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SCREENING AND ISOLATION OF MARINE BACTERIA WITH BIOLOGICAL ACTIVITY FROM OIL CONTAMINATED SEA WATER

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INTRODUCTION

The Marine ecosystem, which is considered as the 'Mother of origin of life', is also a source of structurally unique natural products which are mainly accumulated in living organisms. Several of these compounds show unique pharmacological activities and are helpful in the invention and discovery of bioactive compounds that are mainly found abundantly in microorganisms, algae and invertebrates and vertebrates. Modern technologies have opened vast areas of research for the extraction of biomedical compounds from marine sources to treat deadly diseases. The number of natural products isolated from marine organisms increases rapidly and now exceeds 18,000, with hundreds of new compounds being discovered every year. A large proportion of these natural compounds have been extracted from marine invertebrates, especially sponges, ascidians, bryozoans and molluscs, and some of them are currently in clinical trials.

Nature has been a source of medicinal agents for thousands of years. An impressive number of modern drugs have been isolated from microorganisms, mainly based on their use in traditional medicine. In the past century, however, an increasing role has been played by microorganisms in the production of antibiotics and other drugs (Fenical, 1993).

Most of the current antimicrobial drugs are the derivatives of the earlier generation and microbial resistance against them further intensified the need for new drug discovery. Acceptable options available are the metabolites of plants or animal origin which are biocompatible, biodegradable and non-toxic in nature.

For more than two decades, there has been an ongoing quest to discover new drugs from the sea (Anand et al., 2006). Most efforts have been directed towards chemical studies of marine invertebrates (Chin et al., 2006). Although these studies have indeed proven that marine invertebrates are an important source of new biomedical leads, a fact well demonstrated by the number of compounds currently in clinical trials, it has proven notoriously difficult to obtain adequate, reliable supplies of these compounds from nature. Because of these problems, a new avenue of study focusing on marine microorganisms has been gaining considerable attention (Faulkner, 2002). At first sight thus, the expectable enormous biodiversity of marine microorganisms might have been the reason for the interest in their study (McCarthy et al., 2004). Although marine microorganisms are not well defined taxonomically, preliminary studies indicate that the wealth of microbial diversity in the world's oceans, make this a promising frontier for the discovery of new medicines (Blunt et al., 2004).

Marine bacteria are most generally defined by their requirements of seawater, or more specifically sodium for growth. In the case of marine fungi, which in general do not display specific ion requirements, obligate marine species are generally considered to be those that grow and sporulate exclusively in a marine habitat. Although such definitions can prove useful, they tend to select for a subset of the microorganisms that can be isolated from any one environment. This problem is compounded in the case of near - shore or estuarine samples where a large percentage of the resident microbes are adapted to varying degrees of marine exposure. For the purpose of microbial drug discovery, it seems only logical to study all microbes that can be isolated from the marine environment. Based on the species studied, most of the new compounds reported from marine microorganisms were obtained from species that can, in principle, be isolated from both land and sea. Although these facultative marine species are clearly a good source of novel metabolites, their ecological roles and degrees of adaptation to the marine environment is largely unknown (Bush, 2004).

Screening of marine bacteria isolated from the surface of marine algae and invertebrates has shown that a high percentage produce antimicrobial metabolites (Bergess et al., 1999). The first antibiotic from marine bacterium was identified and characterized in 1966 (Burkholder et al., 1966). In addition, bacteria in biofilms formed on the surface of marine organisms have been documented to contain a high proportion of antibiotic producing bacteria than some other marine environment (Lemos et al., 1985; Anand et al., 2006). A number of surface associated marine bacteria have also been found to produce antibiotics (Holmstrom and Kjelleberg 1999; Hans et al., 2004).

Searching of novel antimicrobial secondary metabolites from marine environment is gaining momentum in recent years. The Indian marine environment is rich in biodiversity, especially microorganisms. However, the wealth of marine micro-flora has not been fully investigated (Ramesh, 2009). Searching for previously unknown microbial strains is an effective approach, which would yield biologically novel active substances. It is known that the antimicrobial activity of the metabolic products of aquatic bacterial strains is not weaker than the corresponding activity of soil strains (Sponga et al., 1999). In addition, the limited attempts have been made on marine organisms and their metabolites in India (Sivakumar et al., 2007; Ramesh and Mathivanan 2009; Ramesh et al., 2009). Importantly investigation of marine environment with reference to bioactive molecule production in India is still at its infancy. Therefore, exploration of marine microbes for secondary metabolites production is worthy task. The bioactive molecules derived from these microbes could be used as therapeutic drugs for the treatments of various ailments in human and animals and as agrochemicals for the management of insect pests, diseases and weeds in agriculture, etc. as suggested by Lange and Sanchez Lopez (1996) and Prabavathy et al. (2009).

Seas and Oceans receive the brunt of human waste, whether it is by deliberate dumping or by natural run-off from the land. In fact over 80% of all marine pollution comes from land-based activities and many pollutants are deposited in estuaries and coastal waters.

Other major menace of sea pollution is oil spilling from the cargo ships. Oil spills affect many species of plants and animals in the environment, as well as humans. The search for effective and efficient methods of oil removal from contaminated sites has intensified in recent years, in part due to the enormous publicity of the Exxon Valdez spill.

In quantitative terms, crude oil is one of the most important organic pollutants in marine environment and it has been estimated that worldwide somewhere between 1.7- 8.8×10^6 tons of petroleum hydrocarbons impact marine waters and estuaries annually.

In the last two decades there have been increased public concerns on the adverse effect of oil exploration on the environment. The toxic effects of crude oil and refined petroleum oils on plants, animals, humans and the environment are devastating [1]. Oil pollution persistence and its transport in water, subsoil and groundwater aquifers is monitored to predict impacts, assess the impacts and audit such effects with a view to mitigate impacts.

Incidence of spills resulting from auto crash involving trucks carrying refined petroleum products (petrol, kerosene, diesel, etc) are common in Nigeria. The Spilled products are not recovered but find their way to streams and rivers in addition to sinking into soil. The impact of these kinds of spills on microbial components of the ecosystem is largely un-assessed. The ecological roles of bacteria make it important that there should be information on the likely effect of such spill.

Exploration of the sea due to anthropological causes like industrialization, tourism, human inhabitation of the sea coasts etc., has lead to fatally polluted conditions in the sea waters. However, several techniques have been evolved to identify, isolate and extract several industrially useful enzymes

Hence, the present study was undertaken to isolate culturable indigenous marine bacteria from the Bay of Bengal, around the coastal area of Machilipatnam, Andhra Pradesh with an emphasis to isolate and characterize oil degrading bacteria. The isolated organisms were further evaluated for their ability to produce bio-products having antimicrobial activity.

. **Objectives of the study:**

The main objectives of the study are:

1. Isolation of bacteria from marine water bodies from Machilipatnam and study their biodiversity.
2. Study the cultural and biochemical characters and identification of culturable marine bacteria.
3. Screening of the isolates for their antimicrobial, lipolytic and enzyme production abilities.
4. Determination of the enzyme yields by the isolates in defined media.

Biodiversity encompasses the variety of all life forms on earth. It includes variability among living organisms from all sources including interalia, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part. This covers diversity within species, between species and of the ecosystems (The Biological Diversity Act, 2002). Another simple and useful definition for biodiversity given by the International Union for Conservation of Nature and Natural Resources states that biodiversity encompasses all life forms, ecosystems and ecological processes and acknowledges the hierarchy at genetic, taxon and ecosystem levels (Kapur and Jain, 2004).

2.1. Marine biodiversity:

Of the total estimated area of 150 million sq.kms of the earth, about 70.68% is occupied by the oceans. 90% of the world's biodiversity is contributed by the marine forms which represent a vast potential of exploitable resources, most of which is yet to be explored. India is one among the 12-mega diverse countries and 25 hot spots of the highly endangered ecoregions of the world. India has a coastal line of ~8000 Kms., including the Andaman and Nicobar Islands and continental shelf of about 504,000 sq. Kms. Among the Asian countries, India has the longest record of inventories of coastal and marine biodiversity dating back to at least two centuries (Rao, 2005, Venkataraman and Wafar, 2005).

Microorganisms with their diverse structure, physiology and metabolism survive in extreme halophilic, thermophilic and acidophilic conditions. Halophiles are an interesting class of extremophiles, which have adapted to harsh, hypersaline conditions. They are able to compete successfully for water and resist the denaturing effects of salts. Halophiles and Halotolerant microorganisms can grow over a wide range of salt concentrations inhabiting natural hypersaline brine in arid, coastal and deepsea locations as well as in artificial salterns used to mine salts from the sea.
Halophiles include both prokaryotic and eukaryotic microorganisms. Among eukaryotic microforms, algae, protozoa, yeast and fungi are prominent.

An important ecological function of the halophilic bacteria is bioremediation. In marine environment they recycle nutrients, degrade and / or detoxify chemical substances including metals, petroleum products, aliphatic and aromatic hydrocarbons, industrial solvents, pesticides and their metabolites. There has been a great interest in studying the diversity of indigenous halophilic organisms capable of degrading pollutants and also as a source for valuable biomolecules of pharmaceutical importance.

It is increasingly realized that the marine environment is an inexhaustible resource of biomolecules of commercial importance including antibiotics, enzymes and antimicrobial compounds to just name a few. Diverse range of enzymes and novel biomolecules derived from marine sources are capable of functioning under conditions that lead to precipitation of most proteins. The enzymes and antibiotics isolated from halophiles are alsoconsidered to be safer for human therapeutics with less toxicity or side effect as the saline conditions optimum for their activity are closer to blood plasma conditions.

There is vast chemical diversity in biomolecules extracted from marine environment as halophiles often incorporate halogens (like F, Cl, Br, I) into their chemical structures, a feature that is rarely seen in terrestrial organism.

Oil degrading activity:

In 2011, Ian M. Head, et al., worked on micro-organisms degrading oil in marine environment and found that hundreds of millions of litres of petroleum enter the environment from both natural and anthropogenic sources every year. The input from natural marine oil seeps alone would be enough to cover all of the world's oceans in a layer of oil 20 molecules thick.

Nuzhat Ahmed et al in 2008 isolated marine bacteria with potency of free-living and animal and plant associated marine bacteria to produce antimicrobial substances from different samples of Arabian Sea of Pakistan coast and found isolates showing antibacterial activity against Aeromonas punctata, Kokuria marina, Rothia marina, Vibrio cholerae, Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Staphylococcus aureus, methicillin resistant Staphylococcus aureus and Proteus vulgaris.

Kritika Sakalle and Shalini Rajkumar in 2009 isolated Crude Oil Degrading Marine Bacteria from a ship yard at Alang coast, Gujarat and found the isolates belonging to the genus Acinetobacter, Marinococcus,Micrococcus, Planococcus.

In the **Encyclopedia of Earth dated 2010**, Mallory Nomack worked on bioremediation to remove spilled oil in marine environment by naturally occurring indigenous bacteria

Since the contaminants of concern in crude oil are readily biodegradable under appropriate conditions, the success of oil-spill bioremediation depends on our ability to establish those conditions in the contaminated environment. According to him, the most important requirement is that bacteria with appropriate metabolic capabilities must be present. If they are, their rates of growth and hydrocarbon biodegradation can be maximized by ensuring that adequate concentrations of nutrients and oxygen are present and that the pH is between about 6 and 9. The physical and chemical characteristics of the oil are also important determinants of bioremediation success. Heavy crude oils that contain large amounts of resin and asphaltene compounds are

less amenable to bioremediation than are light-or medium-weight crude oils that are rich in aliphatic components. Finally, the oil surface area is extremely important because growth of oil degraders occurs almost exclusively at the oil-water interface.

3.1. SAMPLE COLLECTION:-

Samples were collected from Bay of Bengal near Manginapudi beach about 20 km from Machilipatnam, Andhra Pradesh. Water samples were collected in sterile containers at 20 meters off the shore line at a depth of about 30cm from the top. Also water samples were collected from the surfaces of rocks and invertebrate shells by surface scraping.

3.2. SAMPLE TECHNIQUES:-

Samples collected were of two types.

1. **Sea water:-**
   A half liter oil contaminated sea water sample was collected at a depth of 20 meters (Lat. 14^{o} 45.46'N. Long. 074^{0} 02.84'E) from shoreline manually.
2. **Scrapings:-**
   Algal scrapings from submerged sea rocks at a depth of 10 meters from sea level were scraped and used as test samples.

3.3. TRANSPORTATION OF SAMPLES:-

After sample collection, the samples were carefully added in sterile Artificial Sea Water (ASW) to mimic the marine environment. Samples collected from scrapings were also transported in sterile ASW while surface water samples were transported directly. The samples were kept in icebox and transported to the laboratory for the isolation and characterization of the bacteria.

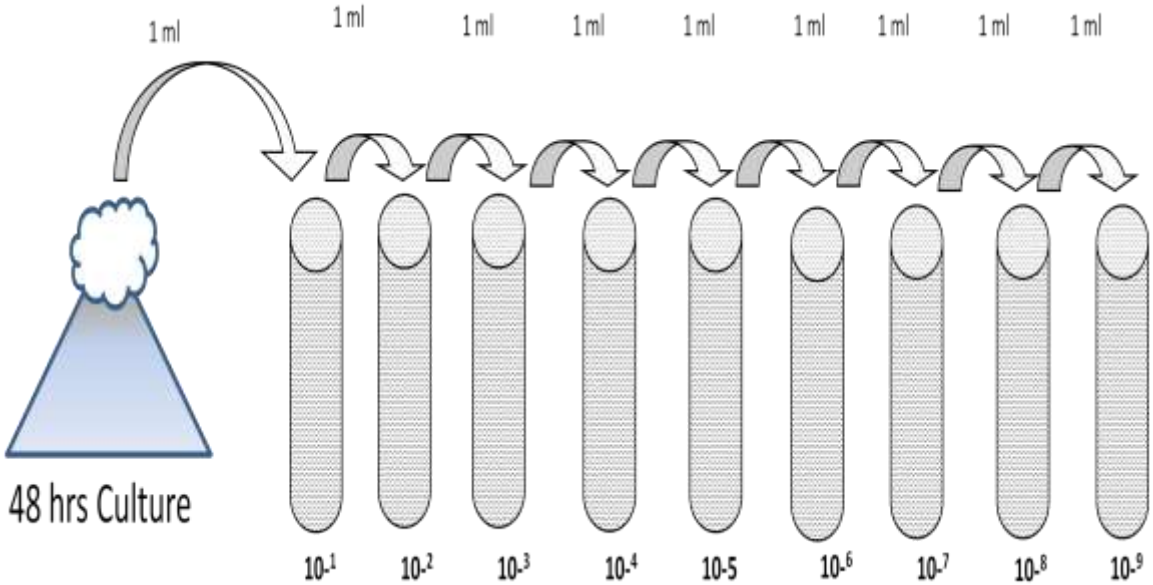
3.4. PROCESSING AND CULTURING OF BACTERIA FROM MARINE SAMPLE:-

About 1ml of the each collected sample was mixed with GP/10 medium and incubated on a rotary shaker set at 200 rpm for 48 hrs.

Serial dilution method:

The cell suspension obtained after 48hrs of incubation of the samples was serially diluted by serial dilution method which was described below.

1 ml of the cell suspension obtained from the above step was diluted into 9 ml of sterile saline. This was further diluted till a dilution of 10^{-9} was achieved.



From the required dilutions 0.1ml of suspension was pipette out and streaked on GP/10 medium in petri plates.

Plates were incubated for 48 hours at room temperature.

3.5. IDENTIFICATION OF BACTERIAL ISOLATES:

Bacterial isolates were characterized based on their morphological, cultural and biochemical properties and biodiversity of the marine samples was studied.

Morphological characterization:

Initial identification of isolates was carried out by studying morphological characters after incubating the plates for 3-5 days at 27°C. The colony morphology, pigment production and its diffusibility were observed and morphologically distinct colonies were selected for staining. The staining properties and bacterial morphology was studied using modified Gram's Staining Method (Leifson, 1951). Pure cultures of morphologically distinguishable isolates were obtained by subsequent sub-culturing thrice and cultures obtained were preserved as stab cultures in GP/10 media.

Gram staining procedure:

a) A loop full of microbial colony from a 24hr culture slant was smeared on clean glass slide and heat fixed.
b) The smear was flooded with Crystal violet and incubated for 30 seconds.
c) The slide is drained off and washed with running tap water.
d) The slide was then flooded with Gram's Iodine and the slide was left undisturbed for 30 seconds and washed with water.
e) The slide was decolorized with Acetone by allowing the solution to drain off by keeping the slide in slanting position.
f) The smear was washed with tap water continuously until no color remains.
g) The slide was counter stained with saffranin for 60 seconds.
h) The smear was washed with tap water and blotted dry.
i) The slide was then observed under a microscope with a 100X oil immersion lens.

Cultural Characteristics:

The cultural characters of the selected isolates were determined by studying the following parameters.

a. Growth at different temperatures
b. Tolerance to pH and salinity
c. Oxygen requirement for growth
d. Motility and spore production.

Growth at different temperatures:

The limits of growth for the isolates were determined at different temperatures between 4°C and 45°C (Baumann et al., 1971). Tubes with 5ml GP/10 broth were inoculated with 0.5ml of 48hrs. Culture and incubated at different temperatures for 3-5 days. The tubes were observed for visible turbidity at 24hr intervals and optimum temperature for growth was recorded.

Growth at different salt concentrations:

The ability of the organisms to grow at different salt concentrations (0%, 1.2%, 2.5%, 5%, 10%, 15% and 20%) was studied by adopting the method of Novick and Tyler, 1985. Tubes of sterile GP/10 broth supplemented with the respective salt concentration were prepared and inoculated with a 48hr culture. After incubation for 3-5 days at 27°C, the turbidity of the tubes was observed. The salt tolerance of the isolates was recorded as increase in the turbidity at different salt concentrations. The tubes showing negative growth were streaked on GP/10 agar media to confirm inhibition of growth. The organisms that could grow upto 5% were categorized as moderately halophilic whereas the other isolates tolerating >10% were considered halophiles.

Growth at different pH:

The ability of the isolate to grow at different pH was tested by inoculating the culture into GP/10 media tubes adjusted to different pH in the ranges of 4.5 - 10.5 (Holmstrom et al., 1998). The samples were incubated at 27°C for 48-72hrs and observed for growth and growth range and optimum pH was determined for each isolate.

Biochemical characterization:

Characterization of the isolates were carried out by performing various biochemical tests adopting standard protocols as described in "Practical Medical Microbiology Manual" (Mackie and McCartney, 1989), unless otherwise specially mentioned. The procedure in all the cases was suitably modified by using GP/10 medium or supplementing the required media with 1.2% NaCl to facilitate the growth of halophilic bacteria. Known positive and negative organisms for the various biochemical tests performed were included as controls.

Sugar utilization test:

The ability of the isolates to utilize different substrates as carbon source was determined by the method of Novick and Tyler, 1985. Phenol red (0.005%) was selected as indicator as it was found to be less toxic effect than bromothymol blue indicator to many marine bacteria. Artificial seawater supplemented with 0.1% NH4Cl, 0.005Mphosphate buffer (pH 7.0) was used as basal medium for sugar utilization and 10µl of Phenol Red was added for every 5ml media. 5% stock solutions of various carbohydrates were prepared and sterilized at 10lbs. The stock solutions were added to 5ml of the basal medium to get a final concentration of 0.5% of the respective sugars. The tubes were inoculated with 0.1ml of 48hr old culture (~105 cells/ml) and incubated at 27°C for 24-48hrs. The utilization of sugars along with the production of acid/gas was recorded.

Indole test:

The ability of the isolates to convert the amino acid tryptophan to indole was tested by adding 0.5ml of Kovac's reagent to 48hr old culture of the isolates in peptone water supplemented with 1.2% NaCl. The production of red color in the alcohol layer indicates a positive Indole reaction.

Methyl Red (MR) test:

The ability of the isolates to ferment glucose and produce acid was determined using Glucose Phosphate Peptone medium (GPP) supplemented with 1.2% NaCl. Methyl red indicator (2-3 drops) was added to 48hr old culture tubes and results were recorded immediately. Appearance of bright red color in the medium indicated a positive reaction. E. coli and K. pneumoniae were used as positive and negative controls respectively for MR and VP tests.

Voges – Proskauer (VP) test:

The ability of the isolates to produce acetoin as the end product was detected by adding 0.5ml of O' meara reagent to 48hr old culture tubes of the marine isolates in GPP media. The development of eosin pink color indicates a positive reaction.

Citrate utilization test:
The ability of the organism to utilize citrate as the sole source of carbon and energy for growth and an ammonium salt as the sole source of nitrogen was determined by streaking Isolates on Simmon's Citrate Agar slant (pH 6.8) and incubating for 96hrs at 27°C. A positive test indicated change in the colour of the medium from green to blue and appearance of a streak of growth. K. pneumoniae and E. coli were used as positive and negative controls for VP and Citrate tests.

Catalase Test:
The production of catalase enzyme, which mediates the release of oxygen from hydrogen peroxide, was tested by picking up single colony from a 48hrs old plate culture and introducing into 1ml of 37% of hydrogen peroxide. The appearance of effervescence / gas bubbles from the surface of the liquid indicated a positive test. Staphylococcus aureus and Streptococcus pneumoniae were used as positive and negative controls for the test respectively.

H_2S production test:
Production of Hydrogen Sulphide was tested by inoculating 48hrs old culture of test organism into GP/10 broth having lead acetate strip inserted from the top of the test tube. The tubes were incubated at 27°C for 48-72 hrs. Blackening of the strip was considered as a positive reaction.

Urease test:
The production of urease enzyme was tested by streaking the culture on Christensen's medium supplemented with 1.2% NaCl (pH 6.8-6.9). The appearance of purple pink color growth indicated a positive reaction. Proteus vulgaris and E.coli were used as positive and negative controls for H_2S production and Urease tests.

Hydrolysis of gelatin:
The hydrolysis of gelatin by the isolates was tested by inoculating the culture onto a nutrient gelatin agar medium supplemented with 1.2% NaCl. The plates were incubated at 27°C for 3-5 days followed by flooding the plates with 1% tannic acid solution. Excess solution was drained off after a few minutes and the plates were observed immediately for reduction of relative opacity around gelatin liquefying colonies.

Hydrolysis of starch:
The ability of the bacterial isolates to hydrolyze starch was tested on nutrient agar plates supplemented with 0.2% soluble starch and 1.2% NaCl. The plates were streaked with the isolates and incubated at 27°C for 3-5 days. After incubation the plates were flooded with Iodine Solution and the zone of clearance around amylase producers was recorded.

Hydrolysis of casein:
Milk agar was prepared by adding 1% sterile skimmed milk (autoclaved at 10lbs for 10min.) to salt supplemented nutrient agar medium cooled at 50°C. The plates prepared were inoculated with single colony of each isolate and incubated for 3-5 days at 27°C. Hydrolysis of milk protein was seen as a zone of clearing around the colony.

The identification of bacteria was carried out using numerical Probabilistic and Taxonomy methods. The initial identification of the organism was performed with the PIBWin version (Bryant, 1995). The computer program includes data matrices suitable for identification of various groups of bacteria. The characters that could not be confirmed just by PIBWin data matrices were compared with the additional data matrices, Bergey's manual of Determinative bacteriology (Sneath et al., 1986) in addition to recent literature searches. The data was analysed using the package Numerical Taxonomy and Multivariant Analysis System (NTSYS 61 package version 2.0) which displays structure in multivariant data, for differential grouping of the isolates into phena using numerical taxonomy approach (Rohlf, 1994, Rohlf, 1998).

3.5. ANTIMICROBIAL ACTIVITY:

The ability of the marine isolates to exhibit antimicrobial activity against both bacterial and fungal strains was tested by using concentrated bacterial supernatant by adopting disc diffusion technique (James et al., 1996, Mearns-Sprag et al., 1998).

Preparation of concentrated Bacterial Pellet:

Concentrated Bacterial Pellet was prepared by modified method of James et al., 1996. Cells from a 48hrs old culture grown in GP/10 medium were pelleted by centrifugation at 9000g for 20 minutes and re-suspended into half strength artificial seawater to get a density of 0.6 g/ml. The suspension was incubated for 24hrs at 27°C. The cells were harvested by centrifuging at 15,000g for 30min. The supernatant was filter sterilized and used for testing antimicrobial and antifouling activities.

Antibacterial activity:

The antibacterial activity was tested against one gram negative test strain (E.coli) and one gram positive strain (B.subtilis) using disc diffusion method (Mearns-Sprag et al., 1998). 100µl of a 24hrs old test culture (~105 CFU/ml) was spread evenly on nutrient agar plates using sterile cotton swab. Whatmann No.1 filter paper discs (sterile) were placed on the plates. Each disc was impregnated with 10µl of the CBP prepared from each marine isolate, so that it is completely absorbed. The plates were incubated for 48hrs at 27°C and observed for the formation of zones. The results were compared with the control disc prepared with distilled water. The relative activity index of the zones was calculated as per the method of Singh et al., 2002 using the formula.

$$\text{Activity index} = \frac{\text{Zone of inhibition in mm of test sample}}{\text{Zone of inhibition in mm of standard organism value}}$$

Antifungal activity:

The antifungal activity against three fungi Aspergillus flavus, Trichoderma viridae, Aspergillus oryzae and Aspergillus niger was studied, by using pour plate method (Aneja, 1993). Inoculum of the fungal test cultures was prepared by growing the strains on potato dextrose broth for 5 days with shaking until the mat formation. Spores of the cultures were collected and suspended in saline to get a final titre of 5 $X10^5$/ml. 500µl of the inoculum was added to 20ml of Potato Dextrose Agar (PDA) medium maintained at 50°C and the plates were prepared. After solidification, sterile Whatmann No.1 filter

paper discs (sterile) were placed on the plates and impregnated with 10µl of the CBS as described above. The growth of the fungi was monitored over a period of 10 days incubation at 27°C and the antifungal activity was determined by measuring the zone of inhibition around the discs.

3.7. ENZYMATIC ASSAY OF THE ISOLATES:

Enzyme assay:

The isolates showing amylase activity were further screened for the amount of enzymatic activity. 48 hours grown cultures were centrifuged at 16000 rpm for 10 minutes and the cell free supernatant (CFS) was used as the enzyme extract. The CFS was diluted 10 times with Buffer and used for enzyme assay.

Alpha amylase enzyme activity in the CFS was determined by the starch digestion method of Windish and Mhatre (1965). In brief 1.0% soluble starch dissolved in phosphate buffer (pH 6.5). One tenth (0.1ml) of test solution was added to 1ml of substrate. After incubation for 30min at 50ºc the reaction mixture was stopped by adding 1 drop of Glacial Acetic acid. The reaction mixture was incubated at 30ºc for 10min. Then 10ml of distilled water was added to the solution. After allowing the reaction mixture to stand for 5 min at room temperature the absorbance was measured at 600nm.One unit of amylase was defined as the amount of enzyme which digested 1milligram of starch per minute in this assay system.

Proteolytic assay:

3.8. DEMONSTRATION OF CRUDE OIL DEGRADING ACTIVITY OF MARINE ISOLATES

Crude oil biodegradation experiment was performed by modifying the technique described by Pirnik et al. (9) by adding the inoculating cells of density 10^8 mL^{-1} to the basal media with 1% crude oil added as sole carbon source.

To study effect of Tween 80 on biodegradation of crude oil an experiment was performed with 0.1% Tween 80and 0.2% oil was added to the flask (10). The estimation of crude oil degradation was accomplished by overlay method by adding 50µl of cells of density 10^8 mL^{-1} was added and incubated for 37^0C for 48hrs.

The oil degrading activity was demonstrated by flooding the plates with 0.5% Congo red and observed for the emulsification of oil around the oil degrading colonies.

Marine samples collected from the coast of Bay of Bengal at Machilipatnam, Andhra Pradesh were processed for isolation of marine microbes. Two samples were collected from oil contaminated sea water and scrapings from submerged rocks.

Sample Collection:

The samples collected were processed to obtain pure cultures of microbes by culturing them on selective media i.e., GP/10 media.

Figure 1: samples

Characterization of the bacterial isolates:

Most of the marine isolates, being slow growers, formed discrete colonies only after incubating for 3-5 days. Morphologically distinguishable colonies were selected and were subjected to morphological characterization.

Morphological characterization:

Based on distinguishable colony morphology and gram staining, 20 isolates were selected from the 2 marine samples. Pure cultures of the isolates were obtained by repeated sub culturing for three times. The isolates were designated as MB1-20, where MB indicated marine bacteria. Based on the colony morphology and pigmentation, the isolates could be broadly grouped into distinct morphological types with colony size ranging from 1-4 mm in diameter in majority of cases where as few punctiforms were also obtained.

Thus among the 20 isolates, 5 were gram positive bacilli, 4 gram positive cocci, 2 gram negative cocci, 2 gram negative coccobacilli, 1 gram positive diplobacilli, 1 gram positive spore forming bacilli, 3 yeast and 1 budding yeast (Table - 1). These isolates were selected for further cultural and biochemical characterization.

Figure 2: Pure cultures of marine isolates

Cultural characterization:

The cultural characters of the distinct morphological isolates were studied to determine the conditions of growth. Growth of the isolates was studied at different temperatures (4-45°C) and pH range (4.5-10.5). The tolerance to salt concentration (in the range 1.2-20%).The results are shown in Table 2.

Among the 20 gram negative isolates, 7 isolates (MB 1, 4, 6, 13, 14, 15 and 17) were able to grow between 4°C – 45°C. 3 isolates (MB 2, 5 and 8) grew between 4°C – 27°C, 2 (MB 3 and 7) isolates showed growth at 37 °C only, 7 isolates (MB 9, 11, 12, 16, 18, 19 and 20)grew between 27°C -37°C and isolate MB 10 was able to grew at 4°C -37°C.

Of the 20 isolates tested for salt tolerance, 8 isolates tolerated upto 1.2%-15% salinity and the remaining isolates tolerated upto only 5% of salt tolerance. In terms of pH 6 isolates were able to grow between pH 5.5-10.5. The remaining 14 isolates exhibited growth over the pH range tested in between 5.5-7.5.

COLONY MORPHOLOGY	ISOLATES	MICROSCOPIC OBSERVATION
White colonies, circular, with entire margins measuring 1-3mm in diameter.	1,4	Gram negative bacilli
White colonies, circular, with entire margins minute in diameter.	5, 7,10,17,20	Gram negative bacilli
White colonies with green pigment, circular, with entire margins measuring 1-2 mm in diameter.	13,18	Gram negative bacilli
Yellow colonies, circular, with entire margins minute in diameter.	2,6,14	Gram negative bacilli
Lemon yellow colonies, circular, with entire margins minute in diameter.	3	Gram negative bacilli
Golden yellow colonies, circular, with entire margins minute in diameter.	9	Gram negative bacilli
Orange colonies, circular, with entire margins measuring 0.5-1mm in diameter.	8,12,15,16	Gram negative cocobacilli
Orange colonies, circular, with entire margins measuring 0.5-3 mm in diameter.	15	Gram negative bacilli
Orange colonies, circular, with entire margins measuring 1-4 mm in diameter	11	Gram negative bacilli

Table 1: Morphological characters of marine isolates

TEST		ISOLATES																			
		SAMPLE 1																SAMPLE 2			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
GROWTH AT DIFFERENT TEMPERATURES	**4**	+	+	-	+	+	+	-	+	-	+	-	-	+	+	+	-	+	-	-	-
	27	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
	37	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
	45	+	-	-	+	-	+	-	-	-	-	+	-	+	+	+	+	+	+	-	+
SALT TOLERANCE IN (%)	**1.2**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	2.4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	5.0	+	+	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
	10.0	+	-	-	+	-	-	-	+	+	-	+	-	+	+	+	+	+	+	-	+
	15.0	+	-	-	+	-	-	-	+	+	-	+	-	-	-	-	+	-	+	-	+
	20.0	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	+
PH	**4.5**	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	-	+	-
	5.5	+	-	+	+	-	-	+	+	+	-	+	+	-	-	+	+	+	+	+	+
	6.5	+	-	+	+	-	-	+	+	+	+	+	+	-	-	+	+	+	+	+	+
	7.5	+	+	+	+	+	-	+	+	+	+	+	+	-	-	+	+	+	+	-	+
	8.5	-	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+
	9.5	-	-	+	-	-	+	+	+	+	-	-	-	+	+	+	-	+	-	-	-
	10.5	-	-	-	-	-	+	-	+	+	-	-	-	+	+	+	-	+	-	+	-

Table 2: Cultural characteristics of marine isolates

Biochemical characterization:

Initial characterizations of the 20 isolates were carried out by conducting sugar utilization tests, IMViC and other biochemical tests. The results are tabulated in Table - 3 and Figure -2,3,4,5, . Based on the morphological and biochemical characters attempts were made to categorize the isolates up to genus level.

The biochemical characters of 20 isolates are given in Table – 3. It was observed that MB 1 was identified as gram negative bacilli, aerobic, sporulating rods. The isolate was positive for catalase, citrate and urease and negative for MR. Based on the morphological and biochemical characters, the isolate

could be characterized to be belonging to the genus Chromobacterium . Further grouping of the isolate was done based on the biochemical characters including sugar fermentation, salt tolerance etc.

It was observed that 2 isolates (MB 2 and 13) were aerobic, gram negative bacilli which were showing positive results for catalase, citrate and urease and negative for H_2s production. Based on these morphological and biochemical characters, the isolates could be characterized to be belonging to the genus Serratia. Further, grouping of the isolates was done based on the biochemical characters including sugar fermentation, salt tolerance etc.

The isolate MB 3 was observed as gram negative bacilli. The isolate showed negative results for all the biochemical tests. Based on these morphological and biochemical characters, the isolates could be characterized to be belonging to the genus Edwarsiella.

It was observed that isolate (MB 4) were gram negative motile rods, which were indole positive, and catalase positive ,citrate positive, VP positive and MR positive. Based on these morphological and biochemical characters, the isolates could be characterized to be belonging to the genus Aeromonas. Further grouping of the isolates was done based on the biochemical characters including sugar fermentation, salt tolerance etc.

was observed that 4 isolates (MB 5, 9, 15 and 16) were identified as gram negative rods which were positive for indole, catalase, citrate and MR and negative for VP negative. Based on these morphological and biochemical characters, the isolates could be characterized to be belonging to the genus Acinetobacter. Further grouping of the isolates was done based on the biochemical characters including sugar fermentation, salt tolerance etc.

The 2 isolates (MB 6 and 18) were identified as gram negative rods which were showing positive for urease, catalase, citrate and VP and showing negative reactions for H2S production, indole and MR. Based on these morphological and biochemical characters, the isolates could be characterized to be belonging to the genus Klebsiella. Further grouping of the isolates was done based on the biochemical characters including sugar fermentation, salt tolerance etc.

5 isolates (MB 7, 10, 14, 17 and 19) were gram negative rods which were showing positive results for indole, catalase, citrate, H2S production and VP and negative for MR. Based on these morphological and biochemical characters, the isolates could be characterized to be belonging to the genus Klebsiella. Further grouping of the isolates was done based on the biochemical characters including sugar fermentation, salt tolerance etc.

It was observed that isolate (MB 8) was identified as gram negative coccobacilli rods which was positive for indole, catalase and negative for citrate MR and VP. Based on these morphological and biochemical characters, the isolate could be characterized to be belonging to the genus Pasteurella. Further grouping of the isolates was done based on the biochemical characters including sugar fermentation, salt tolerance etc.

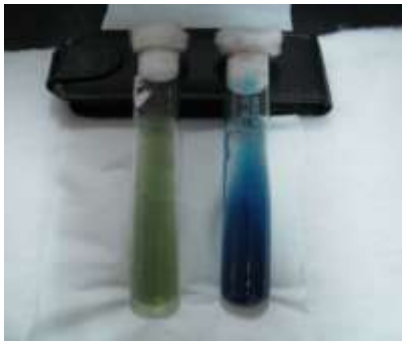
The isolate MB 11 was identified as gram negative rods which was urease, MR and VP negative and positive for indole, catalase, citrate. Based on these morphological and biochemical characters, the isolates could be characterized to be belonging to the genus Providencia. Further grouping of the isolates was done based on the biochemical characters including sugar fermentation, salt tolerance etc.

It was observed that isolate, MB 12 was gram negative coccobacilli rods which was positive for urease, indole and catalase and negative for citrate MR and VP. Based on these morphological and biochemical characters, the isolates could be characterized to be belonging to the genus Pasteurella . Further grouping of the isolates was done based on the biochemical characters including sugar fermentation, salt tolerance etc.

It was observed that isolate MB 20 was gram negative rod which was indole, citrate, MR and VP negative and positive for catalase. Based on these morphological and biochemical characters, the isolates could be characterized to be belonging to the genus Actinobacillus. Further grouping of the isolates was done based on the biochemical characters including sugar fermentation, salt tolerance etc.

Negative Positive

Figure 3: MR test



Negative Positive

Figure 4: citrate utilization test

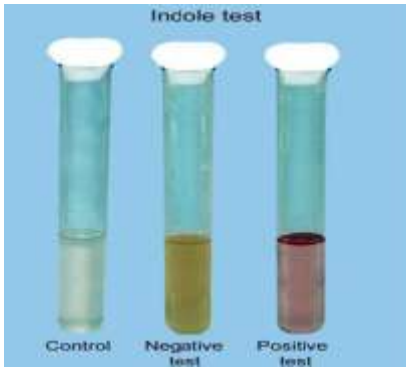


Figure 5: Indole test

Negative Positive

Positive Negative

Figure 6: Urease test **Figure 7: H_2S pr**

TEST	ISOLATES																			
	SAMPLE 1																SAMPLE 2			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
INDOLE	+	-	-	+	-	-	+	+	-	+	+	+	-	+	+	+	+	-	+	-
METHYL RED	-	-	-	+	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-
VOGES PROSKAEUR	+	-	-	+	-	+	+	-	-	+	-	-	+	+	-	-	+	+	+	-
CITRATE	+	+	-	+	+	-	+	-	+	+	+	-	+	+	-	-	-	+	+	-
UREASE	+	+	-	-	+	+	+	-	+	+	-	+	+	+	-	+	+	+	+	+
H_2S PRODUCTION	-	+	-	+	-	-	-	-	-	-	+	-	-	-	+	-	+	-	-	-
CATALASE	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 3: Biochemical characters of marine isolates

Figure 8: Sugar fermentation test

TEST		ISOLATES																			
		SAMPLE 1																SAMPLE 2			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
DEXTROSE	A	+	-	-	+	+	+	+	-	-	+	-	+	+	-	-	+	-	-	+	+
	G	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MANNITOL	A	+	-	-	+	-	+	-	-	-	-	+	+	-	-	+	-	+	-	-	-
	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
STARCH	A	+	-	-	+	+	+	-	+	+	-	-	-	+	+	+	+	+	+	+	-
	G	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SUCROSE	A	+	-	-	+	-	+	+	-	-	+	+	-	-	-	-	-	-	+	-	+
	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GLYCEROL	A	-	-	-	+	-	+	-	+	+	+	-	-	-	+	-	-	-	-	-	+
	G	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 4: SUGAR FERMENTATION TEST

Identification of the isolates to probable species level:

Further characterization of the isolates to species level was carried out using PIBWin package, which is windows based software with the major function of identification of unknown isolates. To identify the isolates using this package, identification matrix for each of the isolate was generated by entering the results of characteristics obtained by experimental test as given in Tables1,2,3,and 7. The parameters of "select best tests" and "calculate matrix ID scores" were used for distinguishing one taxon from another taxa or all taxa from each other. The reliable identification was indicated when the ID score was ≥0.999 and model likelihood was ≥0.01. The matrix ID scores obtained reflect percentage probability for each taxon identification and these were used to assess reliability of identification. The field area entry of an organism and the parameters used for analysis are shown in Figures – 7-8.

Among the 20 isolates MB 1 belonging to Chromobacterium, was further characterized based on PiBWin package to species level based on biochemical characterization like catalase positive, indole negative and non fermentative as Chromobacterium violaceum.

The isolates MB 2 and 13 belonging to Serratia genus were grouped to species level based on catalase positive,H_2S production negative, enzymatic characters positive and fermentative organism as Serratia mercescens.

The isolate MB 3 Edwardsiella was characterized as Edwarsiella ictaluri based on catalase negative, non fermentative, enzymatic characters negative.

MB 4 belonging to the genus Aeromonas was grouped upto species as Aeromonas hydrophila based on urease negative, fermentative organism, VP and MR positive.

The isolates MB 5, 9, 15 and 16 were grouped to Acinetobacter calcoaceticus based on urease positive,H_2S production negative and non fermentative.

The isolates MB 6 and 18 were grouped to Klebsiella pneumonia subsp aerogenes based on fermentative for sugar production and indole negative and positive for enzyme production.

The isolates MB 7, 10, 14, 17 and 19 were grouped from genus Klebsiella to species level as Klebsiella oxytoca based on H_2S production and enzymatic activity negative and indole positive.

MB 8 was further characterized from genus Pasturella to the species Pasteurella multocida based on urease, H_2S production and enzyme production negative.

MB 11 was grouped to species level Providencia alcalifaciens based on biochemical characters like negative for enzyme production, urease and VP and positive for indole.

The isolate MB 12 was characterized to be belonging to the species Pasteurella pneumotropica based on negative for enzyme production,VP and MR and positive for urease.

Finally, MB 20 was grouped to Actinobacillus suis based on negative for enzyme production and H_2S production and positive for catalase.

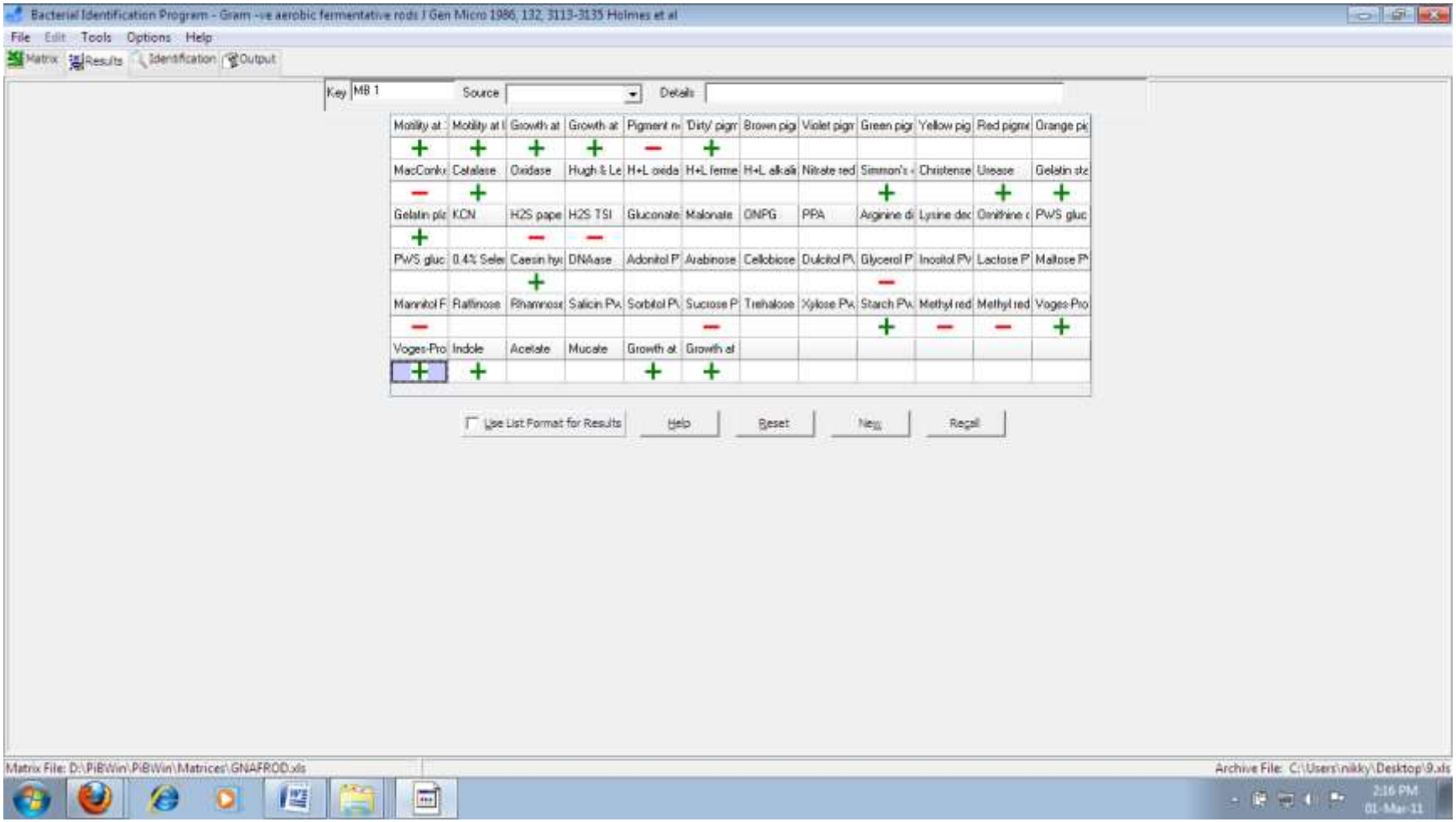


FIGURE 9: Field areas entry for generation of identification matrix

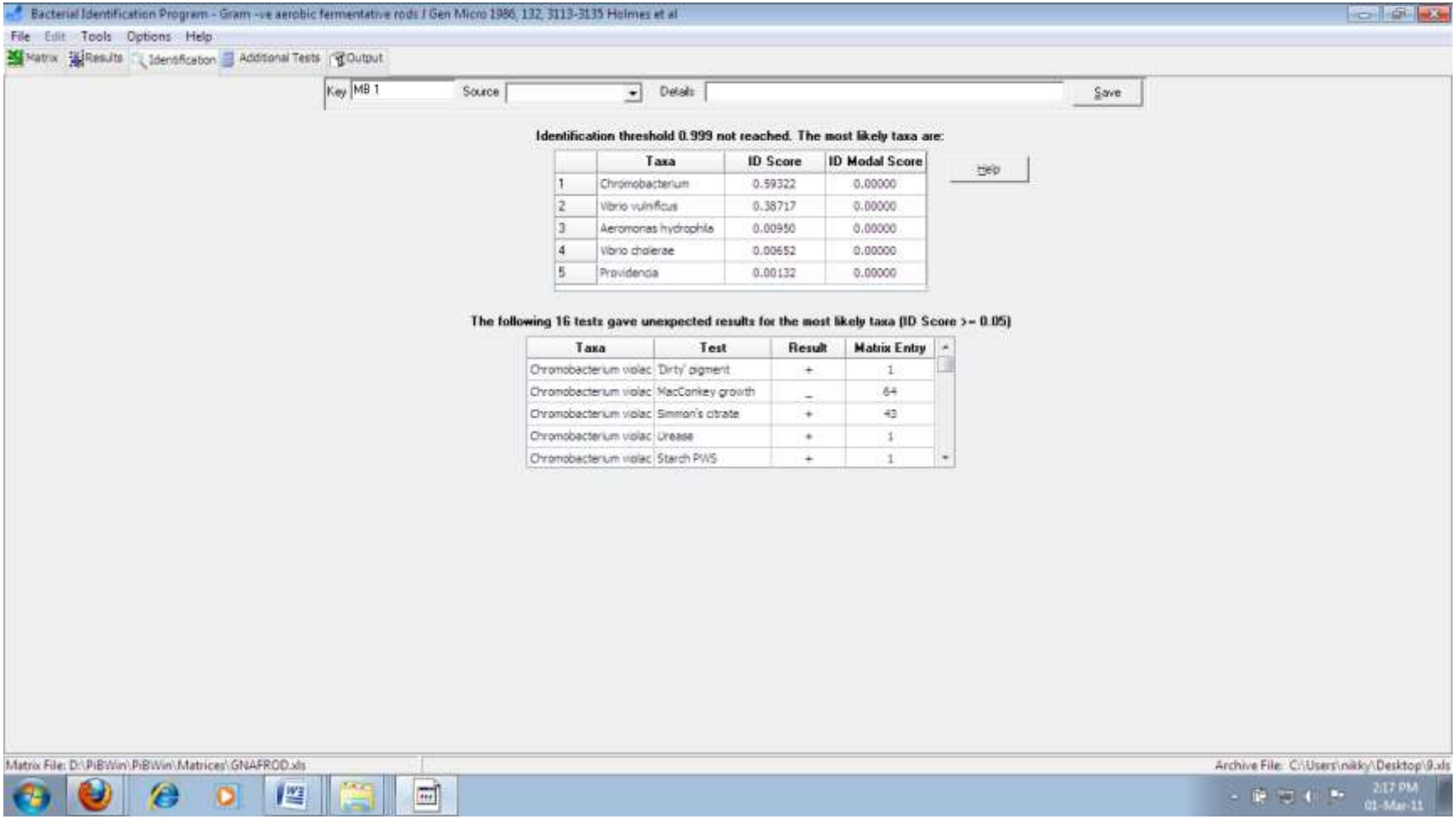


Figure 10: Identification thresholds and score of MB 1

Antimicrobial activity:

The antimicrobial activity of the 20 isolates was tested against 2 bacterial and 3 fungal species and the results are given in Tables 4 & 5and Figures – 10,11,12,13and 14.

Among the 20 isolates, 13 isolates did not showed any antimicrobial activity.5 of them have shown antibacterial activity against E.coli (gram negative bacteria), while 3 of them exhibited antibacterial activity against B.subtilis (gram positive bacteria). The isolates, MB 13 and 18, showed the ability to degrade both E.coli and B.subtilis. MB 10 & 13 isolates exhibited antimicrobial activity against both bacteria and fungi. 7 isolates showed only antifungal activity. MB 10 showed highest activity against bacteria and fungi (Table - 26a and b).

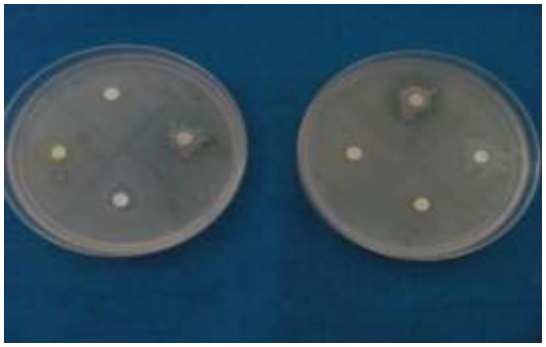


Figure 11: Antibacterial activity against B.subtilis

Figure 12: Antifungal activity of A.oryzae

Figure 13: Antifungal activity of A. flavus

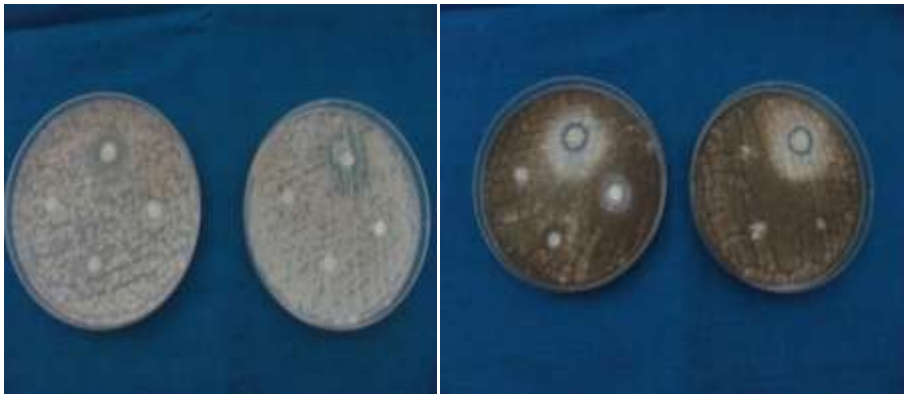


Figure 14: Antifungal activity of T.viridae Figure 15: Antifungal activity of A.niger

TEST	ISOLATES																			
	SAMPLE 1																SAMPLE 2			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Escherichia coli	-	-	-	-	-	0.7	-	-	-	-	-	-	1.4	1.6	-	-	0.5	0.7	-	-
Bacillus subtilis	-	-	-	-	-	-	-	-	-	2.5	-	-	1.8	-	-	-	-	0.6	-	-

Table 5: Antibacterial activity of marine isolates

TEST	ISOLATES																			
	SAMPLE 1																SAMPLE 2			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Aspergillus niger	-	-	-	1.2	-	-	-	-	-	1.5	-	-	1.5	-	-	-	-	-	-	-
Aspergillus flavus	-	-	-	1.1	-	-	-	-	-	-	-	-	0.8	-	-	-	-	-	-	-
Aspergillus oryzae	-	-	3.5	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-
Trichoderma viridae	-	-	-	-	-	-	-	-	-	1.8	-	-	1.6	-	-	-	-	-	-	-

Table 6: Antifungal activity of marine isolates

Enzymatic characters of marine isolates:

The enzymatic characters of 20 isolates were tested for the production of enzymes like amylase, caseinase, gelatinase and the results are given in Tables 4 & 5and Figures – 15, 16 and 17. Among the 20 isolates, MB 1, 4 and 13 showed enzymatic activity on all three substrates, whereas MB 2,10,14,17 and 18 showed activity on only one substrate.

ASSAY FOR α AMYLASE ACTIVITY BY AMYLASE PRODUCING ISOLATES:

Among the 20 isolates identified for the production of amylase activity only six isolates (MB 1, 4, 10, 13, 14, 18) were identified to be positive for amylase production. Of them only three isolates showed (MB 1, 4, 18) the maximum zones of hydrolysis in the range of 1.3-1.8cm .

The isolates with maximum activity were further selected and tested for their enzymatic activity using **Starch iodide method**, among which only isolates MB 1 and 18 have shown the maximum amount of enzymatic activity of 230.522 U/ml, 104 U/ml, respectively.

CALCULATION:

MB 1:

Abs. of SB	=	0.415
Abs. of Sample	=	0.128
Δ Abs	**=**	**0.287**

Therefore total starch digested = 69.15 mg by 0.01 ml of enzyme.
Alpha Amylase activity (SDU) = 230.522 U/ml.

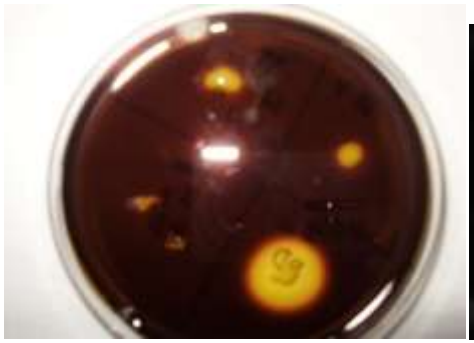


Figure 16: Hydrolysis of starch

Figure 17: Hydrolysis of gelatin

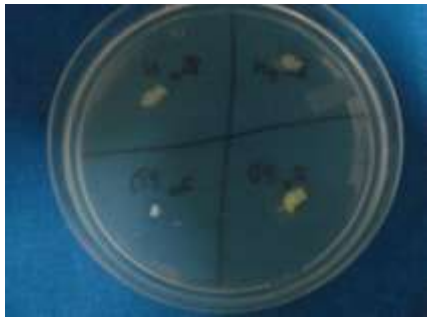


Figure 18: Hydrolysis of casein

TEST	ISOLATES																			
	SAMPLE 1																SAMPLE 2			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
AMYLASE	**1.8**	**-**	**-**	**1.6**	**-**	**-**	**-**	**-**	**-**	**0.6**	**-**	**-**	**0.8**	**0.6**	**-**	**-**	**-**	**1.3**	**-**	**-**
CASEINASE	**0.9**	**-**	**-**	**0.8**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**1.1**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
GELATINASE	**1.8**	**1.6**	**-**	**2.5**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**3.0**	**-**	**-**	**-**	**2.4**	**-**	**-**	**-**

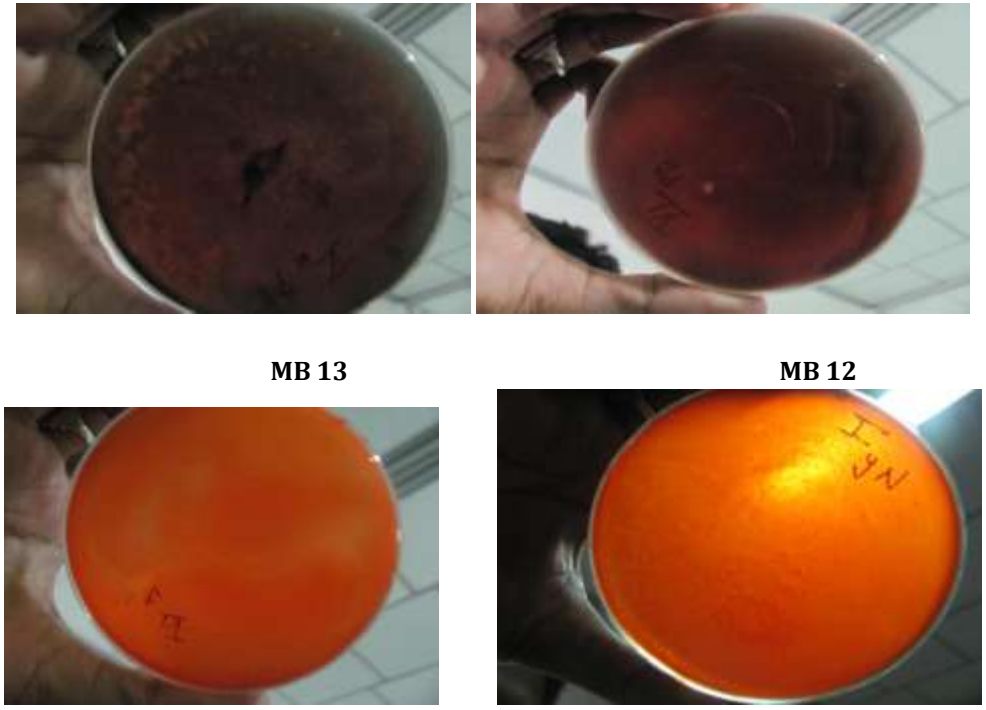
Table 7: Enzymatic characters of marine isolates

Sample id	Buffer(ml)	Starch(ml)	Enzyme(ml)	Reagent(ml)	Acetic acid	Distilled water	OD at 595nm	Enzyme activity(u/ml)
Reagent blank	2	-	-	3	1-2 drops	10		
Enzyme blank	1.9	-	0.1	3	1-2 drops	10		
Substrate blank	1	1	-	3	1-2 drops	10	0.415	
Sample 1	0.9	1	0.1	3	1-2 drops	10	0.128	230
Sample 2	0.9	1	0.1	3	1-2 drops	10	0.425	
Sample 3	0.9	1	0.1	3	1-2 drops	10	0.285	104

Table 8: ASSAY FOR α AMYLASE ACTIVITY BY AMYLASE PRODUCING ISOLATES

Estimation of crude oil biodegradation:

The results from experimental flasks indicated 40% isolates were showing oil degradation activity of crude oil from the medium. Highest crude oil biodegradation was observed with isolates MB 7, 12, 13, 14 and 15 with 60% degradation with 1% crude oil in the medium. A comparatively lower biodegradation of 30-40% was found in MB 8 and MB 9 isolates. Least biodegradation of 20% was recorded with isolates MB11 and MB 16.



MB 13 **MB 12**

MB 14 **MB 9**

Figure 19: Oil degrading activity of oil degrading bacteria

Marine bacteria are isolated from surfaces of oil contaminated sea water from 20 meters depth of the open ocean and also from marine living or non-living marine surfaces. Halophiles have emerged as a renewed source for the discovery of novel biologically active compounds (Fenical, 1993 and 1997, Burgess et al., 2003, Longeon et al., 2004). Many free living and sediment inhabiting bacteria have been shown to produce secondary metabolites that display antimicrobial activity and several other biological properties.

Characterization and identification of marine isolates:

Two water samples were collected from 20 m depth from oil contaminated sea and from scrapings from living and non-living surfaces.

Morphologically distinct colonies were characterized by Gram staining and 20 isolates were selected from the 2 marine samples.

The characterization of the MB isolates was carried out, by studying their cultural and biochemical characters. Biochemical characterization based on the carbohydrate metabolism tests is vital to distinguish groups of marine bacteria for many years. Bacterial metabolism of carbohydrates can be broadly divided into two different mechanisms. The oxidation of carbohydrates can be strictly aerobic process or an anaerobic process, the later also called as fermentation (Hugh and Leifson, 1953).

Seawater normally has a pH of around 8 and as its buffer potentials are low, the pH variations are considerable on storage. Hence, it was found advantageous to buffer the medium slightly with 0.005M phosphate buffer (Leifson, 1963). Further the composition of seawater varies with the place of collection and season. Hence, to get uniformity, media prepared in ASW are preferred over those made with seawater (Novick and Tyler, 1985). Studies of Leifson, (1963), showed that for the sugar utilization tests of halophilic bacteria, preparation of O-F medium using either natural sea water or artificial sea water containing bromothymol blue failed to support the growth of a number of marine bacteria. The bromothymol blue indicator was found to be toxic for many of marine organisms. Several indicators were compared and Andrade's and phenol red indicator were found to be satisfactory, as they appeared to have least toxicity on halophiles. These factors were taken into consideration while performing the sugar utilization test and media prepared in ASW with Phenol red indicator were used in the present study.

Initial characterization of the 20 isolates was carried out, by conducting sugar utilization tests, IMViC and other biochemical tests. Based on the morphological, cultural and biochemical characters, attempts were made to categorize the isolates upto genus level.

It was observed that among the 20 isolates obtained, gram negative bacilli constituted 80% followed by gram negative coccobacilli rods (20%)

Biological activity of the isolates:

The growing interests in marine microbiology is partly due to the existence of species with varied metabolic properties that enable them to survive and grow under unfavorable conditions and produce several secondary metabolites. Rosenfeld and Zobell (1947) first demonstrated that marine bacteria produce antimicrobial substances. The first documented bioactive marine bacterial metabolite was the highly brominated pyrrole antibiotic, isolated from a bacterium obtained from the surface of the Caribbean Sea grass Thalassia (Burkholder et al., 1966). In recent years it has been increasingly realized that biofilms contain a higher proportion of often pigmented, antibiotic producing bacteria than other regions (Lemos et al., 1986, Burgess et al., 2003).

Clinical microbiologists use paper disk method as a popular means to determine susceptibility of bacteria to antibiotics and chemotherapeutic agents. The standard filter paper used for the antibiotic disc assays is composed of cellulose. The free hydroxyl groups present on each of the glucose residue render the surface of the disc hydrophilic. Studies of Braithwaite and Smith (1990) showed that as bioactive compounds produced by most of the marine bacteria are cationic, they have better adsorption to surface of the disc and slowly diffuse into the medium. Hence, disc diffusion method was found to be quite suitable for initial screening of marine isolates. However, as more than one type of bioactive compounds of different nature can be produced by same organism, further characterization of the potential organisms may need testing using other approaches or employing different disc materials to facilitate discovery of any additional compounds (Braithwaite and Smith, 1990).

Antimicrobial activity:

Among the 20 isolates, 65% isolates haven't shown any antimicrobial activity.25 % of them have shown antibacterial activity against E.coli (gram negative bacteria),while 15% of them exhibited antibacterial activity against B.subtilis (gram positive bacteria).The isolates MB 13 and 18 showed antibacterial activity on both E.coli and B.subtilis. MB 10 & 13 isolates exhibited antimicrobial activity against both bacteria and fungi. 35% isolates showed only antifungal activity. MB 10 showed highest activity against bacteria and fungi.

Isolation and screening of crude oil degrading bacteria:

Complex processes of oil transformation in the marine environment start developing from the first seconds of oil's contact with seawater. The progression, duration, and result of these transformations depend on the properties and composition of the oil itself, parameters of the actual oil spill, and environmental conditions.

Spills of non-petroleum hydrocarbons including vegetable oils and fish oils are of environmental concern because of their potential to cause serious effects on marine life and coastal environments. Biodegradation by indigenous microorganisms is an important and potentially ubiquitous process affecting both the chemical composition and physical properties of contaminant oils. Data on the environmental persistence of non-petroleum oils is now required for risk assessments and decision making by spill responders. This article investigates the biodegradability of various vegetable and fish oils under the influence of natural bacteria in seawater.

The influence of nutrients and microbial environment on changes in bacterial numbers and the extent and rate of degradation for various test oils (olive, mustard, canola and cod liver oils) were studied over time. Time-series visual and microscopic observations were made to characterize physical changes in the residual oils, formation of floating and precipitate particles, oil droplets and dispersion.

The fate of most petroleum substances in the marine environment is ultimately defined by their transformation and degradation due to microbial activity. About a hundred known species of bacteria and fungi are able to use oil components to sustain their growth and metabolism. In pristine areas, their proportions usually do not exceed 0.1-1.0% of the total abundance of heterotrophic bacterial communities. In areas polluted by oil, however, this portion increases to 1-10%.

When petroleum is spilled into the sea, it spreads over the surface of the water. It is subjected to many modifications, and the composition of the petroleum changes with time. This process is called weathering, and is mainly due to evaporation of the low- molecular-weight fractions, dissolution of the water-soluble components, mixing of the oil droplets with seawater, photochemical oxidation, and biodegradation.

Oil aggregates look like light gray, brown, dark brown, or black sticky lumps. They have an uneven shape and vary from 1 mm to 10 cm in size (sometimes reaching up to 50 cm). Their surface serves as a substrate for developing bacteria, unicellular algae, and other microorganisms. Besides, many invertebrates (e.g., gastropods, polychaetes, and crustaceans) resistant to oil's impacts often use them as a shelter.

Many oil spills in the sea cause shoreline pollution, despite efforts to prevent the drift of a spill toward the coastline. Cleaning up a polluted coastline by enhancing microbial activities was first attempted in 1989 after the spill from the Exxon Valdez. The initial measure taken after this accident was physical washing with high-pressure water.

Biochemical processes of oil degradation with microorganism participation include several types of enzyme reactions based on oxygenases, dehydrogenases, and hydrolases. These cause aromatic and aliphatic hydrooxidation, oxidative deamination, hydrolysis, and other biochemical transformations of the original oil substances and the intermediate products of their degradation.

In conclusion, application of oil degrading bacteria in the contaminated sea water is a proven alternative treatment tool that can be used to treat certain aerobic oil-contaminated environments. Typically, it is used as a polishing step after conventional mechanical cleanup options have been applied. It can be very cost – effective, although an in depth economic analysis has not been conducted to date. It has the advantage that the toxic hydrocarbon compounds are destroyed rather than simply moved to another environment. This method is not considered a primary response tool, although it could be so used if the spilled oil does not exist as free product and if the area is remote enough not to require immediate cleanup to satisfy a tourism industry. If the affected environment is a high energy shore line, the usage of degrading bacteria will be less likely effective than on a lower shoreline.

In conclusion the results obtained during this study indicated that the antimicrobial activity was due to the production of antibacterial compound in case of B. subtilis and fungal cultures of Aspergillus flavus, Aspergillus oryzae, Aspergillus niger, Trichoderma viridae. The compound could be extracted from growth medium by extraction with organic solvents whereas crude extract of antibacterial activity producing strain E.coli did not show any activity. This investigation reveals the importance of marine bacteria in the evaluation of antibacterial activity.

The biodegradation process of oil contaminated sea water was significantly influenced by environmental conditions, with a higher rate and extent of biodegradation observed in seawater amended with nutrients and wastewater that contained elevated numbers of bacteria and nutrients. All results clearly revealed a significant response of the samples to oil-contaminated seawater environments. Observations on changes in the physical properties of the residual oil may be important in the context of oil spill response strategies.
Further it may be investigated that different oils respond in different rates and extents to biodegradation depending on their stability, viscosity and compositions.

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Artificial Sea water (ASW)

Sodium chloride	-	6 g
Potassium chloride	-	0.170 g
Magnesium chloride	-	1.3 g
Magnesium sulphate	-	1.75 g
Distilled water	-	1000 ml

Christensen's medium

Peptone	-	0.1 g
Sodium chloride	-	1.2 g
Dipostassium hydrogen phosphate	-	0.2 g
Phenol red	-	0.002%
Glucose 10%	-	1 g in 10 ml - 1 ml
Urea 20%	-	2 g in 10 ml - 2 ml pH: 6.8-6.9
Agar	-	2 g
Distilled water	-	100 ml

Glucose phosphate peptone medium

Peptone	-	0.5 g
Dipotassium hydrogen phosphate	-	0.5 g
NaCl	-	1.2 g
Glucose 10%	-	5 ml (1 g in 10 ml)
Distilled water	-	100 ml

GP/10 medium

Gelatin	-	2.5 g
Tryptone	-	0.5 g
Yeast extract	-	0.05 g
ASW	-	1000 ml

GP/10 Agar

GP/10 media with 2% Agar

Milk medium

Nutrient agar with 1.2% NaCl	-	100 ml – (87.5 ml)
Skimmed milk	-	1%
NaCl	-	1.2 g
		pH - 7.0 + 0.2

Nutrient agar

Peptone	-	5 g
Beef extract	-	3 g
NaCl	-	12 g
Agar	-	20 g
Distilled water	-	1000 ml

Nitrate broth

Potassium nitrate	-	0.02 mg
Sodium chloride	-	1.2 g
Peptone	-	0.5 mg
Distilled water	-	100 ml

Nutrient-Gelatin agar medium

Potassium dihydrogen phosphate	-	0.5 g
Dipotassium hydrogen phosphate	-	1.5 g
Gelatin	-	4 g
Glucose	-	0.05 g
NaCl	-	12 g
Nutrient agar	-	1000ml

Nutrient - Starch agar medium

Soluble starch	-	0.2g
NaCl	-	1.2 g
Nutrient agar	-	100 ml

Peptone water

Peptone	-	2 g
Sodium chloride	-	1.2 g
Distilled water	-	100 ml

Potato dextrose agar

Potatoes	-	200 g
Dextrose	-	20 g
Agar	-	20 g
Distilled water	-	1000 ml

Simmon's citrate

Magnesium sulphate	-	0.20 g
Ammonium hydrogen phosphate	-	1.0 g
Dipotassium phosphate	-	1.0 g
Sodium citrate	-	2.0 g
Sodium chloride	-	12 g
Bromo thymol blue	-	0.08 g
Agar	-	15 g

Distilled water	-	1000 ml

REAGENTS:

Andrade's Indicator

Acid fuschin	-	0.5 g
Distilled water	-	100 ml

1N NaOH solution was added until the colour changes to yellow.

Gram's Stain

(i) Crystal violet

Sol A - Crystal violet	–	1 g
Alcohol 95%	-	20 ml
Sol B - Ammonium oxalate	–	0.8 g
Distilled water	–	80 ml

Mix A + B

(ii) Gram's iodine

Iodine	-	1 g
Potassium iodide	-	2 g
Distilled water	-	300 ml

(iii) Saffranin solution

Saffranin	-	1 g
Distilled water	-	100 ml

Kovac's reagent

P-dimethyl amino benzaldehyde	-	5 g
Amyl alcohol	-	75 ml
Conc. HCl	-	25 ml

Lead acetate strips

Whatmann No : 1 filter paper strips were soaked in freshly prepared lead acetate strips (0.5 g of lead acetate in 10 ml of distilled water and allow them to dry and store).

Methyl red indicator

Methyl red	-	0.1 g
Ethanol	-	300 ml
Distilled water	-	200 ml

O' meara reagent

40% Potassium hydroxide	-	0.5 ml
Creatine	-	0.3 g

Sabrauds Broth

Dextrose	-	2g
Yeast extract	-	1g
Peptone	-	1.5g
Distilled water	-	100ml

Tannic acid solution

Tannic acid	-	1 g
Distilled water	-	100 ml