

# Bioprospecting the *lat* gene in soil samples

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Twenty soil communities from the northeastern forests (Assam) and the Western Ghats (Maharashtra) were screened for the presence of the lysine aminotransferase (*lat*) gene from *Nocardia*. Hybridization probes and primers were synthesized in accordance with the reported sequence of the *Nocardia lat* gene from GenBank (number: G1 49355). Seven positives were obtained from the 20 soils. Six of the seven positive were from the Western Ghats and one from the northeast Assam forests. Eighteen actinomycete isolates from the 7 positive soils showed the presence of the *lat* gene. Only 9 isolates actually produced an antibiotic. These results are discussed.

[Dharwadkar A, Gupta V and Pant A 2003 Bioprospecting the *lat* gene in soil samples; *J. Biosci.* **28** 597–604]

## 1. Introduction

**b**-lactams inhibit the transamidation reaction which leads to the crosslinking of peptidoglycans (Travis *et al* 1994). Substituting the reactive groups on the **b**-lactam ring results in products like penicillin and cephamycin, which are important antibiotics. Therefore, the isolation of organisms producing **b**-lactam-type antibiotics is an important area of bioprospecting. **b**-lactams were initially obtained from fungi; but after Nagarajan *et al* (1971) isolated three novel cephalosporin molecules from *Streptomyces*, the actinomycetes were also recognized as important sources as has been mentioned by Gordon and Sykes (1982). Until 1972, penicillin and cephalosporin formed the two major substituted products of **b**-lactams and the discovery of cephamycins (Coque *et al* 1991) expanded the spectrum of **b**-lactams by introducing high stability to **b**-lactamases. Cephamycins are produced by *Streptomyces* and *Nocardia* and lysine aminotransferase is an important enzyme in the biosynthesis of **b**-lactams. The gene database reports numerous sequences from organisms producing **b**-lactams, which include fungi and actinomycetes.

Lysine aminotransferase enzyme of *Candida guilliermondii*, *Rhodotorula glutinis*, *Flavobacterium fuscum*, *Pseudomonas aeruginosa* and *Achromobacter liquidum* occurs in the lysine catabolism pathway (Fothergill and Guest 1977; Kinzel *et al* 1983; Der Garabedian and Ver-

meersch 1989; Fujii *et al* 2000). Lysine aminotransferase of actinomycetes is involved in the production of antibiotics.

The lysine aminotransferase (*lat*) gene is located in the cephamycin gene cluster in *Streptomyces clavuligerus* (Tobin *et al* 1991) and *Nocardia lactamdurans* (Kern *et al* 1980; Coque *et al* 1991). The enzyme converts lysine into **a**-amino adipic semialdehyde by deamination, which is spontaneously cyclized to piperidine-6-carboxylic acid (Kern *et al* 1980; Madduri *et al* 1989). The piperidine-6-carboxylic acid synthesized is a precursor of the **b**-lactam ring (Alexander and Jensen 1998; Martin 1998). The object of the work reported here was to discover the presence of the *lat* gene in soil samples collected from various parts of the country (figure 1).

## 2. Materials and methods

### 2.1 Soil sampling

The soil samples were collected from different locations in India with a majority 13 from the Western Ghats; 5 from the northeast Assam forests and Brahmaputra valley. These soil samples include the megadiversity areas in these regions (Myres *et al* 2000). Single soil samples were obtained from the Eastern Ghats and Sikkim. The soil

**Keywords.** Bioprospecting; community DNA; **b**-lactam

samples were collected (figure 1, table 1) from a depth of 5 cm, sealed in sampling bags and brought to the laboratory for further analysis. Analysis of the soil characteristics was done by Zuari Industries Pvt. Ltd. using standard protocols.

## 2.2 Probe preparation

A nucleotide sequence corresponding to the conserved pyridoxal phosphate-binding site of lysine aminotransferase was used as a probe (Coque 1991). The probe 5'CAGGCGATGCAGGACCTCTGCCA3' was synthesized in a Gene assembler Plus synthesizer.

## 2.3 Extraction of community DNA from soil

Community DNA from soil was extracted using the following protocol. To 5 g of a soil sample 10 ml of NaCl-EDTA buffer at pH 8.0 was added. Lysozyme (25 mg) was added to each sample for cell lysis and the mixture was incubated on ice for an hour after vortexing (Hilger and Myrold 1991). Five ml of lysis buffer was added (25 mM Tris, 100 mM EDTA, 2% SDS and 1% CTAB) and the mixture was incubated at 65°C for an hour. On reaching room temperature, the contents of the tube were centrifuged at 13,000 g for 10 min. To the supernatant, an equal amount of chloroform : isoamyl alcohol (24 : 1) was added. After mixing, the contents were centrifuged at



**Figure 1.** Map of India showing the different sampling locations.

13,000 *g* for 10 min. The treatment was repeated twice. To the supernatant of the chloroform treatment, an equal amount of isopropanol was added. The mixture was incubated overnight at room temperature allowing the DNA to precipitate. The precipitated DNA was pelleted by centrifugation at 13,000 *g* for 10 min. The pellet was kept in 70% ethanol for 4 h at room temperature. Ethanol (70%) was removed by centrifugation at 13,000 *g* for 10 min. The DNA pellet was then dried under low vacuum and dissolved in 1 ml of T<sub>10</sub>E<sub>1</sub> buffer (10 mM Tris and 1 mM EDTA of pH 8.0). One hundred **ng** RNase, from a 10 mg/ml stock was added to the DNA pellet and reaction was allowed to proceed at 37°C for 2 h (Tsai and Olson 1991). The DNA was purified using Promega DNA Purification Kit (Wizard Plus Minipreps DNA Purification System). The DNA concentration was determined on 0.8% agarose gel in 0.5XTAE buffer after staining with ethidium bromide.

#### 2.4 DNA extraction efficiency

Ten g of each soil were autoclaved twice with an interval of 24 h at room temperature. A single colony of *Escherichia coli* (ATCC: 8739) was inoculated into 50 ml of

nutrient broth and was allowed to grow for 24 h at 37°C. Triplicate TVC determinations from appropriately diluted samples showed that the 50 ml culture contained  $160 \times 10^9$  cells to  $180 \times 10^9$  cells. The theoretical yield of DNA was therefore 1440 µg to 1620 µg, considering that a single cell of *E. coli* contains  $9 \times 10^{-9}$  µg of DNA. This 50 ml nutrient broth was added to the autoclaved soil. The yield of DNA obtained from the seeded soil sample was determined spectrophotometrically at A<sub>260 nm</sub> and quantified using a standard curve made with calf thymus DNA (Tsai *et al* 1991).

#### 2.5 Hybridization

Two hundred ng of community DNA was dissolved in 1 µl of T<sub>10</sub>E<sub>1</sub> buffer. Hybond nylon membrane (15 × 14 cm) was pre-wetted in sterile double distilled water and then rinsed in 2X SSPE (sodium chloride sodium phosphate EDTA). The community DNA was spotted onto the membrane. The membrane was air dried and baked at 80°C for 2 h. It was rolled in a silicone-coated bottle and given 2 washes, each of 15 min – of denaturing solution (0.5 M NaOH.1.5 M NaCl); 2 washes – each of 15 min of neutralizing solution (0.5 M Tris, 1.5 M NaCl) and a single wash

**Table 1.** Analysis of soil data.

Sample	Latitude (°N)	Longitude (°E)	Recovery of DNA per 5 g soil	Recovery per gram of soil	Percent recovery
Western Ghats					
1	17.42	74.02	200	40	13.88
2	17.42	74.02	340	68	23.61
3	17.42	74.02	300	60	20.83
4	18.31	73.55	140	28	9.72
5	18.31	73.55	140	28	9.72
6	18.31	73.55	60	12	3.7
7	18.31	73.55	60	12	3.7
8	18.31	73.55	160	32	9.88
9	18.31	73.55	140	28	8.64
10	20.02	73.50	140	28	9.72
11	18.31	73.55	500	100	34.72
12	17.33	74.23	160	32	11.11
13	17.33	74.23	140	28	8.64
Northeast and Brahmaputra valley					
14	26.11	91.47	680	136	41.97
15	26.11	91.47	360	72	22.22
16	26.11	91.47	33	60	18.52
17	26.11	91.47	60	12	3.70
18	26.11	91.47	460	92	28.39
Sikkim					
20	27.20	88.40	700	140	48.61
Eastern Ghats					
19	18.07	83.27	850	170	59.03

of 5 min of equilibrating solution (6X SSPE) (Maniatis *et al* 1982). Pre-hybridization was then carried out in 50 ml of pre-hybridization buffer (1% milk powder, 5X Denhardt's solution, 5X SSPE and 0.1% SDS) at 60°C for 3 h at 6 rpm (Maniatis *et al* 1982; Vollossiouk *et al* 1995). The probe DNA (100 ng) was end labelled. A 10 µl end-labelling reaction was set-up as 1U of T4 polynucleotide kinase, 10X kinase buffer and 1.25 mCi ( $g^{32}P$ ) dATP at 37°C for an hour. The reaction was stopped using 1 µl of 0.5 M EDTA. Hybridization was carried out overnight in 10 ml of fresh hybridization buffer (same as pre-hybridization buffer) at 60°C. Washes were given to the membrane with the washing solution (3X SSPE, 0.5 M SDS). One hot wash at the hybridization temperature and two washes at room temperature – each of 15 min – ensured complete removal of the background. Hybridization was detected by autoradiography using Konica medical X-ray films (20.3 × 25.4 cm/8 × 10 in). A clear hybridization signal was obtained after 72 h. Prolonged exposure of one week confirmed the results.

## 2.6 Isolation of actinomycetes from soil and extraction of actinomycete DNA

Of the total 20 soil samples, the 7 isolates which gave a positive signal from the hybridization experiments, were used to isolate actinomycetes. One hundred mg of the

original soil sample was suspended in 1 ml of sterile distilled water and vortexed for 1 min. Eight µl of the soil suspension was added to 1 ml of 1 : 140 diluted redistilled phenol and the mixture was incubated for 20 min. Serial dilutions followed by plating led to the isolation of 45 actinomycete strains which could be distinguished on the basis of pigmentation pattern and morphological characteristics. None of the strains was taxonomically identified. Each isolate was purified using standard microbiological techniques. Each isolate was grown in nutrient broth for 48 h at 30°C. DNA was extracted from each of the 45 strains using the same protocol given earlier and used in polymerase chain reaction (PCR).

## 2.7 PCR amplification

The hybridization probe was used as the forward primer. The reverse primer was constructed from the terminal 23-nucleotide residues of the *lat* gene from *N. lactamdurans* (Coque *et al* 1991). The sequence is: 5'TCAGCCGCGTGAGGCGAGGCTGGCCGCC3'. The primers were used to amplify a region of DNA corresponding to 590 bases. The PCR reaction was carried out in 25 µl volume containing : 30 ng actinomycete genomic DNA from each of the above strains, 0.1 mM dNTPs, 30 pmol of each primer, 2U of Taq DNA polymerase (Perkin-Elmer, USA), Taq DNA polymerase buffer (10 mM Tris-HCl, pH 8.3,

**Table 2.** Analyses of soil samples.

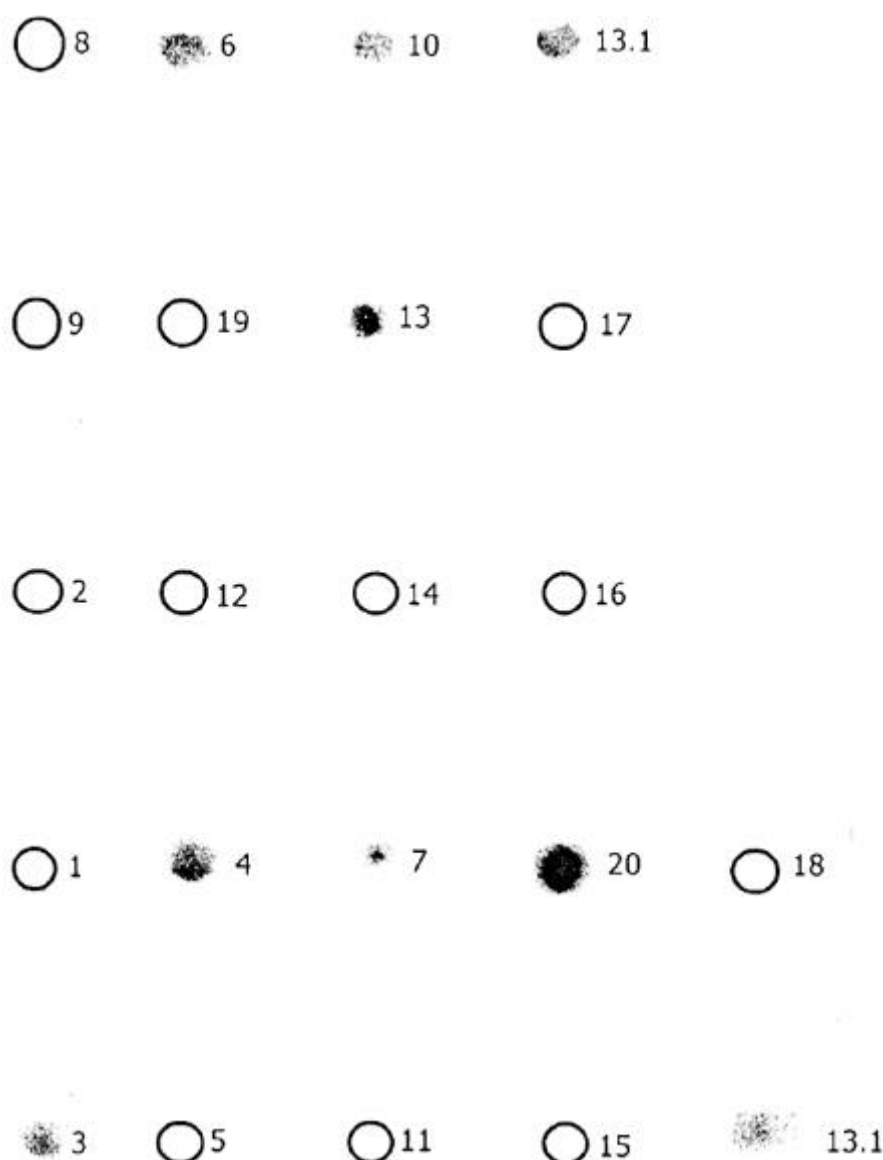
Soil sample	pH	Redox potential	Organic carbon (% values)	Phosphorous content (kg/0.405 hectare)	Potassium content (kg/0.405 hectare)	Micronutrient analyses in PPM			
						Zn	Cu	Fe	Mn
1	6.50	0.08	0.32	4.00	> 200	1.08	2.11	75.85	27.54
2	6.20	0.70	0.70	17.00	155.00	> 2.56	2.39	> 32.07	> 44.55
3	7.30	1.45	1.45	22.00	173.00	0.89	2.72	> 47.57	30.55
4	6.80	2.25	2.25	16.00	202.00	> 14.12	3.48	5.02	33.62
5	7.90	1.56	1.56	12.00	> 200	1.25	8.51	5.73	44.59
6	7.90	0.68	3.30	18.00	> 200	> 8.87	5.17	12.62	> 31.86
7	6.80	0.13	0.96	7.00	130.00	> 2.51	4.58	11.10	20.56
8	6.40	0.86	2.16	28.00	> 200	> 7.39	11.59	17.82	> 24038
9	7.40	0.28	2.10	12.00	> 200	> 3.02	9.55	12.38	> 45.75
10	6.20	0.36	1.80	12.00	96.00	> 2.77	6.38	51.62	> 42.70
11	6.20	2.20	3.24	16.00	> 200	> 2.92	4.31	21.39	> 57.61
12	7.50	1.09	2.40	14.00	160.00	> 4.12	4.28	4.95	> 48.82
13	6.90	0.99	2.35	13.00	> 200	1.51	4.15	2.82	20.20
14	7.10	0.73	0.96	20.00	67.00	> 6.18	11.57	19.05	15.80
15	6.10	0.26	1.08	24.00	35.00	> 13.11	1.60	> 33.80	12.00
16	6.10	0.19	3.46	19.00	57.00	0.81	0.97	> 62.11	> 27.80
17	6.00	0.42	3.29	30.00	52.00	0.78	0.60	> 35.8	> 38.20
18	6.80	0.29	4.32	24.00	18.00	> 2.51	4.58	11.10	> 46.06
19	7.10	0.40	1.68	12.00	124.00	1.95	1.87	> 50.92	> 25.80
20	8.00	0.27	2.22	7.00	> 200	3.95	2.93	56.34	20.95

ANOVA results: Between regions – *F* value: 1.3 (degrees of freedom: 17 and 95). Expected *F* value: 3.5 (degrees of freedom: 17 and 95).

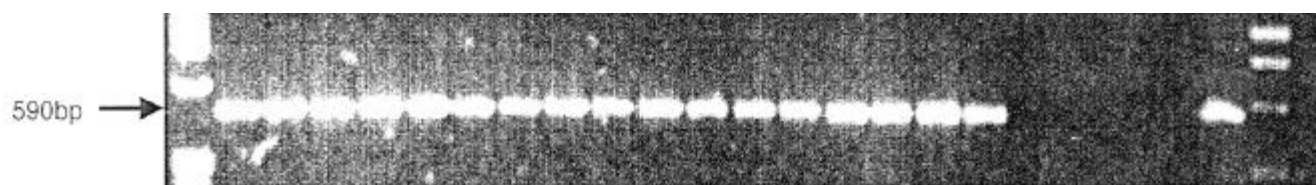
1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.1% Triton X100). PCR amplification was performed in MJ Research Incorporation's Peltier Thermal Cycler PTC-200 with initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 20 s, annealing at 68°C for 20 s; and extension at 72°C for 45 s. The final extension was at 72°C for 5 min. The amplified products were separated on 2% agarose gels in 0.5X TAE buffer and bands were detected by staining with ethidium bromide. The reproducibility of the amplification was confirmed by repeating each experiment three times.

## 2.8 Production of antibiotic

Those cultures, which gave an amplification product in PCR, were used to test for the production of antibiotic. Cultures of *E. coli* (ATCC: 8739) (18 h old) were plated onto nutrient agar plates. Sterile Whatman paper discs, wetted with 10 µl broth from the stationary phases of these cultures, were placed on these *E. coli* mats (Hansen *et al* 2001). The plates were incubated at room temperature for 24 h and the zone of inhibition was noted.



**Figure 2.** Autoradiogram of the results of the hybridization experiment. The numbers denote the soil DNA samples and the dot after the soil sample number indicates the isolate number whose genomic DNA has been loaded.



**Figure 3.** The results of the isolate DNA PCR reaction. Lanes on the extreme left and extreme right show the markers *fx HaeIII* digest and the 1 kb ladder respectively. Lanes in between the marker lanes show the results for the DNA's of the following isolates – 3-1, 3-2, 4-2, 4-4, 6-2, 6-3, 6-4, 7-2, 10-2, 20-2, 20-5, 20-7, 20-10, 13-4, 13-7, 13-8, 13-9, 13-2, 6-7, 20-8, 1-1, 13-1 respectively where the number before the dot indicates the soil sample number and that after the dot indicates the isolate number. The two 13-1 samples indicate the PCR amplified DNA and the genomic DNA respectively. The band and its size are indicated by an arrow, which indicates the positive results.

## 2.9 Statistical analysis of data

The dependence of the DNA recovery (table 1) on the physical parameters of soil (table 2) was analysed using multiple regression method in the MINITAB software. Variation in the DNA recovery between the different sampling locations and within the same area of sampling was compared using ANOVA analysis in the MINITAB software programme.

## 3. Results and discussion

The first 13 of the 20 soil samples (table 1) were collected from soil zones in the Western Ghats. Samples 1 and 2 were from evergreen forests. Samples 3 to 12 were from disturbed and scrub areas from several locations. Sample 13 was from a seasonal lake (latitude 17-33°N and longitude 74-23°E). The soils in the Western Ghats were shallow and medium black and were classified as vertisols, which is a subtype of the type invertisols (FAI Yearbook of India 1985). The northeast Assam forests and the Brahmaputra valley soils (samples 14–18, table 1) on the other hand varied in colour from yellow brown to oily black (sample 18). They were loamy with appreciably higher clay content than those of the Western Ghats. These were classified as antisols (FAI Yearbook of India 1985). The only sample from Sikkim (sample 20, table 1) was brown and shiny with high mica content. The soils of Sikkim were types of inceptisols (FAI Yearbook of India 1985). There was only one sample from the Eastern Ghats (sample 19, table 1) from an undisturbed forest ecosystem. The soil type belongs to the alsisols, which are lateritic soils (FAI Yearbook of India 1985).

Identifying special genotypes in soil, which is the basic aim of bioprospecting, requires efficient extraction of DNA. The soil itself adsorbs DNA (Steffan *et al* 1988) and humic acids interfere with the purification of DNA and PCR protocols (Steffan *et al* 1998). Extraction protocols reviewed by Steffan *et al* (1988) and by Frostegard (1999) indicate that the recovery of soil DNA depends on

**Table 3.** Data on positive isolates.

Soil sample	Total number of isolates	Number of isolates reported positive for the <i>lac</i> gene
3	7	2
4	6	2
6	4	3
7	1	1
10	4	1
13	13	5
20	10	4

the soil type and is almost never 100%. Miller *et al* (1999) reported 1.5 µg to 53 µg DNA per gram of soil dependent on the soil type. The null hypothesis that DNA recovery is not dependent on the physical properties of soil was accepted based on a MINITAB program (MINITAB multiple regression analysis).

The Western Ghat soils showed an average recovery of 13%. This may have been due to the humic acid content of these soils. An average recovery of about 28% was obtained from the loamy soils of the northeastern region and the Brahmaputra valley, omitting a single value of 3.7% (sample 17, table 1). It was possible to recover more than 40% DNA from the single soil samples collected from the Eastern Ghats and Sikkim (table 1). The null hypothesis, that there was no difference in the DNA recovery between regions, was rejected based on ANOVA (analysis of variance test) at the 99% confidence limit.

The varying humic acid content resulted in losses during the purification of DNA. The purified DNA did not amplify repeatably with the PCR primers. Tsai and Olson (1992) have shown that humic acids interfere with PCR amplification whereas DNA probes have been used efficiently in the detection of specific microorganisms (Holben *et al* 1998). Therefore, in the succeeding experiments DNA was hybridized with the 23-mer probe synthesized in accordance with the sequence stated earlier.

**Table 4.** Production of antibiotic.

Isolate checked for the production of antibiotic	Zone of inhibition	Pigmentation pattern
3-1	–	Pink
3-2	+	Brownish orange
4-2	+	Pinkish red
4-4	+	White
6-2	+	Orange
6-3	–	Red
6-4	–	Brown
7-2	+	Yellow
10-2	–	Green
13-1	+	Magenta
13-4	–	Grey
13-7	+	Yellow
13-8	–	White
13-9	–	Grey
20-2	+	Faint brown
20-5	–	Dark brown
20-7	+	Pink
20-10	–	Black

Humic acids do not interfere with hybridization (Steffan *et al* 1998). Hybridization of each soil DNA with the probe showed that of the 20 soil samples only 7 tested positive for the presence of the *lat* gene (figure 2). Six out of the seven positive samples were from disturbed ecosystems in the Western Ghats. Samples 1 and 2, which were from forest ecosystems did not show the presence of the *lat* gene. The seventh positive sample was from Sikkim, which was also collected from an area influenced by human activities. None of the 5 soil samples from northeastern region and the Brahmaputra valley tested positively for the lysine aminotransferase gene with this hybridization probe despite the higher recovery of DNA from these soils.

Lysine aminotransferase is known to be involved in the synthesis of *b*-lactam antibiotics in the actinomycete group of organisms. Therefore, actinomycetes from the 7 positive samples were isolated by pretreatment of the soil with phenol (Lawrence 1956). Forty five strains were isolated from these 7 soil samples. The 45 strains were grown in nutrient broth medium for 48 h and DNA was extracted from each. The purified DNA was amplified using PCR primers synthesized as given in §2. Only 18 of the 45 isolates responded positively to the amplification resulting in a 590 bp band in each case (figure 3, table 3). The band obtained probably corresponds to the latter half of the gene reported by Coque *et al* (1991). The occurrence of the gene indicated the ability to synthesize an antibiotic. The 18 isolates were individually grown in nutrient broth medium for 48 h. *E. coli* plated onto nutrient agar and treated with the broth from each of the 18 isolates showed that only 9 isolates actually produced an antibiotic (table 4).

The present study shows that highly focused bioprospecting is possible with known gene sequences; although organisms which produce antibiotics through other pathways, or produce dissimilar enzymes performing the same function, will not be accessed in these assays. In conclusion, it is noted that antibiotic producers were obtained more frequently in disturbed soil communities than in the undisturbed soil community of the forests.

### Acknowledgements

We thank R A Bahulikar and M C Rahalkar for their help and Dr Sharayu Paranjape, University of Pune, for the statistical analysis. We would also like to acknowledge the Department of Biotechnology, New Delhi, for the financial support.

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MS received 4 December 2002; accepted 20 May 2003

Corresponding editor: VIDYANAND NANJUNDIAH