

Molecular Profiling Of Rhizosphere Bacterial Communities of Plants Grown In Soils with Petroleum Contaminants

Ayser Ihsan Falih

M.Sc Biotechnology, Department of Biotechnology

University College of Science, Osmania University

ABSTRACT

Rhizosphere, a thin layer of soil that surrounds the root and is under the joint, direct influence of the root, root hairs, and AM hyphae adjacent to the root. In the rhizosphere, microbial processes include exudation, water uptake, nutrient mobilization, soil organic matter (SOM) decomposition, and respiration. The diversity of microbes associated with plant roots is enormous, in the order of tens of thousands of species. This complex plant-associated microbial community, also referred to as the second genome of the plant, is crucial for plant health. Recent advances in plant-microbe interactions research revealed that plants are able to shape their rhizosphere microbiome, as evidenced by the fact that different plant species host specific microbial communities when grown on the same soil. Understanding the rhizosphere interactions between microbes and plants is a critical first step in moving toward sustainable production of biofuel crops. In this project soil samples are procured from rhizosphere soil of soils having various petroleum contaminants. These soils are screened for isolation of bacteria and further studies are performed. DNA is isolated from the soil collected. The DNA is then amplified with the help of selected primers and agarose gel

electrophoresis is run and then the target DNA is extracted from the gel. Biochemical analysis of the bacteria is done to know the biochemical properties of the bacterial colonies from the soil. Further analysis is to be done.

Keywords: Rhizosphere, DNA, Biochemical properties, Agarose gel electrophoresis, Soil organic matter (SOM).

INTRODUCTION

Atmospheric concentrations of CO₂, CH₄, and N₂O have increased dramatically since the beginning of the industrial revolution largely due to human activities such as fossil fuel combustion and land-use change. These gases have the capacity to trap heat in the atmosphere by absorbing infrared radiation reflected by the earth's surface. Considerable evidence is now available showing that greenhouse gases have increased the global mean surface temperature by 0.3–0.6°C over the last century. Current predictions based on general circulation models indicate that the mean global temperature will increase by an additional 1–3.5°C in the next 50–100 years, with greater warming occurring at higher latitudes. Temperature is a key factor that regulates many terrestrial biogeochemical processes, such as soil respiration, litter



decomposition, N mineralization and nitrification, denitrification, CH₄ emission, fine root dynamics, plant productivity, and plant nutrient uptake. Despite the relatively robust literature on the response of individual ecological processes to changing temperature, a comprehensive understanding of whole ecosystem response to global warming remains elusive.

As some of these experiments are nearing completion, and others are being planned, there is a clear need to synthesize the existing data on ecosystem response to experimental warming in order to understand differences in response to date, to develop and refine current conceptual and quantitative frameworks for evaluating the response of ecosystems to elevated temperature, and to provide direction for future work in this relatively new field. Meta-analysis is a technique developed specifically for the statistical synthesis of independent experiments. Although meta-analysis has generally been applied to the analysis of data gathered from published literature, it is also well suited for combining the results of experiments carried out by individual researchers or by networks of research groups. Formal meta-analysis statistical techniques have numerous advantages over older approaches to quantitative data synthesis, such as “vote counting”, and have increasingly been used in the ecological literature.

Rhizomicrobial

Respiration

Microbial respiration of plant residues
Priming effect respiration Basal respiration

Root-derived respiration Plant-derived respiration Microbial-derived respiration. Total CO₂ efflux from soil (Rs) Turnover rate Residence time in soil Root respiration Rhizo-microbial respiration Microbial respiration of plant residues Priming effect respiration Basal respiration Root-derived respiration Plant-derived respiration Microbial-derived respiration Total CO₂ efflux from soil (Rs) Turnover rate Residence time in soil conditions, like soil type, plants cover, equipment, environmental conditions etc. Comprehensive reviews of these methods are given. Most methods involve a certain degree of disturbance of the soil system that changes natural fluxes to an uncertain degree. Increasing number of studies have explored soil respiration in relation to environmental factors and across bioclimatic area, pointing out a different role of various ecosystems in the terrestrial C cycle and its feedbacks to climate change. Whilst soil respiration has been well characterized for range of forest ecosystems comparatively little is known about grasslands.

Importance of Grassland ecosystems in global C balance.

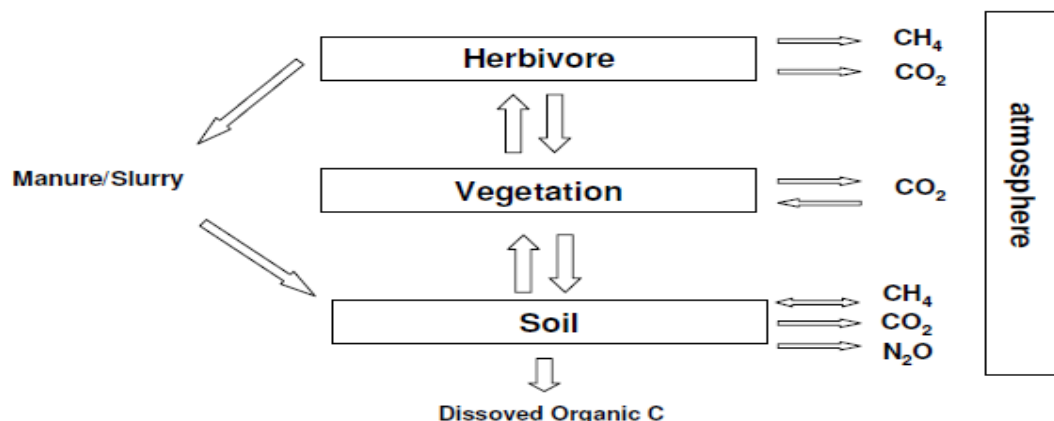
Grasslands are one of the world's most widespread vegetation types and comprise 32% of the earth's area of natural vegetation. Grasslands play a significant role in carbon storage and is an important component of the global carbon cycle. Even so, there have been relatively few long-term studies of grassland at the ecosystem level. At least in part, this is caused by the focus of many scientists on forests. Some researchers



have tried to assess the carbon budget in grassland. These studies suggest that grassland ecosystems can be a sink of CO₂ during their growing periods. However the grassland estimate, which is derived from a simple model CESAR, is the most uncertain (coefficient of variation of 130%) among all land-use types. And the contribution of this sink to the global carbon budget has not been adequately clarified. Grassland ecosystems are particularly complex and difficult to investigate because of the wide range of management and environmental conditions to which they are exposed. Currently, the net global warming potential (in terms of CO₂ equivalent) from the greenhouse gas exchanges with grasslands is not known. It is clear that an integrated approach, that would allow quantifying the fluxes from all three radiatively active trace gases (CO₂, CH₄, N₂O), would be desirable. Besides their natural aspect, grasslands have a pure agricultural destination as a primary food source for wild herbivores and domesticated ruminants. Actually, grasslands being a mixture of different grass species, legumes and herbs may act as carbon sinks, erosion preventives, bird directive areas, habitat for small animals, nitrogen fixation.

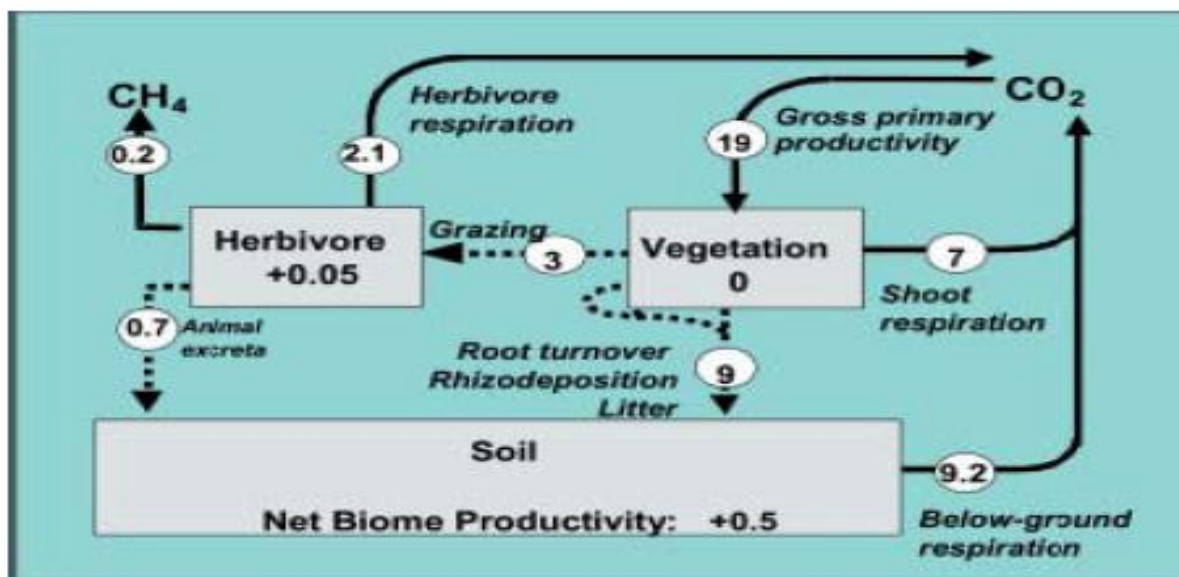
The grassland's carbon cycle integrates exchanges of carbon in the form of organic matter among three compartments (soil, vegetation, herbivores) and under inorganic form as CO₂ between each of these and

the atmosphere (Fig. 5 and 6). The vegetation exchanges actively CO₂ with the atmosphere through the biological processes of photosynthesis and respiration and contributes to inputs of organic matter into the soil by the decomposition of the dead tissues. The herbivores consume grass matter, return part of the ingested carbon through excrements, which naturally serves as fertilizing substrate for grasses, and emit CO₂ to the atmosphere as a result of respiration. In managed grasslands the excreted carbon may be incorporated directly into soil as manure by farming practices. For natural steppe ecosystems, in absence of livestock, the fraction of primary productivity consumed by herbivores, typically rodents, is very small and generally does not exceed 1-2% of NPP. Up to 98% of the total carbon store in temperate grassland ecosystems can be found sequestered in the below ground pool [37] which generally has much slower turnover rates than aboveground C [51]. Carbon dioxide is lost from grassland soils by root respiration and microbial respiration from decomposition of soil organic matter. Changes in inorganic carbon content is a function of the balance between inputs to soil of carbon fixed by photosynthesis and losses of soil carbon via decomposition. Soil erosion can also result in the loss (or gain) of carbon locally, but the net effect of erosion on carbon losses as CO₂ for large areas on a national scale is unclear.



Schematic diagram of the greenhouse gas fluxes and main organic matter (OM) fluxes in a grazed grassland. Moreover, grasslands contribute to the biosphere – atmosphere exchange of non CO_2 greenhouse gases, with fluxes intimately linked to management practices. Of the three greenhouse gases that are exchanged by grasslands, CO_2 is exchanged with the soil and vegetation, N_2O is emitted by soils and CH_4 is emitted by livestock at grazing and can be exchanged with the soil. For grasslands, the nature, frequency and intensity of disturbance plays a key role in the C balance. In agricultural systems, land use and management act to modify both the input of organic matter via residue production, organic fertiliser application, grazing management and the rate of decomposition (by modifying microclimate and soil conditions through crop selection, soil tillage, mulching, fertiliser application, irrigation and liming). Management practices that increase soil and root respiration cause short-term effluxes of CO_2 to the atmosphere, whilst practices that increase the rate of decomposition of organic matter lead to longer-term losses of soil organic carbon in the form of carbon

dioxide. Herbage harvesting by cutting also results in carbon exports from grassland plots. Most of the carbon harvested and stored in hay or silage will be released as CO_2 to the atmosphere shortly after harvest. In a cutting regime, a large part of the primary production is exported from the plot as hay or silage, but part of these C exports is compensated for by farm manure and slurry application. Under intensive grazing, up to 60 % of the above ground dry matter production is ingested by domestic herbivores. However, this percentage can be much lower during extensive grazing. The largest part of the ingested carbon is digestible (up to 75% for highly digestible forages) and, hence, is respired shortly after intake. Only a small fraction of the ingested carbon is accumulated in the body of domestic herbivores or is exported as milk. Large herbivores, such as cows, respire approximately one ton C per year. Additional carbon losses occur through methane emissions from the enteric fermentation. However, grazing practices which increase grassland productivity have the potential to increase SOC and C sequestration.



Carbon cycle in grazed grassland. The main carbon fluxes ($\text{t C ha}^{-1}\text{yr}^{-1}$) are illustrated for grassland grazed continuously by cattle at an annual stocking rate of two livestock units per ha. Soil respiration (R_s) is an important component of the ecosystem C budgets. It is a major source of CO_2 released by terrestrial ecosystems and after the photosynthesis, CO_2 efflux from soil remains the second largest C flux accounting for 60-90% of total ecosystem respiration [60]. R_s is known to experience high spatial and temporal variation with different controlling factors involved on different time-scales. However, up to now not so many studies have dealt with the interannual variability of soil respiration and only a few of them were performed in grassland ecosystems despite the fact that it is one of the world's most widespread vegetation types which comprises 32% of the earth's area of natural vegetation.

R_s integrates the CO_2 produced by soil microorganisms in the root-free and root-affected soil and actual root respiration.

Microbial respiration in the root-affected soil, so called rhizomicrobial respiration, is closely coupled to roots distribution and activity and could be hardly separated from the last one [23]. We will call this type of respiration as 'root-derived' (R_a), and the CO_2 originated from the root-free soil as 'microbial-derived' respiration (R_h). According to recent reviews the relative contribution of R_a and R_h generally accounts for approximately one half of the total CO_2 efflux [52], but varies significantly among studies (10-90%). Quantifying the contribution of these two major respiratory sources to the total CO_2 efflux and understanding the seasonal and interannual variability and their response to climate change is very important for successful modelling and prediction of the ecosystem C cycling. The potential change in soil CO_2 efflux will largely depend on the relative contribution of R_a and R_h to the total CO_2 efflux. Isotope studies, applying the isotope pulse labeling techniques have demonstrated that the processes of photosynthetic C uptake and its following



evolution through the rooting systems are coupled with a time lag in the range of minutes to days, suggesting the diurnal variation in soil respiration is also affected by photosynthetic C supply.

Factors Influencing Soil Respiration

At the beginning of the twentieth century, some of the major factors that influence soil respiration had been established. These included the role of soil moisture in microbial activity, the primary role of bacterial decomposition as a source of CO₂ efflux, importance of soil diffusion kinetics in determining efflux and the correlation of CO₂ production with the rate of diffusion through the soil. More recently, estimates of global terrestrial CO₂ flux to the atmosphere have improved substantially, in accuracy and number, especially in relation to different biomes [20]. The mean rates of soil respiration (g C m⁻² yr⁻¹) for a variety of vegetation-based, global biomes have been tabulated by Raich and Schlesinger [29]. Examples include Tundra (60 ± 6), northern bogs and mires (94 ± 16), desert scrub (224 ± 38), temperate grasslands (442 ± 78), temperate deciduous forests (647 ± 51), and tropical moist forests (1,260 ± 57). With increasing climate change, current evidence indicates there has been a substantial increase in terrestrial CO₂ flux to the atmosphere during the period of 1960 to present, especially for temperate and tropical biomes compared to high latitude biomes. Based on data analyzed by Bond-Lamberty and Thomson, the recent annual global soil respiration (R_s) is estimated to be 98 ± 12 Pg C; or if agricultural areas are excluded, 85 Pg C. The contribution to total

R_s by boreal, temperate and tropical biomes is 13%, 20% and 67%, respectively. Although the largest contribution is from temperate and tropical biomes, the most significant relative change in recent years (7%) has been in the polar biomes. There are less dramatic increases (2-3%) in lower latitudes. This is further supported by meta-analyses of large networks of data sources. Furthermore, as may be expected, the Bond-Lamberty and Thomson analyses indicate increasing R_s can be partially attributed to increasing global climate change. Laboratory studies of the effects of warming on soil respiration also indicate that the response of microbial respiration to warming as assessed by Q₁₀ measurements may differ substantially for soils from different latitudes [43]. As climate patterns change, including variations in temperature and precipitation patterns, major shifts in biome boundaries are expected to occur. Among these are likely transitions between grasslands and forests. Some current evidence suggests that mean soil organic carbon in forested sites can be as much as two-times larger than in remnant grasslands (e.g. 3,382 vs. 1,737 gC m⁻²), including increased R_s in forested sites compared to grasslands (745 vs. 611 gC m⁻² yr⁻¹). Microbial biomass carbon was also higher in the woodlands compared to grasslands (444 vs. 311 mg C kg⁻¹ soil, respectively). Transitions between grasslands and woodland ecosystems can occur in either direction, depending on climatic factors, particularly changes in precipitation patterns, with less precipitation favoring transitions from woodland to grassland regimes. Soil Respiration,



Precipitation Patterns and Soil Moisture Among major climatic variables, patterns of precipitation and soil moisture are likely to have significant effects on soil microbial communities and their respiratory responses. Therefore, a survey of some pertinent published research on the response of soil respiration to variations in precipitation is presented as background for the more focused analysis of the role of microbial communities in soil respiration presented later.

In tundra, moss-rich surface soil that has thawed, and is sufficiently moist to support microbial activity, the CO₂ efflux is higher for mesic sites compared to wet sites where water-logging and anaerobic conditions can suppress aerobic respiration [55]. Illeris et al. [24], working with subarctic heath soil, report that optimum moisture content for CO₂ efflux was in the moderate range of 240% soil dry weight, consistent with a range between 200 and 500% reported by Heal et al. [32]. Laboratory measurements of tundra soil respiration from a mesic upslope location compared to a wetter downslope location [10] also supported the conclusion that respiratory efflux (nmol min⁻¹ cm⁻³) was greater at the mesic site relative to the wetter site when measured at two different temperatures of 15°C (9.1 ± 0.6 vs. 4.1 ± 0.7) and 25°C (21.4 ± 0.2 vs. 7.8 ± 0.5). With increased evidence of global warming, and increasing annual temperatures in polar regions, substantial stores of organic compounds in the permafrost may be released supporting microbial respiratory growth and CO₂ efflux to the atmosphere. There are millions of square kilometers of circumpolar tundra, and

estimates of respiratory CO₂ emissions can become as high as 5 to 10 kmol km⁻² h⁻¹, assuming continued climate change warming, a 10-cm thaw depth, and suitable patterns of precipitation [28]. This is based, however, on a model that assumes only bacterial and protist contributions - estimates could change substantially in the future, depending on differences in soil physical characteristics, percent active bacteria, and a better estimate of contributions by fungi. However, the above estimates are consistent with current evidence based on field sampling [5]. In addition to estimates of tundra protist contributions to respiratory CO₂, the carbon content of the protist community can be as much as 25% of the amount in the bacteria in the sampled Alaskan tundra soil [30].

BIOCHEMICAL ASSAYS

MATERIALS AND METHODS:

Soil Dilution to isolate wild strain

1. Place 1 gram of your soil sample into a culture tube containing 10 ml of sterile water; cap the tube and shake vigorously.
2. Using a serological pipette, remove 1 ml of the soil/water mixture and place into a fresh culture tube.
3. Add 9 ml of fresh sterile water to this second tube; cap and shake vigorously.
4. Repeat step 2 using the second, diluted tube and then repeat step 3 with this third tube.
5. Continue step 4 with each additional tube until you have diluted the original soil/water mixture a

minimum of four times (a 10-4 dilution).

6. Plate 100 µl samples from the 4th and 5th tubes (dilutions 10-3 & 10-4) onto their own separate, individual petri plates filled with nutrient agar and allow to incubate at room temperature over night.
7. Examine each of the plates for individual bacteria colonies and choose the plate with the fewest colonies to make your estimates of the number of bacteria in the original 1 cc soil sample (# colonies on plate C 102 = # of bacteria in dilution tube; # of bacteria in dilution tube C 10 |# of dilutions| = # of bacteria in original sample tube).
8. If there are not individual colonies but still a "lawn" at the 10-4 dilution, repeat steps 1-6, adding a 5th dilution, 6th dilution, etc. as necessary until individual colonies are observed.

Media Composition isolation of wild strain:

| | |
|-------------------------------------|---|
| Brain Heart Infusion 17.5 g | - |
| Enzymatic Digest of Gelatin 10 g | - |
| Dextrose 2 g | - |
| Sodium Chloride 5 g | - |
| Disodium Phosphate 2.5 g | - |

Distilled water -
1000 liter

Wild strain isolation by Quadrant Streak Method

1. Sterilize the inoculating loop in the bunsen burner by clicking on the loop and dragging it to the burner. Put the loop into the flame until it is red hot. Allow it to cool.
2. Pick an isolated colony from the agar plate culture and spread it over the first quadrant (approximately 1/4 of the plate) using close parallel streaks.
3. Flame the loop.
4. Turn the plate 90° and lightly sweep the loop 1-2 times through the inoculated area, then streak into the next quadrant without overlapping the previous streaks.
5. Flame the loop.
6. Turn the plate 90°, overlap the previous area 1-2 times, and streak into the next quadrant as in step 4.
7. Flame the loop.
8. Repeat #6, streaking the remainder of the plate.
9. Invert the plate and incubate at 37°C for 24 hr.

BIOCHEMICAL ASSAYS FOR ISOLATED WILD STRAIN

MOTILITY TEST

AIM:

Motility test is used to determine the motility of microorganisms.

PRINCIPLE:

Bacterial motility can be observed directly from examination of the tubes following incubation. Growth spreads out from the line of inoculation if the organism is motile. Highly motile organisms provide growth throughout the tube. Growth of nonmotile organisms only occurs along the stab line.

MATERIALS REQUIRED:

- 24 h old culture
- LB Agar
- Test tubes
- Inoculation loop

LURIA BROTH AGAR MEDIA:

- Tryptone - 10grams
- Yeast extract- 5grams
- Sodium chloride - 5 grams
- Agar - 5 grams
- Distilled water 1000ml

Indole production test

Tryptophan an essential amino acid is oxidized by some bacteria by the tryptophanase resulting in the formation of indole, pyruvic acid and ammonia. The indole test is performed by inoculating a bacterium into tryptone broth, the indole produced during the reaction is detected by adding Kovac's reagent (Dimethyl amino benzaldehyde) which produces a cherry-red reagent layer.

Requirements

1. Nutrient broth cultures
2. Tubes containing 1% Tryptone broth, 5 ml/tube
3. Kovac's reagent
4. Dropper bottle
5. 1 ml pipette
6. Bunsen burner

7. Inoculating needle

Preparation of tryptone broth (pH 7.0)

1. Tryptone 10 g
2. Calcium chloride 0.01-0.03 M
3. Sodium chloride 5 g
4. Distilled water 1000 ml

Methyl red and Voges proskauer tests

The methyl red test and VP test are used to distinguish between bacteria that produce large amount of acid and those that produce the neutral product acetoin as an end product. Both these are performed simultaneously because they are physiologically related and are performed on the same medium MR_VP broth. In these tests, if an organism produces large amount of organic acids (end products) from glucose the medium will remain red (a positive test) after the addition of methyl red a pH indicator (pH <4.4).

MRVP test is of value in the separation of E.coli and Enterobacter aerogenes which appear virtually identical except for certain physiological differences that are used as indicators of the sanitary quality of water, foods, food production and eating establishments.

Requirements

- Nutrient broth cultures
- V. P broth tubes – 5 ml/tube
- Methyl red PH indicator
- V. P reagent 1 (naphthol solution)

- V. P reagent 2 (40% potassium hydroxide)
- Clean empty test tubes
- Bunsen burner
- Inoculating loop

Preparation of MRVP broth (pH 6.9) tubes

1. Peptone - 7 g
2. Dextrose/Glucose - 5 g
3. Potassium phosphate - 5 g
4. Distilled water - 10000 ml

Gelatin Hydrolysis (Production of Gelatinase)

Gelatin is a protein produced by hydrolysis of collagen a major component of the connective tissue and tendons in humans and other animals, Hydrolysis of gelatin is brought about by microorganisms capable of producing proteolytic exoenzyme known as gelatinase, which acts to hydrolyze this protein to amino acids, Hydrolysis of gelatin can be identified by growing the microorganisms in the nutrient gelatin. If the degradation of gelatin occurs in the medium by an exoenzyme produced by the bacteria , it can be detected by lequification by flooding the plates with the protein precipitating material (By adding the gelatin plates with mercuric chloride solution and observing for the clearing around the line of growth).

Materials required

1. 24 h culture
2. Gelatin agar medium (Hi Media)
3. Mercuric chloride solution
4. Sterile petri dishes
5. Inoculating loops

Procedure

- The gelatin agar medium was prepared according to standard protocol and sterilized at 121°C for 15 minutes and cooled to 45 to 50°C.
- The sterilized medium was poured in to sterile petri dishes and allowed to solidify.
- The culture was inoculated by streaking on to the gelatin agar plates.
- The plates were incubated at 37 °C for 4 to 7 days.
- The plates after incubation period were flooded with mercuric chloride solution and the plates were allowed to stand for 5 to 10 minutes.

Starch Hydrolysis

Amylase is an exoenzyme produced by the bacteria the hydrolysis starch a polysaccharide, the ability of the bacteria to produce amylase is determined by adding a indicator iodine solution starch in the presence of iodine produces dark blue coloration of the medium and an yellow zone around the colony which indicate the amylolytic activity

Requirements

1. 24 h old culture.
2. Starch Agar Medium (Hi Media).
3. Grams iodine solution.
4. Sterile petri dishes.
5. Dropper.
6. Inoculating loop.

Oxidase Production



The production of oxidase is one of the most significant tests we have for differentiating certain groups of bacteria. For example, all the Enterobacteriaceae are oxidase-negative and most species of Pseudomonas are oxidase-positive. Another important group, the Neisseria, are oxidase producers. This test: The first method utilizes the entire TSA plate; the second method is less demanding in that only a loopful of organisms from the plate is used. Both methods are equally reliable. Oxidase positive bacteria possess cytochrome oxidase or indophenol oxidase (An iron containing haeme protein). These both catalyse the transport of electrons from donor compounds (NADH) to electron acceptors (usually oxygen). The test reagent, N, N, N', N'-tetra-methyl-p-phenylenediamine dihydrochloride acts as an artificial electron acceptor for the enzyme oxidase. The oxidised reagent forms the coloured compound indophenol blue. The cytochrome system is usually only present in aerobic organisms which are capable of utilizing oxygen as the final hydrogen receptor. The end product of this metabolism is either water or hydrogen peroxide (Broken down by catalase).

Requirements

1. 24 h old culture.
2. Nutrient Agar Plates
3. N, N, N', N'-tetra-methyl-p-phenylenediamine dihydrochloride
4. Bunsen burner.
5. Inoculating loop.

Protease Assay

- 0.10 to 0.25 ml culture supernatant is diluted with resp. 0.90 to 0.75 ml

Tris-buffer (100 mM, pH 7.2) and equilibrated at 30°C in a tube.

- 0.5ml of a pre-warmed 1.25% (w/v) azoalbumine (Sigma) solution is added and the contents are mixed.
- After 30-60 minutes incubation at 30°C the reaction was stopped by the addition of 1.0 ml 10% (w/v) trichloroacetic acid. The precipitate is removed by centrifugation. To 1.0 ml of the supernatant is added 1.0 ml 0.5 M NaOH immediately before reading the absorbance at 440 nm.
- The protease activity in the supernatant is considered to be due to alkaline serine protease and expressed in absorbance units per ml \times hour. Blanks are prepared for every sample, with the same amounts of sample with buffer, after which is added the TCA and only then followed by the azoalbumine.

Cellulase Assay

- Required: 1% Sodium carboxymethylcellulose, 10% NaCl in 0.01 N acetate buffer pH 5.5 (HAc and NaAc) and 1% 3,5 dinitrosalicylic acid CM-cellulose solution and sample are preheated at 30°C and mixed by putting together 0.5 ml of each solution.
- They were incubated for 10' at 30°C. 1% 3,5 dinitrosalicylic acid reagent (1.0 ml) is then added, mixed and the tube immersed in boiling water for 5 min.
- The tube is cooled with running tap water and 10 ml water is added, the

whole mixed and the absorbance is measured at 540 nm.

- The control is a sample which contained instead of enzyme, 0.5 ml water. Reducing sugars in the extract were determined separately and subtracted from the results obtained in the assay.
- 1 unit is that amount of enzyme that produces 1.0 mg of reducing sugar calculated as glucose under the above conditions.

Assay of α -AMYLASE

- 20 mM Sodium Phosphate Buffer with 6.7 mM Sodium Chloride, pH 6.9 at 20 °C (Prepare 100 ml in deionized water using Sodium Phosphate, Monobasic, Anhydrous, Sigma Prod. No. S-0751, and Sodium Chloride, Sigma Prod. No. S-9625. Adjust to pH 6.9 at 20°C with 1 M NaOH.)
- 1.0% (w/v) Soluble Starch Solution (Starch) (Prepare 25 ml in Reagent A using Starch Potato Soluble, Sigma Prod. No. S-2630. Facilitate solubilization by heating the starch solution in a glass beaker directly on a heating/stir plate using constant stirring.
- Bring to boil and maintain the solution at this temperature for 15 minutes.
- Allow the starch solution to cool to room temperature with stirring. Return the starch solution to its original volume (25 ml) by the addition of water and dispense samples for assay while stirring.)

Sodium Potassium Tartrate Solution

Dissolve 12.0 grams of Sodium Potassium Tartrate in 8.0 ml of 2 M NaOH. Heat directly on a heating/stir plate using constant stirring to dissolve.

96 mM 3,5-Dinitrosalicylic Acid Solution

Prepare 20 ml in deionized water using 3,5-Dinitrosalicylic Acid, Heat directly on a heating/stir plate using constant stirring to dissolve.

Color Reagent Solution

With stirring, slowly add Reagent C to Reagent D. Dilute to 40 ml with deionized water. If not completely dissolved, the reagents should dissolve when mixed. The solution should be stored in an amber bottle at room temperature. The Color Reagent Solution is stable for 6 months.

F. 0.2% (w/v) Maltose Standard Solution

Prepare 10 ml in deionized water using Maltose, Monohydrate.

α -Amylase Solution

Immediately before use, prepare a solution containing 1 unit/ml of α -Amylase in cold deionized water.

PROCEDURE:

Pipette (in milliliters) the following reagents into suitable containers:

| | Test | Blank |
|--------------------|------|-------|
| Reagent B (Starch) | 1.00 | |
| | 1.00 | |

Mix by swirling and equilibrate to 20 °C.
Then add:

Reagent G (Enzyme Solution) 1.00

Mix by swirling and incubate for exactly 3.0 minutes at 20 °C. Then add:

Reagent E (Clr Rgt Soln) 1.00
1.00

Reagent G (Enzyme Solution) -----
1.00

Cap and place in a boiling water bath for exactly 15 minutes, then cool on ice to room temperature and add:

Deionized water 9.00
9.00

Mix by inversion and record the A_{540nm} for both the Test and Blank using a suitable spectrophotometer.

Laccase assay:

ABTS Plate Screen

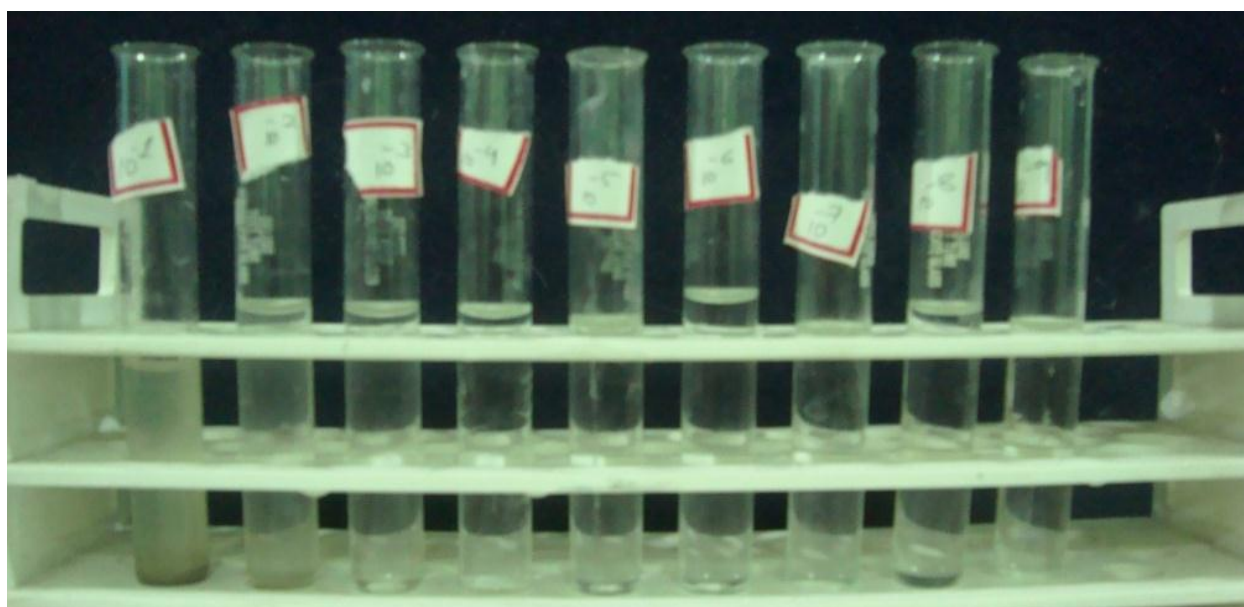
Requirements:

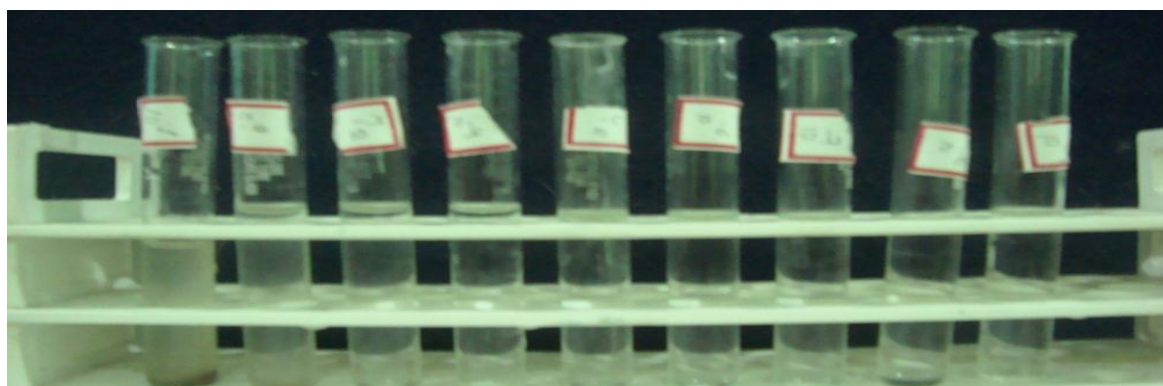
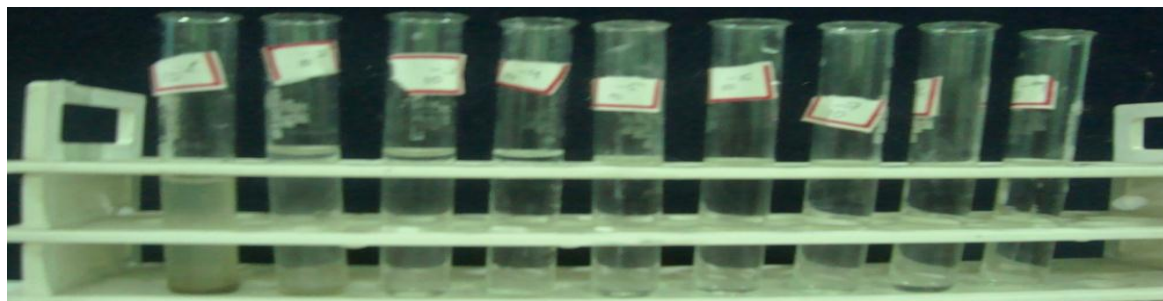
- ABTS 20mM (10mg/mL) stock solution
- CuSO₄ 100mM (25mg/mL) stock solution

Procedure

- Make a liter of your favorite agar growth medium and autoclave.
- After cooling to 65°C add:
 - 10ml/ABTS (0.2 mM)
 - 1mL CuSO₄
- Swirl to mix and pour plates.
- Once plates are hard:
 - Drop 5μL of overnight culture onto the plate (when this dries it will form a little circle).
 - Culture plate 24-48 hours
 - Observe green halos around ABTS oxidizing cultures.

Serial Dillution





Sprea plate of serial diluted samples



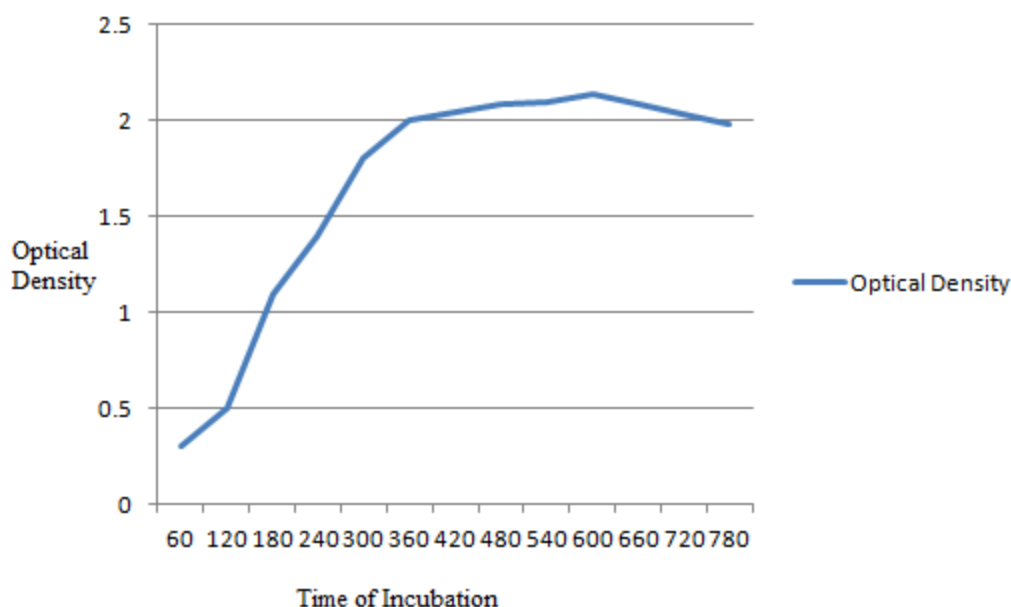
Quadrant streaking of





Bacterial Growth Curve

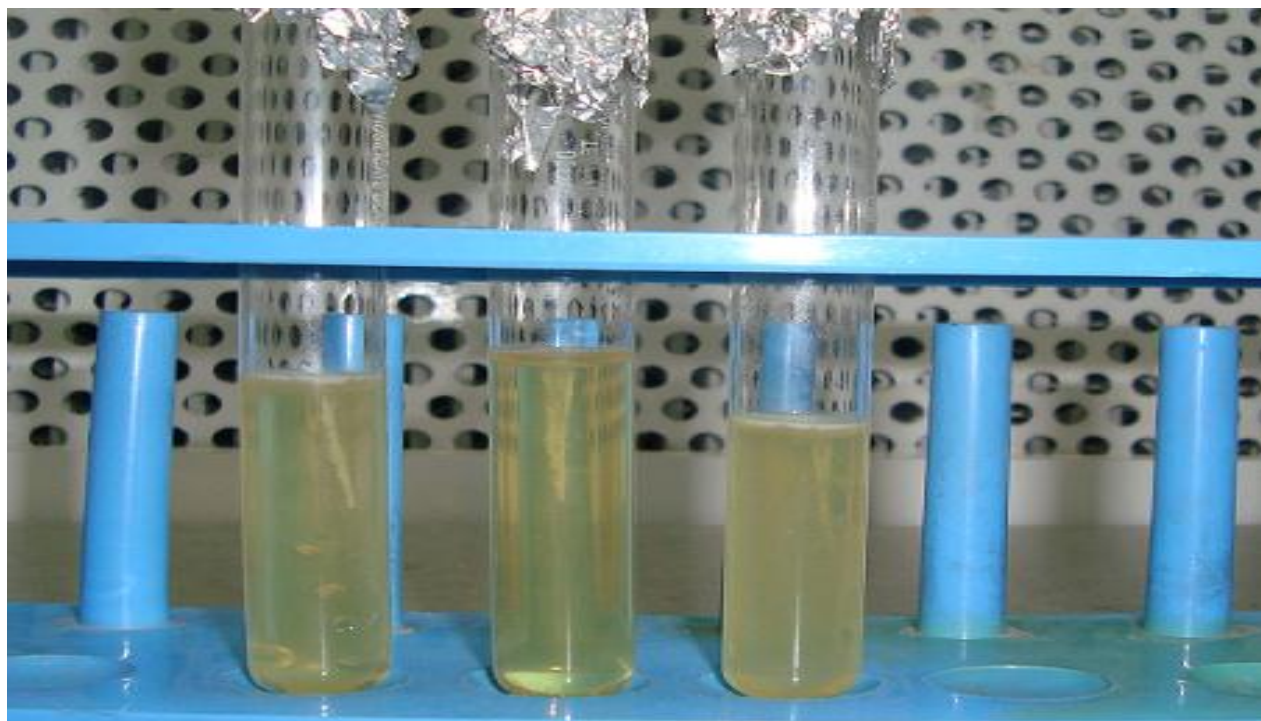
| Optical Density | Time of Incubation |
|-----------------|--------------------|
| 0.3 | 60 |
| 0.5 | 120 |
| 1.1 | 180 |
| 1.4 | 240 |
| 1.8 | 300 |
| 2 | 360 |
| 2.04 | 420 |
| 2.09 | 480 |
| 2.1 | 540 |
| 2.14 | 600 |
| 2.09 | 660 |
| 2.03 | 720 |
| 1.98 | 780 |

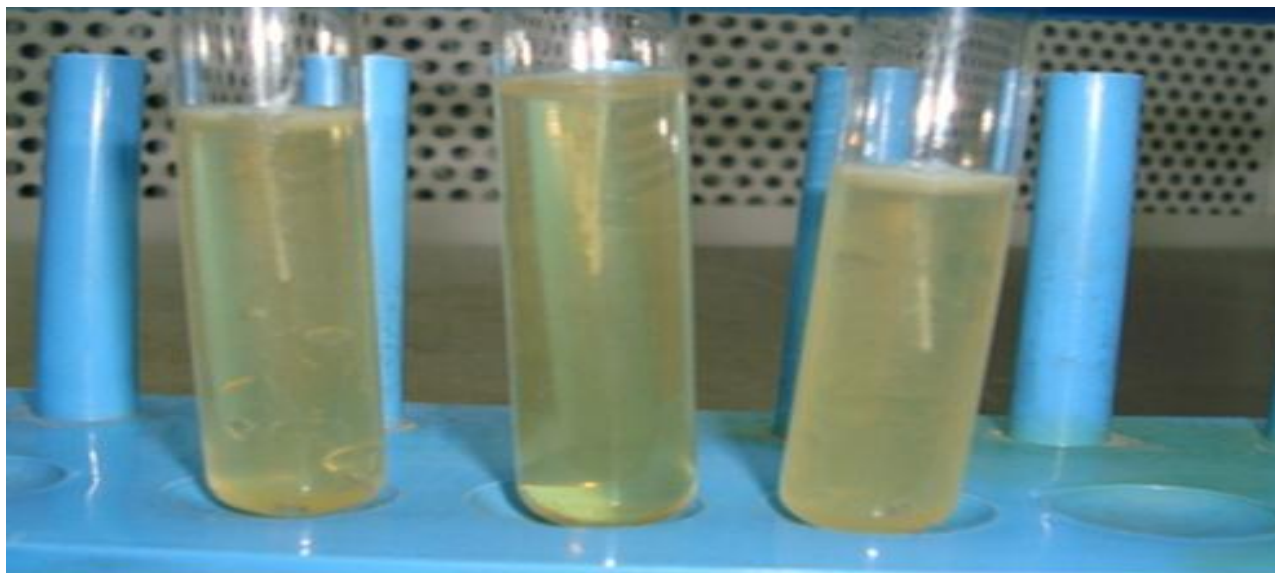


The dynamics of the microbial growth can be charted by means of a population growth curve, which is constructed by plotting the increase in cell numbers versus time of incubation and can be used to delineate

stages of the growth cycle. It also facilitates measurement of cell numbers and the rate of growth of a particular organism under standardized conditions

Motility Test





Motility Agar is soft agar in a test tube (without a slanted surface). Cells are stab-inoculated into the agar (the top surface is not inoculated). Non-motile bacteria will only grow where they were inoculated. Motile bacteria will grow along the stab and

will also swim out away from the stabbed area. Thus, a negative result is indicated by growth in a distinct zone directly along the stab. A positive result is indicated by diffuse (cloudy growth), especially at the top and bottom of the stab.

Catalase Test:



Many aerobic bacteria and most of those which are facultatively anaerobic produce the enzyme catalase. The function of this enzyme is to detoxify hydrogen peroxide (H_2O_2), which is formed from the superoxide radical by superoxide dismutase.

Many aerotolerant anaerobic bacteria have peroxidase (which is not the same enzyme as cytochrome c oxidase) instead of catalase. Obligate anaerobic bacteria lack superoxide dismutase and catalase.

Indole Test



Bacteria, which express the enzyme tryptophanase can hydrolyze the amino acid tryptophan to indole, pyruvic acid and ammonia. Presence of indole can be shown

by means of Kovác's reagent in the so-called indole test. Kovác's reagent contains p-dimethylaminobenzaldehyde, which forms a red complex with indole

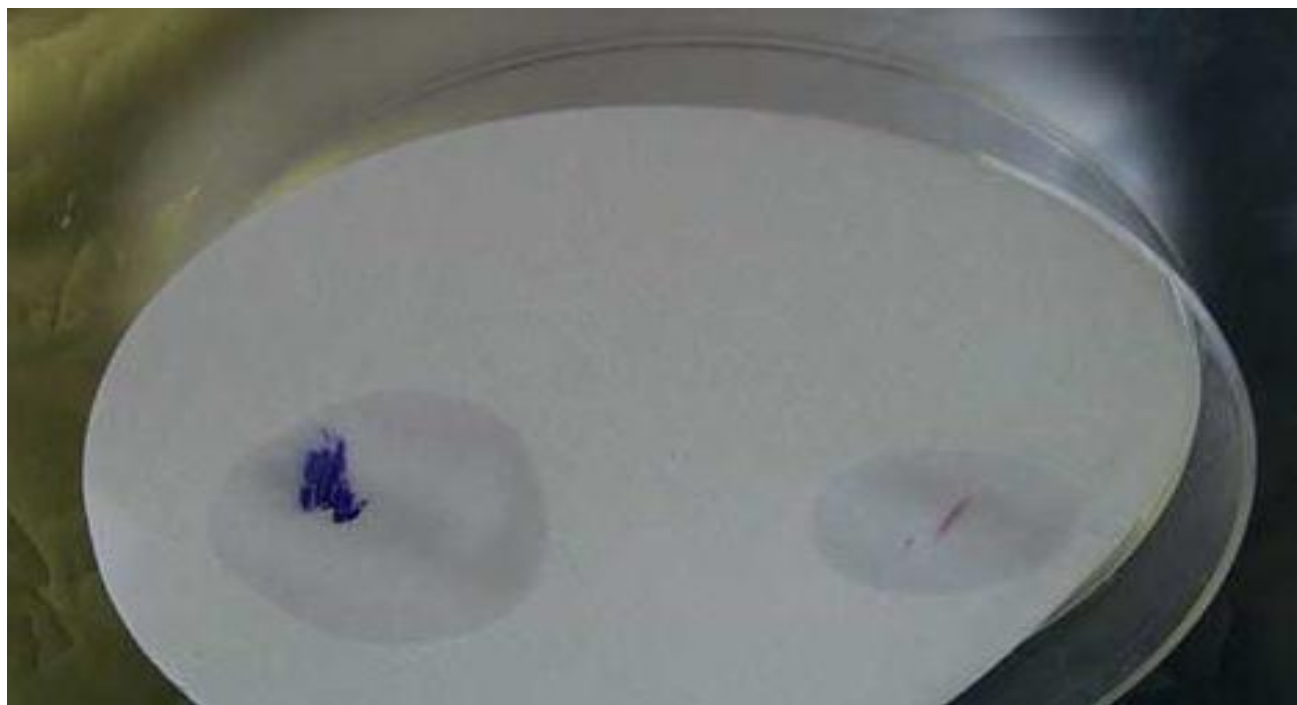
MR-VP Test



MRVP media contains glucose, peptone and phosphate buffer. Many enteric organisms can overcome the buffering capacity of the media by producing large quantities of a stable acid end product, thus lowering the

pH. Acid production is detected using the pH indicator methyl red (red pH < 4.4, yellow pH > 6).

Oxidase test:



Bacteria, which have aerobic respiration, often have cytochrome c and a cytochrome c oxidase. The presence of these components can in combination with other methods be used for typing. A commercial test, which contains an artificial electron acceptor (N, N, N', N'-tetramethyl-p-phenylenediamine, see Fig. 1), is often used. This artificial electron acceptor change colour depending

upon redox state. The substance is also referred to as a redox indicator and it can be oxidized by the oxidized form of cytochrome c. Cytochrome c oxidase is the last enzyme of the electron transport chain, where it normally reduces oxygen to water and pump protons to the outside

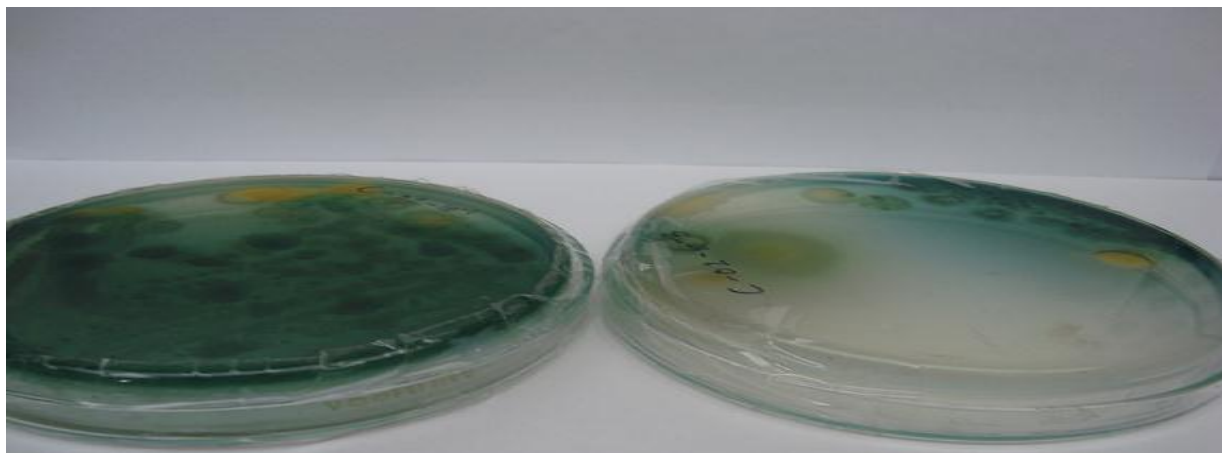
Starch hydrolysis:



The purpose is to see if the microbe can use starch, a complex carbohydrate made from glucose, as a source of carbon and energy

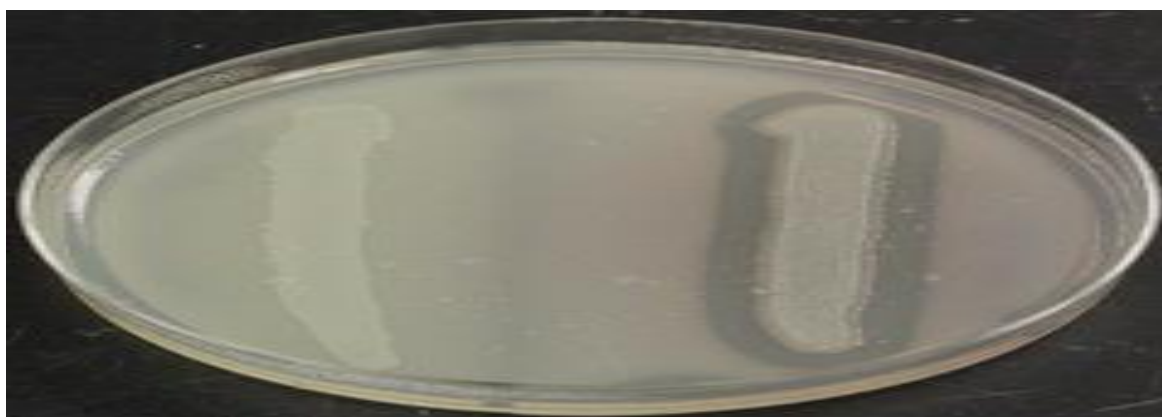
for growth. Use of starch is accomplished by an enzyme called alpha-amylase.

Laccase activity:



supernatant was able to oxidize ABTS and guaiacol the above image shows the enzyme solution with these two substrates and the ability of laccase to oxidize them.

Protease activity:



Bacteria secrete proteases to hydrolyse (digest) the peptide bonds in proteins and therefore break the proteins down into their constituent monomers(amino acids)

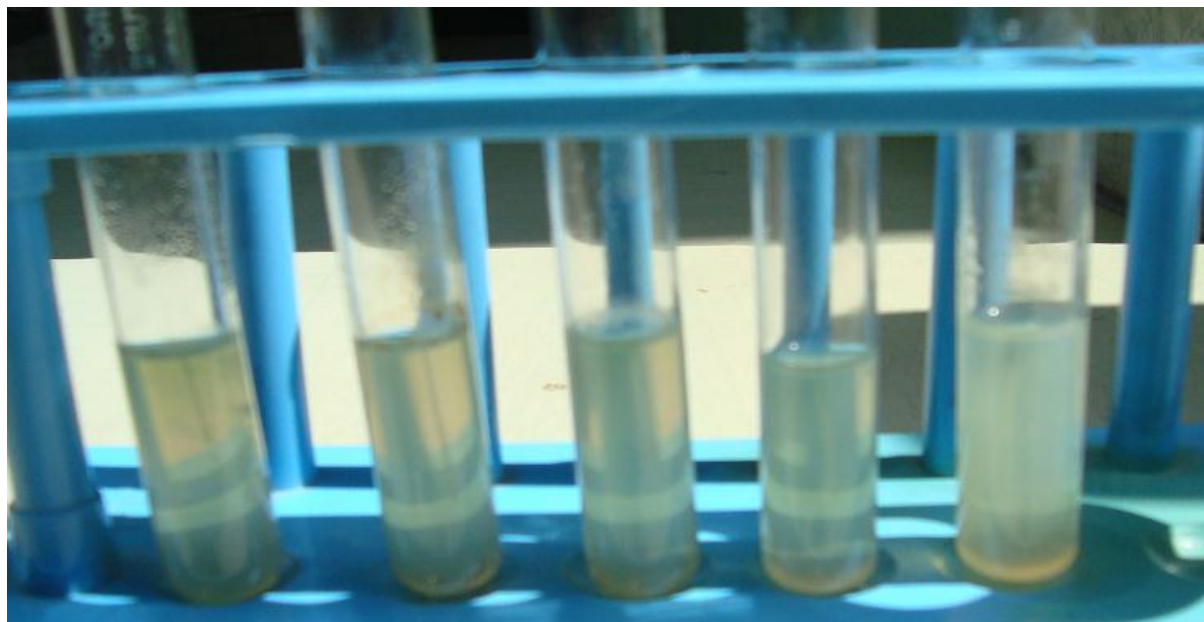
Amylase test:



This assay is a liquid phase enzymatic assay. The salivary alpha Amylase metabolizes specifically the substrate. The intensity of

the colour developed is proportional to the activity of alpha amylase in the sample

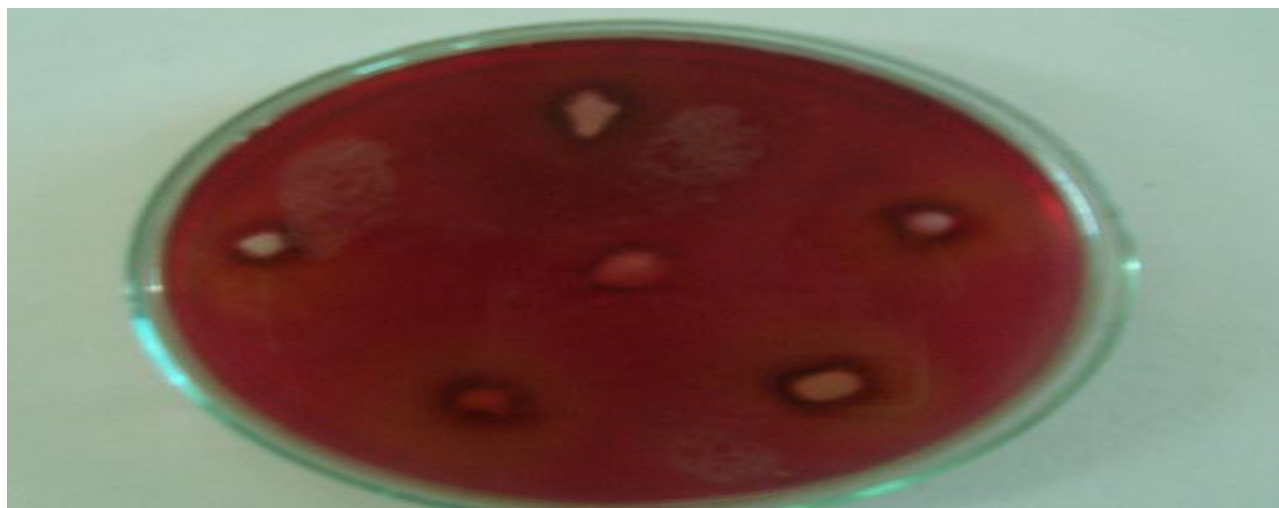
Gelatin hydrolysis:



the purpose is to see if the microbe can use the protein gelatin as a source of carbon and energy for growth. Use of gelatin is accomplished by the enzyme gelatinase. A medium containing gelatin is used. We have all seen how gelatin forms a semisolid substance. In the medium it serves as the

solidifying agent (substituting in place of agar) in addition to its role as a source for carbon and energy. When gelatin is used, the medium changes from semisolid to liquid and cannot be resolidified.

Cellulose test:



Cellulase activity is determined by its effect on microcrystalline cellulose with respect to glucose formation. Released glucose is determined in a hexokinase/glucose-6-phosphate dehydrogenase system

Primer Designing:

Seq

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>Escherichia coli
AAATTGAAGAGTTTATCATGGCTCAGATTGAACGCTGGCGGCAGGCCAACACATGCAAGTCGAACGGTAACAGGAAGAAGCTT
GCTTCTTTTGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAACTGCCGTATGGAGGGGATAACTACTGGAAACGGTAGCT
AATACCGCATAACGTCGCAAGACCAAGAGGGGTACCTTCGGGCCTCTTGCCATCGGATGTGCCAGATGGGATTAGCTAGTAGG
TGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAAGTGAACACGGTCCAGACT
CCTACGGGAGGCGAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCCTTCGGG
TTGTAAAGTACTTTTCAGCGGGGAGGAAGGGAGTAAAGTTAATACCTTTGCTCATTGACGTTACCCGCGAGAAGAAGCACCGGCTAA
CTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGACGAGCGGTTTGTTA
AGTCAGATGTGAAATCCCGGGCTCAACCTGGGAAGTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCC
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GGCTTCCGGAGCTAACGCGTTAAGTCGACCGCTGGGGAGTACGGCCGCAAGGTTAAACTCAAATGAATTGACGGGGGGCCGCA
CAAGCGGTGGAGCATGTGGTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACGGAAGTTTTTCAGAGATGAG
AATGTGCCTTCGGGAACCGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTGTGAAATGTTGGGTTAAGTCCCGCAACGA
GCGCAACCCCTTATCCTTTGTTGCCAGCGTCCGGCCGGGAAGTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATG
ACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAA
GCGGACCTCATAAAGTGCCTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCA
GAATGCCACGGTGAATACGTTCCCGGGCCTTGACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTT
AACCTTCGGGAGGGCGCTTACCACCTTTGTGATTCATGACTGGGTGAAGTCGTAACAAGGTAACCGTAGGGGAACCTGCGGTTGG
ATCACCTCCTTA
```

Pair 1:

☒ Left Primer 1:

Sequence:

Start: 308 Length: 20 bp Tm: 60.0 °C GC: 55.0 % ANY: 3.0 SELF: 1.0

☒ Right Primer 1:

Sequence:

Start: 511 Length: 20 bp Tm: 60.0 °C GC: 55.0 % ANY: 4.0 SELF: 1.0

Product Size: 204 bp Pair Any: 4.0 Pair End: 2.0

Screenshot of Primer for Escherichia_coli

Pair 1:

☒ Left Primer 1:

Sequence:

Start: 08 Length: 20 bp Tm: 60.0 °C GC: 55.0 % ANY: 3.0 SELF: 1.0

☒ Right Primer 1:

Sequence:

Start: 1491 Length: 20 bp Tm: 60.0 °C GC: 55.0 % ANY: 4.0 SELF: 1.0

Product Size: 204 bp Pair Any: 4.0 Pair End: 2.0

Screenshot of Primer for Escherichia_coli

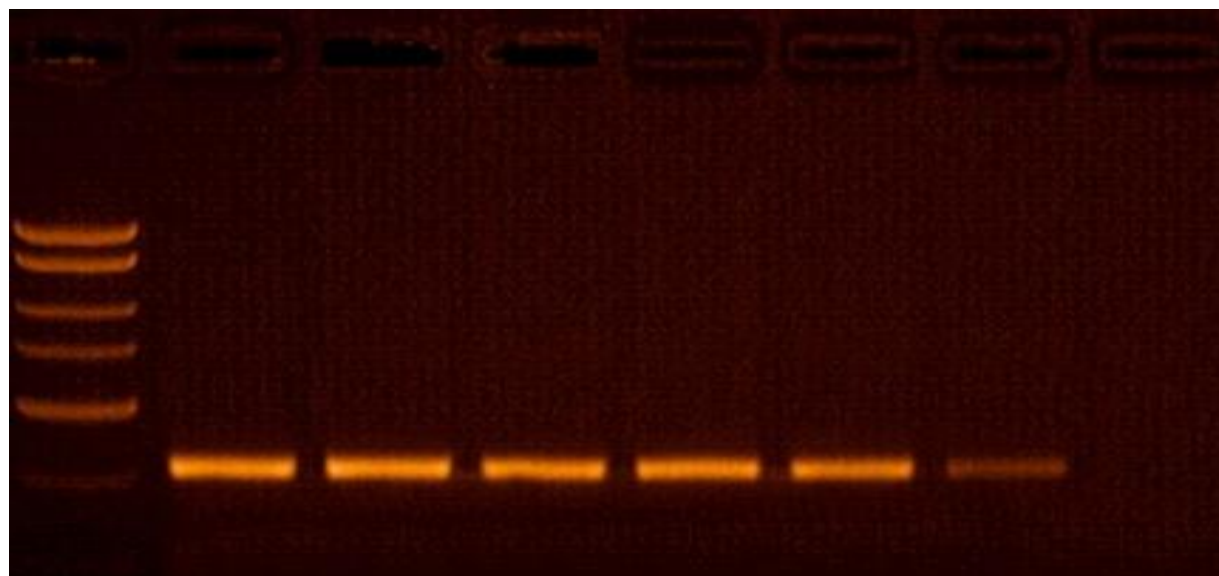
Escherichia_coli_F CAGCCACACTGGAAGTGA

Escherichia_coliR GTTAGCCGGTGCTTCTTCTG

Escherichia_coli_F AGAGTTTGATCCTGGCTCAG

Escherichia_coliR TACCTTGTTACGACTT

Agarose Gel Electrophoresis:



The target genes are amplified in PCR and the result is seen in agarose gel electrophoresis.

DISCUSSION

Soil is a limiting natural resource, and the rhizosphere is the primary site of nutrient uptake for the plant as well as a site of infection. Root exudates are a complex mixture of sugars, organic acids, hormones, and other small molecules whose abundance and ratios are modulated depending upon the plant and environmental conditions. By examining the microbial communities in bulk and rhizosphere soil, the effect of these temporary feast conditions created by the root moving through a pocket of soil on microbial growth and activity. Climate change is manifested as warming, elevated CO₂ and increased nitrogen deposition, and will have potentially large effects on greenhouse gas production, as well as destabilization of nitrogen cycling and stored carbon in soil. In this project the DNA from bacteria present in the soil rhizosphere from the soil containing petroleum contaminants. Primers are selected and primer designing is done. The primers are used for the amplification of the target DNA and the result is visualized in agarose gel electrophoresis. The biochemical properties of the isolated bacteria are determined using several assays like indole, MR-VP, etc.

CONCLUSION

Soil is a limiting natural resource, and the rhizosphere is the primary site of nutrient uptake for the plant as well as a site of infection. Root exudates are a complex mixture of sugars, organic acids, hormones, and other small molecules whose abundance and ratios are modulated depending upon the plant and environmental conditions. After the isolation of the DNA from the soil. The

target DNA was isolated from the agarose gel. Further analysis is to be done in the direction of gene sequencing of the target sequence.

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