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A Simplistic Screening Assay of Antimicrobial Compounds and Enzymatic Activity from Local Soil Microbes

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ABSTRACT

Antibiotics production is the most emerging field worldwide with a constant need for the new ones to fight the microbial resistance. In this context, the research was pursued to isolate, characterize and screen for promising antibiotic-producing microbes from local soil. The soil bacterial isolates (S1, S2, S3,S4, and S5) and fungal isolates (F1, F2, F4, F6, and F7) were selected and screened for antimicrobial activity against the Test bacteria by agar well diffusion and disc diffusion methods. Assays for Extracellular enzymes including Protease, Lipase, Lecithinase, Cellulase, and Amylase following the substrate hydrolysis were performed on different Agars such as Casein Agar, Tween 80 Agar, Egg Yolk Agar, Carboxymethylcellulose Agar, and Starch Agar respectively. The isolated microorganisms which produced antimicrobial compounds were identified as Bacillus, Actinomycetes, Streptomycetes, *H. werneckii, A. niger, A. flavus, A. fumigatus* and *P. notatum* on the basis of their cultural and microscopic characteristics and their optimum growth. The antimicrobial activity was determined by varying pH and NaCl concentrations. The research work revealed that among all isolates *Actinomycetes* (21%), *P. notatum* (29%) and *H. werneckii* (21%) showed maximum bioactivities against the test organisms and all isolates exhibited at least four of the tested enzymes.

Keywords:
Antibiotics, Soil microorganisms, Enzymatic assay, Optimization.

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INTRODUCTION

The term 'antibiotic' means 'against life'. An antibiotic was initially characterized as a substance, produced by microorganism [1, 2], which at low concentrations can inhibit the growth and development of other microorganisms[1,3]. Soil is a primary source of diverse type microorganisms. Most of the novel antibiotics have been detected by screening of "wild isolates" from the soil. First best-known and most broadly exploited antibiotic was Penicillin[2,3]. After the discovery of penicillin, different antibiotics were procurer. In 1939, work begins on the isolation of potential anti-microbial products from the soil microorganisms such as Streptomycin. Other antibiotics that have been discovered since including Bacitracin, Polymyxin, Chloramphenicol, and Tetracycline. Scientists have discovered several mechanisms of action of antibiotics. These antibiotics target cell wall, proteins, and nucleic acid synthesis[1,5]. Soil microorganism synthesizes antibiotics and show excellent enzymatic activity by consuming nutrients degraded by various commercially important enzymes. Enzymes are biocatalysts produced by living cells for specific biochemical reactions and metabolic processes of the cell. Enzymes are present in

each living cell, including all microorganisms[4]. Single strain of microorganisms may produce a galaxy of enzymes, hydrolyzing, oxidizing or reducing, and are metabolic in nature. Consequently, it is logical to choose strains for the industrial enzymes which are produced insignificant amount. Commercial enzymes are produced by molds, bacteria, and yeast etc[3]. Ever since the possibilities of industrial uses of microbial enzymes have increased significantly in 21st century increasing as such enzymes have great potential for many industries to meet the demand of humans[1].

Among hydrolytic enzymes, Proteases play a pivotal role with respect to their applications in both physiological and commercial fields. Proteolytic enzymes catalyze the cleavage of peptide bonds in proteins, and microorganisms produce a large array of intracellular and/or extracellular proteases. Casein Agar is a medium utilized for the recognition of hydrolytic microorganisms. Proteins are comprised of different chains of amino acids held together by peptide bonds, and hydrolytic enzymes hydrolyze these peptide bonds[10].

Starch particles are hydrolyzed by amylases to yield assorted products, like dextrin and dynamic polymers composed of the units of glucose[10]. Alpha-amylases are the starch-converting enzymes which have the great importance in industries. For amylases, Starch Agar is widely used that manifest the capacity of a microorganism to produce certain hydrolytic exo-proteins, including alpha-amylase and oligo-1, 6-glucosidase[8].

Carboxymethylcellulose (CMC) including (cellobiohydrolase and beta-glucosidases, which are broadly known as cellulases, hydrolyze the glycosidic bonds of cellulose molecules. CMC screening by microorganisms was performed on different agar plates having selective substrates like CMC. [4] In these cases the cellulolytic actions were checked by staining or having zones like precipitation of substrate observed in CMC plates. Whereas clear zones of restraint encompassing the wellspring of the enzymes. An assortment of colors has been utilized for the differential staining, the most well-known stain being the Congo red[8,10].

Bacterial lecithinases are of extraordinary intrigue on account of the conceivable part of these proteins in pathogenicity. Probably, the most critical contaminants associated with nourishment poisonous quality are lecithins. The bacterial compound is a zinc protein. Egg Yolk Agar (EYA) is a differential medium. The incorporation of lecithin in the egg yolk brings about a misty precipitation around the colonies[10].

Lipases catalyze the hydrolysis of long-chain triglycerides. Tweens, for example, Tween 80 (unsaturated fat esters of polyoxyethylene sorbitan) sought after have been the most extensively sought after substrates for the area of lipolytic microbes in a chromogenic culture media and as fluoro-genic substrates. The methodology relies upon the precipitation, (as the calcium salt), of the unsaturated fat released out of hydrolysis of Tween. Concerning the character of Tweens as lipolytic substrates, there are a few reports of Tweens for measuring the lipase trial of esterase, and at times for a "Tweenase" or "Tween-hydrolyzing" activity. Enzymes applications in pharmaceutical industry are as broad and fast developing[8].

The biosynthesis of anti-infection agents like other microbial metabolites is controlled by various factors like growth conditions, carbon, nitrogen, mineral salt levels and physical parameters like temperature, pH, and agitation during production[9]. Moreover, the evolution of drug-resistant microorganisms warrants for an enhanced search for new secondary drugs with the new structure[6]. In this manner, new antibiotic-producing microorganisms and new resources must be tapped the screening program. In this context, local soil samples were collected and analyzed[7].

MATERIALS AND METHODS

***Sample Collection*:**

A total of 10 soil samples (20 grams of each) collected from different locations in Karachi. Each sample was scooped from a larger volume and was put in a separate plastic bag under aseptic conditions. The plastic bags containing soil samples were marked and stored at 4°C for further work[1]. Test microorganisms included: *Escherichia coli* (Gram-negative), *Proteus vulgaris* (Gram-negative), *Pseudomonas aeruginosa* (Gram-negative), *Staphylococcus aureus* (Gram-positive), *Candida albicans* (yeast). All cultures were preceded from *DR. ESSA LABORATORY AND DIAGNOSTIC CENTRE*[1].

Isolation of Soil Microorganisms:

One gram of soil sample was diluted into 15 ml falcon tube to which 9 ml of distilled water for serial dilution. Ten-fold serial dilution was carried out, 0.1 ml of 10^{-3} and 10^{-4} were poured in respective plates of Potato Dextrose Agar, Nutrient Agar, Czapek Dox Agar. The plates were incubated at 37°C, 25°C for 24 hours and 72 hours respectively[1,2].

Screening for Antibiotic Producing Bacteria:

Soil microorganisms which were grown on Potato Dextrose Agar and Nutrient Agar were overlaid by soft agar (0.75%) seeded with test strains. Incubated the plates at 37°C for 24 hours. The zones of inhibition (mm) were observed after 24 and 48 hours. The strains which inhibited the test strains by forming clear zones were isolated, purified and maintained in nutrient agar slants at 40°C for further use. [2] Based on the zones of inhibition in preliminary screening of isolates having potential antimicrobial activity were selected for further work. Solvent extraction was done by centrifugation and bioactivity of the extracts was assessed following Agar well diffusion method. The lawns of each test organisms were prepared on MHA plates. The wells of (6mm) were made by using sterile Borer on MHA. A volume of 100µL of the supernatant of the culture as added into wells and left for 30 minutes until it was diffused. The plates were incubated for respective time (For bacteria 24 hours at 37°C and 7 days for fungi at 28°C). Zones of inhibition were recorded[3,4].

Enzymatic assay of isolated stains:

For fast track assay for monitoring for production of extracellular enzymes by microbes, distinctive substrates were added into agar medium. Presence of Extracellular enzymes namely protease, lipase, lecithinase, cellulose, and amylase following the substrate hydrolysis was monitored on different Agars such as Casein Agar, Tween 80 Agar, Egg Yolk Agar, Carboxymethylcellulose Agar, and Starch Agar respectively. For this a sterile wire loop was used to pick colonies from a pure culture, streaked on selected agar plate by dividing it into four quadrants followed by incubation (37°C for 48°C hours)[1,10].

Optimization of growth and antibiotic production:

This was done in broth isolates by varying their physical and chemical properties such as pH, NaCl (percentile) etc. Then the growth and antibacterial activity of the isolates were observed. For this 25ml of Nutrient broth and SDA broth were prepared in different tubes with the pH values changed in the tubes[7,8]. The pH varies from 6.0-9.0 and the NaCl concentrations of 0.5%, 1.0%, 3.0% and 5.0% were used. Then added culture in equal amount in all tubes and incubated for 48hrs at 37°C. After incubation, the growth (O.D) was measured by a spectrophotometer. Then centrifuged the broth and examined the bioactivity of supernatant by Agar well diffusion method[6-8].

Statistical Analysis:

All the data obtained from secondary screening were analyzed by one way ANOVA. The level of significance was determined using SPSS version 15 and the results having a P-value <0.05 were defined to be significant.

RESULTS

Bioactive compounds such as antibiotics are widely used. Basically, this study is focused on the isolation of antibiotic-producing microorganisms from soil. Initial isolation of soil microorganisms was done by spared plate method on NA, SDA, PDA, CZA, TSA. Screening of antibiotic-producing bacteria was done by agar overlay method. Soil microorganism that suppresses the growth of test bacteria by (as the zone of inhibitions) were selected for further confirmation of antimicrobial activity measured by agar well diffusion method (as shown in Fig. **1** interestingly). Enzyme activity by bacterial assay was performed on different agars (as shown in Table **1**) all the isolated strains did hydrolyze four of the given substrates.

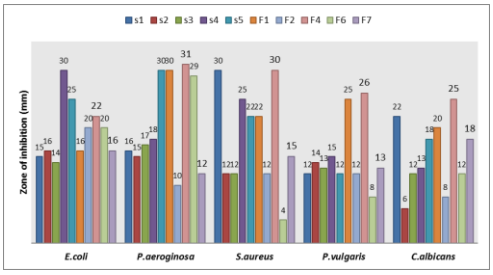

Fig. 1: Bar diagram of Agar well diffusion method, in which different colors represent isolated strains activity against test microorganisms *E. coli, P. aeroginosa, S. aureus, P. vulagris, C. albicans* (on the basis of their zones of inhibition in (mm). Among all isolates S5 (21%), F7(29%) and F1(21%) showed maximum zones of inhibition against the test organisms.

Table 1: Enzymatic activities of the isolated strains on different Agars Substrates.

ISOLATES	CELLULASE ACTIVITY CMC AGAR	AMYLASE ACTIVITY STARCH AGAR	LIPASE ACTIVITY TWEEN 80	PROTEASE ACTIVITY CASEIN AGAR	LECITHINASE ACTIVITY EGG YOLK AGAR
S1	+++	+++	+++	+++	+++
S2	+++	+++	+++	+++	+
S3	+++	+++	+	+++	+
S4	+	++	-	+++	++
S5	+++	+++	+	-	+++
F1	+++	+++	+++	++	-
F2	++	++	+	+++	-
F4	++	+++	-	+++	+
F6	++	++	+++	+++	+
F7	+++	+++	+++	+++	++

-: no growth, +: growth, ++: growth and zone, +++ growth and zone very good.

Optimization for antibiotic production was done and the growth of the isolates was studied by a spectrophotometer while antibacterial activity was monitored by using supernatant in agar well diffusion. It was found that optimal pH for the antimicrobial activity of the isolated strains ranged from pH 6 to pH 7 (as shown in Figs. **2** and **3**). The antibiotic-producing microbes *Actinomycetes, Streptomyces, Bacillus, Hortea werneckii, Aspergillus flavus, Aspergillus fumigatis, Penicillium notatum, Aspergilus niger* were identified on the basis of their growth characteristics (as shown in Figs. **4** and **5**).

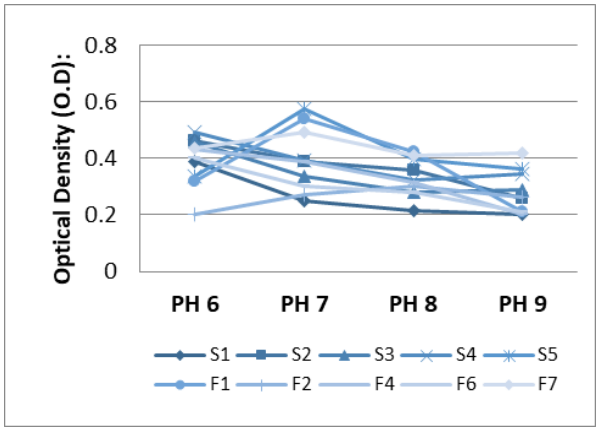

Fig. 2: Effect of pH on Antibiotic-producing microbes.

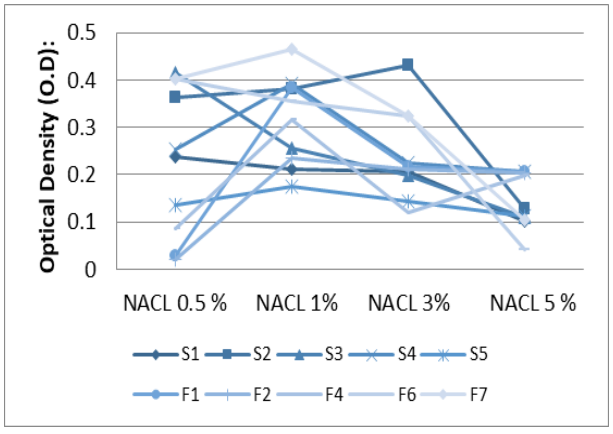

Fig. 3: Effect of NaCl conc. on Antibiotic-producing microbes.

Table 2: Culture characteristics of the isolated antibiotic-producing soil microbes.

ISOLATES	GROWTH CHARACTERISTICS	MICROSCOPIC CHARACTERISTICS	MICROBIAL STRAINS IDENTIFICATION
S1	White creamy, Large opaque, raised and margined colonies	Gram-positive rods in chains	*Bacillus*
S2	Off-white, Large opaque, raised, irregular colonies	Gram-positive rods in short chains	*Bacillus*
S3	Thin, transparent colonies with red soluble pigment	Gram-positive filamentous rods	*Streptomyces*
S4	White Thin, transparent colonies	Gram-positive branches, spider-like	*Streptomyces*
S5	White, powdery pinpoint colonies	Gram-positive, diphtheroid or filamentous rods	*Actinomycetes*
F1	Shiny black, slimed or mucoid colonies	Round yeast. Aerial mycelia. Septate hyphae and hyphae conidia	*Hortea werneckii*
F4	Greenish-yellow color, overall velvety to woolly texture.	Vesicles are spherical, Septate hyphae, long conidiophores, and biseriate structure.	*Aspergillus flavus*
F6	Blue-green color, white edged. Powdery texture.	Hyphae septate, hyaline. Phialides brush like conidia unicellular, ovoid in chains.	*Penicillium notatum*
F6	Blue-green color, white border powdery texture	Subclacate vesicle, hyphae septate smooth walled	*Aspergillus fumigatus*
F7	Black color with velvety or cottony texture.	Terviticillate, conidia vary in shape i.e. ovoid to fusiform.	*Aspergillus niger*

DISCUSSION

Evolution emergence and widespread dissemination of multi-resistant pathogens genuinely warrant novel and sustainable methodologies and approaches for the development of new antibiotics with a wide range of activities, against infectious agents[1,3,7]. The study illustrates that the antimicrobial substances give promising results against pathogens. It is essential to search for antibiotics and metabolite producing microbes from different ecological niches such as soil[1,2].

In the present research work, different microorganisms were isolated from soil. A sample collected from various locations of Karachi. Primary screening (using agar overlay method methods) that 10 isolates showed manifested antimicrobial activity against test isolates that have potent antimicrobial activity were selected then the antimicrobial activity of the extracts was examined following Agar well diffusion methods[1,9].

Extracellular enzymes produced by microorganisms play a vital role in the cycling of biological compounds[10]. Functional enzymes potentially can be determined by estimating of enzymatic activities by using target substrates. Assays of extracellular enzymes protease, lipase, lecithinase, cellulase, and amylase following the substrate hydrolysis were performed on different Agars. All bacterial and fungal strains hydrolyze the provided substrates as given in Table **1**[1,10].

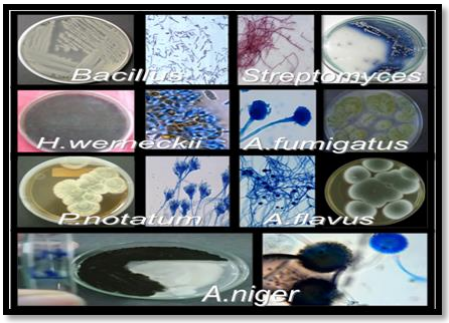

Fig. 4: Macroscopic and microscopic characteristics of isolated antibiotic-producing microbial isolates.

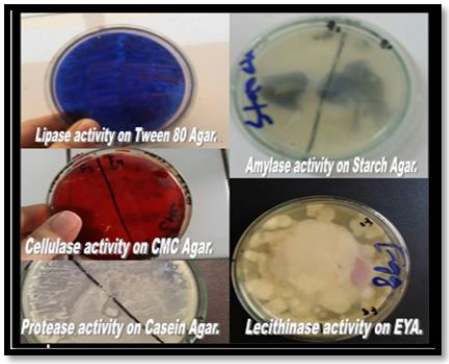

Fig. 5: Enzymatic activity of the isolated strains on different substrate less agar medium.

All the bacterial and fungal isolates exhibited at least four of the tested enzymes[1,9]. The optimum NaCl concentrations for the antibacterial activity for all the isolated strains range from (1% to 3%) as shown in Fig. **3** & discussed in Table **2**[6]. Revealed that among all isolates *Actinomycetes* (21%), *P. notatum* (29%) and *Hortea werneckii* (21%) showed appreciable bioactivity against test organisms and all the isolates produces at least three of the tested enzymes such as amylases, lipases, and proteinases. So, the isolated strains carry commercial value and their bioactivity potential as important for industrial point of view[1,2,10].

CONCLUSIONS

The potential of local soil microbial isolates to produce antimicrobial substances hydrolyze the enzymes that can utilize varied substrates has been demonstrated. These isolates were daily identified and characterized. Further studies will be used by analysis of protein electrophoresis and MS/MS Mass-spectrometry that may help to identify and characterize the enzyme protein[1,10].

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