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Isolation of Soil Bacteria for Potential Production of Antibiotics and their Inhibitory Effect on Growth of Pathogens

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A B S T R A C T

Soil is a richest source of microorganisms. These have played a significant role in antibiotic discovery. Though, thousands of antibiotics were discovered from soil microorganisms, very few were potent or old antibiotics loss their potency or few pathogens became multiple drug resistance. To solve these problems one has to discover new antibiotics to control pathogens. Hence, selective isolation of rare microorganisms from soil may allow the discovery of new antibiotics. Therefore they were isolated and screened for antibiotic production & to find their inhibitory effects on pathogens. It was observed that, two colonies (C2 & C4) of seven antibiotic producing bacterial colonies were identified as *P. aeruginosa strain-1*(C2) *& strain-2*(C4), and remaining unknown colonies were named as C1, C3, C5, C6 & C7. Out of seven bacteria isolated, antibiotics of C1 has inhibition effect on growth of *E. coli*; C2 on *E. coli*, *P. aeruginosa* & *S. aureus*; C3 & C5 on *S. aureus;* C4, C6 & C7 on *E . faecalis*, Further, each of soil bacteria antibiotics shown varied degree of antibiotic effects on different pathogens. C4 has highest inhibitory effect of the growth *of E. faecalis*; followed by C5 on *S. aureus* and C2 on *P. aeruginosa.* The antibiotic effect of some selected soil bacteria, and commercial streptomycin antibiotic has almost equivalent affect on four studied pathogens.

Introduction

Bacteria found in soils can be bacilli, cocci and spirilla. Bacilli are more numerous than others in the soil and are widely distributed (Brock *et al.,* 1991). Many antibiotics are known to exist in nature, but still efforts to discover new antibiotics are continued. In fact bacillus species have produced antibiotics in soluble form and these antibiotics have been found to be cheaper and more effective, hence these microorganisms are preferable for commercial production (Sandhya *et al.,* 2015). The significance of these is mainly in the biotransformation of various raw materials into compost & bioremediation, however their intensive applications in

clinical practices like virulence, production of antibiotics, resistance etc., are still need to be studied.

Pseudomonas aeruginosa is a common gram negative bacillus bacterium that can cause disease in plants and animals, including humans. It is a prototypical "Multidrug resistant (MDR) pathogen" recognized for its ubiquity, its intrinsic advanced antibiotic resistance mechanisms, and its association with serious illnesses such as ventilator-associated pneumonia and various sepsis syndromes. *P. aeruginosa* is not extremely virulent in comparison with other major pathogenic bacteria Sps such as *Staphylococcus aureus* and *streptococcus pyogenes*. Further, among various strains, some are pathogenic, some are antibiotic resistant & some are free living saprophytic bacteria.

P. aeruginosa is the quintessential opportunistic pathogen of humans that can invade virtually any tissue. The most virulent Pseudomonas species produce mucoid colonies and green pigments (Fig.1a & b).

Antibiotics

Antibiotics are medicines that kill bacteria or slow the growth of bacteria. They are used to cure diseases. Antibiotics are natural drugs that are produced by several fungi or bacteria. Over five thousand antibiotics have been identified from the culture of Gram positive, Gram negative and filamentous fungi but only hundred antibiotics have been commercially used to treat human, animal and plant disease. Therefore screening of new antibiotics is still needed. Further, certain pathogenic bacteria are multidrug & antibiotic resistant. This problem can be solved only when new antibiotics are discovered. A major feature of industrial antibiotics production is directed to screening of new potent antibiotics producing organism either from natural sources or from established culture. Screening for antibiotics producing microorganism, can be detected and isolated by the use of highly selective procedure which allows detection and isolation of only those microorganism of interest from a large population is possible. Soil is the largest source of microorganism, & majority of antibiotics so far isolated were produced from Streptomycetes, which are inhabitants of the soil (Srividya *et al.,* 2008). Selective isolation of rare microorganisms may allow the discovery of new bioactive metabolites (Vineeta *et al.,* 2009).

The problem of resistance against the present antibiotics in bacteria increases day by day. So here is an urgent need to search new antibiotics or the sources of new antibiotics. A lot of work has been done during last few decades, that has witnessed the production of novel antibiotics from different microorganisms. Soil is a primary source of microorganisms. Soil bacteria & fungi have played a significant and an important role in antibiotic discovery. The numbers & species of microbes in soil is depend on environmental conditions like nutrient availability ,soil texture ,presence of moisture in soil and type of vegetation cover, and their number varies according to type of environmental condition (Atlas and Barhta,1998) . From ancient times is well understood that, natural products have a key role in the discovery in development of many antibiotics (Newman and Cragg, 2007). One of the best approach to the discovery of new antimicrobial agents natural sources has been to use folklore or historical records to guide the collection of samples or a good research work on the soil of that area (Cordell *et al*, 1994).

Antibiotics are one of the important pillars of modern medicines (Ball *et al*., 2004), but old antibiotics lose their efficiency and they are necessarily replaced with new ones for many species of pathogenic bacteria (Hancock, 2007). Microorganisms that are able to producing secondary metabolites have adverse chemical structure and biological activities and are produced only by some species of genus Bacillus (Stachelhaus *et al*., 1995). Therefore the present research study was carried out to survey on antibiotic producing bacteria from soil of Bidar district (Karnataka state, India) & to find their inhibitory effect on the growth & reproduction of selected pathogens such as S*taphylococcus aureus, Eschereschia.coli, Enterococcus faecalis and* Pseudomonas *aeruginosa.*

Materials and Methods

Soil sample collection

Four different types of soil samples collected based on texture & vegetation covered over the soil from different localities of Bidar such as i) Sacred groove of Papanash temple (forest soil), ii)Near pond of fort (Pond soil), iii) Near Sri Sai chemical Industry, Kolar (Industrial soil), and iv) Agriculture land of Janawada (Rhizosphere soil). They were used immediately for serial dilution & isolation of soil microbes (Fig.2).

Collection of test organisms

Fresh culture of pathogenic bacteria such as *Eschereschia coli, Enterococcus feacalis, Pseudomonas aeruginosa & Staphylococcus aureus* were collected from Azyme Biosciences, Bangalore and sub cultured in nutrient broth for pure line growth.

Preparation of culture plates

Nutrient agar medium & nutrient broth of 500ml each were prepared as per table 1. They were autoclaved, & nutrient agar medium was poured into sterilized petridishes, and nutrient broth was poured into test tubes, and then allowed to cool.

Serial dilution of soil samples & inoculation

Freshly collected each of soil samples was weighed 1gm. Six test tubes were taken and each filled with 9ml of 0.9% NaCl solution & labeled serially such as 1,2,3,4 etc. 1gm of soil was added into first test tube, shaken well & allowed to settle the soil particles at bottom. 1ml of supernatant solution from first test tube was added into the second tube & shaken well. Similarly, 1ml from 2nd tube added into the 3rd tube & these steps repeated till 6th tube to get serial dilutions of 1/10, 1/100, 1/1000, 1/10000, and 1/100000.

The soil sample from last test tube was pour plated on nutrient agar medium for microbial growth for 2 days & number of bacterial colonies was observed & each bacteria was selected based on colour, shape & texture of colony for pure line culture & these unknown colonies named as C1, C2, C3, C4, C5, C6 & C7 (Fig.3). Each selected colony was inoculated in to nutrient broth medium for mass-culture.

Inoculation of test organisms (Pathogens)

The test organisms such as *Eschereschia coli, Enterococcus faecalis, Pseudomonas aeruginosa* & *Staphylococcus aureus* were separately inoculated by swabbing the microorganisms by sterilized cotton on to the culture medium to grow the microbes into lawns. Each of mass cultured pure line bacteria of soil was inoculated into the separate wells & simultaneously, commercial streptomycin disc (as control) was used for comparative study. Culture plates were incubated at 37°C in the incubator for 24hrs to observe the results.

Determination of Antibacterial effect of isolated soil bacteria

After 24hrs of incubation, culture plates were observed for clear inhibitory zone formation around each well (Fig.4). The rate of effect of isolated bacteria on each test organism was detected by measuring the diameter of each growth inhibition zone around well.

Identification of antibiotics producing bacteria

Out of seven colonies selected, two colonies were temporarily named as C2 (round, mucoid, creamy white, raised, smooth & dull colony) & C4 (creamy white,non-mucoid, cloudy, rough colony) based on color, texture (Sandhya *et al*., 2015) & thier maximum effect on pathogens (*E.coli, E. faecalis, S. aureus* & *P. aeruginosa*) and sent them for species idenification to Chromus Biotech, laboratory, Bengaluru (Karnataka). Remaining five colonies were named as such C1 (white, cloudy & rough colony), C3 (orange, smooth & shiny), C5 (Creamy white, cloudy & smooth colony, C6 (white, cloudy & rough colony) & C7 (white, smooth & shiny colony) (Fig.3).

Detection of strains of isolated C2 & C4 bacteria

C2 & C 4 bacteria were detected as *P.aeruginosa* strains (1 & 2) with the help of Chromus Biotech laboratory, B'luru. But their strain name is detected by following DATA BASE. Chart & key points (5a, b, c & d).

Results and Discussion

Various pathogens have got affected by antibiotics of different soil bacteria (table 2). *E. coli* growth was inhibited by soil organisms named C1 & C2 and others like C3, C4, C5, C6 & C7 have not shown any effect on growth of *E.coli.* Pathogen *P. aeruginosa* growth was inhibited by the effect of C2 (strain-1) whereas C1, C4, C6 & C7 have not shown any effect. *E. faecalis* was affected by the antibiotics of C4, C6 & C7 & remaining C1, C2 & C3 have not shown the effect. These above results merely say that, the different pathogens have effected by different antibiotics secreted by different soil inhabiting free bacteria.

Table.1 Composition of nutrient agar medium & nutrient broth medium

Chemicals	**Composition g/1**	
	Nutrient agar medium	**Nutrient broth medium**
Peptone	10	10
Beef Extract	3	3
Sodium Chloride	5	5
Agar	20	--
Distilled Water	1000	1000
P^H	7	7

Table.2 Antibacterial activity of isolated soil bacteria strains against different pathogenic bacteria

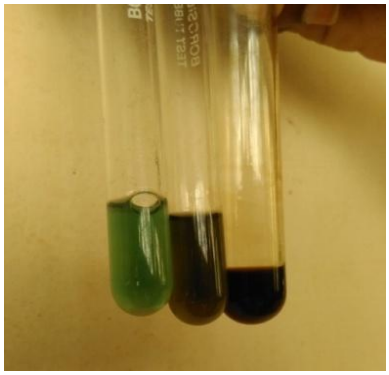
Names of pathogens	**Name of the isolated colonies**							
	C1	C2	C3	C4	C5	C6	C7	Control
Eschereschia coli	+	+	–	–	–	–	–	+
Enterococcus faecalis	–	–	–	+	–	+	+	+
Stapylococcus aureus	–	+	+	–	+	–	–	+
Pseudomonas aeruginosa	–	+	–	–	–	–	–	+

Table.3 Diameter of zone of inhibition of isolated colonies on respective pathogens

Antibiotic producing bacteria	**Sources of soil sample**	**Effects on pathogen**	**Zone of inhibition (cm)**	**Control**
C1	Rhizosphere soil	*E. coli*	0.5	1.7
C2 (identified as *P.aeruginosa*)	Rhizosphere soil	*E. coli*, *P. aeruginosa* *S. aureus*	1.2 1.6 1.0	1.7 1.5
C3	Rhizosphere soil	*S. aureus*	0.6	1.6
C4 (identified as *P.aeruginosa*)	Forest soil	*E. faecalis*	2.0	1.5
C5	Pond soil	*S. aureus*	1.6	1.6
C6	Rhizosphere soil	*E. faecalis*	1.5	1.5
C7	Industrial soil	*E. faecalis*	1.2	1.5

Fig.1 Colonies of *P. aeruginosa* grown on a) agar plate & b) broth, with coloured pigments

a



b

Fig.2 Collected soil samples from different localities of Bidar

Fig.3 Bacterial colonies of different soil samples & round marked selected species.

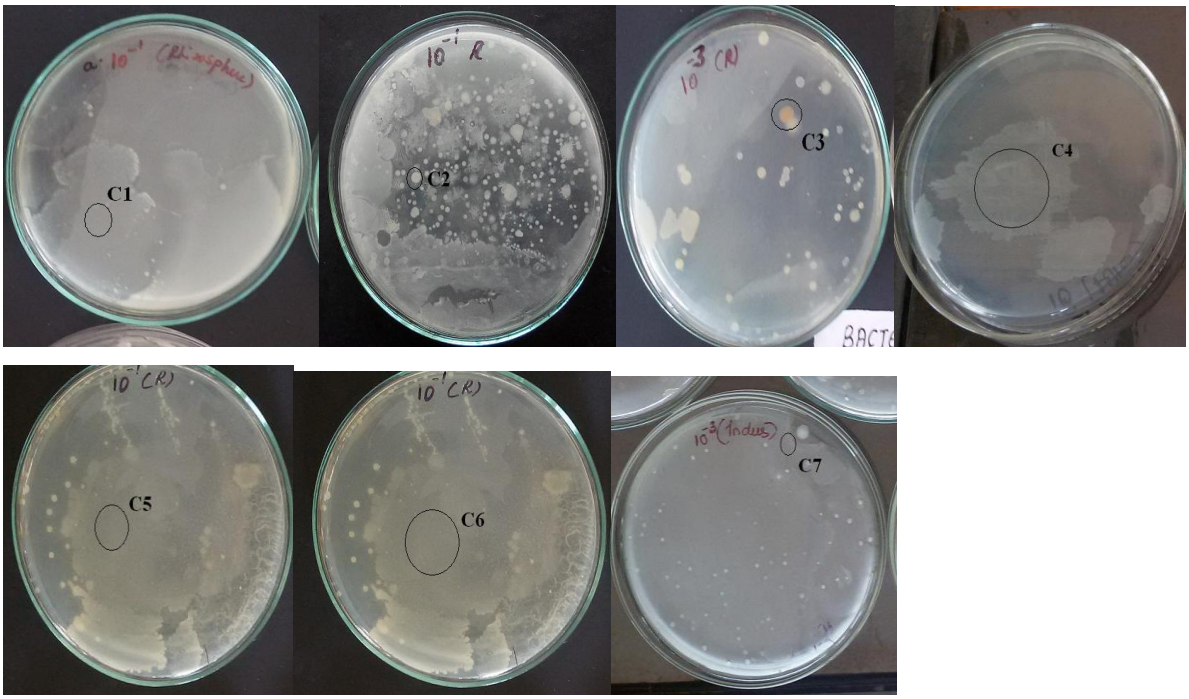


Fig.4 Antibacterial effect of isolated soil bacteria on cultures of a) *E.coli*, b) *S.aureus*, c) *E.faecalis* & d) *P. aeruginosa*

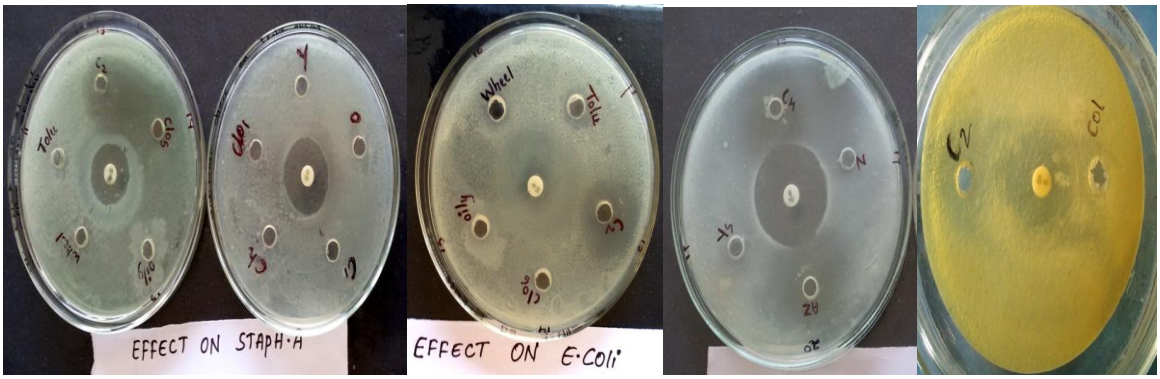


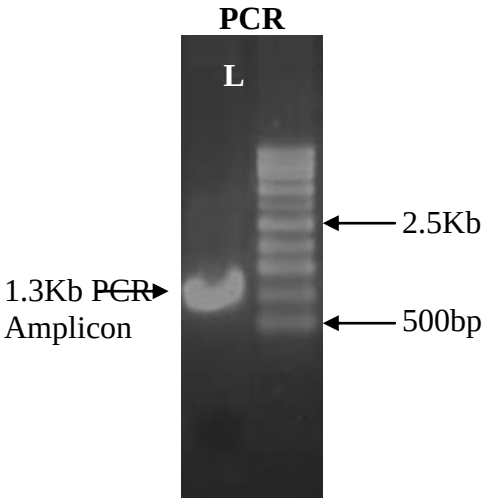
Fig.5a gDNA of the given sample C2

1 .Aligned data of C2 Sample: (1230bp)

GCTGGCAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACGGGCGCTAATACC
GCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGGAT
TAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTC
ACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGC
GAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGG
AGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCG
TGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGT
AGGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTACTGAG
CTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAAC
ACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACA
GGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCCTTGAGATCTTA
GTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAA
TTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTG
GCCTTGACATGCTGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCAGACACAGGTGCTGCA
TGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAG
TTACCAGCACCTCGGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGA
CGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGC
CAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGAATCAGTAGTCCGGATCGCAGTCTGCAACTCGAC
TGCGTTGAAAAA

Gel Photographs:
Genomic DNA:

Fig.5b PCR Amplicon loaded on Agarose Gel



Lane Description:1 – 16s rDNA PCR amplicon of C2 sample (~1.3 Kb)L – 500bp DNA Ladder

Fig.5c Genomic DNA Loaded on 1% Agarose Gel

2. Aligned Data of C4>: (1242bp)

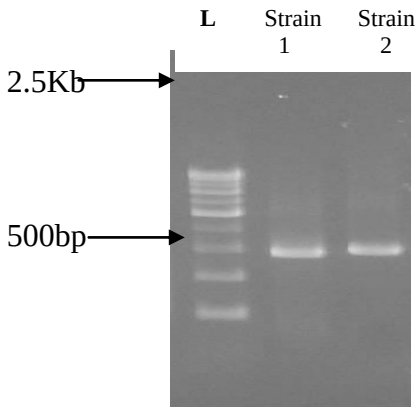
TGCAAGGGTAAATCGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACGGGCGCT
AATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCC
TAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGA
GAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGG
GAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGA
TTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCA
ACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAA
TCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGC
TCAACCTGGGAACTGCATCCAAAACTACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCT
GTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACT
GATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACG
CCGTAAACGATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCGATAA
GTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCAC
AAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCT
GAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCAGACACAGGTGCTGCATGGCTGTCG
TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTAC
CAGCACCTCGGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGA
CGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGG
TTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAA
CTCGACTGCGTGAAGTCGGGAAAATTCCGG

Genomic DNA:

1

Fig.5d PCR Product loaded on 1% Agarose Gel

PCR Product:



Lane Description 1 .C4;

Lane Description: L - 500bp DNA Ladder 1 – C4 (1.3Kb) 2- C2(1.3Kb)

Phylogenetic tree of C2 &C4:

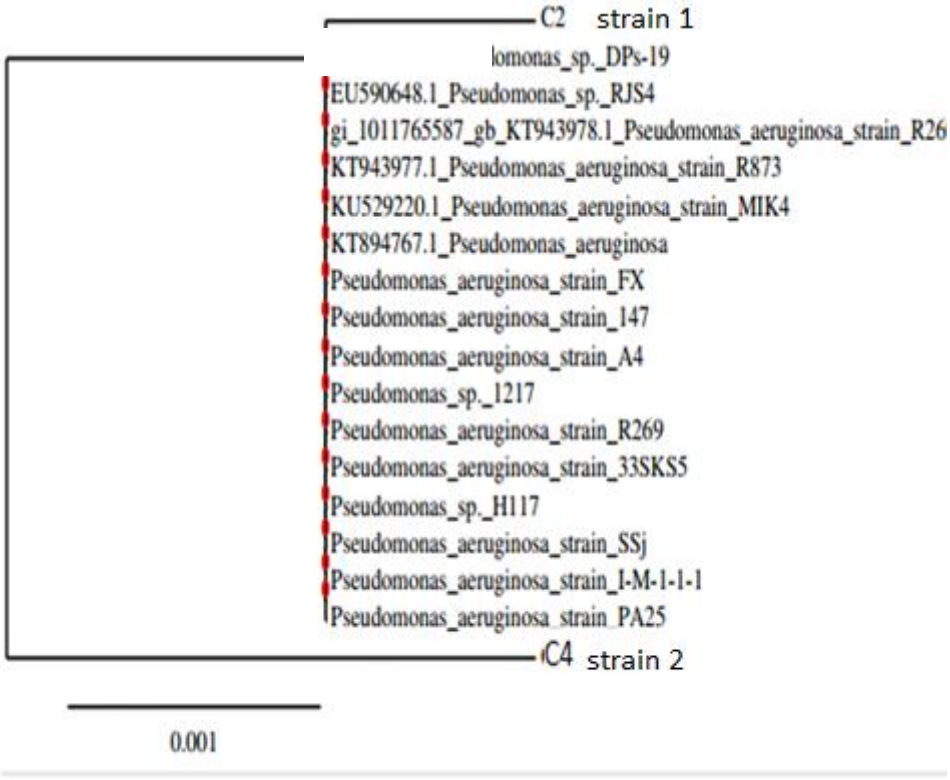


Fig.6 a, b, c &d: Comparison of effect of antibiotics soil bacteria on test organisms

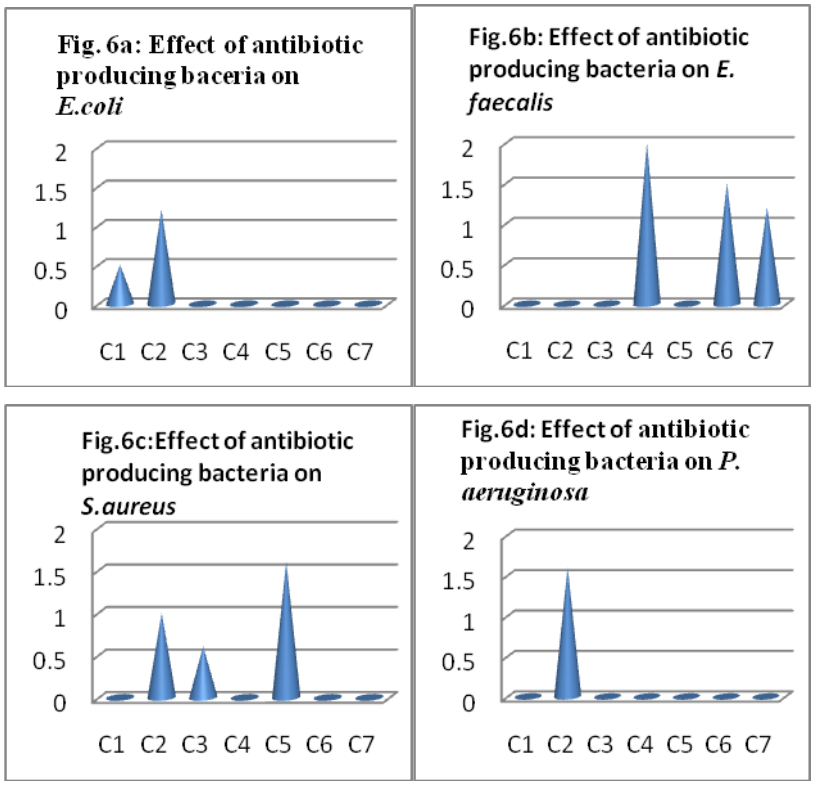
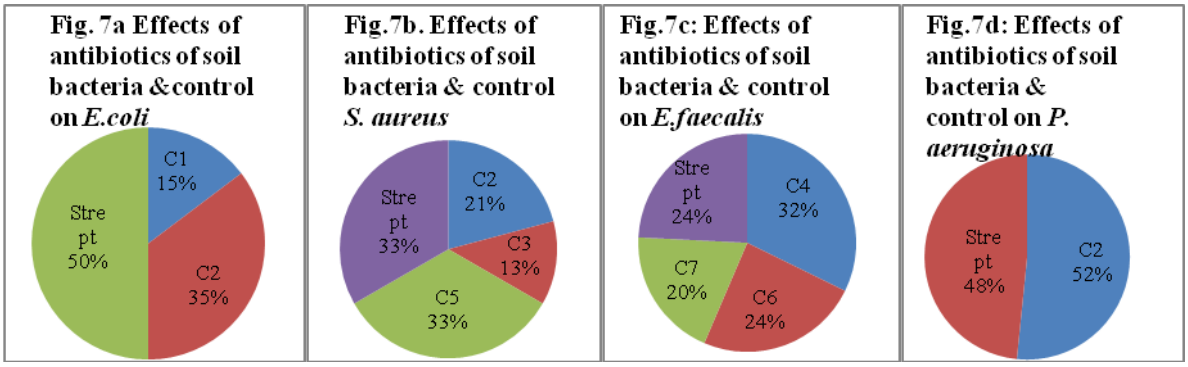


Fig.7 a,b,c&d: Comparisons of effects of antibiotics of soil bacteria & Streptomycin antibiotic on pathogens



Further each of soil bacterial antibiotics shown varied degree of antibiotic effects on different pathogens (Table 3 & Figs. 6a, b, c & d). Soil bacteria C2 on *P. aeruginosa*, C4 & C6 on *E. faecalis* and C5 on *S. aureus* have shown significant antibacterial effect as shown in figs.6a, b, c & d. However, *E. coli* has found to be more resistant to all antibiotics of soil bacteria except C1 which also has less effect on *E. coli*, *E. faecalis* & *S. aureus* pathogens were found more effective (susceptible) organisms by soil bacteria (Figs.9a,b,c & d).

On *E.coli* the C2 is more effective than C1. On *E. faecalis* C6 is more effective than C4 followed by C7. On *S. aureus*, C5 is more effective than C2 followed by C3. On *P. aeruginosa* only C2 has shown growth inhibitory effect.

Antibiotics of soil bacteria C1 and C2 have less inhibition effect on growth of *E. coli* when compared to control (streptomycin antibiotic) fig.7a. Antibiotics of soil bacteria C2 and C3 have less inhibition effect and C5 has equal effect on growth of *S. aureus*, when compared to control (Streptomycin) fig.7b. Antibiotics of soil bacteria C7 has less inhibition effect and C6 has equal inhibition effect and C4 has more inhibition effect on growth of *E. faecalis* when compared to control (Streptomycin) fig.7c. Antibiotics of soil bacteria C2 have more inhibition effect on growth of *P. aeruginosa* when compared to control (Streptomycin) fig.7d.

In conclusion, antibiotics are one of the important pillars of modern medicines (Ball *et al*., 2004), but either old antibiotics lose their efficiency or pathogens were becoming multidrug resistant, and hence they are necessarily replaced with new ones for many species of pathogenic bacteria (Hancock, 2007). From ancient times it is well understood that, natural products have a key role in the discovery in development of many antibiotics (Newman and Cragg, 2007). Majority of antibiotics so far isolated were produced from Streptomycetes, which are inhabitants of the soil (Srividya *et al.*, 2008). Microorganisms that are able to produce efficient secondary metabolites have adverse chemical structure and biologically active are only by some species of genus bacillus (Stachelhaus *et al*., 1995). Pseudomonas species found predominant antibiotic producing bacteria from soil microorganisms. Majority of isolated bacteria were bacillus sps. and they have been found with different coloured pigments such as green, blue-green, brown, creamy white, pure white. Phenazines are heterocyclic compounds that are produced naturally by different bacterial species (Price-Whelan *et al*., 2006). Pyocyanin is water soluble blue green phenazine pigment produced in large quantities by *P.*

aeruginosa. Pyocyanin is chemically N-methyl-1-hydroxyphenazine has antibiotic activity against wide variety of microorganisms. Out of seven antibiotic producing bacteria C1 antibiotic has inhibition effect on growth of *E. coli*; C2 (*P. aeruginosa* Strain-1) antibiotic has inhibition effect on growth of *E. coli*, *P. aeruginosa* & *S. aureus*; C3 antibiotic has inhibition effect on growth of *S. aureus*; C4 (*P. aeruginosa* Strain-2) antibiotic has inhibition effect on growth of *E. faecalis*; C5 antibiotic has inhibition effect on growth of *S .aureus*; C6 antibiotic has inhibition effect on growth of *E. faecalis*; C7 antibiotic has inhibition effect on growth of *E. faecalis.* C2 & C4 colonies were detected as *P. aeruginosa* with two strains. Each of soil bacterial antibiotics shown varied degree of antibiotic effects on different pathogens. C4 has highest inhibitory effect of the growth *of E. faecalis*; followed by C5 on *S. aureus* and C2 on *P. aeruginosa*. The antibiotic of some selected soil bacteria and commercial antibiotics have almost similar & equivalent affect on four studied pathogens.

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