Screening of Thermophiles From Municipal Solid Waste and Their Selective Antimicrobial Profile

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Abstract: The main object of this study was to screen thermophilic bacterial strains with selective antimicrobial potential from garbage dump site. Six thermophilic bacterial strains were isolated from municipal solid waste by enrichment technique. All these strains were screened for their antimicrobial activity by using agar well diffusion assay against Gram-negative and Gram-positive bacteria. The methanol extracts of Cell Free Supernatant (CFS) of four bacterial strains Brevibacillus borstelensis, Bacillus galactosidilyticus and Bacillus licheniformis RH101 and RH104 were found to inhibit the growth of test organisms. The strain Brevibacillus borstelensis showed maximum inhibition against Micrococcus flavus, Dietzia sp. K44 and Staphylococcus aureus while Bacillus licheniformis strains and Bacillus galactosidilyticus inhibited the growth of at least one gram-positive test organism. The methanol extract of Brevibacillus borstelensis showed very selective antimicrobial activity against Gram-positive pathogens.

Key words: Antimicrobial activity, 16S rRNA gene sequencing, fractions, pathogens, cell free supernatants

INTRODUCTION

New emerging pathogens with increasing antibiotic resistance, along with the susceptibility of immunocompromised to common diseases has become an alarming problem world wide. In the current scenario, microorganisms are still a dominant source for antimicrobial compounds. But in spite of technology advancement, the existing microbes fail to deliver new effective antimicrobial compounds. Therefore, it is urgently needed to identify new microbial sources for effective antagonistic compounds (Horikoshi, 1995). In recent years, identifying new ecological niches like thermophiles, insects, endosymbionts are providing access to new organisms and novel bioactive chemicals (Gerard and Arlene, 2007). Thermophiles have been recognized as a valuable source of new antimicrobial molecules. However, these thermophiles are not exploited for the production of the same. It is well known that thermophiles have a very rich diversity but the traditional culture dependent methods are insufficient to isolate these microorganisms (Allan et al., 2005). Therefore it is necessary to explore thermophiles using modern molecular approach and understand the limitation factors involved in poor cultivability of these groups (Phoebe et al., 2001).

In order to explore the microbial sources from environment, we selected the municipal solid waste for isolation of thermophilic microorganisms. Solid waste contains a number of natural and synthetic materials like food and plant residues, human waste, waste paper, wet biomass and oils with some
toxic materials and is being continuously exposed to sun and natural climate throughout the year. The initial phase of the degradation process is characterized by activity and growth of mesophilic microbes, which in turn leads to a rapid increase in temperature. At the next stage, thermophilic microbes become responsible for the degradation process and both the growth and activity of non-thermotolerant microbes are inhibited (Finstein and Morris, 1975). The objective of the present study was therefore to isolate and characterize thermophilic bacterial strains from municipal solid waste and screen for their antimicrobial activity.

MATERIALS AND METHODS

Isolation and Identification of Bacteria

Samples were collected from different sites of municipal solid waste Delhi, India in 2006. All the collected samples were mixed thoroughly and divided into four parts. Each part was suspended in sterilized water and serially diluted with distilled water for isolation of thermophilic bacterial strains. Aliquots of each diluted sample were spread in nutrient agar plate and incubated at 60°C for 48 h. Individual colonies were picked and purified by streaking on nutrient agar plate.

Each strain was grown in 200 ml of nutrient broth at pH 8.0 for 24 h and was harvested at 8000 g for 25 min. Supernatant of the cultures were also screened for antimicrobial activity. Cultures were maintained in nutrient agar slants and preserved in nutrient broth containing 15% (v/v) glycerol at -70°C.

Microbiological properties of the isolated strains were determined according to the methods described in Bergey’s Manual of Systematic Bacteriology (Sneath et al., 1986). The identification of isolates was performed by 16S rRNA gene sequence analysis.

16S rRNA Gene Sequencing

Total DNA preparation was carried out from the strains according to Takagi et al. (1993). Amplification of 16S rRNA gene was performed as described by Weisburg et al. (1991) using universal primers fD1 (5’ AGTTTGATCCTGGCTCA 3’) and rP2 (5’ ACGGCTACCTTGTTACGACTT 3’). In a 100 µL reaction volume, 20 pmol of each primer was used for 20 ng genomic DNA template. Amplification was performed on an automated thermocycler (MJ Research, USA) using 1 U Taq polymerase (NEB) and the recommended buffer system. Amplification profile is as follows: 95°C for 5 min followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min and a final extension of 7 min. Amplified DNA was purified using QIAGEN PCR purification kit (QIAGEN, Germany) and was sequenced at TCGA (New Delhi, India). Similarity searches of the sequences obtained were performed using BLAST algorithm of NCBI (Altschul et al., 1990).

Test Organisms

To screen for antimicrobial activity Escherichia coli NCIM 2739, Pseudomonas aeruginosa NCIM 2053, Bacillus subtilis NCIM 2063, Micrococcus flavus NCIM 2378, Staphylococcus aureus NCIM 2794, Dietzia sp. K44 MTCC 7402 were used.

Extraction of Active Compounds

Cell Free Supernatants (CFS) of all the strains which were grown at 60°C for 24 h were lyophilized and extracted separately with petroleum ether, ethyl acetate, chloroform and methanol. Seventy milliliter of each organic solvent were used in extraction of lyophilized powder obtained from 200 ml CFS. Extracts were evaporated to dryness using rotavapor at 40°C (BUCHI Labortechnik AG, Switzerland) and dissolved in suitable solvents to screen for antimicrobial activities. All solvents were purchased from SRL, India.
Antimicrobial Assay

Antimicrobial activity of CFS and the extracts were carried out by well agar assay as described by Gillespie et al. (2002). Cell free supernatants and solvent extracts were applied as 100 µL drops in 6 mm diameter wells of petri plates containing lawn of test organisms and incubated at 30°C. Inhibition zones were measured after 48 h in case of Dietzia sp. K44 and after 24 h with other organisms.

The methanol extract of Br. borstelensis was further studied for comparative analysis with commonly available antibiotics by disc method. Antibiotics discs were used in this study were Vancomycin 30 mcg, Tetracycline 25 mcg, Ampicillin 10 mcg, Streptomycin 10 mcg, Penicillin G 10 units, chloramphenicol C 30 mcg, and Erythromycin 15 mcg. Br. borstelensis disc contained crude methanol extracts of the concentration equivalent to 280 mcg. All antibiotic discs were purchased from Hi Media Laboratories India.

All the assays were performed in duplicates and the antimicrobial activity was expressed as mean of the diameter of zones of inhibition (mm) produced by extracts. Inhibition was reported as diameter of the clear zone around the well in mm and was scored using ProtoCOL System, Synoptics Limited, United Kingdom.

RESULTS AND DISCUSSION

Isolation and Characterization of Bacteria

All isolated bacterial strains were able to grow on nutrient agar media at 60°C. These bacterial strains were identified as Bacillus licheniformis RH101, Brevibacillus borstelensis RH102, Bacillus galactosidlyticus RH103, Bacillus licheniformis RH104, Bacillus sp. RH105, and Bacillus amyloliquefaciens RH106 by biochemical characterization. The strain Bacillus licheniformis RH101, Brevibacillus borstelensis RH102 and Bacillus licheniformis RH104 were further identified by molecular 16S rRNA gene sequencing analysis (Table 1).

Nucleotide Sequence Accession Number

GenBank accession numbers DQ144419, DQ144420 and DQ144421 were assigned to Bacillus licheniformis RH101, Brevibacillus borstelensis RH102 and Bacillus licheniformis RH104, respectively.

Antimicrobial Profile

All six strains were screened for their antimicrobial activity against E. coli, P. aeruginosa, M. flavus, B. subtilis, S. aureus and Dietzia sp. K44. Cell free supernatant, the petroleum ether and ethyl acetate fractions of CFS did not show any antimicrobial activity (data not shown). However, methanol extracts were found to have good activity against gram positive bacteria M. flavus, S. aureus, B. subtilis and Dietzia sp. K44 (Table 2). As the methanol extracts were found to be mostly soluble in water, antimicrobial activity was ascertained with aqueous solution. Antimicrobial activity of the methanol extract and its solubility in aqueous solvents suggest the polar nature of the active compound(s).

<table>
<thead>
<tr>
<th>Strains</th>
<th>Species</th>
<th>Optimum growth temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus licheniformis</td>
<td>RH101</td>
<td>60</td>
</tr>
<tr>
<td>Br. borstelensis</td>
<td>RH102</td>
<td>60</td>
</tr>
<tr>
<td>Bacillus galactosidlyticus</td>
<td>RH103</td>
<td>60</td>
</tr>
<tr>
<td>Bacillus licheniformis</td>
<td>RH104</td>
<td>60</td>
</tr>
<tr>
<td>Bacillus sp.</td>
<td>RH105</td>
<td>60</td>
</tr>
<tr>
<td>Bacillus amyloliquefaciens</td>
<td>RH106</td>
<td>60</td>
</tr>
</tbody>
</table>
Table 2: Antimicrobial profile of isolated thermophilic strains on test organisms

<table>
<thead>
<tr>
<th>Test organism/ Isolated strains</th>
<th>Inhibition zone diameter against test organism (mm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M. flavus</td>
</tr>
<tr>
<td>B. licheniformis RH 101</td>
<td>24</td>
</tr>
<tr>
<td>Br. borstelenisis RH 102</td>
<td>28</td>
</tr>
<tr>
<td>B. galactosidilytica RH 103</td>
<td>20</td>
</tr>
<tr>
<td>B. licheniformis RH 104</td>
<td>14</td>
</tr>
<tr>
<td>Bacillus sp. RH 105</td>
<td>NI</td>
</tr>
<tr>
<td>B. amylobacter RH 106</td>
<td>NI</td>
</tr>
</tbody>
</table>

*Mean of inhibition zone diameters including cup diameter, NI-No Inhibition

Table 3: Comparative study of commonly used antibiotics and methanol extract of thermophilic bacteria against test organisms

<table>
<thead>
<tr>
<th>Test organisms</th>
<th>P</th>
<th>V</th>
<th>A</th>
<th>T</th>
<th>S</th>
<th>C</th>
<th>ME</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>R</td>
<td>R</td>
<td>17</td>
<td>22</td>
<td>13</td>
<td>24</td>
<td>ND</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>R</td>
<td>R</td>
<td>13</td>
<td>16</td>
<td>24</td>
<td>28</td>
<td>ND</td>
</tr>
<tr>
<td>M. flavus</td>
<td>R</td>
<td>19</td>
<td>ND</td>
<td>24</td>
<td>18</td>
<td>26</td>
<td>28</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>R</td>
<td>16</td>
<td>8</td>
<td>22</td>
<td>12</td>
<td>19</td>
<td>18</td>
</tr>
<tr>
<td>S. aureus</td>
<td>R</td>
<td>19</td>
<td>26</td>
<td>22</td>
<td>25</td>
<td>28</td>
<td>24</td>
</tr>
<tr>
<td>Dietzia sp. K44</td>
<td>30</td>
<td>22</td>
<td>32</td>
<td>22</td>
<td>28</td>
<td>24</td>
<td>22</td>
</tr>
</tbody>
</table>

ME: Methanol Extract of Br. borstelenisis, R: Resistant, ND: Not Detectable, P: Penicillin, V: Vancomycin, A: Ampicillin, T: Tetracycline, S: Streptomycin, C: Chloramphenicol

The methanol extracts of Br. borstelenisis was compared with the commonly used antibiotics against all test organisms. It was found that all bacterial strains except Dietzia sp. K44 were resistant to at least one of the antibiotics. Dietzia sp. K44 strain used in this study was isolated in the lab from natural sources. This strain was never exposed to antibiotics. This could be the reason for its sensitivity against all antibiotics. The inhibition zone of crude methanol extract of Br. borstelenisis (28 mm) is quite comparable to routinely used antibiotics (4-32 mm) (Table 3). It appears that the thermophilic Br. borstelenisis strain has good promising antimicrobial activity against new emerging microbes.

Interestingly, the four strains which showed promising antimicrobial activity were specific against gram positive bacteria. This is also in concurrence to other reports suggesting better antagonistic activity of Bacillus sp. against gram positive bacteria (Yilmaz et al., 2006).

In the search of new antimicrobial compounds, it is necessary to follow new strategies for isolation of bacteria with unique characteristics from novel sources (Chopra et al., 1997). The bacterial community inside the municipal waste is capable of surviving under harsh conditions. Production of bioactive compounds seems to be the adaptation technique developed by these microbes to survive in such environment. The limited spectrum antimicrobial activity of these thermophiles further illustrates this survival strategy.

The antimicrobial resistance in gram-positive bacteria are becoming more prevalent and are responsible for number of infections. Antagonism displayed by thermophilic RH 102 against M. flavus, S. aureus and Dietzia sp. K44 can have potential applications with regard to its selective nature against gram positive pathogens (Davis and Webb, 1998). Dietzia sp. K44 bears close similarity to Dietzia maris, an actinomycetes that has been twice reported as nosocomial pathogen (Pidoux et al., 2001) and as an aetiological agent of reticulated papillomatosis (Natarajan et al., 2005).

Research on narrow spectrum antibiotics would be more advantageous as they are effective against only a narrow class of pathogens. This approach becomes more rational with rapid advances in real time diagnosis, which would pave away for faster diagnosis of diseases in the near future. Availability of narrow spectrum antibiotics would prevent misuse of broad-spectrum antibiotics, thereby slowing the emergence of clinically significant resistant pathogens (Walsh, 2003).
The bacillus species are widely recognised as a rich source of antimicrobial compounds with a number of antimicrobial compounds being reported from them (Nagai et al., 2003). But thermophilic bacillus strains are yet to be exploited properly for novel and stable antimicrobial compounds (Gebhardt et al., 2002).

In this study, four thermophilic bacterial strains showing antimicrobial activity against gram positive organisms were isolated. On the basis of their activities, Brevibacillus borstelensis RH 102 seems to be promising. This is the first study to report the production of antimicrobial compounds from Brevibacillus borstelensis which was isolated from municipal waste. This study demonstrates that municipal waste might be a good source for isolating thermophilic microorganisms with novel characteristics.

The antimicrobial activity from thermotolerant Brevibacillus species might be considered for further investigations for the production of useful antimicrobial compounds.

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REFERENCES


