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Lipase-producing microorganisms from a Kenyan alkaline soda lake

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Abstract

Lipolytic enzyme production of 150 isolated strains from samples of Lake Bogoria (Kenya) was examined. Among these, fifteen isolates were selected on the basis of their lipolytic activities and subjected to morphological and 16S rRNA gene sequencing analyses for their identification. All the microorganisms have been selected under culture conditions with pH ranges between 7–10 and temperatures of 37–55 °C. Most of them showed optimal growth at 37 °C and tolerated salinity up to 10% (w/v). Ten of the isolates were Gram-negative, nine of which were closely related to the *Pseudomonas* cluster and one to the *Halomonas* cluster sharing high similarity profile with *Halomonas desiderata*. The remaining Gram-positive isolates were closely related to the *Bacillus* cluster, and were grouped with *Bacillus halodurans*, *Bacillus alcalophilus* and *Bacillus licheniformis*. Four members of the *Bacillus* cluster and the *Halomonas* sp. produced lipolytic activity under alkaline conditions, while others did so at neutral pH values.

Introduction

Soda deserts and soda lakes are naturally occurring, highly alkaline environments with unique conditions. The most extensively studied soda lakes are those of the East African Rift Valley where detailed limnological and microbiological investigations have been performed over many years (Jones *et al*. 1998, Grant *et al*. 1990). The Great Rift Valley is an arid tropical zone where in many areas dissolved minerals have concentrated into alkaline brines with carbonate as the major anion, forming shallow soda lakes. The salinities of these lakes vary from around 5% (w/v) total salts to saturation. Soda lakes contain dense populations of aerobic organotrophic and alkaliphilic bacteria, some of which are potential sources of alkali-stable enzymes (Gessesse & Gashe 1997, Martins *et al*. 2001).

Microbial lipases are among the most important group of enzymes with applications in detergents, manufacture of food ingredients, and pitch control in the pulp and paper industry. These enzymes are also interesting as biocatalysts in organic media, for transesterification reactions, synthesis of chiral compounds, etc. (Jaeger & Reetz 1998). Members of diverse genera have been reported to produce different types of lipolytic enzymes. So far not many lipases with optimum activity under alkaline conditions have been studied. Very recently, lipase/esterase positive clones from genomic DNA libraries made from DNA or enrichment cultures isolated from Lake Elementeita soda lake in Kenya have been reported (Rees *et al.* 2003). The present report concerns the isolation and characterization of lipolytic enzyme producing microorganisms from water samples from Lake Bogoria, a soda lake in the East African Rift Valley.

Materials and methods

Screening for microorganisms with lipolytic activity

Samples, collected from Lake Bogoria (Kenya) (0°20′ N and 36°5′ E), were enriched in a liquid basal medium composed of olive oil (Sigma) 3% (v/v) and trace elements (% w/v) (K_2HPO_4 0.08, KH_2PO_4 0.06, $(NH_4)_2SO_4$ 0.1, $MgSO_4 \cdot 7H_2O$ 0.02, $CaCl_2 \cdot 2H_2O$ 0.005, NaCl 0.3, $FeCl_3$ 0.0001), adjusted to pH 7 and 10, respectively. After 5 d of incubation on an orbital shaker (200 rpm) at 37 and 55 °C, respectively, lipase producers were screened on Rhodamine B (Sigma) agar plates at the same temperatures. Production of lipase was monitored under UV light at 350 nm as clear halos around the colonies.

Culture conditions for lipase production

The selected isolates were grown in 1 l flasks containing 200 ml liquid basal medium at pH 7 and 10, respectively. Effect of different concentrations of olive oil (2–5% v/v), yeast extract (0.1–0.5% w/v) and also the presence of arabic gum (1% w/v) on production of lipase activity was studied.

Phenotypic characterization

Characterization of the isolates as gram positive or gram negative was done by Gram's stain reaction (Gerdhardt *et al*. 1994). Biochemical characteristics were screened by API 20E system (bioMérieux) according to Logan & Berkeley (1984).

The effects of temperature on the growth of the isolates was tested by plating out the cells on agar medium and incubating at 25, 37, 45 and 55 °C, respectively, for 48 h. The tolerance to NaCl was studied by plating out the cells on an agar medium containing 0–10% (w/v) NaCl and incubating at their respective optimum temperature for 24 h.

16S rDNA amplification and sequencing

Genomic DNA was extracted and purified according to Marmur (1961), and its purity was assessed from the A_{260}/A_{280} and A_{260}/A_{230} ratios (Johnson 1994). Universal primers (8-27F: AGAGTTTGATCCTGGCTCAG and 1422: GGTTACCTTGTTACGACTT) were used to amplify 16S rDNA sequences (Weisburg *et al*. 1991). PCR products were purified using QIAquick PCR Purification kit (Quiagen) and then resuspended in 40 μl deionised water. DNA sequencing on both strands was performed by the dideoxy chain termination method with an ABI Prism 3100 DNA Analyzer, using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Biosystems) according to the protocol provided by the manufacturer. GenBank and RDP databases were used to search for 16S rDNA sequence similarities (Maidak *et al*. 2000).

Phylogenetic analysis

16S rDNA sequence analysis was performed with the aid of DNAMAN 4.03 software package by using the neighbour-joining method and the Jukes-Cantor distance correction matrix method (Saitou & Nei 1987). For the phylogenetic trees, only sequences from the type strains whose names have been validly published were taken into account.

Plate assay for bacterial lipases

To the basal liquid medium (see above) was added 3% (v/v) olive oil, 0.25% (w/v) arabic gum and agar to a concentration of 1% and 1.5% (w/v), respectively, for different cultivation temperatures. After autoclaving, the medium was cooled down to 60 °C and stirred vigorously for 1 h, before pouring the plates. Rhodamine B was added to give 0.001% (w/v) and the pH adjusted using sterile Na_2CO_3 solution (Kouker & Jaeger 1987).

Lipase activity assay

Lipase activity in the culture supernatant was assayed using *p*-nitrophenylpalmitate (Sigma) as substrate (Kouker *et al*. 1987). 2-Propanol, 10 ml, containing 30 mg *p*-nitrophenylpalmitate was mixed with 90 ml 0.05 M Sörensen phosphate buffer containing 207 mg sodium deoxycholate and 100 mg arabic gum. To the freshly prepared substrate solution (2.4 ml) pre-warmed at 37 °C, was added 0.1 ml the enzyme solution and optical density at 410 nm was measured against an enzyme-free control. One enzyme unit was defined as the activity needed to release 1 μmol *p*-nitrophenol per min under standard assay conditions.

Results

Morphological and physiological characteristics of the isolates

Soil and water samples taken directly from Lake Bogoria (Kenya) were stored at 4 °C before use. The samples were enriched on minimal medium containing

Table 1. General characteristics of the isolated lipolytic enzyme producing microorganisms from Lake Bogoria, Kenya.

Isolates	LBA5	LBA14	LBA17	LBA18	LBA19	LBA22	LBA34	LBA36	LBA38	LBB1	LBB2	LBB3	LBC2	LBD1	LBD3
Morphological characteristics															
Size	0.6–1.1	0.5–2.2	0.3–1.7	0.3–1.7	0.3–1.7	0.6-0.6	0.6–1.7	0.5–2.2	0.6–3	0.3–1.7	0.3–1.1	0.5–3	0.3–1.1	0.3–0.6	1.1–1.4
Colony colour	C	C	C	C	C	C	W	C	C	W	C	W	W	Y	C
Colony shape															
Circular/entire margins	+	–	–	–	–	–	–	–	–	–	–	–	–	–	
Rhizoid/filamentous margins	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Physiological characteristics															
Growth at															
25 °C	+	+	+	+	+	+	+	+	+	+	+[a]	+[a]	+	+[a]	+[a]
37 °C	++	++	++	++	++	++	++	++	+	++	++	++	+	+	+
45 °C	+	+	+[a]	+[a]	+[a]	+[a]	–	–	++	+	+	+	+	+	+
55 °C	–	–	–	–	–	–	–	–	–	–	+	–	++	++	++
Growth in presence of NaCl															
2.5%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5%	+[a]	+	+[a]	+[a]	+	+	+	+	+	+	+	+	+	+[a]	+
10%	–	–	–	+[a]	–	–	+[a]	+	+[a]	+[a]	+	+	+	–	+[a]
Biochemical characteristics															
Gram staining	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+
β-Galactosidase	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+
Arginine dihydrolase	+	–	+	+	+	+	+	+	–	–	–	–	+	–	–
Lysine decarboxylase	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Tryptophan deamidase	+	–	–	–	–	+	+	–	+	–	–	+	–	–	–
Acetoin production	–	+	+	–	–	–	+	+	+	–	+	–	–	+	–
Gelatinase	+	–	–	–	–	–	–	–	–	–	+	–	+	–	+
Growth on															
Citrate	–	+	+	+	+	+	+	+	+	+	–	–	+	–	–
Glucose	–	+	+	+	+	–	+	–	–	–	–	–	–	–	–
Manitol	–	+	+	+	+	–	+	–	+	–	–	–	+	–	–
Inositol	–	+	+	+	+	–	+	–	–	–	–	–	–	–	–
Sorbitol	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–
Rhamnose	–	+	+	+	+	–	–	–	–	–	–	+	–	–	–
Sucrose	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
Melibiose	–	+	+	+	+	+	–	+	+	–	–	–	–	–	–
Arabinose	–	+	+	–	+	+	–	+	+	–	–	+	–	–	–

C: Cream; W: White; Y: Yellow.
[a]Weak growth.
++: Optimum growth.

only olive oil as carbon source during 5 d. Appearance of cloudiness and disappearance of oil were the principal indicators for the presence of lipase producing microorganisms. Even though all the cultures under different conditions showed some degree of growth in liquid medium, only few were positive on specific lipase agar plates. The 15 best lipase producers were selected according to the size of halos formed in the specific plate assay. Morphological, biochemical and growth characteristics of these isolates are summarized in Table 1. The microorganisms produced cream to white circular opaque colonies after 24 h. Microscopic examination showed bacilli shaped cells with the exception of isolate LBA22 that were coccoid. The cell size varied in the range of 0.3–0.8 $\times$ 0.3–2.8 μm. Majority of the isolates showed optimum growth at 37 °C and poor growth at 25 °C and only isolates LBC2, LBD1 and LBD3 grew at 55 °C. Ten of the isolates were Gram negative, nine of which and one Gram-positive isolate, LBC2, had optimum growth at pH 7 and exhibited growth up to pH 9. One remaining Gram-negative and four Gram positive isolates LBB1, LBB2, LBB3, LBD1 and LBD3 grew optimally at pH 10 with no growth below pH 8. Most of the isolates were able to grow in presence of 5% (w/v) NaCl, while isolates LBA18, LBA34, LBA38, LBB1 and LBD3 could grow even at 10% (w/v) NaCl. Biochemical tests revealed that all the isolates were incapable of producing H_2S, ornithine descarboxylase, indole, and to reduce nitrate. Only isolates LBA5, LBB2, LBC2 and LBB3 were able to liquefy gelatine. None of them were able to grow in amygdaline and the majority were able to use different sugars for growth.

Phylogenetic analysis

The 16S rDNA sequences obtained for the selected strains were aligned to those available in the Ribosomal database. Gram-positive isolates were mainly associated with members of the diverse *Bacillus* spectrum showing sequence similarities between 96.2 and 99% (Figure 1). Isolates LBB2 and LBD3 revealed 99.8% sequence identity to *Bacillus halodurans*, LBC2, 99.7% identity to *Bacillus licheniformis*, LBD1, 98.4% identity to *Bacillus flavothermus* and LBB3, 96.2% identity to *Bacillus alcalophilus*. The Gram-negative isolates with optimal growth at pH 7 were placed in the *Pseudomonas* spectrum, being closely related (98–99.8%) to *Pseudomonas pseudoalcaligenes* and *stutzeri*, respectively, while the one growing optimally at pH 10 was 99% related to *Halomonas desiderata* (Figure 2).

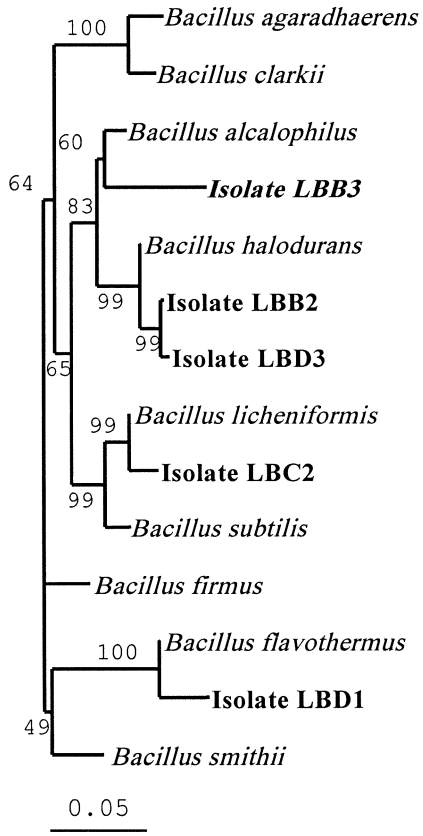

Fig. 1. Unrooted phylogenetic tree constructed from analysis of the 16S rDNA sequences of Gram-positive isolates. The accession numbers of the sequences of the microbial strains used in the phylogenetic analyses are: AY334094, LB B2; AY334095, LB B3; AY334096, LB C2; AY334097, LB D1; AY334098, LB D3; X76445, *Bacillus agaradhaerens* DSM 8721; X76444, *Bacillus clarkii*; X60616, *Bacillus firmus*; Z26932, *Bacillus flavothermus*; X60623, *Bacillus licheniformis*; X60643, *Bacillus smithii*; AY310301, *Bacillus subtilis*.

Lipolytic activity

The isolates that tested positive on rhodamine plates were grown in a minimal medium consisting of basal salts and olive oil as carbon source at their respective optimum pH and temperature, and samples were taken during 24 h. The level of enzyme production in liquid medium did not always correspond to the size of the halos produced by the isolates on the solid media. Lipolytic activity in the culture supernatant was decreased very early during the cultivation in some cases, due to formation of calcium soaps and to the presence of proteases. *Pseudomonas* sp. LBA34 exhibited

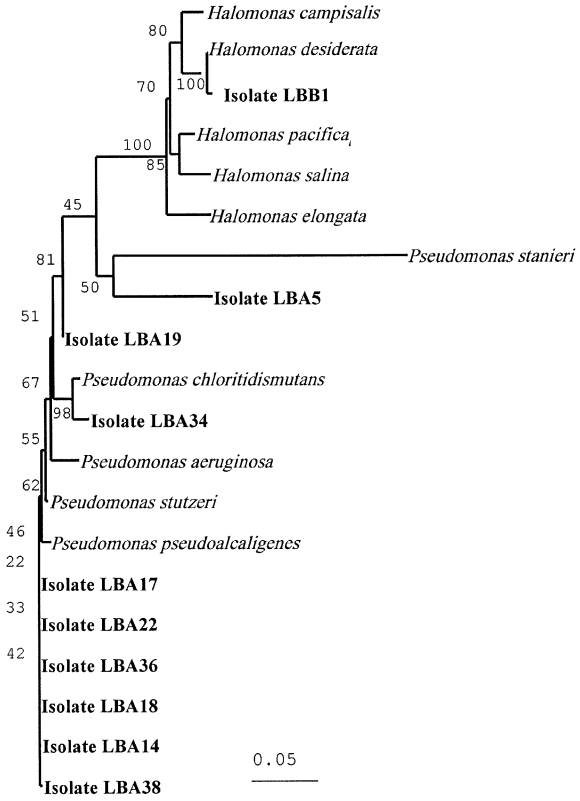

Fig. 2. Unrooted phylogenetic tree constructed from analysis of the 16S rDNA sequences of Gram-negative isolates. The accession numbers of the sequences of the strains used in the phylogenetic analyses are: AY334084, LB A14; AY334085, LB A17; AY334086, LB A18; AY334087, LB A19; AY334088, LB A22; AY334089, LB A34; AY334090, LB A36; AY334091, LB A38; AY334092, LB A5; AY334093, LB B1; *Pseudomonas stutzeri*, AB098613; *Pseudomonas stanieri*, X92176; *Pseudomonas Aeruginosa* ATCC 27853, AY268175; *Pseudomonas pseudoalcaligenes*, AB 109012; *Pseudomonas chloritidismutans*, AY017341; *Halomonas campisalis*, AF54286; *Halomonas desiderata*, X92417; *Halomonas elongata* DSM 3043, AJ295146; *Halomonas pacifica*, L42616; *Halomonas salina*, AJ295145.

the highest lipolytic activity among all the isolates, while *Halomonas* sp. LBB1 exhibited highest activity in the alkaline range among the organisms growing at pH 10 (Table 2). There was no notable influence of the variations in olive oil and yeast extract amount in the medium on expression of lipolytic activity by the isolates. Addition of the emulsifier to the medium led to poor growth of the isolates in general which in turn affected the enzyme production.

Table 2. Maximal lipolytic activity and cell dry wt of the isolates obtained during cultivation on basal liquid medium containing 3% (w/v) olive oil.

Isolate	pH	Dry wt (mg ml^{-1})	Lipase activity (U l^{-1})
LBA5	7	0.61 ± 0.08	25.1 ± 3.15
LBA14	7	2.14 ± 0.91	69.89 ± 1.62
LBA17	7	0.35 ± 0.12	4.13 ± 0.86
LBA18	7	0.27 ± 0.05	15.13 ± 1.71
LBA19	7	1.02 ± 0.17	62.28 ± 19.42
LBA22	7	0.82 ± 0.19	2.56 ± 0.46
LBA34	7	1.37 ± 0.45	104.66 ± 22.91
LBA36	7	0.97 ± 0.19	24.26 ± 13.02
LBA38	7	0.41 ± 0.07	5.68 ± 0.38
LBB1	10	0.53 ± 0.08	53.17 ± 11.76
LBB2	10	1.32 ± 0.14	2.16 ± 0.47
LBB3	10	1.17 ± 0.11	9.28 ± 0.98
LBC2	7	1.68 ± 0.16	22.63 ± 2.49
LBD1	10	1.29 ± 0.22	19.82 ± 1.19
LBD3	10	1 ± 0.08	20.07 ± 3.07

Cultivation temperature was 37 °C. Only for isolates LVC2, LBD1 and LBD3 was 55 °C.

Discussion

Soda lakes are unique environments due to their phylogenetic diversity of aerobic population composed mainly of organotrophic bacteria. This study was carried out with the aim of finding potential sources of lipolytic enzymes active under alkaline conditions. Screening and isolation of microorganisms for lipolytic activity is relatively easy and is most frequently performed by employing agar plates containing triglycerides. The screening system used in this study was based on a chromogenic substrate. All the microorganisms isolated showed a high sensitivity towards the emulsifier (arabic gum) used. More than 150 microorganisms were isolated on liquid basal medium with olive oil. In most of the cases supplementation of the solid medium with an extra carbon source as glucose decreased the expression of the lipolytic activity. Both alkalitolerant and obligate alkaliphiles were found among the isolates and were identified by phylogenetic analysis as the microbial species found in soda lake microbial population and known for being good lipase producers. The alkalitolerant group was largely constituted by members of the genus *Pseudomonas*, a very large and important group of Gram-negative bacteria. Members of this family comprise a substantial proportion of the microflora of free-living saprophytes in soil, fresh water, marine environments and many

other natural habitats. Many lipases from this genus have been extensively studied and are commercially available (Rathi *et al.* 2000, Litthauer *et al*. 2002). One other alkalitolerant bacterium was closely related to *Bacillus licheniformis*. Lipolytic activity of these isolates at pH 10 was below detection limit.

Majority of the obligate alkaliphiles were identified as different species of the genus *Bacillus*, which are known to produce a wide variety of enzymes with resistance to thermal and alkaline conditions (Bell *et al*. 1999, Lee *et al*. 1999, Nthangeni *et al*. 2001). One isolate showed maximal sequence identity of 96.2% to *Bacillus alcalophilus*, and is being investigated as a new species candidate (unpublished work). Only one alkaliphilic isolate was identified as Gram-negative *Halomonas* sp. and produced maximal extracellular lipolytic activity at pH 10. Members of this genus have been isolated from a range of terrestrial, marine, hyper saline and also soda lake environments (Fransmann *et al*. 1988). So far however, there have not been many studies on the production of lipolytic enzymes by this class of organisms.

Currently, lipase enzymes from representative organisms belonging to the different groups are being cloned, expressed and characterized.

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References

Bell PJL, Nevalainen H, Morgan HW, Bergquist PL (1999) Rapid cloning of thermoalkalophilic lipases from *Bacillus* sp. using PCR. *Biotechnol. Lett.* **21**: 1003–1006.

Franzmann PD, Wehmeyer U, Stackebrandt E (1988) *Halomonadacea* fam. nov., a new family of the class proteobacteria to accommodate the genera *Halomonas* and *Deleya*. *Syst. Appl. Microbiol.* **11**: 16–19.

Gerhardt P, Murray RGE, Wood WA, Krieg NR (1994) *Methods for General and Molecular Bacteriology*. Washington, DC: American Society for Microbiology.

Gessesse A, Gashe BA (1997) Production of alkaline protease by an alkaliphilic bacteria isolated from an alkaline soda lake. *Biotechnol. Lett*. **19**: 479–481.

Grant WD, Jones BE, Mwatha WE (1990) Alkaliphiles: ecology, diversity and applications. *FEMS Microbiol. Rev.* **75**: 255–270.

Jaeger KE, Reetz MT (1998) Microbial lipases form versatile tools for biotechnology. *Trends Biotechnol.* **16**: 396–403.

Johnson JL (1994) Similarity analysis of DNAs. In: Gerhart P, Murray RGE, Wood WA, Krieg NR, eds. *Methods for General and Molecular Bacteriology*. Washington, DC: American Society for Microbiology, pp. 655–682.

Jones BE, Grant WD, Duckworth AW, Owenson GG (1998) Microbial diversity of soda lakes. *Extremophiles* **2**: 191–200.

Kouker G, Jaeger KE (1987) Specific and sensitive plate assay for bacterial lipases. *Appl. Environ. Microbiol.* **53**: 211–213.

Lee D, Koh S, Kim K, Kim B, Choi H, Kim D, Suhartono M, Pyun Y (1999) Isolation and characterization of a thermophilic lipase from *Bacillus thermoleovorans* ID-1. *FEMS Microbiol. Lett.* **179**: 393–400.

Litthauer D, Ginster A, van Eeden Skein E (2002) *Pseudomonas luteola* lipase: a new member of the 320-residue *Pseudomonas* lipase family. *Enzyme Microb. Technol.* **30**: 209–215.

Logan NA, Berkeley CW (1984) Identification of *Bacillus* strains using the API System. *J. Gen. Microbiol.* **130**: 1871–1882.

Maidak BL, Coloe JR, Lilburn TG, Parker Jr CT, Saxman PR, Stredwick JM, Garrity GM, Li B, Olsen GJ, Pramanik S, Schmidt TM, Tiedje JM (2000) The RDP (Ribosomal Database Project) continues. *Nucl. Acids Res.* **28**: 173–174.

Marmur J (1961) A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J. Mol. Biol.* **3**, 208–218.

Martins R, Davids W, Al-Soud WA, Levander F, Rådström P, Hatti-Kaul R (2001) Starch-hydrolyzing bacteria from Ethiopian soda lakes. *Extremophiles* **5**: 135–144.

Nthangeni M, Patterton, van Tonder A, Vergeer W, Litthauer D (2001) Over-expression and properties of a purified recombinant *Bacillus licheniformis* lipase: a comparative report on *Bacillus* lipases. *Enzyme Microb. Technol.* **28**: 705–712.

Rathi P, Bradoo S, Saxena RK, Gupta R (2000) A hyperthermostable, alkaline lipase from *Pseudomonas* sp. with the property of thermal activation. *Biotechnol. Lett.* **22**: 495–498.

Rees HC, Grant S, Jones B, Grant WD, Heaphy S (2003) Detecting cellulase and esterase enzyme activities encoded by novel genes present in environmental DNA libraries. *Extremophiles* **7**: 415–421.

Saitou N, Nei M (1987) The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**: 406–425.

Weisburg WG, Barns SM, Pelletier D, Lane DJ (1991) Ribosomal DNA amplification for phylogenetic study. *J Bacteriol.* **173**: 697–703.