ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 5 (3): 252-256, 2006
ISSN 1682-296X

Production of Antimicrobial Agents from Thermophilic *Yersinia* sp.1 and *Aeromonas hydrophila* Isolated from Hot Spring in Jordan Valley

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Abstract: Two Thermophilic bacteria which exhibit antimicrobial activity were isolated from hot spring Jordan Valley. Those Gram-negative rods shaped were found to be *Yersinia* sp.1 and *Aeromonas hydrophila*. Extracts from aqueous phases of the tow strains were tested for antimicrobial activity by agar well diffusion method against 6 microorganisms (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia*, *Enterobacter* sp. and *Candida albicans*). The highest antimicrobial activity was recorded by *Yersinia* sp.1 giving (16 mm) inhibition zone against *Pseudomonas aeruginosa*, where *Aeromonas hydrophila* produce no activity against the test organisms. Extracts from the two thermophilic strains showed relatively high antibacterial activity against *Staphylococcus aureus*. Partial purification of antimicrobial agents by Thin Layer Chromatography (TLC) gave two inhibitory bands with Rf values of 0.22 for extract from *Yersinia* sp.1 and 0.25 for extract from *Aeromonas hydrophila*.

Key words: Thermophilic bacteria , *Yersinia* sp.1, *Aeromonas hydrophila*, antimicrobial agents

INTRODUCTION

Researchers are investigating microorganisms that live in hot springs as potential sources of valuable biochemicals, screening marine microorganisms known first for their environmental potential showed some interesting biological molecules including unusual enzymes, antibiotics, anti-algal compounds, anti-cancer substances and secreted sugars (Landenstein and Antranikian, 1998).The practical applications of microorganisms from extreme environments vary from the production of low-volume, high-value products such as thermostable DNA polymerase for DNA amplification in the Polymerase Chain Reaction (PCR), to large-scale industrial processes in competition with more tradition technologies (Costa *et al*., 1989).

Resistance of several pathogenic bacteria to antimicrobial agents is an emerging problem that has required medical sector to alter empirical therapy for diseases and has prompted laboratory researchers to rethink testing strategies. Twenty years ago, bacteria that were resistant to antimicrobial agents were easy to detect in the laboratory because the concentration of drug required to inhibit their growth was usually quite high and distinctly different from that of susceptible strains. Newer mechanisms of resistance, however, often result in much more subtle shifts in bacterial population distributions, for example one of the most difficult phenotypes to detect, is decreased susceptibility to β-lactams . There is certainly an urgent need for new antimicrobial agents, given the increase in drug resistance in many common bacterial pathogens and changes in the spectrum of pathogens, together with the emergence of new diseases (Daveis and Webb, 1998).

As researchers have acknowledged that extreme environments were capable of sustaining biological life, increasing numbers of these novel microorganisms had been isolated since mid 1970s (Horikoshi and Grant, 1998) lactacin F has been purified and characterized, a bacteriocin produced by *Lactobacillus acidophilus* 11088 (NCK88) also has been purified. Mersacidin , a peptide antibiotic produced by a species of *Bacillus* was active against gram-positive organisms including methicillin-resistant *Staphylococcus aureus*, but had no activity against gram-negative bacteria or fungi (Chatterjee *et al*., 1992). Thermoleovorin-S2 and thermoleovorin-N9 produced by *Bacillus thermoleovorans* S-II and *B. thermoleovorans* NR-9, respectively, were effective against all but the producing strain of *B. thermoleovorans*.

Agar-diffusion tests with *S. thermophilus* strains as targets identified 13 out of 41 strains as producers of

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antibacterial activity. Thermophilin A, the bacteriocin-like substance present in the culture supernatant of *S. thermophilus* ST134 was purified to homogeneity by ammonium sulfate precipitation and ion-exchange chromatography, followed by ultrafiltration. Forty-seven isolates of thermophilic bacteria isolated from water and soil samples collected from Sankhamphang Hot Spring were capable to inhibit the growth of test organisms. *B. subtilis* was the most susceptible followed by *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Partial purification of the antibiotic by thin layer chromatography gave two inhibitory bands with Rf values of 0.25 and 0.48 (Naphrae, 1999). Paper disc diffusion method was used to confirm the result of the 35 isolates that were found to have inhibitory activity against four organisms i.e., *Bacillus cereus*, *Staphylococcus aureus*, *Salmonella enteritidis* and *Vibrio parahaemolyticus* (Lapmak, 1999).

The main objective of this study was to test these thermophilic bacterial isolates for the ability to produce antimicrobial agents, testing the effect of those antimicrobial agents on pathogenic microorganisms and partial purification of antimicrobial agents produced from such bacterial strains.

MATERIALS AND METHODS

Sampling sites processing: Water samples were collected from Zara hot spring in the Jordan Rift Valley near the Dead Sea. To reduce temperature loss of collected samples 500 mL sterile thermal glass containers were used to transfer water samples from the thermal springs which keep the temperature of the water constant. Samples were inoculated to thermus agar (ATCC medium 697) TT media (for 1 L, 2 g NaCl, 4 g Yeast extract, 8 g peptone and 18 g agar) in duplicates and incubated at 42 and 55°C for 24 h. Samples were also inoculated to thermus liquid TT media. Purified colonies were continually maintained on the same medium either on plates or slants. For growth experiments, strains were inoculated in 250 mL flasks containing 50 mL of TT broth and incubated at 55°C in a shaking incubator (GFL 3033, Germany) at 200 (rpm).

Determination of the optimal growth temperature: Each of the pure culture was incubated (Memmert SV1422, Germany) at different temperatures, i.e., 25 to 65°C with a 5°C difference and then 1°C, to find out the maximum and minimum temperature for their growth. The optimal growth temperatures were taken for each pure culture by comparing the number of bacterial colonies grown at different temperatures (Khalil *et al*., 1998).

Extraction of bacterial culture supernatant: Thermophilic bacterial isolates exhibiting antimicrobial activity were grown on 50 mL TT broth using 250 mL flasks overnight at their optimum growth temperatures, until they reached stationary phase ($OD_{600\ nm}$ = 1.5-2.00). Cells were pelleted by centrifugation at 5000 rpm. (Biofuge 37520, Kendro, Germany).

Bacterial supernatant (50 mL) was extracted using 100 mL (50×2) petroleum ether, followed by 100 mL (50×2) chloroform and finally with 100 mL (50×2) ethyl acetate using aspirating funnel. Anhydrous sodium sulfate was used to remove any traces of water from the organic extracts. TT liquid media was extracted similarly to be used as control.

Test for antimicrobial activity

In the organic extracts: Antimicrobial activity of bacterial supernatant extracted by organic solvents was tested by paper disc assay. TT agar plates were inoculated with *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Candida albicans* and *Enterobacter* sp. using a cotton swap. After paper discs were sterilized by UV light (UVi CN15, Cole Palmar, USA) for 30 min, they were dipped in the supernatant organic extracts and then transferred to TT plates inoculated with the test organisms. The plates were incubated at 35°C for 24 h. Paper discs socked in 40 μL of the organic solvents only were transferred to TT plates inoculated with the test organism and used as controls. Controls were made of filter papers without any solvent; to make sure that the composition of the filter paper did not interfered with the inhibition of the test organisms.

In the aqueous phase: The aqueous phases were tested for their antimicrobial activities by agar well diffusion test (ADT). One hundred microliters of each aqueous residue were dispensed in 7 mm pre-cut wells on TT plates inoculated with each test strain. Plates were incubated at 35°C overnight and the activity of each extract was estimated by measuring the inhibition zones in (mm). Inhibition zone 2 mm or more were considered as positive result (Aktypis *et al*., 1998). Controls (TT liquid media) were applied for each of the tested organisms.

Thin layer chromatography: Silica gel plates (Merck60, F254, 0.1 mm thick, 20×10 cm) were used to carry out Thin Layer Chromatography (TLC). Forty microliter of the aqueous residues of each extract was spotted on silica gel plate. Spotting the same amount of the extracted TT liquid media was used as control. The mobile phase used was composed of 20% methanol: 80% chloroform. The

aqueous residues of each extract were detected by UV light (Kalil, 2002) and Rf values were calculated as the ratio between the distance traveled by inhibitory bands (observed under UV light) and the distance traveled by the solvent (mobile phase).

RESULTS

Determination of morphological and biochemical of bacterial isolates: The two isolates were found to be strictly aerobic further supported by the presences of carbohydrate fermentation, citrate utilization but the absence of catalase and urease activity. Formation of indole react with *p*-diethyl amino benzylaldehyde to give red complex was observed in one of the isolates but not in the other (Table 1).

Both isolates were gram-negative rods and Sporulation was clearly detected under light microscope for all rod shaped isolates. The elongated structure observed was a common feature in all rod isolates. (Fig. 1and 2).

Based on the battery of additional biochemical and morphological tests (Table 1) and other identification

Table 1: Identification of bacterial isolates using biochemical and morphological characteristics

	Bacterial isolate	
Characteristics	*Aeromonas hydrophila*	*Yersinia* sp.1
Biochemical		
CHO fermentation	+	+
Oxidase	-	-
Triple sugar iron(TSI)	R/Y	R/Y
Urease	-	-
Catalase activity	-	-
Indole	+	-
Methyl red	+	-
Vogas-Proskauer	+	+
Citrate utilization	+	+
Gram stain	-	-
Morphological		
Cell morphology	Rod shaped	Rod shaped
Spore formation	Former	Former
Colony color	White	Light red

(+) For positive reaction, (-) for negative reaction, (R/Y) Red slant/Yellow butte, (R/R) Red slant and butte, (Y/Y) Yellow slant and butte

Table 2: Antimicrobial activity of extracts from aqueous phase of *Yersinia* sp.1 and *Aeromonas hydrophila*

	Inhibition zone* (mm) of antimicrobial activity exhibited by:	
Test organism	*Yersinia* sp.1	*Aeromonas hydrophila*
Staphylococcus aureus	13	16
Escherichia coli	10	12
Pseudomonas aeruginosa	16	(-)**
Klebsiella pneumonia	10	9
Enterobacter sp. 1	12	12
Candida albicans	13	12

* Well diameter used is (7) mm.**(-) No activity is recorded against the tested organism

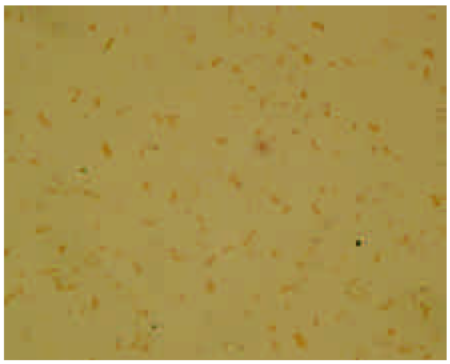

Fig. 1: Morphological structure of *Aeromonas hydrophila*, as appear under the light microscope, with magnification power of 10×100 (Nikon, DXM1200, Japan)

Fig. 2: Morphological structure *Yersinia* sp.1, as appear under the light microscope, with magnification power of 10×100 (Nikon, DXM1200, Japan)

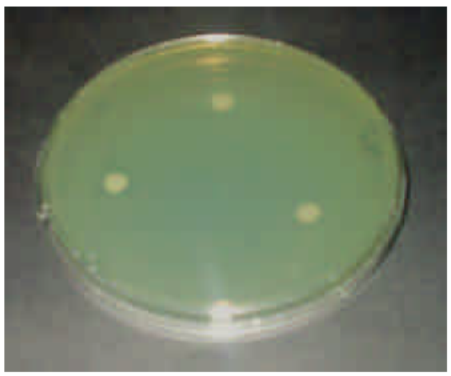

Fig. 3: Antimicrobial activity in the organic extracts of *Aeromonas hydrophila*; chloroform extract (1), ethyl acetate extract (2) and petroleum ether extract (3) against the test organism *Pseudomonas aeruginosa* (Nikon, CoolPix 995, Japan)

Fig. 4: Inhibition zone by *Aeromonas hydrophila* against *Staphylococcus aureus*, (3) well No (3). Wells 1 and 2 is represent TT media control and empty well respectively (Nikon, CoolPix 995, Japan)

kits (API NE 20 and Rap ONE ID) the isolates were further more identified for *Aeromonas hydrophila* and *Yersinia* sp.1

Growth temperatures: Minimum growth temperature for *Aeromonas hydrophila* and *Yersinia* sp.1 reached 26°C and 28°C, respectively, with an optimum temperature exceeding 46°C. All bacterial isolates were thermotolerent since they grow at temperatures above 45°C and were adapted to water temperature from which they were isolated.

Testing the antimicrobial activity following extraction process

Antimicrobial activities in the organic phases: Organic extracts resulted from the extraction process of bacterial broth, showed no antimicrobial activity against any tested organism. This indicates that organic phases have no active agents against any of the tested organisms (Fig. 3).

Antimicrobial activities in the aqueous phase: Extracts from aqueous phases showed antimicrobial activity were tested against at least 5 of the 6 test microorganisms. Results were recorded by measuring the inhibition zone in mm, where only 2 mm or more of inhibition zone (Aktypis *et al*., 1998) were considered as positive result. Controls (TT liquid media) were applied for each of the tested organisms (Fig. 4).

All tested organisms showed various degrees of sensitivity to extracts from the aqueous phase (Table 2).

For *Yersinia* sp.1 extract the highest inhibitory effect was against *Pseudomonas aeruginosa* (16 mm inhibition zone). Extracts from *Aeromonas hydrophila* showed 16 mm zone of inhibition against *Staphylococcus aureus* (Fig. 4), while was unable to inhibit *Pseudomonas aeruginosa*.

Partial purification of antimicrobial agents gave inhibitory bands with Rf values of 0.22 and 0.25 for *Aeromonas hydrophila* and *Yersinia* sp.1, respectively.

DISCUSSION

The maximum growth temperatures were closely related to those of thermophilic bacteria obtained by Khalil *et al*. (1998) and were less than those of various *Thermus* species of Beffa *et al*. (1996). Minimum growth temperatures were found to be less than *Thermus thermophilus* and *Thermothrix azorensis*, but closely related to thermophilic isolates of Khalil *et al*. (1998).

Both isolates were found to be carbohydrate fermenters without the production of gas, able to utilize citrate and have a negative catalase activity, these results are in conflict with results of *Proteobacteria* isolated from Alcafache in central Portugal were oxidase and catalase positive (Rainey *et al*., 2003). *Aeromonas hydrophila* was indole and methyl red positive. Most isolates were able to ferment glucose. The slants remain red (alkaline) because of limited glucose (0.1%) in the medium and limited acid, which is oxidized rapidly and does not persist. Because of depletion of glucose bacteria decompose peptone producing NH_3 and NH_4OH.

The absence of antimicrobial activity in the organic phases (after the extraction) suggested that these molecules (agents) are water-soluble molecules. Extracts from *Aeromonas hydrophila* and *Chryseomonas luteola* has no effect on *Pseudomonas aeruginosa,* this result agrees with the results presented by Martirani *et al*., (2002). Extracts from both bacterial isolates showed relatively high antibacterial activity against *Staphylococcus aureus*.

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