SCREENING OF ENVIRONMENTAL BACTERIA HAVING POTENTIALLY ACTIVE CHARACTERS FOR INCREASING SOIL BIOLOGICAL ACTIVITIES

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ABSTRACT

Sustainable agriculture involves successful management of agricultural resources to satisfy human needs while maintaining or enhancing environmental quality and conserving natural resources for future generations. Improvement in agriculture sustainability will require the optimal use and management of soil fertility and soil physical properties. Both rely on soil biological processes, which are influenced by, soil bacterial diversity, diversified bacteria increase soil biological activity and build up long-term soil productivity and health. Bacterial strains from different environments (soil, air and water) were screened for characters, which have potential for increasing soil fertility. The studied characters were solubilization (P and S in soil) bioabsorbent (water retaining biopolymers) and production of antimicrobial compounds (bactericides and fungicides i.e. biocontrol of plant pathogens).

Keywords: Solubilization, Antibacterial Activity, Bioabsorbent, environment

INTRODUCTION

Bacteria have been on the earth 2 to 3 billion years longer than any other life. They are one of the most diverse life forms on earth and may consist of more than one million species. Only a fraction of these species have been identified, even fewer have been studied and are available in culture collections centers (Hill et al. 2000). The vastness of bacterial diversity is a concept that is overwhelming to the human mind, and our knowledge of the genetic diversity within the bacterial genome is limited (Colwell 1997). The bacterial diversity (especially the genomic diversity) of the earth which although is important but is still untapped and unknown resource of the planet. Bacterial diversity influences nutrient cycling and decomposition, soil structure and biological interactions (De weger et al. 1995). The identification of obvious bacterial functions is attainable, but it is more difficult to further dissect species function and relationships. The present proposal addressed the critical issues of understanding diversity of bacteria in a soil system. The challenge ahead is to identify the level of bacterial diversity, species composition and distribution to maintain the ecosystem and withstand environmental stress. Bacteria possess characters, which are of remarkable scientific and economic interests (Vuichiro et al. 1994).

In Pakistan, agriculture provides a major share of national income and export earning, which ensures food security, income and employment to a large proportion of the population. Deterioration of soil fertility is a major worry while the central paradigm for the biological management of soil fertility is Soil biota. Some bacteria that are associated with the roots of crop plants can exert beneficial effects on their hosts and they are often collectively referred to as PGPR (Plant Growth Promoting Rhizobacteria). PGPR are being exploited commercially for plant protection to induce systemic resistance against various pest and diseases. Biocontrol of phytopathogens appears to be a major mechanism of plant growth promotion by these bacteria. Suppression of phytopathogens results from the production of bacterial secondary metabolites or can be mediated by the plant's own defence system that is elicited by the root-associated bacteria (Induced Systemic Resistance, ISR). The

fluorescent pseudomonads constitute a major group among these PGPR. (http://www.agr.kuleuven.ac.be/)Seed treatment with PGRP causes cell wall structural modification and biochemical physiological changes leading to synthesis of proteins and chemicals involved in plant defense mechanisms. Lipopolysaccharides, siderophores and salicylic acid are the major determinants of PGPR mediated ISR (Introduction of Systematic Resistance) (Rammamoorthy et al., 2001). PGPR affect plant growth by synthesizing phytochromes, increasing availability and uptake of nutrients to enhanced plant height and productivity, decreasing heavy metals toxicity, antagonizing plant pathogens and including systemic resistance in plants to pathogens (Burd et al, 2000).

Plant diseases, caused primarily by fungal and bacterial pathogens, cause severe losses to agriculture and horticultural crops every year. These losses result in reduced food supplies poorer-quality agricultural products, economic hardships for grower and processes and ultimately, higher price. Traditional chemical control methods are not always economical or effective for many diseases, in some chemical controls may have unwanted health, safety and environmental risks (Rovera *et.al.*1998).

Biological control involves the use of beneficial microorganisms, such as specialized fungi and bacteria, to attack and control plant pathogens and the disease they cause. Biological control offers an environmentally friendly approach to the management of the plant disease and can be incorporated with cultural and physical controls and limited chemical usage for an effective and integrated disease management system (Rovera *et.al.*1998). These natural 'antibiotics' creating a zone of inhibition of varying diameter where certain pathogens cannot exist surround the colonies of *L. acidophilus* and *bulgarius*. *Acidophilin* and *bulgarican* have been shown to inhibit the growth of the food poisoners *Clostridium botulinum, Staphylococcus aureus, Escherichia coli* and some *Salmonella* species. It is important to note that unlike penicillin and other pharmaceutical antibiotics that destroy both pathogenic and friendly bacteria, the natural 'antibiotics' produced by lactic bacteria do not attack friendly microbes. It is also interesting to note that the virulent strains of bacteria that are becoming increasingly resistant to commercially produced antibiotics do not mutate against natural intestinal flora like Lactobacilli (http://www.nrdc.org). Today as a result of extensive research, the study of microbial products were recognized as an integral component of natural products chemistry and as well as they are significant resource for environment friendly compounds. Eco-friendly compounds have multiple industrial and agricultural applications. This work was carried out to characterize the bacteria from different environmental sources for the production of commercially and agricultural important products.

MATERIALS AND METHODS

Isolation, purification and growth conditions

Five bacterial strains were selected which were from the CMG stock; CMG645, CMG646 and CMG648 these strains were from marine origin and one was isolated from the drain water, which has later on given the code of CMG649, one was isolated from the garden mud which was later on given the code of CMG650. These isolates were grown on the nutrient agar and the purification was done by streaking and restreaking and the cultures were preserved on nutrient agar slants at 4ºC. The bacterial strains were grown on nutrient agar plates supplemented with the commercially available antibiotics like Kanamycin (Km), Tetracycline (Tc), Chloramphenicol (Cm), Streptomycin (Sm) and Ampicillin (Am), with varying concentrations such as 25µg/ml, 50µg/ml, 100µg/ml, 200µg/ml, 300µg/ml. Plates were incubated at 37ºC for 48-72 hours and their maximum tolerable concentrations (MTC) were determined. Stock solutions of antibiotics were prepared as described by Maniatis (1992).

Screening of Bacterial isolates for plant growth promoting characters

1. Solubilization

All the bacterial strains were characterized and screened for different properties like bioabsorbent production, solubilization of insoluble inorganic metal salts and antibacterial activity. Bacterial strains were screened for ability to solubilize, insoluble inorganic metal compounds, based on clear haloes

around bacterial colonies. Tris media (liquid and solid) amended with in-soluble inorganic metal salts were used to detect the solubilization activity. Solubilization was checked on Tris minimal media having composition as follows, (gm/L) Tris HCl, 6.06; NaCl, 4.68; KCl, 1.4; NH_4Cl, 1.07; Na_2SO_4, 0.43; $MgCl_2.6H_2O$, 0.7; $CaCl_2.2H_2O$, 0.003; Carbon source 0.2%. pH adjusted at 7.00 with the HCl. After autoclaving the media Zinc salts were added in the medium. (Fasim *et. al* 2002)

2. Bioabsorbent biopolymers

Bacterial isolates, producing bioabsorbent polymer, were screened by ethanol precipitation method. Broths of all bacterial isolates were treated with ethanol. All strains were grown in *E. coli* medium. Cultures were grown at 37ºC at 180 rpm on shaker in an incubator for 24 hours. After 24 hours these cultures were treated with 70% ethanol to detect the presence or absence of the bioabsorbent polysaccharide production.

3. Antibacterial activity

Bacterial isolates were studied for their antibacterial activity by a method known as Spot-on-the-lawn deferred antagonism method (Haris *et al.,* 1989). Antibacterial activity was checked on nutrient agar. Lawn of a sensitive strain was spread over the nutrient plate and a drop of the culture (to be tested) was placed in the center of the plate. Plates were incubated at 37°C for 24 hours. Antibacterial activity was measured by the appearance of zone of inhibition around the culture. The strains scored positive by the deferred antagonism method were then tested for direct or well diffusion assay (Muriana *et al.,* 1987).

Effect of Different Factors on Antibacterial Activity

After two hours difference, culture broth of the selected bacterial strains were removed and supernatant was inoculated to the nutrient agar plates having the lawn of the sensitive strain to check the production of antibacterial activity appears in the medium. Supernatant was obtained by centrifugation, and then supernatant was filtered by millipore filter paper (0.2µm) and used for antibacterial activity testing.

For the identification of the nature of antibacterial compound/s and to check the effect of enzymes the culture of selected strains was treated with the enzymes. For this purpose the culture of selected bacterial strains were picked up from center of a plate of deferred antagonistic assay (Naz *et al*, 1993) and inoculated to 5 ml luria broth and after 24 hours this 5 ml of each strain was distributed in five eppendorfs. These cultures were centrifuged at 14,000 rpm for 5 minutes. Supernatant was drawn out from the eppendorfs tubes and then it was filtered with millipore filter paper (0.4 µm) and then this filtrate was treated with enzymes. Enzymes, which were used, were protease P, protease K, pepsin, lysozyme and RNase. The activity of enzyme treated supernatant was tested by the "agar-well diffusion method". To know whether the antibacterial activity was heat sensitive or not the supernatant was heated at 121°C for 5 minutes. Later the activity of heat-treated supernatant was tested by the agar-well diffusion method.

Crude Extraction of Antibacterial Compound

Bacterial isolate CMG 646 having antibacterial activity was inoculated on the lawn of a sensitive strain and after 24 hours the zone of inhibition was observed. Agar piece having zone of inhibition was cut and dipped in diethyl ether in schott duran bottle to get a crude extraction of antibacterial compound. As the piece of agar was also added so in another schott duran bottle only agar was added to the diethyl ether to use it as a negative control. Extracted samples were air dried for further analysis.

Analytical Chromatography

Crude extract having antibacterial compound were chromatographed on T.L.C aluminum sheets (20x20 cm, silica gel 60 F254 MERCK), to get separate spots of different compounds present in the crude extraction, by ascending method using a solvent system (v/v) of diethyl ether: water in 1:1 ratio and detected by spraying with aniline phthalate solution. And then it was observed under U.V lamp.

Silica gel was developed twice to get better separation. Control was also run to exclude the similar bands appeared in the crude extract and the control.

Purification of Antibacterial Compound

Crude extract was developed twice on a large silica gel cards to get bands clear apart, these bands from the silica cards were cut separately and marked as spot 1to 6. Square pieces of silica cards were placed in diethyl ether to allow the compound to get dissolve in the diethyl ether, while silica cards without compound were also dipped in diethyl ether and it was used as a negative control.
After the complete dissolution of compound/s Whatman filter paper discs were soaked in the diethyl ether having dissolved compound and in the control also. A filter paper was also soaked in the simple culture as a positive control. All these filter papers were then placed on a plate having lawn of a sensitive strain (*Staphylococcus aureus*) and the plate was incubated at room temperature.

RESULTS AND DISCUSSION

Pakistan is an agriculture –based country about 60% of our population is currently related to the agriculture and it provides a major share of national income and export earning, which ensures food security, income and employment to a large proportion of the population. Improvement in agriculture sustainability will require the optimal use and management of soil fertility and soil physical properties. Soil bacterial diversity, increase soil biological activity and build up long-term soil productivity and health. It is believed that their diversity with respect to selected characters (antibacterial activity, solubilization and bioabsorbent biopolymer) could be used for improving fertility of agricultural lands.

Bacteria at different stages during their growth produce different types of compounds mainly divided into primary and secondary metabolites. Bacterial metabolites are important not only in biotechnological research but also gaining importance for commercial purposes. Some of the bacterial products are considered as the best substitute of the synthetic products, because synthetic products are not eco-friendly and can not be easily degraded or when degraded increase pollution. There fore those bacterial products, which can replace the synthetic products, is gaining importance in industrial and agriculture areas.

In this context, in this study, bacteria from different environments were isolated to analyze for the production of secondary metabolites, which have potential to be used as bioinoculants. Center for Molecular Genetics culture stock was screened for selected characters and three bacterial strains were selected form CMG stock i.e. CMG645, CMG646 and CMG648. All of them were marine and collected from different sites in the sea. As all these strains selected from CMG stock were marine so some terrestrial strains were also isolated to study some of the terrestrial bacteria for the production of commercially important bacterial products.

Bacteria isolated were given the name of the environment from where they were isolated i.e. drain water isolate (DWi), garden mud isolate (Mi) later these were given the codes CMG649 and CMG650 respectively. All these selected bacterial strains were grown on nutrient media Streaking and restreaking was done in order to obtain pure culture. CMG stock was already identified and CMG645 and CMG646 were found to be *Pseudomonas sp;* CMG648 was found to be *Klebsiella sp.*

CMG645, CMG646, CMG649 produced green pigment on nutrient agar medium while other gave off white colonies on nutrient agar. All these strains were stained with Gram staining procedure described by Gram (1994) to study cellular morphology and purity of the culture. Out of three isolates from CMG stock and isolated all were Gram-ve. All of these bacterial strains were grown in nutrient broth and after 24 hours they were used in screening for the production of bioabsorbent polysaccharide for water absorbing capacity, solubilization compounds for solubilizing insoluble inorganic metal salts and antibacterial compounds active against disease causing bacterial strains.

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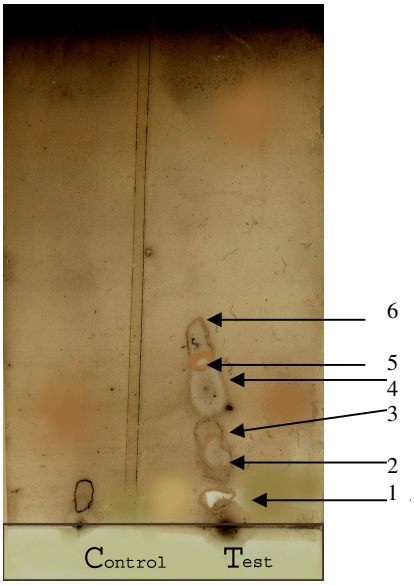


Fig. 1 Thin Layer Chromatography
Cont. (Plane agar piece treated with solvent and run as control),
Test Sample, (Crude Extract from Deferred Antagonistic
Activity Plate of CMG646 against lawn of *Staph. aureus.*),
Numbers 1to 6 show the spots (compound) present in crude extract.

Table 4 Rf values of the spots observed by Thin Layer Chromatography
for Antibacterial Activity of CMG646

Isolate CMG 646	**Distance**	**Solvent front =6.3cm**	**Rf value**
	4mm		0.063
	8mm		0.126
	11mm		0.174
	13mm	***Rf = b/a**	0.206
	25mm		0.396
	27mm		0.428

***Rf = Distance starting line - solvent front = a**
Distance starting line - middle of spot = b

All of these bacterial strains were grown in nutrient broth and after 24 hours they were used in screening for the production of bioabsorbent polysaccharide for water absorbing capacity, for solubilizing insoluble inorganic metal salts and antibacterial compounds active against disease causing bacterial strains.

MTC's of isolates were checked for five antibiotics on asset of nutrient agar plates containing different concentrations of antibiotic compound. The antibiotic compounds tested were ampicillin, tetracycline, chloramphenicol, streptomycin and kanamycin. CMG649 and CMG645 andCMG650 proved to be more resistant to most of the antibiotics. MTC's of antibiotics for the selected strains CMG645, CMG646, CMG648, CMG649 and CMG650 were determined. CMG645 was found to be resistant to Tc, Cm, Amp, up to 200µg/ml, 300µg/ml, and 300µg/ml respectively. It is sensitive to Km, and Sm, CMG646 was resistant to Cm, and Amp, both up to 300µg/ml. CMG648 was found to be resistant to only Amp, up to 300µg/ml. CMG649 was resistant to Sm, Cm, Km, Amp, up to

200µg/ml, 300µg/ml, 100µg/ml, 300µg/ml respectively. CMG650 was found resistant to all five antibiotics used i.e. Sm, (300µg/ml), Tc, (100µg/ml), Cm, (100µg/ml), Km, (200µg/ml), Amp, (300µg/ml). These suggest that except CMG648 all the selected bacterial strains are resistant to drugs belong to β-lactum group. High resistance like CMG645 to Tc, (200µg/ml), Cm, (300µg/ml), CMG646 Cm, (300µg/ml), CMG649 Cm, (300µg/ml), CMG650 Tc, (100µg/ml), Cm, (100µg/ml) shown by most of the selected strains to Tc, And Cm, is might be due to an alternative metabolic pathway or enzymes reaction. Second possibility is that these organisms acquired the resistance genes are present on the plasmids (Gale *et al*. 1981).

Biopolymers are large molecules formed by the polymerization of many identical or two or more kinds of fundamental units under biological process by living organisms. They can be biologically active molecules and offer a number of novel material properties and commercial opportunities. The ability to produce biopolymer is direct and logical response to selective pressure in natural environment (Weiner *et al.*1997). Extracellular polymers are usually composed of high molecular weight capsular polysaccharides these include cellulose, (Carpita and Vergar, 1998) xanthane (Sutherland, 1998) dextrane (Aslop 1983) gellan (Pollock 1993), a pullalan etc. Bacteria especially of marine and soil origin have been reported for bioabsorbent polymers. These have the ability to absorb water significantly more than its own weight. Bioabsorbent biopolymers are polysaccharides extracted from bacterial sources having high water absorbing capacity and can retain water for a long period of time (Weiner 1997). When treated with water, they form gel like substances they easily degradable and do not cause pollution problems. They can be used in agriculture they enhance water holding capacity of the soil and in sanitary products especially baby diapers. Their production is effected by growth condition. They have application in food industry, oil industry and in medicine. Bioabsorbent biopolymer production was checked in all selected bacterial strains, which were grown in nutrient broth and after 24 hours they were used in screening for the production of bioabsorbent polysaccharide for water absorbing capacity, production of biopolymer were checked by treating the culture with ethanol, which indicates that these bacterial strains produce exopolysaccharides (EPS) because ethanol lyses the bacterial cell wall thus making the attached EPS to precipitate and become visible. These selected bacterial strains produce off-white precipitates after treating with ethanol. CMG646 gave a bit different results in a sense that it not only produced the off-white precipitates but also a gel like material given the name of gelatin, this showed that probably CMG646 produced two types of polysaccharides, one was in the form of a gel and another was in the form of precipitates. These precipitates were then analyze for water absorbing capacity and showed 13% water absorbance as compare to their relative weight. Time of incubation is directly proportion to production rate and water absorbance capacity (Fig 3). On Tris minimal media all the selected bacterial strains showed solubilization zone for their respective metals. Solubilization activity was detected by the disappearance of added mineral particles i.e. insoluble inorganic Zn and production of clear zones around the growth.

Table 1. Quantitative measurements of Antibacterial Activity by Bacterial cells Inoculation Antibacterial Activity after 24 hours.

S.No	Lawns → ↓ Inoculums	CMG 641	CMG 642	CMG 644	CMG 645	CMG 646	CMG 647	CMG 904	CMG 1306	K2
1	CMG645	-	+	-	-	-	-	-	-	-
2	CMG646	-	-	-	+	-	-	-	-	-

Analysis of antibacterial activity

Antibacterial activity of all bacterial strains was checked using the preliminary screening test for bacteriocins i.e. "Deferred antagonistic assay" (lawn of a sensitive strain with drops of culture to be tested as antibacterial compound producing strain)(Naz *et al.* 1993). Eight strains showed antagonistic activity (antibacterial activity) against indicator strains, the strains that were used as indicator are *Bacillus subtilis* (CMG904), *Bacillus polymxa* (CMG644), *E.coli* (CMG642), *Klebsiella oxytoca* (CMG641), CMG1306 (Uncultured bacterium clone), and *Staphylococcus aureus* (CMG1025).

Strains which scored positive by the deferred antagonism assay to detect by "Agar well diffusion assay" to detect the inhibitory activity in liquid cultures (Table 1 & 2). Antibacterial activity of the culture was tested through agar well diffusion method, and readings were taken after 12 hours interval up to 3days.It was observed that the zone appeared within 8 hours after inoculation, but it was very small, and it remain increasing till 24 hours rapidly, after which it slows down which indicate that the organism has stopped producing the products not essential for its own growth, due to the nutrients depletion in the medium, however when the same organisms were inoculated in the fresh medium they again start producing the antibacterial compound. Same procedure was done with the supernatant of these bacterial strains. Quantitative analysis of antibacterial compound showed that it is present in the supernatant and can produce zone of inhibition as in the case of culture it self. Two sets of plates were used; one was inoculated with 12 hours old supernatant and one set of plates were inoculated with 24 hours old supernatant this was done to check that up to which time the antibacterial compound remains in the supernatant, and this was observed that zones appeared in the 12 hours old supernatant and also increased up to a certain level when checked after 12 hours of inoculation but in case of 24 hours old supernatant this was observed that either the zone was not produced or if otherwise produced were not increased significantly only CMG645 and CMG646 gave zones against CMG644 (0.7 cm) and CMG641 (0.4 cm) respectively (Table 2).

Table no. 2 Antibacterial Activity after 48 hours.

S.No	Lawns → ↓ Inoculums	CMG 641	CMG 642	CMG 644	CMG 645	CMG 646	CMG 647	CMG 904	CMG 1306	K2
1	CMG645	0.3	1.9	1.06	-	-	-	-	-	-
2	CMG646	0.4	4.3	0.7	1.4	-	-	-	-	-

Digits in columns show the size of zone of inhibition in cm

Supernatant inoculations at two hours interval during growth showed that antibacterial activity does not appear in the supernatant before 4 hours after inoculation. By filtering the culture with Millipore filter paper the bacteria free supernatant was inoculated and which showed the antibacterial activity thus it means that the antibacterial compound is released by the bacterial strains in the supernatant. In CMG649 this activity appear in the supernatant round 6^{th} hours of the growth and persists up to 18^{th} hours of the growth. In CMG 646 it appears at 8^{th} hours. of growth and persists up to 34^{th} hours of the growth. While in CMG 645 it appears at 8^{th} hours. and persists up to 24^{th} hours. CMG646 shows the maximum retention of the antibacterial compound in the supernatant. Treatment with enzymes showed different results in different strains. In case of CMG646 positive control plate showed the appearance of the zone of inhibition that is a positive result. In case of CMG646 only pepsin has stopped the activity of CMG646, while protease K, protease E, RNase, Lysozyme had no effect on the activity (Table 3).

Table 3. Qualitative Analysis of Antibacterial Activity.
Antibacterial Activity of enzymes treated Supernatant of CMG646
Inoculum CMG646

Lawn →	With out any inoculum (+ve control) CMG1306		CMG 1025				CMG 645				CMG 641			
Hrs →	12	24	12	24	36	48	12	24	36	48	12	24	36	48
-ve Control	-	-	+	+	+	+	-	-	-	-	+	+	+	+
Protease K (10ug/ml)	-	-	+	+	+	+	-	-	-	-	-	-	-	-
Protease E (10ug/ml)	-	-	+	+	+	+	-	-	-	-	-	-	-	-

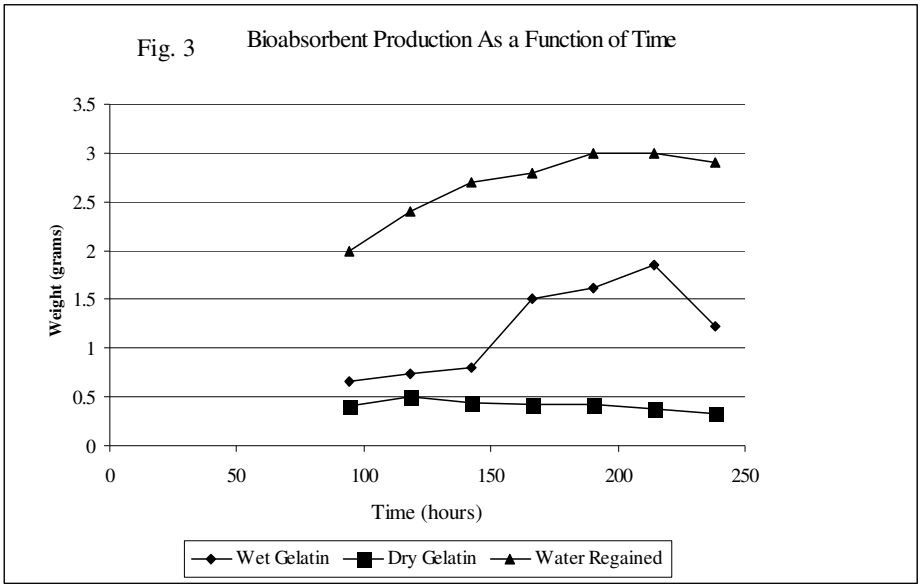
RNase (1ug/ml)	-	-	+	+	+	+	-	-	-	-	-	-	-	-
Lysozyme (10ug/ml)	-	-	+	+	+	+	-	-	-	-	+	+	+	+
Pepsin (10ug/ml)	-	-	-	-	-	-	-	-	-	-	-	-	-	-

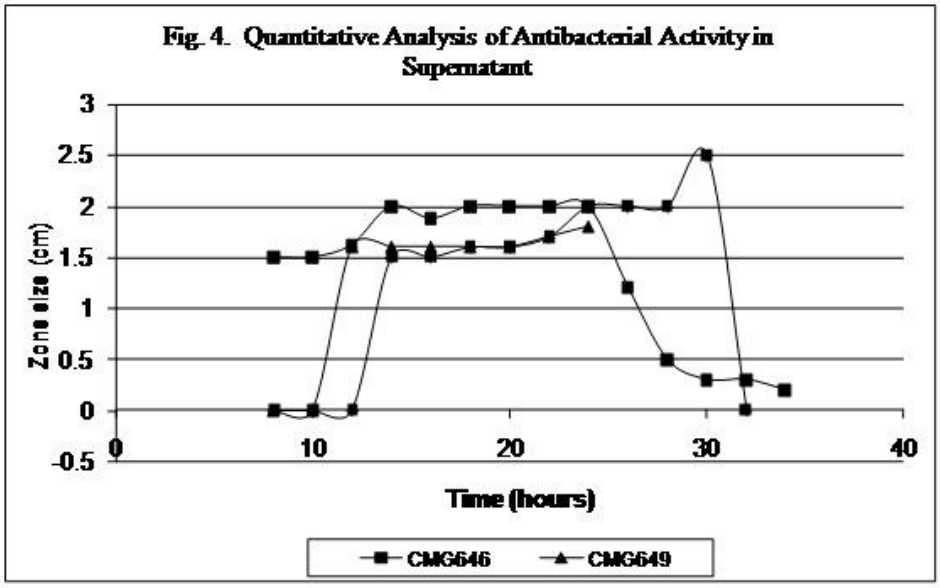
+ Sign shows the appearance of zone of inhibition, - Sign shows the absence of zone of inhibition

Supernatant of the bacterial strains cultures were heated and it was observed that heat destroyed the antibacterial activity of all the five selected bacterial strains. Which showed that the compound was heat sensitive (Table 3). For the chemical analysis T.L.C was done and for this purpose bacterial strain was grown on the nutrient agar having lawn of sensitive strain and after 24 hours the piece of gel from the zone of inhibition was dissolved in the diethyl ether and then this diethyl ether was allowed to evaporate and this was done twice and a thick mixture was prepared, which was yellow in color. T.L.C. or thin layer chromatography is similar to paper chromatography, but instead of a paper, an aluminum sheet coated with a thin layer of an absorbent material, such as finely powdered silica gel or aluminum oxide, is used. Spots of solution are applied onto a base line near the slide's lower edge, and the slide is then placed into a shallow bath of solvent. The solvent travels upward by capillary action, passing through the point at which the sample was applied, carrying the component with it at different rates. The T.L.C. plate is developed with aniline pthylate spray and viewed under ultraviolet light to reveal each component separated by T.L.C cards (Fig. 1).

After the development of the silica gel card, the card was divided into six portions with respect to the spots, each spot was separated from other by cutting the silica card in form of silica strips, then each strip was cut into small pieces which were then dip into diethyl ether to extract the individual spot into the solvent and solvent was air dried and finally all the silica gel got dissolved. Now filter paper strips were dipped in the purified extracts along with the positive control i.e. the pure culture and negative control i.e. the silica gel dissolved in the diethyl ether. These filter paper strips were placed on the plate having bacterial lawn of *Staphylococcus aureus* and it was observed that within six hours the zones of inhibition were produced. And it was observed that two spots showed antibacterial activity not all. T.L.C of crude extract from CMG646 showed six spots with different Rf values (Table 4) three of which were aniline pthylate spray sensitive and three were U.V sensitive (Fig. 1) and two of which showed antibacterial activity upon inoculation in the plate having *staphylococcus aureus* lawn. As the bacterial strains CMG646, CMG645, CMG649 showed antibacterial activity against different indicator strains with the same intensity, so it seems that they show broad-spectrum antagonistic antibacterial activity and hence they are the producers of strong antibacterial compound.

Fig. 3 Bioabsorbent Production As a Function of Time





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