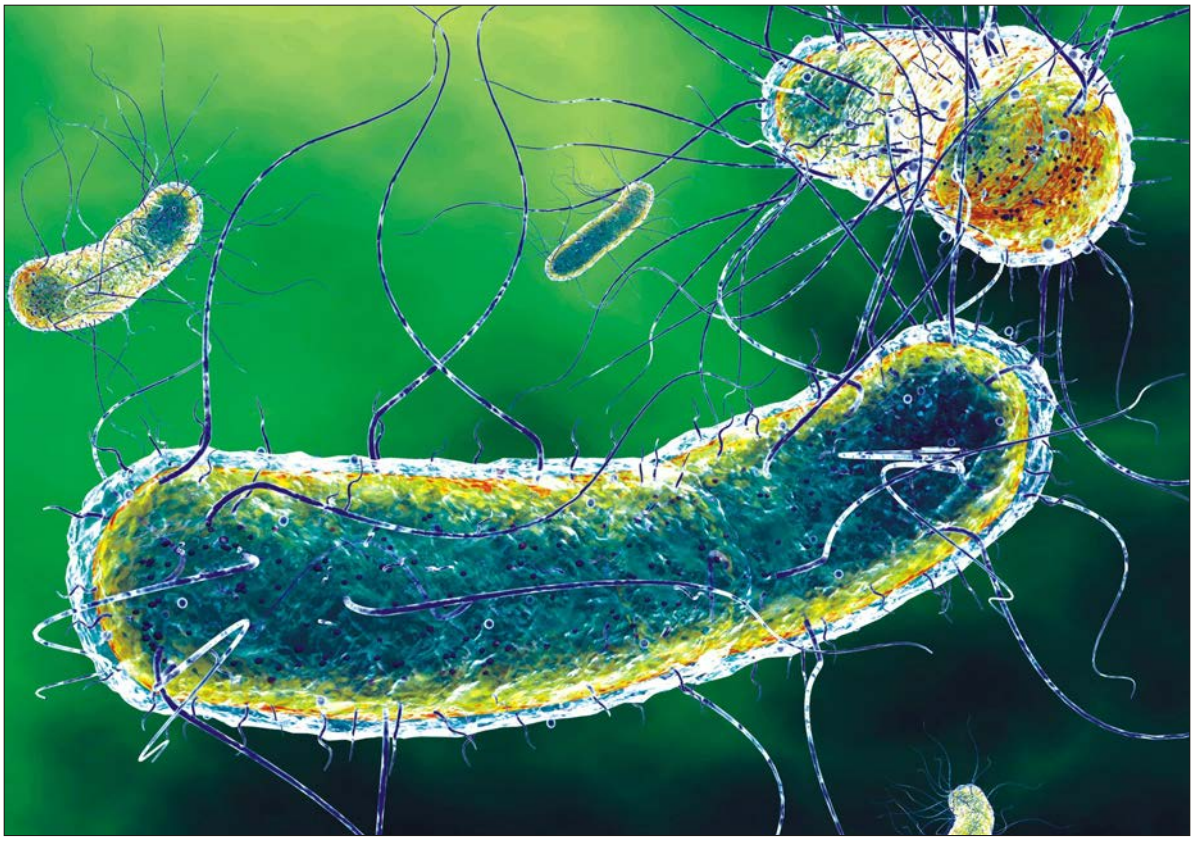

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Screening of Thermophiles From Municipal Solid Waste and Their Selective Antimicrobial Profile

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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, micro organisms 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

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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 (BÜCHI 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 well 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 galactosidilyticus* 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. aeroginosa*, *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 1: Thermophilic microorganisms isolated from municipal solid waste

Strains	Species	Optimum growth temperature (°C)
Bacillus licheniformis	RH 101	60
Br. borstelensis	RH 102	60
Bacillus galactosidilyticus	RH 103	60
Bacillus licheniformis	RH 104	60
Bacillus sp.	RH 105	60
Bacillus amyloliquefaciens	RH 106	60

Table 2: Antimicrobial profile of isolated thermophilic strains on test organisms

Test organism/ isolated strains		Inhibition zone diameter against test organism (mm)*					
		M. flavus	*Dietzia* sp.	*B. subtilis*	*S. aureus*	*E. coli*	*P. aeruginosa*
B. licheniformis	RH 101	24	26	NI	NI	NI	NI
Br. borstelensis	RH 102	28	22	NI	18	NI	NI
B. galactosidilyticus	RH 103	20	NI	NI	NI	NI	NI
B. licheniformis	RH 104	14	08	10	NI	NI	NI
Bacillus sp.	RH 105	NI	NI	NI	NI	NI	NI
B. amyloliquefaciens	RH 106	NI	NI	NI	NI	NI	NI

*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

Test organisms	Antibiotics							
	P	V	A	E	T	S	C	ME
E. coli	R	R	17	R	22	13	24	ND
P. aeroginosa	R	R	R	13	8	16	R	ND
M. flavus	R	19	ND	20	24	18	26	28
B. subtilis	R	16	8	06	22	R	04	ND
S. aureus	26	19	26	22	12	ND	19	18
Dietzia sp. K44	30	22	32	25	22	28	24	22

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

The methanol extracts of *Br. borstelensis* 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. borstelensis* (28 mm) is quite comparable to routinely used antibiotics (4-32 mm) (Table 3). It appears that the thermophilic *Br. borstelensis* 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 way 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 anti microbial 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

Allan, R.N., L. Lebbe, J. Heyrman, P. de Vos, C.J. Buchanan and N.A. Logan, 2005. *Brevibacillus levickii* sp. nov. and *Aneurinibacillus terranovensis* sp. nov. two novel thermoacidophiles isolated from geothermal soils of Northern Victoria Land, Antarctica. Int. J. Syst. Evol. Microbiol., 55 (3): 1039-1050.

Altschul, S.F., W. Gish, W. Miller, E.W. Myers and D.J. Lipman, 1990. Basic local alignment search tool. J. Mol. Biol., 215: 403-410.

Chopra, I., J. Hodgson, B. Metcalf and G. Poste, 1997. The search for anti-microbial agents effective against bacteria resistant to multiple antibiotics. Antimicrobiol. Agents Ch., 41: 497-503.

Davis, J. and V. Webb, 1998. Antibiotic Resistance in Bacteria. In: Emerging Infections, Krause, R.M. (Ed.). Academic Press, New York, pp: 239.

Finstein, M.S. and M.L. Morris, 1975. Microbiology of municipal solid waste composting. Adv. Applied Microbiol., 19: 113-151.

Gebhardt, K., J. Schimana, J. Muller, H.P. Fielder, H.G. Kallenborn, M. Holzenkampfer, P. Krastel, A. Zeeck, J. Vater, A. Holtzel, D.G. Schmid, J. Rheinheimer and K. Dettner, 2002. Screening for biologically active metabolites with endosymbiotic bacilli isolated from arthropods. FEMS. Microbiol. Lett., 217: 199-205.

Gerard, D.W. and D.S. Arlene, 2007. New strategies for combating multi-drug resistant bacteria. Trends Mol. Med., 13 (6): 260-267.

Gillespie, D.E., S.F. Brady, A.D. Betlermann, N.P. Cianciotto, M.R. Liles, M.R. Rondon, J. Clady, R.M. Goodman and J. Handelsman, 2002. Isolation of antibiotics turbomycin A and B from a metagenomic library of soil microbial DNA. Applied Environ. Microbiol., 68: 4301-4306.

Horikoshi, K., 1995. Discovering novel bacteria with an eye to biotechnological applications. Curr. Opin. Biotechnol., 6: 292-297.

Nagai, K., N. Kamigiri, K. Arao, Y. Suzumura, M. Kawano, H. Yamaoka, M. Zhang, K. Watanabe and Y. Suzuki, 2003. YM-266183 and YM-266184, novel thiopeptide antibiotics produced by *Bacillus cereus* isolated from marine sponge. J. Antibiotics, 56: 123-128.

Natarajan, S., D. Milne, A.L. Jones, M. Goodfellow, J. Perry and R.J. Koerner, 2005. *Dietzia* strain X: A newly described Actinomycete isolated from confluent and reticulated papillomatosis. Br. J. Dermatol., 153: 825-827.

Phoebe, Jr. C.H., J. Combie, F.G. Albert, K. Van Tran, J. Cabrera, H.J. Correira, Y. Guo, J. Lindermuth, N. Rauert, W. Galbraith and C.P. Selitrennikoff, 2001., Extremophilic organisms as an unexplored source for antifungal compounds. J. Antibiotics (Tokyo), 54: 56-65.

Pidoux, O., J.N. Aragenson, V. Jacomo and M. Drancourt, 2001. Molecular identification of a *Dietzia maris* hip prosthesis infection isolate. J. Clin. Microbiol., 39: 2634-2636.

Sneath, P.H.A., N.S. Mair, M.E. Sharpe and J.G. Holt, 1986. Bergey's Manual of Systematic Bacteriology. Vol. 2, Williams and Wilkins, Baltimore, US., pp: 965-1599.

Takagi, H., O. Shida, K. Kadowaki, K. Komagata and S. Udaka, 1993. Characterization of *Bacillus brevis*, with descriptions of *Bacillus migulanus* sp. nov., *Bacillus choshinensis* sp. nov., *Bacillus parabrevis* sp. nov. and *Bacillus galactophilus* sp. nov. Int. J. Syst. Bacteriol., 43: 221-231.

Walsh, C., 2003. Where will new antibiotics come from? Nat. Rev. Microbiol., 1: 65-70.

Weisburg, W.G., S.M. Barns and D.J. Lane, 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol., 173: 697-703.

Yilmaz, M., H. Soran and Y. Beyatli, 2006. Antimicrobial activities of some *Bacillus* sp. Strains isolated from the soil. Microbiol. Res., 161: 127-131.