

Full Length Research Paper

Isolation and characterization of three thermophilic bacterial strains (lipase, cellulose and amylase producers) from hot springs in Saudi Arabia

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Three strains of thermophilic bacteria were isolated from two different hot springs in Saudi Arabia. These strains were designated M4 and M5 which were isolated from Al-Khoba hot spring and Sh3 which was isolated from Al-Arida hot spring. Cells are Gram positive-stain, strictly aerobic, grew optimally at pH 7.5 to 8.5 and temperature of 55 to 60°C, and tolerated maximally 10% (w/v) NaCl. The three isolates were lipase, cellulose, and amylase producers with variable degrees of enzymatic activity. They grew on various complex substrates such as yeast extract, carbohydrates and organic acids, which included starch, d-galactose, glutamate, fumarate and acetate. Microscopic observations showed that M4 had a long thick rod and spore former, M5 had a very small short rod, and Sh3 had rod and spore former. Phylogenetic analysis of 16 S rRNA sequence for the 3 strains (M4, M5 and Sh3) revealed that these strains have high sequence similarity with *Bacillus* sp., *Brevibacillus borstelensis* and *Deinococcus geothermals*, respectively.

Key words: Thermophilic bacteria, amylase, lipase, cellulase, hot springs, Saudi Arabia.

INTRODUCTION

Extremophiles are microbes that can live and reproduce in harsh environments. In the past 40 years, they have been found in hot springs, volcanic areas, deep sea, in Antarctic biotopes and in other particular geothermal sites, all places earlier believed to be too severe to support life. More recently, the search for novel species was fuelled by industries because it was realized that the ability of these microorganisms to survive in such extreme conditions was strictly related to special features, which mainly consists of novel enzymes and biochemical pathways (Herbert, 1992). In fact, it is generally true that the enzymes of an organism are adapted to function optimally at or near its growth conditions, and so the range of extremes at which life is found defines the range of conditions at which enzyme activity might be detected. In particular, it is believed that the outstanding stability of extremophilic enzymes will contribute to filling the gap between chemical and biological processes (Madigan and Marrs, 1997). To date, there are only two major examples of actual applications of these biocatalysts that have reached the

market and can be outlined: Taq polymerase, isolated from *Thermus aquaticus*, and cellulase 103 from alkaliphiles. Taq polymerase opened new frontiers in molecular biology by becoming the key element of the polymerase chain reaction and prompted reinforcement of the research activities on DNA-polymerases from hyperthermophiles. Cellulase 103 was isolated from the bacteria living in soda lakes, and breaks down the microscopic fuzz of cellulose fibers that traps dirt on the surface of cotton textile without harming the natural fabric. This biocatalyst was introduced in 1997 by Genencor International (Rochester, NY, USA) as a novel detergent agent that helps to keep cotton fabric looking 'as new' even after thousands of washing cycles (Pennisi, 1997). It is established that the use of thermophilic enzymes in polysaccharides processing could deliver major benefits related to the reduced risk of contamination, the lowering of the inlet stream viscosity and the possibility of keeping the same pH during the whole biotransformation, thus avoiding the salt addition that is necessary in modern processes (Di Leri et al.,

1998). In fact, several research groups have focused their attention on the search and characterization of this type of biocatalyst (Di Lerni et al., 1998; Duffner et al., 2000; Moracci et al., 2001). Enzymes that degrade polymers, such as amylases, pullulanases, xylanases, proteases and cellulases, could potentially have an important role, for example in food, chemical, pharmaceutical, paper, pulp and waste-treatment industries. Low molecular weight metabolites of industrial interest are cyclodextrins, compatible solutes and unusual lipids (Schiraldi et al., 2002; Hough and Danson, 1998; Horikoshi, 1999).

The aim of the present study was to isolate, characterize and identify a thermophilic bacterial strain that produces thermostable enzymes such as lipases, amylases and cellulases.

MATERIALS AND METHODS

Sampling and enrichment

Water samples were collected from two thermal hot springs located in Gazan area in Saudi Arabia, these are Al-Ain Alhara (or Al-Khawaba) located at a distance of about 50 km southeast of Gazan city. The second hot spring called Al-Aridah is at a distance of about 53 km northeast of Gazan city.

Samples were collected in 500 ml sterile thermal glass containers which keep the temperature of the water samples constant (Khalil et al., 1998). Five milliliter from each water sample was inoculated to 500 ml Erlenmeyer flask containing 250 ml TT media (ATCC medium 697). The inoculated flasks were incubated at 55°C with shaking at 300 rpm for 48 h. Ten milliliter of each cultured flask were centrifuged and bacterial pellet were spread on TT agar media, and then incubated at 55°C in a bench top incubator for 24 to 48 h. For the purpose of getting pure culture, distinctive colonies from TT agar, plates were picked up and transferred to a fresh TT agar plates, then incubated at 55°C in a bench top incubator for 24 and 48 h.

Isolates growth temperature

Isolated pure cultures were incubated at different temperatures, that is, 20 to 65°C with a 5°C difference and then 1°C difference, to find out the maximum temperature for their growth. The optimal growth temperature was taken for each isolated pure culture by comparing the total number of bacteria grown at different temperatures.

Detection of hydrolases secretion

Amylases

The ability of the thermophilic isolates to produce amylase was tested by culturing each isolate on a minimal medium composed of 1% (w/v) soluble starch (GCC, UK), 1% (v/v) Castenholz salts and 1.8% (w/v) agar (Idg, UK). The mixture of agar and salts was autoclaved separately at 121°C for 20 min. Starch was filter-sterilized and then added to the autoclaved agar-salts mixture to have a final concentration of 1% (w/v). The pH was adjusted to 7.0 with 1 M NaOH. Twenty-four hour old cultures growing on TT agar plates, which were incubated at 55°C, were used as culture sources to inoculate the Petri plates with the starch medium by placing a

loopful of bacteria of the different thermophilic isolates into the centre of the plates and then incubated for 72 h at 55°C. After the incubation period, plates were flooded with Lugol's iodine to detect the presence of clear halos, as opposed to the blue-black color typical of the reaction of Lugol's solution to starch, around those bacterial colonies capable of secreting amylase.

Cellulases

The ability of the thermophilic isolates to produce cellulose was tested by placing a loopful of bacteria of each isolate into the centre of a TT agar plate containing 1% (w/v) carboxymethyl cellulose (CMC) (Sigma, USA). The pH was adjusted to 7.0 with 3 M NaOH. Petri plates with this medium were inoculated from 16 h old cultures growing on TT agar plates which were incubated at 55°C by placing a loopful of each of the thermophilic isolates into the centre of every plate and then incubated for 72 h at 55°C. After the incubation period, plates were flooded with 0.1% (w/v) Congo red solution for 1 to 2 min followed by washing the plate with 1 M NaCl to detect the presence of clear halos around bacterial colonies that secrete cellulases according to the method of (Teather and Wood, 1982).

Lipases

The ability of the thermophilic isolates to produce lipases was tested, using the method described by Choo et al. (1998) with a slight modification, by culturing each isolate on a TT agar plate containing 1% (v/v) tributyrine and 0.01% (w/v) $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Merck). The pH was adjusted to 7.5 with 3 M NaOH. The medium was autoclaved at 121°C for 30 min. It was stirred after autoclaving for around 30 min and then heated in the microwave (Philips) for 20 min at 80°C; this was followed by stirring again for about 10 min. The purpose of these heating and stirring steps was to emulsify the medium. A loopful of each of the different thermophilic isolates was spotted into the centre of each plate and then incubated at 55°C. Plates were followed up for 182 h of incubation to check the formation of the clearing zones around the bacterial colonies that secrete lipases. A positive control *Escherichia coli* (BL21) was used (incubated at 37°C).

pH effect on growth

In order to test their alkalitolerance/alkaliphilicity, all the isolates were cultured into TT agar plates with pH 9.0 and 11.0 and compared with their growth at pH 7.0 and 6.0. Plates were incubated aerobically at 55°C for 20 h.

Testing for halotolerance

In order to detect the presence of halotolerant thermophiles, each isolate was cultured in 500 ml flask containing 200 ml of TT media with final NaCl concentration of 0.25, 1, 2.5, 5, 10, 12.5 and 15 (w/v) %, and incubated at 55°C for 24 h. Viable cell counts were determined by carrying out serial dilutions and plating them on TT agar plate for 24 to 48 h.

Profiling of the bacterial carbohydrates metabolism

Sugar utilization experiment was carried out in M9 Minimal Media (6 g/L Na_2HPO_4 , 3 g/L KH_2PO_4 , 1 g/L NH_4Cl , 0.5 g/mL NaCl and 3 mg/L CaCl_2) supplemented with trace elements and vitamins with the addition of 1% (w/v) of the sugars used (Table 3) as a sole carbon source.

Table 1. Primers used in the 16S rRNA PCR analysis.

Primer name	Primer sequence 5'-3'	Primer size	Tm (°C)	GC (%)	Sequence-amplified
8-27 F	5'-AGA GTT TGA TCC TGG CTC AG-3'	20	61.5	50.0	The whole gene
1492 R	5'-GGT TAC CTT GTT ACG ACT T-3'	19	53.7	42.1	The whole gene

Table 2. Detection of extracellular enzymes for the 3 isolates at 55°C.

Isolate	Analyse activity at 55°C	Cellulase activity at 55°C	Lipase activity at 55°C
M4	Very good	Very good	Good
M5	Excellent	Very good	Excellent
Sh3	Excellent	good	No activity

16s rDNA sequence determination and phylogenetic analysis

DNA extraction

Bacterial genomic DNA was extracted from the 3 isolates using NORGEN BIOTEK bacterial genomic DNA isolation kit.

PCR amplification of 16S rRNA genes

The amplification was conducted with universal primers (Thermo) designed to anneal the conserved regions of bacterial 16S rRNA genes. The properties of these primers are shown in Table 1.

The PCR reaction contain 10 µl 5x Phusion™ GC buffer (contains 7.5 mM MgCl₂, which provides 1.5 mM Mg Cl₂ in the final reaction conditions) (Finnzymes, Finland), 0.5 µl dimethylsulfoxide (DMSO) (aids in the denaturing of templates with high GC content) (Finnzymes, Finland), 5 µl of 2 mM of deoxyribonucleotides triphosphates mix, (BioLabs, UK) (provides 0.2 mM of each deoxyribonucleotide triphosphate in the final reaction conditions), 0.25 µl of each primer (provides 0.5 µM of each in the final reaction conditions), 0.5 µl of Phusion™ DNA polymerase (provides 0.02 U/µl) (Finnzymes, Finland), and 2 µl of DNA template with a final concentration ranging from 15 to 30 ng in a total volume of 50 µl. Amplification was performed with the thermocycler (Whatman Biometra, Germany). PCR was heated to 98°C for 4 min to achieve the initial DNA denaturation, followed by 30 cycles with the following cycling profile: 98°C for 30 s, for denaturation; 53°C for 30 s, for annealing; and 72°C for 1 min, for extension. A final extension was carried out after the amplification reaction at 72°C for 10 min.

Detection of the PCR products

PCR products, stained with 6x orange loading dye (fermentas), were electrophoresed through a 1.2%(w/v) agarose gel (Saveen Werner, Sweden), in 1X TAE buffer, stained with ethidium bromide (Mercury, USA). 10 µl of 1Kb DNA marker (O'gene ruler; Fermentas) was loaded to detect the 16S rRNA bands which were then visualized by UV transillumination. The amplified 16S rRNA bands were cut and stored at -20°C.

Purification of PCR products

The PCR products were purified using QIAEX® II Gel extraction Kit (150) (QIAGEN). This was followed by measuring the concentration

of the purified DNA using the NanoDrop (NanoDrop Technologies, INC. Wilmington, USA, NanoDrop® ND-1000; full-spectrum U.V/vis spectrophotometer). A concentration of 20 ng/100 base (300 ng of the 1.5 kb 16S DNA) is required for sequencing.

Sequencing

In order to be sequenced, the purified DNA samples were dried using the freeze-dryer (Labconco Lyph-Lock, model 7750, Ab Ninolab, and Stockholm, Sweden). Samples were labeled and kept in the refrigerator until they were sent for sequencing. Sequencing was carried out by MWG Biotech AG, Martinsried, Germany.

With the dideoxy method according to Sanger et al. (1981), sequencing was conducted in one direction using the forward primer (8-27 F) used in the PCR. Sequencing results were sent by e-mail after 4 working days.

RESULTS

Detection of extra cellular enzymes

The three isolates showed variable degrees of activities for amylase, cellulose and lipase at 55°C. Both strains M5 and Sh3 showed excellent amylase activity. Strain M4 showed very good amylase and cellulase activity. Regarding lipase activity, Sh3 showed no activity, while M5 expresses excellent activity and M4 showed good activity (Table 2).

The more the width of the clearing zone, the more activity reordered (excellent > very good > good). This indicates that the activity is reflected on the width of the clearing zone.

16 S rRNA sequence analysis

Phylogenetic analysis of 16 S rRNA sequence for the 3 strains (M4, M5 and Sh3) revealed that, these strains have high sequences similarity with *Bacillus* sp., *Brevibacillus borstelensis* and *Deinococcus geothermals*, respectively (Figure 2).

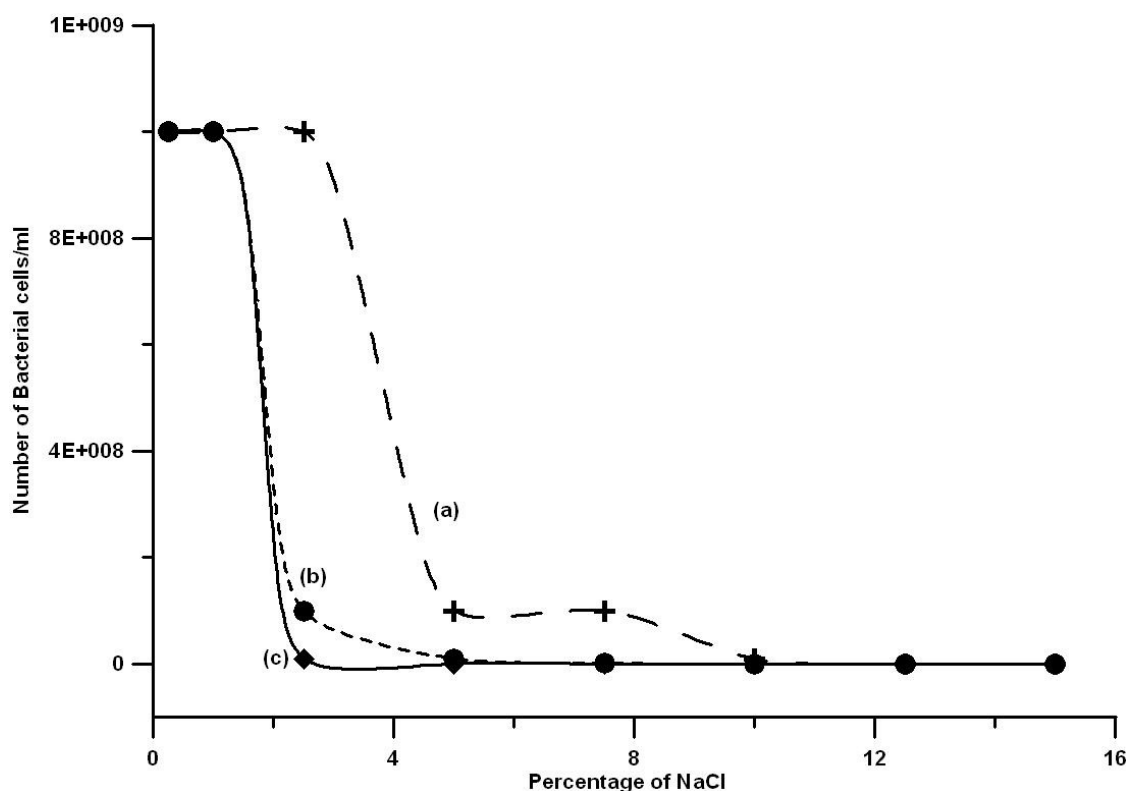


Figure 1. Growth rate of the three isolates on different concentrations of NaCl. (a) = M4, (b) = M5 and (c) = Sh3

Halophilic thermophiles detection

Halotolerance was tested by increasing the NaCl-concentration in the TT-medium. The three isolates grew well in the standard TT-medium (including 0.25% (w/v) NaCl). After 24 h of incubation at different concentrations of NaCl and 55°C, all the isolates were able to grow normally in the presence of 4 to 5% (w/v) NaCl, showing that they can all be halotolerant. When the concentration was increased further, most of the isolates showed very weak growth at 7.5 to 10% NaCl. At 12.5 and 15%, all three isolate showed no growth at all (Figure 1).

Sugar utilization

All isolates showed very good growth on wide range of sugars used as a sole carbon source when incubated at 55°C for 24 to 48 h (Table 3). Strain M4 was unable to grow on D-mannose, D-sorbitol and inositol, M5 were able to grow on all carbon sources tested (Table 3), except L-glutamine and D-arabitol, strain Sh3 which was unable to grow on L-rhamnose, L-arginine and D-arabitol but grew on the remaining sugars (Table 3).

DISCUSSION

Minimum, optimum and maximum growth temperature for

all isolates were determined. All three isolates showed very good to excellent growth between 50 and 65°C (optimum temperatures). All isolates showed their ability to grow at their minimum temperature which varies between 30 and 40°C.

The three isolates were either alkalitolerant (showed very good to excellent growth from pH.7.0 to 9.0, but no growth at pH.11) or alkaliphilic (showed very good and excellent growth between pH.7.0 and 11.0), while they show less cellular yield at pH.6.0 (Dugler et al., 2004).

The three isolates grew well in the standard TT-medium (including 0.25 % (w/v) NaCl). After 24 h of incubation at different concentrations of NaCl and 55°C, all the isolates were able to grow normally in the presence of 4 to 5% (w/v) NaCl, showing that they can all be halotolerant. When the concentration was increased further, most of the isolates showed very weak growth at 7.5 to 10% NaCl. At 12.5 and 15%, all three isolate showed no growth at all (Figure 1).

According to the definition of halotolerant microorganisms, they are identified as microorganisms that have no specific requirement for salt other than the usual NaCl needed by all (non-halotolerant) organisms but they are able to grow in up to 1.25 M NaCl (slightly halotolerant), up to 3.0 M NaCl (moderately halotolerant), or up to saturated (5.2 M) NaCl (extremely halotolerant) (Russell, 1989). It was previously reported that thermotolerant/thermophilic *Bacillus licheniformis* strains

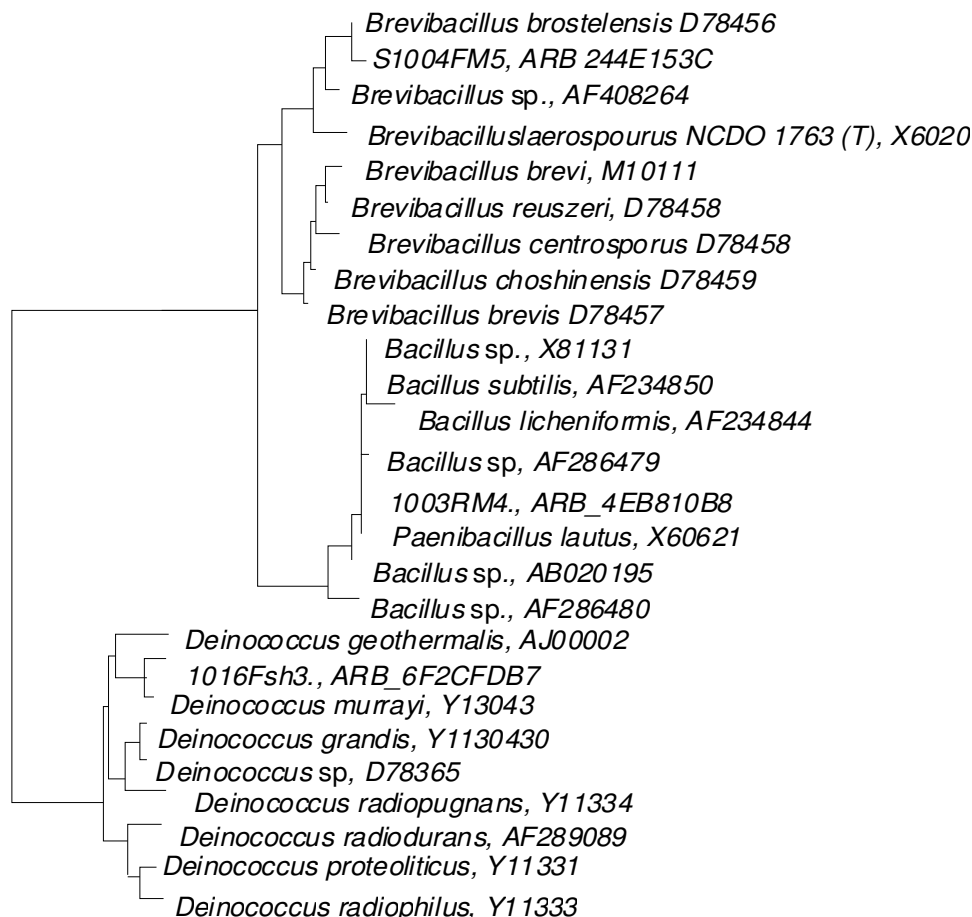


Figure 2. Phylogenetic analysis of the 3 isolated strains.

Table 3. Growth of the isolates on various complex substrates.

Utilized Sugar	M4	M5	Sh3
D-Cellobiose	+	+	+
Lactose	+	+	+
D-Galactose	+	+	+
D-Mannose	-	+	+
L-Rhamnose	+	+	-
D-Xylose	+	+	+
D-Manitol	+	+	+
D-Sorbitol	-	+	+
L-Arginine	+	+	-
L-Glutamine	+	-	+
Malate	+	+	+
Succinate	+	+	+
Glutamate	+	+	+
Fumarate	+	+	+
Acetate	+	+	+
Inositol	-	+	+
D-Arabitol	+	-	-

+: Sugar was utilized, -: sugar was not utilized.

could tolerate different NaCl concentrations; such as 7% (w/v) NaCl (Tharek et al., 2006), 10 and 13% (w/v) NaCl (Yakimov et al., 1995).

Detection of hydrolases secretion

Amylase

The isolates showed different amylase activity at 55°C (Table 1). Clearing zones were different in sizes which indicated enzymatic activities within these isolates (Rawana, 2007).

Cellulase activity detected in the isolates was very good for M4 and M5 and good for strain Sh3. For lipase activity, strain Sh3 showed no activity at all, while M5 showed excellent activity and M4 showed good activity. Previous studies showed that many *Bacillus* species express activity for thermostable cellulases (Dihlln et al., 1985). Although, CMC has often been used as an index of cellulase activity due to its high solubility, some investigations indicated that many organisms that cannot degrade cellulose can hydrolyze CMC via mixed β -glucan enzymes (Fields et al., 1998).

Lipase

The expansion of the clearing zone varied with the incubation time for the same isolates and among the different isolates as well. Within 180 h of incubation, the expansion of the clearing zones continued to increase with time but to certain limit where it showed stability for most of the isolates. This expansion of the clearing zones was due to the consistency of the homogeneity of the lipase medium. Therefore, it is not recommended to exceed that time of incubation. On the other hand, to get prominent clearing zones, it is better not to reduce the incubation time to less than 140 h.

Profiling of the bacterial carbohydrates metabolism

All isolates showed very good growth on all sugars used as a sole carbon source when incubated at 55°C for 24 to 48 h. Presence of different species which were able to degrade different carbohydrates between the isolates could be explained by trend of microbial societies toward surviving at low organic content in such niches and development of adaptable systems for uptake of any available food (Deekova et al., 2008). Investigation on their carbohydrate degrading activity demonstrated presence of biotechnologically valuable enzyme producers.

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