Indian Journal of Pharmaceutical Education and Research | Association of Pharmaceutical Teachers of India

16S rDNA sequencing of Antibiotic Producing Bacterial Strain *Lysinibacillus* sp.(BP-3) from Nagavali River Basin of Srikakulam.

Praveen.B, Bisht S.P.S* and Ashok Kumar Panigrahy[1]

Department of Biotechnology, Roland Institute of Pharmaceutical Sciences, Berhampur-760010, Orissa, India

[1]Professor Department of Botany Berhampur University, Orissa.

ABSTRACT

Antibiotic producing bacteria *Lysinibacillus* sp.(BP-3) was isolated from the Nagavali river basin, Srikakulam, Andhra pradesh, India. The whole cell protein extract of BP-3 strain was found to have significant inhibitory effect on the test strains viz: *E.coli* (MTCC 40), *Staphlococcus aureus* (MTCC87),*Proteus vulgaris*(MTCC426), and *Pseudomonas aeruginosa* (MTCC 424). Identification of the bacteria was carried out using biochemical tests, 16SrRNA sequencing and sequences submitted to the NCBI GenBank Maryland USA and awarded with the accession number HM359123. Strain BP-3 exhibited maximum inhibitory effect and was identified as *Lysinibacillus* sp. Nagavali river basin receives a very high number of microbes from the dense virgin forests of Orissa and Andhra Pradesh during the first monsoon therefore it is of a great importance to screen the microbes for the hunt of a novel antibiotic producing strain from the site and characterize it at molecular level to evaluate its potential as candidate strain for antibiotic production.

Submitted: 20/05/2011 Revised: 25/06/2011 Accepted: 18/10/2011

Keywords: Nagavali, antibiotic, 16SrRNA sequencing, Lysinibacillus

INTRODUCTION

Soil is a diverse medium composed of many minerals and substrates essential for metabolic pathways of prokaryotic and eukaryotic inhabitants. The abiotic and biotic diversity present in this medium makes it difficult for the isolation of all the microbial community present. Research has demonstrated that not even 1% of the entire soil microbial community has been identified. [1, 2] There is great opportunity for discovering new groups of microorganisms with industrial and clinical importance present in soil.

Lysinibacillus sphaericus is a naturally occurring soil bacterium. It is a Gram-positive, mesophilic, rod-shaped bacterium. Under harsh conditions, *Lysinibacillus sphaericus* can form dormant endospores that are resistant to heat, chemicals, and ultraviolet light. These spores may remain viable for a longer time. Although it is typically a facultative anaerobe, *L. sphaericus* may be anaerobic under certain conditions. It is a common environmental organism which produces an insecticidal toxin similar to that produced by *Bacillus thuringiensis*. Another name for this organism is *Bacillus sphaericus*. The genus change was based on distinctive peptidoglycan composition of the cell wall, as well as phylogenetic and physiological analyses.[3]

Lysinibacillus sphaericus is an important organism to study because it can be used as an insecticidal toxin that controls mosquito growth. This organism, along with similar organisms, is utilized in insect control programs to reduce the population of disease vector species that transmit diseases such as malaria, yellow fever, and West Nile virus. *Lysinibacillus sphaericus* C3-41 is one such strain and has been used to control mosquito larvae population in China for more than 10 years.[4] Commercially, *Lysinibacillus sphaericus* is known as VectoLex. It is highly toxic to the *Culex* genus of mosquitoes and somewhat toxic to the *Anopheles* and *Aedes* genera of mosquitoes.[5] Genome sequencing of this organism is useful because it increases the knowledge of the bacilli and also offers insight for future improvement of important biological control agents.[6] Standard microbiological techniques and innovative molecular genetic technology are being designed[6,7,8] and by applying these techniques to a given environment, one can obtain large quantities of genomic material and study a vast part of a given microbial community.

MATERIAL & METHODS

Sampling and screening for antibiotic producing bacteria

The strain BP-3 was isolated from Nagavali river basin (18° 10'to 19° 44' 0N lat and 82° 53' to84° 05' 0E long) of Srikakulam District, Andhra pradesh, India, maintained on nutrient agar and stored at 4°C. The soil suspensions were homogenized by shaking at 200 rpm for 15 minutes at 30°C and serial dilutions were carried up to 10^{-3}. Two repetitions of each of the dilutions were inoculated on Nutrient agar and the cultures (or master plates) were incubated at 37°C for 24 hours. After the incubation period, colonies that exhibited antagonism were designated as Antimicrobial Agent Producing Microbes (AAPM) and were sub cultured and purified by streaking them on a Nutrient agar plate. After

***Address for Correspondence:**

Bisht S.P.S, Department of Biotechnology, Roland Institute of Pharmaceutical Sciences, Berhampur-760010, Orissa, India

E-mail: sps_bisht@yahoo.com, sps.bisht@gmail.com

purification, the isolated three AAPM's were preserved and stored at -20°C for further tests and strain BP-3 was further characterize at molecular level.

Conventional identification tests:

The isolate was initially evaluated by conventional tests i.e. Gram stain, growth and morphometric characteristics on nutrient agar, growth at 37°C, cataslase, oxidase, motility, indole production, gelatin liquefaction, oxidative fermentative carbohydrate utilization, decarboxylation of lysine, urease activity. Additional tests included phenylalanine deamination, nitrate reduction, citrate utilization and H_2S production.

Antibiograms :

Susceptibility Test with Whole cell extract:

Cultures were centrifuged at 3000g for 15 min, then the cell pellets were resuspended in 15% glycerol, 1% SDS, 0.1M Tris-Hcl pH-6.8 and denatured by treatment at 100°C for 20 min. Non solubilized material was removed by centrifugation at 3000g for 15 min and the resulting supernatant was used as crude whole cell extract. The target microorganisms used were *Escherichia coli* MTCC 40, *Pseudomonas aeruginosa* MTCC 424, *Proteus vulgaris* MTCC 426 and *Staphylococcus aureus* MTCC 87. AAPM's were incubated 24 hours at 37°C and the targets were incubated also for 24 hours at 37°C. In order to create a bacterial lawn of the targets on nutrient agar, the spread plate technique was employed by using 200 µl of each target. By using a sterile borer with the 5mm diameter, wells were prepared and loaded with 20 µl of the AAPM's supernatant and placed over the bacterial lawns. A positive control was included by using a sterile tetracycline antibiotic disc. Antibiograms were incubated for 24-48 hours at 37°C. After this period, inhibition zones were measured with a ruler having a cm scale.

DNA preparation and PCR amplification:

Genomic DNA was extracted from the isolates using Chromous Genomic DNA isolation kit (RKT09). Each genomic DNA used as template was amplified by PCR with the aid of 16SrDNA primers (16S Forward Primer: 5'-AGAGTRTGATCMTYGCTWAC-3' 16S Reverse Primer: 5'-CGYTAMCTTWTTACGRCT-3' with the programme consisted of denaturation at 94°C for 5 min and subsequent 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 2 min followed by final extension at 72°C for 5 min. The presence of PCR products was determined by electrophoresis of 10 µl of the reaction product in 1% agarose gel.

16S rRNA sequencing and data analysis:

Sequencing analysis was performed on a 1500 bp PCR product. The sequence analysis was performed using the ABI 3130 genetic analyzer and Big Dye Terminator version 3.1 cycle sequencing kit. The three 16SrRNA sequences were aligned and compared with other 16SrRNA genes in the GenBank by using the NCBI Basic Local alignment search tools BLAST. A distance matrix was generated using the Jukes-cantor corrected distance model. The phylogenetic trees created using Weighbor (Weighted Neighbor Joining: A Likelihood-Based Approach to Distance-Based Phylogeny Reconstruction) with alphabet size 4 and length size 1000. The 16SrRNA gene sequences have been deposited to Genbank using BankIt submission tool and has been assigned with NCBI accession number (HM359123).

RESULTS & DISCUSSION

The bacterial strain was isolated and identified as *Lysinibacillus* sp. based on the results of 16SrRNA sequencing. The 16S rDNA amplification by Forward Primer:5'

AGAGTRTGATCMTYGCTWAC-3', Reverse Primer: 5'-CGYTAMCTTWTTACGRCT-3' followed by sequencing reveals strain BP-3 had highest homology (99%) with *Lysinibacillus sphaericus*; 2317-2 ;(GenBank entry:DQ286297). More than 1400bp of the 16S rRNA genes of the strains BP-3 was sequenced. Analysis of the 16S rRNA sequences confirmed the strain.BP-3 was found to be most similar to *Lysinibacillus sphaericus*; 2317-2;(GenBank entry:DQ286297). (Fig 1). The 16SrRNA gene sequence has been deposited to Genbank using BankIt submission tool and has been assigned with NCBI (National Centre for Biotechnology information) accession number HM359123. Fig. 2 shows the sequence comparison of strain BP-3 and *Lysinibacillus sphaericus*; a 2317-2 .The result of inhibition zones against the test microorganisms is shown in Table 1. The strain BP-3 is found to be gram negative rod shaped bacterium with a circular, opaque, raised colony morphology with rhizoidal margin. Table 2 shows the Biochemical identification test results.

CONCLUSION

The screening of microbes from the Nagavali river basin during first monsoon rain was carried out very first time and screening of soil and mud samples revealed that Nagavali river basin is having a huge microbial biodiversity and its metagenomic studies are required to understand the microbial flora of biomedical importance more specifically the production of bacteriocins at commercial scale.

ACKNOWLEDGEMENTS

The authors are thankful to Administration and Management of Roland Institute of Pharmaceutical Sciences, Berhampur, Orissa, India for financial support.

Fig. 1: Phylogenetic position based on 16S rRNA gene sequence analysis of strain BP-3

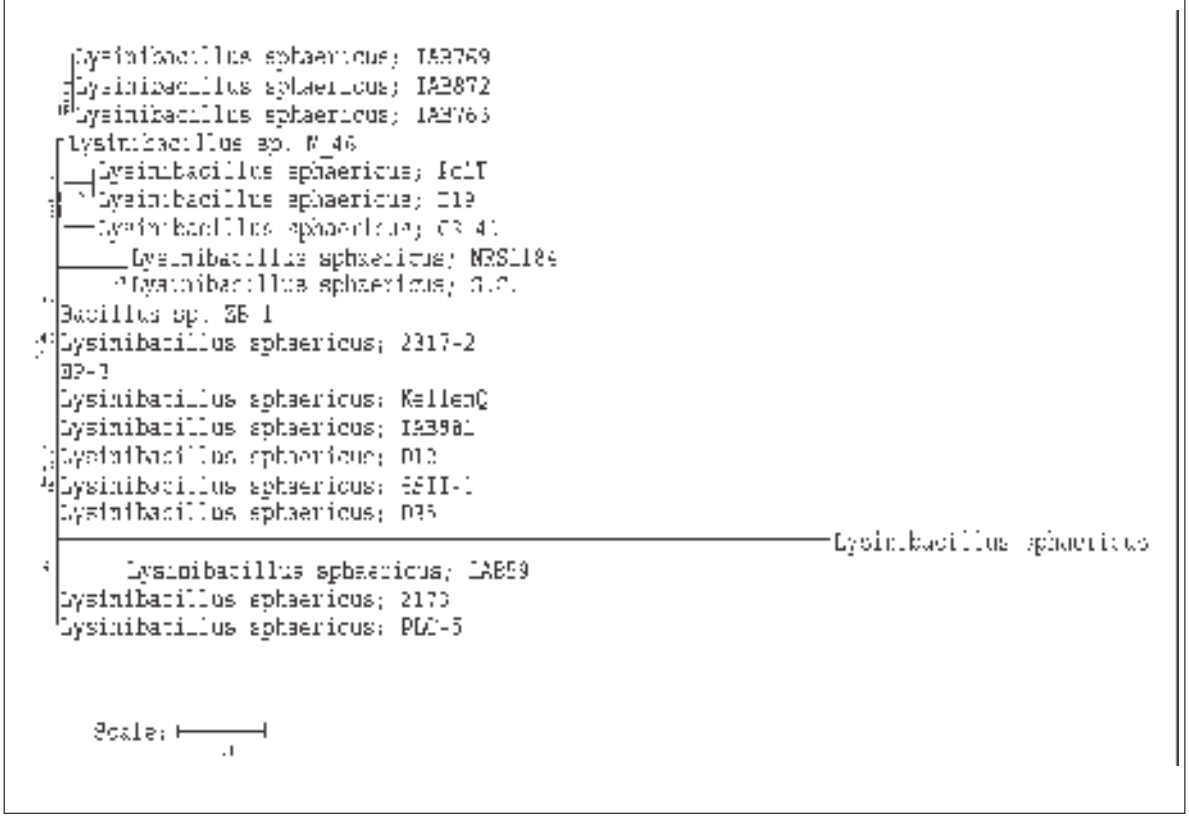

Table. 1. Susceptibility test using un-filtered supernatants

Specimen	*Escherichia coli*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Proteus vulgaris*
BP-3	**18**	**12**	**18**	**10**

The isolated AAPM (BP-3) and its diameter (mm) of the inhibition zone produced towards the test microorganism it was exposed to.

Table. 2: Biochemical test result of the candidate strain

Biochemical tests	Results BP-3
Carbohydrate fermentation	
Glucose	-ve
Adonitol	-ve
Lactose	-ve
Arabinose	-ve
Sorbitol	-ve
Citrate utilization	-ve
Nitrate reduction	-ve
Lysine utilization	+ve
Ornithine utilization	+ve
Phenylalanine deamination	-ve
Urease production	+ve
H_2S production	-ve
Indole Test	-ve
MR-VP Test	-ve

Fig. 2: *Bacillus sphaericus* strain 2317-2 16S ribosomal RNA gene, partial sequence

Bacillus sphaericus strain 2317-2 16S ribosomal RNA gene, partial
Sequence
Length=1419

Score = 2621 bits (1419), Expect = 0.0
Identities = 1419/1419 (100%), Gaps = 0/1419 (0%)
Strand=Plus/Plus

```
Query  25    TGCAAGTCGAGCGAACAGAGAAGGAGCTTGCTCCTTTGACGTTAGCGGCGGACGGGTGAG  84
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1     TGCAAGTCGAGCGAACAGAGAAGGAGCTTGCTCCTTTGACGTTAGCGGCGGACGGGTGAG  60

Query  85    TAACACGTGGGCAACCTACCTTATAGTTTGGGATAACTCCGGGAAACCGGGGCTAATACC  144
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61    TAACACGTGGGCAACCTACCTTATAGTTTGGGATAACTCCGGGAAACCGGGGCTAATACC  120

Query  145   GAATAATCTGTTTCACCTCATGGTGAAATATTGAAAGACGGTTTCGGCTGTCGCTATAGG  204
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121   GAATAATCTGTTTCACCTCATGGTGAAATATTGAAAGACGGTTTCGGCTGTCGCTATAGG  180

Query  205   ATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGT  264
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181   ATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGT  240

Query  265   AGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGG  324
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241   AGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGG  300

Query  325   GAGGCAGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGA  384
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301   GAGGCAGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGA  360

Query  385   GTGAAGAAGGATTTCGGTTCGTAAAACTCTGTTGTAAGGGAAGAACAAGTACAGTAGTAA  444
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361   GTGAAGAAGGATTTCGGTTCGTAAAACTCTGTTGTAAGGGAAGAACAAGTACAGTAGTAA  420

Query  445   CTGGCTGTACCTTGACGGTACCTTATTAGAAAGCCACGGCTAACTACGTGCCAGCAGCCG  504
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421   CTGGCTGTACCTTGACGGTACCTTATTAGAAAGCCACGGCTAACTACGTGCCAGCAGCCG  480

Query  505   CGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTG  564
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481   CGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTG  540

Query  565   GTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGG  624
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541   GTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGG  600

Query  625   AGACTTGAGTGCAGAAGAGGATAGTGGAATTCCAAGTGTAGCGGTGAAATGCGTAGAGAT  684
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601   AGACTTGAGTGCAGAAGAGGATAGTGGAATTCCAAGTGTAGCGGTGAAATGCGTAGAGAT  660

Query  685   TTGGAGGAACACCAGTGGCGAAGGCGACTATCTGGTCTGTAACTGACACTGAGGCGCGAA  744
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  661   TTGGAGGAACACCAGTGGCGAAGGCGACTATCTGGTCTGTAACTGACACTGAGGCGCGAA  720
Query  745   AGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCT  804
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  721   AGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCT  780

Query  805   AAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGG  864
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  781   AAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGG  840

Query  865   GGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGA  924
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  841   GGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGA  900

Query  925   GCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCGTTGAC  984
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  901   GCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCGTTGAC  960

Query  985   CACTGTAGAGATATGGTTTCCCCTTCGGGGGCAACGGTGACAGGTGGTGCATGGTTGTCG  1044
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  961   CACTGTAGAGATATGGTTTCCCCTTCGGGGGCAACGGTGACAGGTGGTGCATGGTTGTCG  1020

Query  1045  TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAG  1104
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1021  TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAG  1080

Query  1105  TTGCCATCATTTAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGG  1164
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1081  TTGCCATCATTTAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGG  1140
Query  1165  GGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACG  1224
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1141  GGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACG  1200
Query  1225  ATACAAACGGTTGCCAACTCGCGAGAGGGAGCTAATCCGATAAAGTCGTTCTCAGTTCGG  1284
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1201  ATACAAACGGTTGCCAACTCGCGAGAGGGAGCTAATCCGATAAAGTCGTTCTCAGTTCGG  1260
Query  1285  ATTGTAGGCTGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCATG  1344
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1261  ATTGTAGGCTGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCATG  1320
Query  1345  CCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTA  1404
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1321  CCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTA  1380
Query  1405  ACACCCGAAGTCGGTGAGGTAACCTTTTGGAGCCAGCCG  1443
             ||||||||||||||||||||||||||||||||||||||||
Sbjct  1381  ACACCCGAAGTCGGTGAGGTAACCTTTTGGAGCCAGCCG  1419
```

REFERENCES

1. Ahmed I, Yokota A, Yamazoe A, Fujiwara T. Proposal of Lysinibacillus boronitolerans gen. nov. sp. nov., and transfer of *Bacillus fusiformis* to *Lysinibacillus fusiformis* comb. nov. and *Bacillus* sphaericus *to* Lysinibacillus sphaericus *comb. nov. Int J Syst Evol Microbiol. 2007 May; 57(Pt 5):1117-25.*
2. Xiaomin Hu, Wei Fan, Bei Han,et al**.** "Complete Genome Sequence of the Mosquitocidal Bacterium *Bacillus sphaericus* C3-41 and Comparison with Those of Closely Related Bacillus Species." Journal of Bacteriology. April 2008; 190(8): 2892-902
3. Courtis S., Cappellano C., Ball M., et al. Recombinant environmental libraries provide access to microbial diversity for drug discovery from natural products. Applied and Environmental Microbiology.2003;69:49-55
4. Hackl E., Boltenstern S.,, Bodrossy L., and Sessitsch A. Comparison of diversities and compositions of bacterial populations inhabiting natural forest soils. Applied and Environmental Microbiology. 2004; 7:5057-65.
5. Rondon M., August P., Bettermann A., et al. Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. Applied and Environmental Microbiology.2000; 66:2541-47.
6. Satoshi O., Iked H., Ishikawas J., et al. Genome sequence of an industrial microorganisms *Streptomyces avermitilis*: deducing the ability of producing Secondary Metabolites. Applied and Environmental Microbiology.2004; 70:5522-27.
7. Sprusansky O., Zhou L., Jordan S., White J., and Westpheling J. Identification of three new genes involved in morphogenesis and antibiotic production in *Streptomyces coelicolor*. Journal of Bacteriology.2003; 185:6147-57.
8. Zhou J., M. Brunsm, and J. Tiedje. DNA recovery from soils of diverse composition. Applied and Environmental Microbiology. 1996: 62:316-22

****