Ann Microbiol (2013) 63:649–659
DOI 10.1007/s13213-012-0515-7

ORIGINAL ARTICLE

Lipase production by *Serratia marcescens* strain SN5gR isolated from the scat of lion-tailed macaque (*Macaca silenus*) in Silent Valley National Park, a biodiversity hotspot in India

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Received: 26 December 2011 / Accepted: 17 July 2012 / Published online: 9 August 2012

Abstract An extracellular lipase-producing bacterium was isolated from a fecal sample of lion-tailed macaque (*Macaca silenus*), an endangered Old World monkey that is endemic to the Western Ghats of South India. Morphological, biochemical and molecular analyses identified the bacterium as *Serratia marcescens*. Production of lipase was investigated in shake-flask culture. Optimum tributyrin concentration of 1.5 % was found to be the most suitable triglyceride to increase lipase production (13.3 U ml^{-1}). The next best lipid source observed was olive oil (11.94 U ml^{-1}), followed by castor oil, coconut oil and palm oil. Analyzing the effect of different carbon sources on lipase production revealed that 2 % glucose yielded higher lipase production than the other tested carbon sources. Investigations on suitable nitrogen source for lipase production revealed that 2 % meat extract yielded higher lipase production. The most suitable trace element for maximum lipase production was zinc sulfate, followed by magnesium sulfate and copper sulfate. Partial characterization of the crude lipase revealed that pH 7.0 and a temperature of 40 °C gave optimal lipase activity. Enzymatic activity of the crude sample was retained over a wide temperature range (20–75 °C), and 70 % of enzyme activity was retained at 60 °C. Testing the effect of various organic solvents on lipase activity revealed that hexadecane increased lipase activity by 85 % over the control.

Keywords Extracellular lipase · Lion-tailed macaque · Nilgiri Biosphere Reserve · Scat · *Serratia marcescens* strain SN5gR

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Introduction

Environmental samples present a vast reservoir of microbes and microbial products that could be harnessed to revolutionize the productivity of white and red biotechnologies. It has been estimated that 1 g of a pristine soil might contain up to 10^4 different species, which potentially represent over 1 million open reading frames encoding putative enzymes. Recent advancements in molecular technologies offer not only an opportunity to understand the fundamental aspects of evolution and community formation but also provide an excellent opportunity to exploit the uncultivable microbes for biotechnological processes (Singh 2009).

Microbes produce different classes of lipolytic enzymes, including carboxylesterases (EC 3.1.1.1), which hydrolyze small ester-containing molecules at least partly soluble in water, true lipases (EC 3.1.1.3), which display maximal activity towards water-insoluble long-chain triglycerides, and various types of phospholipase (Songer 1997; Titball 1998; Arpigny and Jaeger 1999). Our knowledge of the structure of lipases and esterases has increased considerably in recent years through the elucidation of many gene sequences and the resolution of numerous crystal structures (Cygler and Schrag 1997; Schrag and Cygler 1997; ESTHER database at bioweb.ensam.inra.fr/esther/). They are produced by several microorganisms, namely bacteria and archaea as well as members of the eucarya. In particular,

lipases from fungi are important in biotechnological applications (Essamri et al. 1998). Compared to plant and fungal lipases, bacterial lipases have been well studied. Nowadays, organic solvent-tolerant enzymes, especially lipases, have come into focus because of their credibility to effectively improve the production of biodiesel. It has been found that some lipases from the genera *Bacillus* and *Pseudomonas* have the ability to tolerate alcohol, and hence could be used for the production of biodiesel from low cost precursors and waste lipids (Fang et al. 2006). Because microbial lipases have considerable industrial potential, additional microbial lipases with different characteristics are sought. The extracellular bacterial lipases are of considerable commercial importance, as their bulk production is much easier. Although a number of lipase-producing bacterial sources are available, only a few are commercially exploited as wild or recombinant strains (Jaeger et al. 1994, 1999; Palekar et al. 2000; Jaeger and Eggert 2002; Lorenz and Eck 2005). Of these, the important ones are *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Chromobacterium*, *Pseudomonas* and *Staphylococcus*. Amongst them, the lipases from *Pseudomonas* are widely used for a variety of biotechnological applications (Jaeger et al. 1994; Pandey et al. 1999; Gupta et al. 2004; Adham and Ahmed 2009; Imandi et al. 2010).

The use of lipases as catalysts in organic synthesis has gained much interest due to their broad substrate specificity, high regio-specificity and high enantioselectivity. Owing to their stability at elevated temperatures and a wide range of pH values in organic solvents, lipases from thermophiles have received even more attention for use in detergents, oil and fat, and in the dairy and pharmaceutical industries (Jaeger et al. 1999; Jaeger and Eggert 2002; Treichel et al. 2010). There is a steadily increasing demand to identify and characterize new lipases, with special emphasis on their application for enantioselective biotransformations. Metagenomics, an approach to access global microbial genetic diversity, has also been used to discover novel, potentially important enzymes, including lipases (Lorenz and Eck 2005; Singh 2009). Several products based on bacterial lipases have been launched successfully in the market in the past few years and a number of such products are from *Pseudomonas* species, such as Lumafast and Lipomax with their major application as detergent enzymes, while Chiro CLEC-PC, Chirazyme L-1 and Amano P, P-30 and PS have tremendous potential in organic synthesis (Gupta et al. 2004; Esakkiraj et al. 2010; Kantak et al. 2011).

In view of the above, the present work was undertaken to evaluate different culture conditions for lipase production by lion-tailed macaque (*Macaca silenus*) fecal isolate *Serratia marcescens* strain SN5gR, which produces an organic solvent-tolerant lipase. Most of the experiments were carried out in accordance with Esakkiraj et al. (2010). The novelty of this study is the isolation of this lipase-producing microbe from the scat of a critically endangered mammalian species endemic to the tropical rain forests of Western Ghats in south-west India, which is one of the world's biodiversity hotspots (Nilgiri Biosphere Reserve). Given the endangered status and uncertain future of the lion-tailed macaque in the wild, scientific interest in the species has increased from the early 1980s and to our knowledge there is no previous report of similar study.

Materials and methods

Bacterium isolation, characterization and identification

Morphological and biochemical characterization

The bacterium used in this study was isolated from the scat of lion-tailed macaque (LTM) an endangered Old World monkey that is endemic to the Western Ghats of South India. Fresh LTM scat samples were collected from the Silent Valley National Park (11°03′–11°13′N, 76°21′–76°35′E) in March 2007 and stored in liquid nitrogen.

Biochemical characterization was performed for the following enzymatic assays: protease, DNase, catalase and oxidase (Adarsh et al. 2007). Commercially available readymade media were used for detecting the presence of enzymes, like DNase (Media No. M482 for DNase; Himedia Laboratories, India). The results were assessed as per the manufacturer's instructions.

Oxidase test Isolated single colonies from nutrient agar plate (pH 7.0) were picked up using a tooth pick and were gently scratched on the oxidase disks (DD 018; Himedia Laboratories). Oxidase discs act as an electron donator to cytochrome oxidase. As the bacteria oxidize the disc (remove electrons), the disc turns purple (observation noted within 60 s), indicating a positive test.

Catalase test On isolated single colonies grown on nutrient agar plate, 1 % hydrogen peroxide (H_2O_2) (Merck Chemicals) was dropped using a glass capillary tube and the appearance of effervescence demonstrated the presence of catalase enzyme. The enzyme catalyzes the breakdown of H_2O_2 with the release of free oxygen. The evolution of oxygen gas results in the formation of bubbles and is indicative of a positive test.

DNase test DNase agar with toluidine blue (Himedia FD05) added as a supplement was used for the detection of the enzyme DNase. The isolates were streaked on these plates and left overnight at their respective optimum temperature. DNase positive organisms produce enzyme, which hydrolyzes DNA present in the medium. Toluidine blue present as an indicator forms a complex with hydrolyzed DNA to produce bright zones surrounding colonies on a royal blue background.

Protease assay A protease test was done in milk agar medium containing 10 % double-toned milk, 0.3 % yeast extract, and 1.5 % agar. The milk contains casein which is broken down by protease into lower subunits. The cells producing protease would give a clear transparent zone around them in an otherwise opaque milk media.

Molecular characterization

PCR amplification of 16S rRNA genes Bacterial genomic DNA was isolated from overnight cultures grown at 42 °C in nutrient broth using the genomic DNA isolation kit from Himedia. The isolated genomic DNA was subjected for PCR amplification of 16S rRNA gene. Each PCR consisted of 1 Ready-to-Go PCR bead (Amersham Pharmacia), 2 μl of forward primer 27f (5′-AGAGTTTGATCMTGGCTCAG-3′), 2 μl of reverse primer 1492r (5′-GGT TAC CTT GTT ACG ACT T -3′), 2 μl of sample DNA (20 ng), enough sterile H_2O to bring the reaction to 25 μl, and 30 μl of mineral oil. Each PCR was 25 cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1.45 min for denaturation, annealing, and extension steps, respectively, in a MJ Mini™ 48-Well Personal Thermal Cycler (Bio-Rad, Hercules, CA, USA). The mineral oil was removed, and the products were visualized on a 1 % agarose gel. PCR products were gel purified by Himedia gel purification kit and sequenced by using the paid sequencing facility from Xceleris (Ahmedabad, India).

Analysis of 16S rDNA data The 16S rDNA nucleotide sequence, thus obtained, was edited with Chromas Lite software (v.2.01) for further DNA analysis. The 16S rDNA sequence was used for BLASTn search at the NCBI genome database server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the nearest neighbors. Multiple alignments were generated with the CLUSTALW2 program from the EMBL Nucleotide Sequence Database at the EBI server (http://www.ebi.ac.uk/Tools/clustalw2/), and a phylogenetic tree was generated with MEGA5 software on the basis of the evolutionary distances calculated by the Neighbor-Joining Method using Maximum Composite Likelihood model. GenBank accession number for the nucleotide sequence is JQ278601.

Bacterium and lipase activity

The bacterium was identified as a strong lipase producer when streaked on spirit blue agar medium (Himedia Laboratories) supplemented with tributyrin.

For the enrichment of the culture, the bacterium was inoculated in enrichment medium containing beef extract (0.3 %), peptone (1 %), NaCl (1 %) and glucose (0.5 %) at pH 7 for 24 h; 10 % of enriched culture was then inoculated into a 250-ml flask containing 45 ml basal medium (g l^{-1}: glucose, 10; peptone, 10; yeast extract, 1; $MgSO_4$, 0.5 g; K_2HPO_4, 1; and NaCl, 10; pH 7).

The bacterial culture was incubated in a shaker incubator at 32 °C for 2 days. The cells were then harvested by centrifugation at 10,000 rpm for 15 min and the supernatant was used for lipase assay. The lipase assay was done by the following method. Agar plates were prepared with tryptone (1 %), yeast extract (1 %), tributyrin (0.25 %), Tween 80 (0.25 %) and agar (2 %). The strains were inoculated on the agar plate to check whether the strains were able to produce halo surrounding the colony, which indicates lipase production after incubating at 37 °C for 18–24 h. Cell-free supernatant from the previously described liquid culture was also checked for lipase activity. Then, 100 μl of the supernatant was added to each well and the plate was incubated overnight at 37 °C.

Lipase assays

Culture supernatant lipase activity was determined by the titrimetry method (olive oil emulsion method) and by hydrolysis of p-nitrophenyl palmitate (pNPP). In the titrimetry procedure, triacylglycerol (tributyrin) is hydrolyzed to yield fatty acids. Subsamples are withdrawn from reactive mixtures at predetermined intervals, and reactivity is quenched by the addition of ethanol. The amount of fatty acids released during the reaction is determined by direct titration with NaOH to a phenolphthalein end point. One unit of enzyme activity is defined as the amount of enzyme required to liberate 1 μmol equivalent fatty acid (ml min^{-1}) under the standard assay conditions (Musantra 1992). Relative activity was calculated by comparing the enzyme activity at the particular temperature or pH with that of the maximum activity of the related variable (100 %). Extracellular lipase activity was measured by hydrolysis of p-nitrophenyl palmitate (pNPP). The reaction mixture in the hydrolysis experiment of p-nitrophenyl palmitate contained 180 μl of solution A (0.062 g of p-nitrophenyl palmitate in 10 ml of 2-propanol, sonicated for 2 min before use), 1.62 ml of solution B (0.4 % triton X-100 and 0.1 % gum arabic in 50 mM Tris–HCl, pH 8.0), and 200 μl of culture supernatant. The product was detected at a wavelength of 410 nm after 15 min of incubation at 30 °C. Under these conditions, the molar extinction coefficient (ε410) of p-nitrophenol (pNP) released from p-nitrophenyl palmitate (pNPP) was 15,000 M^{-1}. One unit of lipase activity was defined as the quantity of enzyme required to release 1 μmol of pNP from pNPP mg $protein^{-1}$ min^{-1} under the assay condition (Mongkolthanaruk and Dharmsthiti 2002).

Effect of nutritional factors on lipase production

The effects of nutritional factors such as triglycerides, carbon and nitrogen sources and metal ions on lipase production were

studied by both titrimetry and pNPP hydrolysis methods. All experiments were carried out in triplicate, and average values are presented. The glucose, peptone, and yeast extract media (basal medium) was used as the control medium.

Effect of triglycerides on lipase production Triglycerides (tributyrin, olive oil, coconut oil, castor oil, and palm oil) were tested individually at a concentration of 1 %. Thereafter, different concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 %) of the best lipase-producing triglyceride were studied to determine the optimum concentration for maximum lipase production. The basal medium (glucose, peptone, and yeast extract media) was used as control.

Effect of carbon and nitrogen sources on lipase production Different carbon sources (glucose, fructose, lactose, maltose, sucrose, and galactose) were added individually to the basal medium at a concentration of 0.5 %. Different concentrations (1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 %) of the best carbon source were then studied to determine the most favorable concentration for maximum lipase production. The glucose, peptone, yeast extract media was used as the basal media. Glucose in the basal media was used as the experimental control for studying the effect of different carbon sources on lipase production.

Different organic and inorganic nitrogen sources (beef extract, meat extract, yeast extract, ammonium chloride, peptone, ammonium sulfate, and sodium nitrate) were tested individually at a concentration of 0.5 % in basal medium. After screening, various concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 %) of maximum lipase-producing nitrogen source were studied to determine the optimum level for higher lipase production. Peptone, in the basal medium, was used as the experimental control.

Effect of trace elements on lipase production Seven different trace elements (zinc sulfate, copper sulfate, calcium chloride, ferrous sulfate and magnesium sulfate) were added individually in the basal medium at a concentration of 0.02 %. Culture medium devoid of trace elements was used as the experimental control.

No triglycerides were added in the media, when effects of carbon and nitrogen sources and trace elements were determined.

Partial characterization of crude lipase

To characterize the crude lipase, the effects of pH, temperature and organic solvents on lipase activity were studied by both titrimetry and pNPP hydrolysis methods. In both of the experiments, the glucose, peptone, yeast extract media was used as the basal media. Temperature was maintained at 37 °C (reaction time 1 h) in a rotary shaker (120 rpm).

Effect of pH and temperature on lipase activity Optimum pH for lipase activity was ascertained by using different pH buffers in the reaction. The assay was carried out individually at various pH values (3, 4, 5, 6, 7, 8, 9 and 10). An amount of 0.2 mol l^{-1} citrate buffer (Gomori 1955) was used for pH values of 3.0, 4.0 and 5.0; 0.1 mol l^{-1} Sørensen's phosphate buffer (Sorenson 1909) was used for pH values of 6.0, 7.0 and 8.0; and 0.1 mol l^{-1} Tris- HCl buffer was used for pH 9.0.

The effect of temperature on lipase activity was studied by incubating the enzyme and substrate solution at various temperatures (20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70 and 75 °C).

While testing the optimum pH, all experiments were done at 37 °C, and the pH of the reaction mixture was maintained at 7.0, when effect of temperature was being tested.

Effect of various organic solvents on lipase activity The effect of organic solvents on lipase activity was determined by incubation of enzyme for 1 h in 0.05 mol l^{-1} Tris–HCl buffer (pH 7) at 37 °C containing organic solvents (50 %v/v). The tested solvents were octane, paraffin liquid, hexadecane, tetradecane, dodecane and DMSO. The control experiment had no organic solvent.

Statistical analysis

At least three repetitions with individual biological sample sets were used for the statistical treatment of the data. The data are expressed as mean values; error bars indicate the standard error.

Results and discussion

Bacteria isolated from soil, water, and other environmental samples are regularly being explored for prospective biotechnological, pharmaceutical, and industrial applications. Thus, this study focused on the partial characterization of organic solvent tolerant and thermoactive extracellular lipolytic enzyme from bacteria isolated from the scat of a critically endangered monkey living only in the forests of Nilgiri Biosphere Reserve (south-west India). To verify the systematic position of this bacterium, a study of morphological, biochemical and 16S rDNA analyses were undertaken. The biochemical and morphological characterization of the bacterial isolate can be seen in Table 1. The 16S rDNA sequence of the bacteria was subjected to megablast (GenBank accession number JQ278601).

Table. 1 Result of biochemical tests for strain SN5gR

Tests	Strain SN5gR
Gram staining	−
Shape	Small rod
Colony morphology	Pinkish to red (after 24 h incubation on nutrient agar, smooth surface)
Acid from glucose	+
Acid from maltose	+
Acid from sucrose	+
Acid from lactose	−
H_2S production	−
Gas	−
Methyl red	−
Voges-Proskauer	+
Indole production	−
Citrate	+
Catalase	+
Oxidase	−
Amylase	−
MacConkey Agar	White colony
Gelatinase	+
Motility	+
DNase	+
Protease	+

Evolutionary analyses were conducted in MEGA5. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 21 nucleotide sequences. The strain showed 99–100 % homology to different strains of *Serratia* sp. (Fig. 1). Since it was found to be closest to *Serratia marcescens,* the isolated bacteria was named *S. marcescens* strain SN5gR.

Microbial lipases are mostly extracellular and their production is greatly influenced by media composition besides physico-chemical factors such as pH, temperature, dissolved oxygen and the presence of inducers. The rate determining factor for the expression of lipase activity has always been reported as the carbon source, since lipases are inducible enzymes. These compounds, such as oils and some surfactants, have been described as agents that greatly enhance the production of enzymes with lipolytic activity. Thus, these lipolytic enzymes are generally produced in the presence of a lipid such as oil or some other inducer, such as fatty acids, hydrolysable esters, triacylglycerols, tweens, bile salts, and glycerol (Esakkiraj et al. 2010; Gupta et al. 2004). Lipidic carbon sources seem to be essential for obtaining a high lipase yield. However, nitrogen sources and essential micronutrients should also be carefully considered for growth and production optimization. These nutritional requirements for microbial growth are fulfilled by several alternative media as those based on defined compounds (synthetic medium) like sugars, oils, and complex components such as yeast extract, malt extract media, peptone, and also agroindustrial residues containing all the components necessary for microorganism development . The present work has been done keeping these facts into consideration allowing us to identify optimum conditions for bacterial growth and/or enzyme secretion and the research findings corroborate earlier published results.

Effect of various triglycerides on lipase production

Several methods can be used for microorganism screening based on the determination of the presence of extracellular lipases. The use of a solid medium with inducer substrates such as vegetable oils, standard triglycerides (tributyrin, triolein), Tween 80, and dyes (Esakkiraj et al. 2010; Wang et al. 1995; Cardenas et al. 2001; Ko et al. 2005) has been widely described in the literature.

Five different lipid sources were tested to study their effects on lipase production (Fig. 2a). All the lipid substrates enhanced lipase production over the control (7.75 U ml^{-1}) with particularly high values being obtained with tributyrin (13.3 U ml^{-1}). The next best lipid source observed was olive oil (11.94 U ml^{-1}), followed by castor oil, coconut oil and palm oil. The production of lipase on basal medium (control) indicates a constitutive lipase production. Similar lipase activity has been reported by *Serratia rubidaea* and *Staphylococcus epidermidis*, which are induced by gingelly oil (Immanuel et al. 2008) and cod liver oil (Esakkiraj et al. 2010), respectively. Bora and Kalita (2002) observed that lipase productivity by *Bacillus* sp. DH 4 was higher when cultured in medium supplied with vegetable oils. The present findings are similar to the inducible effects of olive oil, tributyrin, coconut oil and castor oil on lipase production by *Rhizopus* sp. BTNT-2 (Bapiraju et al. 2005). The effect of different concentrations of tributyrin on lipase production showed an increase (8.25–14.3 U ml^{-1}) as the concentration of tributyrin increased from 0.5 to 1.5 %. A further increase in concentration from 1.5 % up to 3.5 % resulted in decreased lipase production to 9.75 U ml^{-1} (Fig. 2b).

Effect of various carbon sources on lipase production

Using different carbon sources for bacterial culture and measuring lipase production revealed that all the supplementary

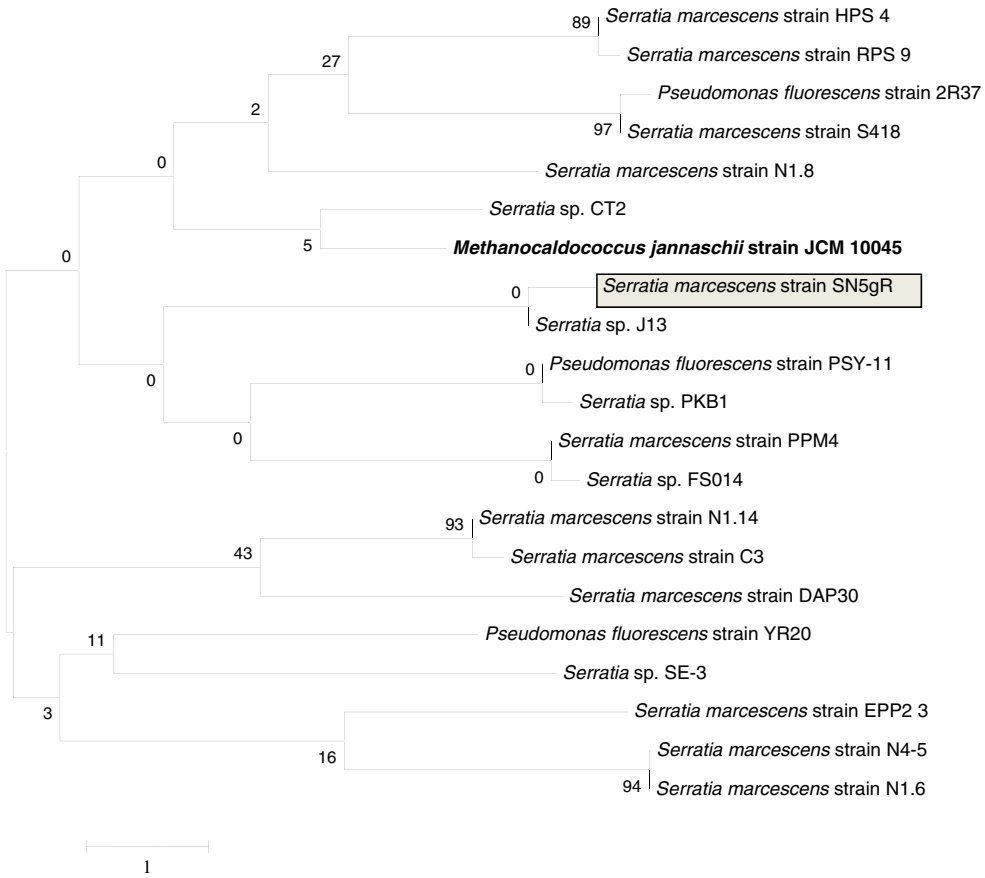

Fig. 1 The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length= 43.50359217 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 21 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions with less than 95 % site coverage were eliminated. That is, fewer than 5 % alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 1,157 positions in the final dataset. *Methanocaldococcus jannaschii* strain JCM 10045 16S rDNA sequence was used as an outgroup. Evolutionary analyses were conducted in MEGA5

carbon sources had some influence on lipase production, with maximum production of 8.9 U ml^{-1} being observed with glucose, followed by fructose and galactose (Fig. 3a). When optimizing the glucose concentration, lipase synthesis increased gradually up to 2 % (10.12 U ml^{-1}) (Fig. 3b). A number of varied carbon sources have been reported as best for maximum lipase production using different bacteria and fungi. Rodriguez et al. (2006) and Esakkiraj et al. (2010) reported fructose to be the most suitable carbon source for maximum lipase production by *Rhizopus homothallicus* and *Staphylococcus epidermidis* CMST-Pi 1, respectively. Lin and Ko (2005), on the other hand, reported that lipase production by *Antrodia cinnamomea* was highly stimulated by the addition of glucose. Rajendran et al. (2008) applied the Plackett–Burman statistical experimental design to evaluate the fermentation medium components. Glucose, olive oil, peptone, and $FeCl_3 \cdot 6H_2O$ were found to have more significant influence on lipase production by *Candida rugosa*. Pareto graph was used to show the effect of five different carbon sources on lipase production by Imandi et al. (2010). A p value less than 0.05 for the three variables, viz., glucose, sucrose, and fructose, indicates that they are significant. Glucose had a confidence level of above 95 % in comparison to other variables and thus considered to be highly significant for lipase production.

Effect of various nitrogen sources on lipase production

A range of inorganic and complex nitrogen sources were tested to evaluate their capacity to support lipase production. Among the tested nitrogen sources, lipase production was the highest in medium supplied with meat extract (12.9 U ml^{-1}), with peptone and beef extract yielding the next highest production levels (12.35 and 11.7 U ml^{-1}, respectively) (Fig. 4a). Medium supplied with 2.0 % meat extract produced the highest amount of lipase enzyme

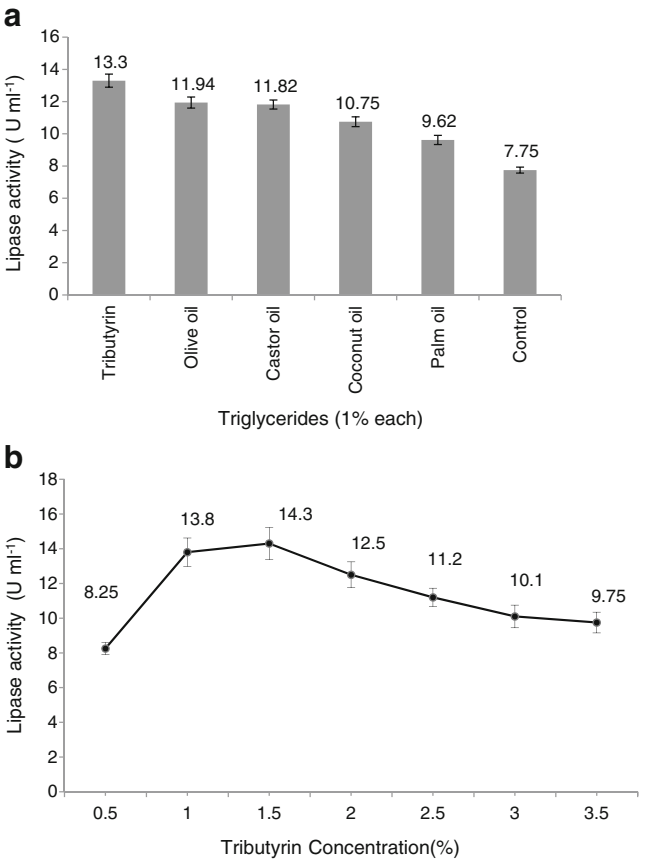

Fig. 2 Effect of **a** various triglycerides and **b** different concentrations of Tributyrin on lipase production by *Serratia marcescens* strain SN5gR. Lipolytic activity is expressed in U ml^{-1}. Each point represents the mean of values obtained from three separate experiments (n=3)

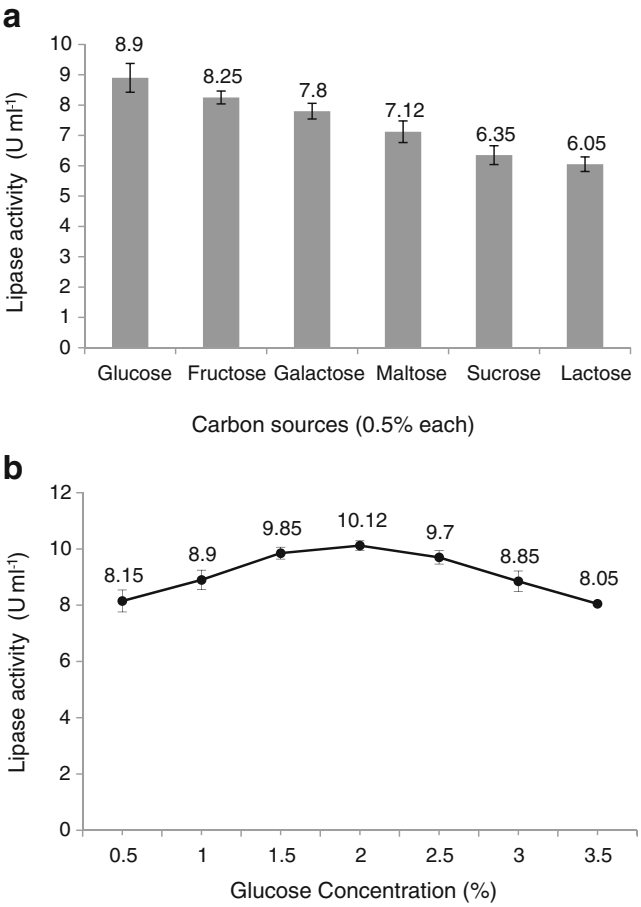

Fig. 3 Effect of **a** various carbon sources and **b** different concentrations of glucose on lipase production by *Serratia marcescens* strain SN5gR. Lipolytic activity is expressed in U ml^{-1}. Each point represents the mean of values obtained from three separate experiments (n=3). No triglycerides were added in the basal media

(12.9 U ml^{-1}) compared with the other tested concentrations (Fig. 4b). Ruchi et al. (2008) reported that lipase production by *Pseudomonas aeruginosa* was specifically higher on organic nitrogen sources than on inorganic sources, which is in agreement with the present study. Also, Bapiraju et al. (2005) reported that organic nitrogen sources, specifically meat extract, peptone and corn steep liquor, yielded more lipase production by *Rhizopus* sp. BTNT-2 than inorganic nitrogen sources. Kantak et al. (2011) reported a lipolytic mesophilic soil fungus (*Rhizopus oryzae*) which produces lipase extracellularly. They optimized the culture conditions to demonstrate that the highest lipase production was observed in basal medium with corn steep liquor as nitrogen source and glucose as carbon source. Esakkiraj et al. (2010) showed that the lipase production by *Staphylococcus epidermidis* CMST-Pi 1 was highest in medium supplied with meat extract, with ammonium sulfate and sodium nitrate yielding the next highest production levels, thus showing its preference towards complex nitrogen sources over inorganic ones.

Effect of trace elements on lipase production

Trace elements such as metal ions are one of the most important cofactors for enzyme production and activity. In the present study, testing the effect of various trace elements on lipase production revealed that all the supplied trace elements increased lipase production over control values (7.75 U ml^{-1}) and, of these, zinc sulfate induced most lipase production (12.95 U ml^{-1}) (Fig. 5). Lipase production by this bacterium also increased when the medium was supplemented with magnesium sulfate, copper sulfate, ferrous sulfate and calcium chloride. The significance of metal ions for lipase productivity has been demonstrated by many earlier researchers. Similar results were obtained by Esakkiraj et al. (2010), although in their case the lipase production was enhanced when the medium was supplemented with EDTA. Similarly, calcium chloride, zinc sulfate, magnesium sulfate, and copper sulfate also elevated lipase production in *Bacillus coagulans* (Alkan et al. 2007).

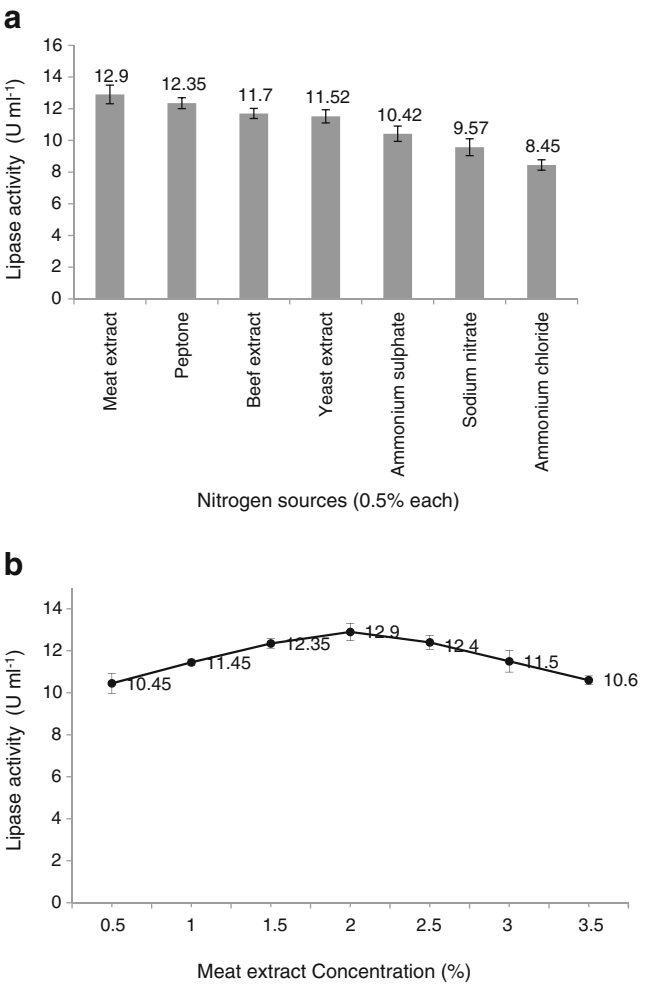

Fig. 4 Effect of **a** various nitrogen sources and **b** different concentrations of meat extract on lipase production by *Serratia marcescens* strain SN5gR. Lipolytic activity is expressed in U ml^{-1}. Each point represents the mean of values obtained from three separate experiments (n=3). No triglycerides were added in the basal media

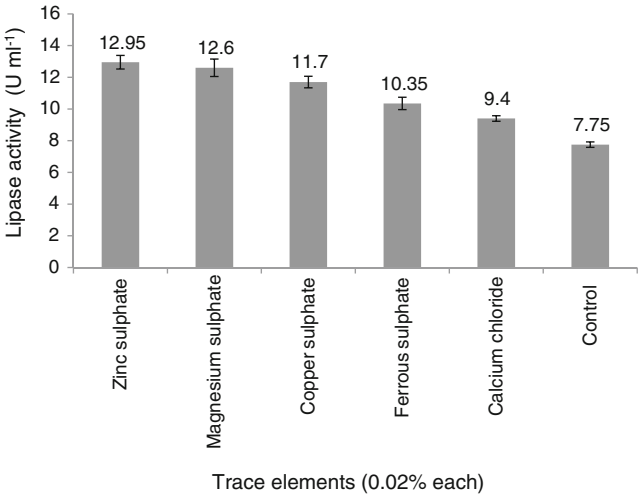

Fig. 5 Effect of trace elements on lipase production by *Serratia marcescens* strain SN5gR Lipolytic activity is expressed in U ml^{-1}. Each point represents the mean of values obtained from three separate experiments (n=3). No triglycerides were added in the basal media

Partial characterization of crude lipase

Effect of pH and temperature on lipase activity

Analyzing the effect of pH on lipase activity showed that, at pH 7.0, strain SN5gR retained 100 % activity; it also revealed relatively high activity over a pH range of 6–8 (Fig. 6). The enzyme activity at pH 6.0 and pH 8.0 were 89.7 and 90.4 %, respectively. A literature survey found that lipases from *Bacillus megaterium* AKG-1 (Sekhon et al. 2005), *Staphylococcus epidermidis* (Joseph et al. 2008), *Candida antarctica* (Pfeffer et al. 2006) and *Staphylococcus epidermidis* CMST-Pi 1 (Esakkiraj et al. 2010) showed maximum activity at pH 7. Kumar et al. (2012) described the lipase from *Bacillus pumilus* RK31 showed that the enzyme was most active at pH 6, retaining about 84 % of its original activity, while at pH 8.0 about 76 % of its original specific activity get destroyed. Akbari et al. (2011) on the other hand reported a lipase, produced by *Pseudomonas* sp., which showed optimum activity at pH 10.

Regarding the effect of temperature on lipase activity of strain SN5gR, maximum lipolytic activity was found at 40 °C (Fig. 7), although the enzyme showed quite high relative activity over a range of temperature from 30 to 50 °C. The enzyme retained 81.3 and 88.4 % activity in 30 and 50 °C temperatures, respectively, but any further decrease or increase in temperature decreased the enzyme activity considerably. Lipase from this new strain of *S. marcescens* is thermophilic in nature and will thus be very useful in many industrial processes. Some bacterial and fungal species such as *Bacillus megaterium* AKG-1 (Sekhon et al. 2005), *Acinetobacter* sp. DYL129 (Kim et al. 2008) and *Fusarium oxysporum* (Prazeres et al. 2006) exhibited optimum lipase activity at the temperature range of 50–55 °C. Thermophillic lipases from *Bacillus stearothermophilus* L1 (Kim et al. 1998)

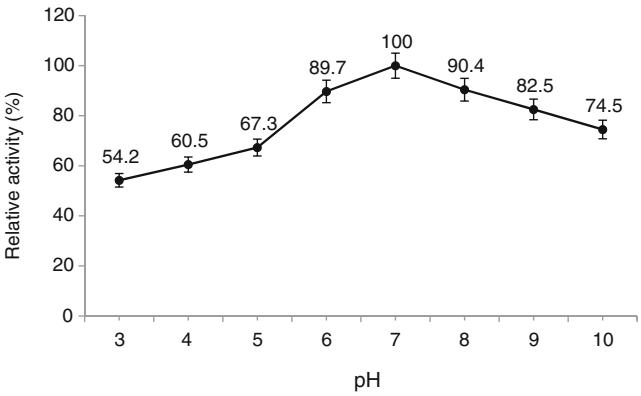

Fig. 6 Effect of pH on lipase activity by *Serratia marcescens* strain SN5gR. Each point represents the mean of values obtained from three separate experiments (n=3)

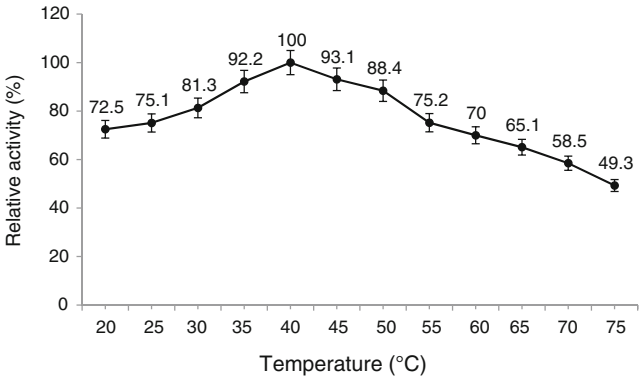

Fig. 7 Effect of temperature on lipase activity by *Serratia marcescens* strain SN5gR. Each point represents the mean of values obtained from three separate experiments (n=3)

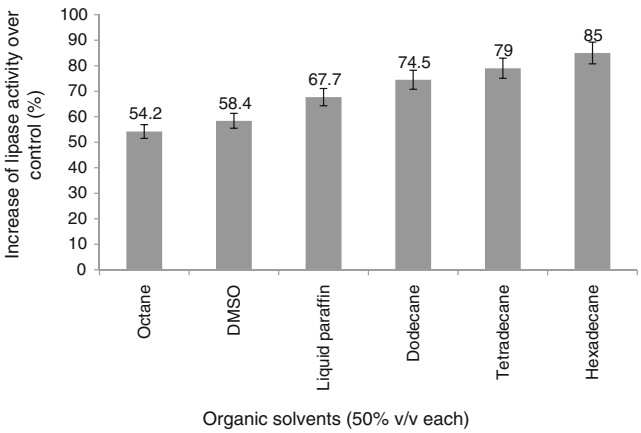

Fig. 8 Effect of organic solvents on lipase activity by *Serratia marcescens* strain SN5gR. Each point represents the mean of values obtained from three separate experiments (n=3)

and *Bacillus thermocatenulatus* (Schmidt-Dannert et al. 1994) showed optimum activity at 60 °C. Although strain SN5gR showed maximum lipase activity at 40 °C, it was found to retain 70 % of enzyme activity at 60 °C temperature. The discovery of a variety of new and more active enzyme has led to expanded growth in the industrial enzyme market. Many of the newly developed enzymes have enhanced thermostability as compared to previously available enzymes. Interest in thermostable enzymes has grown, mainly due to the fact that most of the existing industrial enzyme processes are run at high temperatures and use enzymes from mesophilic sources.

Effect of organic solvents on lipase activity

Various organic solvents were analyzed for their effect on lipase activity. The results showed that the lipase from this bacterium was stable in all the organic solvents tested (Fig. 8). Lipase activity was increased considerably over control values by hexadecane (85 %), tetradecane (79 %) and dodecane (74.5 %). Liquid paraffin, DMSO and octane also showed increased lipase activity over control (52.5 %). Similar organic solvent tolerant lipases have been reported by many workers. Zhao et al. (2008) showed that lipase from *Serratia marcescens* ECU1010 is stable in dimethylsulfoxide (DMSO). Similarly, Baharum et al. (2003) reported that lipase from *Pseudomonas* sp. strain S5 is stable in organic solvents like n-hexane, cyclohexane, toluene and 1-octanol. Akbari et al. (2011) have reported the isolation, characterization, and catalytic properties of a novel thermophilic lipase from *Pseudomonas* sp., which is activated in ionic liquids and organic solvents. The enzyme exhibited excellent stability in the presence of 25 % of n-hexane, toluene, acetone, and t-butanol. The optimum values of pH and temperature were determined at 10 and 55 °C, respectively. Gaur et al. (2008) reported a lipase from *Pseudomonas aeruginosa* which is stable when cultured in the presence of p-xylene, hexane, cyclohexane, n-heptane and dodecane. Whangsuk et al. (2012) have recently described a novel highly organic solvent tolerant extracellular lipase from *Proteus* sp. SW1 and have highlighted its potential application for biodiesel production.

Conclusion

The present study reports the characterization of a novel extracellular lipase by strain SN5gR isolated from the fecal sample of lion-tailed macaque (*Macaca silenus*), an endangered Old World monkey that is endemic to the Western Ghats of South India. The DNA sequencing and homology analysis showed it to be a close relative of *Serratia marcescens* (100 % homology). Our investigation showed that the addition of lipid compounds, different carbon and nitrogen sources and trace elements in the culture greatly influenced the behavior of the microorganism. Moreover, the effect of the addition of several inducers on lipase production showed differential behaviors. Optimum tributyrin concentration of 1.5 % was found to be the most suitable triglyceride to increase lipase production (13.3 U ml^{-1}). The next best lipid source observed was olive oil (11.94 U ml^{-1}), followed by castor oil, coconut oil and palm oil. Analyzing the effect of different carbon sources on lipase production revealed that 2 % glucose yielded higher lipase production than the other tested carbon sources. Investigations on suitable nitrogen source for lipase production revealed that 2 % meat extract yielded higher lipase production. The most suitable trace element for maximum lipase production was zinc sulfate, followed by magnesium sulfate and copper sulfate. The results obtained in this study permit us to conclude that pH and temperature are highly significant factors in the activity of the crude lipase from the newly isolated *S. marcescens* strain SN5gR, with an optimal crude enzyme activity at pH 7.0 and 40 °C, although the enzyme showed quite high

relative activity (above 80 %) over a pH range of 6–8 and temperature range (30–50 °C). Interestingly the enzyme retained 70 % of its activity even at 60 °C. This crude lipase showed resilience to a variety of different organic solvents (hexadecane, tetradecane, dodecane, liquid paraffin, DMSO and octane). The finding that the investigated lipase is active at elevated temperatures and is resistant to large number of organic solvents will make this enzyme an attractive and useful candidate for many industrial purposes including organic synthesis.

Acknowledgments B.G., S.M. and K.G. acknowledge the support of technical facilities available in Presidency University and Bethune College (Govt. of West Bengal, India) respectively. B.G., K.G. and S.M. also acknowledge the financial assistance from the University Grants Commission (Govt. of India) for carrying out this research program. The authors also wish to express their sincere regards to the anonymous referees and the editorial board for the helpful comments to improve the manuscript of the research paper.

Competing interests The authors declare that they have no conflict of interest.

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