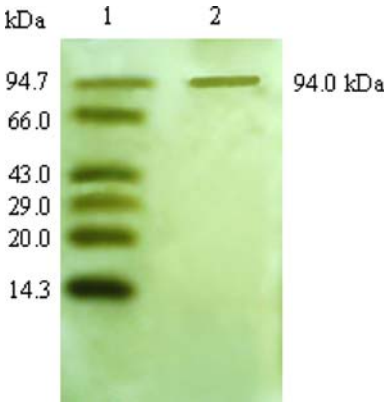

Fig. 2 SDS-PAGE of purified acidic lipase from *P. gessardii*. *Lane 1*: Molecular mass standards (14.3 kDa to 94.7 kDa). *Lane 2*: Lipase (10 μg), purified by ammonium sulfate precipitation in combination with ion exchange chromatography and gel filtration chromatography

column chromatography. A final yield of 16.2%, i.e., 8.1-fold purified acidic lipase, was obtained after the purification steps. The specific activity of the purified acidic lipase was found to be 1,473 U/mg protein. The molecular mass of the protein was found to be 94 kDa based on SDS-PAGE (Fig. 2), suggesting that the enzyme is a monomer. The purified acidic lipase from a fungal source (*Aspergillus niger*) was found to have only a lower molecular weight, 32.2 kDa [3], compared to *P. gessardii* acidic lipase. Moreover, the acidic lipase produced from *P. gessardii* in the presence of goat tallow as a substrate is found to be a high molecular weight acidic lipase.

The amino acid composition analysis by HPLC showed that the purified acidic lipase contained 94.27 and 9.41% of polar and apolar amino acids, respectively. It may be illustrated that aspartic acid and glutamic acid, the polar acidic amino acids in acidic lipase, are 10.82% and 15.42%, which is much higher than in other *Pseudomonas* lipases (5.8%) reported in the literature [13]. The ratio of polar/apolar amino acid of acidic lipase is 10.02, while the other *Pseudomonas* lipase reported the same as 1.59.

Effect of pH on purified acidic lipase activity and stability

The produced purified *P. gessardii* acidic lipase could tolerate a broad range of pHs from 1.0 to 7.5 (Fig. 1b). The values presented in Fig. 1b were compared with the basic statistical analysis; namely Duncan's multiple range test, applied as one-way ANOVA, revealed significant differences ($P \leq 0.05$). All statistical analyses were performed with SPSS 10.0 version (SPSS Inc., Chicago, IL). Although there were no remarkable differences observed at pH range 1.0 to 6.0, the maximal lipase activity was observed at pH 3.5. However, beyond pH 6.5, the activity rapidly dropped

down, reaching a value of about 32% at pH 7.0. The stability of the enzyme remained relatively stable within the pH, ranging from 1.0 to 5.5 with comparatively lower values of stability, i.e., 56 and 54% at pH 1.0 and 5.5, respectively. On the other hand, Rahman et al. [14] reported that the *Pseudomonas* sp. strain S5 had the maximal lipase activity at pH 9.0 and lost its lipase activity below 6.0. Other *Pseudomonas* lipase LST-03 [15] has the maximal activity at pH values ranging from 6.0 to 10.0.

Enzyme kinetic parameters

The Michaelis-Menten enzyme kinetic parameters, K_m and V_{max}, for the purified acidic lipase were calculated from the Lineweaver-Burk plot (figure not shown). The purified acidic lipase showed a lower K_m value (0.66 mM) and higher V_{max} value (0.29 mM/min) than the other *Pseudomonas* sp. lipase reported in the literature. The comparatively lower value of K_m represents higher affinity between enzymes and substrates, whereas V_{max} represents the higher catalytic efficiency of lipase [16].

Acknowledgments The author K. Ramani is thankful to the Council of Scientific and Industrial Research (CSIR), New Delhi, India, and the Central Leather Research Institute (CLRI), India, for awarding a research fellowship and providing the facilities needed to carry out this work.

References

1. Crine DG, Paloumet X, Bjornsson L, Alves MM, Mattiasson B (2007) Anaerobic digestion of lipid rich waste effects of lipid concentration. Renewable energy 32:965–975
2. Yamamoto K, Fujiwara N (1995) The hydrolysis of castor oil using a lipase from *Pseudomonas* sp. f-B-24: positional and substrate specificity of the enzyme and optimum reaction conditions. Biosci Biotech Biochem 59:1262–1266
3. Mhetras NC, Bastawde KB, Gokhale DV (2009) Purification and characterization of acidic lipase from *Aspergillus niger* NCIM 1207. Biores Technol 100:1486–1490
4. Mahadik ND, Puntambekar US, Bastawde KB, Khire JM, Gokhale DV (2002) Production of acidic lipase by *Aspergillus niger* in solid state fermentation. Process Biochem 38:715–721
5. Mahadik ND, Puntambekar US, Bastawde KB, Khire JM, Gokhale DV (2003) Process for the preparation of acidic lipase US Patent No. 06, 534: 303
6. Hassan F, Shah A, Hameed A (2006) Industrial application of microbial lipases. Enzyme Microb Technol 39:235–251
7. Sani DG (2006) Step up: bacterial lipase to substitute pancreatic lipase for enzyme therapy. Available from: http://microbepundit.blogspot.com/2006/10/step-up-bacterial-lipase-to-substitute.html
8. Papanikolaou S, Chevalot I, Panayotou MG, Komaitis M, Marc I, Aggelis G (2007) Industrial derivative of tallow: a promising renewable substrate for microbial lipid, single cell protein and lipase production by *Yarrowia lipolytica*. Electronic J Biotechnol 10:425–435
9. Marmur J (1961) A procedure for the isolation of deoxyribonucleic acids from microorganisms. J Mol Biol 3:208–218
10. Reddy GSN, Aggarwal RK, Matsumoto GI, Shivaji S (2000) *Arthrobacter flavus* sp. nov., a psychrophilic bacterium isolated from a pond in McMurdo Dry Valley, Antarctica. Int J Syst Evol Microbiol 50:1553–1561
11. Deuerlu N, Akpinard MA (2002) Partial purification of intestinal triglyceride lipase from *Cyprinion macrostomus* Heckel, 1843 and effect of pH on enzyme activity. Turk J Biol 26:133–143
12. Laemmli UK (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227:680–685
13. Jinwal U, Roy U, Chowdhury A, Bhaduri A, Roy PK (2003) Purification and characterization of an alkaline lipase from a newly isolated *Pseudomonas mendoncina* PK-12Cs and chemoselective hydrolysis of fatty acid ester. Bioorg Med Chem 11:1041–1046
14. Rahman RNZRA, Baharum SN, Basri M, Salleh AB (2005) High-yield purification of an organic solvent-tolerant lipase from *Pseudomonas* sp. strain S5. Anal Biochem 341:267–274
15. Kilbanov AM (2001) Improving enzymes by using them in organic solvents. Nature 409:241–246
16. Sharma R, Chisti Y, Chand BU (2001) Production, purification, characterization and applications of lipases. Biotech Advances 19:627–662