Extremophiles (2001) 5:135–144
Digital Object Identifier (DOI) 10.1007/s007920100183

ORIGINAL PAPER

Rita F. Martins · Wagied Davids · Waleed Abu Al-Soud
Fredrik Levander · Peter Rådström · Rajni Hatti-Kaul

Starch-hydrolyzing bacteria from Ethiopian soda lakes

Received: November 20, 2000 / Accepted: February 6, 2001 / Published online: April 6, 2001

Abstract Alkaliphilic bacteria were isolated from soil and water samples obtained from Ethiopian soda lakes in the Rift Valley area – Lake Shala, Lake Abijata, and Lake Arenguadi. Starch-hydrolyzing isolates were selected on the basis of their activity on starch agar plate assay. Sixteen isolates were chosen, characterized, and subjected to 16S rRNA gene sequence analysis. All the isolates were gram positive and catalase- and β-galactosidase positive. All isolates except one were motile endospore-forming rods and were found to be closely related to the *Bacillus* cluster, being grouped with *Bacillus pseudofirmus*, *Bacillus cohnii*, *Bacillus vedderi*, and *Bacillus agaradhaerens*. The one exception had nonmotile coccoid cells and was closely related to *Nesterenkonia halobia*. The majority of the isolates showed optimal growth at 37°C and tolerated salinity up to 10% (w/v) NaCl. Both extracellular and cell-bound amylase activity was detected among the isolates. The amylase activity of two isolates, related to *B. vedderi* and *B. cohnii*, was stimulated by ethylenediaminetetraacetic acid (EDTA) and inhibited in the presence of calcium ions. Pullulanase activity was expressed by isolates grouped with *B. vedderi* and also most of the isolates clustered with *B. cohnii*; cyclodextrin glycosyltransferase was expressed by most of the *B. agaradhaerens*-related strains. Minor levels of α-glucosidase activity were detected in all the strains.

Key words Alkaliphiles · Soda lake · Starch hydrolysis · 16S rRNA gene · *Bacillus* species · *Nesterenkonia halobia*

Communicated by T.A. Krulwich

R.F. Martins (✉) · W. Davids[1] · R. Hatti-Kaul
Department of Biotechnology, Center for Chemistry and Chemical Engineering, Lund University, P.O. Box 124, S-221 00 Lund, Sweden
Tel. +46-46-2224741; Fax +46-46-2224713
e-mail: rita.martins@biotek.lu.se

W. Abu Al-Soud[2] · F. Levander · P. Rådström
Department of Applied Microbiology, Center for Chemistry and Chemical Engineering, Lund University, Lund, Sweden

Present address:
[1]Department of Biochemistry, Stockholm Bioinformatics Center, Stockholm University, Stockholm, Sweden
[2]Department of Bacteriology, Lund University, Lund, Sweden

Introduction

Naturally occurring alkaline environments, such as carbonate springs, alkaline soils, and lakes, are characterized by their highly basic pH values ranging from 8 to 11. Soda lakes, which represent stable and extremely productive aquatic ecosystems, exhibit ambient pH values around 10 or higher. They are characterized by the presence of a high concentration of sodium carbonate formed by evaporative concentration, and are also associated with varying degrees of salinity and low concentration of both Mg^{2+} and Ca^{2+} ions (Grant and Tindall 1986; Grant and Horikoshi 1992).

Soda lakes are widely distributed (Grant and Jones 2000); however, as a result of their inaccessibility, few such lakes have been explored from the microbiological point of view. The East African Rift Valley is a volcanically active region with an abundance of soda lakes, many of which are fed by hot springs of varying temperature. In recent years, much effort has been dedicated to biological and geochemical studies of Kenyan soda lakes in the Rift Valley (Grant and Horikoshi 1992). The microbial population of these lakes, which is considerably diverse phylogenetically, includes Archaea, Cyanobacteria, gram-negative organisms affiliated with Proteobacteria (*Halomonas/Deleya* and the *Pseudomonas* spectrum), and gram-positive organisms that are mostly related to members of the genera *Bacillus* and *Arthrobacter* (Duckworth et al. 1996; Grant et al. 1990; Jones et al. 1998).

Alkaliphilic microorganisms, in particular *Bacillus* species, have attracted much interest in the past few decades

because of their ability to produce extracellular enzymes that are active and stable at high pH values. The unusual properties of these enzymes offer a potential opportunity for their utilization in processes demanding such extreme conditions. An application of great impact has been the inclusion of enzymes in laundry and dishwashing detergents. Leather tanning, paper pulp bleaching, production of cyclodextrins, and treatment of agricultural waste and wastes from food processing industries constitute some other areas of application for alkaline-stable enzymes (Aguilar et al. 1998; Horikoshi 1996; Sharp and Munster 1986).

This article reports a study on screening and characterization of starch-degrading alkaliphiles isolated from samples around the soda lakes Lake Shala, Lake Abijata, and Lake Arenguadi in the Rift Valley area of Ethiopia. Ethiopian soda lakes have been characterized with respect to chemical and algal compositions (Wood and Talling 1988; Kebede et al. 1994); however, reports on the prokaryotic population of the lakes are limited. The few reports concerned with the enzymes from microorganisms isolated from these soda lakes reveal rather exceptional catalytic features that render them promising candidates for biotechnological applications (Gessesse and Gashe 1997a, 1997b).

Materials and methods

Source of organisms

Soil (approximately 50 g) and water samples (35 ml) were collected in sterile tubes from Lake Shala, Ethiopia, and transported to Lund for enrichment and cultivation. Fifteen isolates from Lakes Abijata and Arenguadi, Ethiopia, were kindly given by Dr. Amare Gessesse. A detailed description of these lakes and other lakes in the Ethiopian area of Rift Valley was reported by Wood and Talling (1988) and more recently by Kebede et al. (1994); some of their characteristics are listed in Table 1. Isolates from Lake Shala, Lake Abijata, and Lake Arenguadi were designated by LS, AL, and AR, respectively. Reference strains *Bacillus pseudofirmus* DSM 2516 and *Bacillus sp*. DSM 2519 were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM), Braunschweig, Germany. All chemicals were obtained from standard sources.

Table 1. Ethiopian soda lakes

	Location	Salinity (g l^{-1})	pH
Lake Abijata[a]	7°60′ N; 38°62′ E	26.4	9.85
Lake Arenguadi[b]	8°69′ N; 39°31′ E	5.54	10.3
Lake Shala[a]	7°28′ N; 38°32′ E	18.1	9.65

[a]Kebede et al. (1994)
[b]Wood and Talling (1988)

Media, cultivation, and isolation

Soil samples were suspended in 50 mM glycine-NaOH buffer pH 10 (approximately 10% w/v), inoculated in an alkaline medium containing 1% (w/v) starch, and incubated aerobically at 37°C, 180 rpm, until growth appeared. Water samples were directly inoculated into the alkaline medium and incubated under the same conditions. The alkaline medium (pH 10.2) contained (in g/l) soluble starch (10; Merck, Darmstadt, Germany), yeast extract (5; Difco, Detroit, MI, USA), peptone (5; Difco), casamino acids (5; Difco), and Na_2CO_3 (10). A trace elements solution containing (g/l) $CaCl_2{\cdot}2H_2O$ (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), $CoNO_3{\cdot}6H_2O$ (0.23), and 2.5 ml 95%–97% H_2SO_4 was added in the proportion of 300 µl/l of the medium. Na_2CO_3 and trace elements stock solutions were autoclaved separately before addition to the medium. Different dilutions of the cultures were prepared in 0.9% (v/v) NaCl and then plated out on alkaline agar medium containing 1.5% (w/v) agar (Difco). The inoculated plates were incubated at 37°C for 24 h. Individual colonies were isolated, subcultured in the alkaline medium, and streaked onto the agar medium until single, uniform colonies were obtained. The pure isolates were ultimately grown in the alkaline medium for 24 h, and 30% (v/v) glycerol stocks were prepared and stored at –80°C.

Plate tests for screening of amylase and cyclodextrin glycosyltransferase (CGTase) producers

Screening for starch hydrolysis activity among the isolated colonies was performed by plating them on the alkaline agar medium and incubating for 24 h at 37°C. Staining of the plates with iodine reagent was carried out to reveal any starch hydrolysis halos (Castro et al. 1993). The diameter of the halo zone versus the diameter of the colony was used as a semiquantitative method for the selection of the starch-hydrolyzing strains.

Screening for CGTase producers was performed by growing single colonies on alkaline agar medium containing 0.02% (w/v) phenolphthalein (Park et al. 1989) and incubating for 24 h at 37°C. Formation of any halo zone around the colony, resulting from the phenolphthalein–cyclodextrin inclusion complex, was an indication of the presence of enzyme activity. The strains DSM 2519 (CGTase producer) and DSM 2516 (α-amylase producer but non-CGTase) were grown under the same conditions as positive and negative controls, respectively.

Characterization of the isolates

Classification of the isolates as gram positive or gram negative was done by Gram's stain reaction (Gerhardt et al. 1994) and KOH sensitivity test (Gregersen 1978). The catalase and oxidase reactions were performed as described by Gerhardt et al. (1994).

The oxygen requirement test was carried out in tubes containing the alkaline medium with 0.6% (w/v) agar medium for 24 h at 37°C. For the alkaliphily test, the nutrient agar (Difco) medium was adjusted to pH 7 using 1 M NaOH. No growth at this pH value, indicated that the particular strain was an obligate alkaliphile, not merely alkalitolerant.

The effect of temperature on growth was studied by plating out the cells on alkaline agar medium and incubating at 25°C, 37°C, 45°C, and 65°C for 48 h, respectively. To study the effect of salinity on cell growth, alkaline agar medium containing 0%, 2.5%, 5%, and 10% (w/v) NaCl, respectively, was inoculated with the cells and incubated at 37°C for 48 h. In all cases, cells were initially cultured in the alkaline medium for 24 h and further diluted in 0.9% (w/v) NaCl before streaking onto the plates.

Analytical methods

Cell density and dry weight determination

Cell density of the culture broths was determined by measuring the absorbance at 600 nm. For dry weight determination, the cells were grown in alkaline medium and harvested by centrifugation at 5,000 *g* for 30 min at 4°C and washed twice with 50 mM glycine-NaOH buffer, pH 10. Finally, the cells were resuspended in the same buffer, and 200 µl of the suspension was filtered through a preweighed 0.2-µm cellulose acetate membrane (Sartorius AG, Goettingen, Germany). Membranes were dried overnight at 105°C and then equilibrated at room temperature before weighing.

Enzyme assays

Cultures grown for 24 h in the alkaline medium at 37°C were tested for amylolytic activities. Amylase and pullulanase activities were measured by incubating 50 µl of the enzyme sample with 0.45 ml 0.3% (w/v) soluble starch (Merck) and 0.5% (w/v) pullulan (ICN Biomedicals, Aurora, OH, USA), respectively, in 50 mM glycine-NaOH buffer, pH 10, at 40°C. The release of reducing sugars was followed by the dinitrosalicylic acid method (Miller 1959). One unit of amylase or pullulanase activity was defined as the amount of enzyme releasing 1 µmol of reducing sugars as glucose per minute under the assay conditions.

CGTase activity was measured as β-cyclodextrin-forming activity by a modification of the phenolphthalein method described by Mäkelä et al. (1988). To 750 µl 5% (w/v) maltodextrin solution (Aldrich, Milwaukee, WI, USA) prepared in 50 mM glycine-NaOH buffer, pH 10, preincubated at 50°C for 5 min, was added 25 µl of the enzyme sample. After 10 min incubation at 50°C, the reaction was quenched by adding 375 µl 0.15 M NaOH. Subsequently, 100 µl 0.02% (w/v) phenolphthalein prepared in 5 mM Na_2CO_3 was added, and after standing at room temperature for 15 min, the color intensity was measured at 550 nm. A calibration curve was made using 0.006–0.22 µmol of β-cyclodextrin (Sigma, St. Louis, MO, USA) solution in 50 mM glycine-NaOH buffer, pH 10, as standard. One unit of CGTase activity was defined as the amount of enzyme releasing 1 µmol of β-cyclodextrin per minute under the assay conditions.

The β-galactosidase activity was measured according to the established procedure (Worthington 1993) with a slight modification; 50 µl of the enzyme sample was added to 950 µl of the reaction mixture containing 450 µl 50 mM glycine-NaOH buffer, pH 10, 200 µl 0.04 M *o*-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma) in 0.01 M Tris-acetate buffer, pH 7.5, and 300 µl water. Initial increase in absorbance at 405 nm at room temperature, resulting from the formation of *o*-nitrophenol, was used as a measure of the enzyme activity.

The α-glucosidase activity was measured according to the established procedure (Worthington 1993) with a slight modification; 100 µl of the enzyme sample was added to 900 µl of 0.06% (w/v) *p*-nitrophenyl-α-D-glucopyranoside (PNPG; ICN Biomedicals, Costa Mesa, CA, USA) in 50 mM glycine-NaOH buffer, pH 10. Initial increase in absorbance at 400 nm was followed at room temperature. One unit of enzyme activity was defined as the amount required to produce 1 µmol of *p*-nitrophenol per minute under the specified assay conditions.

PCR amplification of 16S rRNA genes

Bacterial strains were grown in the alkaline medium for 24 h, and the cells were harvested by centrifugation at 5,000 *g* for 10 min and then washed once in sterile water; finally, the cells were resuspended in sterile water and stored at –20°C. The two degenerate primers used in this study were selected on the basis of published alignment of bacterial 16S rRNA gene sequences (Rådström et al. 1994). The forward primer targets the bases 247 to 270 (5′-AACA(A/G)GATTAGATACCCTGGTAGT-3′) and the reverse primer targets the bases 649 to 668 (5′-A(C/T)TTGAC GTCATCCCCACCT-3′). The polymerase chain reaction (PCR) mixtures, in a total volume of 50 µl, contained 0.5 µM each of the forward and reverse primers, 0.2 mM each of the deoxyribonucleoside triphosphates, 1×*Pwo* buffer [10 mM Tris-HCl (pH 8.85, 20°C), 25 mM KCl, 5 mM $(NH_4)_2SO_4$, and 1.5 mM $MgCl_2$], and 1.25 U of *Pwo* DNA polymerase (Roche Molecular Biochemicals, Basel, Switzerland). A 10-µl cell suspension was added to the reaction mixture, which was subjected to cell lysis at 94°C for 5 min, followed by 35 cycles of heat denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and DNA amplification at 72°C for 30 s. Final extension of the amplified DNA was done at 72°C for 7 min. Incubation was carried out in a model 2400 thermal cycler (Applied Biosystems, Foster City, CA, USA). The 422-bp PCR product was visualized by electrophoresis on 1.3% (w/v) agarose gel containing ethidium bromide. The gel was analyzed by the gel documentation system (Bio-Rad, Hercules, CA, USA).

DNA sequencing

The PCR products were purified from agarose gel using a QIAquick gel extraction kit according to the instructions of the manufacturer (Qiagen, Dorking, UK). Sequencing of PCR products was carried out by using the same primer pair already described and the Prism ready reaction dRhodamine terminator cycle sequencing kit according to the instructions of the manufacturer (Applied Biosystems). Analysis of the reaction mix was performed in a DNA sequencer (Applied Biosystems).

Phylogenetic analysis

The 16S rRNA gene sequences of the alkaliphilic bacterial strains and the sequences obtained from the GenBank and EMBL were aligned by the CLUSTAL X program. A phylogenetic tree was also constructed from the aligned sequences with the neighbor-joining method (Saitou and Nei 1987).

Accession numbers

The GenBank/EMBL 16S rRNA gene sequences used in the phylogenetic analysis are shown in Fig. 1. The 16S rRNA gene sequences determined in this study have been deposited in the GenBank database under the accession numbers AF335253 to AF335268.

Results

Morphological and physiological characteristics of starch-hydrolyzing soda lakes strains

Inoculation of the soda lakes samples on 1% (w/v) starch-containing alkaline medium and incubation at 37°C produced colonies that could use starch as the carbon source. The colonies were isolated and grown individually on solid alkaline medium and subjected to iodine staining, which confirmed starch hydrolysis by revealing halos around the colonies. Of the 89 isolates obtained, 16 showed a ratio of the diameter of the halo zone to that of the colony of more than 1.0 and were selected for further studies (Table 2). The colonies were predominantly circular and entire while some were rhizoid and filamentous; all of them were smooth after 24 h at 37°C (Table 2). The colonies of LS-1C, LS-2C, LS-3C, LS-7C, and AL-125 were observed to grow into the agar surface. Cultures grown in liquid medium for 24 h showed motile rod-shaped cells under the microscope, with the exception of the isolate LS-12C, which showed nonmotile coccus cells.

Cultures grown in liquid and agar media for 24 h at 37°C were used for characterization. All the isolates were gram positive and, with the exception of LS-12C, were endospore-forming bacteria. Normal Gram staining procedures were not entirely reliable, as also reported earlier (Duckworth et al. 1996; Agnew et al. 1995); therefore, the KOH test was used to classify the isolates according to differences in the cell-wall groups. Both strict aerobes and facultative anaerobes were distinguished among the isolates (Table 3). All the isolates tested positive for catalase and β-galactosidase whereas about 50% of the isolates were positive for the oxidase reaction (see Table 2).

The strains LS-6C, LS-8C, LS-9C, LS-10C, LS-12C, and AR-199 were alkalitolerant as seen by their ability to also grow on neutral nutrient agar, while the remaining isolates were obligate alkaliphiles (Table 3). The majority of the strains showed optimum growth at 37°C while the strains LS-8C and LS-12C grew better at 45°C. The isolates LS-4C, LS-6C, and AL-127 showed no growth at 45°C up to 48 h (Table 3). At 25°C, poor growth of the isolates was observed after 24 h but was better when the incubation period was extended to 48 h. None of the strains grew at 65°C during 48 h of incubation.

Most of the isolates were able to grow in the presence of 10% (w/v) NaCl, implying their tolerance to the salinity usually associated with soda lakes (Grant and Tindall 1986). The only exceptions were the strains LS-6C and LS-9C, which showed growth at salt concentration up to 5%, and the strains LS-8C and LS-10C, which showed weak growth in the presence of 10% salt only after 48 h incubation (see Table 3).

Amylolytic activities

Amylolytic activities of the culture samples were determined at pH 10. Both the culture supernatant after cell harvesting and the cells were tested for enzymatic activity. For the latter, cell pellets were washed twice with 50 mM glycine-NaOH buffer, pH 10, and resuspended in the same buffer. Most of the strains showed some cell-bound amylase activity along with the extracellular activity, whereas only cell-bound amylase was detectable in the cultures of the strains LS-8C, LS-9C, LS-10C, and AR-199. However, the isolates LS-11C, AL-125, AL-127, and AR-188 had no detectable amylase activity under the assay conditions (Table 4).

The amylase activity of the strains was further determined by including 0.1–10 mM ethylenediaminetetraacetic acid (EDTA) or $CaCl_2$ in the assay mixture. Activity of the strains LS-4C and LS-6C was seen to be stimulated with 5–10 mM EDTA, the former displaying higher activity, i.e., about 120%, with 10 mM EDTA. On the other hand, their activity was inhibited in the presence of Ca^{2+} ions. A decrease in activity by 15% and 30% for the strains LS-4C and LS-6C, respectively, was noticed at $CaCl_2$ concentration of 0.5 mM with complete inactivation at 5 mM ion concentration (data not shown). The enzymatic activity of the strains LS-1C, LS-3C, LS-5C, and LS-10C was reduced by 50%, while that of the remaining isolates was completely inhibited in the presence of 5 mM EDTA.

The culture samples were also tested for pullulanase and α-glucosidase activities. Pullulanase activity was detected in

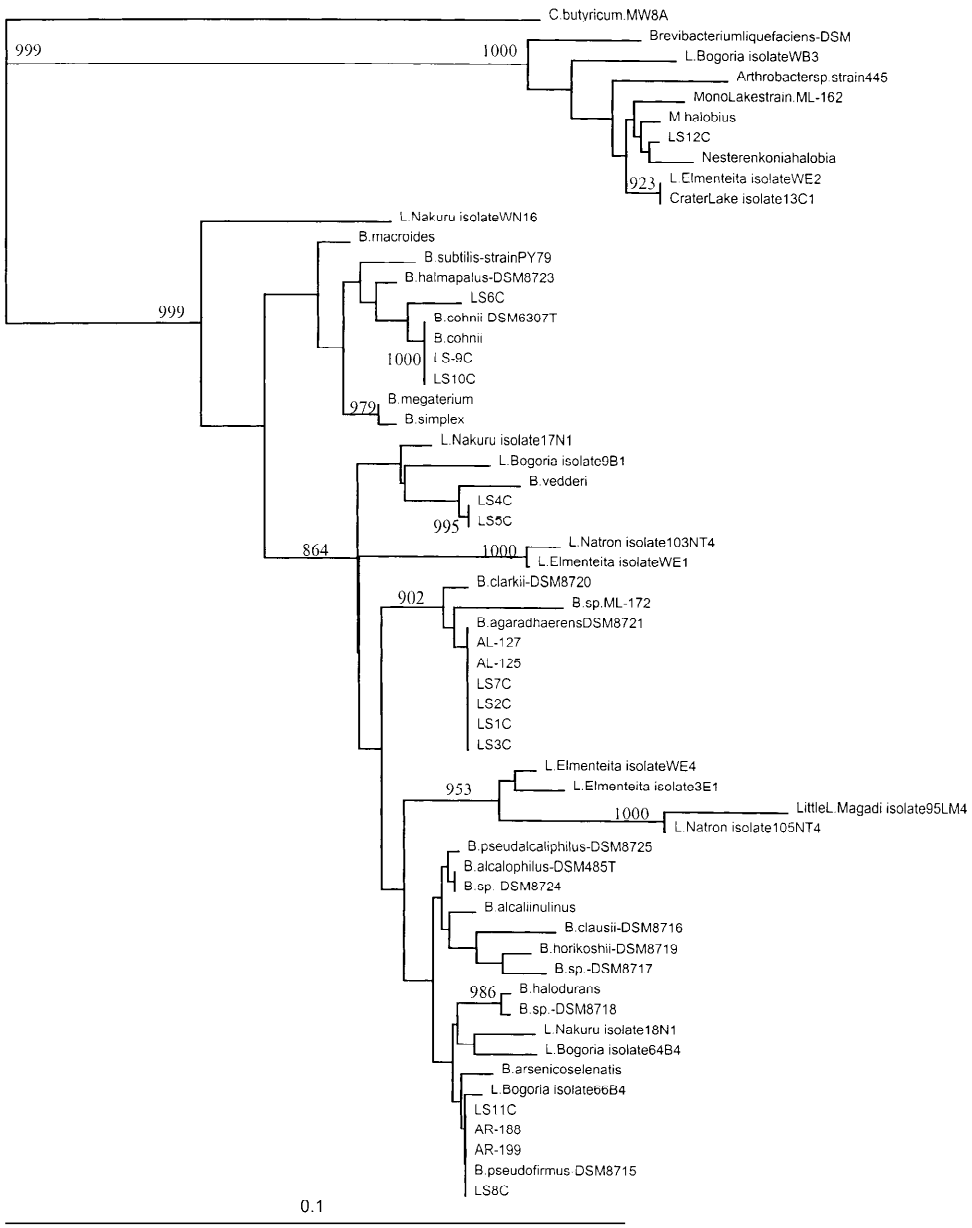

Fig. 1. Phylogenetic tree of the soda lake isolates based on 16S rRNA gene sequence using the neighbor-joining method (Saitou and Nei 1987). *Clostridium butyricum* MW8A was used as the outgroup. *Scale* represents the average number of nucleotide substitutions per site. Bootstrap values for 1,000 estimations are shown at *nodes*. The accession numbers of the additional 16S rRNA sequences used are as follows: *Arthrobacter* sp. X93354, *Arthrobacter* sp. X93354, *Bacillus agaradhaerens* DSM8721 X764445, *Bacillus agaradhaerens* DSM8721 X764445, *Bacillus alcaliinulinus* AB018595.1, *Bacillus alcaliinulinus* AB018595.1, *Bacillus alcalophilus* DSM485T X76436, *Bacillus alcalophilus* DSM485T X76436, *Bacillus arsenicoselenatis* AF064705, *Bacillus arsenicoselenatis* AF064705, *Bacillus clarkii* DSM8720 X76444, *Bacillus clarkii* DSM8720 X76444, *Bacillus clausii* DSM8716 X76440, *Bacillus clausii* DSM8716 X76440, *Bacillus cohnii* AF1400114.1, *Bacillus cohnii* AF1400114.1, *Bacillus cohnii* DSM6307T X76437, *Bacillus cohnii* DSM6307T X76437, *Bacillus halmapalus* DSM8723 X76447, *Bacillus halmapalus* DSM8723 X76447, *Bacillus halodurans* AB013373.1, *Bacillus halodurans* AB013373.1, *Bacillus horikoshii* DSM8719 X76443, *Bacillus horikoshii* DSM8719 X76443, *Bacillus macroides* AF157696, *Bacillus macroides* AF157696, *Bacillus megaterium* D16273, *Bacillus megaterium* D16273, *Bacillus pseudalcaliphilus* DSM8725 X76449, *Bacillus pseudalcaliphilus* DSM8725 X76449, *Bacillus pseudofirmus* DSM8715 X76439, *Bacillus pseudofirmus* DSM8715 X76439, *Bacillus simplex* D78478, *Bacillus simplex* D78478, *Bacillus* sp. DSM 8717 X76441, *Bacillus* sp. DSM 8717 X76441, *Bacillus* sp. DSM 8718 X76442, *Bacillus* sp. DSM 8718 X76442, *Bacillus* sp. DSM 8724 X76448, *Bacillus* sp. DSM 8724 X76448, *Bacillus* sp. ML-172 AF140005.1, *Bacillus* sp. ML-172 AF140005.1, *Bacillus subtilis* PY79 AF142577, *Bacillus subtilis* PY79 AF142577, *Bacillus vedderi* Z48306, *Bacillus vedderi* Z48306, *Brevibacterium liquefaciens* DSM 20579 AJ251417.1, *Brevibacterium liquefaciens* DSM 20579 AJ251417.1, *Clostridium butyricum* MW8A AJ002592, *Clostridium butyricum* MW8A AJ002592, Crater Lake isolate 13C1 X92155, Lake Bogoria isolate 64B4 X92160, Lake Bogoria isolate 66B4 X92158, Lake Bogoria isolate 9B1 X92167, Lake Bogoria isolate WB3 X92153, Lake Elmenteita isolate 3E1 X92162, Lake Elmenteita isolate WE1 X92164, Lake Elmenteita isolate WE2 X92154, Lake Elmenteita isolate WE4 X92161, Lake Nakaru isolate 17N1 X92168, Lake Nakaru isolate 18N1 X92159, Lake Nakaru isolate WN16 X92169, Lake Natron isolate 103NT4 X92163, Lake Natron isolate 105NT4 X92166, Little Lake Magadi isolate 95LM4 X92165, *Micrococcus halobius* X80747, Mono Lake ML-162 AF140001.1, *Nesterenkonia halobia* Y13857

Table 2. Morphological characteristics of the starch-hydrolyzing soda lake isolates

Isolate	Approximate cell size (μm)[a]	Colony shape	Colony color	Motility[b]	Ratio halo/colony	Oxidase[b]
LS-1C	6.2 ×0.5	Rhizoid with filamentous margins	White	+	2.4	–
LS-2C	3.4 ×0.6	Rhizoid with filamentous margins	White	+	2.2	–
LS-3C	4.5 ×0.5	Rhizoid with filamentous margins	White	+	2.2	–
LS-4C	3.4 ×0.8	Circular with entire margins	Yellow	+	1.5	–
LS-5C	3.9 ×0.8	Circular with entire margins	Yellow	+	2.0	–
LS-6C	4.5 ×0.6	Circular with entire margins	White	+	2.3	+
LS-7C	6.7 ×0.5	Rhizoid with filamentous margins	White	+	1.7	+
LS-8C	4.5 ×0.6	Circular with entire margins	White	+	1.1	+
LS-9C	6.2 ×0.5	Circular with entire margins	White	+	1.5	+
LS-10C	5.6 ×0.6	Circular with entire margins	White	+	1.6	+
LS-11C	3.4 ×0.8	Circular with entire margins	White	+	1.4	–
LS-12C	0.8 ×1.1	Circular with entire margins	White	–	2.3	–
AL-125	4.5 ×0.6	Rhizoid with filamentous margins	White	+	1.6	–
AL-127	2.8 ×0.6	Circular with entire margins	White	+	1.7	+
AR-188	4.5 ×0.8	Circular with entire margins	White	+	1.3	+
AR-199	5.6 ×0.6	Circular with entire margins	White	+	1.7	+

All isolates showed gram-positive and rod-shaped cells, with the exception of isolate LS-12C, which showed coccus cells
With the exception of LS-12C, all isolates were endospore forming. All isolates were catalase- and β-galactosidase positive
[a]Cells grown in alkaline medium for 24 h
[b]+, positive reaction; –, negative reaction

cultures of only four isolates. The strains LS-4C and LS-5C presented extracellular activity, whereas the strains LS-9C and LS-10C showed only cell-bound activity, similar to their amylase activity. Minor levels of α-glucosidase activity were detected in all the strains in the range of 1–6 mU/ml (data not shown).

Producers of CGTase were screened among the selected isolates by growth in alkaline agar medium containing phenolphthalein. The two reference strains, DSM 2516 and DSM 2519, were also included in the study. Both are amylase positive and generate halo zones after iodine staining on starch agar plates; however, only the strain DSM 2519 had CGTase activity as shown by phenolphthalein inclusion. Five isolates, LS-1C, LS-2C, LS-3C, LS-7C, and AL-125, exhibited production of CGTase by this method. CGTase activity in their culture supernatants was in the range 0.32–0.50 U/ml (Table 4).

Phylogenetic analysis

The 16S rRNA gene sequences obtained for the isolated strains were aligned to those available in EMBL/GenBank database, including those of the isolates from soda lakes in the Kenyan region of the Rift Valley (Duckworth et al. 1996). The alignment placed the isolates in the *Bacillus* spectrum (see Fig. 1), except for the strain LS-12C, which showed a close identity (99%) to *Micrococcus halobius*, renamed as *Nesterenkonia halobia* (Stackebrandt et al. 1995), and to the soda lake members of this group, i.e., Lake Elmenteita isolate WE2 and Crater Lake isolate 13C1. The strains LS-1C, LS-2C, LS-3C, LS-7C, AL-125, and AL-127 were grouped with *Bacillus agaradhaerens* with a sequence identity of 100%. The closest relative of this group from the soda lakes isolates reported by Duckworth and coworkers (1996) was the Lake Nakuru isolate 17N1, with 97% identity. The latter isolate was closer (98% identity) to strains

Table 3. Physiological characteristics of the starch-hydrolyzing soda lake isolates

Isolate	Oxygen requirement	Growth[a] on nutrient agar (pH 7)	Growth[a] in presence of NaCl:		Growth[a] at:	
			5% (w/v)	10% (w/v)	37°C	45°C
LS-1C	Facultatively anaerobic	–	+	+	+	+
LS-2C	Facultatively anaerobic	–	+	+	+	+
LS-3C	Facultatively anaerobic	–	+	+	+	+
LS-4C	Strictly aerobic	–	+	+	+	-
LS-5C	Facultatively anaerobic	–	+	+	+	+
LS-6C	Strictly aerobic	+	+	–	+	–
LS-7C	Facultatively anaerobic	–	+	+	+	+
LS-8C	Strictly aerobic	+	+	+[b]	+	+[c]
LS-9C	Strictly aerobic	+	+	–	+	+
LS-10C	Strictly aerobic	+	+	+[b]	+	+
LS-11C	Strictly aerobic	–	+	+	+	+
LS-12C	Strictly aerobic	+	+	+	+	+[c]
AL-125	Facultatively anaerobic	–	+	+	+	+
AL-127	Facultatively anaerobic	–	+	+	+	–
AR-188	Strictly aerobic	–	+	+	+	+
AR-199	Strictly aerobic	+	+	+	+	+

[a]+, growth; –, no growth
[b]Weak growth visible after 48 h incubation
[c]Showed better growth at 45°C

Table 4. Amylolytic activities of the isolates

Isolate	OD_{600}[a]	Amylase activity		Pullulanase activity	CGTase activity (U/ml)
		Supernatant (U/ml)	Cells (U/mg of cells)[b]		
LS-1C	5.1	1.43	0.03	[c]	0.43
LS-2C	5.1	0.48	[c]	[c]	0.42
LS-3C	5.2	1.72	0.04	[c]	0.35
LS-4C	4.9	0.59	[c]	0.16[d]	ND
LS-5C	5.1	1.17	[c]	0.18[d]	ND
LS-6C	4.1	3.21	0.16	[c]	ND
LS-7C	4.8	1.95	0.03	[c]	0.32
LS-8C	4.1	[c]	0.11	ND	ND
LS-9C	3.6	ND	0.13	0.06[e]	ND
LS-10C	3.7	[c]	0.14	0.05[e]	ND
LS-11C	12.4	ND	[c]	[c]	ND
LS-12C	7.3	0.92	0.12	[c]	ND
AL-125	2.3	[c]	[c]	[c]	0.5
AL-127	2.7	[c]	[c]	[c]	ND
AR-188	11.9	[c]	[c]	[c]	ND
AR-199	3.3	[c]	0.12	ND	ND

CGTase, cyclodextrin glycosyltransferase; ND, not detected under the standard assay conditions used
[a]OD_{600} was used as a measure of cell density
[b]The amount of cells is in terms of dry weight
[c]Minor activity expressed (in the lower detection limit of the assay)
[d]Activity expressed in U/ml of cell-free supernatant
[e]Activity expressed in U/mg of cells (in terms of dry weight)

LS-4C and LS-5C, which had a similar percentage of identity with *Bacillus vedderi*. LS-9C and LS-10C had 100% sequence identity with *Bacillus cohnii* DSM 6307 T, while LS-6C was close, with 98% identity. Significantly lower identity of these isolates was found with the Kenyan Lake Nakuru isolate WN16, i.e., LS-9C and LS-10C with 94% and LS-6C with 93%. Finally, LS-8C, LS-11C, AR-188, and AR-199 were 100% identical to *Bacillus pseudofirmus* DSM 8715 and showed 99% identity with Lake Bogoria isolate 66B4.

Discussion

The few reports available on the microbial diversity of soda lakes have shown the existence of a wide range of physiological groups (Imhoff et al. 1979; Duckworth et al. 1996; Jones et al. 1998). The aerobic microbial population is composed of organotrophic bacteria, including *Bacillus* species, which produce a wide variety of enzymes including proteases, lipases, cellulases, and amylases for recycling of biopolymers. This study was performed to isolate starch-degrading alkaliphilic microorganisms, and was confined to a few soda lakes in Ethiopia that are less well studied than the Kenyan lakes of the Rift Valley (Duckworth et al. 1996; Jones et al. 1998). Starch-degrading enzymes from a few alkaliphilic *Bacillus* species isolated from different environments have been reported during the last two decades (Takami and Horikoshi 2000). Production of high-temperature alkaline active amylases has even been reported from mesophilic *Bacillus licheniformis* and *Bacillus coagulans* (Bajpai and Bajpai 1989; Medda and Chandra 1980).

Selection of the starch-degrading isolates grown on solid starch alkaline medium was based on the extent of decolorization obtained after iodine staining. It should be stressed here that the bacterial populations isolated are of course biased by the manner of sampling and the culture conditions employed. According to the 16S rRNA gene analysis, the isolates were clustered into five groups, four of which were *Bacillus* species: *B. pseudofirmus*, *B. cohnii*, *B. vedderi*, and *B. agaradhaerens*, while one was *Nesterenkonia halobia*. All these *Bacillus* species are known to be alkaliphilic (Spanka and Fritze 1993; Agnew et al. 1995; Nielsen et al. 1995), while *N. halobia* was reported as a moderately halophilic organism exhibiting good growth at pH values between 6 and 10 (Onishi and Kamekura 1972; Mota et al. 1997). These and related species have also been isolated from the soda lakes in the Kenyan region of the Rift Valley (see Fig. 1) (Duckworth et al. 1996). Even though several isolates in this study have been clustered with a specific *Bacillus* species, they appear to be unique isolates according to their physiological differences (e.g., oxidase reaction and growth at pH 7.0), as well as variance in their enzyme production levels.

Earlier reports have shown *B. pseudofirmus* and *B. agaradhaerens* to be organisms with industrial potential. Alkaline amylases from the former species have been isolated and characterized (Horikoshi 1971; Takami and Horikoshi 2000). A variety of polysaccharide-degrading enzymes including cellulase, xylanase, xyloglucanase, mannanase, and pectate lyase from *B. agaradhaerens* have been studied for different applications (Outtrup et al. 1998; Busch et al. 1999; Kauppinen et al. 1999; Schulein et al. 1999; Xu 2000). In comparison, studies on *B. vedderi* and *B. cohnii* have been limited. Isolation of *B. vedderi* was reported from bauxite processing waste (Agnew et al. 1995) and that of *B. cohnii* from Wadi Natrum in Egypt and Mono Lake, California (Spanka and Fritze 1993). Work on *N. halobia* has also been restricted, although recently expression of both alkaline amylase and pullulanase by an isolate has been reported (Devi and Yogeeswaran 1999).

In contrast with the majority of the isolates, which grew well up to a salinity of at least 10% NaCl, strains related to *B. cohnii* showed weak or no growth at that salt concentration (see Table 3), in accordance with the earlier report by Spanka and Fritze (1993) suggesting a relatively low salt tolerance of this species. None of the isolates obtained appeared to be thermophilic in character. With the exception of LS-4C and AL-127, the isolates clustered with *B. agaradhaerens* and *B. vedderi* showed some flocculation when grown in liquid medium.

Despite significant growth of all the isolates within 24 h at 37°C, the overall amylase activity levels in liquid medium were quite low and did not seem to correlate with the activity detected on agar plates. Such an observation was reported earlier by Castro et al. (1993), who attributed this difference to different environmental conditions of liquid and solid media involving different transport phenomena for nutrients and metabolites. Alternatively, it could be the variation in the detection principle in the two media; in solid medium, substrate utilization is measured, whereas in the liquid medium, the product formed is measured. Hence, if the starch is partially degraded, the amount of reducing sugars formed has not reached the theoretical maximum. Both cell-bound and extracellular amylase activity was found among the organisms (see Table 4). The presence of intracellular, membrane-bound, and extracellular amylases in alkaliphilic *Bacilli* with varying pH optima has been reported earlier (Ikura and Horikoshi 1992).

The majority of the amylases known from mesophilic and extremophilic organisms are dependent on calcium ions for their activity and stability, which leads to increased process costs (Janecek and Baláz 1992). Hence, it has been of industrial interest to obtain amylases that do not have requirement for calcium ions, either by way of protein engineering or by screening for novel amylases from environmental samples. So far, only two alkaline amylases from *Bacillus* species have been reported not to show inhibition in the presence of EDTA (Horikoshi 1971; Boyer and Ingle 1972). Organisms living in the soda lakes are adapted to an environment deficient in calcium and magnesium ions, which precipitate as carbonates. However, EDTA had an inhibitory effect on amylase activity of a majority of the isolates, indicating their requirement for the metal ions. However, the activity of two organisms related to *B. vedderi* and *B. cohnii*, respectively, was stimulated by EDTA and correspondingly inhibited by Ca^{2+} ions. This feature would be interesting to investigate further at the molecular level.

The strains clustered with these two *Bacillus* species, *B. cohnii* and *B. vedderi*, were also seen to express pullulanase activity at pH 10, suggesting their ability to hydrolyze the α-1,6-linkage in starch. Alkaline debranching enzymes are very effective in removing starch-based stains, especially when used in combination with α-amylase in a detergent. Some alkaliphilic *Bacillus* strains producing highly alkaline pullulanase have been isolated earlier from other environments (Takami and Horikoshi 2000).

Yet another industrially significant amylolytic enzyme studied is CGTase, which converts starch into cyclodextrins, ring structures composed of six or more glucose molecules. Commercial production of cyclodextrins became feasible only with the use of CGTase from an alkaliphilic *Bacillus*, although its low thermostability presents a considerable disadvantage in the process (Starnes 1990). The isolates related with *B. agaradhaerens*, with the exception of AL-127, were CGTase producers; this *Bacillus* species is so far not known to express this enzyme. CGTase from LS-3C has been purified and characterized.

We believe that at least some of the alkaliphilic strains, especially those related to *B. vedderi* and *B. cohnii,* and their enzymes, which thus far are less studied, could have promising industrial potential. Our current work is concerned with structural and functional characterization of starch-hydrolyzing enzymes from some of the strains isolated during this study. Both the organisms and the enzymes are also being evaluated as catalysts in biotechnological applications involving both hydrolytic and synthetic reactions.

Acknowledgments. This work was supported by the Swedish International Development Cooperation Agency (Sida), Swedish National Board for Industrial and Technical Development (NUTEK), and by the Portuguese Fundação para a Ciência e Tecnologia (FCT) through grant BD/18134/98 of the program PRAXIS XXI. The authors are grateful to Dr. Amare Gessesse for providing us with the isolates and for his comments on the manuscript. Special thanks are also due to Dr. Osvaldo Delgado for his valuable help, and to Dr. Javier Breccia for his suggestions.

References

Agnew MD, Koval SF, Jarrell KF (1995) Isolation and characterization of novel alkaliphiles from bauxite-processing waste and description of *Bacillus vedderi* sp. nov., a new obligate alkaliphile. Syst Appl Microbiol 18:221–230

Aguilar A, Ingemansson T, Magnien E (1998) Extremophile microorganisms as cell factories: support from the European Union. Extremophiles 2:367–373

Bajpai P, Bajpai PK (1989) High-temperature alkaline α-amylase from *Bacillus licheniformis* TCRDC-B13. Biotechnol Bioeng 33:72–78

Boyer EW, Ingle MB (1972) Extracellular alkaline amylase from *Bacillus* species. J Bacteriol 110:992–1000

Busch A, Bettiol JP, Smets J, Boyer SL (1999) Laundry detergents and/or fabric care compositions comprising a modified cellulase. PCT Int Appl WO9957256, United States, November 11, 1999

Castro GR, Ferrero MA, Méndez BS, Sineriz F (1993) Screening and selection of bacteria with high amylolytic activity. Acta Biotechnol 13:197–201

Devi KPR, Yogeeswaran G (1999) Co-expression of saccharifying alkaline amylase and pullulanase in *Micrococcus halobius OR-1* isolated from tapioca cultivar soil. World J Microbiol Biotechnol 15:199–204

Duckworth AW, Grant WD, Jones BE, Steenbergen R (1996) Phylogenetic diversity of soda lake alkaliphiles. FEMS Microbiol Ecol 19:181–191

Gerhardt P, Murray RGE, Wood WA, Krieg NR (1994) Methods for general and molecular bacteriology. American Society for Microbiology, Washington, DC

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

Gessesse A, Gashe BA (1997b) Production of alkaline xylanase by an alkaliphilic *Bacillus* sp. isolated from an alkaline soda lake. J Appl Microbiol 83(4):402–406

Grant WD, Horikoshi K (1992) Alkaliphiles: ecology and biotechnological applications. In: Herbert RA, Sharp RJ (eds) Molecular biology & biotechnology of extremophiles. Blackie, New York, pp 143–162

Grant WD, Jones BE (2000) Alkaline environments. In: Lederberg J (ed) Encyclopedia of microbiology, 2nd edn, vol 1. Academic Press, New York, pp 126–133

Grant WD, Tindall BJ (1986) The alkaline saline environment. In: Herbert RA, Codd GA (eds) Microbes in extreme environments. Academic Press, London, pp 25–54

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

Gregersen T (1978) Rapid method for distinction of gram-negative from gram-positive bacteria. Eur J Appl Microbiol 5:123–127

Horikoshi K (1971) Production of alkaline enzymes by alkalophilic microorganisms. Part II. Alkaline amylase produced by *Bacillus* no. A-40-2. Agric Biol Chem 35:1783–1791

Horikoshi K (1996) Alkaliphiles–from an industrial point of view. FEMS Microbiol Rev 18:259–270

Ikura Y, Horikoshi K (1992) pH optima of intracellular, membrane-bound and extracellular amylases from alkaliphilic *Bacilli*. Biosci Biotechnol Biochem 56:2064–2065

Imhoff JF, Sahl HG, Soliman GSH, Trüper HG (1979) The Wadi Natrun chemical composition and microbial mass developments in alkaline brines of eutrophic desert lakes. Geomicrobiol J 1:219–234

Janecek S, Baláz S (1992) α-Amylases and approaches leading to their enhanced stability. FEBS Lett 304:1–3

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

Kauppinen MS, Schulein M, Schnorr K, Andersen LN, Bjornvad ME (1999) Bacterial mannanases and their gene sequence and industrial applications. PCT Int Appl WO9964619, Denmark, December 16, 1999

Kebede E, G-Marian Z, Ahlgren I (1994) The Ethiopian Rift Valley lakes: chemical characteristics of a salinity-alkalinity series. Hydrobiologia 288:1–12

Mäkelä MJ, Korpela TK, Puisto J, Laakso SV (1988) Nonchromatographic cyclodextrin assays: evaluation of sensitivity, specificity, and conversion mixture applications. J Agric Food Chem 36:83–88

Medda S, Chandra AK (1980) New strains of *Bacillus licheniformis* and *Bacillus coagulans* producing thermostable α-amylase active at alkaline pH. J Appl Bacteriol 48:47–58

Miller G L (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426–428

Mota RR, Márquez MC, Arahal DR, Mellado E, Ventosa A (1997) Polyphasic taxonomy of *Nesterenkonia halobia*. Int J Syst Bacteriol 47:1231–1235

Nielsen P, Fritze D, Priest FG (1995) Phylogenetic diversity of alkaliphilic *Bacillus* strains: proposal for nine new species. Microbiology 141:1745–1761

Onishi H, Kamekura M (1972) *Micrococcus halobius* sp. nov. Int J Syst Bacteriol 22:233–236

Outtrup H, Dambmann C, Olsen AA, Bisgard-Frantzen H, Schulein M, Jorgensen PL (1998) Gene sequence and cloning for production of xylanolytic enzyme from *Bacillus agaradherens*. U.S. Patent 343,000, June 23, 1998

Park CS, Park KH, Kim SH (1989) A rapid screening method for alkaline β-cyclodextrin glucanotransferase using phenolphthalein-methyl orange containing solid medium. Agric Biol Chem 53:1167–1169

Rådstrom P, Bäckman A, Qian A, Kragsbjerg P, Påhlson C, Olcén P (1994) Detection of bacterial DNA in cerebrospinal fluid by an assay for simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae*, and streptococci using a seminested PCR strategy. J Clin Microbiol 32:2738–2744

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

Schulein M, Outtrup H, Jorgensen PL, Bjornvad ME (1999) Alkaline xyloglucanases from *Bacillus* suitable for fabric detergents. PCT Int Appl WO9902663, Denmark, January 21, 1999

Sharp RJ, Munster MJ (1986) Biotechnology implications for microorganisms from extreme environments. In: Herbert RA, Codd GA (eds) Microbes in extreme environments. Academic Press, London, pp 215–295

Spanka R, Fritze D (1993) *Bacillus cohnii* sp. nov., a new, obligately alkaliphilic, oval spore-forming *Bacillus* species with ornithine and aspartic acid instead of diaminopimelic acid in the cell wall. Int J Syst Bacteriol 43:150–156

Stackebrandt E, Koch C, Gvozdiak O, Schumann P (1995) Taxonomic dissection of the genus *Micrococcus*: *Kocuria* gen. nov., *Nesterenkonia* gen. nov., *Kytococcus* gen. nov., *Dermacoccus* gen. nov., and *Micrococcus* Cohn 1872 gen. emend. Int J Syst Bacteriol 45:682–692

Starnes RL (1990) Industrial potential of cyclodextrin glycosyl transferases. Cereal Foods World 35:1094–1099

Takami H, Horikoshi K (2000) Analysis of the genome of an alkaliphilic *Bacillus* strain from an industrial point of view. Extremophiles 4:99–108

Wood RB, Talling JF (1988) Chemical and algal relationships in a salinity series of Ethiopian inland waters. Hydrobiologia 158:29–67

Worthington V (1993) Worthington enzyme manual: enzymes and related biochemicals. Worthington Biochemical, Freehold, NJ, USA

Xu F (2000) Methods for using pectate lyases in baking. PCT Int Appl WO0015038, United States, March 23, 2000