BIOPROSPECTING OF BACTERIA FOR VARIOUS BIOTECHNOLOGICAL APPLICATIONS

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I hereby declare that the data presented in this Dissertation report entitled, Bioprospecting Of Bacteria For Various Biotechnological Applications is based on the results of investigations carried out by me in the Microbiology programme at the School Of Biological Sciences and Biotechnology, Goa University under the Supervision ofDr. Trupti Shyamsundar Asolkar, and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness Of observations / experimental or other findings given the dissertation.

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This is to certifr that the dissertation / internship report "Bioprospecting Of Bacteria For Various Biotechnological Applications" is a bonafide work carried out by Ms. Snehal Babi Velingkar under my supervision/mentorship in partial fulfilment ofthe requirements for the award of the degree of Master's in Science in the Discipline Microbiology Programme at the School Of Biological Sciences And Biotechnology, Goa University.

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INTRODUCTION

The existence of a large microbial community in the soil is supported by an enormous group of organic matters in the earth. Most of these microorganisms are bioactive and survive at the top few inches of the agricultural soils.(Prashanthi*, 2021). Abiotic and biotic factors are involved in regulating the activity and diversification of soil microorganisms. The presence of microbes in soil is based on the existence of ambient conditions provided by the types of vegetation, the texture and chemical nature of the soil, nutrients availability, pH, moisture content, climate, and temperature. The physiology of the soil is also determined by all these conditions as it varies across the same place between different seasons. (Prashanthi*, 2021).

Agriculture plays a vital role in the Indian economy. Over 70 per cent of the rural households depend on agriculture(Arjun, 2013).Economic transformation of a developing country like India crucially depends on performance of its agriculture and its allied sector. This sector plays a significant role in rural livelihood, employment and national food security.

Microbial communities play a pivotal role in the functioning of plants by influencing their physiology and development. Many members of the rhizosphere microbiome are beneficial to plant growth, also plant pathogenic microorganisms colonize the rhizosphere striving to break through the protective microbial shield and to overcome the innate plant defense mechanisms in order to cause disease.(Rodrigo Mendes, 2013)

Rhizosphere organisms that have been well studied for their beneficial effects on plant growth and health are the nitrogen-fixing bacteria, mycorrhizal fungi, plant growth promoting rhizobacteria (PGPR), biocontrol microorganisms, mycoparasitic fungi, and protozoa. (S. Sivasakthi, 2013)

Rhizosphere organisms that are deleterious to plant growth and health include the pathogenic fungi, oomycetes, bacteria, and nematodes.

PGPR involves the protection of plants from biotic and abiotic stresses. The major indirect mechanisms adopted by PGPR are hydrolytic enzyme production, exo-polysaccharide production, bioremediation of heavy metals, and stimulation of induced systemic resistance (ISR)(Anelise Beneduzi, 2012)

In plants, PGPR activates the immune response by stimulating an induced systemic resistance (ISR) through strengthening the physical and biochemical responses of the plant cell towards environmental stresses (Choudhary, 2007).Association of PGPR with host plants has often been found to enhance the biosynthesis of defense-related molecules in the later. The elevated levels of the defense proteins thus provide the host plant a better chance of survival under stress conditions.(Kumar, 2018).

Biocontrol broadly refers the use of one living organism to curtail the growth and proliferation of another, undesirable one. Biocontrol can be defined as “any condition under which a practice whereby survival or activity of a pathogen is reduced through the agency of another living organisms with the result there is a reduction in incidence of disease caused by pathogens (Anelise Beneduzi, 2012)

PGPR also improve seed germination, root development, mineral nutrition and water utilization. The manipulation of the crop rhizosphere by inoculation with PGPR for biocontrol of plant pathogens has shown considerable promise (Kay Thi Oo, 2020)

Commercialized PGPB strains *include Agrobacterium radiobacter, Azospirillumbrasilense, Azospirillumlipoferum, Azotobacter chroococcum, Bacillus fimus, Bacillus licheniformis,*

Bacillus megaterium, Bacillus mucilaginous, Bacillus pumilus, Bacillus spp., Bacillus subtilis, Bacillus subtilis var. amyloliquefaciens, Burkholderiacepacia, Delftiaacidovorans, Paenobacillusmacerans, Pantoeaagglomerans, Pseudomonas aureofaciens,

Pseudomonas chlororaphis, Pseudomonas fluorescens, Pseudomonas solanacearum, Pseudomonas spp., Pseudomonas syringae, Serratia entomophilia, Streptomyces griseoviridis, Streptomyces spp., Streptomyces lydicus and various Rhizobia spp(Glick, 2012)

CLASSIFICATION OF PGPR

PGPR has been more recognized as biocontrol of plant diseases than on growth promotion, andinvolved bacteria like Bacillus subtilis and fluorescent pseudomonas that are antagonistic to soil-borne plantpathogens in earlier studies (Chen, et al., 2006).The bacterial species identified as PGPR increased because of the advances made in bacterial taxonomy and the progress in our understanding of the different mechanisms of action of PGPR from very diverse bacterial taxa (Glick, 2012)

1. ***Rhizobia***

Rhizobia and *Bradyrhizobia* are well known as symbiotic microbes of legumes, forming N2-fixing nodules. *Rhizobia* can produce phytohormones, siderophores, HCN; they can solubilize sparingly soluble organic and inorganic phosphates, and they can colonize the roots of many non-legume plants (Shachi Shah, 2018).

Strains of*Bradyrhizobium japonicum, Rhizobium leguminosarumbv. phaseoli, R. leguminosarumbv. trifolii, R. leguminosarumbv. viciae and Sinorhizobiummeliloti.* obtained under field conditions the stimulation of growth of maize and lettuce (Lactuca sativa L.) by inoculation with dicalcium phosphate solubilizing strains of R. *Leguminosarumbv. phaseoli*.

In regions where legumes are cultivated in rotation with non-legumes, rhizobia are frequently found as endophytes of the nonlegume plant involved in the rotation. *S. meliloti, R. leguminosarumbv. viciae, and B. japonicum* used either as seed dressing or as soil drench reduced infection of *Macrophominaphaseolina*, *Rhizoctonia solani and Fusarium spp*., in both leguminous (soybean; Glycinemax and mungbean; Vigna radiata) and non-leguminous (sunflower; Helianthus annuus and Okra; Abelmoschus esculentus) plants.(Shachi Shah, 2018)

2. Bacilli

The majority (95%) of Gram-positive bacteria in soils under different types of management regimes (permanent grassland, grassland turned into arable land, and arable land), were putative Bacillus species; *B. mycoides, B. pumilus, B.megaterium, B. thuringiensis, and B. firmus*, as well as related taxa such as *Paenibacillus*, were frequently identified by sequencing the DNA bands obtained on PCR-denaturing gradient gel electrophoresis (DGGE) gels (Garbeva et al., 2003).

Other Gram-positive bacteria including *Arthrobacter spp*. and *Frankia* spp. were a minority (less than 6% of the clones obtained). The ubiquity and the importance of *B. benzoevorans* in soils throughout the world were proved by using molecular methodology developed to identify non-culturable bacteria.(Park, 2011).

Bacillus spp. are able to form endospores that allow them to survive for extended periods under adverse environmental conditions. *B. subtilis* was isolated from the rhizosphere of a range of plant species at concentration as high as 107 per gram of rhizosphere soil .

3. Pseudomonads

The beneficial effect of seeds or seed pieces bacterization were first made with *Pseudomonas spp*. isolates, on root crops. By treating potato (*Solanum tuberosum L*.) seed pieces with suspensions of strains of *Pseudomonas fluorescens and P. putida,* (Burr et al.1978).

Many strains of pseudomonads can indirectly protect the plants by inducing systemic resistance against various pests and diseases (Van Loon et al., 1998). Production of indole acetic acid (IAA) by *Pseudomonas putida GR12-2* plays a major role in the root development of canola (*Brassica rapa*) root system as evidenced by the production of roots 35 to 50% shorter by an IAA-deficient mutant (Bhattacharyya PN, 2012).

The antagonism of some strains of *P. aeruginosa* against Pythium spp. the causal agents of damping-off and root rot of many crops(JM, 2007).

Anti-fungal (antagonistic) activities

In phytopathology, antagonism refers to the action of any organism that suppresses or interferes with the normal growth and activity of a plant pathogen, such as the main parts of bacteria or fungi.These organisms can be used for pest control and are referred to as biological control agents. They may be predators, parasites, parasitoids, or pathogens that attack a harmful insect, weed, or plant disease or any other organism in its vicinity. The inhibitory substance is highly

specific in its action, affecting only a specific species. Many soil microorganisms are antagonistic. They secrete a potent enzyme which destroys other cells by digesting their cell walls and degrade the cellular material as well as released protoplasmic material serves as a nutrient for the inhibitor organism, for example Aspergillus has an antagonistic effect on Penicillium and Cladosporium. Trichoderma has an effect on actinomycetes(Xiao, et al., April 2021).

Biological control relies on screening for pest or pathogen antagonists or natural enemies, originating from its area of spread or areas that closely match the climate and soil type of the infested region by the pest or pathogen (al, 2018). The application of PGPR to mitigate biotic stress can help both in growth promotion as well as in disease control within the host plant, thereby increasing crop productivity to meet the global demand.

Agriculturally important enzymes

Cellulase

Cellulose is a linear polysaccharide of glucose residues with β-1,4-glycosidic linkages. Abundant availability of cellulose makes it an attractive raw material for producing many industrially important commodity products.

Cellulolysis is basically the biological process controlled and processed by the enzymes of cellulase system. Cellulase enzyme system comprises three classes of soluble extracellular enzymes: 1, 4-β-endoglucanase, 1, 4-β-exoglucanase, and β-glucosidase (β-D-glucoside glucohydrolase or cellobiase)(Pratima Gupta 1. K., Isolation of Cellulose-Degrading Bacteria and Determination of Their Cellulolytic Potential, 2011).

Cellulases are the enzymes that hydrolyze β -1,4 linkages in cellulose chains. They are produced by fungi, bacteria, protozoans, plants, and animals. The catalytic modules of cellulases have been classified into numerous families based on their amino acid sequences and crystal structures

Pectinase

Pectin is a polymeric material having carbohydrate group esterifies with methanol. It is an important component of plant cell wall. Plant pathogen attack target cell by producing number of cell degrading enzymes which facilitate the entry and expansion of pathogen in host tissue. Pectinases are group of enzyme that break down pectin.

Pectinases catalyze the degradation of pectic polysaccharides into simpler molecules like galacturonic acids.(Pedrolli, Monteiro, Gomes, & Carmona, 2009).Pectinases are found in bacteria, fungi, yeasts, plants, and insects.(Frati, Galletti, De Lorenzo, Salerno, & Conti, 2006) Pectinases are widely used in industrial applications such as processing fruits and vegetables, production and clarification of juice, fermentation of tea and coffee. In addition, pectinases are exploited for bleaching pulp and recycling wastepaper, animal feed, vegetable oil extraction, and pretreatment of wastewater produced from different fruit juice industries(faiza amin, 2022)

Xylanase

Xylan is the major component of the plant cell wall and the most abundant renewable hemicellulose (Beg QK, 2011). the enzymatic degradation of the substrate to its monomer, xylose, is a complex process involving a battery of enzymes (Polizeli ML, 2005). Endoxylanase and P-xylosidase have the most important activities among the xylanolytic enzymes involved in xylan hydrolysis.

Xylans are broadly categorized into four major groups based on its substituents, viz., homoxylan, arabinoxylan, glucuronoxylan, and glucuronoarabinoxylan. Homoxylans contain xylose residues only, and can be either linear or branched (Sun et al. 2011). Arabinoxylans consist of a (1, 4) b xylan main chain, but is substituted with a-arabinosyl

residues. The b-(1, 4)-linked D-xylopyranosyl residues are substituted with one a-(1,2)-linked 4-O-methyl-D-glucuronic acid in the case of glucuronoxylan; while in glucuronoarabinoxylans, the same backbone is linked to arabinofuranose and uronic acid (Polizeli ML, 2005). The side chains determine the solubility, physical conformation, and reactivity of xylan molecule with other hemicellulosic components, and, hence, greatly influence the mode and extent of enzymatic cleavage.

Phosphatases

Phosphatases have been extensively studied in soil, because they catalyze the hydrolysis of ester–phosphate bonds, leading to the release of phosphate (P), which can be taken up by plants or microorganisms (Antoun, 2001) P deficiency in the plant-soil system, enabling the mineralization of organic P to increase P availability for both plants and soil organisms.

Phosphatases are enzymes catalyzing the hydrolysis of both esters and anhydrides of phosphoric acid.Phosphatases can also be subdivided according to their regulation (e.g., calmodulin), the requirements of metal cations for their activity (e.g., Mg2+ and Ca2+) and their sensitivity to various phosphatase inhibitors.(de Freitas, 1997)

Urease

Urease break down urea-based fertilizers in the soil. They hydrolyze and degrade urea into carbon dioxide and ammonia particles. These are easily absorbed by the soil and lead to Nitrogen mineralization of soil. It also leads to rise in soil pH value

Soil urease originates mainly from the plants and microorganisms found as both intra and extracellular enzyme. The stability of this enzyme in the system is affected by the several factors. For example, studies have shown that extracellular urease associated with the soil organo-mineral complexes is more stable than urease in the soil solution and those humus urease complexes extracted from the soil are highly resistant to denaturing agents such as extreme temperatures.(Jabri E, 1995)

Nitrate reductase

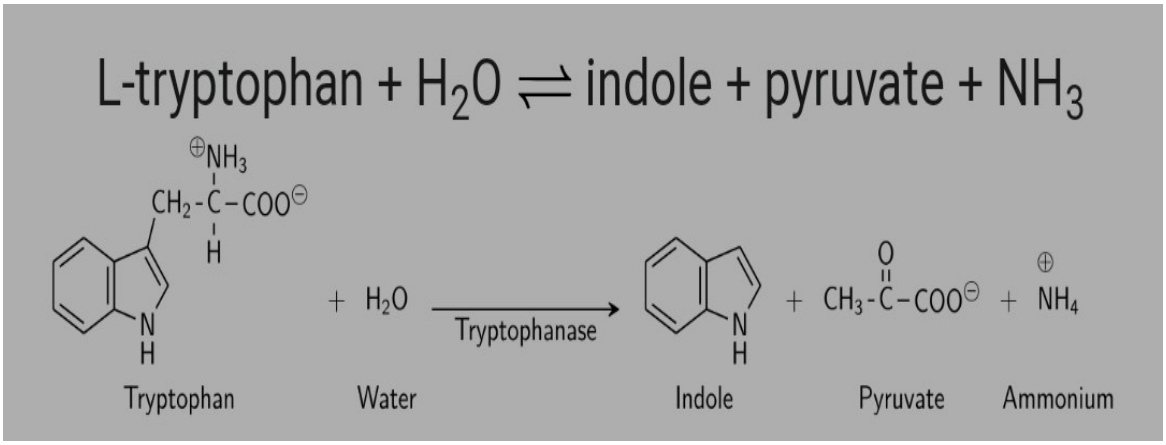
Nitrate reductase (NR) is the key enzyme for nitrogen assimilation in multiple organisms (Tille, 2014), which catalyzes the nitrate to nitrite reduction in plant cell cytoplasm(Skerman, 1967). Nitrate, its main substrate, has been shown to be necessary for cellular-signaling and commonly distributed in multiple plant tissues.

NO functions as a signaling molecule, regulating plant development and growth, resistance to abiotic and biotic stresses and metabolic repartition for optimizing nutrient utilization.

In green plants, the enzyme nitrate reductase appears to be the rate limiting enzyme in the series of reaction whereby nitrogen is utilized for protein synthesis.

Tryptophanase

Tryptophanase is a bacterial enzyme involved in the degradation of tryptophan to indole, pyruvate and ammonia.



In many bacteria, the degradation products of tryptophan are biologically essential compounds that are necessary for survival. In Escherichia coli and in several other species of pathogenic bacteria such as Vibrio cholerae, indole generated by the action of Tnase acts as a cell-to-cell signalling molecule in quorum sensing, biofilm formation and the expression of multidrug-exporter genes(K, 2003).

PLANT GROWTH PROMOTING ACTIVITIES

IAA Production

Auxins were the first plant hormones discovered. In 1880, Charles Darwin and his son Francis Darwin reported that some plant growth responses are regulated by "a matter which transmits its effects from one part of the plant to another." In the 1930s, the term "auxin" was coined by biochemists (Darwin C, 1880) . Indole-3-acetic acid (IAA) is the most common plant hormone of the auxin class and it regulates various aspects of plant growth and development.

Production of IAA by microbial isolates varies greatly among different species and strains and depends on the availability of substrate. Indole-3-acetic acid (IAA) is the main auxin in plant controlling many important physiological processes including cell enlargement and division, tissue differentiation, and response to light and gravity.

IAA is responsible for the phototropism and response to gravity, it initiate the primary and lateral root formation, and also regulates the leaf morphogenesis. IAA is involved in plant- pathogen interaction and the defence mechanism of plants. IAA is involved in the plant gene regulation and has important role to play in the stress response.

Phosphate solubilization

Phosphorus (P) is one of the major growth-limiting macronutrients required for proper plant growth, particularly in tropical areas, due to its low availability in the soil.(E. B. Santana, 2016).

Phosphate solubilizing bacteria (PSB) are beneficial bacteria capable of solubilizing inorganic phosphorous from insoluble compounds (Chen, et al., 2006).

P-solubilization ability of rhizosphere microorganisms is considered to be one of the most important traits associated with plant phosphate nutrition. It is generally accepted that the mechanism of mineral phosphate solubilization by PSB strains is associated with the release of low molecular weight organic acids, through which their hydroxyl and carboxyl groups chelate the cations [an ion that have positive charge on it.] bound to phosphate, thereby converting it into soluble forms.

Phosphorus (P) is one of the major essential macronutrients for plants and is applied to soil in the form of phosphate fertilizers. However, a large portion of soluble inorganic phosphate which is

applied to the soil as chemical fertilizer is immobilized rapidly and becomes unavailable to plants (Malboobi, et al., 2009).

Phosphate (P) compounds are capable of immobilizing heavy metals, especially Pb, in contaminated environments through phosphate-heavy metal precipitation. However, most P compounds are not readily soluble in soils so it is not readily used for metal immobilization. Phosphate solubilizing bacteria (PSB) have the potential to enhance phosphate-induced immobilization of metals to remediate contaminated soil. However, there is a limit on the amount of phosphate which can be added to the environment due to the issue of eutrophication(Park, 2011).

Siderophore production

Siderophores are small, high-affinity iron-chelating compounds that are secreted by microorganisms such as bacteria and fungi. They help the organism accumulate iron.(JB, 1995).

Production of siderophores (Fe3+ binding agents) involves the preventing of harmful effects by phytopathogens should be assessed. For example, plant pathogen inhibition includes production of ammonia, hydrogen cyanide and chitinases while ammonia involves in the nitrogen supply for plants.

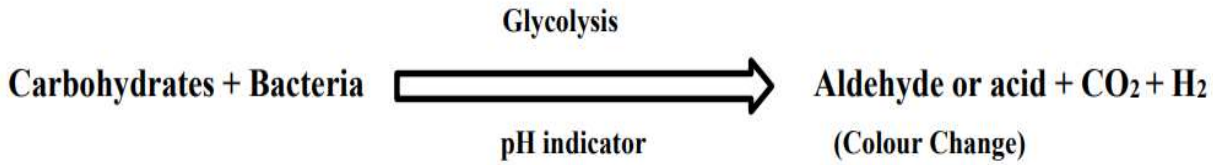
The role of siderophores is primarily to scavenge Fe(III) and make it available to the microbial cell, siderophores could also have other functions. The production of the siderophores could help the microorganisms in their competition for mineral nutrients, in addition to function as a virulence factor to protect the microorganisms against other harmful microorganisms inhabiting in their environment.(Alexander DB, 1991)

Siderophores produced by the several *spp of Pseudomonas* play important role in the biological control of plant pathogen and in plant growth promotion through competition for iron (CO, 2014)(Fang T, 2013).

BIOCHEMICAL TEST

1. CARBOHYDRATE FERMENTATION

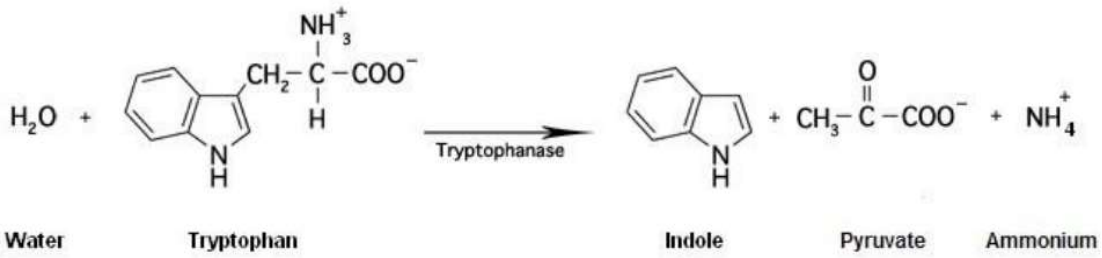
Most microorganisms use carbohydrate differently depending on their enzymecomponents. In fermentation, substrate and alcohols undergo anaerobic dissimilation and produce an organic acid (For example lactic acid, formic acid or acetic acid). The pH indicator Phenol Red is used to detect the production of acid, which is red at a neutral pH 7 and changes to yellow at a slightly acidic pH of 6.8. This indicates a positive reaction.



In some cases, acid production is accompanied by the evaluation of gas such as Hydrogen or Carbon dioxide. To detect the presence of gas produced or Durham's tube (an inverted inner vial) is placed in the fermentation broth, in which the evaluation of gas will be visible as a bubble. Cultures that are not capable of fermenting any carbohydrate and not producing concomitant evolution of gas are noted. This is a negative reaction.

2. INDOLE PRODUCTION TEST

Tryptophan an essential amino acid oxidized by some bacteria by the enzyme tryptophanase resulting in the formation of indole, pyruvic acid and ammonia. In this experiment, the medium contains the substrate tryptophan which is utilized by themicroorganisms.



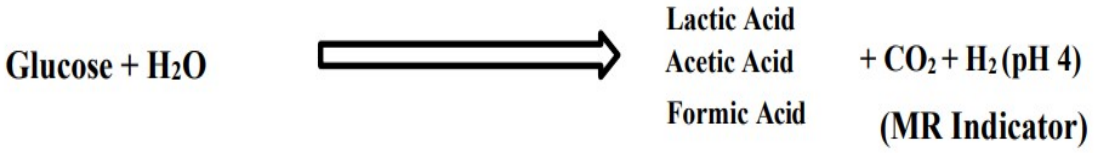
Enzymatic Degradation of Tryptophan

This ability to hydrolyze tryptophan with the production of indole is not a characteristic of all microorganisms and therefore serves as a biochemical mask. The presence of indole is detected by adding Kovac's reagent, which produces a cherry red reagent layer. This colour is produced by the reagent which is composed of Paradimethylaminobenzaldehyde yielding the cherry red colour.

3. METHYL RED TEST

All enteric organisms oxidize glucose for energy production and the end products of this process will vary depending on the specific enzymatic pathway present in the bacteria. In this test, the pH indicator methyl red detects the presence of large concentrations of acidic red detects the presence of large concentrations of acidic products. The test can be used in differentiating Escherichia coli and Enterobacter aerogenes (both coliform bacteria) that are used as indicator of

the sanitary quality of water, foods etc.Both of these organisms initially produce organic acid end productsduring the early incubation period. The low acid end products produce acidic pH 4 which is stabilized and maintained by E. coli at the end of incubation. During the later incubation period Enterobacter aerogenes enzymatically converts these acids into nonacid end products such as 2,3 butanedial and acetyl methyl carbinol (pH 6).

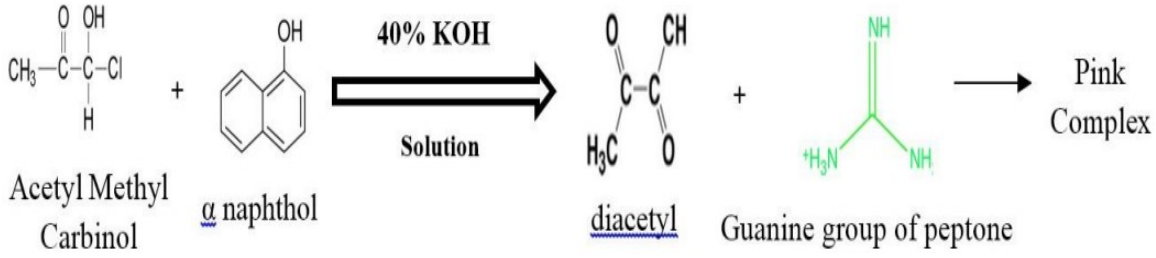


At a pH of 4, Methyl red indicator will turn red throughout the tube, which is indicating of a positive test. At pH 6, still indicating the presence of acid but with a lower hydrogen ion concentration, the indicators turn Yellow, which is indicating the negative test.

4. VOGES – PROSKAUER TEST

This determines the ability of many bacteria to ferment carbohydrates with the production of non- acidic / neutral end products, acetyl methyl carbinol or its reduction product, acetyl methyl carbinol or its reduction products, acetyl- methyl carbinol or its reduction product 2,3 Butylene glycol from the organic acids.The reagent used in this test, Barrett's reagent, consists of a mixture of alcoholic α- naphthol and 40% potassium hydroxide solution. Detection of the acetyl methyl carbinol requires this end product to be oxidized to a diacetyl compound. This reaction will occur in the presence of α- naphthol catalyst and a guanidine group that is present in the peptone. At a result, a pink complex a guanidine group that is present in the peptone. As a result,

a pink complex is complex is formed imparting a rose colour to the medium.Acetyl Methyl Carbinol reaction with Barrett's reagent.



Development of deep rose colour in culture with in a minute following the addition of Barrett's reagent is indicative of presence of the acetyl methyl carbinol and represents a positive result. The absence of rose colouration is a negative result.

5. CITRATE UTILIZATION TEST

Citrate test is used to differentiate among enteric bacteria on the basis of their ability to utilize / ferment citrate as the sole carbon source. In the absence of glucose or lactose some microorganisms utilize citrate as a carbon source. This ability depends on the presence of citrate enzyme that facilitates the transport of citrate in the cell. Citrate, the first major intermediate in Krebs's cycle is produced by the condensation of active acetyl CoA with oxalo-acetic acid and acetate. These products are then enzymatically converted to pyruvic acid andcarbon dioxide. During this reaction themedium becomes alkaline; CO2 combines with sodium and water to form carbonate, an alkaline product. This changes the bromothymol blue indicator in the medium from green to Prussian blue.

Citrate test is preferred / performed by inoculating the microorganisms in to an organic synthetic medium. Simmons citrate agar (solid) or Koser's citrate medium (liquid) in which sodium citrate is the only source of carbon and energy.Bromothymol blue is green when acidic (pH 6.8 and below). When alkaline (pH 7.6 and above). Formation of blue colour constitutes a positive test. Citrate negative culture will show no growth and the medium will remain green.

6. NITRATE REDUCTION TEST

The reduction of nitrate by some aerobic and facultative anaerobic microorganisms occur in the absence of molecular oxygen an anaerobic process whereby the cell uses in organic substances such as nitrates or sulphates to supply oxygen that is subsequently utilized as a final hydrogen acceptor during energy formation. The biochemical transformation may be utilized as follows:

$$NO_3^- + 2H^+ + 2e^- \xrightarrow{\text{Nitrate reductase}} NO_2 + H_2O$$

Nitrate reduction can be determined by cultivating organisms a nitrate broth medium. The medium is basically a nutrient broth supplemented with 0.1% potassium nitrate (KNO3) as the nitrate substrate. In addition, the medium is made into a semisolid by the additional of 0.1% agar. The semisolid impedes the diffusion of oxygen in to the medium, there by favoring the anaerobic requirement necessary for nitrate reduction. An organisms ability to reduce nitrate to nitrite is determined by the addition of two reagent solution A, which is sulphanillic acid followed by

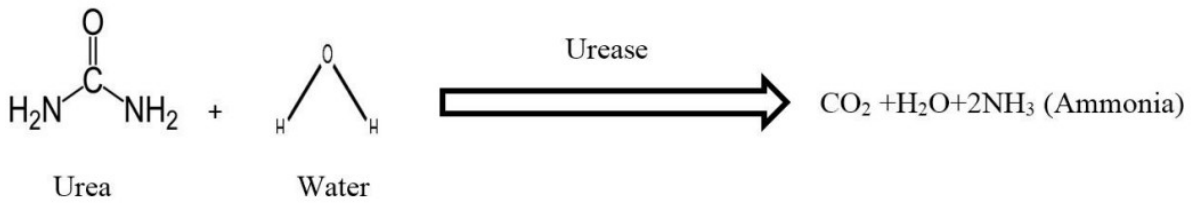
solution B, which is α-napthylamine followed reduction, the addition of solution A and B will produce an immediate cherry red colour.

This test determines the production of an enzyme called nitrate reductase, resulting in the reduction of nitrate (NO3). With this enzyme, nitrate is reduced to nitrite (NO2). It then forms nitrous acid that reacts with the first reagent sulphanillic acid, and that reacts with the other reagent α-napthylamine to form a red colour. The development of red colour, therefore, verifies that nitrates were not reduced to nitrites by the organism.

7. UREASE TEST

Urease is a hydrolytic enzyme that attacks nitrogen and carbon bond in amide compounds such as urea and forms the alkaline end products ammonia. The presence of urease is detectable when the organisms are grown in a urea broth medium containing the pH indicator phenol red. As the substrate urea is split into its products, the phenol red to turn to a deep pink. This is a positive reaction for the presence of urease. Failure of deep pink colour to develop is evidence of

negative reaction.



8. CATALASE TEST

Catalase is an enzyme, which is produced by microorganisms that live in oxygenated environments toneutralize toxic forms of oxygen metabolites and H2O2. The catalase enzyme neutralizes the bactericidal effects of hydrogen peroxide and protects them. Anaerobes generally lack the catalase enzyme.

Catalase mediates the breakdown of hydrogen peroxide H2O2 into oxygen and water. To find out if a particular bacterial isolate is able to produce catalase enzyme, small inoculums of bacterial isolate is mixed into hydrogen peroxide solution (3%) and the rapid elaboration of oxygen bubbles occurs. The lack of catalase is evident by a lack of or weak bubble production.

Catalase-positive bacteria include strict aerobes as well as facultative anaerobes. They all have the ability to respire using oxygen as a terminal electron acceptor.Catalasenegative bacteria may be anaerobes, or they may be facultative anaerobes that only ferment and do not respire using oxygen as a terminal electron acceptor (i.e. Streptococci).

AIM AND SCOPE

AIM

Bioprospecting of bacteria for various biotechnological applications.

OBJECTIVES

i. Isolation and purification of soil bacteria

ii. Screening of bacteria for industrially important enzymes

iii. Screening of bacteria for antimicrobial activity

MATERIALS AND METHODS

2.2 COLLECTION OF THE SAMPLES

2.1 (a) Collection of the samples

Three soil samples were collected from Ponda i.e.

i. Soil from vegetable market (Ponda)
ii. Soil from plastic waste dump (Ponda)
iii. Sewage soil sample (Ponda)

The samples were serially diluted using 0.85% of saline upto 10^8 and 100µl (0.1ml) of each dilution was plated out in triplicates on Nutrient agar plates in sterile condition using alcohol and spreader.

The plates were then incubated at 30°C for 2-3 days. The colonies obtained were streaked on to the fresh Nutrient agar plates and were further purified until isolated colonies were obtained.

2.1 (b) Maintenance of the isolates

The isolated colonies were streaked on Nutrient agar plates and were incubated at room temperature for 24 – 48 hours. The isolated cultures were stored for longer time by preparing their glycerol stocks. The cultures were revived one day prior conducting tests.

2.1 (c) Glycerol stocks

To prepare 60% glycerol stock solution, 30 ml of glycerol were measured in cylinder and 20 ml of distilled water was added to it in a screw cap bottle. And was autoclaved at 121°C for 10 min. (60% glycerol). In another screw cap bottle nutrient media was prepared and autoclaved at 121°C for 20 min.

In a sterile 2ml eppendroftube, 600µl of 50% Glycerol was added and 300µl of Nutrient media was added and using a sterile nichrome loop a loopful of bacterial cultures was added were stored in -20°C freezer for future use.

2.2 Production of hydrolytic enzymes

I. Qualitative analysis

2.2(a) Cellulase production

Cellulose-degrading ability of bacterial isolates was performed by streaking on the cellulose Congo Red agar media

The use of Congo-Red as an indicator for cellulose degradation in an agar medium provides the basis for a rapid and sensitive screening test for cellulolytic bacteria. Colonies showing discoloration of Congo-Red were taken as positive cellulose-degrading bacterial colonies. (Pratima Gupta 1. K., 2011)

2.2(b) Pectinase production

Screening of pectinase producing bacteria was carried out in YEP agar medium containing yeast extract 1%, pectin 1%, agar 1.5%, and NaCl 0.5% (pH 7.0) at 37∘C for 48 hours of incubation. After incubation, the colonies showing clear zones upon flooding with iodine-potassium iodide solution (1.0 g iodine, 5.0 g potassium iodide, and 330 mL H2O) were selected as pectinase producers.(Karabi Roy, 2018)

2.2(c) Xylanase production

Spot inoculate the cultures on Xylan agar medium and were incubated at 37°C for 3 days. Flood the plates with the 1% Congo red solution for 10 -15 minutes and was destained using 1M NaCl solution and the zone of clearance was measured. (Sarita Shrestha, 2021) (Antranikian, 1997).

II. Quantitative analysis

(a) For cellulase activity

Cellulose-degrading ability of bacterial isolates was performed by well diffusion method on Congo red agar medium. The plates were allowed to solidify and the wells were punched. 30µl of crude culture suspension was added to the wellincubated overnight at 37°C and observed forthediscoloration of Congo red around the bacterial colony.

(b) For pectinase activity

YEP, agar medium was prepared. The plates were solidified and the wells were punched. 30µl crude culture suspension was added to the well and then the plates were incubated at 37°C for 48 hours. After incubation the plates were flooded with iodine-potassium iodide solution and were observed for the colonies showing clear zones around the colonies.

(c) For xylanase activity

Xylan agar medium was prepared. The plates were solidified and the wells were punched. 30µl crude culture suspension was added to the well and then the plates were incubated for 3 days. After an incubation period of 3 days the plates were floodedwith 1% Congo red solution and kept for 10-15 minutes and destained with 1M NaCl solution.(Vincent, 2013)(Amara, 2008)

2.3 Antagonism using fungal plant pathogen

2.3i. (a) Isolation of *Aspergillus flavus*

The bread sample (1g) was homogenized in 9 ml sterile peptone water (0.1%) to give a stock solution. A ten-fold serial dilution was carried out and was placed on Potato dextrose agar (PDA) supplemented with 50µg streptomycin per litre. The inoculum (0.1 ml) was spread on the medium using sterile bent glass rod. The plates were incubated at a temperature of 27 + 2°C for 3 to 5days. The isolates obtained were subcultured on fresh PDA plates.

2.3 ii (a) Isolation of *Fusarium*

The wilted plant or rotten vegetable were taken. And was washed with saline followed by the hypochlorite solution. Using a sterile blade or cutter the infected part of the plant was chopped in small pieces and was placed on the PDA plate and were incubated at room temperature for 3 to 5 days. The obtained fungi were subculted and were examined by microscopic characteristics.

2.3(b) Identification of isolates

The microscopic examination of the fungal isolates were done using lactophenol cotton blue stain. The fungal mycelium was picked up in sterile condition and place on the grease free slide. Then the mycelia was teased using needle and a drop of lactophenol cotton blue stain was placed and the coverslip was placed. The slide was observed under 10X and 40X.

For *Aspergillus flavus,*thread like branching mycelia and septate hyphae.

For *fusarium,* thickened basal cells and tapered, rounded apical cells. Microconidia are oval or cylindrical, hyaline and smooth. They lack septa

2.3(c) Antifungal activity

All isolated bacteria were tested for antifungal activity as follows: a 1-cm^2 fungal plug was inoculated in the center of a plate with PDA, each isolate were revived from the stock and pure isolated bacterial colony wasstreaked using a sterile loop at a distance of 2.5 cm from the fungal disc. The plates were then incubated at 28 C for 72 h and verified every 12-48 h. The strains with capacity to inhibit proliferation of the fungus were selected.

(Iván Petatán-Sagahón 1, 2011)(A.-L. Moyne1, 2009)

2.4 Screening of isolates for plant growth promoting activity

2.4(a) i. Indole-3- acetic acid production

Nutrient broth media (25ml) supplemented with 50u/ml of Tryptophan was inoculated with the bacterial isolate. The flask was then incubated at 28C for 24 hours on rotary shaker.

Cultures were centrifuged at 10,000 rpm for 15 min. 2 ml of supernatant was taken and 2 to 3 drops of ortho- phosphoric acid was added. 4 ml of Salkowski reagent was added and incubated for 25 min. at room temperature and development of pink colour indicates the IAA production.

The absorbance of the positive cultures was taken at 540nm. Auxin production was determined by using a standard graph.

2.4 (a)ii Standard graph

The standards were made in culture medium at 0, 5, 10, 20, 50, and 100 µg/ml (ppm)

IAA is not soluble in water but it is in acetone. To a glass beaker of 10 ml acetone in the fume hood, add 10 mg IAA. Stir with metal spatula until completely dissolved. This is the 1000 µg/ml stock.

Label a series of amber vials with the dilution series.

- Add 1 ml of the 1000 µg/ml stock to the first vial, add 9 ml medium. Mix well by inversion. This is the 100 µg/ml standard.
- Transfer 5 ml of the 100 µg/ml standard to another vial and add 5 ml of media. Mix well by inversion. This is the 50 µg/ml standard.
- Transfer 1 ml of the 100 µg/ml standard to another vial and add 9 ml media. Mix well by inversion. This is the 10 µg/ml standard.
- Transfer 2 ml of the 100 µg/ml standard to another vial and add 8 ml media. Mix well by inversion. This is the 20 µg/ml standard.
- Transfer 1 ml of the 50 µg/ml standard to another vial and add 9 ml media. Mix well by inversion. This is the 5 µg/ml standard.
- Transfer 2 ml of the Salkowski Reagent into 6 test tubes labeled with each standard.
- Transfer 1 ml of each standard, including a no-IAA control of pure media, into the test tubes.
- Incubate at room temperature for 25minutes in dark and then read the absorbance at 540nm.

Record the absorbance and enter into an Excel spreadsheet. Use the “scatter” function and then add trendline. Click on the options to display equation and R2 value. Solve for x and then check the equation using the standards.

((L.) (R.Wilczek K.Geetha1, 2014)(Weber, 1951)

2.4(b) Phosphate solubilizing activity

The plates were prepared with Pikovaskya's medium. The cultures were streaked on the plates and incubated in an incubator at 28°C for 7days. The plates were then examined for clear zone around the bacterial colony and data were recorded.

(Pikovskaya, 1948)((L.) R.Wilczek K.Geetha1*, 2014)

2.4(c) Siderophore production

The bacterial isolates were screened for siderophore production by Chrome azurol S(CAS) plates assay method.

The isolates giving a yellow-orange zone around the colony indicated positive result. The zone diameter was measured at 48hr, 72hr and 96hr (OS, pp. 47-56)

2.5 Biochemical test

a. Carbohydrate Fermentation

Prepare peptone broth and add phenol red which act as a pH indicator. Insert an inverted durham's tube and autoclave at 121°C for 20 mins. Autoclave sugars separately for 10 mins.

Add sugars aseptically and a loopful of cultures in broth and incubate the tubes at 35-37°C for 18-24 hours.

b. Indole test

Tryptone broth was inoculated with the loopful of culture and was incubated at 35°C for 18-24 hours. 5 drops of Kovác'sreagentwas added directly to the tube. A positive indole test is indicated by the formation of a pink to red color.

c. Methyl red test

Organisms were inoculated into appropriately labeled tubes containing MR broth by means of loop inoculation.Uninoculated tube was kept as control. Both tubes were incubated at 37°C for 24-48 hours. After proper incubation 5 drops of MR indicator was added to both tubes including control.It was mixed well andwas observed for red colour which indicates the positive test for MR test.

d. Voges Prokauer's test

Organism was inoculated into VP broth by means of loop inoculation. One tube is kept uninoculated as control. The tube will be incubated at 37°C for 24-48 hours. 2ml of Omera's reagent was added. The tubes were shaken gently for 30 seconds with the caps off to expose the media to oxygen.The reaction was allowed to complete in 15 – 30 minutesand tubes were observedfor development of deep rose colour.

e. Citrate test

Simmons citrate agar slant was inoculated with the test organism by means of a stab and streak inoculation.An uninoculated tube was kept as control.Both tubes were incubated at 37°C for 24 – 48 hours & was observed for formation of blue colour constitutes a positive test.

f. Nitrate Reduction Test

Organism was inoculated in to nitrate broth by means of loopinoculation.An uninoculated broth was kept as control.Both tubes were incubated at 37°C for 24-48 hours.After proper incubation equal amounts of nitrate reagent (solution A & B) were added to nitrate broth Cultures and to the control tube and the reaction was observed for a red colourdevelopement.

g. Urease Test

Organism was streaked on Christener's urea agar using loop. The plates were then incubated at 34°C for 24-48 hours. The plates were observed for the pink colour.

h. Catalase test

Transfer a small amount of bacterial colony to a surface of clean, dry glass slide using a loop or sterile wooden stick. Place a drop of 3% H2O2 on to the slide and mix.A positive result is the rapid evolution of oxygen (within 5-10 s) as evidenced by bubbling.A negative result is no bubbles or only a few scattered bubbles.

RESULTS

3.1 Isolation and purification of the bacterial isolates

In total three samplings were done. Each sample was serially diluted upto 10^8 and 100ul of last three dilution were spread plated out on sterile Nutrient agar and were incubated for 24-48 hours at 28°C. The colonies obtained were streaked on nutrient agar till purified isolates were obtained.

Forty-two isolates were obtained and were sub-cultured on fresh nutrient media until the pure isolated colonies were obtained.

Sample	Dilution factor	No. of colonies obtained	Total no. of colonies obtained	Selected isolates
1	10^6	92	160	14
	10^7	44		
	10^8	24		
2	10^6	83	184	19
	10^7	64		
	10^8	37		
3	10^6	90	143	9
	10^7	34		
	10^8	19		

TABLE 3.1

The glycerol stocks of the all forty-two isolates were prepared under sterile conditions and were kept in -20°C freezer for future use.

3.2 Screening for enzyme activity

All forty-two were screened qualitative and quantitatively for cellulase, pectinase and xylanase activity respectively.

3.2.1 **Qualitative screening**, cultures were spot inoculated.

(a) For **cellulase activity**, all forty-two isolates were screened for the cellulase activity and it was observed that thirteen isolates were able to produce cellulase extracellularly and were able degrade the substrate cellulose supplemented in the growth medium. The Isolate 8, 33 and 40 were able to produce cellulase maximally which was indicated by the zone of clearance around the spotted colony and the isolate 2 and 35 were the least cellulase producers.

Isolate 8 Zone – 2.1cm	Isolate 33 Zone – 2.0 cm

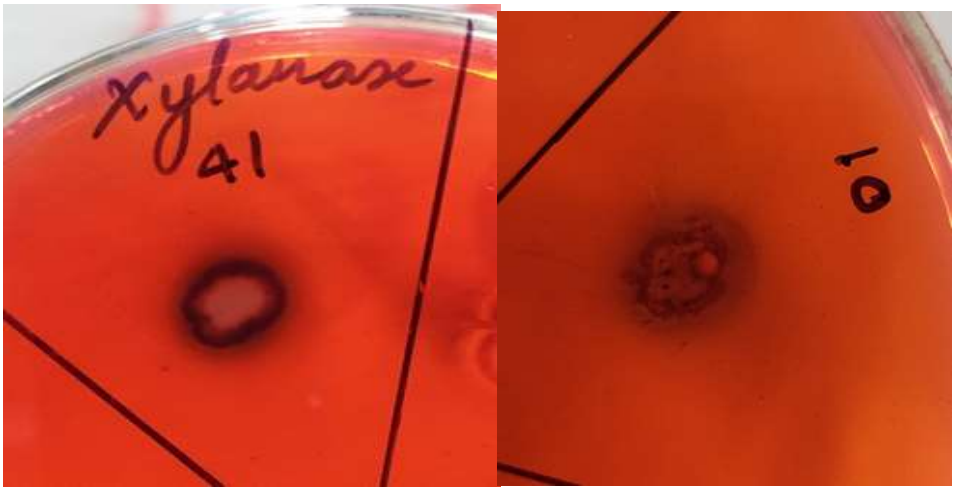
FIGURE 3.2.1 (a) Cellulase activity showed by isolates indicated by clear zone around the colonies

(b) For **pectinase activity**, all forty-two isolates were screened for the pectinase activity and were observed that the fourteen isolates were able to produce pectinase extracellularly and was degrade the substrate pectin provided in the growth medium. Isolate 26 and 35 were able to producer of pectinase maximally which was indicated by the zone of clearance around the colonies. And the isolate 21 and 37 were the least pectin degrader.

Isolate 26 Zone – 4.0 cm	Isolate 35 Zone – 3.5

FIGURE 3.2.1 (b) Pectinase activity showed by isolates indicated by clear zone around the colonies

(c) For **xylanase activity**, all forty-two isolates were screened for xylan degrading ability of the isolates bacteria and two isolates showed the positive results by showing the halozone around the spotted colonies i.e; isolate 10 and 41.



Isolate – 40 Zone – 2.0 cm	Isolate – 10 Zone – 0.8cm

FIGURE 3.2.1 (c) xylanase activity showed by isolates indicated by halozone around the colonies

3.2.2 In **Quantitative screening**, isolates showing positive test in qualitative screening were used to screen using well diffusion method.

(a) For **cellulase**, thirteen isolates showed the positive results for the spot inoculation test on Congo Red agar media containing 1% Cellulose were screened quantitatively by agar well diffusion method and was observed that out of thirteen isolates screened for quantitative test two isolates were showing the maximum clearance around the well then other isolatesi.e. 33 and 40

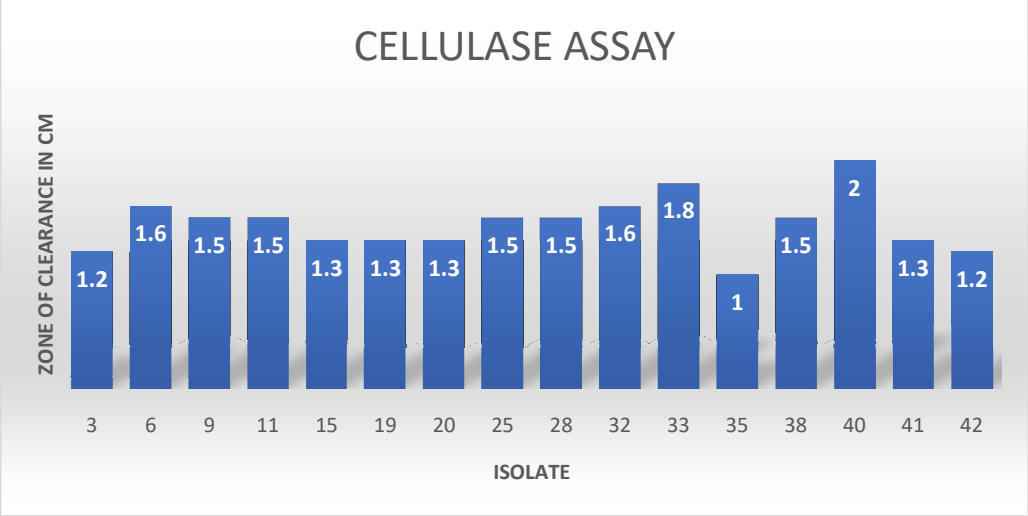


FIGURE 3.2.2 (a) CELLULASE ASSAY BY WELL DIFFUSION METHOD

(b) For **pectinase**, fourteen isolates were showing the positive results for the spot inoculation test on YEP agar media containing 1% pectin. This fourteen isolates were screened for quantitative test by agar well diffusion method.Where In isolate 4 and 22 showed the maximum clearance around the well after it was flooded with iodine solution and washed with distilled water.

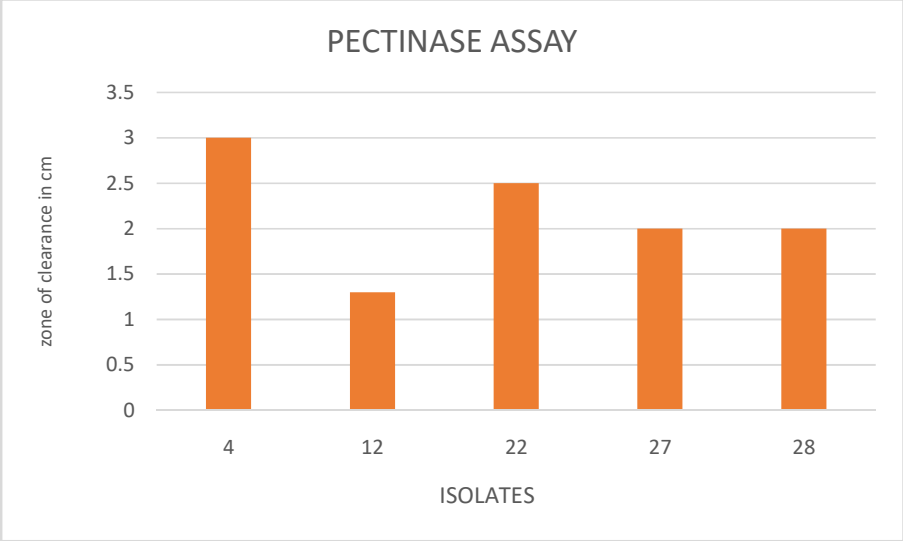


FIGURE 3.2.2 (b) PECTINASE ASSAY BY WELL DIFFUSION METHOD

(c) For **xylanase**, from all forty- two isolates only two isolates were able to show positive result for the spot inoculation test on media contained 1% of xylan birchwood.This two isolates were screened for quantitative test by agar well diffusion.Where in isolate 10 and 41 showed the halo-zone around the well after being flood with 1% Congo red solution and then destained using 1M NaCl solution.

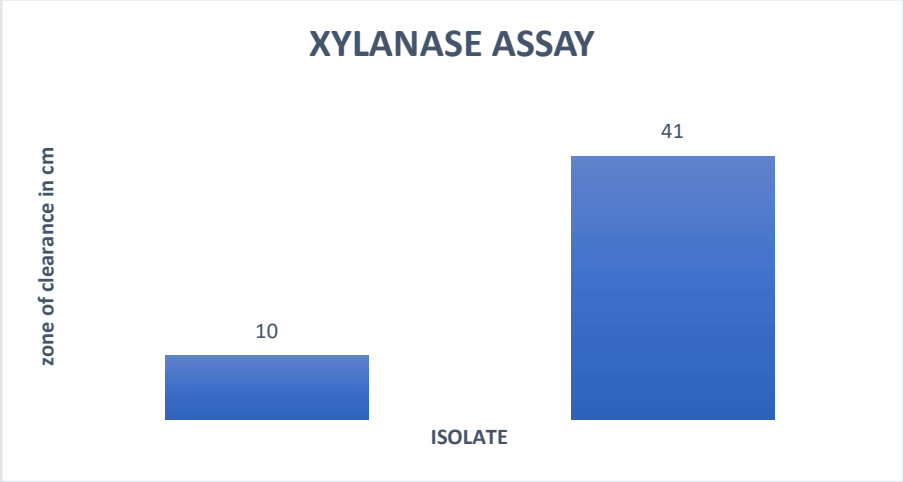


FIGURE 3.2.2 (c) XYLANASE ASSAY BY WELL DIFFUSION METHOD

3.3 Antagonism using plant pathogenic fungi

For antagonistic activity using plant pathogenic fungi against all forty-two isolates were carried out using two plant pathogenic fungi Aspergillus flavus and Fusarium respectively. Aspergillus flavus is saprotrophic and pathogenic fungi. It mainly colonizes the cereal grains, legumes and tree nuts. Fusarium, is a filamentous fungi, saprophytic, which colonies the dead decaying plant tissue

For Aspergillus flavus, isolates 6, 28, 32, 33, 36, 40 Showed the positive result by inhibiting the growth of fungi Aspergillus flavus.

ISOLATE 33 | ISOLATE 36 | ISOLATE 32

CONTROL

3.3 (a) Antagonism using *Aspergillus flavus*

Isolates 10, 11, 12, 16, 19, 21, 41, 30. Showed the inhibition of fungi Fusarium.

ISOLATE 10 ISOLATE 41

ISOLATE 30 CONTROL

3.3 (b) Antagonism using *Fusarium*

3.4 Plant growth promoting ability of the bacterial isolates

(a) For **Phosphate solubilization**, all forty-two isolates were screened for the phosphate solubilization on Pikovskaya's agar containing 0.1% of Bromophenol blue. Each isolate were spot inoculated. Isolate 1, 26, 32,38 and 40 showed the clear zone around the colony which indicated the positive result. Isolate 32 showed the maximum clearance around the colony.

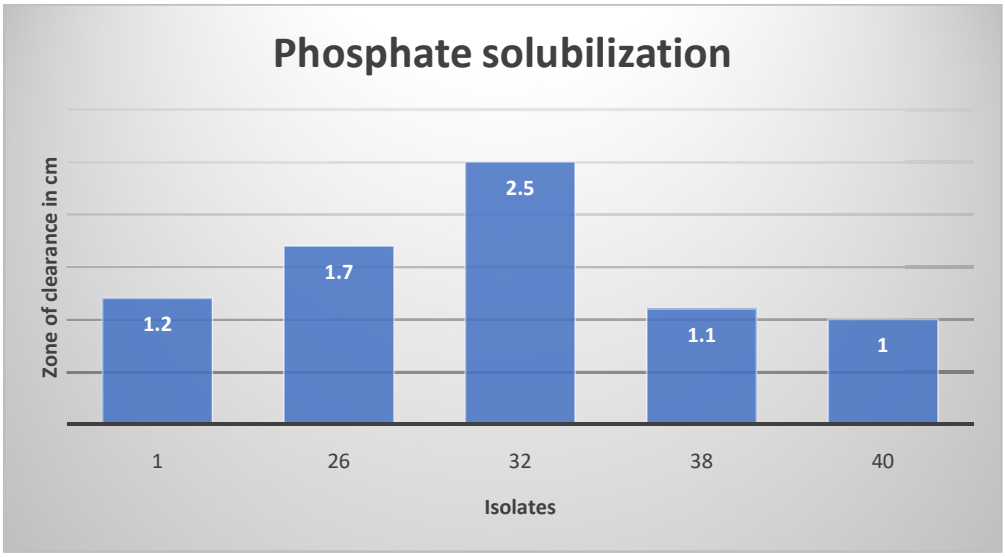
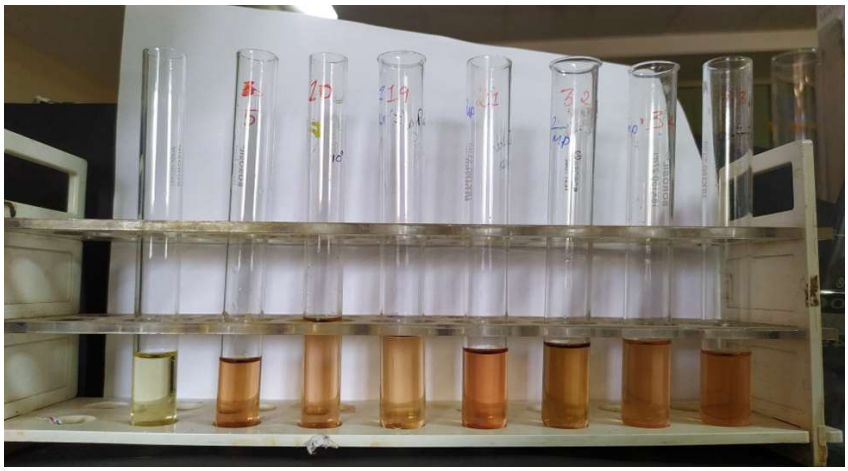


FIGURE 3.4 (a) PHOSPHATE SOLUBILIZATION

ISOLATE 32

3.4 (a) Phosphate solubilization by isolate indicated by the clearance around the colonies on Pikovskya's agar

(b) IAA production was observed in seven isolates. Isolate 5, 10, 19, 21, 32, 34, and 38. The standard graph was plotted. The standard graph using the commercially available Indole-3-acetic acid was plotted and compared. The bacteria promote the plant growth by various numbers of mechanisms, including the solubilization of phosphorous and production of phytohormones, such as indole-3- acetic acid (IAA) (Weber, 1951)(Choudhary, 2007)



(i) IAA Production by the bacterial isolates indicated by the pinkish-brown colouration

(ii) Standards of IAA using commercially available Indole-3- acetic acid

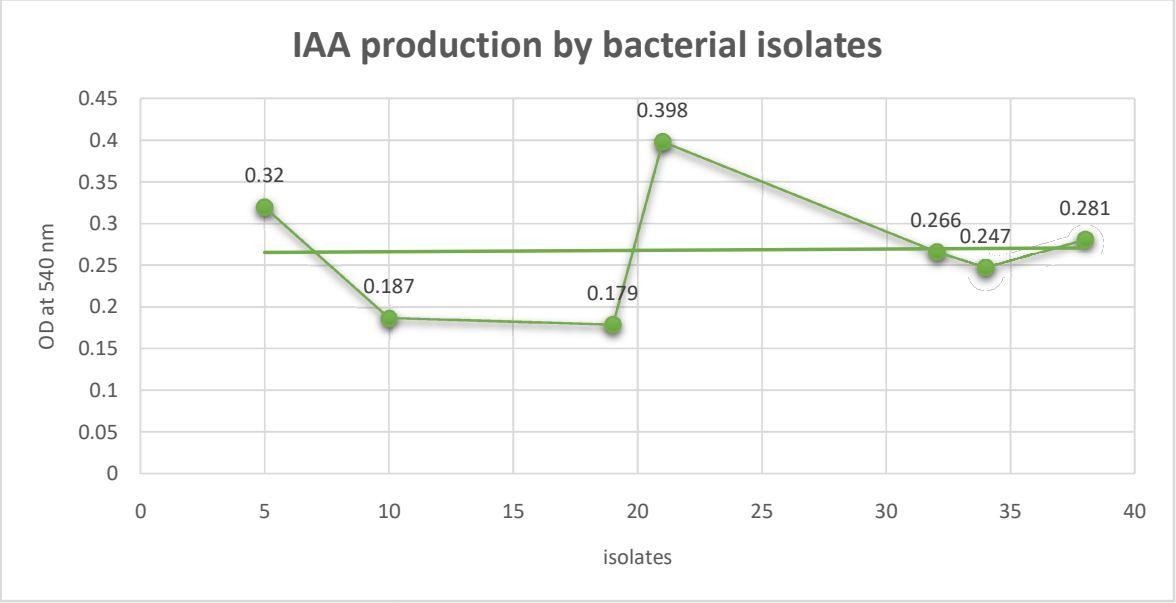


FIGURE 3.4 (b) (i) IAA Production by the bacterial isolates

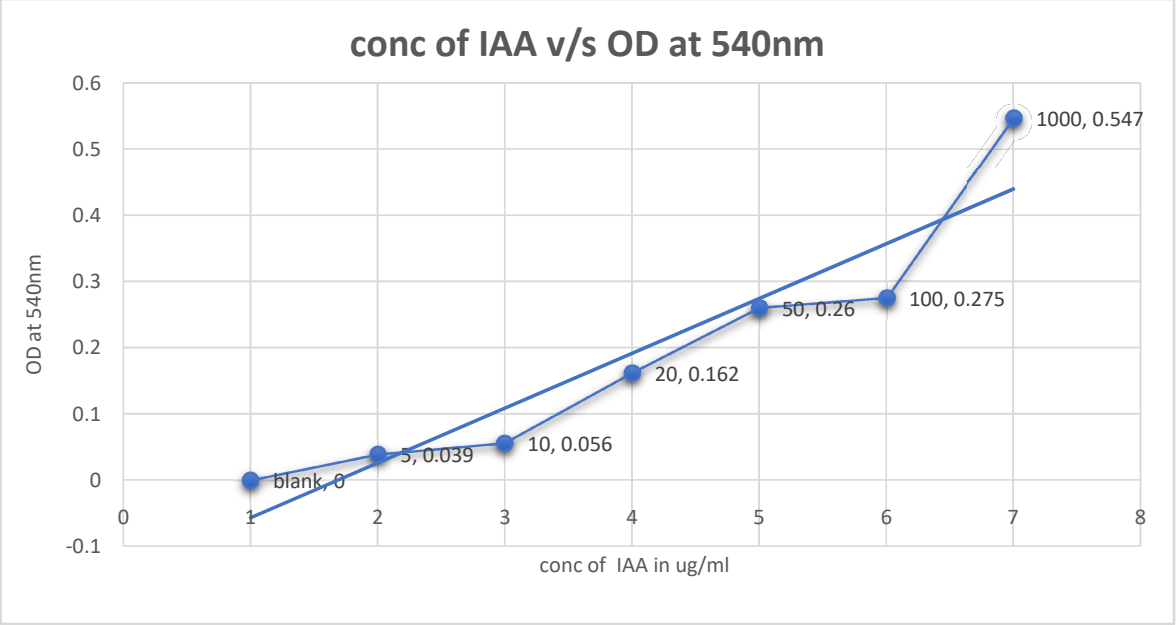


FIGURE 3.4 (b) (ii) Estimation of IAA production

(c) Siderophore are the low molecular weight molecules that chelate iron with a very high and specific affinity. Fe is an essential plant micronutrient required for plant growth. All

forty-two isolates were screened for the siderophore production on CAS agar and only isolate 32 were able produce siderophore indicated by yellow colored zone around the colonies.

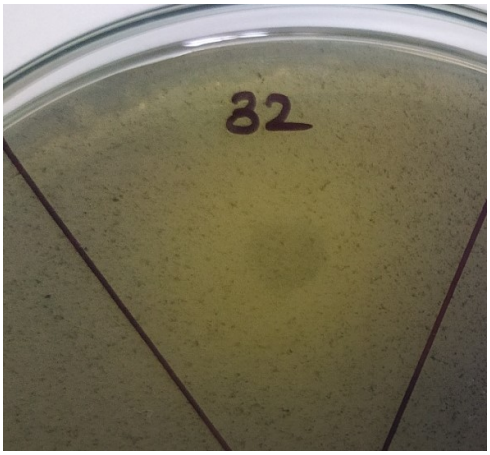
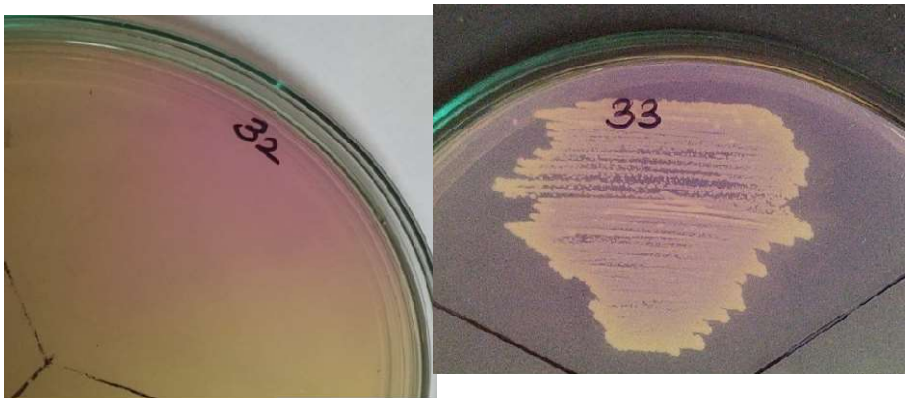


FIGURE 3.4 (c) Siderophore Production by the isolate 32 on CAS agar

3.5 BIOCHEMICAL TEST

For screening of urease and nitrate reductase activity isolate showing best results for enzyme activity, plant growth promoting activity and antagonistic activity using fungal plant pathogens were selected and screened.

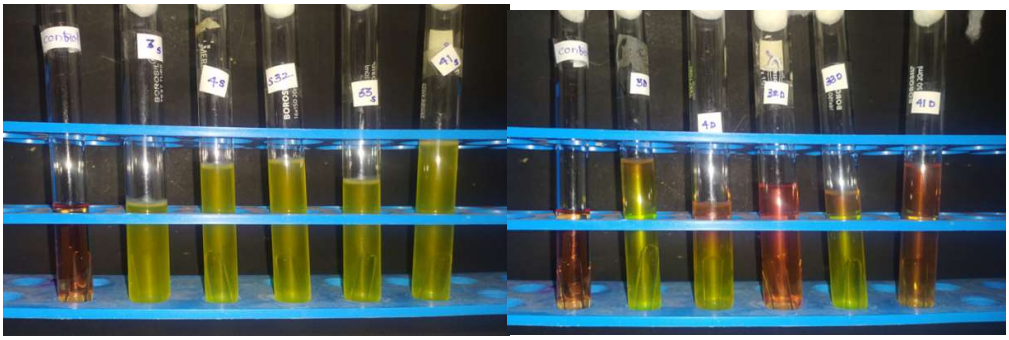
For Urease, 6 isolates were selected from the forty-two isolates and screened were for urease activity. Out of six, two isolates showed positive result for urease i.e; isolate 32 and 33 which was indicated by the pink coloration. Urease is considered to be the virulence factor in many pathogenic bacteria. It is essential for the colonization in the host organism and in the maintenance of the bacterial cells in tissues. Urease catalyzes the hydrolysis of urea into ammonia and carbon dioxide.



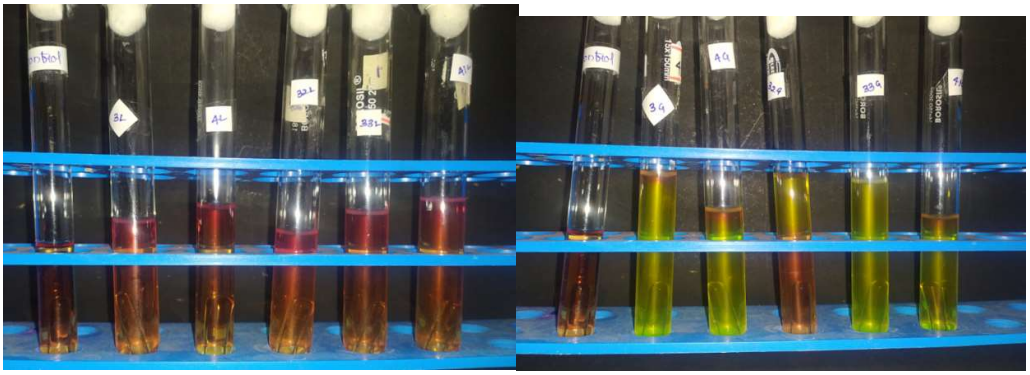
Isolate 32 Urease positive	Isolate 33 Urease positive

FIGURE 3.5.1 Urease Test by bacterial isolates indicated by development of pink colour.

Sugar utilization/ fermentation, Carbohydrates are degraded in central metabolic pathway, namely, glycolysis, pentose phosphate pathway, and tri carboxylic acid pathway, to fuel cell with the energy and build block to synthesize all biomolecules.



SUCROSE	DEXTROSE



LACTOSE

GLUCOSE

FIGURE 3.5.2 Sugar Fermentation by bacterial isolates indicated by color change of the pH indicator phenol red..

Isolate	Sugar fermentation			
	Glucose	Dextrose	Sucrose	Lactose
3	+	+	+	-
4	+	+	+	-
32	+	-	+	-
33	+	+	+	-
40	+	+	+	-
41	+	-	+	-

TABLE 3.5.2

For nitrate reductase, only isolate 32 showed the colour change from yellow transparent liquid to the red-colored solution. Nitrate reductase catalyzes the reduction of nitrate to nitrite. Nitrate is the predominant source of nitrogen in the soil.

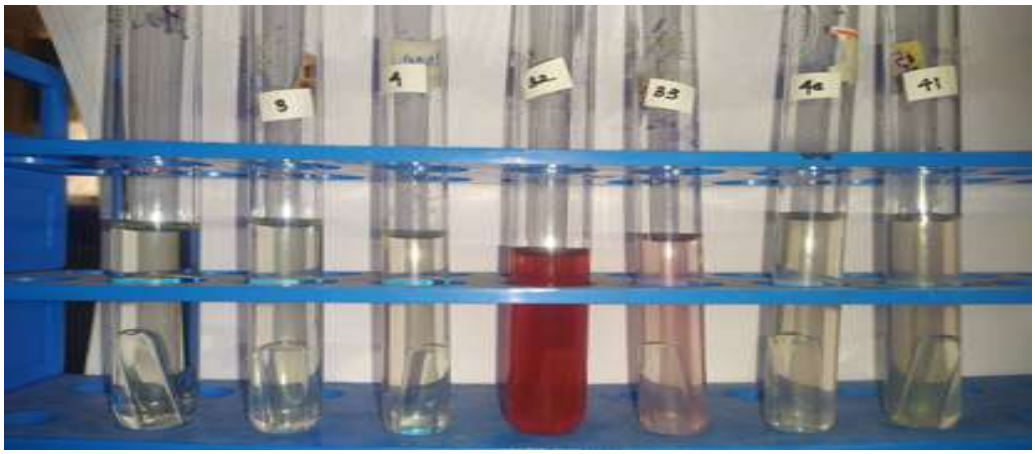


FIGURE 3.5.3 Nitrate Reductase Test indicated by colour change from yellow to red due to production of an enzyme called nitrate reductase.

Isolates 32 Catalase positive	Isolates 41 Catalase positive	Isolates 4 Catalase positive

FIGURE 3.5.4 Catalase Test indicated by efferverscence formed after H2O2.

It demonstrate the presence of catalase enzyme that catalyzes the releases of oxygen from hydrogen peroxide. Isolate 4, 32 and 41 showed the positive result.

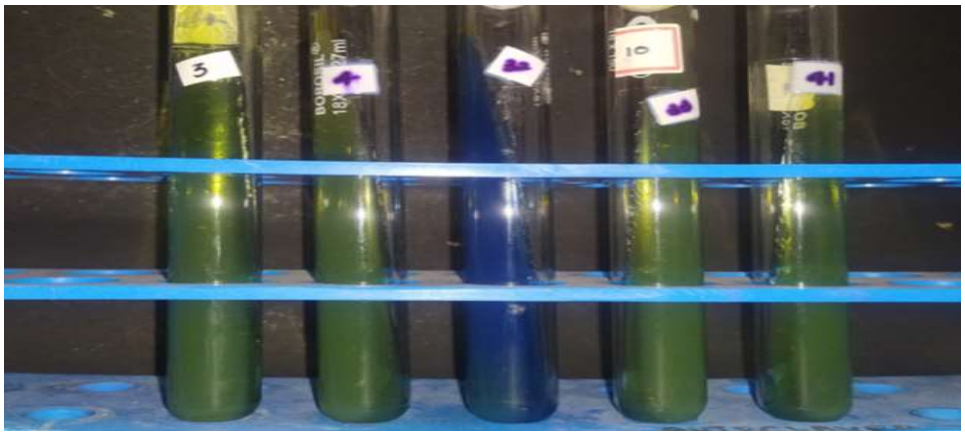


FIGURE 3.5.5Citrate utilization Test indicated by colour change from green to deep blue due to pH change.

Citrate test screens the bacteria for the ability to utilize citrate as its carbon and energy source. Isolate 32 shows the ability to utilize citrate as the source carbon and energy.

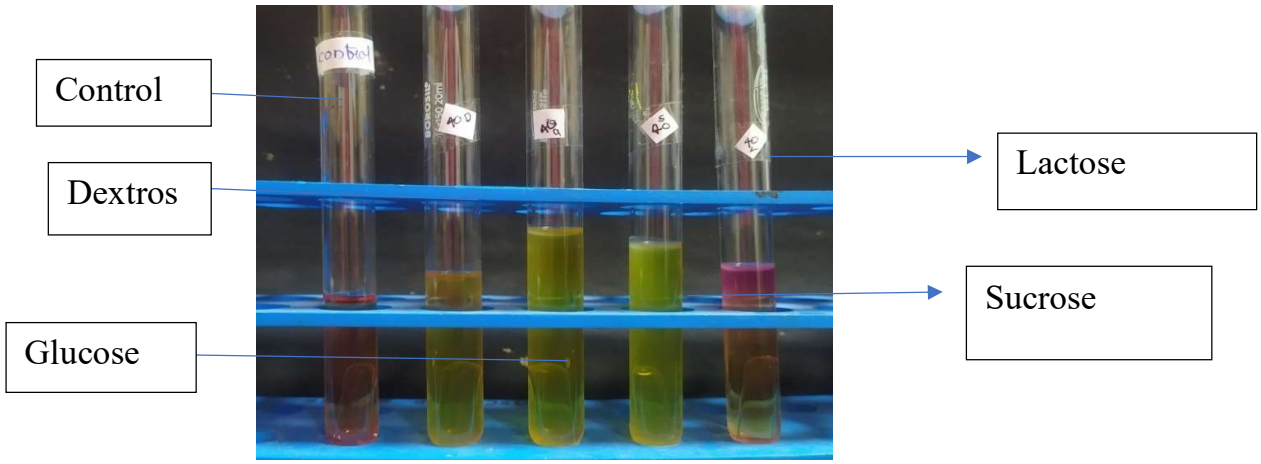


FIGURE 3.5.6 SUGAR FERMENTATION FOR ISOLATE 40

BIOCONTROL USING FUNGAL PLANT PATHOGENS

Isolate	*Aspergillus flavus*	*Fusarium*
1	-	-
2	-	-
3	-	-
4	-	-
5	-	-
6	+	-
7	-	-
8	-	-
9	-	-
10	-	+
11	-	+
12	-	+
13	-	-
14	-	-
15	-	-
16	-	+
17	-	-
18	-	-
19	-	+
20	-	-
21	-	+
22	-	-
23	-	-
24	-	-
25	-	-

26	+	-
27	-	-
28	-	-
29	-	-
30	-	+
31	-	-
32	+	-
33	-	-
34	-	-
35	-	-
36	-	-
37	-	-
38	+	-
39	-	-
40	+	-
41	-	+
42	-	-

Keys

+ positive test

- negative test

PRODUCTION OF PLANT GROWTH PROMOTING FACTORS

Isolate	*Siderophore production* *(zone diameter in cm)*	*IAA production*	*Phosphate solubilization* *(zone diameter in cm)*
1	-	-	1.2cm
2	-	-	-
3	-	-	-
4	-	-	-
5	-	+	-
6	-	-	-
7	-	-	-
8	-	-	-
9	-	-	-
10	-	+	-
11	-	-	-
12	-	-	-
13	-	-	-
14	-	-	-
15	-	-	-
16	-	-	-
17	-	-	-
18	-	-	-
19	-	+	-
20	-	-	-
21	-	+	-
22	-	-	-
23	-	-	-
24	-	-	-

25	-	-	-
26	-	-	1.7cm
27	-	-	-
28	-	-	-
29	-	-	-
30	-	-	-
31	-	-	-
32	2.3cm	+	2.5cm
33	-	-	-
34	-	+	-
35	-	-	-
36	-	-	-
37	-	-	-
38	-	+	1.1cm
39	-	-	-
40	-	-	1.0cm
41	-	-	-
42	-	-	-

Keys

+ positive test

- negative test

DISCUSSION

Forty-two isolates were screened for the presence of Cellulase, Pectinase, and Xylanses which help in degrading complex carbohydrate substrates thereby by making the nutrient available for the plants.

All the forty-two isolates were screened for the biocontrol or antagonistic activity using fungal plant pathogen. (*Aspergillus flavus and Fusarium*).

The bacterial isolates were screened for the plant growth promoting factors viz phosphate solubilization, Siderophore production, and IAA production.

In primary screening of enzyme activity

For cellulase activity, all forty-two isolates were screened for the cellulase activity and it was observed that thirteen isolates were able to produce cellulase extracellularly and were able degrade the substrate cellulose supplemented in the growth medium. The Isolate 8,33 and 40 were able to produce cellulase maximally which was indicated by the zone of clearance around the spotted colony and the isolate 2 and 35 were the least cellulase producers.

For pectinase activity, all forty-two isolates were screened for the pectinase activity and were observed that the fourteen isolates were able to produce pectinase extracellularly and was degrade the substrate pectin provided in the growth medium. Isolate 26 and 35 were able to producer of pectinase maximally which was indicated by the zone of clearance around the colonies. And the isolate 21 and 37 were the least pectin degrader

For xylanase activity, all forty-two isolates were screened for xylan degrading ability of the isolates bacteria and two isolates showed the positive results by showing the halozone around the spotted colonies i.e; isolate 10 and 41.

For Phosphate solubilization, all forty-two isolates were screened for the phosphate solubilization and isolate 1, 26, 32,38 and 40 showed the zone of clearance around the colony.Isolate32 showed the maximum clearance around the colony.

IAA production was observed in seven isolates. Isolate 5, 10, 19, 21, 32, 34, and 38. The standard graph was plotted. The standard graph using the commercially available Indole-3-acetic acid was plotted and compared. The bacteria promote the plant growth by various numbers of mechanisms, including the solubilization of phosphorous and production of phytohormones, such as indole-3- acetic acid (IAA) (Choudhary, 2007).

Siderophore are the low molecular weight molecules that chelate iron with a very high and specific affinity is an essential plant micronutrient required for plant growth. All forty-two isolates were screened for the siderophore production and only isolate 32 were able produce siderophore indicated by yellow coloredzone around the colonies.

For Urease, 6 isolates were selected from the forty-two isolates and screened were for urease activity. Out of six, two isolates showed positive result for urease i.e; isolate 32 and 33 which was indicated by the pink coloration. Urease is considered to be the virulence factor in many pathogenic bacteria. It is essential for the colonization in the host organism and in the maintenance of the bacterial cells in tissues. Urease catalyzes the hydrolysis of urea into ammonia and carbon dioxide.

For nitrate reductase, only isolate 32 showed the color change from yellow transparent liquid to the red-colored solution. Nitrate reductase catalyzes the reduction of nitrate to nitrite. Nitrate is the predominant source of nitrogen in the soil. For antagonistic activity using plant pathogenic fungi against all forty-two isolates were carried out using two plant pathogenic fungi

*Aspergillus flavus*and *Fusarium* respectively. *Aspergillus flavus* is saprotrophic and pathogenic fungi. It mainly colonizes the cereal grains, legumes and tree nuts.*Fusarium*, is a filamentous fungus, saprophytic, which colonies the dead decaying plant tissue

For *Aspergillus flavus,* isolates 6, 28, 32, 33, 36, 40 Showed the positive result by inhibiting the growth of fungi *Aspergillus flavus.* And isolates 10, 11, 12, 16, 19, 21, 41, 30. Showed the inhibitionoffungi*Fusarium*

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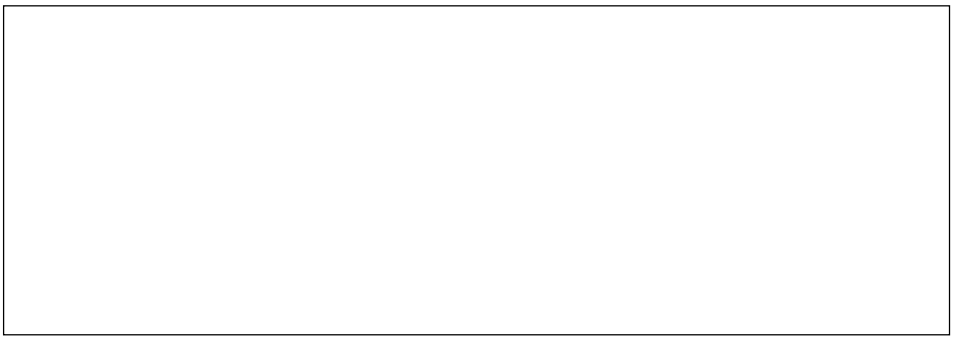

Biofertilizers consist of the microorganisms that bring about the improvement of the nutrient availability in the soil by enhancing their accessibility to the plants. Thus, Biofertilizer proves to be an effective way of supplementing the plants with the soluble form of the essential nutrients for the sake of good growth and benefit.

Understanding the role of the soil enzymes essential for soil health that are involved in the transformation of organic matter, nutrient cycling, nitrogen fixation, etc., and thus help in regulating ecosystem. Enzyme like cellulase play important role in Carbon cycling, urease play important role in Nitrogen Cycling etc., which will sufficiently help to improve the soil fertility.

However, different crop requires different microbes to help them in production of plant growth promoting factors any controllers for the phytopathogens. Microbial technology is a technology I which the strength of the beneficial microbe is used to enhance the plant growth or crop protection by enriching the indigenous microorganisms.

Soil enzymes increases the reaction rate at which plant residue decompose and release plant available nutrients. The soil enzymes are vital to soil health and fertility management in ecosystems. These enzymes have significant effect on soil biology, environmental management, growth and nutrient uptake in plants growing in ecosystem.

Biological control of plant disease is the suppression of populations of plant pathogen by living organisms. Amongst beneficial microorganisms' isolates can be selected which are highly effective against pathogen. Application of such selected and mass-produced antagonistic activity. Microbial biological control agents protect crops from damage by diseases via different modes of actions. They may induce resistance or prime enhanced resistance against infections by a pathogen in plant tissues without direct antagonistic interactions with the pathogen.

The soil microbes are the active elements for the soil developments and the basis of sustainable agriculture. Form the point of sustainable agricultural development and good eco-environment establishment.

ANNEXURE

ANNEXURE-1

Media composition

1. Nutrient agar (g/l)

Peptone 10g

Beef extract 3g

NaCl 35g

Distilled water 1000ml

Agar powder 25g

pH 7.4

2. Media used for Antifungal Activity

Potato Dextrose Agar (g/l)

Potatoes infusion 200g

Dextrose 20g

Agar powder 30g

pH 5.6

3. Media used for Enzyme Activity

i. Cellulase activity

KH_2PO_4 0.5 g

$MgSO_4$ 0.25 g

Cellulose 2 g

Agar 15 g

Congo-Red	0.2 g
Gelatin	2 g
distilled water	1 L
pH	6.8–7.2

ii. Pectinase activity

YEP Broth/agar

Peptone	10g
Yeast extract	10g
NaCl	5g
pH	7.2

Add agar agar accordingly

iii. Xylanase activity

Yeast extract	3.0
Peptone	1.5
NaCl	3.5
$NaNO_3$	1.0
KH_2PO_4	1.0
$MgSO_4$	0.3
Agar powder	20
Birch wood xylan	1.0
Distilled water	1000ml

4. Media used for the screening plant growth promoting factors

a. Chrome Azurol S Agar g/100ml

Peptone 1

NaCl 0.5

Beef extract 0.3

CAS indicator 10ml

Agar 2

Distilled water 90ml

pH 7

b. Pikovskaya media g/L

Yeast extract 0.5

Dextrose 10

Calcium phosphate 5.0

Ammonium sulphate 0.5

Potassium chloride 0.2

Magnesium sulphate 0.1

Manganese sulphate 0.0001

Ferrous sulphate 0.0001

Bromophenol blue 0.4%

Agar 15

pH 7

c. Tryptophan medium g/l

Tryptophan 0.5%

Nutrient broth 25

pH 7

*Filter sterilize 0.5% of tryptophan using 0.22µ filter

5. Biochemical test

a. Tryptone water g/l

Tryptone 10

NaCl 5

pH 7.5±0.2

b. Peptone water g/l

Peptone 10

NaCl 5

c. Simmons citrate agar g/l

Magnesium sulphate 0.2

Ammonium dihydrogen phosphate 1

Dipotassium phosphate 1

Sodium citrate 2

Sodium chloride 5

Bromothymol blue 0.08

Agar	15
pH	6.8±0.2

d. Christensen urea agar g/l

Protease peptone	1
Dextrose	1
Sodium chloride	5
Disodium phosphate	1.2
Monopotassium phosphate	0.8
Phenol red	0.012
Agar	15
pH	6.8±0.2

e. Nitrate broth g/l

KNO3	1
Nutrient broth	25
pH	7

ANNEXURE-2

Composition for stains and reagents

1. Phenol red

   0.2% of aqueous phenol red solution was prepared by adding 0.2g of phenol red to 100 ml water

2. Congo red

   1% of aqueous Congo red solution was prepared by dissolving 0.1g of Congo red in 100ml water

3. Iodine-potassium iodide solution

   1.0 g iodine and 5.0 g potassium iodide was dissolve in 330 mL H2O

4. NaCl solution

   To prepare 1 molar solution of NaCl, 58.44g of NaCl was dissolved 1000 ml of water

5. Reagent for Nitrate reductase

   a. Solution A (sulphanilic acid)

Sulphanilic acid	8g
Acetic acid (5N)	one part of glacial acetic acid to the 2.5 part of d/w

Solution B (α – naphthylamine)

α – naphthylamine	5g
Acetic acid (5N)	1L

6. Bromophenol blue indicator

   0.4g in 100ml of distilled water

7. Salkowsky's reagent

0.5 M FeCl3	40g
70% Perchloric acid	50%

8. Chrome Azurol S indicator

60.0mg of chrome azurol S was dissolved in 50 ml of milliQ water. 10 ml of Fe III solution (27mg of FeCl3.6H2O and 83.3µl concentrated HCl in 100 ml of milliQ water) was added, along with 72.9mg of hexadecyltrimethyl ammonium bromide (HDTMA) dissolved in 40 ml of milliQ water. The HDTMA solution was added slowly while stirring, resulting dark blue solution (100ml total volume), which was then autoclaved and added to the basal media

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