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Starch hydrolysing *Bacillus halodurans* isolates from a Kenyan soda lake

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Abstract

Fourteen obligate alkaliphilic and halotolerant bacterial isolates, exhibiting extracellular amylase activity at 55 °C and pH 10, were isolated from hot springs around Lake Bogoria, Kenya. From 16S rDNA sequence analysis, nine isolates shared 100% identity with *Bacillus halodurans* strain DSM 497^T, while the rest shared 99% identity with alkaliphilic *Bacillus* species A-59. PCR of the intergenic spacer region between 16S and 23S rRNA genes (ISR-PCR) divided the isolates into two groups, while tDNA-PCR divided them into three groups. *Bacillus halodurans* DSM 497^T had a different ISR pattern from the isolates, while it had a tDNA-PCR profile similar to the group that shared 99% identity with alkaliphilic *Bacillus* species A-59. All isolates hydrolysed soluble starch as well as amylose, amylopectin and pullulan. The amylase activity (1.2–1.8 U ml^{-1}) in the culture broths had an optimum temperature of 55–65 °C, was stimulated by 1 mM Ca^{2+}, and was either partially (16–30%) or completely inhibited by 1 mM EDTA. Activity staining of the cell-free culture supernatant from the isolates revealed five alkaline active amylase bands.

Introduction

Soda lakes are natural alkaline environments, characterised by the presence of large amounts of soda (Na_2CO_3) usually present as natron (Na_2CO_3) or trona ($Na_2CO_3/NaHCO_3$) with substantial buffering capacity (Grant & Jones 2000). In Kenya, soda lakes with pH values ranging from 8–12, are found along the East African Rift Valley and are fed by hot springs, thus providing a potential habitat for alkalithermophilic microorganisms that produce enzymes with novel properties for potential applications in biotechnology. The microbial population of these lakes is phylogenetically diverse and includes archaea, such as halobacteria and methanogens, and eubacteria, such as those belonging to *Bacillus* and *Clostridium*, and Proteobacteria, such as *Pseudomonas, Halomonas*, sulfur oxidisers, nitrifiers, and anoxygenic phototrophic bacteria (Grant & Jones 2000).

Alkaliphilic microorganisms produce extracellular enzymes with potential applications in processes that demand extreme conditions, such as in laundry and dishwashing detergents, leather tanning, paper pulp bleaching and waste water treatment. Recently, isolation of a number of alkaliphiles with starch hydrolysing activity from soda lakes in the Rift Valley in Ethiopia was reported (Martins *et al.* 2001). In this paper, we report on the isolation of *Bacillus halodurans* species from hot springs occurring around Lake Bogoria in Kenya, that produce extracellular amylases active at pH 10 and 55 °C. These isolates exhibit intraspecific variation as shown by the ISR-PCR (intergenic spacer region between 16S and 23S rRNA genes) and tDNA-PCR patterns obtained from the isolates even though they share high identities (99–100%) with known *B. halodurans* species in the databases.

Materials and methods

Screening for amylase producing microorganisms

Water samples were collected from hot springs around Lake Bogoria, Kenya (0°20′ N and 36°15′ E) and put into selective medium (pH 10) containing 10 g soluble starch l^{-1}, 3 g peptone l^{-1} , 1.5 g NaCl l^{-1}, 2 g K_2HPO_4 l^{-1} and 10 g Na_2CO_3 l^{-1}. The latter was autoclaved separately and mixed with the remaining medium prior to sample inoculation. The samples were then incubated at 55 °C with shaking at 200 rpm for up to 48 h. The mixed cultures were plated out on agar plates containing the same medium with 15 g agar l^{-1} and incubated overnight at 55 °C. Single colonies were picked out and purified by repeated streaking on agar plates. They were then replica-plated and one set of the plates was flooded with iodine solution. Presence of a clear zone or 'halo' around the colonies was used as an indication of extracellular amylase production (Castro *et al.* 1993).

Cultivation of isolates

The corresponding positive colonies were grown in Horikoshi II medium (Horikoshi 1971), pH 10. Trace elements solution consisting of (g l^{-1}): $CaCl_2 \cdot 2H_2O$ (1.7), $FeSO_4 \cdot 7H_2O$ (1.3), $MnCl_2 \cdot 4H_2O$ (15.1), $ZnSO_4 \cdot 7H_2O$ (0.25), H_3BO_3 (2.5), $CuSO_4 \cdot 5H_2O$ (0.125), $Na_2MoO_4 \cdot 2H_2O$ (0.125), $Co(NO_3)_2 \cdot 6H_2O$ (0.23), and 2.5 ml of 18.3 M H_2SO_4, was added in the proportion of 300 μl l^{-1} of medium. Cultures were grown overnight at 55 °C with shaking at 200 rpm. The cells were harvested by centrifugation and the cell free culture supernatant was used as the enzyme source.

Cell growth was monitored as the optical density at 600 nm.

Amylase activity determination

Starch hydrolysing activity was determined from the amount of reducing sugars formed using a modification of the dinitrosalicylic acid (DNS) method (Miller 1959) with glucose as the calibration standard. The cell free culture supernatant (50 μl) was incubated with 450 μl 3 g starch l^{-1} in 50 mM glycine/NaOH buffer pH 10 at 55 °C for 10 min. The reaction was stopped by the addition of DNS reagent, after which the samples were placed in a boiling water bath for 10 min. The samples were cooled in water for 10 min and then absorbancy read at 540 nm. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol reducing sugars per min under the standard assay conditions. The culture supernatant was also tested for hydrolysis of amylose (5 g l^{-1}), amylopectin (2.5 g l^{-1}) and pullulan (5 g l^{-1}), respectively.

Activity staining of the cell free culture supernatant on native 12% polyacrylamide gel was carried out as described by Kim *et al.* (1995).

Biochemical tests

Analyses such as Gram-stain reaction, KOH sensitivity, O_2 requirement and catalase activity of the isolates, were carried out using standard procedures (Gerhardt *et al.* 1994). To determine whether the microorganisms were alkaliphilic or alkalitolerant, cultures were plated on to agar plates containing nutrient broth adjusted to pH 7. No growth at this pH indicated that the isolate is an obligate alkaliphile.

The effect of various concentrations of NaCl on the growth of the microorganisms was studied by cultivation on agar plates containing Horikoshi II medium at pH 10 and trace elements, with 0, 2.5, 5, and 10% (w/v) NaCl, respectively. Effect of temperature on growth of the microorganisms was also studied by growing the cultures on agar plates at 37 °C, 45 °C, 55 °C and 65 °C, respectively.

16S rDNA sequencing and analysis

Genomic DNA was extracted and purified according to Sambrook *et al.* (1989), and its purity was assessed from the A_{260}/A_{280} and A_{260}/A_{230} ratios (Johonson 1994).

Universal 16S rDNA PCR forward primer 8-27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer 1492R (5′-CTACGGCTACCTTGTTACGA-3′) were used in the amplification of 16S rDNA genes (Weisburg *et al.* 1991). PCR products were recovered from 0.8% (w/v) agarose gels using a QIAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany). DNA sequencing on both strands was performed by the dideoxy chain termination method with an ABI prism 3100 DNA Analyzer, using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reactions kit V2.1 (Applied Biosystems, Foster City, CA). The sequences for isolates LBK 34 and LBK 314 have been registered in the GenBank Data Library under accession numbers AF542086 and AF542087. Sequences belonging to the same or closely related species available through the public databases were aligned, and a similarity matrix was calculated using Similarity Matrix version 1.1 software (Maidak *et al.*

2000). Only unambiguously aligned positions from all sequences were used to calculate the matrix, and gaps were not included in the match/mismatch count.

ISR and tDNA-PCR amplification

ISR-PCR (PCR amplification of intergenic spacer regions between the 16S and 23S ribosomal RNA genes) and tDNA-PCR (tRNA gene amplification) were conducted according to Daffonchio *et al.* (1998). ISR-PCR was carried out in 25 μl reactions using the Gene Amp PCR 9700 system (Applied Biosystems) with 1 unit of *Taq* DNA polymerase, 2.5 μl 10 $\times$ STR buffer (Promega), 0.1 μg template DNA and 0.3 μM each of forward primer named S-D-Bact-1494-a-S-20 (5′-GTCGTAACAAGGTAGCCGTA-3′) and reverse primer named L-D-Bact-0035-a-A-15 (5′-CAAGGCATCCACCGT-3′). The following temperature profile was used: initial denaturation at 94 °C for 4 min followed by 30 cycles each consisting of 94 °C for 1 min, 55 °C for 7 min and 72 °C for 2 min, with a final extension step at 72 °C for 7 min.

tDNA-PCR was performed in a final reaction volume of 25 μl consisting of 1 unit *Taq* polymerase, 2.5 μl 10 $\times$ STR buffer (Promega), 0.1 μg DNA and 1 μM each of forward primer T3A (5′-AGTCCGGTGCTCTAACCAACTGAG-3′) and reverse primer T5B (5′-AATGCTCTACCAACTGAACT-3′). The reaction was subjected to a hot start PCR, wherein the reaction mix without *Taq* polymerase was first incubated at 75 °C for 3 min, after which the enzyme was added and the mixture subjected to the following thermal programme: initial denaturation at 94 °C for 4 min, followed by 35 cycles each consisting of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 7 min with a final extension at 72 °C for 10 min.

The ISR and tDNA PCR products were subjected to electrophoresis in 2 and 3% (w/v) agarose gels, respectively. The molecular weight markers used were 100 bp and 1 kb DNA ladders (MBI Fermentas GmbH, Heidelberg, Germany).

Results and discussion

Morphological and physiological characteristics of starch hydrolysing isolates

Fourteen isolates with extracellular starch hydrolysing activity were selected by presence of clear zones or 'halos' around the colonies after staining with iodine solution, with the ratio of the diameter of halos to that of the colonies greater than one. All 14 isolates were rod shaped, motile, forming a terminal spore and were Gram positive. They were aerobic, obligate alkaliphiles, and could grow in presence of up to 10% (w/v) NaCl. Growth of the isolates on solid medium was observed up to only 55 °C.

The screening conditions (55 °C and pH 10) resulted in the growth of a limited number of species, typically related to alkaliphilic bacilli. *Bacillus alcalophilus*, *B. halodurans*, *B. agaradhaerens* and *B. clarkii* are among the Gram positive bacterial populations commonly found at the soda lakes along the East African and Rift Valley (Duckworth *et al.* 1996, Martins *et al.* 2001). The isolates in this study exhibited growth properties similar to those of *B. halodurans* species that grow optimally around pH 9–10, although some strains can grow at pH 7 between 15 °C and 55 °C, and tolerate salinity up to 12% (w/v) NaCl (Nielsen 1995).

16S rDNA, ISR and t-DNA PCR analyses

16S rDNA sequence analysis of the fourteen isolates revealed that they shared high identity with different alkaliphilic *Bacillus* 16S rDNA sequences available in databases. The first group consisting of nine isolates (LBK 315, LBK 317, LBK 32, LBK 326, LBK 328, LBK 34, LBK 35, LBK 37 and LBK 39) had highest identity (100%) with *Bacillus halodurans* strain DSM 497^{T}, while the remaining isolates (LBK 312, LBK 313, LBK 314, LBK 318 and LBK 327), had highest identity (99%) with alkaliphilic *Bacillus* species A-59 (Table 1).

The first group of nine isolates had a similar t-DNA band pattern, with a unique band of about 300–400 bp while the second group of isolates had a unique band at around 500 bp. Isolate LBK 318 had both these bands. The three different band patterns are shown in Figure 1, as represented by the isolates LBK 34, LBK 314 and LBK 318, respectively. Interestingly, the tDNA band pattern of the reference strain, *B. halodurans* DSM 497^{T} was found to be similar to that of the group 2 isolates, while it exhibited 100% 16S rDNA sequence identity with group 1 isolates.

ISR-PCR grouped the fourteen isolates into two groups, the grouping corresponding to that of the 16S rDNA sequence analysis. A comparison of the ISR band pattern between representative strain LBK 34 from the first group, LBK 314 from the second group, LBK 318, which had a unique t-DNA band pattern, and the reference strain, *B. halodurans* strain DSM

Table 1. 16S rDNA sequence similarity matrix between Lake Bogoria isolates and other alkaliphilic *Bacillus* species.

Similarity matrix									
No.	Strain	1	2	3	4	5	6	7	8
1	LBK 34	–	0.998	0.981	0.999	0.999	0.996	1	0.981
2	LBK 314		–	0.981	0.997	0.999	0.997	0.998	0.983
3	C-125			–	0.981	0.981	0.978	0.981	0.998
4	202				–	0.998	0.995	0.999	0.980
5	A-59					–	0.996	0.999	0.982
6	AH-101						–	0.996	0.98
7	DSM_497							–	0.981
8	C-3								–

Sequences used in the construction of the similarity matrix and their accession numbers are: [1] Isolate LBK 34, AF542086; [2] Isolate LBK 314, AF542087; [3] *B. halodurans* strain C-125, AB002661; [4] *Bacillus* sp. 202-1, AB043844; [5] *Bacillus* sp. A-59, AB043856; [6] *Bacillus halodurans* strain AH-101, AB027713; [7] *B. halodurans* DSM 497^T, AJ302709; [8] *Bacillus* species C3, AB043847.

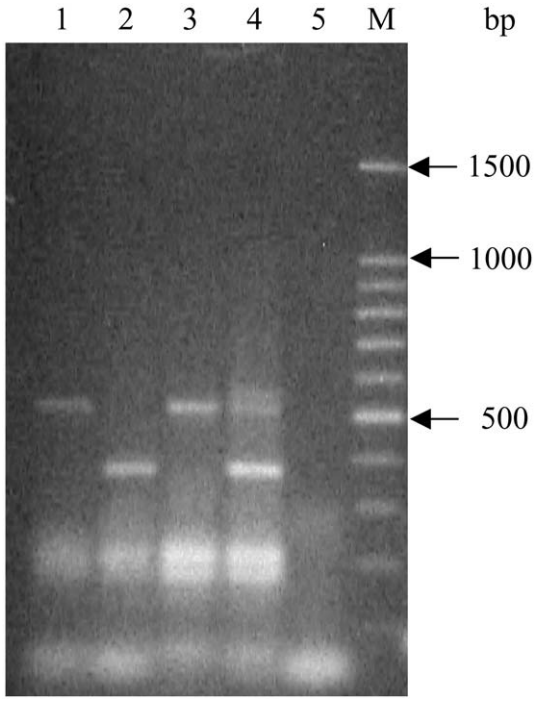

Fig. 1. tDNA-PCR of representative isolates from Lake Bogoria, Kenya and the *B. halodurans* reference strain. Lane 1: *B. halodurans* DSM 497^T; lane 2: LBK 34; lane 3: LBK 314; lane 4: LBK 318; lane 5: negative control; lane M: 100 bp DNA ladder (MBI Fermentas GmbH, Heidelberg, Germany).

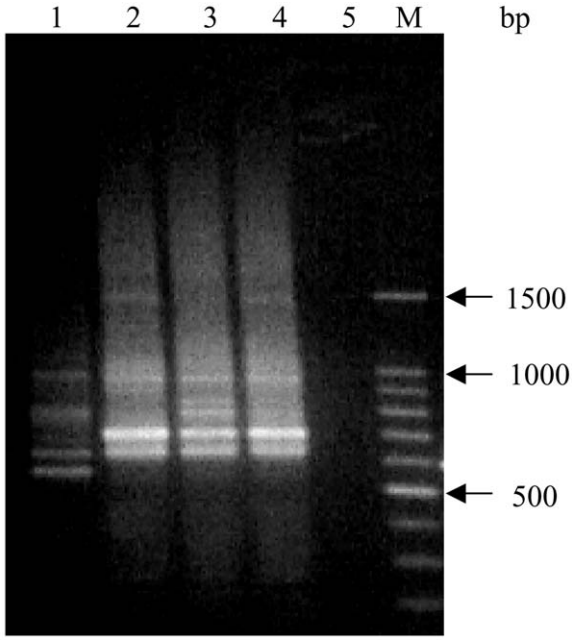

Fig. 2. ISR-PCR of representative isolates from Lake Bogoria, Kenya and the *B. halodurans* reference strain. Lane 1: *B. halodurans* DSM 497^T; lane 2: LBK 34; lane 3: LBK 314; lane 4: LBK 318; lane 5: negative control; lane M: 100 bp DNA ladder (MBI Fermentas GmbH, Heidelberg, Germany).

497^T, revealed that, the latter had a different pattern type from those of the Lake Bogoria isolates and had an extra band of about 550 bp, while group 2 isolates had two more bands of around 800–850 bp which were absent in group 1 isolates and in LBK 318 (Figure 2). Thus, although 16S rDNA sequence analyses of the fourteen isolates showed high identity with known *B. halodurans* species in the databases, differences were observed in the ISR and tRNA gene pattern analyses within the group and between the isolates and the reference strain, *Bacillus halodurans* DSM 497^T. This would indicate that these isolates were indeed different from those already reported in the databases

Table 2. Extracellular starch hydrolysing activity in cell free culture supernatants of Lake Bogoria isolates[a].

Carbon source	O.D. 600 nm	Substrate	Activity (U ml^{-1})
Soluble starch	7–13	Soluble starch	0.5–1.8[b]
		Amylose	0.4–0.9[b]
		Amylopectin	0.4–1.1[b]
		Pullulan	0.1–0.3[b]
Maize bran[c]	10	Soluble starch	1.4
Maize germ[c]	9	Soluble starch	0.9
Wheat bran[c]	12	Soluble starch	0.8

[a]Ten g l^{-1} of carbon source was used during cultivation, while, 2.5 g l^{-1} of amylopectin, 3 g l^{-1} of soluble starch and and 5 g l^{-1} of amylose and pullulan respectively, were used as substrates in the assays.

[b]Range of activity obtained in culture supernatants of the 14 isolates after cultivation in presence of soluble starch.

[c] Only isolate LBK 34 was cultivated in presence of maize bran, maize germ and wheat bran, respectively. In comparison, the isolate grown in the presence of starch as carbon source exhibited maximal O.D. 600 nm of 13 and amylase activity of 1.73 U ml^{-1}, respectively.

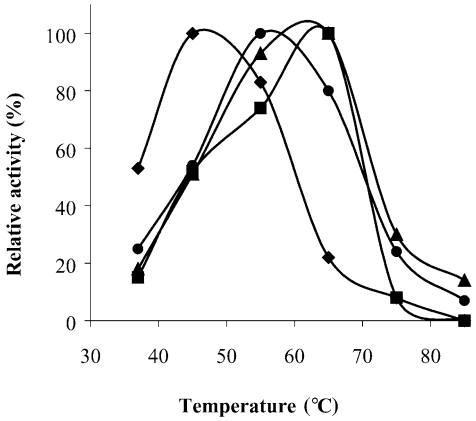

Fig. 3. Effect of temperature on starch hydrolyzing activity of the cell free culture supernatant of the three representative isolates LBK 34 (●), LBK 314 (▲) and LBK 318 (■) from Lake Bogoria, Kenya, and the reference strain, *B. halodurans* DSM 497^T (♦). The 100% activity corresponds to 2.3 U ml^{-1} for DSM 497^T, 1.73 U ml^{-1} for LBK 34, 1.7 U ml^{-1} for LBK 314 and 1.41 U ml^{-1} for LBK 318, respectively. One unit of enzyme activity is defined as the amount of enzyme releasing 1 μmol of reducing sugars per min under the standard assay conditions. Assays were carried out in duplicates.

and showed intraspecific variation. It is interesting to note that, although the 16S rDNA sequence analysis showed 100% identity between *B. halodurans* DSM 497^T and isolate LBK 34, the tDNA and ISR band patterns differed between the two.

Extracellular starch hydrolysing activities of Lake Bogoria isolates

Cultivation of isolates from Lake Bogoria in presence of starch as the sole carbon source resulted in the growth of these microorganisms which reached the maximal optical density at 600 nm of 7–13. The cell free culture supernatants from the fourteen isolates possessed varying levels of starch hydrolysing activity ranging from 0.5 to 1.8 U ml^{-1} (Table 2). Amylase activity of most of the isolates was stimulated by 10–30% in presence of 1 mM Ca^{2+}. Calcium ions are required for maintenance of enzyme activity and structural integrity of amylases (Vihinen & Mäntsälä 1989). Presence of 1 mM EDTA completely inhibited amylase activity of about half of the isolates while isolates LBK 34, LBK 314, LBK 315, LBK 318, LBK 326, and LBK 327 still retained 16–30% of the original activity in presence of the chelator. Alkaline active α-amylases from alkaliphilic *Bacillus* species have shown differences in their response to the chelator, some being completely inhibited (Kim *et al.* 1995), while others exhibiting significant resistance to inactivation in presence of the chelator (Hagihara *et al.* 2001).

The culture supernatants from all the isolates could hydrolyse amylose (0.4–0.9 U ml^{-1}), amylopectin (0.35–1.1 U ml^{-1}) and pullulan (0.1–0.3 U ml^{-1}) (Table 2), thus possessing the ability to hydrolyse both α-1,4 and α-1,6 glycosidic bonds. Pullulanase activity was much lower compared to the activities with other substrates. Alkaline debranching enzymes, when used in combination with alkaline active α-amylase, are very effective in removing starch based stains, making them useful in detergent formulations.

Relative activity of the three representative isolates LBK 34, LBK 314, and LBK 318 (from t-DNA grouping) at various temperatures showed that the optimum temperature was between 55 °C and 65 °C (Figure 3), which was higher than that of the reference strain, *B. halodurans* DSM 497^T (45 °C). Isolate LBK 34 could grow and produce amylases when cultivated in presence of by-products (10 g l^{-1}) of the milling industry, such as maize bran maize germ and wheat bran, with maximum activity in the culture supernatant of 1.39, 0.94 0.77 U ml^{-1}, respectively (Table 2), hence providing economical and readily available substrates for cultivation of these isolates and also potential uses of these isolates in the conversion of these wastes into useful products.

Activity staining of cell-free culture supernatant on native PAGE from the three representative isol-

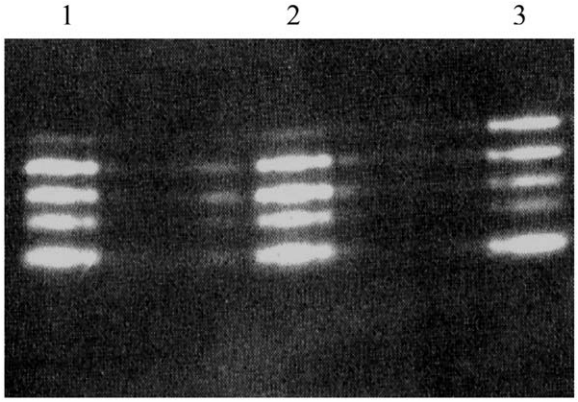

Fig. 4. Activity staining of cell free culture supernatant of the three representative isolates. Lane 1: LBK 34; lane 2: LBK 314; lane 3: LBK 318. Cell free culture supernatant was run on native 12 % polyacrylamide gel (Biorad, Richmond, CA), which was then soaked in 10 g starch l^{-1} in 50 mM glycine-NaOH pH 10 and incubated with shaking (80 rpm) at 55 °C for 1 h. The gel was then stained with a solution containing 5 g KI l^{-1} and 0.5 g I_2 l^{-1}. The clear bands observed indicate amylolytic activity.

ates LBK 34, LBK 314, LBK 318, revealed five amylase active bands throughout the cultivation period (Figure 4). Presence of multiple forms of amylolytic enzymes from alkaliphilic *Bacillus* species has been previously reported and has been attributed to proteolysis of the mature peptide (Shirokizawa *et al.* 1990, Kim *et al.* 1995). For the isolates in this study, the band corresponding to the largest amylase decreased in intensity with time, while the amylase band with lowest molecular weight, increased in intensity with time, during cultivation. This may be an indication of proteolytic degradation of the mature protein with time. However, cultivation in presence of a protease inhibitor (PMSF) up to 5 mM did not alter the production of the different amylase variants (data not shown).

The amylase with highest molecular weight from isolate LBK 34 has now been characterised and is shown to be a maltohexaose forming α-amylase (Hashim SO *et al.* submitted).

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