EXOPOLYSACCHARIDE PRODUCING BACTERIA FROM SOIL BASED NESTING STRUCTURES OF INSECTS

By

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THESIS

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DECLARATION

I, hereby declare that this thesis entitled “Exopolysaccharide producing bacteria from soil based nesting structures of insects” is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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LIST OF ABBREVIATIONS AND SYMBOLS USED

et al.	And other co-workers
cm	Centimeter
cfu	Colony forming unit
CRD	Completely randomized design
CD	Critical difference
°C	Degree celsius
Fig.	Figure
g	Gram
hrs	Hours
kg	Kilogram
L	Litre
µg	Microgram
µL	Microliter
µm	Micrometer
mg	Milligram
mL	Milliliter
mm	Millimetre
min	Minutes
M	Molar
viz.	Namely
nm	Nanometre
pH	Negative logarithm of hydrogen ions
No.	Number
OD	Optical density
%	Per cent
rpm	Rotations per minute
Sl.	Serial
sp. or spp.	Species (singular and plural)
SE (m)	Standerd error (Mean)
subsp.	Subspecies

i.e.	That is
EPS	Exopolysaccharide
TTM	Termitarium collected from Vellayani
KTM	Termitarium collected from Kollam
TWP	Wasp nest collected from Vellayani
KWP	Wasp nest collected from Kollam

Introduction

1. INTRODUCTION

Termites and mud dauber wasps make nests from the soil by cementing the soil particles using secretions, excreta, and saliva. These nesting structures have good aggregate stability and strength. Termites have been employed as a biological indicator to assess soil health and fertility because they are involved in methanogenesis, nitrogen fixation, acetogenesis, and nutrient circulation, all of which improve soil water content, pH, porosity, and organic carbon content (Dawes, 2010). Some termite societies like *Macrotermes* spp. and *Coptotermes* spp. are found in mounds that they built by excavating soil particles from depths (Enagbonma and Babalola, 2019). Termite mound soils provide unique natural habitats for a variety of microorganisms, the most prominent of which are bacteria, and study has revealed that termite mound soil is a bacterial "gold mine" (Devi and Thakur, 2018). The mud dauber wasp nests are also found to harbor a multitude of microorganisms which mainly include actinomycetes. Though termite mound soils and mud dauber nests are abundant in bacteria, there is a scarcity of information on the EPS-producing bacteria from these structures.

Extracellular polymeric substances, which are highly hydrated polymers mostly made up of polysaccharides, proteins, and DNA, are synthesized by a diverse spectrum of microorganisms. Extracellular matrix has water holding capacity, protecting microbes, adhesion, biocrust formation, interaction with microbes and plants, aggregation, carbon storage, entrapment of nutrients and protection against abiotic and biotic stress (Costa *et al*., 2018). Exopolysaccharide helps in bacterial adhesion and biofilm formation and also protects the bacteria from a variety of environmental stresses, antimicrobial compounds, antibodies, and bacteriophages. Most of the roles attributed to EPSs are defensive in nature, and their precise duties are reliant on the ecological activities that the microorganisms perform (Moghannem *et al*., 2018).

Microorganisms may benefit from the production of extracellular polymeric compounds in water stress habitats. Due to the production of EPS, some EPS-producing bacteria, such as *Pseudomonas*, may thrive even in drought environment. EPS can protect

these bacteria from desiccation during drought stress by increasing water retention and regulating the diffusion of organic carbon sources (Zethof *et al*., 2019). Plants treated with the EPS-producing bacterium *Azospirillum* demonstrated improved soil structure and aggregation, indicating tolerance to water stress (Naseem *et al*., 2018). Exopolysaccharide is vital for the development of a symbiotic relationship between nitrogen-fixing rhizobia and plants. The polymer synthesis by the bacterial isolates of NaCl-tolerant can reduce Na uptake in plants by trapping and decreasing the amount of ions available (Upadhyay *et al*., 2011). In thermophilic bacteria EPS can be a protection factor from very high temperatures by shielding microorganisms.

Soil microorganisms play an important role in improving the quality and health of soil (Jeffries *et al.,* 2003). Rhizosphere microorganisms play an important role in maintaining the productivity of the land by means of microbiological conservation. They can improve soil structure by means of aggregating soil, by exopolysaccharide production and enzymes (Mu'minah *et al*., 2015). EPS aids in the initial adherence of bacteria to solid surfaces as well as they secure anchoring (Boyd and Chakrabarty, 1995). Biofilm can retain and capture nutrients, ions and other compounds. A good soil structure, which is based on aggregation, is essential for preserving agricultural production, environmental quality, and the long-term usage of soil. The adhesive, viscous properties of exopolysaccharide (EPS), bind microbial cells to mineral surfaces in the soil and improve aggregation of soil by binding soil particles together. The formation of microaggregate and macro-aggregates is mediated by organic materials, polysaccharide and various kinds of organisms such as bacteria, fungi, worms, ants and insects. During the growth of bacteria, exopolysaccharide is released into the soil. Capsular and slime polysaccharides soak on clay surfaces and aid in the development of stable soil aggregates. (Arfarita *et al.,* 2016; Cheng *et al*., 2020). Only few researches have been carried on the impact of exopolysaccharide producing bacteria on stable soil aggregation

Recognizing the importance of exopolysaccharide producing bacteria in plant protection against stress conditions and in role of soil aggregation , the current study was taken up with the objectives .

1. Isolate and characterize exopolysaccharide producing bacteria from mud wasp nests and termitaria.

2. Qualitative and quantitative assessment of exopolysaccharide production.

3. Effect of carbon source and carbon nitrogen ratio on EPS production.

4. Effect of temperature and salinity on survival of EPS producing bacteria

5. Study the effect of the two high exopolysaccharide producing isolates on soil aggregate stability

Review of literature

2. REVIEW OF LITERATURE

2.1. EXOPOLYSACCHARIDE: COMPOSITION AND IMPORTANCE

Extracellular polymeric substances are produced by microorganisms and they are secreted in to their surrounding environment. These high molecular weight substances are natural polymers. They are the key component in bacterial biofilm development and help in establishing the integrity by both structurally and functionally, and physiochemical properties of bacterial biofilms. They are aptly represented as the "house of the biofilm cells" (Flemming *et al*., 2007). Extracellular polymeric substances comprises of polysaccharides, proteins, lipids, DNA and some humic substances. They can host a huge number of bacteria and mostly attached to the outer surface of cells, or are released into growth medium suitable for it. Biofilm formation and cells' attachment to surfaces are mainly depend on these substances. In biofilm's total organic matter, Exopolysaccharides (EPS) contribute in an average of 70 percentage. The term 'exopolysaccharide' was proposed by Sutherland (Wingender *et al*., 1999).

Exopolysaccharides are the major part of extracellular matrix that shows 40-95 percentage of bacterial biomass (More *et al*., 2014). There are two types of EPS produced by bacteria *viz.*, Slime EPS and capsular EPS. Different types polysaccharides, proteins, nucleic acids of bacteria, and lipids make up this diverse combination. They are lengthy chain molecules, having molecular weight of about 10-30 KDa. Bacterial exopolysaccharide secretion is promoted in a high carbon to low nitrogen ratio conditions of growing media (Naseem *et al*., 2018).

Exopolysaccharides are homo- or heteropolymers that are typically adorned with non-carbohydrate substituents such as acetyl, pyruvyl, or succinyl groups, which give the polysaccharide anionic characteristics. Homopolysaccharides are made up of repeating units of single sugar monomers. Monomers includes six carbon compounds group such as hexoses and five carbon compounds such as pentoses or amino sugars joined by glycosidic bonds, whereas heteropolysaccharides comprises more than one different sugar monomers in repeating units (Saha *et al.,* 2020). Most of the heteropolysaccharides

are anionic in nature, whereas majority of homopolysaccharides have neutral charge. Anionic nature of heteropolysaccharides are due to the availability of more than one monomeric sugars and some additional constituents viz., pyruvic acid, lactic acid, uronic acids, succinic acid, phosphates, hexosamine, and sulfate esters. Some of the plant-associated microbial communities have capability to produce exopolysaccharides during stress conditions. Physical and chemical properties of EPS can help microbial cells to mitigate moisture stress and nutritional deficiency. EPS are able to alleviate the effect of reducing water potential. The binding ability, gelatinous characteristics of EPS help microbes to attach to nutritional surfaces in the soil. It will improve soil-aggregation by helping to bind soil particles altogether (Naseem *et al*., 2018).

2.2. RELATIONSHIP WITH BIOFILM, EXTRACELLULAR MATRIX AND EXOPOLYSACCHARIDES

Soil is growing medium for a huge range of microorganisms. Many of microorganisms are able to for structures called as biofilms. It includes superficial microbial cells attached in hydrated extracellular polymeric substance. These structures enhance the adhesion and growth by the production of exopolysaccharide (Cia *et al*., 2019). Rhizosphere bacteria such as *Pseudomonas*, *Azospirillum*, *Bacillus* and *Rhizobium* that associate with plant roots are found in the mucigel of the root or may be found interior to the extra cellular matrix produced by bacteria (Naseem *et al*., 2018).

Attachment of bacteria to the surface of the plant root is the initiation step in biofilm formation. Bacterial biofilm formation around the plant roots depends on the different bacterial communities and load of population present inside the polymeric extracellular matrix. Initiate biofilm development in response to specific environmental conditions. At the process of colonization, bacteria produce extracellular polymeric substance which constructs the biofilm matrix (Serra *et al*., 2019). Extracellular polymeric matrix mainly comprises by exopolysaccharide**,** Protein**,** Nucleic acids**,** Lipids. Exopolysaccharide contribute up to 95 percentage in this structure, followed by protein (1-60%), nucleic acid (1-10%) and lipid (up to 40%) (Cia *et al*., 2019).

The EPS biosynthesis promote the initial adhesion of bacterial cells to root surfaces. It also helps to form a microcolony in rhizosphere region. Physical and chemical nature of EPS helps the cells to withstand different environmental stresses.

2.1.PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR)

The plant-associated microbial communities have appreciable capabilities to negotiate many of the abiotic, biotic and nutritional stress effects on plants. The root -soil amalgamate comprises a compelling microcosm known as the rhizosphere, where microorganisms, plant roots, and soil components interact each other. Rhizobacteria are beneficial and free-living microorganisms located near the plant root and strive to make a significant impact on plants ranging from direct influence to indirect effects against environmental stresses. The use of these rhizobacteria on plants or seeds promotes the vegetative growth and enhances the resistance against different biotic and abiotic stresses. Many of the plant growth promoting bacteria are able to produce biofilm and it will help bacteria for root adhesion and increasing the population (Pankaj *et al*., 2019). Exopolymeric matrix help to increase in the quantity of root adhering soil and improve water-holding capacity. It also reduces water loss during desiccation and protect the roots from the soil hardness in the rhizosphere region (Rolli *et al*., 2015).

2.2. RHIZOBACTERIAL EXOPOLYSACCHARIDE PRODUCTION

Rhizobacterial EPS in the form of a capsule or slime is discharged into the soil, comprising homopolysaccharides or heteropolysaccharides. Through different intermolecular forces EPS aquire an anionic form, which improves its chemical characters and influences its interactions with cations. Various parameters such as the phase of bacterial growth, the composition of the medium and the environmental conditions are responsible for the production and release of EPS (Brahmhachari *et al*., 2018). As EPS maintain hydrated microenvironment, it holds more water and dries very slowly compared to the surroundings. It plays a important role in enhance the soil permeability. Formation of micro and macro-aggregates in the soil increases the nutrient

uptake and water movement through the plant, thereby improving the growth of the plants (Selvakumar *et al*., 2012; Rossi *et al*., 2012). The hygroscopic nature of rhizobacteria strains changes according to the polysaccharide content of EPS. Azospirillum improves soil structure and aggregation through EPS production (Kaushal, 2019). EPS producing drought tolerant *Pseudomonas* and *Bacillus* spp. increases the RAS / RT (root-adhering soil/root tissue) ratio and macroaggregate stability and increases the uptake of water and nutrients from the rhizospheric soil (Vardharajula *et al*., 2011).

2.3. FACTORS AFFECTING EPS PRODUCTION

2.3.1. Nutrient Content:

Nutrient levels have a big impact on EPS generation and composition. Excess carbohydrate medium, such as glucose, has been demonstrated to promote EPS synthesis (Fleming Wingender, 2001). Microorganisms can synthesize EPS from a variety of nitrogen sources, including ammonium, nitrate, nitrite, and amino acids. Among these, ammonium salts and amino acids are the most frequent (Sutherland, 1990). The amount of EPS known to be present is determined by the nitrogen substrates used. Low nitrogen content in the growing medium effects EPS synthesis as well (Sleytr, 1997). Under nitrogen-limited conditions, sixty percentage of the glucose was transformed into EPS in various bacterial specieses *viz.*, *Aureobasidium*, *Escherichia*, *Pseudomonas* and *Sinorhizobium* (Sutherland, 2001; Lee *et al*., 1999). Microbial cells produce extracellular proteins in response to the medium's high nitrogen level. Sanin *et al*. (2003) discovered an increase in exogenous protein production in *Pseudomonas* sp and *Rhodococcus* sp cells cultured in media with high ammonium salts.

The carbon content of the termite mound soil was about 0.60 percent. The nearby soil was about 0.50 percent. It was increased about 11.80 percent as compared to the control according to Dembare (2013). Soil carbon is the greatest carbon reservoir on the environment. It is crucial in global climate models and have a significant role in the carbon cycle. The physical qualities of soil are improved by adding carbon to it. It improves sandy soil's cation exchange capacity and water holding capacity. Nitrogen levels were

found to be 23.01 g kg^{-1} in the surrounding soil and 22.09 g kg^{-1} in the termite mound soil. The nitrogen content of mound soil was declined. Nitrogen is a major regulator of ecosystem processes and an essential macronutrient for plant growth.

2.3.2. Growth Phase

The generation of EPS during a specific growth phase is a characteristic of certain bacterial genera. EPS generation has been seen in some strains of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* during the end of exponential and in the beginning of stationary phases of bacterial growth (Sutherland, 2001). Growth and exopolysaccharide production occur concurrently in many bacterial species, with EPS biosynthesis being growth correlated (Datta and Basu, 1999; De Vuyst *et al*., 1998; Bryan *et al*., 1986). The optimal period for maximum EPS production differs depending on the bacterial type. In some species, EPS production is maximized during the exponential phase (Bramhachari and Dubey, 2006; Macedo *et al*., 2002; van de Berg *et al*., 1995), whereas in others, it is maximized during the stationary phase (Datta and Basu, 1999; Petry *et al*., 2000; Wilkinson *et al*., 1955; Williams and Wimpenny, 1977).

2.3.3. Temperature

Many authors have explored the effect of cultivation temperature on EPS biosynthesis. The best production temperature for the majority of EPS molecules was 26ºC to 31ºC (Lory, 1992; Gandhi *et al*., 1997). However, as stated in Listeria, in some circumstances, low temperatures were shown to be more conducive for EPS formation. Briandet *et al*. (1999) discovered that a culture temperature of l0ºC stimulates the synthesis of extracellular cold shock protein in *Listeria monocytogenes* cells.

2.4. PROPERTIES OF EPS AGAINST STRESS CONDITIONS

The soil has the greatest microbial variety, with numerous prokaryotes and fewer eukaryotes per gram of soil. Extracellular polymeric substances, which are highly hydrated polymers mostly made up of polysaccharides and many macromolecules such as proteins and DNA. EPS matrix has water holding capacity, protecting microbes,

adhesion, biocrust formation, microbe- microbe interaction, microbe-plant interaction aggregation, carbon sequestration, nutrient immobilization and protection against different environmental stresses (Costa *et al*., 2018).

2.5. ACTION OF EPS PRODUCING BACTERIA IN SALINITY CONDITION

Salt tolerance in plants is mostly determined by the ability of roots to take in restricted or controlled amounts of Na^+ and Cl^- while continuing to take in important elements such as K^+ and NO_3^- (Upadhyay *et al*., 2011). Plants growing in saline soils will be in saline stress due to increased uptake of sodium ions. Exopolysaccharide producing bacteria will decrease the uptake of sodium ions and help to withstand salt stress (Ashraf *et al*., 2004).

Bacillus tequilensis UPMRB9 and *Bacillus aryabhattai* UPMRE6 strains can act as good biofertilizer in saline-affected coastal rice agriculture (Shultana *et al*., 2020). The salinity of the medium had a significant effect on bacterial EPS production. UPMRB9 had the maximum EPS production (31.50 mg mL^{-1}) at a concentration of 1.5 M NaCl. UPMRB9, UPMRE6, and UPMRG1, produced higher EPS compared to UPMRA4 and UPMRE3 in the highest NaCl concentration. The plant inoculation research concluded that bacterial inoculation has a considerable beneficial effect on different metabolism of plants. This ultimately leads to a greater yield.

Qurashi *et al*. (2012) studied the influence of exopolysacharide and biofilm formation in response to varying salt stress in two bacterial strains, *Halomonas variabilis* (HT1) and *Planococcus rifietoensis* (RT4). Higher salinity increased biofilm development and exopolysaccharide accumulation. When compared to untreated controls, the mass of soil particle adhering to root and rhizosphere was increased in higher salt concentrations. Under salinity, soil aggregation was greater at plant roots. The fresh weight and dry weight values of EPS are significant with increasing salt stress, indicating that it has a better water retention ability. Soil aggregates adhering to plant roots and present in soil were weighed to determine the effect of bacterial inoculation under varied

salt stress concentrations. At 100 mM NaCl, *Planococcus rifietoensis* (RT4) generated the greatest rise (808%), while *Halomonas variabilis* (HT1) caused the greatest increase (666%). The increase in aggregate weight at 200 mM NaCl with *Planococcus rifietoensis* (RT4) was 130 % more than the noninoculated control. The weight of aggregate production in soil was smaller than that of roots. Soil aggregation was more apparent with *Planococcus rifietoensis* (RT4), which created seventy five percent more soil aggregates at 200 mM NaCl stress.

2.6.ACTION OF EPS PRODUCING BACTERIA IN TEMPERATURE STRESS

Elevated temperatures have a significant impact on agricultural yield, which varies depending on the intensity and duration of heat stress. Extracellular polymeric compounds can protect thermophilic bacteria by sheltering them from extremely high temperatures (Costa *et al*., 2018).

Bacillus cereus revealed the potential for exopolysaccharides (EPS) synthesis in a study conducted by Mukhtar *et al*. (2020), which considerably reduced the undesirable effects of heat on tomato development. EPS was much higher in bacterial culture under stressed conditions compared to normal conditions. This research indicates that microbes such as *Bacillus cereus* help to withstand heat stress on crop growth and development, and this strategy can lead to the new mitigation technology to reduce heat stresses.

Under temperature stress, the thermotolerant *Pseudomonas* sp. strain AKM-P6 produced EPS, allowing the plants to endure high temperatures. All of the selected thermotolerant isolates produced EPS at both low and high temperatures, with the production of EPS being substantially higher at high temperatures. Under both normal and stressful conditions, the isolate AKM-P6 produced the most EPS. Inoculation of *Pseudomonas* sp. strain AKM-P6 improved biomass under elevated temperature conditions compared to uninoculated plants. Microorganisms may have a role in alleviating the negative effects of environmental stress on crop growth, which may lead to the development of microbial inoculation to offset such effects (Ali *et al*., 2009)

2.7.ACTION OF EPS PRODUCING BACTERIA IN DROUGHT CONDITION

Drought stress can alter soil physicochemical and biological properties, making them unsuitable for agriculture. EPS can protect bacterial cells from desiccation by forming biofilms, increasing the likelihood of bacterial cells colonizing specific ecological niches. Water availability regulates bacterial protein and polysaccharide production and consumption, and thus indirectly influences soil structure (Sandhya *et al*., 2009). Exopolysaccharide production can benefit microorganisms in drought-stressed environments. EPS protects bacteria from desiccation by different metabolic adjustments (Caruso *et al*., 2018). During drought stress, EPS has the ability to make nutrients available in the bacterial microenvironment (Vurukonda *et al*., 2016).

According to the conclusions of Sandhya *et al*. (2009), bacteria such as *Pseudomonas* produces exopolysaccharide (EPS), which help to withstand different environmental stresses by various physiochemical characteristics. The EPS-producing *Pseudomonas* strain GAP-P45 acts as a plant growth promoting rhizobacteria and can mitigate the effects of drought stress in *Helianthus* sp. Drought stress had a significant impact on the growth of *Helianthus* sp seedlings as it showed a poor growth, decreased vigour, and leaf necrosis. The percentage of water stable aggregates in RAS was used to assess the effect of *P. putida* GAP-P45 on rhizosphere soil aggregation.

According to a study conducted by Vardharajula (2014), Bacillus spp. help to soil aggregation by secretion of EPS under drought stress. *Bacillus* spp strains HYD-B17, HYTAPB18, and RMPB44 increased aggregate stability under normal and drought stress condition, whereas stability was higher in drought condition due to higher EPS production.

2.10. PROPERTIES OF WASP NEST AND TERMITE MOUND SOIL:

Termite mounds are structures in tropical ecosystems that were primarily built by the eusocial insect termites (*Isoptera* sp.) (Jouquet *et al*., 2015). Soil is a blend of various minerals that make up the nutrient status, organic content, water, air and the organisms

that dwell in the soil. Termites are the best soil fauna and also omnipresent as that of ants and earthworms since it spends part of its life cycle in or on the soil. Termites have a higher impact on chemical properties of soil viz., soil carbon and nitrogen presence within their nest materials. In due course of its life cycle, the termites highly influence the physical, chemical and biological properties of the soil by the interaction of termite's gut microflora. Termite mounds have characteristic structural morphology when compared to the surrounding soil in terms of volume and growth of the mound (Abe *et al*., 2009).

Termites were recognized as 'soil engineers', the disturbance process they made is highly notable which allows the soil transformations. Termites transport organic matter and other minerals from various locations and deposit them in mounds, increasing clay level, carbon content of soil and nutritional level. Clay content of termite mound soil is always higher when comparing to neighboring soil. The pH of the termite mound soil and the surrounding soil, respectively, was 7.17 and 7.67. Termites changed the pH of the mound up to 12.5 and the pH of the mound changes depending on the termite species and soil type. The electrical conductivity of surrounding soil and soil mound was 0.29 dS/m and 0.31 dS/m respectively. Organic carbon, phosphorus, potassium, magnesium, iron, zinc, and Copper were high in mound soil, whereas N, Ca, S, and Mn were low in quantity (Dhembare (2013). The soil qualities of termite mounds are often better than those of the surrounding environment. Soils having electrical conductivity higher than 4 dS/m can call as saline. The termite mound was not saline. *Macrotermes michaelseni* made their habitat with high clay content and higher Cation Exchange Capacity than surrounding soils. It also increased soil fertility (Dangerfield *et al*., 1998).

Termite mounds had been found to have high bulk densities, resulting in decreased porosity and increased hardness (Holt *et al*., 1980). Native soil microorganisms' structure and functions are well understood for their significance as a crucial indicator of soil health (Zhu *et al*., 2017). The termite mound soils are highly

fertile as nutrients required for plant growth are higher in it. Higher carbon content as well as nutrient availability makes it suitable for microbial population development (Nithyatharani, 2018). Soil enzymes were important participants in soil fertility and microbial activity indicators. Because of the action of termite gut microflora and other associative microbes in the termite ecosystem, the abundance of enzymes associated to lignocellulosic biomass degradation was higher in termite mound soil.

Cellulase activity, saccharase activity, and -glucosidase activity of collected materials were examined in the study conducted by Santhoshkumar *et al*. (2020). They reported that termites are important players in the carbon mineralization and immobilization proccess of soil ecosystems and consume a lot of polysaccharides viz., cellulose and hemicellulose. The microflora of termite soil and their stomach plays an important role in dissimilatory action. Thus, termites and associated ecosystem microflora had a huge ecological impact on the bio-recycling of lignocelluloses.

Higher levels of carbon and nitrogen in mound may be due to consumption of organic material and soil particles by termites (Brauman, 2000). They can reduce the litter size into small particles, enhance the mineralization by fungi and soil bacteria and make carbon rich humic substances. As different organic matters passing through the digestive tract was subjected to different types of chemical and biological mechanisms. These proccesses modify the amount of organic matter, and the degree of humification and metal ion complexation (Brauman, 2000). Termites' soils have higher resistance from weathering (Sarcinelli *et al*., 2009).

Wasps belong to order Hymenoptera and suborder Apocrita. They develop a mixture that is both more sticky and lighter than clay, which is their basic structural material for nest. Clay soils are aluminium polysaccharides that are hydrous and include varying amounts of iron, magnesium, alkali metals, and other cations. Wasps grab mud, hydrate it, and combine it with their saliva, which serves as a cement. The saliva of mud dauber wasp contains phosphate, magnesium, sulphur, chlorine, potassium, calcium, and other unknown components, according to researchers (Jayawardhana *et al*., 2020). Calcium,

magnesium, total iron, chloride, sulphate, and phosphates were all found in large amounts in wasp nest clay, according to Kamalu *et al*. (2015). When compared to the nearby soil, potassium and aluminium were insignificant.

An assessment was carried out by Susheela *et al*., 2020 to analyse the nest building patterns, elemental analysis, and physico-chemical features of the nests of the paper wasp, *Ropaldia marginata*, and mud wasp, *Sceliphron caementarium*, from the Coimbatore district of Tamil Nadu. The pH value of 5.69 for *Sceliphron caementarium* (mud wasp) nest suggested that the material was acidic, but the pH value of mud wasp indicated that the nest was alkaline. High bulk density values indicate high quality adsorbents. The bulk density of the studied paper wasp nest was 0.25 g cm^{-3}, while that of the mud wasp nest was 1.55 gcm^{-3}. The moisture availability of mud-wasp nest was determined to be 1.26, implying that the mud-wasp nest can be preserved for lengthy periods of time without significant microbiological activity. The organic matter in mud wasp was 0.34, which is a higher value than the organic matter in paper wasp. The chemical compound responsible for the stronger and light weight construction, and adhesive properties of *S. caementarium* nest is composed of varied concentrations of Ca, k, and Mg (Kulkarni 2020).

2.11. ROLE OF EXOPOLYSACCHARIDE IN WASP NEST & TERMITE MOUND SOIL

Termites are the only social insect detritivores with a capability of lignocellulosic processing (Grzimek, 2003). Digestion of organic matter begins with the help of termite saliva. Water, the most abundant component in saliva, is primarily responsible for lubrication. Termite saliva is rich in digestive enzymes. Digestive enzymes to degrade starch, sugars, proteins and lipids are present in termite saliva (Resh and Carde, 2009). Termites have some intestinal enzymes which help for cellulose degradation. Termite saliva contains cellulose digesting enzymes: α-1-4-glucanase followed by glucosidase that degrades the cellobiose to six carbon glucose (Nakashima *et al*., 2002).

2.12.MICROORGANISMS IN WASP NEST AND TERMITE MOUND SOIL

A diverse group of microorganisms viz., bacteria, fungi, actinomycetes can be found in termite nests and neighbouring soils (Paul and Verma 1992).

Termite nests were revealed to be a rich source of actinobacteria providing 118 actinobacterial isolates in research by Sujada *et al*. (2014). *Streptomyces* was the dominant genus. *Nocardia* and *Pseudonocardia* were the important genus found in carton nest and subterranean nests respectively. In the termite nests studied, novel species were also detected. Each termite nest contained more than 20% bioactive actinobacteria capable of inhibiting the growth of minimum of one test organism. About 12 isolates from the genera *Streptomyces, Amycolatopsis, Pseudonocardia, Micromonospora*, and *Nocardia* showed inhibitory activities for different microbes.

As feeding materials of termites are rich in mineral content, termite nest host for a plenty of microorganisms. A study was performed with the aim of enumerating microorganisms and studying fungi found in termite mound soil micro-biome. The researchers compared microorganisms found in termite mound soil to those found in surface soil near the mound. The study found significant differences in the number of colony forming units (CFUs). Lower CFUs were found in mound soils as compared to soil taken from the surrounding area. Because of the alkaline pH (8), both soil samples contained larger amounts of actinomycetes than bacteria and fungi. Fourteen fungus species were found in termite mound soil, while 15 were found in surface soil around the mound. *Acremonium* sp, *Aspergillus niger*, *Humicola* sp and *Myrothecium* sp were discovered to be prominent in mound soil. In contrast, *A. niger, A. terreus*, and a *Penicillium* species were more common in soil around the mound (Ganesan *et al*., 2010).

2.13. BACTERIA PRESENT IN WASP NEST & TERMITE MOUND SOIL

Antimicrobial generation, biocontrol, bioremediation, and bio-filtering are all the key functions of bacteria isolated from termite nests. Higher nutrient and organic carbon content of termite mound made it as an alternative for biofertilizer. These kinds of nests

have the potential to help the ecosystem (Fang *et al*., 2020). Chestnut gall wasp hosts different bacterial isolates. It includes both spore forming and non-spore forming bacteria (Iskender *et al*., 2017)

Aribisala *et al*. (2018) isolated *Bacillus* spp. and *Clostridium* spp. from the termitarium were used in this study. Gram staining revealed that the organisms were gram positive. Following biochemical tests, they were discovered to be motile, catalase positive, coagulase negative, and consistent in carbon source use.

2.14. ROLE OF EXOPOLYSACCHARIDE PRODUCING BACTERIA IN SOIL AGGREGATE STABILITY

Termites have a different eating behavior, in which it will consume organic matters. Excretory substances of termite will preserve these organic matters, therefore organic carbon and nitrogen concentration of termite mound will be higher compared to nearby areas. Termites are key organisms to form stable soil aggregates. The organic matter breakdown helps to increase soil porosity and hygroscopic potential of soil (Miklós, 1995; Schaefer, 2001; Balbino *et al*., 2002).

Stability of soil aggregate was determined by organic carbon content of soil and clay particles (Amezketa, 1999). Different disturbances in cathedral mounds leads to less stable soil aggregation, therefore in the surface area of cathedral mounds are formed with impervious soil layer (Traoré, *et al*., 2019). Vardharajula (2011) found that high carbon content in microbial exopolysaccharides causes soil aggregation. Bacterial EPS forms aggregation of microbes and helps in adhering soil particles together. It is mixture of high molecular weight polymers. Because of different inter molecular forces viz., cation bridges, hydrogen bonding, Van der Waals forces, and anion adsorption mechanisms, clay particles in the mounds will absorb the different forms and produces a protective capsule around soil aggregates (Selvakumar *et al*., 2012).

Soil bacteria such as *Bacillus and Pseudomonas* are able to produce biofilms and EPS, which aid in soil structure stabilization (Costa *et al*., 2018). Plant's rhizospheres

produce EPS, which protects its nearby zones from moisture loss and changes in water potential while also enhancing nutrient intake and stimulating plant growth. It guards seedlings against drought stress and increase the root exudate production. The better soil aggregation and improved structure will accelerate the growth of seedlings as it will ensure optimum nutrient and water uptake (Sandhya *et al*., 2009). Qurashi *et al.* (2012) explained soil aggregation by EPS producers in their study. Under elevated salt stress, inoculating chickpea plants with the EPS-producing strains *Halomonas variabilis* and *Planococcus rifietoensis* protected the plants from salinity. It also helped in vegetative growth of plant. Soil aggregation was significantly changed. The findings showed that biofilm forming bacteria can be used to improve vegetative growth and nutrition in excess salt environments.

2.15. ROLE OF EXOPOLYSACCHARIDE PRODUCING BACTERIA IN SUSTAINABLE AGRICULTURE

Soil structure preservation is an important aspect of sustainable agriculture. It influences a variety of mechanisms that influence crop yield (Lal, 1991). Microbial EPS form a substrate that hosts different microbes altogether in biofilms. Biofilm attachment to periphery protects microorganisms from extreme environmental drought conditions, trap nutrients, and reinforce tolerance to toxigenic elements (Silambarasan *et al*., 2019). Exopolysaccharides are sometimes referred to as "adhesive polymers" (Czaczyk *et al*., 2007).

Extracellular polymeric substances lead to cohesiveness of bacteria and the attachment of biofilms to surfaces. It also alters the spatial organization and help in the interactions between microorganisms, and functioning as binding agent between cells (Costa *et al*., 2018). Through different inter and intramolecular forces viz., dispersion forces, electrostatic interactions, and hydrogen bonding, these functionalities are critical for the development and biological process of biofilms. As a result of the creation of a jelly like structure around the cells, the microorganisms are tightly packed and form stable consortia gel matrix (Flemming *et al*., 2007). Extra cellular polymeric substances

have a significant role in formation positive obligatory relationship between rhizobium and plants. The complete mechanism for nodule development is yet unknown.

EPS producing bacteria was shown to have a high water-holding capacity due to their ability to produce EPS. EPS-producing bacteria such as *Pseudomonas* strain GAP-P45 can survive even in drought conditions (Sandhya *et al*., 2009). Sandy soil have high porosity and cannot hold water. EPS was inoculated in to sandy soil and it showed a differents in moisture content. Sandy soils were able to trap more water when EPS was inoculated (Roberson *et al.*, 1992). Furthermore, by acting as a protective sponge, the EPS protects the bacteria from desiccation, allowing them to make metabolic changes (Caruso *et al*., 2018). Salt tolerant bacterial isolates will reduce the sodium absorbtion by immobilizing and reducing the ion availability (Upadhyay *et al*., 2011). The polymer helps bacteria survive by preventing nutritional imbalance and osmotic stress (Etesami *et al*., 2018). Thermophilic bacteria can get benefit from extracellular polymeric compounds that protect them from extreme heat. Under extreme temperatures, the thermotolerant *Pseudomonas* sp. strain AKM-P6 produced EPS, allowing the plants to survive (Ali *et al*., 2009).

Materials and Methods

3. MATERIALS AND METHODS

The experiment on "Exopolysaccharide producing bacteria from soil based nesting structure of insects" was carried out in the Department of Agricultural Microbiology, College of Agriculture, Vellayani during 2019-2021. The details of the materials used and methods followed in the study are described below.

3.1. GENERAL

3.1.1. Composition of media, reagents and solutions

The compositions of different media, reagents and solutions used are given in Annexure 1

3.1.2. Sterilization techniques

All growth media, broth and glassware were sterilized in an autoclave at 121˚C for 20 min at 15 psi pressure. Isolation, purification, inoculation and other microbiological works were carried out in a laminar airflow chamber.

3.1.3. Collection of samples from termitarium and mud dauber wasp nest

Soil samples from termitaria and mud dauber wasp nests were collected from Thiruvananthapuram at 8.5^0 North latitude and 76.9^0 East longitude, at an altitude of 29 m above mean sea level and Kollam at an altitude of 19.2 m above mean sea level, 8.56^0 North latitude and 76.37^0 East longitude. Ten soil samples each were collected from termitaria and mud dauber wasp nests (size ranging from 2cm-5cm) (Plate 1). Samples were collected from newly built nests where active workers were found in great numbers during sample collection as well as from already stable nests which were recently abandoned by newly emerging wasp. The adhering dust and debris were removed using a clean brush the samples were separately packed in sterile polythene bags, brought to the laboratory and stored 28±2 ^{0}C for further studies.

3.2. ISOLATION OF EXOPOLYSACCHARIDE PRODUCING BACTERIA

3.2.1. Isolation of bacteria from termitaria and mud dauber wasp nest

Isolationof bacteria was carried out from the samples by serial dilution and plating technique using nutrient agar as medium. Spread plate technique was used for plating the diluted soil samples (Reynolds, 2005).

Ten gram of sample was weighed and transferred to 250 mL conical flask containing 90 mL sterile water blank and vortexed to mix the contents well to get 10^{-1} dilution. One mL of this dilution was transferred to 9 mL sterile water blank followed by vortexing to get 10^{-2} dilution. Similarly, dilutions were made up to 10^{-5} and 100 µL of the serially diluted soil samples were spread uniformly using a sterilized glass spreader on previously prepared nutrient agar plates. The plates were incubated at 28±2^{o}C for 24 hours and observed for bacterial colonies. Morphologically different colonies were picked and streaked on nutrient agar plates for purification. Pure cultures thus obtained were stored in nutrient agar slants at 4^{o}C

3.2.2. Isolation of EPS producing bacteria in selective medium

The isolates were evaluated for their ability to produce exopolysaccharides by plate assay. The EPS selective medium proposed by Tallgren *et al*. (1998) was modified by replacing soft brown sugar with sucrose. The isolates were streaked onto solidified agar plates and incubated for 24 to 48 h at 28±2^{o}C. The isolates having mucoidal colonies were selected for further studies.

3.2.3. Purification of EPS producing bacteria

Exopolysaccharide producing bacteria were further purified by streak plate method on modified EPS selective medium, EPS production was confirmed and the isolated colonies were preserved on Nutrient agar slants at 4°C in a refrigerator for further use.

Plate 1. Mud dauber wasp nest (A to F) and termitaria (G to L) collected from different locations.

3.3. QUALITATIVE AND QUANTITATIVE ESTIMATION OF EXOPOLYSACCHARIDE PRODUCTION

3.3.1. Qualitative assessment of EPS production

The qualitative determination of exopolysaccharide production was performed according to Paulo *et al*. (2012). The bacterial isolates were streaked on modified EPS selective agar plates. EPS production was characterized by the size and appearance of the slime produced around the colonies. Mucoidal colonies were selected and a portion of the mucoidal substance was mixed with 2 mL of absolute ethanol. The presence of a precipitate confirmed EPS production by the isolate.

3.3.2. Quantitative estimation of EPS production

3.3.2.1. Quantitative estimation of EPS production by gravimetric method

Bacterial EPS content was estimated according to the protocol of Celik *et al*. (2008). The bacterial isolates were grown in modified EPS selective broth. Fifteen mL broth was centrifuged at 12,000 rpm for 20 minutes at 4˚C. The supernatant was pooled with 98% cold ethanol (1:2) and kept overnight in refrigerated condition. Afterwards the mixture was centrifuged at 12,000 rpm for 20 minutes at 4˚C. The resulting pellet was air dried and the weight was recorded.

3.3.2.2. Carbohydrate estimation using phenol sulphuric method

Total carbohydrate was estimated by phenol sulphuric acid method using glucose as standard (Dubuois *et al.*, 1956). The isolates were grown in modified EPS selective broth and incubated at 28±2^{o}C for 4 days. Two ml of the broth was mixed with 1 ml freshly prepared 5% aqueous phenol in a test tube. Subsequently 5 ml concentrated H_2SO_4 was added rapidly to the solution and allowed to stand for 10 min. This mixture was vortexed for 30 seconds and kept in a water bath at room temperature for 20 min for the reaction to take place. Absorbance of the solution at 490 nm was recorded using a spectrophotometer and compared with that of a standard solution of glucose. Reference

solutions were prepared in identical manner as above except for replacing 2 ml broth with double distilled water for preparing the standard curve. The results were expressed as total carbohydrates in ppm.

3.4. CHARACTERIZATION OF THE ISOLATES

3.4.1. Morphological Characterization of Superior EPS Isolates

Ten isolates showing maximum EPS production were streaked on nutrient agar plates and the morphological characters of the colonies like colour, texture, margin, and elevation were recorded. .

3.4.1.1. Gram staining

Gram staining was carried out as per modified (Cappuccino and Sherman, 1999). Gram positive stained was performed and cells were observed under a microscope.

3.4.1.2. KOH test

A loopful of 24h old culture of isolate was mixed with one drop of freshly prepared 3 % potassium hydroxide solution placed on a clean glass slide. The loop was occasionally raised 1- 2 cm from the surface of the slide. Positive reaction was indicated by stringing within the first 30 seconds of mixing.

3.4.1.3. Endospore staining

Schaeffer Fulton method was used for endospore staining. Malachite Green 5% was added to heat-fixed bacterial smear and heated for a few minutes over a steam bath. Safranin was added as counter stain and observation recorded.

3.4.1.4. Capsule staining

Negative staining using nigrosin provided a dark background against which the shape of unstained cells was clearly visible.

3.4.2. Biochemical test

Biochemical tests of the Gram-positive bacteria were conducted using HiBacillus biochemical kit (HIMEDIA KB013). HiAssorted kit (HIMEDIA KB002) was used to study biochemical reactions of Gram-negative bacteria.

3.4.3. Sequencing of isolates

Molecular characterization of the best isolates selected by qualitative and quantitative screening was done at Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram. The following procedures were carried out.

3.4.3.1. Isolation of Bacterial Genomic DNA

The isolation of bacterial genomic DNA was done using NucleoSpin® Tissue Kit (Macherey-Nagel).

A loopful of bacterial culture was taken in a microcentrifuge tube to which, 180 µL of T1 buffer and 25 µL of proteinase K was added and incubated at 56^{o}C. The incubation was carried out in a water bath until the cells were completely lysed. On completion of lysis, five µL of RNase A (100 mg mL^{-1}) was added and incubated at 28±2^{o}C for five minutes. Following this, 200 µL of B3 buffer was added and incubated at 70^{o}C for ten minutes. Further, 210 µL of 100 per cent ethanol was added and mixed thoroughly using vortex mixer. The obtained mixture was then pipetted into NucleoSpin® Tissue column placed in a two ml collection tube and centrifuged at 11000 rpm for one minute. The NucleoSpin® Tissue column was washed with 500 of BW buffer by transferring it to a new two ml tube. This was again washed with 600 µL of B5 buffer. After completion of washing the NucleoSpin® Tissue column was kept in a clean 1.5 ml tube. The elution of DNA was done using 50 µL of BE buffer.

3.4.3.2. DNA Quality and Quantity Check Using Agarose Gel Electrophoresis (AGE)

The quality of the isolated DNA was checked using agarose gel electrophoresis. To five µL of DNA one µL of 6X gel-loading buffer (0.25 per cent

bromophenol blue, 30 per cent sucrose in TE buffer pH-8.0) was added. The samples were loaded onto a 0.8 per cent agarose gel prepared in a 0.5X TBE (Tris-Borate-EDTA) buffer with 0.5 g mL^{-1} ethidium bromide and electrophoresis was carried out with 0.5X TBE buffer at 75 V for 30 min. The gel was visualized and DNA was quantified using Gel documentation system (Bio-Rad).

3.4.3.3. PCR Analysis

PCR analysis was done with 2x Phire Master Mix (5 µL), 4 µL of distilled water, 0.25 µL of forward primer, 0.25 µL of reverse primer and one µL of obtained DNA.

Target	Primer name	Direction	Sequence (5'→ 3')
16S rRNA	16S-RS-F	Forward	CAGGCCTAACACATGCAAGTC
	16S-RS-R	Reverse	GGGCGGWGTGTACAAGGC

3.4.3.4. PCR Amplification

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). The PCR amplification profile as follows,

PCR Amplification profile of 16S rRNA:

95 °C	-	5 minutes	
95 °C	-	30 seconds	35 cycles
60 °C	-	40 seconds	
72 °C	-	60 seconds	
72 °C	-	7 minutes	
4 °C	-	∞	

3.4.3.5. Agarose Gel Electrophoresis of PCR Products

The PCR products were examined using 1.2 per cent agarose gels prepared in 0.5X TBE buffer containing 0.5 µg mL^{-1} ethidium bromide. Electrophoresis was

performed at 75V by loading the mixer of 1 µL of 6X loading dye and 4 µL of PCR products with 0.5X TBE as electrophoresis buffer for about 1-2 h till the bromophenol blue front had migrated nearly to the bottom of the gel. A 2-log DNA ladder (NEB) was used as molecular standard. Gel documentation system (Bio-Rad) captured the image of the gels under UV light using UV transilluminator (Genei).

3.4.3.6. ExoSAP-IT Treatment

ExoSAP-IT (USB) is a specifically prepared buffer containing two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), for the elimination of undesired primers and dNTPs from a PCR product combination with no interference in downstream applications.

Five micro litres of PCR product were mixed with 0.5 µL of ExoSAP-IT and incubated at 37^{o}C for 15 minutes followed by 5 minutes of enzyme inactivation at 85°C.

3.4.3.7. Sequencing Reaction

In PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) sequencing reaction was carried out by using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA).

The Sequencing PCR mix consisted of

MilliQ water	6.6µL
5X Sequencing Buffer	1.9µL
Forward Primer	0.3µL
Reverse Primer	0.3µL
Sequencing Mix	0.2µL
Exosap treated PCR product	1µL

3.4.3.8. Sequencing PCR Amplification Profile

96°C	-	2 minutes	
96°C	-	30 seconds	30 cycles
50°C	-	40 seconds	
60°C	-	4 minutes	
4°C	-	∞	

3.4.3.9. Post Sequencing PCR Clean-up

A mixer of distilled water, 125mM EDTA, 3M sodium acetate (pH 4.6) and ethanol were prepared. In each well of the sequencing plate containing sequencing PCR product, 50 µL was added and mixed by vortex mixer and incubated at 28±2°C for 30 minutes. After incubation, the mixer was centrifuged at 3700 rpm for 30 minutes. The supernatant was decanted and 50 µL of 70 per cent ethanol was added to each well and spun at 3700 rpm for 30 minutes. The ethanol wash was repeated twice by decanting the supernatant and adding 70 per cent ethanol. After final wash, the supernatant was allowed to air dry. The cleaned up and air-dried product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems) using Sanger DNA sequencing method.

3.4.3.10. Sequence Analysis

Sequence Scanner Software v1 (Applied Biosystems) was used to check the sequence quality. Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 (Drummond *et al.,* 2010). The consensus sequence was created using the software BioEdit 7.2 (Hall *et al.,* 2011).

NCBI-BLAST module was used to compare the sequence similarity and the best match in GenBank database of obtained sequence database of unknown culture (Altschul *et al*., 1990).

3.5. PHOSPHATE SOLUBILIZATION OF BACTERIAL ISOLATES:

The isolates were evaluated for their ability to dissolve insoluble tricalcium phosphate present in Pikovskaya's agar medium (Pikovskaya, 1948; Gupta *et al.,* 1994).

A loopful of culture grown in Pikovskaya's broth was spot inoculated at each quadrant of the Pikovskaya's agar plates (3 mm thickness). Thus, five EPS producing bacteria were compared and observed for P solubilization in a single plate with three replications and incubated at 28±2°C for 5 days. Phosphorous solubilization was observed as clear zone around the bacterial colonies and the diameter of the zone was measured in millimetres.

3.6. POTASSIUM SOLUBILIZATION OF BACTERIAL ISOLATES

The ability of the bacterial isolates to solubilize potassium was evaluated using Aleksandrov medium amended with insoluble potassium aluminosilicate (Lu and Huang's method, 2010). The plates were spot inoculated with the bacterial isolates and incubated at 28°C for 7 days. Three replications were kept for each isolate. The potassium solubilization zone was measured.

3.7. SILICON SOLUBILIZATION OF BACTERIAL ISOLATES

Silicon solubilization capability of the bacterial isolates was assayed by the method described by Vasanthi *et al*. (2013). The bacterial isolates were spot inoculated on plates containing 3mm thick Bunt and Rovira agar with 0.25 percent magnesium trisilicate. Three replications were maintained. The plates were examined for a clear zone of solubilization after a 7-day incubation period.

The two best isolates *Klebsiella pneumoniae* KWP23 and *Paenibacillus polymyxa* KTM17, having highest exopolysaccharide production and carbohydrate content was taken for further studies.

3.8. EFFECT OF CARBON SOURCE AND CARBON: NITROGEN RATIO ON EPS PRODUCTION

The effect of three concentration levels of two different carbon sources viz., glucose and sucrose on the exopolysaccharide production by each isolate was assessed by growing in modified EPS selective broth amended with the two different carbon sources and yeast extract as nitrogen source in two different experiments. 100 ml

aliquots of sterile broth containing each carbon source at concentration levels of 10:1, 30:1, 50:1(C:N ratio) were taken in conical flasks, inoculated with a loopful of pure bacterial culture and incubated for 72h. The growth was observed visually. Quantity of exopolysaccharide produced was recorded according to the method described by Fang and Jia (1996). Five replications were maintained for each isolate. 15 ml broth was centrifuged at 12,000 rpm for 20 min at 4˚C. The supernatant was pooled with 98% cold ethanol (1:2) and kept overnight in refrigerated condition. Afterwards the supernatant was centrifuged at 12,000 rpm for 20 minutes at 4˚C. The resulting pellet was air dried and weight was recorded.

3.9. EFFECT OF TEMPERATURE AND SALINITY ON EPS PRODUCTION

The effect of temperature on the growth of EPS producing bacterial isolates was tested in modified EPS selective broth. Each conical flask containing 100 mL of the sterile broth was inoculated with a loopful of pure bacterial culture and incubated at four different levels of temperature *i.e.*, 20°C, 30°C, 40°C, 50°C for 72h and the growth was observed visually. Five replications were maintained for each isolate. Quantity of exopolysaccharide produced was recorded by centrifugation according to Fang and Jia, (1996) as described above.

The effect of salinity on the growth of EPS producing bacterial isolates were tested in modified EPS selective broth. Each conical flask containing 100 mL of three different sterile broth of salinity; 0, 3, 6 % was inoculated with a loopful of pure bacterial culture and incubated for 72 h and the growth was observed visually. Seven replications were maintained for each isolate. Quantity of exopolysaccharide produced was determined as above.

3.10. *IN VITRO* ANTIFUNGAL ACTIVITY AGAINST SELECTED BACTERIAL ISOLATES.

The antifungal activity of the bacterial isolates was assayed by dual culture method. The fungal pathogens, *Rhizoctonia solani, Sclerotium rolfsii* and *Helminthosporium* sp.

available at the Department of Agricultural Microbiology, College of Agriculture were used for the assay. Fungal discs were cut using corkborer and placed on the centre of the PDA agar plates. *Klebsiella pnuemoniae* KWP23 and *Paenibacillus polymyxa* KTM17 were streaked onto the plate equidistantly at 1 cm from the edge. Inoculated plates were incubated at 25 °C for 72 hours. The inhibition zone was measured and recorded.

3.11. SOIL AGGREGATE STABILITY STUDY

The isolates showing maximum EPS production were evaluated for their capacity for soil aggregation. Soil sample was collected from Vellayani area at 8.5^0 North latitude and 76.9^0 East longitude, at an altitude of 29 m above mean sea level. The soil was sandy clay loam in texture. Soil was sieved using 2 mm sieve. The bacterial isolates were grown in the broth of modified EPS selective media, washed and resuspended in sterile distilled water (10^8 cfu ml^{-1}) and inoculated to 1 Kg soil sample for each treatment (T_1 with Isolate 1 & T_2 with Isolate 2), 150g were stored in each container with lid with five replications with a control for each treatment. The inoculated soil sample was incubated for three months.

3.12. ANALYSIS OF PHYSICOCHEMICAL PROPERTIES OF SOIL

3.12.1. Soil reaction (pH)

The pH of the soil was determined using the potentiometer with soil and water in the ratio of 1: 2.5 (Jackson, 1973).

3.12.2. Soil Salinity (EC)

Electrical conductivity was measured using soil suspension (soil: water 1:2.5) prepared for measuring soil reaction. EC was determined at 28±2^{o}C by using a direct reading conductivity meter (Jackson, 1973).

3.12.3. Bulk density and Porosity

Bulk density of the soil sample was estimated according to tamping method described by Ahmedna *et al*. (1997). Twenty gram of soil sample was taken in 100 mL measuring cylinder followed by continuous gentle tapping. The volume of the soil was noted (V1). Fifty mL of distilled water was added (V2) along the sides of the cylinder and the apparatus was set aside undisturbed for 30 minutes. The final volume was noted as V3.

Pore space volume = $(V1 + V2) - V3$ ml

Per cent pore space = $\frac{(V1 + V2) - V3}{V1} \times 100$

Bulk density = $\frac{W}{V1}$ mg m^{-3}

Where W – Weight of the soil taken

V1 – Volume of soil taken

V2 – Volume of water added

V3 – Volume of soil and water after 30 minutes

3.12.4. Water Holding Capacity

Thirty gram of soil sample was taken in a plastic container with holes which provided proper drainage. The soil samples were saturated by adding water and the container was kept aside till the excess water drained out completely. The soil was weighed immediately. The soil sample was kept in a hot air oven and dried at 105^{o}C for 48h. Then the soil sample was cooled in a desiccator and the weight was recorded (Vengadaramana *et al.*, 2012). The same procedure was carried out for the samples treated with the bacterial isolates.

Water Holding Capacity of soil $= \frac{Wd - Wf}{Wi} \times 100$

where,

W_i - Weight of initial soil (g)

W_f - Weight of soil after 48h at 105^{o}C (g)

W_d -Weight of soil after water drained off (g)

3.13. AGGREGATE WEIGHT

One hundred grams of soil amended with the bacterial isolates and the soil sample kept as control was sieved using 2 mm and 5 mm sieves. This approach involved horizontally vibrating 100 g of dry soil, on 2 mm and then 5 mm sieve.

3.13.1 Total aggregate weight

Weight of the aggregates from the respective sieves was recorded.

3.13.2. Single aggregate weight

Mean weight of the single aggregate from the 5 mm sieve was recorded.

3.13.3. Random ten aggregate mean weight

Ten soil aggregates were randomly selected from the respective sieves and their total weight was recorded.

3.13.4. Mean-Weight Diameter of Dry Aggregate (MWDD) (Kemper & Chepil, 1965)

The mean-weight diameter of dry aggregates was determined using the Kemper and Chepil method (1965). This approach involved horizontally vibrating 150 g of dry soil aggregates which is amended with the bacterial isolates, using the sieves of 5 mm, 2

mm and 0.25 mm and determining the mass of aggregates that resisted break down. Thus, the mean weight diameter of dry aggregate (MWDD) was calculated.

$$MWDD = \sum_{i=1}^{n} Xi\ di$$

where,

MWDD = Mean - Weight Diameter of Dry Aggregates (mm)

Xi = Mean Diameter of each size fraction (mm)

di = proportion of total sample weight occurring in the corresponding size fraction

3.13.5. Influence of Time of Immersion on Size-distribution of Aggregates

Aggregate stability by water immersion based on time was estimated as per Kawaguchi and Kita (1957) method with slight modifications. Hundred grams soil was sieved using 2 mm sieve and 5 mm sieve. Aggregates from 5 mm sieve were placed in Petri plates placed above a graph paper so that the size of the aggregates could be recorded. The size distribution of the aggregates was recorded. The plates were filled with water in such a way that the aggregates were fully immersed in water. The difference in size distribution was recorded at immersion, and at intervals of 10 min, 30 min, 1 hour and 24 hours. Size distribution and stability of the aggregates were recorded.

3.13.5. Effect of Wet Sieving on Stability of Soil Aggregates.

Aggregate stability by water immersion based on oscillations was conducted as per modified Kawaguchi and Kita (1957) method. Hundred gram soil amended with the bacterial isolates was sieved using 2mm sieve. Ten grams of › 2 mm size aggregate were placed on a fine sieve. A container with a diameter of 8 cm and a height of 4 cm was filled with water. The fine sieve with the aggregates was placed in the container. The sieve was moved up and down 120 times, at a frequency of 60 cycles per minute within a distance of 8 cm (including movement of 4 cm under water taken in the container), so that the aggregates of various size disintegrated inside the container based on their

stability. Oscillations were carried out for 2 minutes and disintegration of the aggregates was recorded. Time taken for initiation of disintegration, weight of disintegrated soil, turbidity of water and changes in stability of aggregates were recorded.

3.14. STATISTICS

Data were analyzed by one-way ANOVA and means were compared with the software GRAPES (General R-shiny based Analysis Platform Empowered by Statistics) developed by Department of Agricultural Statistics, Kerala Agricultural University (Gopinath *et al*., 2021).

Results

4. RESULTS

The results of the study entitled on “Exopolysaccharide producing bacteria from soil based nesting structure of insects” was carried out in the Department of Agricultural Microbiology, College of Agriculture, Vellayani during 2019-2021 is presented in this chapter. Studies were carried out to isolate and screen bacteria which are capable of producing exopolysaccharide. The experimental data from the above mentioned study were statistically analyzed and the results are presented in this chapter.

4.2. ISOLATION OF EXOPOLYSACCHARIDE PRODUCING BACTERIA

4.2.1. Isolation of Bacteria from Termitaria and Mud Dauber Wasp Nest

Bacteria were isolated from termitaria and mud dauber wasp nest, by serial dilution and plate count method with Nutrient agar. Thirty three bacterial isolates were obtained from different locations (Table 1). Colony morphology of the bacterial isolates on nutrient agar medium is given in Table 2.

4.2.2. Isolation of Exopolysaccharide Producing Bacteria in Selective Medium

Among the thirty three colonies, fifteen isolates had slimy mucoidal appearance and were identified as exopolysaccharide producers (Plate 2).

4.3 QUALITATIVE AND QUANTITATIVE ESTIMATION OF EXOPOLYSACHHARIDE PRODUCTION

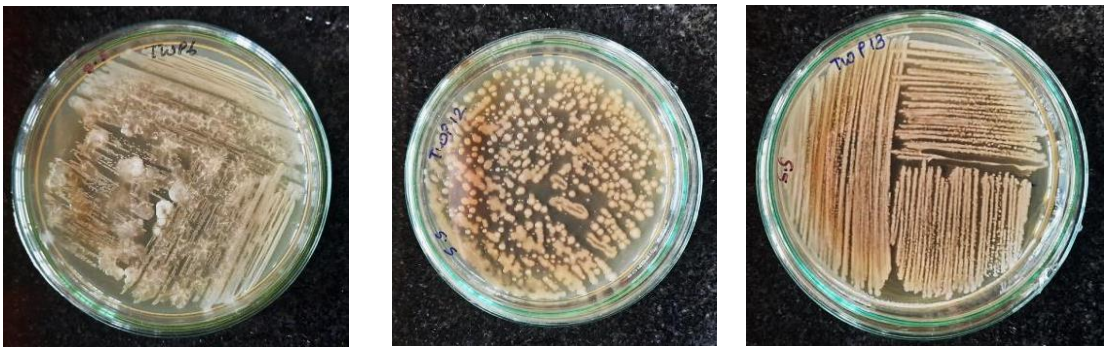
4.3.1. Qualitative Assessment of Exopolysaccharide Production

The qualitative assessment of the EPS producing bacterial isolates were done on the basis of visual observation of the mucoidal appearance of the colony in EPS selective medium as shown in the Table 3. Of the fifteen EPS producing bacterial isolates, twelve which showed better EPS production were selected for further quantitative estimation of EPS production.

Table 1. List of bacterial isolates obtained from termitaria and mud dauber wasp nest soil samples collected from different locations

Sl No	Isolate Code No:	Source	Places
1	TWP1	Wasp nest	Vellayani, Thiruvananthapuram
2	TWP2	Wasp nest	Vellayani, Thiruvananthapuram
3	TWP3	Wasp nest	Vellayani, Thiruvananthapuram
4	TWP4	Wasp nest	Vellayani, Thiruvananthapuram
5	TWP5	Wasp nest	Vellayani, Thiruvananthapuram
6	TWP6	Wasp nest	Vellayani, Thiruvananthapuram
7	TWP7	Wasp nest	Vellayani, Thiruvananthapuram
8	TWP8	Wasp nest	Vellayani, Thiruvananthapuram
9	TWP10	Wasp nest	Vellayani, Thiruvananthapuram
10	TWP11	Wasp nest	Vellayani, Thiruvananthapuram
11	TWP12	Wasp nest	Vellayani, Thiruvananthapuram
12	TWP13	Wasp nest	Vellayani, Thiruvananthapuram
13	TWP14	Wasp nest	Vellayani, Thiruvananthapuram
14	TWP16	Wasp nest	Vellayani, Thiruvananthapuram
15	TWP17	Wasp nest	Vellayani, Thiruvananthapuram
16	TWP18	Wasp nest	Vellayani, Thiruvananthapuram
17	KWP21	Wasp nest	Eravipuram, Kollam
18	KWP23	Wasp nest	Eravipuram, Kollam
19	TWP24	Wasp nest	Vellayani, Thiruvananthapuram
20	TWP25	Wasp nest	Vellayani, Thiruvananthapuram
21	KTM1	Termitarium	Eravipuram, Kollam
22	KTM2	Termitarium	Eravipuram, Kollam
23	KTM3	Termitarium	Eravipuram, Kollam
24	KTM4	Termitarium	Eravipuram, Kollam
25	KTM5	Termitarium	Eravipuram, Kollam
26	KTM6	Termitarium	Eravipuram, Kollam
27	KTM7	Termitarium	Eravipuram, Kollam
28	KTM8	Termitarium	Eravipuram, Kollam
29	KTM9	Termitarium	Eravipuram, Kollam
30	KTM10	Termitarium	Eravipuram, Kollam
31	KTM15	Termitarium	Eravipuram, Kollam
32	KTM17	Termitarium	Eravipuram, Kollam
33	KTM18	Termitarium	Eravipuram, Kollam

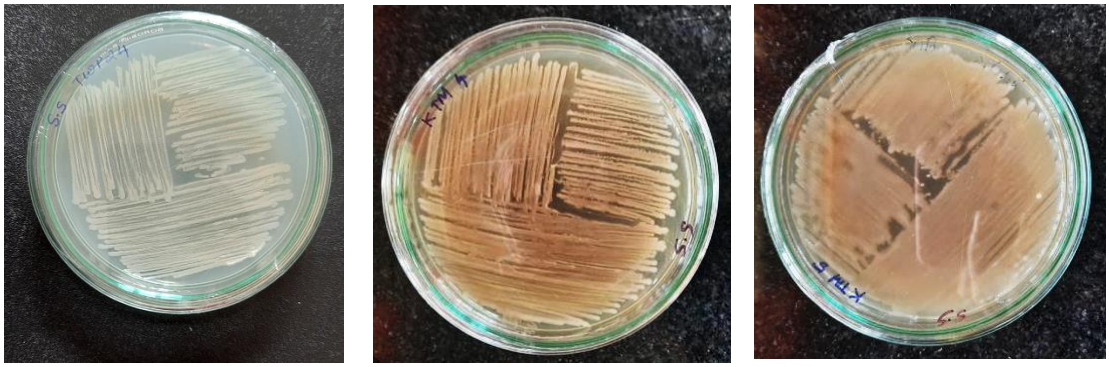
TWP1 **TWP2** **TWP5**



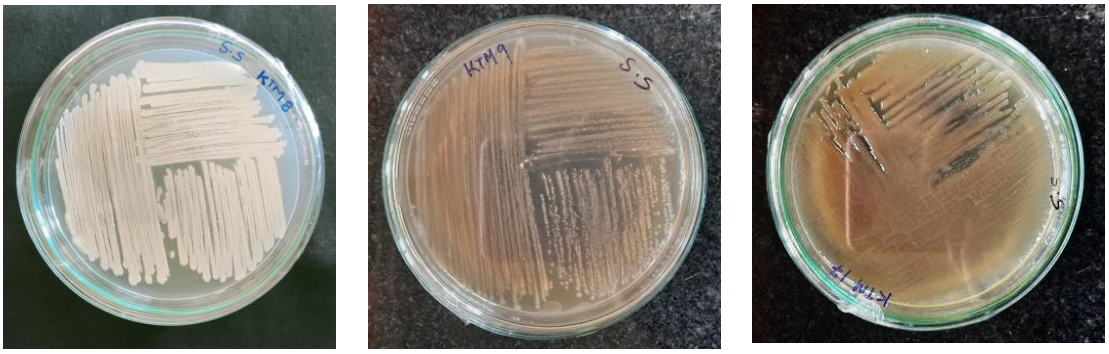
TWP6 **TWP 12** **TWP 13**

TWP16 **KWP 21** **KWP 23**

Plate 2. Exopolysaccharide producing bacterial isolates in EPS selective medium.



TWP24 **KTM 4** **KTM 5**



KTM 8 **KTM 9** **KTM 17**

Plate 2. Exopolysaccharide producing bacterial isolates in EPS selective medium.

Table 2. Colony characters of bacterial isolates on nutrient agar medium.

Sl No	Isolate code No:	Color	Elevation	Margin
1	TWP1	Creamy white	Raised	Circular, entire
2	TWP2	Creamy white	Raised	Circular, entire
3	TWP3	Brownish	Raised	Undulated
4	TWP4	Creamy white	Raised	Undulated
5	TWP5	Brownish	Raised	Undulated
6	TWP6	Creamy white	Raised	Undulated
7	TWP7	Creamy white	Raised	Undulated
8	TWP8	Creamy white	Flat	Undulated
9	TWP10	Creamy white	Raised	Undulated
10	TWP11	Brownish	Raised	Undulated
11	TWP12	Creamy white	Raised	Undulated
12	TWP13	Creamy white	Raised	Undulated
13	TWP14	Brownish	Raised	Undulated
14	TWP16	Creamy white	Raised	Undulated
15	TWP17	Creamy white	Raised	Undulated
16	TWP18	Creamy white	Flat	Undulated
17	KWP21	Creamy white	Raised	Undulated
18	KWP23	Creamy white	Raised	Circular, entire
19	TWP24	Creamy white	Raised	Undulated
20	TWP25	Creamy white	Raised	Undulated
21	KTM1	Creamy white	Flat	Undulated
22	KTM2	Creamy white	Flat	Undulated
23	KTM3	Creamy white	Raised	Undulated
24	KTM4	Creamy white	Raised	Undulated
25	KTM5	Creamy white	Raised	Undulated
26	KTM6	Creamy white	Raised	Undulated
27	KTM7	Creamy white	Raised	Undulated
28	KTM8	Creamy white	Raised	Undulated
29	KTM9	Creamy white	Raised	Undulated
30	KTM10	Creamy white	Raised	Undulated
31	KTM15	Creamy white	Raised	Undulated
32	KTM17	Creamy white	Raised	Undulated
33	KTM18	Creamy white	Raised	Undulated

Table 3: Qualitative Assessment of Exopolysaccharide production of selected isolates on modified EPS medium

Sl. No	Isolate Code No:	EPS production
1	TWP1	+ +
2	TWP2	+ + +
3	TWP5	+ +
4	TWP6	+ + +
5	TWP12	+ + +
6	TWP13	+ +
7	TWP16	+
8	KWP21	+ +
9	KWP23	+ + +
10	TWP24	+
11	KTM4	+ +
12	KTM5	+ +
13	KTM8	+
14	KTM9	+ +
15	KTM17	+ + +

(+) less mucoidal **(++)** moderate mucoidal **(+++)** more mucoidal

4.3.2. Quantitative Estimation of Exopolysaccharide Production

4.3.2.1. Quantitative Estimation of Exopolysaccharide Production by gravimetric method

The weight of EPS produced by the selected twelve isolates was recorded in μgmL^{-1}. Maximum EPS production was recorded in the isolate KWP23 (25.78 $\mu g\ mL^{-1)}$, followed by KTM17 (22.45 µg mL-1) which is on par with TWP13 (20.18 $\mu g\ mL^{-1}$), KTM4 (19.75 $\mu g\ mL^{-1}$), TWP12 (19.73 $\mu g\ mL^{-1}$). The lowest EPS production on weight by volume basis was recorded TWP5 (2.58 $\mu g\ mL^{-1}$) (Table 4).

4.3.2.2. Carbohydrate Estimation Using Phenol Sulphuric Method

The total carbohydrate content in exopolysaccharide was reported to be maximum in bacterial isolate KTM17 (14.48 μgmL^{-1}) which is on par with KWP23 (13.35 μgmL^{-1}). Among this the least carbohydrate content was recorded for KWP21 (1.61 μgmL^{-1}) (Table 5).

4.4 CHARACTERIZATION OF THE ISOLATES

The isolates TWP5 and KTM9 having least EPS production and total carbohydrate content were excluded. The remaining ten isolates were selected for characterization and biochemical studies.

4.4.1. Morphological Characterization of Superior exopolysaccharide producing Isolates

The colony characters of the isolates are given in Table 2. All nine isolates were creamy white except TWP13 which was brownish with raised colony. All the isolates except KWP23 and TWP2, were gram negative which was confirmed by KOH test. TWP6 was spherical while other bacterial isolates were rod shaped (Plate 3). TWP2, TWP12, TWP13, KTM4, KTM5, KTM17 were endospore formers. However, all the isolates were capsule formers (Table 6).

Table 4: Quantitative estimation of exopolysaccharide production of the selected isolates

Sl. No.	Isolate Code No:	EPS production (µg mL^{-1})*
1	TWP1	4.25^{cde}
2	TWP2	5.83^{cd}
3	TWP5	2.58^{e}
4	TWP6	4.35^{b}
5	TWP12	19.73^{b}
6	TWP13	20.18^{b}
7	KWP21	7.03^{c}
8	KWP23	25.78^{a}
9	KTM4	19.75^{b}
10	KTM9	2.95^{de}
11	KTM5	5.83^{cd}
12	KTM17	22.45^{b}
CD (0.05)		2.878
SEm (±)		0.986

*Mean of 3 replications. Figures in a column followed by the same letter do not differ significantly (p>0.05)

Table 5: Estimation of total carbohydrates in exopolysaccharides of the selected isolates

Sl. No.	**Isolate Code No.**	**Total carbohydrate (µg mL^{-1})**
1	TWP1	3.24^{e}
2	TWP2	1.85^{f}
3	TWP5	2.30^{ef}
4	TWP6	2.14^{ef}
5	TWP12	6.45^{c}
6	TWP13	9.027^{b}
7	KWP 21	1.61^{f}
8	KWP23	13.35^{a}
9	KTM4	4.69^{d}
10	KTM5	2.13^{ef}
11	KTM9	2.21^{ef}
12	KTM17	14.42^{a}
	CD (0.05)	1.312
	SEm (±)	0.45

*Mean of 3 replications. Figures in a column followed by the same letter do not differ significantly (p>0.05)

Table 6: Cell morphology and staining reactions of selected exopolysaccharide producing bacterial isolates

Sl No	Isolate code No:	Cell shape	Gram reaction	Endospore staining	Capsule staining
1	TWP1	Rod	G+	-	+
2	TWP2	Rod	G-	+	+
3	TWP6	Rod	G+	-	+
4	TWP12	Rod	G+	+	+
5	TWP13	Rod	G+	+	+
6	KWP21	Rod	G+	-	+
7	KWP23	Rod	G-	-	+
8	KTM4	Spherical	G+	+	+
9	KTM5	Rod	G+	+	+
10	KTM17	Rod	G+	+	+

4.4.2. Biochemical Characterization of Selected Exopolysaccharide Producing Isolates

Biochemical characterization of selected EPS isolates was conducted using biochemical kit. HiAssorted biochemical kit was used for Gram negative isolates (Table7) (Plate 4) and HiBacillus kit was used for gram positive isolates (Table 8) (Plate 5).

4.4.3. Molecular Characterization of Superior Exopolysaccharide Producing Isolates

Based on EPS production**,** five best isolates viz. TWP12, TWP13, KWP23, KTM4 and KTM17 were selected for 16S rRNA gene sequencing (Table 9). The results obtained by sequencing after contig alignment and BLASTN query are presented in the Table 10. The maximum score, query cover, percent identity and the best match in GenBank database of the selected isolates are presented.

4.5. PHOSPHATE SOLUBILIZATION OF BACTERIAL ISOLATES

Among the five isolates tested, *Klebsiella pneumoniae* KWP23 alone could solubilize phosphate in Pikovskaya's agar medium. The solubilization zone was (4 mm) around their colony (Plate 6).

4.6. POTASSIUM SOLUBILIZATION OF BACTERIAL ISOLATES:

The isolate *Klebsiella pneumoniae* KWP23 developed a distinctive zone of solubilization (1 mm) around their colony in Aleksandrov medium (Plate 7).

4.7. SILICON SOLUBILIZATION OF BACTERIAL ISOLATES:

There was no indication of zone of silicate solubilization in Bunt and Rovira medium. None of the isolates chosen were silicate solubilizers.

Table 7: Biochemical characterization of exopolysaccharide producing Gram negative isolates

Sl.No	**Test**	**TWP2**	**KWP23**
1	Citrate utilization	-	-
2	Lysine utilization	+	+
3	Ornithine utilization	+	+
4	Urease	-	-
5	Phenyalanine Deaminase	-	-
6	Nitrate Reduction	+	+
7	H_2S production	-	-
8	Glucose	-	+
9	Adonitol	-	-
10	Lactose	-	+
11	Arabinose	-	-
12	Sorbitol	-	-

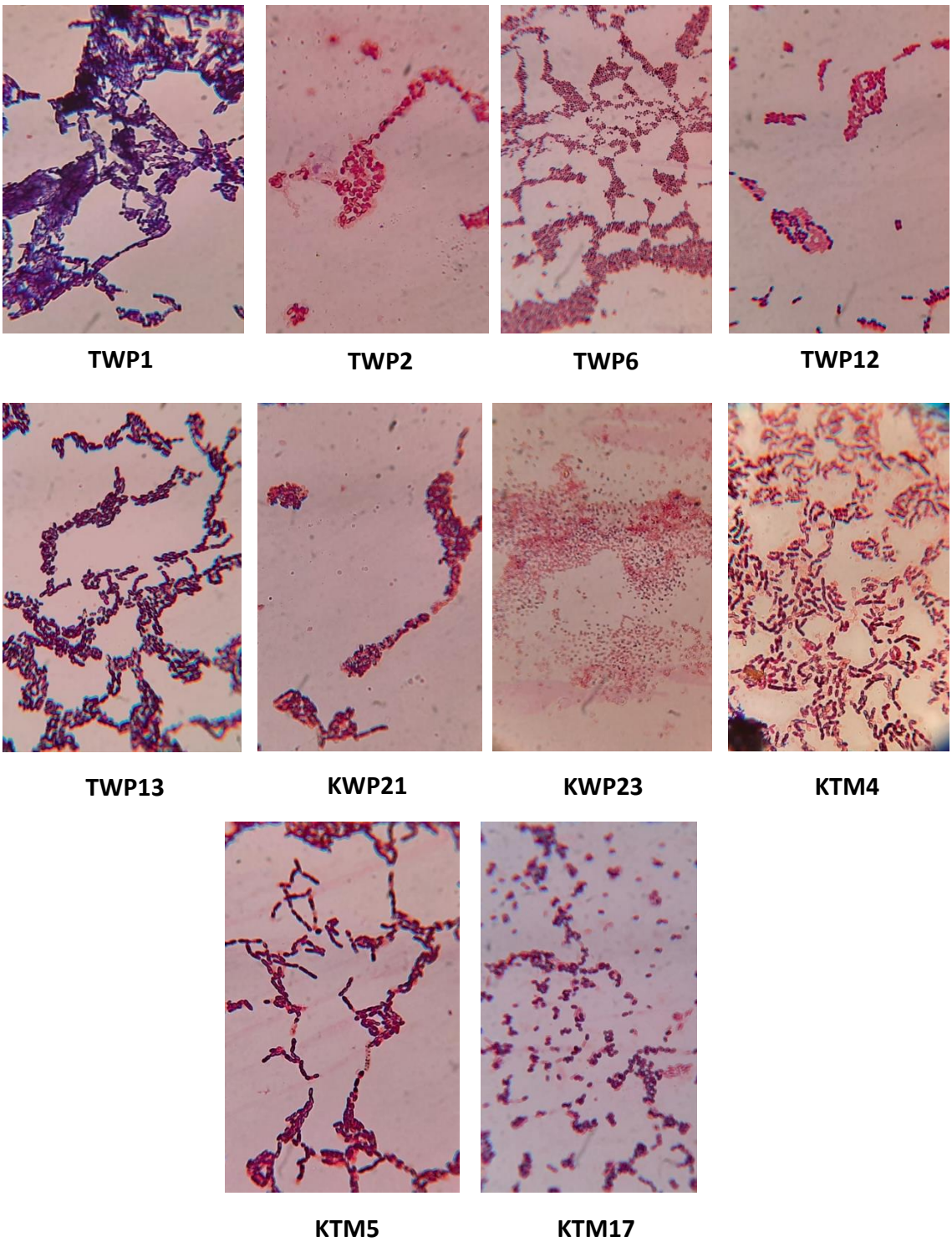


Plate 3. Gram staining of exopolysaccharide producing bacterial isolates

Plate 4: Biochemical characterization of Gram positive bacterial isolates using HiBacillus Kit- KB013

Plate 5. Biochemical characterization of Gram negative bacterial isolates using HiAssorted Kit-KB002

Table 8: Biochemical characterization of exopolysaccharide producing Gram positive isolates.

Sl.No	Test	TWP1	TWP6	TWP12	TWP13	KWP21	KTM4	KTM5	KTM17
1	Malonate	-	-	-	-	-	-	-	-
2	Voges - Proskauer‘s	-	-	-	-	-	-	-	-
3	Citrate	-	-	-	-	-	-	-	-
4	ONPG	-	-	+	+	+	-	-	-
5	Nitrate reduction	+	+	-	-	+	-	-	-
6	Catalase	-	+	+	+	+	-	+	-
7	Arginine	-	-	-	-	-	+	-	+
8	Sucrose	+	+	+	+	+	-	+	+
9	Mannitol	+	+	-	+	+	-	-	+
10	Glucose	+	+	-	-	+	-	-	+
11	Arabinose	-	-	-	-	-	-	-	-
12	Trehalose	+	-	+	+	-	-	-	-

Table 9. 16S rRNA gene sequence of the exopolysaccharide producing bacterial isolates obtained with universal primer

Isolate code	**Sequence**	**GenBank Acession No.**
TWP12	ACGTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAACTTCGGGA AACCGAAGCTAATACCGGATAGGATCTTCTCCTTCATGGGAGATGATTGAAAGATGGTTTCGGCTA TCACTTACAGATGGGCCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGAT GCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG AGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATG AAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACGAGAGTAACTGCTCGTACCT TGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGC AAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGC CCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGAAAAGCGGAA TTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTTTTTGG TCTGTAACTGACGCTGAGGCGCGGAAAGCGTGGGGAGCAAACGGGAATTTATTGGGCGTAAAGCG CGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAAC TGGGGAACTTGAGTGCAGAAGAGAAAAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATG TGGAGGAACACCAGTGGCGAAGGCGGCTTTTTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGG GGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGG TTTCCGCCCTTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTG AAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACG CGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACTCTAGAGATAGAGCGTTCCCCTTCGGGGG ACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA ACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTTAGTTGGGCACTCTAAGGTGACTGCCGGTGACA AACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCT ACAATGGATGGTACAAAGGGCTGCAAGACCGCGAGGTCAAGCCAATCCCATAAAACCATTCTCAGT TCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGT	OM232813

TWP13	TCTATGACGTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAACT TCGGGAAACCGAAGCTAATACCGGATAGGATCTTCTCCTTCATGGGAGATGATTGAAAGATGGTTTC GGCTATCACTTACAGATGGGCCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCA ACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTA CGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGT GATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACGAGAGTAACTGCTCGT ACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG TGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAA AGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGAAAAGCG GAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTTTT TGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGGAGCAAACAGGATTAGATACCCTGGTAGG TCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTGGGAATTATTGGGCGTAAAGCGCGCG CAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGG GAACTTGAGTGCAGAAGAGAAAAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGA GGAACACCAGTGGCGAAGGCGGCTTTTTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAG CAAACAGGATTTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTT CCGCCCTTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAA ACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGA AGAACCTTACCAGGTCTTGACATCCTCTGACAACTCTAGAGATAGAGCGTTCCCCTTCGGGGGACAG AGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAG CGCAACCCTTGATCTTAGTTGCCAGCATTTAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCG GAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAAT GGATGGTACAAAGGGCTGCAAGACCGCGAGGTCAAGCCAATCCCATAAAACCATTCTCAGTTCGGA TTGTAGGCTGCAACTCGCCCTACCATGAAGCTGGAATCGCT	OM232814

KWP23	CTCGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTA CTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCATGC CATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTA GCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAG CAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCT TCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCGTTGAGGTTAATAACCTCGTCGATTGACGTTA CCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTA ATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTC AACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGT AGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGCGTTAATCGGAATTACTGGGCGTAAAGCGC ACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACATGGGAACTGCATTCGAAACTG GCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGG AGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGA GCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGTTGTGCCCTTG AGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAAC TCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAG AACCTTACCTGGTCTTGACATCCACAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTGTGAG ACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCA ACCCTTATCCTTTGTTGCCAGCGGTTAGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAG GAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGC ATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGGATTG GAGTCTGCAACTCGACTCCATGAAGTCGGAATCGC	OM232812

KTM4	TTCTATGACGTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAACT TCGGGAAACCGAAGCTAATACCGGATAGGATCTTCTCCTTCATGGGAGATGATTGAAAGATGGTTTC GGCTATCACTTACAGATGGGCCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAA CGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC GGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGA TGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACGAGAGTAACTGCTCGTACC TTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGG CAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGC CCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGAAAAGCGGAAT TCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGTAAAGCGCGCGCAGGCGGT TTTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAG TGCAGAAGAGAAAAGCGGAATTCCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCA GTGGCGAAGGCGGCTTTTTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGA TTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAG TGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAA TTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACC AGGTCTTGACATCCTCTGACAACTCTAGAGATAGAGCGTTCCCCTTCGGGGGACAGAGTGACAGGTG GTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTG ATCTTAGTTGCCAGCATTTAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGG GGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAAA GGGCTGCAAGACCGCGAGGTCAAGCCAATCCCATAAAACCATTCTCAGTTCGGATTGTAGGCTGCAA CTCGCCTACATGAAGCTGGAATCGCTA	OM232810

KTM17	ACCTAGCGGCGGACGGGTGAGTAACACGTAGGCAACCTGCCCACAAGACAGGGATAACTACCGGAA ACGGTAGCTAATACCCGATACATCCTTTTCCTGCATGGGAGAAGGAGGAAAGGCGGAGCAATCTGTC ACTTGTGGATGGGCCTGCGGCGCATTAGCTAGTTGGTGGGGTAAAGGCCTACCACGGCGACGATGCG TAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGC AGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGT TTTCGGATCGTAAAGCTCTGTTGCCAGGGAAGAACGTCTTGTAGAGTAACTGCTCTTGAAGTGACGG TACCTGAGAAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGT TGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGCTCTTTAAGTCTGGTGTTTAATCCCGAGGC TCAACTTCGGGTCGCACTGGAAACTGGGGAGCTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTG TAGCGGTGACCGGGTCGCACTGGAAACTGGGGAGCTTGAGTGCAGAAAGAGGAGAGTGGAATTCCA CGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGGCTGT AACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGT AAACGATGAATGCTAGGTGTTAGGGGTTTCGATACCCTTGGTGCCGAAGTTAACACATTAAGCATTC CGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCAGTGG AGTATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCTCTGACCGGTCT AGAGATAGATCTTTCCTTCGGGACAGAGGAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTG AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATGCTTAGTTGCCAGCAGGTCAAGCTGGGC ACTCTAAGCAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCT TATGACCTGGGCTACACACGTACTACAATGGCCGGTACAACGGGAAGCGAAGGAGCGAGGTGGAGC CAATCCTAGAAAAGCCGGTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAA	OM232811

Table 10. Details of BLASTN search of 16S rRNA gene sequence of selected exopolysaccharide producing isolates showing best match in GenBank data base.

Sl No.	**Sample Code**	**Scientific Name**	**Max Score**	**Total Score**	**Percentage Identity (%)**	**Query Cover (%)**	**Accession No.**
1.	TWP12	*Priestia aryabhattai*	1465	2731	99.87	100	MK543014.1
2.	TWP13	*Priestia aryabhattai*	1448	2829	99.62	100	MK543014.1
3.	KWP23	*Klebsiella pneumoniae*	1467	28806	99.87	100	CP001982.1
4.	KTM4	*Priestia megaterium*	1434	2543	99.87	97	MF624838.1
5.	KTM17	*Paenibacillus polymyxa*	1319	2425	99.86	99	FJ178378.1

The best two isolates *Paenibacillus polymyxa* KTM17 and *Klebsiella pneumoniae* KWP23 were selected for the further studies on the basis of exopolysaccharide production

4.8. EFFECT OF CARBON SOURCE AND CARBON: NITROGEN RATIO ON EXOPOLYSACCHARIDE PRODUCTION

The effect of different carbon sources viz. glucose and sucrose on EPS production was evaluated by varying their concentration levels in broth. Glucose was found to be the best carbon source for *Klebsiella pneumoniae* at 10:1 level based on EPS production (34.25 µg mL^{-1}) (Plate 8). Sucrose had less influence on EPS production of *Klebsiella pneumoniae*. Lowest EPS production was recorded at sucrose 50:1 level (10.50 µg mL^{-1}) and the highest was at 10:1level (13.75 µg mL^{-1}) (Table 11).

For *Paenibacillus polymyxa* KTM17, highest EPS production was recorded when sucrose was used as carbon source at 50:1 (32.875 µg mL^{-1}) and the minimum obtained when the carbon source was glucose at 50:1 (5.800 µg mL^{-1}). So the best source for *Paenibacillus polymyxa* KTM17 was sucrose at 50:1 and glucose utilization was minimum (Table 12) (Plate 8).

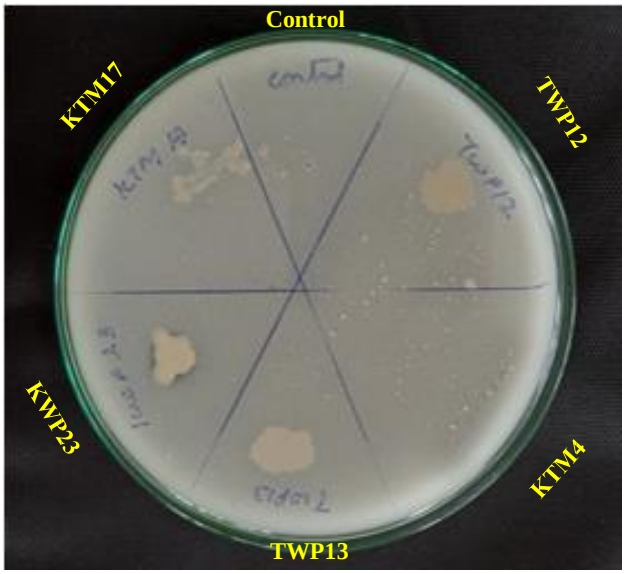


Plate 6. Phosphate solubilization by selected isolates in Pikovskaya‘s medium amended with tricalcium phosphate.

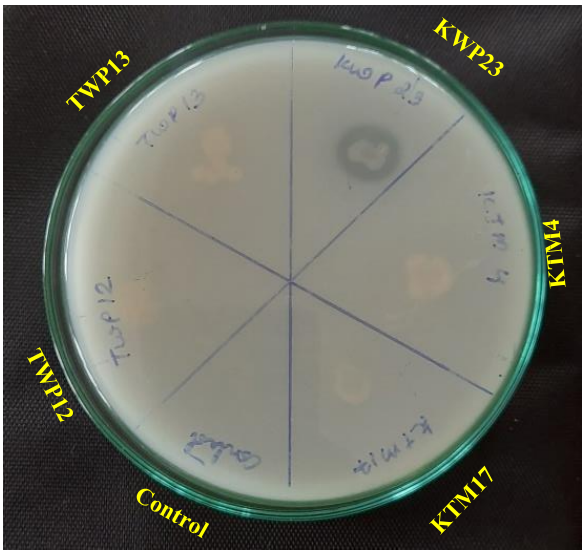


Plate 7. Potassium solubilization by selected isolates in Aleksandrov medium amended with potassium aluminosilicate

Table 11.Effect of different carbon sources on EPS production of *Klebsiella pneumoniae* KWP23

Sl. No	Carbon Source	Carbon Level	EPS Production (µg mL^{-1})*
1	Glucose	10:1	34.25^{a}
		30:1	15.55^{cd}
		50:1	21.95^{b}
2	Sucrose	10:1	13.75^{d}
		30:1	16.50^{c}
		50:1	10.50^{e}
	CD (0.05)		1.888
	SEm (±)		0.647

*Mean of 5 replications. Figures in a column followed by the same letter do not differ significantly (p>0.05)

Table 12. Effect of different carbon sources on EPS production of *Paenibacillus polymyxa* KTM17

Sl .No	Carbon Source	Carbon Level	EPS Production (µg mL^{-1})*
1	Glucose	**10:1**	19.00^{b}
		30:1	8.75^{d}
		50:1	5.80^{e}
2	Sucrose	**10:1**	16.48^{c}
		30:1	20.38^{b}
		50:1	32.88^{a}
	CD (0.05)		0.672
	SEm (±)		1.963

*Mean of 5 replications. Figures in a column followed by the same letter do not differ significantly (p>0.05)

4.9. EFFECT OF TEMPERATURE AND SALINITY ON SURVIVAL OF EPS PRODUCING BACTERIA

4.9.1. Effect of temperature on survival of EPS producing bacteria:

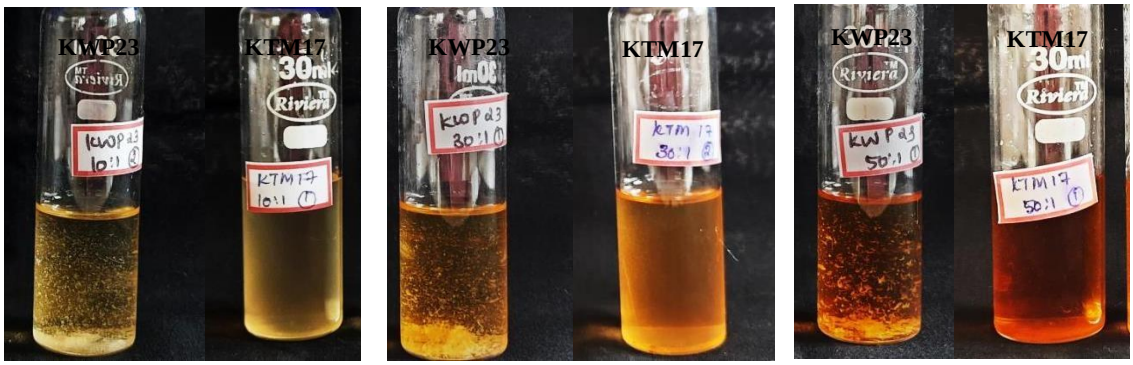
The effect of different temperature was evaluated by providing required temperature individually (Plate 10). Increasing the temperature level (from 20^{o}C to 50^{o}C) resulted in an increase in EPS production that was directly proportionate to the rise in temperature. The highest EPS production by *Klebsiella pneumonia*e KWP23 was (23.00 µg mL^{-1}) at 50^{o}C (Table 13).

In case of *Paenibacillus polymyxa* KTM17, EPS production was maximum at 30^{o}C (31.50 µmL^{-1}) followed by 50^{o}C (17.40 µg mL^{-1}) and 40^{o}C (12.75 µg mL^{-1}).
Minimum EPS production was obtained at 20^{o}C (8.25 µg mL^{-1}) (Table 14).

4.9.2. Effect of salinity on survival of EPS producing bacteria

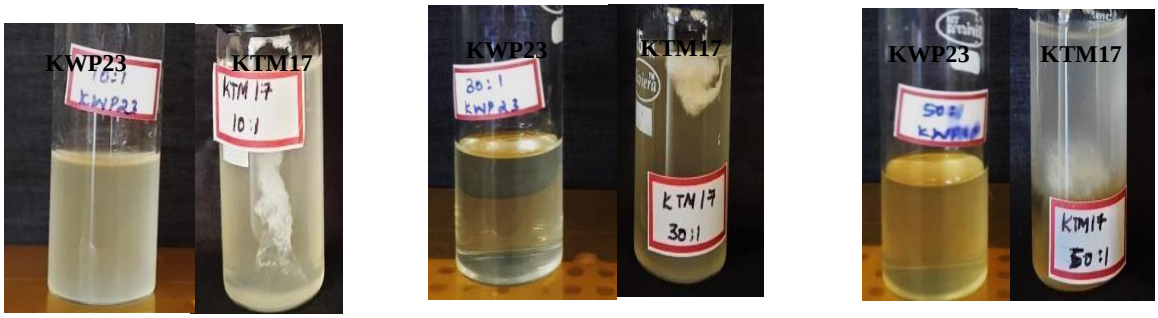
The effect of altering salinity on EPS production by *Klebsiella pneumonia*e KWP23 and *Paenibacillus polymyxa* KTM17 isolates was assessed (Plate 11). For the isolate *Klebsiella pneumonia*e KWP23, the EPS production was maximum without NaCl (18.0 µg mL^{-1}) followed by 3% (0.5 M) NaCl (27.5 µg mL^{-1}). Minimum EPS production was recorded at 6% (1 M) NaCl (20.5 µg mL^{-1}) (Table 15).

For the isolate *Paenibacillus polymyxa* KTM17, the EPS production was maximum at 6% (1 M) (26.25 µg mL^{-1}) NaCl. Minimum EPS production was recorded at 3% (0.5 M) NaCl (22.75 µg mL^{-1}) which is on par with the EPS production without salinity (Table 16).



10:1 **30:1** **50:1**

Plate 8: EPS production by *Klebsiella pneumoniae* KWP23 and *Paenibacillus polymyxa* KTM17 with glucose as carbon source at different C: N ratio.



10:1 **30:1** **50:1**

Plate 9: EPS production by *Klebsiella pneumoniae* KWP23 and *Paenibacillus polymyxa* KTM17 with sucrose as carbon source at different C: N ratio.

Table 13. Effect of different levels of temperature on EPS production by *Klebsiella pneumonia* KWP23

Sl. No	Temperature Level	EPS Production* (µg mL^{-1})
1	20^0C	7.50^d
2	30^0C	11.25^c
3	40^0C	13.75^b
4	50^0C	23.00^a
	CD (0.05)	0.42
	SEm (±)	0.14

*Mean of 5 replications.

Table 14. Effect of different levels of temperature on EPS production of *Paenibacillus polymyxa* KTM17

Sl. No	Temperature Level	EPS Production* (µg mL^{-1})
1	20^0C	8.25^d
2	30^0C	31.50^a
3	40^0C	12.75^c
4	50^0C	17.40^b
	CD (0.05)	1.463
	SEm (±)	4.386

*Mean of 5 replications.

Table 15. Effect of different levels of salinity on EPS production of *Klebsiella pneumoniae*

Sl. No	**Salinity Level % (W/W)**	**EPS Production* (µg mL^{-1})**
1	0	18.00^{a}
2	3	7.00^{b}
3	6	5.25^{b}
	CD (0.05)	3.132
	SEm (±)	1.017

*Mean of 7 replications. Figures in a column followed by the same letter do not differ significantly (p>0.05)

Table 16. Effect of different levels of salinity on EPS production by *Paenibacillus polymyxa* KTM17

Sl.No	**Salinity Level % (W/W)**	**EPS Production * (µg mL^{-1})**
1	0	22.50^{b}
2	3	22.75^{b}
3	6	26.25^{a}
	CD (0.05)	0.752
	SEm (±)	0.244

*Mean of 5 replications. Figures in a column followed by the same letter do not differ significantly (p>0.05)

4.10. *IN VITRO* ANTIFUNGAL ACTIVITY AGAINST SELECTED BACTERIAL ISOLATES.

A clear inhibition zone was observed in dual culture assay of *Paenibacillus polymyxa* KTM17 with *Rhizoctonia solanii* (Plate 12). With the progression of time, the colour of fungal hyphae near the margin of the inhibition zone darkened. *Klebsiella pneumoniae* KWP23 did not inhibit the growth of *Rhizoctonia solanii*. *Klebsiella pneumoniae* KWP23 and *Paenibacillus polymyxa* KTM17 inhibited the growth of *Helminthosporium* sp. (Plate 12). However there was no inhibition of growth of *Sclerotium rolfsii* by *Paenibacillus polymyxa* KTM17 and *Klebsiella pneumoniae* KWP23.

4.11. ANALYSIS OF MECHANICAL AND PHYSICOCHEMICAL PROPERTIES OF SOIL SAMPLE

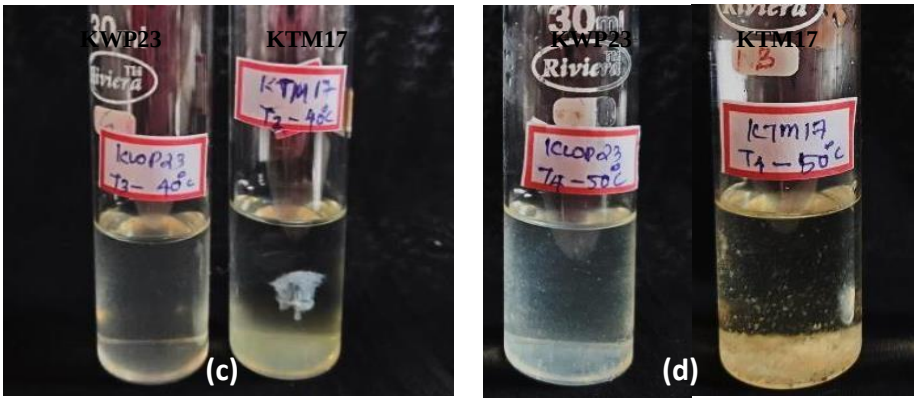
The soil of the experimental site was sandy clay loam which belongs to oxisols, Vellayani series. The data of physicochemical properties of the soil sample taken are presented in Table 17.

4.12. ANALYSIS OF PHYSICOCHEMICAL PROPERTIES OF TREATED SOIL SAMPLE

Highest porosity (56.63 %) was obtained in soil treated with *Klebsiella pneumoniae* KWP23 whereas the bulk density was reported minimum (1.103 g/mL) which was on par with control (1.13 g/mL). Minimum porosity (47.53 %) was obtained in treatment with *Paenibacillus polymyxa* KTM17 and the maximum bulk density (1.31 g/mL) was observed in this isolate. Water holding capacity was maximum in the soil treated with *Paenibacillus polymyxa* KTM17 (38.150 %) and minimum in *Klebsiella pneumoniae* KWP23 (33.967 %) (Table 18). Chemical properties of the soil after treatment are presented in Table 19.

Table 17. Analysis of physicochemical properties of soil sample

Sl. No	Parameters	Value
I	Physical properties	
1	Mechanical composition	
a.	Coarse sand	47.01
b.	Fine sand	16.64
c.	Silt	4.34
d.	Clay	29.41
2	Texture	Sandy clay loam
3	Bulk density	1.247 g cm^{-3}
4	Water holding capacity	31.967%
5	Porosity	37.603 %
II	Chemical properties	
1	pH	6.81
2	EC	0.51 dS/m



Plates 10. EPS production by *Klebsiella pneumoniae* KWP23 and *Paenibacillus polymyxa* KTM17at different temperatures (a) 20 ^{0}C (b) 30 ^{0}C (c) 40 ^{0}C (d) 50^{0}C

Plate 11. EPS production by *Klebsiella pneumoniae* KWP23 and *Paenibacillus polymyxa* KTM17 at different salinity levels (0%, 3% and 6%)

Table 18. Physical properties of soil after treatment with *K. pneumoniae* KWP23 and *P. polymyxa* KTM17.

Sl No	Treatment	Porosity (%)*	Bulk density * (g cm^{-3})	Water holding capacity (%)*
1	*K. pneumoniae* KWP23	56.63^{a}	1.10^{b}	33.97^{b}
2	*P. polymyxa* KTM17	47.53^{b}	1.31^{a}	38.15^{a}
3	Control	43.32^{c}	1.13^{b}	33.15^{c}
	CD (0.05)	3.70	0.07	0.57
	SEm (±)	1.06	0.02	0.16

*Mean of 8 replications. Figures in a column followed by the same letter do not differ significantly (p>0.05)

Table 19. Chemical properties of soil after treatment with *K. pneumoniae* KWP23 and *P. polymyxa* KTM17

Sl. No	Treatment	pH	EC (dS/m)
1	*K. pneumoniae* KWP23	7.24	0.2
2	*P. polymyxa* KTM17	7.20	0.1
3	Control	6.82	0.3

4.13. AGGREGATE WEIGHT

4.13.1. Total aggregate weight.

Maximum aggregates were obtained from 2mm sieve for the both the soil samples amended with bacterial isolates (Plate 13). Soil treated with *P. polymyxa* KTM17 yielded more aggregates in 2mm sieve (13.357 g) and also in 5mm sieve (1.417 g). In case of *Klebsiella pneumoniae* KWP23, minimum aggregates were obtained in 2mm (11.140 g) and in 5 mm sieve (1.261 g). The 2 mm aggregate weight was significantly different for the isolates while the 5mm aggregates were on par (Table 20).

4.13.2. Single aggregate weight

Single aggregate weight of *Paenibacillus polymyxa* KTM17 (0.224 g) and *Klebsiella pneumoniae* KWP23 (0.190 g) respectively. There was no significance difference (Table 21)

4.13.3. Random ten aggregate weight

Random ten aggregate weight recorded maximum for *Paenibacillus polymyxa* KTM17 and minimum for *Klebsiella pneumoniae* KWP23 as (0.424 g) and (0.356 g) respectively (Table 21).

4.13.3. Mean weight diameter of dry aggregates (MWDD)

When the data were analyzed, the mean weight diameter of dry aggregates obtained was highest for the soil amended with *Paenibacillus polymyxa* KTM17 (1.068 mm) which is on par with *K. pneumoniae* KWP23 (1.065 mm) (Table 22).

Plate 12. *In vitro* antagonistic activity of *Klebsiella pneumoniae* KWP23 and *Paenibacillus polymyxa* KTM17 against *Rhizoctonia solani* and *Helminthosporium* sp

Table 20. Total aggregate weight

Sl No	Treatment	Weight of aggregates of 2mm size* (g)	Weight of aggregates of 2mm-5mm size (g)	Total aggregate weight* (g)
1	*K. pneumoniae* KWP23	11.140[b]	1.261	12.557[b]
2	*P. polymyxa* KTM17	13.357[a]	1.417	14.618[a]
3	Control	8.020[c]	0.670	8.690[c]
	CD (0.05)	2.186	NS	1.908
	SEm (±)	0.632	0.221	0.551

*Mean of 8 replications.

Table 21: Aggregate mean weight

Sl. No	Treatment	*Ten aggregate weight (g)	Single aggregate weight (g)
1	*K. pneumoniae* KWP23	0.356[b]	0.190
2	*P. polymyxa* KTM17	0.424[a]	0.224
3	Control	0.135[c]	0.138
	CD (0.05)	0.058	NS
	SEm (±)	0.017	0.024

*Mean of 8 replications.

Table 22: Mean weight diameter of dry aggregates (MWDD)

Sl. No	Treatment	MWDD (mm)
1	*K. pneumoniae* KWP23	1.065[a]
2	*P. polymyxa* KTM17	1.068[a]
3	Control	0.901[b]
	CD (0.05)	0.017
	SEm (±)	0.052

*Mean of 8 replications. Figures in a column followed by the same letter do not differ significantly (p>0.05)

4.13.4. Influence of Time of Immersion on Size distribution of Aggregates.

A comparison was made on influence of time on water immersed soil aggregates. The study revealed that the best soil aggregation stability was given by the isolate *Paenibacillus polymyxa* KTM17 (Table 23) (Plate 14). The stability of the aggregate lasted for more than 24 hours when immersed in water whereas that in the aggregate kept as control disintegrated immediately on pouring water. There was no change in the dimension of the soil aggregate treated with *Paenibacillus polymyxa* KTM17, where the control disintegrated initially. The soil aggregate treated with *K. pneumoniae* KWP23 started to disintegrated slightly after one hour (Table 23) (Plate 15).

4.13.5. Effect of Wet Sieving on Stability of Soil Aggregates.

A comparison study was made on effect of moisture condition on the soil aggregates based on oscillations, the soil aggregates kept as control fully disintegrated and water became turbid (Plate 16). As in the case of soil amended with *Klebsiella pneumoniae* KWP23 and *Paenibacillus polymyxa* KTM17, the aggregates disintegrated partially. The turbidity of water was highest in control (0.254 nm) and lowest in *Paenibacillus polymyxa* KTM17 (0.008 nm) Table 24. The weight of the disintegrated soil was maximum in control (0.908 g) and minimum in the soil aggregate amended with *Paenibacillus polymyxa* KTM17 (0.014 g) Table 25 (Plate 16).

23. Influence of Time of Immersion on Size-distribution of Aggregates.

Sl. No	**Treatment**	**Dimension of aggregate without water (mm × mm)**	**Initial dimension of aggregate with water (mm × mm)**	**After 10 min (mm × mm)**	**After 30 min (mm ×mm)**	**After 1 hour (mm × mm)**	**After 24 hours (mm × mm)**
1	*K. pneumoniae* KWP23	6 × 5	9 × 5	9 × 5 (No change)	9 × 5 (No change)	Slightly disintegrated	Slightly disintegrated
2	*P. polymyxa* KTM17	10 × 7	10 × 7 (No change)	10 × 7 (No change)	10 × 7 (No change)	10 × 7 (No change)	10 × 7 (No change)
3	Control	7 × 5	10 × 7	12 × 10	11 × 12	12 × 12	Fully disintegrated

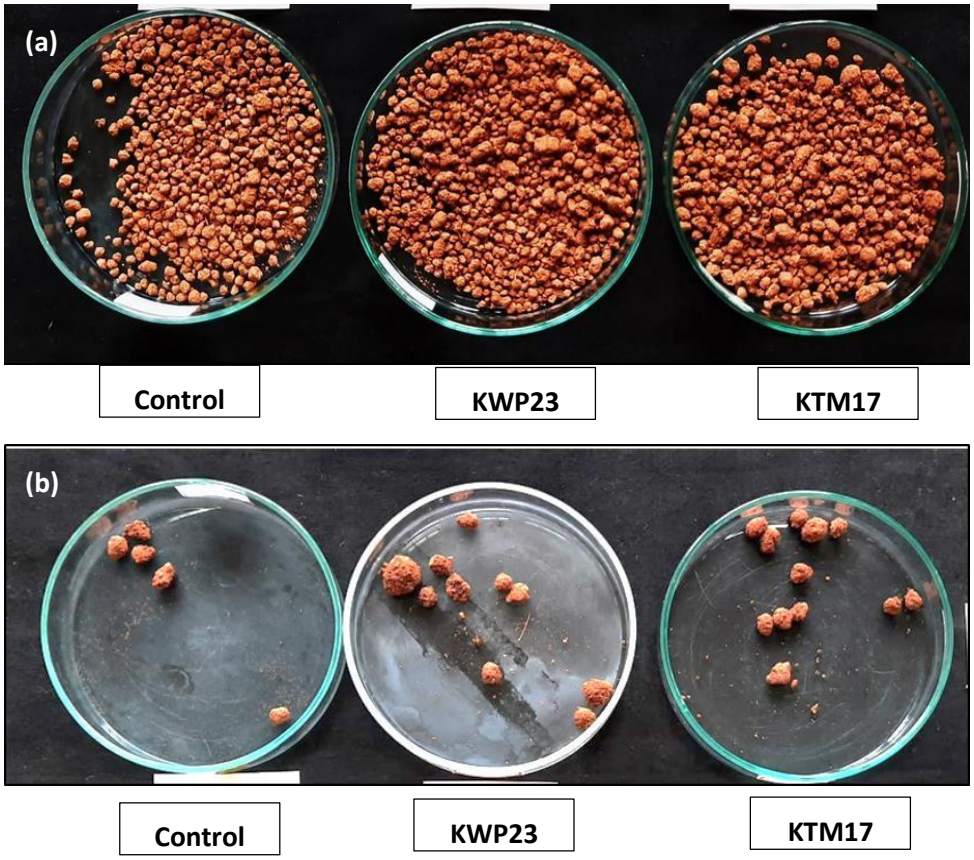


Plate 13. Soil aggreggates separated using (a) 2 mm sieve and (b) 5 mm sieve obtained in soil incubation study after amendment with *Klebsiella pneumoniae* KWP23 and *Paenibacillus polymyxa* KTM17

Table 24. Turbidity of water by soil disintegrated after oscillations in moisture conditions.

Sl No	Treatment	Turbidity (560 nm)
1	*K. pneumoniae* KWP23	0.009[b]
2	*P. polymyxa* KTM17	0.008[b]
3	Control	0.254[a]
	CD (0.05)	0.004
	SEm (±)	0.001

*Mean of 8 replications. Figures in a column followed by the same letter do not differ significantly (p>0.05)

Table 25. Weight of the soil disintegrated after oscillations in moisture conditions.

Sl. No	Treatment	Weight (g)
1	*K. pneumoniae* KWP23	0.040[b]
2	*P. polymyxa* KTM17	0.014[c]
3	Control	0.908[a]
	CD (0.05)	0.005
	SEm (±)	0.017

*Mean of 8 replications.

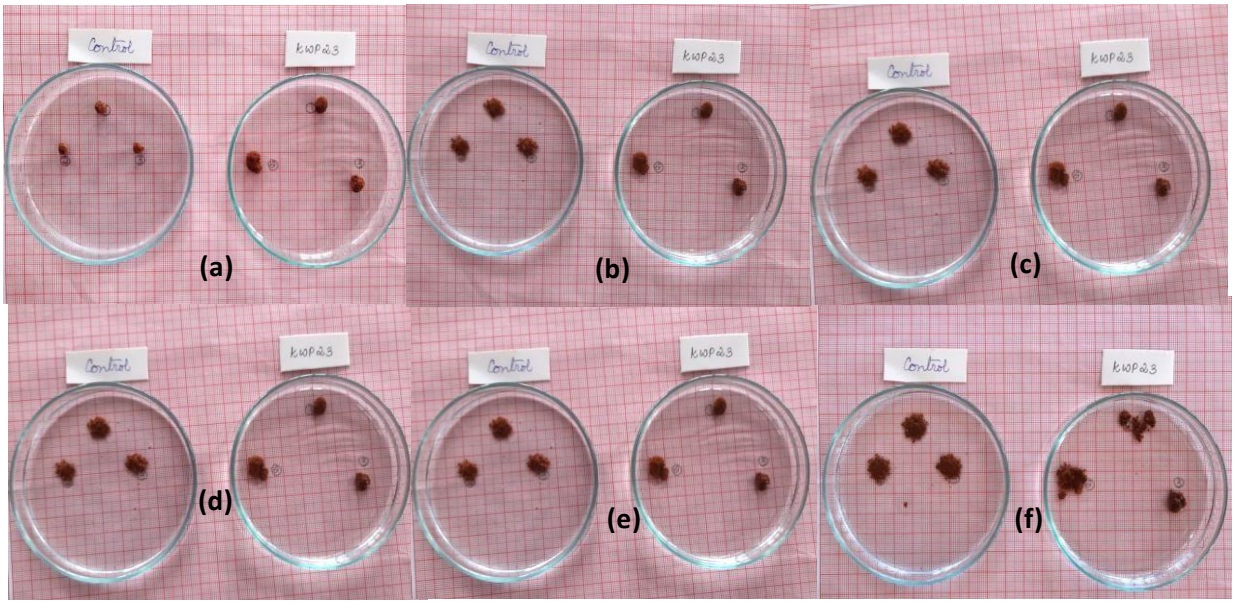


Plate 14. Time based aggregate stability of the soil amended with *Klebsiella pneumoniae* KWP23 on water immersion (a) before adding water (b) immediately after adding water (c) after 10 minutes (d) after 30 minutes (e) after 1 hour (f) after 24 hours.

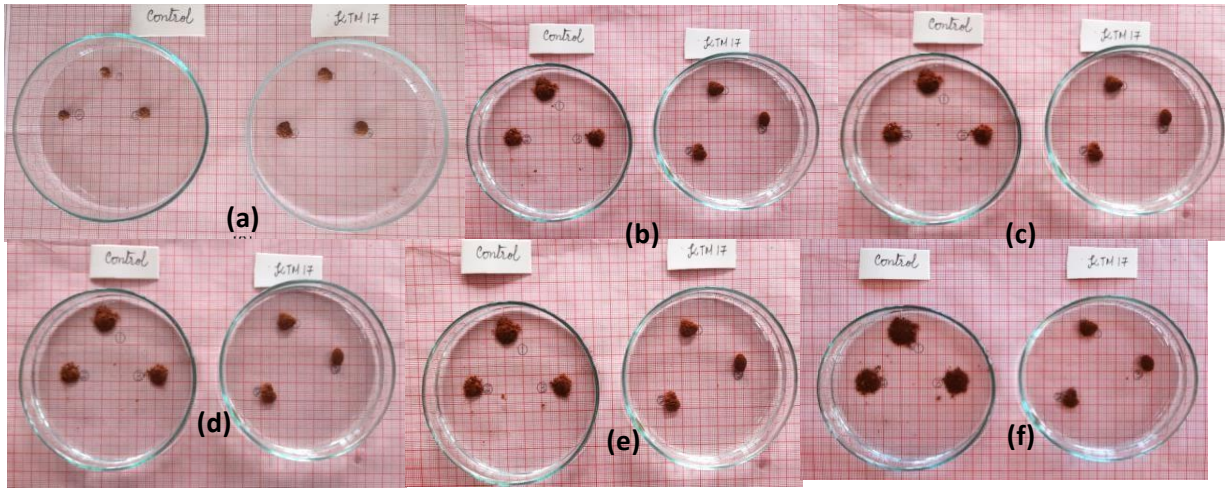


Plate 15. Time based aggregate stability of the soil amended with *Paenibacillus polymyxa* KTM17 on water immersion (a) before adding water (b) immediately after adding water (c) after 10 minutes (d) after 30 minutes (e) after 1 hour (f) after 24 hours.

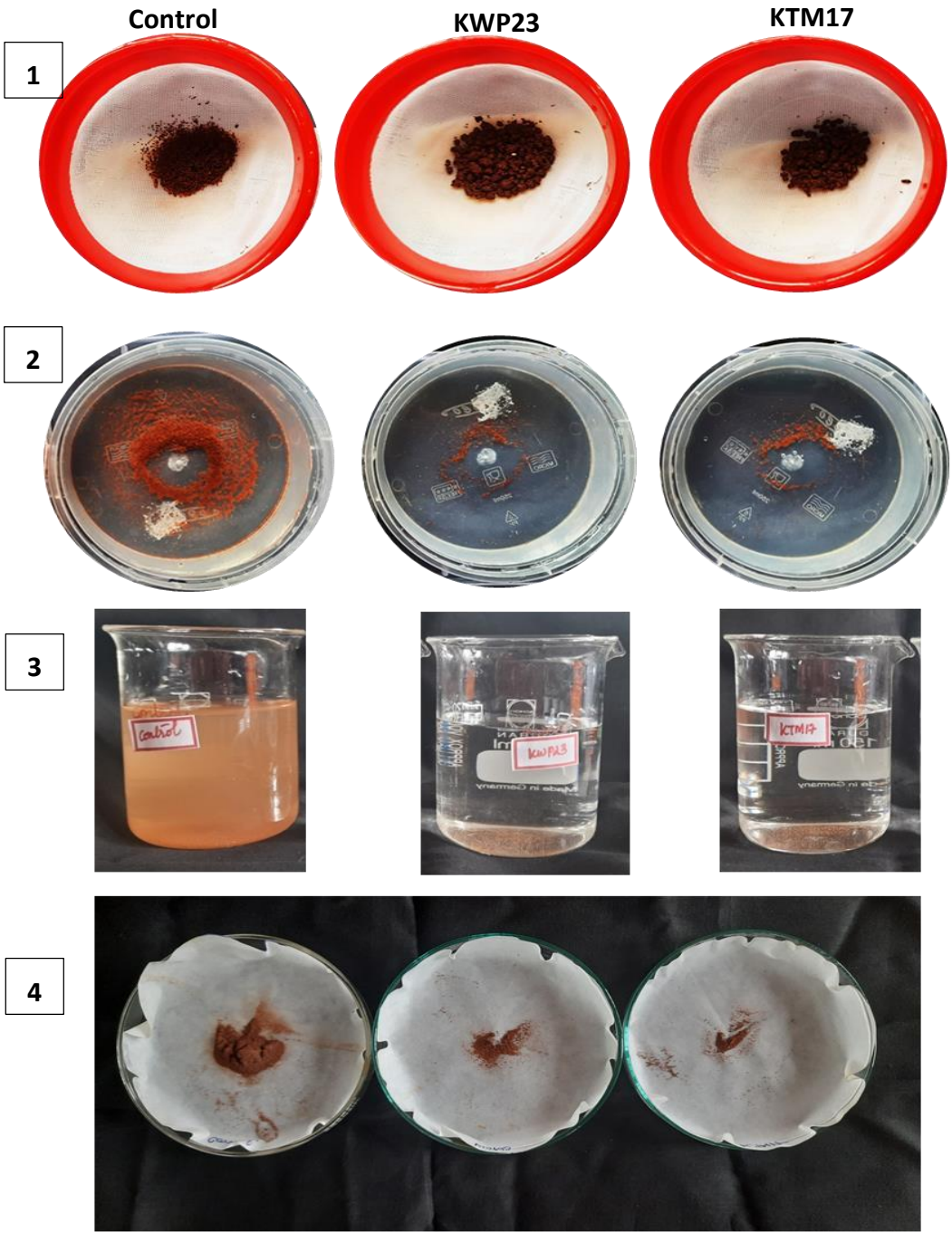


Plate 16. Effect of wet sieving on stability of soil aggregates.(1) aggregates on fine sieve after oscillations (2) soil dispersed in water after oscillations (3) turbidity of water after oscillations (4) soil dispersed in water after oven drying

Discussion

5. DISCUSSION

Extracellular polymeric substances are known to be produced by soil microbes in order to adhere to surfaces and shield themselves from external stresses; such substances may be especially important in the low-nutrient and variable-moisture situations found in marginal soils. They are highly hydrated polymers mostly made up of exopolysaccharides (EPS), proteins, and DNA, produced by a diverse spectrum of microorganisms. Physical and chemical characteristics of EPS can help microbial cells to mitigate moisture and nutrient stress. EPS can alleviate the effect of reduced water potential by increasing the water content they absorb. The sticky, gelatinous properties of EPS bind microbial cells to mineral surfaces in the soil and improve soil-aggregation by binding soil particles together (Sher *et al*., 2020). Compositions of the substrate and the environmental conditions have a considerable impact on production of EPS (Rabha *et al*., 2012).

Exopolysaccharide (EPS) performs a wide range of activities including cell-to-cell interaction, adhesion, and provide excellent protection against severe environments such as salt and high or low temperature (Boukhelata *et al*., 2018). EPS matrix has water holding capacity, adhesion and biocrust formation properties. It helps in carbon storage, entrapment of nutrients and aggregation of microorganisms. It is also involved in interactions of the producers with other microbes and plants and thus protection against abiotic and biotic stress (Costa *et al*., 2018)

Insect nesting structures are usually made of natural materials like soil, fibre, wood pulp etc, processed by the insect using different mechanisms like pulverizing with their mouth parts or by partial digestion in their gut. Mud dauber wasp nest is made of clay, which is levigated by their mandibles and moistened with saliva and made to a fine paste, which is used as a binding material and termitarium is composed of partially digested food materials and termite faecal matter which contain that is filled with minerals and other organic components. These nesting structures have a high probability of containing microorganisms associated with the salivary glands and gut of these insects (Sanghmitra *et al*., 2020; Manjula *et al*., 2016). In addition to that they provide environment for the growth of specific microorganisms capable of adapting to nutrient

and water stress. In the present study, of the 33 bacterial isolates obtained on nutrient agar from different soil-based nesting structures, fifteen were found to be EPS producers by plating on EPS selective medium (Tallgren *et al*., 1999) where brown sugar was replaced with sucrose. Mu'minah *et al*., 2015 have isolated EPS producing bacteria from potato rhizosphere soil in sucrose enriched medium. Tallgren *et al*., 1999 have used EPS selective media amended with brown sugar for the isolation EPS producing microorganism from sugar beet.

Quantitative EPS production and total carbohydrate content were assessed. In the present study, maximum EPS production was recorded for the bacterial isolate KWP23 (25.775 µg $mL^{-1)}$ which was later identified as *Klebsiella pneumoniae* (Figure 1) and total carbohydrate content was maximum for KTM17 (14.42 µg mL^{-1}) which was later identified as *Paenibacillus polymyxa* (Figure 2). Isolation and characterization of some exopolysaccharide producing bacteria from cassava peel heaps was carried out by Ayobami *et al*. (2019) and EPS production by the isolates varied from 12.76 g/L to 0.13 g/L. Of the eighty bacteria isolated, four of the EPS producers were *Klebsiella pneumoniae*.

Based on the quantity of EPS produced ten isolates were selected and subjected to morphological and biochemical characterization. Phenotypic characterization of the selected isolates revealed that there were eight Gram positive and two Gram negative bacteria. In a study conducted on the diversity of exopolysaccharide-producing bacteria isolated from the rhizosphere of wheat (*Triticum aestivum* L.) grown in normal and saline Pakistani soils by Ashraf *et al*., 1999 presence of various EPS-producing Gram positive and Gram negative bacteria was reported. Many bacteria irrespective of their Gram staining nature are capable of producing capsule and slime layer and EPS is a main component of these layers. This helps the bacteria withstand different abiotic stresses including osmotic and temperature stress (Roberson *et al.*, 1993, Kambourova *et al.*, 2009).

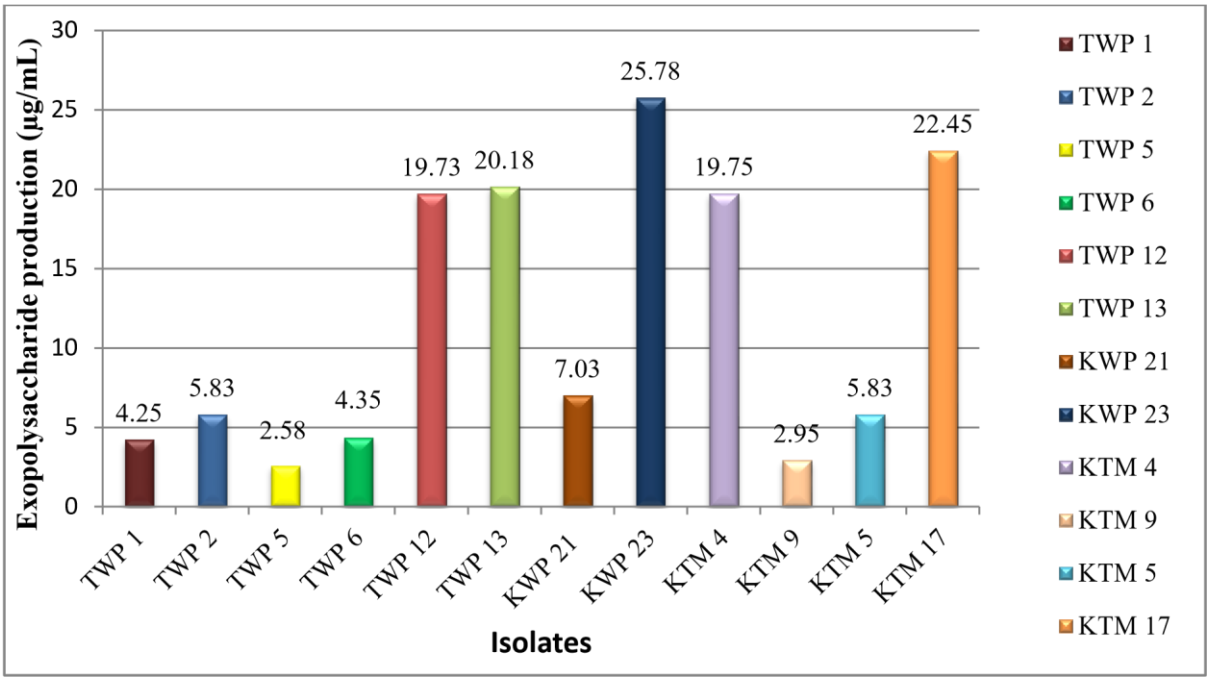


Figure 1. Quantitative assessement of exopolysaccharide production of the selected bacterial isolates.

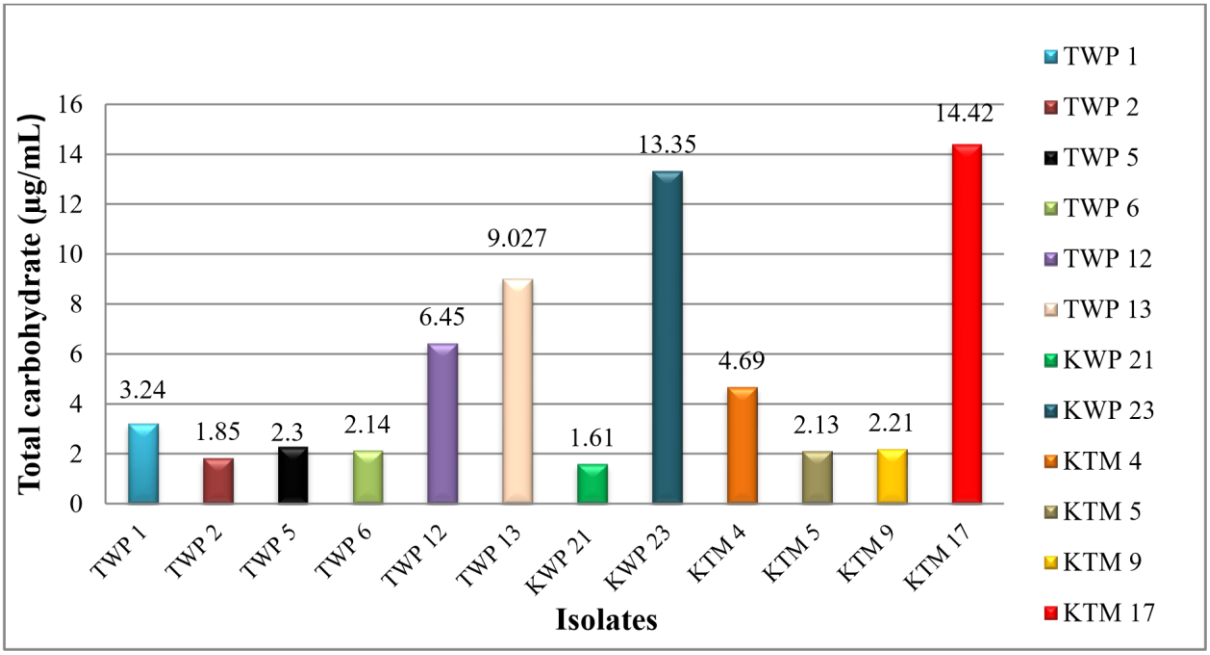


Figure 2. Estimation of total carbohydrate content in exopolysaccharide of selected EPS producing bacterial isolates.

Termitarium and mud dauber wasp nest are dry soil structures. The formation of endospores will help the bacteria tide over unfavourable conditions, especially when the soil of the nesting structure dries. The spore-forming bacteria produces resistant forms termed "spores" to tolerate harsh conditions such as acidity, temperature, and desiccation (Remezie *et al.*,2017). Out of the ten isolates, six were endospore formers of which one was Gram negative in our study. A gram negative endospore former, *Acetonema longum,* was reported to be present in the gut of wood-feeding termite, *Pterotermes occidentis* (Kane and Breznak, 1991).

On analyzing the sugar utilizing pattern of the test isolates, TWP1 was able to utilize sucrose, glucose, mannitol and trehalose. A comparative study by Abe *et al* (1991) revealed that the bacteria isolated from the nesting structures are also found to utilize sugars like glucose and trehalose.

Phylogenetic analysis of our study showed that KWP23 and KTM17 isolates are most closely related to *Klebsiella pneumoniae* (99.87% sequence similarity) and *Paenibacillus polymyxa* (99.86% sequence similarity), respectively. *Klebsiella pneumoniae* and *Paenibacillus polymyxa* strains are reported to produce EPS in large scale and is exploited industrially. *Paenibacillus polymyxa* strain 92 isolated from wheat rhizosphere is reported to produce 38 gL^{-1} of levan in a media containing 10% sucrose (Grinev *et al.,* 2020). Liyaskina *et al.* (2021) also reported that *Paenibacillus polymyxa* strain 2020 showed an EPS production of 24.38 gL^{-1} after 48 h of incubation. Similarly Castillo & Uribelarrea (2004) reported *Klebsiella pneumoniae* sp. *pneumoniae* serotype K63 to produce excess EPS in presence of a carbon source like glucose. The two isolates showing maximum EPS and total carbohydrate content i.e., *Klebsiella pneumoniae* KWP23 and *Paenibacillus polymyxa* KTM17 were used for further study.

Carbon sources such as sucrose, glucose, lactose, maltose, whey, starch, mannitol, sorbitol, or sugar concentrates, as well as nitrogen sources such as ammonium sulphate, peptone, sodium nitrate, urea, and yeast extract, are used to produce microbial polysaccharides, and they have effects on the yield or even the molecular size of the EPS

(Tomulescu *et al*., 2021). The carbon substrate utilization rate is directly proportional to EPS production, and bacterial cells get electron donors from substrates that produce better biomass, which eventually leads to higher EPS production (Laspidou and Rittmann, 2002). Bacteria grown in sucrose produced 5 times more exopolysaccharide dry weight than glucose at the same concentration (2%) (Harahap *et al*., 2018). In the present study screening for the effect on different carbon sources EPS production at revealed that the highest EPS production was recorded at the level 50:1 (C:N) of sucrose by *Paenibacillus polymyxa* KTM17 (32.88 µg mL^{-1}) (Figure 3).

Many *P. polymyxa* strains have been found to contain levansucrase, which has a high sucrose hydrolyzing activity and may be responsible for the high EPS production when sucrose is used as a carbon source (Liang and Wang, 2015). Similar findings were reported by other researchers. In the study conducted by Vermani *et al*. (1997) sucrose at a concentration of 50 g L^{-1} provided maximum polysaccharide recovery, and organism development was good and similar to that obtained with other sucrose concentrations. In the present study there is an increase in EPS production as sucrose increases in case of *Paenibacillus polymyxa* KTM17. Sucrose is frequently used as the best carbon source for EPS manufacturing. It was recently shown that using sucrose in the medium results in high yields of levan-type EPSs (Liang and Wang, 2015). Sathiyanarayanan *et al.* (2014) conducted a study on the effect of carbon source on EPS production by *Bacillus megaterium* MSBN04 and reported that maximum EPS production was observed with sucrose (30 g L^{-1}) followed by glucose as carbon source. The optimum NaCl concentration for EPS production by this bacterium was 3-4 %.

The overall yield of EPS produced by lactic acid bacteria is determined by the medium composition and the conditions under which the organisms develop (i.e., temperature and incubation period). Gram negative bacteria, such as *Klebsiella* sp., *Acinetobacter calcoaceticus*, and *Aeromonas salmonicida*, have shown similar results (Cerning *et al*., 1994). Yuksekdag and Aslim (2008) discovered that *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* produced the most EPS

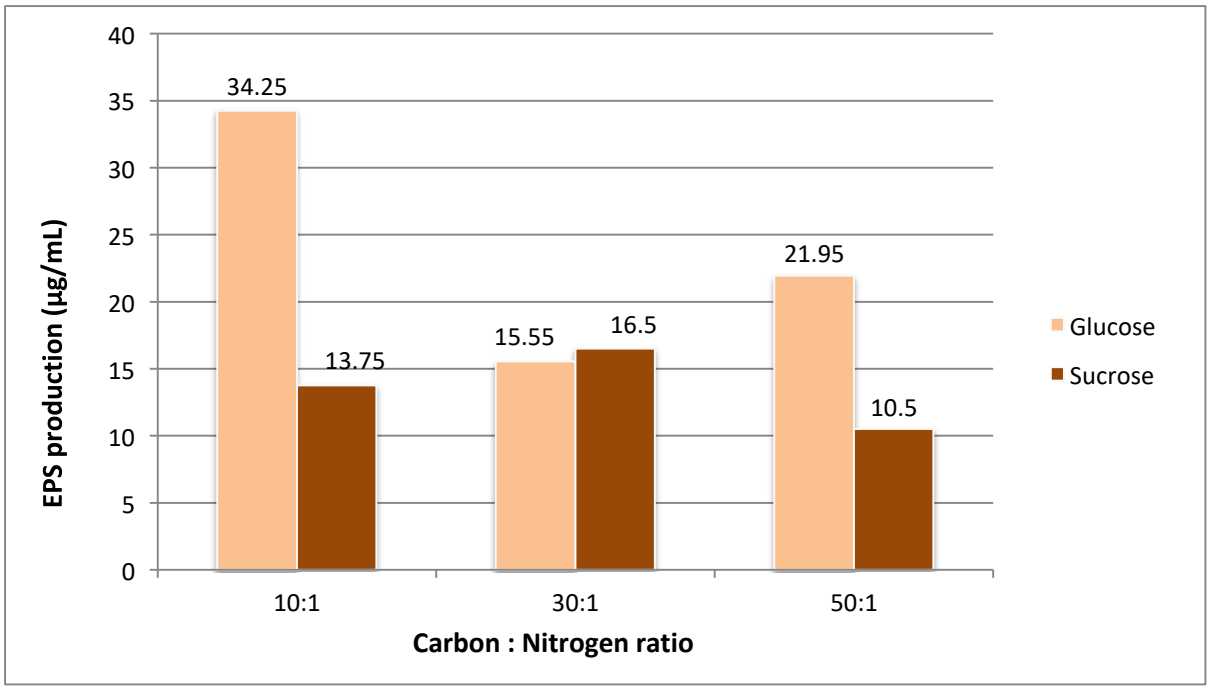


Figure 3.Exopolysaccharide production of *Paenibacillus polymyxa* KTM17 with different carbon source and C:N ratio.

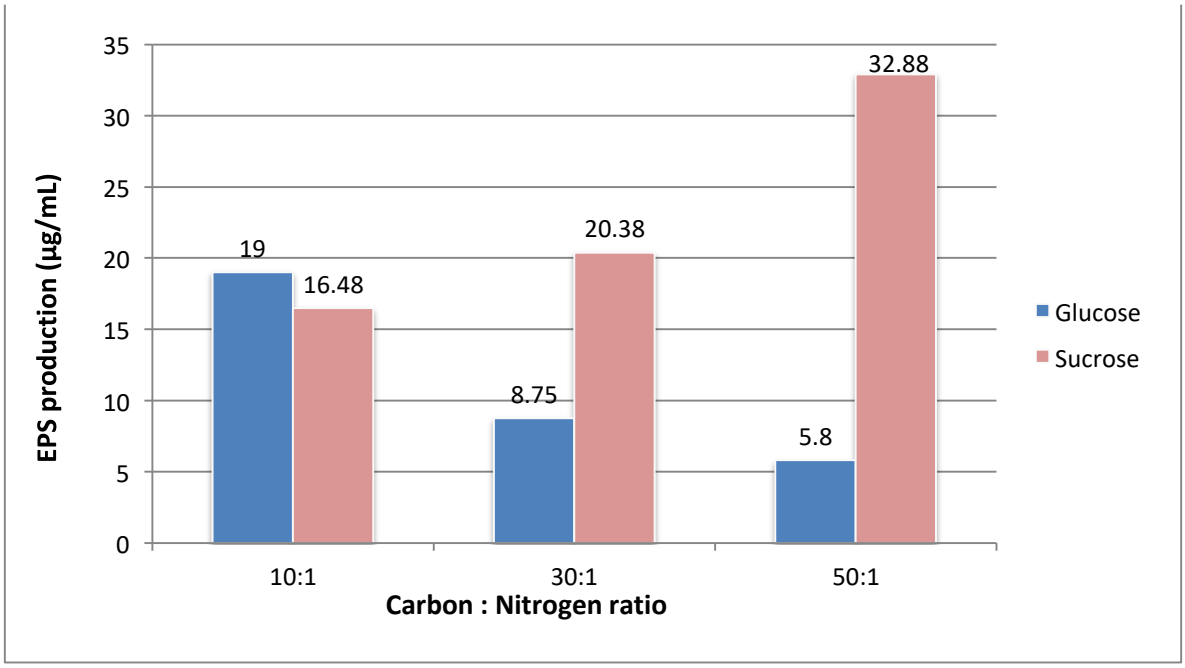


Figure 4. Exopolysaccharide production of *Klebsiella pneumoniae* KWP23 with different carbon source and C:N ratio.

in a glucose-containing medium. Our results showed that the yield of *Klebsiella pneumoniae* KWP 23 depends on the carbon source present in the medium. Glucose was the most efficient carbon source for EPS production. Similar result was obtained by Premnath *et al.* (2020) in *Klebsiella pneumoniae* isolated from marine environment. In the current study *Klebsiella pneumoniae* KWP 23, the highest yield of EPS (34.25 µg mL^{-1}) (Figure 4) was obtained when glucose at 10:1 level was used as carbon source. In the study conducted by Vermani *et al.* (1997) suggested that in case of glucose and lactose at a low concentration (5 g L^{-1}), the growth and EPS production were maximum. Higher levels of glucose and lactose inhibited the growth and EPS production. According to Rubinstein *et al.* (2012), the higher C ratio created osmotic pressure, and this may affect the specific signals that regulate the synthesis of the matrix. Lui *et al.* (2009) reported maximum EPS production in *Paenibacillus polymyxa* supplemented with sucrose rather than glucose. They also reported that as the concentration of sucrose increased the EPS production also increased. Similar results were observed in the present study and it can be suggested that the best carbon source for *Paenibacillus polymyxa* KTM17 and *Klebsiella pneumoniae* KWP 23 was sucrose and glucose respectively. This demonstrates the ability of bacterial isolates not only to grow using a range of carbon sources, but also to use those carbon and nitrogen sources to efficiently build up EPS. EPS production is observed to have a positive correlation with biomass production by bacterial isolates (Rao *et al.,* 2013).

Studies suggested that under stress conditions production of EPS will be higher. Ali *et al*. (2009) conducted research with thermotolerant *Pseudomonas* sp. strain AKM-P6 with the production of EPS being substantially higher at high temperatures. Under both normal and stressful conditions, the isolate AKM-P6 produced the most EPS. In the present study *Klebsiella pneumoniae* KWP23 recorded highest EPS production at 50^{o}C (23.00 µgmL^{-1}). Different strains of *Klebsiella pneumoniae* were found to grow in a temperature range of 17^{o}C to 46^{o}C (Tsuji *et al.*, 1982) above which the growth and survival of the bacteria is affected. The maximum EPS yield was observed at 30 °C for *Alcaligenes faecalis* B14 in the study conducted by Kaur *et al*. (2013). Optimum temperature for EPS production was suggested as 30°C (Harappa *et al*., 2019). In our

data regarding EPS production under different temperature, for *Paenibacillus polymyxa* KTM17 (31.50 µgmL^{-1}) highest EPS production was recorded at 30ºC (Figure 5). Similar results were reported in *Paenibacillus polymyxa* (Liu et al., 2009; Liang and Wang, 2015). Extracellular polymeric compounds can protect thermophilic bacteria by sheltering them from extremely high temperatures (Costa *et al*., 2018).

Plant growth-promoting rhizobacteria that produce EPS can also bind cations such as Na^+. As a result, an increase in the population density of EPS-producing bacteria in the root zone is expected to reduce the amount of Na^+ available for plant uptake, alleviating salt stress in plants growing in saline environments. However, little is known about the role of EPSproducing rhizobacteria in stimulating plant growth under soil salinity. As per our study for *Klebsiella pneumoniae* KWP23, maximum EPS production was noted in the medium without NaCl (18.00 µg mL^{-1}) (Figure 6). As the concentration increases there was a decline in the EPS production. In the study conducted by Vermani *et al*. (1999) it was reported that there was a marginal increase in the exopolysaccharide production at a concentration of 0.1 gL^{-1} NaCl but a decline was observed at higher concentration.

Contradictory to the above study it is suggested that EPS production will be higher at high concentration of NaCl. Ashraf *et al*. (1999) reported that the addition of salt in the medium increased polysaccharide formation in certain strains of *Bacillus* (MAS17), whereas it had an inhibitory effect (MA401) in the production of EPS. In our study maximum EPS production (26.25 µg mL^{-1}) was recorded at 6% NaCl for *Paenibacillus polymyxa* KTM17. Atoue *et al*. (2019) reported that, *Marinobacter lipolyticus* SM19 TP5 isolate was not able to grow in a salt-free medium. However, increasing the salt content to 10% NaCl resulted in the greatest growth. The isolate's capability to thrive in high concentrations of different salts can be related to the excretion of EPS, which sustains bacterial cell viability under salt stress and protects them in the rhizosphere. The isolate's capacity to develop at higher concentrations of a wide range of salts reveals its halophilic physiology and ability to survive under salinity

The isolate's capacity to develop at higher concentrations of

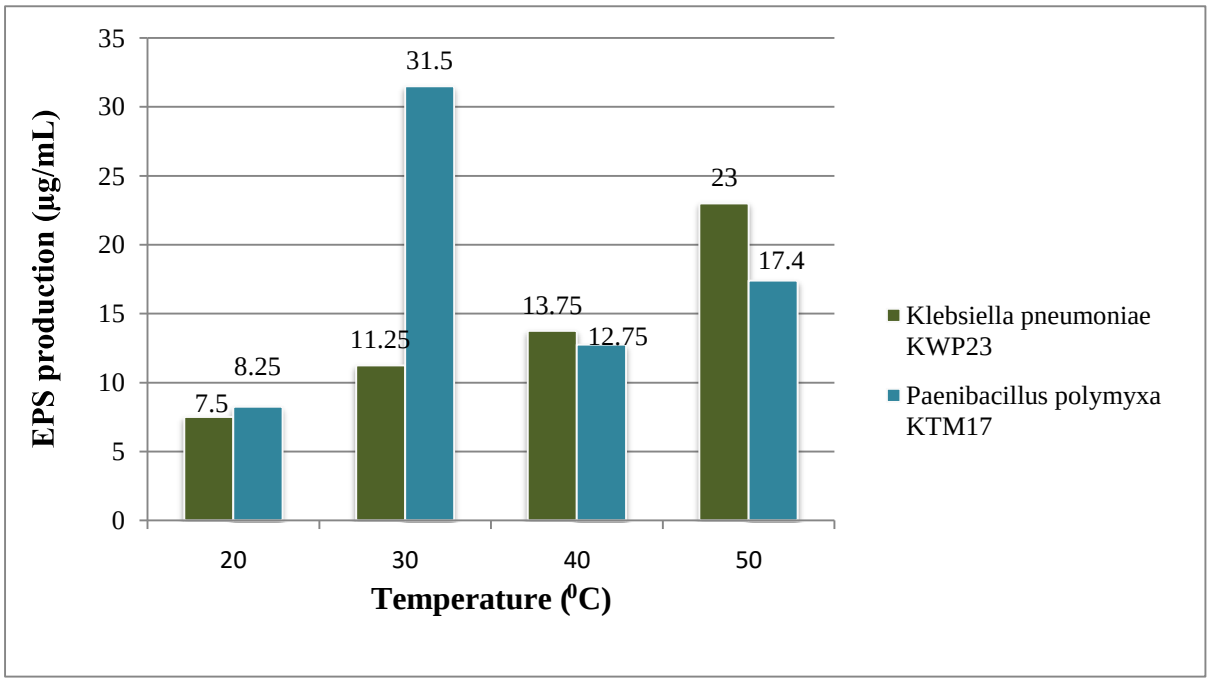


Figure 5. Exopolysaccharide production by *Klebsiella pneumoniae* KWP23 and *Paenibacillus polymyxa* KTM17 at different levels of temperature.

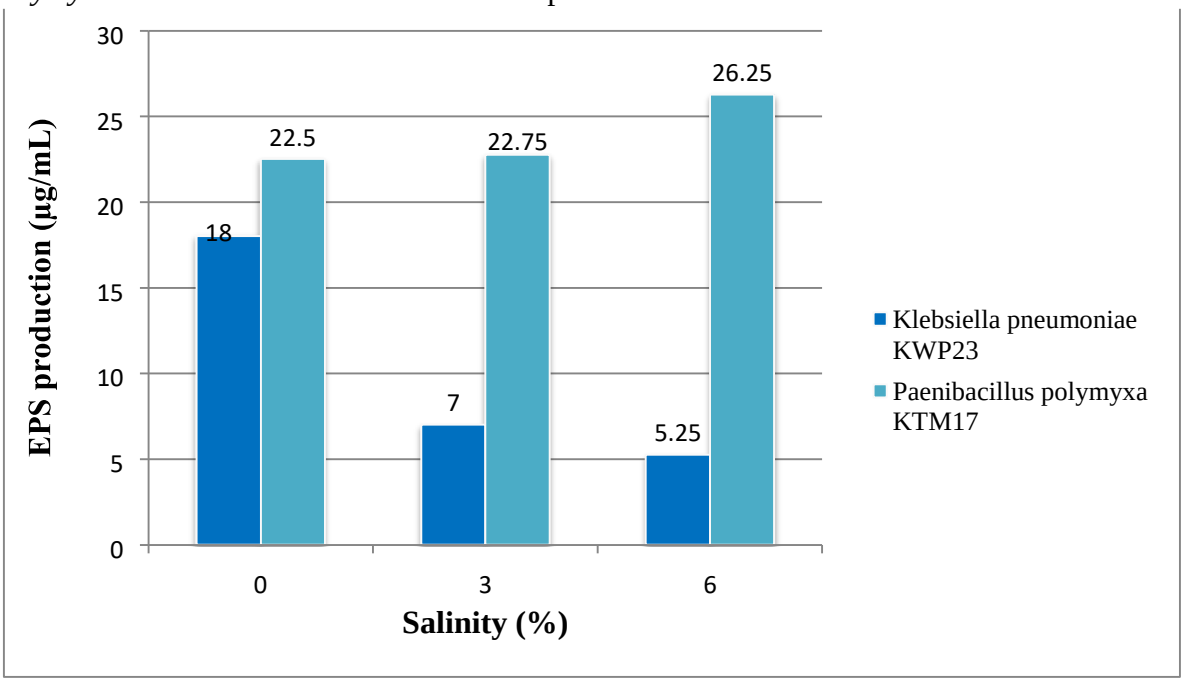


Figure 6.Exopolysaccharide production by *Klebsiella pneumoniae* KWP23 and *Paenibacillus polymyxa* KTM17 at different salinity levels.

stress (Kusale *et al*., 2021). From research work of Rahman *et al*. (2018) he stated that the novel strain *Bacillus aryabhattai* thrived in a spectrum of saline conditions. When cultivated at varying salinity (NaCl) levels, the isolate thrived well within a salinity range of 1% to 5%.

Plant growth promotion activity has been discovered in many bacteria including *Azospirillum, Klebsiella, Burkholderia Bacillus, Paenibacillus,* and *Pseudomonas*. (Kuan *et al.,* 2016). *Paenibacillus polymyxa* is an efficient rhizobacterium that can fix nitrogen (Puri *et al*., 2016), mobilize phosphorus from inaccessible compounds (Wang *et al*., 2012), and synthesize a wide range of physiologically active substances, including phytohormones, antibiotics, enzymes, volatile organic components, and exopolysaccharides (EPSs) (Liang and Wang, 2015). *K. pneumoniae* strains produce EPSs (i.e. fucogel) that are primarily constituted of 3-6 sugar units (galactose, glucose, mannose, galacturonic and glucuronic acids, fucose, rhamnose, glucosamine) and have a variety of characteristics. They produce both acidic capsular polysaccharides and exopolysaccharides for stress mitigation, with increased EPS production when the carbon/nitrogen ratio is higher (under nitrogen limitation and carbon excess conditions) (Tomulescu *et al*., 2021).

Some *Klebsiella* species are known as plant growth promoting rhizobacteria and model systems, and they have been examined for their role in nitrogen fixation as well as phosphate solubilization. The *Klebsiella* genus, along with *Pseudomonas, Serratia, Erwinia, Enterobacter, Rhizobium, Agrobacterium, Phyllobacterium, Variovorax,* and others, is regarded as one of the most proficient in plant-growth promotion, with biostimulation potential due to phosphate solubilization and auxin synthesis (Tomulescu *et al*., 2021). *Klebsiella* strains (SM6 and SM11) isolated from rhizospheric soil are found to solubilize mineral phosphate via oxalic acid release from glucose. Bacteria of the genus *Pseudomonas, Rhizobium, Klebsiella, Enterobacter, Acinetobacter, Bacillus, Erwinia, Bukholderia, Micrococcus, Serratia,* and others have been shown to solubilize P through the secretion of organic acids, resulting in acidification and the release of free P

bound in minerals (Rajput *et al*., 2012). In our study *Klebsiella pneumoniae* KWP23 found solubilized phosphate, produced zone of solubilization indicating $Ca_3(PO_4)_2$ solubilization. *Paenibacillus polymyxa* KTM17 formed a small halo around the colony. Zang and Kong (2014) reported potassium solubilizing *Klebsiella* sp. of strains GL6, GL9, and JM3 showed potassium solubilization activity. In the present study similar findings were obtained, *Klebsiella pneumoniae* KWP23 had shown zone of potassium solubilization activity.

Various antimicrobial substances produced by *Paenibacillus* spp., such as polymyxins by *Paenibacillus polymyxa*, pelgipeptins by *Paenibacillus elgii*, and paenibacillin P and N by *Paenibacillus alvei*, have been reported to affect a wide range of microorganisms, including fungi, soil bacteria, and plant pathogenic bacteria (Godoy *et al*., 2016; Anandaraj *et al*. 2009; Ding *et al*. 2011). The volatiles generated by *Paenibacillus* sp KWN38 may limit the growth of the pathogenic fungus. This is consistent with the observations in which diverse volatile chemicals produced by antagonistic fungi and bacteria shown antifungal activity (Naing *et al*., 2013). From our study it was revealed that *Paenibacillus polymyxa* KTM17 was having antifungal activity against the fungal pathogens *Helminthosporium* sp., and *Ralstonia solanii*.

Soil aggregate stability is an important soil feature that influences soil sustainability and crop yield. Soil aggregation is the process by which aggregates of varying sizes are linked and held together by various organic and inorganic components. As a result, it includes the formation and stabilization processes. It is difficult to assess and evaluate aggregate stability (Amézketa, 1999). Microorganisms produce enzymes that are responsible for mineralizing large molecular weight compounds, as well as extracellular polysaccharides that bind soil particles and stabilize soil aggregates (Chenu, 1993). Amézketa *et al*. (1995) investigated the effect of polysaccharides generated in situ on aggregate stability using artificial aggregates inoculated with bacteria. Water stable aggregates (WSA) of bacteriaincubated aggregates was greater than WSA of aggregates-control.

Klebsiella pneumoniae KWP23 and *Paenibacillus polymyxa* KTM17 were taken for the soil aggregation stability studies. In our study, the pH of the treated soil changed from 6.21 to the range of 7.24 and 7.20 for *Klebsiella pneumoniae* KWP23 and *Paenibacillus polymyxa* KTM17 respectively. Since the experiment was carried out in 1 kg of soil there was significant change in pH of the soil due to the growth of the bacterial isolates. Peiris *et al*. (2004) discovered that an optimal pH of 7 was best for EPS generation by a *Klebsiella oxytoca* strain. Liang *et al.* (2013) reported that culturing the strain *Paenibacillus polymyxa* for more days, makes the medium slightly alkaline.

EPS with a high WHC can protect microorganisms, soil, and plants from drought stress by enhancing hydrating conditions and bridging between soil particles and clay (Costa *et al*., 2018). Kaci *et al*. (2005) reported that *Paenibacillus polymyxa* KTM17 absorbs 100 times more water than their own weight. Our study demonstrated that the EPS amended soil had good water holding capacity. Among the two, *Paenibacillus polymyxa* KTM17 was the best in holding water with an EPS production of 38.15%. Similar finding was reported by Roberson and Firestone (1992) that water holding capacity will be more in EPS amended soil than unamended soil.

Aggregate stability has been considered as an indicator of soil structure and is a key measure of soil quality (Kabir *et al*., 2017). To characterize aggregate stability, several approaches and indices have been developed. Mean weight diameter (MWD) is one among them to characterize the structural integrity of the entire soil by integrating the aggregate size distribution into a single figure (Six *et al*., 2000). Greater structural stability of macro aggregates is indicated by higher MWD values (Piccolo *et al*. 1997). In our study when the data were analyzed, the mean weight diameter of dry aggregates for the soil amended with *Paenibacillus polymyxa* KTM17 was 1.068 mm which was on par with *K. pneumoniae* KWP23 (1.065 mm). The aggregate stability was studied based on time by water immersion and oscillations methods. Both these methods showed that more durable aggregates were present in the soil which is amended with *Paenibacillus polymyxa* KTM17. As the MWD increases the durability of the aggregates also increases.

The polysaccharides produced by the bacteria, as well as the bacteria themselves, were responsible for the higher aggregate stability.

Alami *et al*. (2000) reported on the effect of EPS-producing plant growth-promoting rhizobacteria on the aggregation of root-adhering soils. Plant growth-promoting rhizobacteria that produce EPS can greatly increase the volume of soil macropores and soil aggregation in the rhizosphere, resulting in improved water and nutrient availability to inoculated plants. Several studies pointed out microorganisms can help in the soil aggregation. The use of microorganisms in the rhizosphere of the plant roots can improve soil structure by means of soil aggregation by exopolysaccharide- producing bacteria. (Mu'minah *et al*., 2015). Bacteria and other microorganisms contribute to the formation of soil aggregates. Various organic compounds such as polysaccharides attracts soil particles and binds each other for the formation of soil aggregates (Nimmo, 2013).

The amendment with bacterial isolates *Klebsiella pneumoniae* KWP23 and *Paenibacillus polymyxa* KTM17 improved soil aggregate formation, its stability and water holding capacity of the soil. These organisms have the potential to be utilized as soil amendments for improving soil structures. A good soil structure, which is based on aggregation, is essential for sustaining agricultural production and environmental quality, as well as the sustainable use of soil and agriculture (Amézketa, 1999). Bacteria produce EPS in their rhizospheres to protect the environment from drying and variations in water potential, as well as to increase nutrient intake and promote plant growth. It protects seedlings against drought stress and promotes root exudates. Aggregation and soil structure enhancement promotes seedling growth by promoting efficient nutrient and water uptake (Sandhya *et al*.,2009). *Paenibacillus polymyxa* CF43 was inoculated to the wheat roots, increases the mass of root-adhering soil (Bezzate *et al*., 2000)

Summary

6. SUMMARY

Bacteria which inhabiting the soil, like plant growth-promoting rhizobacteria (PGPR) release extracellular polymeric substances into the environment, which include high molecular weight exopolysaccharides (EPS). The EPS constitute a physiological barrier around the cell and also provides for desiccation prevention, protection from external stresses, adhesion to substances, and symbiosis with other cells. EPS benefits the cells by providing nutrition in starvation, retaining moisture during desiccation, promoting cell adhesion to each other and to the surface, enhancing better signalling between cells, and protecting against extreme temperatures, pH, salinity, and other environmental factors.

Bacteria play an important role in soil aggregation because they can synthesize exopolysaccharides (EPS) and lipopolysaccharides (LPS), which act as "glue" for soil particles. These compounds maintain soil aggregates by "glueing" soil particles together. Bacteria utilize these substances to adhere to mineral surfaces, promoting the production of composite building blocks and microaggregates. The present study entitled ―Exopolysaccharide producing bacteria from soil based nesting structures‖ was conducted in the Department of Agricultural Microbiology, College of Agriculture, Vellayani, Thiruvananthapuram.

The main objective of the current study was to isolate and characterize exopolysaccharide producing bacteria from mud wasp and termitaria and to study the ability of the best two isolates in soil aggregate stability study. Further the selected isolates were subjected to quantitative and qualitative assessment of exopolysaccharide and then identified by molecular characterization. They were tested for their multiple traits such as biocontrol activity, and mineral solubilizing potential such as P, K and Si. The salient findings of the current study are summarized as below.

Thirty three bacterial isolates were obtained from different termitaria and mud dauber wasp nest. Among them colonies of fifteen isolates had slimy mucoidal appearance and were identified as exopolysaccharide producers. Of the fifteen EPS producing bacterial isolates, twelve which showed better EPS production were selected for further quantitative estimation of EPS production. After quantitative and qualitative assessment ten isolates were selected for further study. All ten isolates were characterized by morphology and biochemical tests.

The quantification of exopolysaccharide of twelve isolates by gravimetric method ranged between 25.78 µg mL^{-1} to 2.58 µg mL^{-1}. The isolate KWP23 recorded maximum exopolysaccharide production. The total carbohydrate content in exopolysaccharide was reported to be maximum in bacterial isolate KTM17 (14.48 µgmL^{-1}) and it was ranged between 14.48 µgmL^{-1} and 1.61 µgmL^{-1}.

Of the ten isolates five isolates were selected for molecular characterization based on EPS production. And were identified as *Priestia aryabhattai, Priestia aryabhattai, Klebsiella pneumoniae, Priestia megaterium, Paenibacillus polymyxa* by 16s rRNA sequencing. *Paenibacillus polymyxa* KTM17 and *Klebsiella pneumoniae* KWP23 were selected for the further studies on the basis of exopolysaccharide production.

Five isolates viz. *Priestia aryabhattai* TWP12, *Priestia aryabhattai* TWP13, *Klebsiella pneumoniae* KWP23, *Priestia megaterium* KTM4, *Paenibacillus polymyxa* KTM17 were screened for phospahte solubilization in Pikovaskya medium supplemented with tricalcium phosphate. A solubilization zone (4 mm)was shown by *Klebsiella pneumoniae* KWP23. The isolate *Klebsiella pneumoniae* KWP23 developed a distinctive zone of solubilization (1 mm) around their colony in Aleksandrov medium in the screening of potassium solubilization. There was no indication of zone of silicate solubilization in Bunt and Rovira medium. None of the isolates chosen were silicate solubilizers.

Effect of carbon source and carbon: nitrogen ratio on exopolysaccharide production was assessed. Glucose was found to be the best carbon source for *Klebsiella pneumoniae* at 10:1 C:N ratio, based on EPS production (34.25 µg mL^{-1}) and for *Paenibacillus polymyxa* KTM17, highest EPS production was recorded when sucrose was used as carbon source at 50:1 (32.875 µg mL^{-1}).

The effect of temperature was evaluated by providing required different temperature individually. Increasing the temperature level (from 20^{o}C to 50^{o}C) resulted in an increase in EPS production that was directly proportionate to the rise in temperature in case of *Klebsiella pneumonia*e KWP23. The highest EPS production by *Klebsiella pneumonia*e KWP23 was (23.00 µg mL^{-1}) at 50^{o}C.

The effect of salinity on EPS production by *Klebsiella pneumonia*e KWP23 and *Paenibacillus polymyxa* KTM17 isolates was assessed. For the isolate *Klebsiella pneumonia*

KWP23, the EPS production was maximum without NaCl (18.0 µg mL^{-1}) and for the isolate *Paenibacillus polymyxa* KTM17, the EPS production was maximum at 6% (1 M) (26.25 µg mL^{-1}) NaCl.

*Klebsiella pneumonia*e KWP23 and *Paenibacillus polymyxa* KTM17 were further subjected to dual culture plate assay for testing antagonistic activity against fungal pathogens. A clear inhibition zone was observed in case of *Paenibacillus polymyxa* KTM17 with *Rhizoctonia solani. Klebsiella pneumoniae* KWP23 and *Paenibacillus polymyxa* KTM17 inhibited the growth of *Helminthosporium* sp. However there was no inhibition of growth of *Sclerotium rolfsii* by *Paenibacillus polymyxa* KTM17 and *Klebsiella pneumoniae* KWP23.

Analysis of soil amended with *Klebsiella pneumonia*e KWP23 and *Paenibacillus polymyxa* KTM17 for soil aggregate stability study was carried out. Maximum porosity (56.63 %) was obtained in *Klebsiella pneumoniae* KWP23 amended soil whereas the water holding capacity was maximum in soil treated with *Paenibacillus polymyxa* KTM17 (38.150 %). The maximum bulk density (1.31 g/mL) was observed in soil incorporated with *Paenibacillus polymyxa* KTM17.

Weight of the aggregates was recorded at different size fraction of 2 mm and 5 mm. Maximum aggregates were obtained from 2mm sieve for the both isolates. Soil treated with *P. polymyxa* KTM17 yielded more aggregates in 2 mm sieve (13.357 g) and also in 5 mm sieve (1.417 g). Single aggregate weight of *Paenibacillus polymyxa* KTM17 was 0.224 g and *Klebsiella pneumoniae* was 0.190 g respectively. Random ten aggregate weight recorded maximum for *Paenibacillus polymyxa* KTM17 (0.424 g). The mean weight diameter of dry aggregates obtained were on par for both the soil amended with the bacterial isolates.

A comparison was made on the influence of time on water immersed soil aggregates to study aggregate stability. The study revealed that the best soil aggregation stability was given by the isolate *Paenibacillus polymyxa* KTM17. The stability of the aggregate lasted for more than 24 hours when immersed in water whereas that in the aggregate kept as control disintegrated immediately on pouring water. A study was made on effect of moisture condition on the soil aggregates based on oscillations, the turbidity of water was highest in control (0.254 nm). The

weight of the disintegrated soil was minimum in the soil aggregate amended with *Paenibacillus polymyxa* KTM17 (0.014 g).

Among the selected isolates, *Klebsiella pneumonia*e KWP23 had maximum production of EPS. They could grow at high temperature due to the production of exopolysaccharide. At higher temperature EPS production was higher. *Klebsiella pneumonia*e KWP23 was effective against *Rhizoctonia solani* and *Helminthosporium* sp. under *in vitro* study. *Paenibacillus polymyxa* KTM17 had more water holding capacity and they revealed the best soil aggregation stability. Maximum mean weight diameter was shown by *Paenibacillus polymyxa* KTM17. As the mean weight diameter increases the soil aggregation stability also increases.

Further studies on the selected exopolysaccharide producing bacterial isolates may be done to assess the growth promotion study under stress condition and soil aggregation stability study under field level.

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Appendices

APPENDIX – I

COMPOSITION OF MEDIA USED

1. *EPS selective media* (Tallgren *et al*., 1998) amended with sucrose.

Sucrose – 40 g

Mg SO_4 – 0.2 g

KH_2PO_4 – 3 g

$K_2H_2PO_4$ – 9 g

Yeast extract – 2 g

Agar – 15 g

Distilled water- 1000 ml

Sucrose, Mg SO_4, KH_2PO_4 , $K_2H_2PO_4$, Yeast extract were dissolved in 500 ml distilled water and volume made up to 1000 ml. 20 g agar-agar was added into this mixture and autoclaved at 15 lbs pressure and 1210C for 15 min.

2. ***Potato Dextrose Agar***

Peeled and sliced potatoes - 200g

Dextrose ($C_6H_{12}O_6$) - 20g

Agar-agar - 20g

Distilled water - 1000 ml

Potatoes were boiled in 500 ml of distilled water and the extract was collected by filtering through a muslin cloth. Agar-agar was dissolved separately in 500 ml of distilled water. The potato extract was mixed in the molten agar and 20 g of dextrose was dissolved in to the mixture. The volume was made up to 1000 ml with distilled water and medium was sterilized at 15 lbs pressure and 1210C for 15 min.

3. Pikovskaya's Media

Glucose - 10.0 g

Ca3(PO4)2 - 5.0 g

(NH4)2SO4 - 0.5 g

Yeast extract - 0.5 g

KCl - 0.2 g

MgSO4 - trace

FeSO4 - trace

Agar-agar - 20.0 g

Distilled water - 1000 mL pH - 7.0

Glucose, Ca3(PO4)2, (NH4)2SO4, Yeast extract, KCl, MgSO4, and FeSO4 were dissolved in 500 mL distilled water and volume made up to 1000 mL. Twenty grams agar-agar was added into this mixture and autoclaved at 15 lbs pressure and 121 ºC for 15 minutes.

4. Aleksandrov Media

Aleksandrov agar – 29.6 g

Distilled water - 1000 mL

Agar-agar - 5.0 g

pH -7.2 ± 0.2

Ready-made (Hi-media) Aleksandrov agar was dissolved in 500 mL distilled water and volume made up to 1000 mL and pH was adjusted. Five grams agar-agar was added into this mixture and autoclaved at 15 lbs pressure and 121 ºC for 15 min.

APPENDIX - II COMPOSITION OF STAINS USED *1. Crystal violet*

One volume saturated alcohol solution of crystal violet in four volumes of one per cent aqueous ammonium oxalate.

2. Gram's iodine

Iodine crystals - 1.0g

Potassium iodide - 2.0g

Distilled water - 300ml

3. Safranin

Ten ml saturated solution of safranin in 100 ml distilled water.

4. Malachite Green

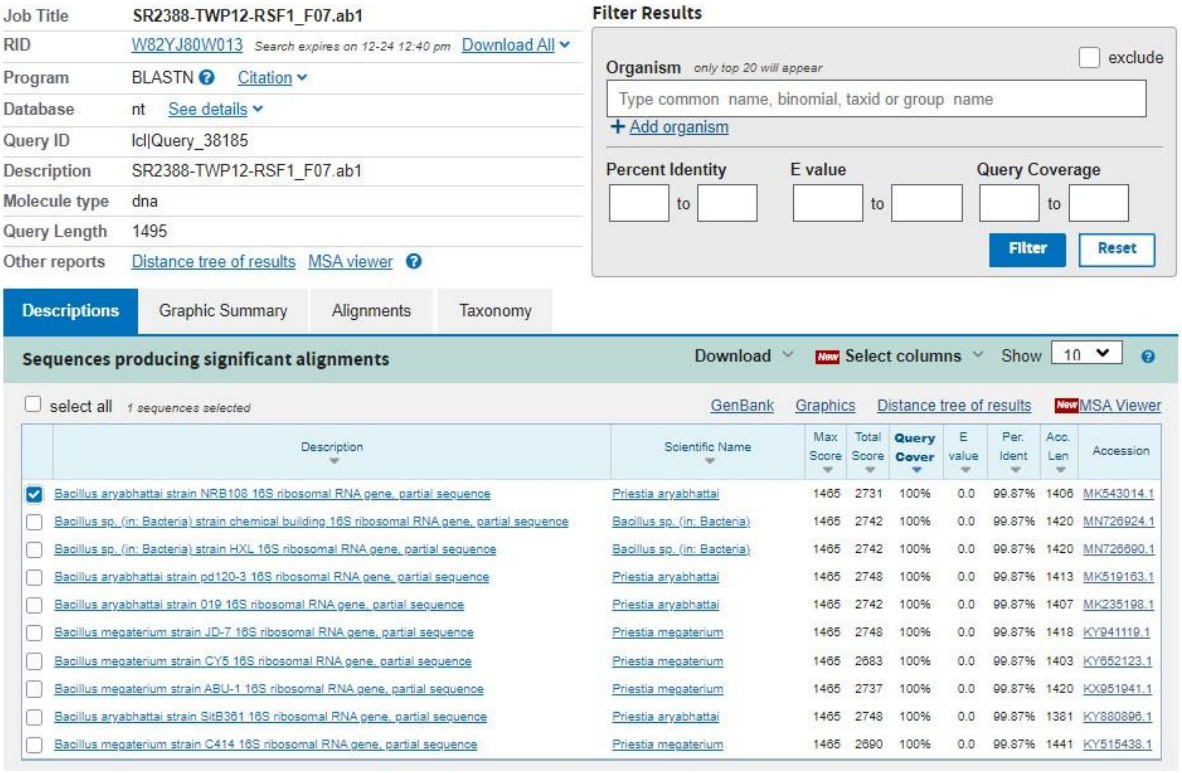
Malachite green - 1.0 gm

Distilled water - 100 ml

APPENDIX – III

SEQUENCE PRODUCING SIGNIFICANT ALIGNMENTS

a) *Priestia aryabhattai* TWP12



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Filter Results

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Type common name, binomial, taxid or group name

+ Add organism

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Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments — Download — Select columns — Show 10

select all 1 sequences selected — GenBank Graphics Distance tree of results MSA Viewer

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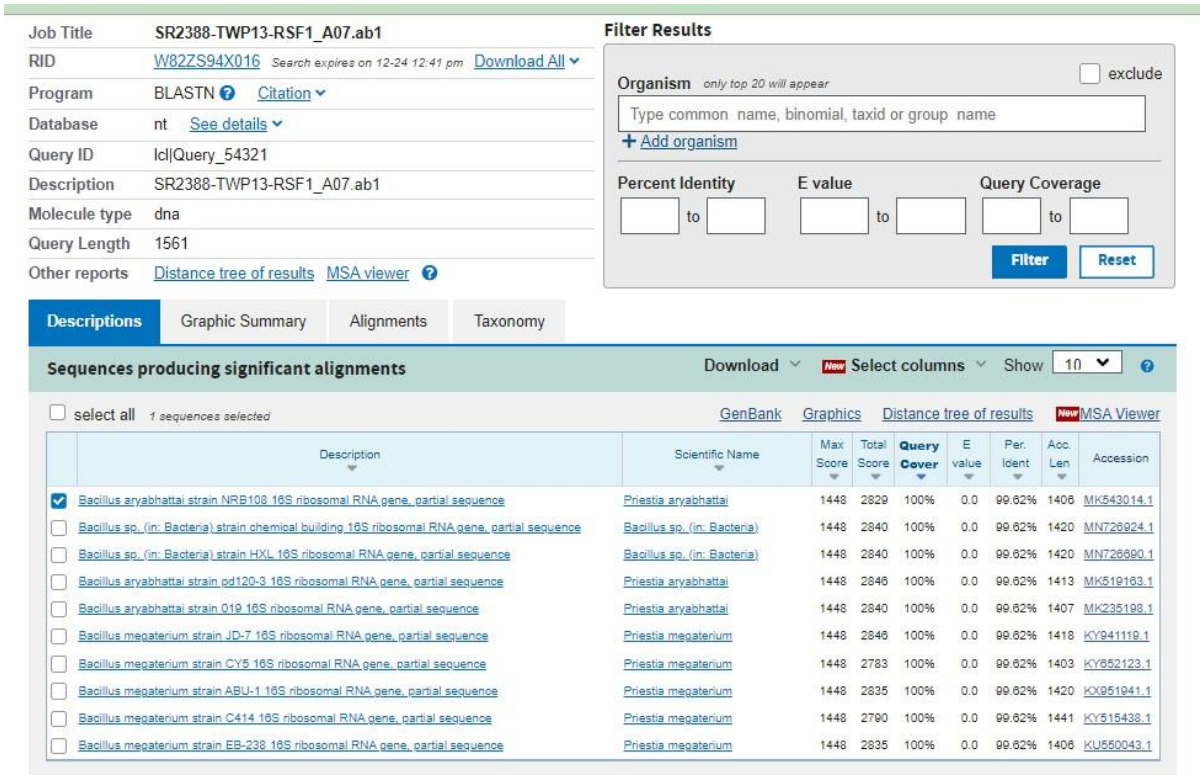
Bacillus aryabhattai strain NRB108 16S ribosomal RNA gene, partial sequence	Priestia aryabhattai	1465	2731	100%	0.0	99.87%	1406	MK543014.1
Bacillus sp. (in: Bacteria) strain chemical building 16S ribosomal RNA gene, partial sequence	Bacillus sp. (in: Bacteria)	1465	2742	100%	0.0	99.87%	1420	MN726924.1
Bacillus sp. (in: Bacteria) strain HXL 16S ribosomal RNA gene, partial sequence	Bacillus sp. (in: Bacteria)	1465	2742	100%	0.0	99.87%	1420	MN726690.1
Bacillus aryabhattai strain pd120-3 16S ribosomal RNA gene, partial sequence	Priestia aryabhattai	1465	2748	100%	0.0	99.87%	1413	MK519163.1
Bacillus aryabhattai strain 019 16S ribosomal RNA gene, partial sequence	Priestia aryabhattai	1465	2742	100%	0.0	99.87%	1407	MK235198.1
Bacillus megaterium strain JD-7 16S ribosomal RNA gene, partial sequence	Priestia megaterium	1465	2748	100%	0.0	99.87%	1418	KY941119.1
Bacillus megaterium strain CY5 16S ribosomal RNA gene, partial sequence	Priestia megaterium	1465	2683	100%	0.0	99.87%	1403	KY852123.1
Bacillus megaterium strain ABU-1 16S ribosomal RNA gene, partial sequence	Priestia megaterium	1465	2737	100%	0.0	99.87%	1420	KX951941.1
Bacillus aryabhattai strain SitB361 16S ribosomal RNA gene, partial sequence	Priestia aryabhattai	1465	2748	100%	0.0	99.87%	1381	KY880896.1
Bacillus megaterium strain C414 16S ribosomal RNA gene, partial sequence	Priestia megaterium	1465	2690	100%	0.0	99.87%	1441	KY515438.1

a) *Priestia aryabhattai* TWP13



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Filter Results

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Type common name, binomial, taxid or group name

+ Add organism

Percent Identity to E value to Query Coverage to

Filter Reset

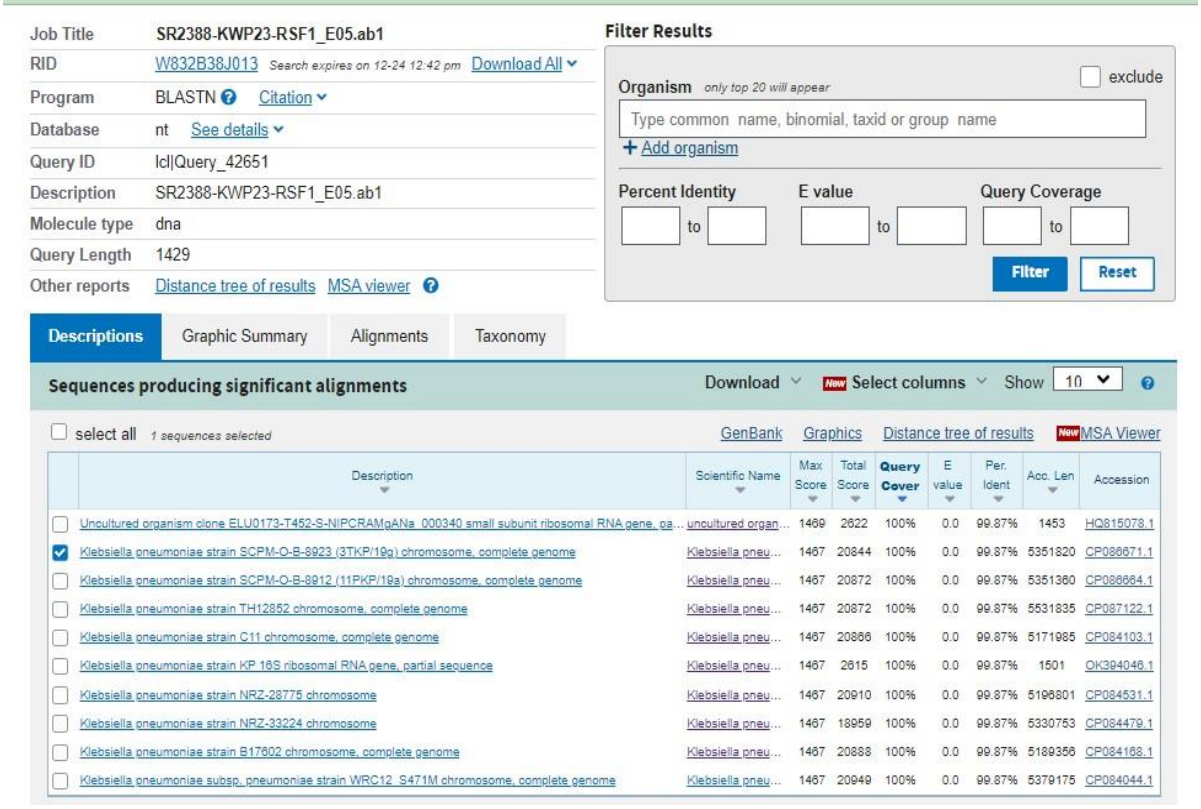
Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments Download New Select columns Show 10

select all *1 sequences selected* GenBank Graphics Distance tree of results New MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bacillus aryabhattai strain NRB108 16S ribosomal RNA gene, partial sequence	Priestia aryabhattai	1448	2829	100%	0.0	99.62%	1406	MK543014.1
Bacillus sp. (in: Bacteria) strain chemical building 16S ribosomal RNA gene, partial sequence	Bacillus sp. (in: Bacteria)	1448	2840	100%	0.0	99.62%	1420	MN726924.1
Bacillus sp. (in: Bacteria) strain HXL 16S ribosomal RNA gene, partial sequence	Bacillus sp. (in: Bacteria)	1448	2840	100%	0.0	99.62%	1420	MN726690.1
Bacillus aryabhattai strain pd120-3 16S ribosomal RNA gene, partial sequence	Priestia aryabhattai	1448	2846	100%	0.0	99.62%	1413	MK519163.1
Bacillus aryabhattai strain 019 16S ribosomal RNA gene, partial sequence	Priestia aryabhattai	1448	2840	100%	0.0	99.62%	1407	MK235198.1
Bacillus megaterium strain JD-7 16S ribosomal RNA gene, partial sequence	Priestia megaterium	1448	2846	100%	0.0	99.62%	1418	KY941119.1
Bacillus megaterium strain CY5 16S ribosomal RNA gene, partial sequence	Priestia megaterium	1448	2783	100%	0.0	99.62%	1403	KY652123.1
Bacillus megaterium strain ABU-1 16S ribosomal RNA gene, partial sequence	Priestia megaterium	1448	2835	100%	0.0	99.62%	1420	KX951941.1
Bacillus megaterium strain C414 16S ribosomal RNA gene, partial sequence	Priestia megaterium	1448	2790	100%	0.0	99.62%	1441	KY515438.1
Bacillus megaterium strain EB-238 16S ribosomal RNA gene, partial sequence	Priestia megaterium	1448	2835	100%	0.0	99.62%	1406	KU550043.1

a) *Klebsiella pneumoniae* KWP23



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Filter Results

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+ Add organism

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Sequences producing significant alignments Download New Select columns Show 10

select all *1 sequences selected* GenBank Graphics Distance tree of results New MSA Viewer

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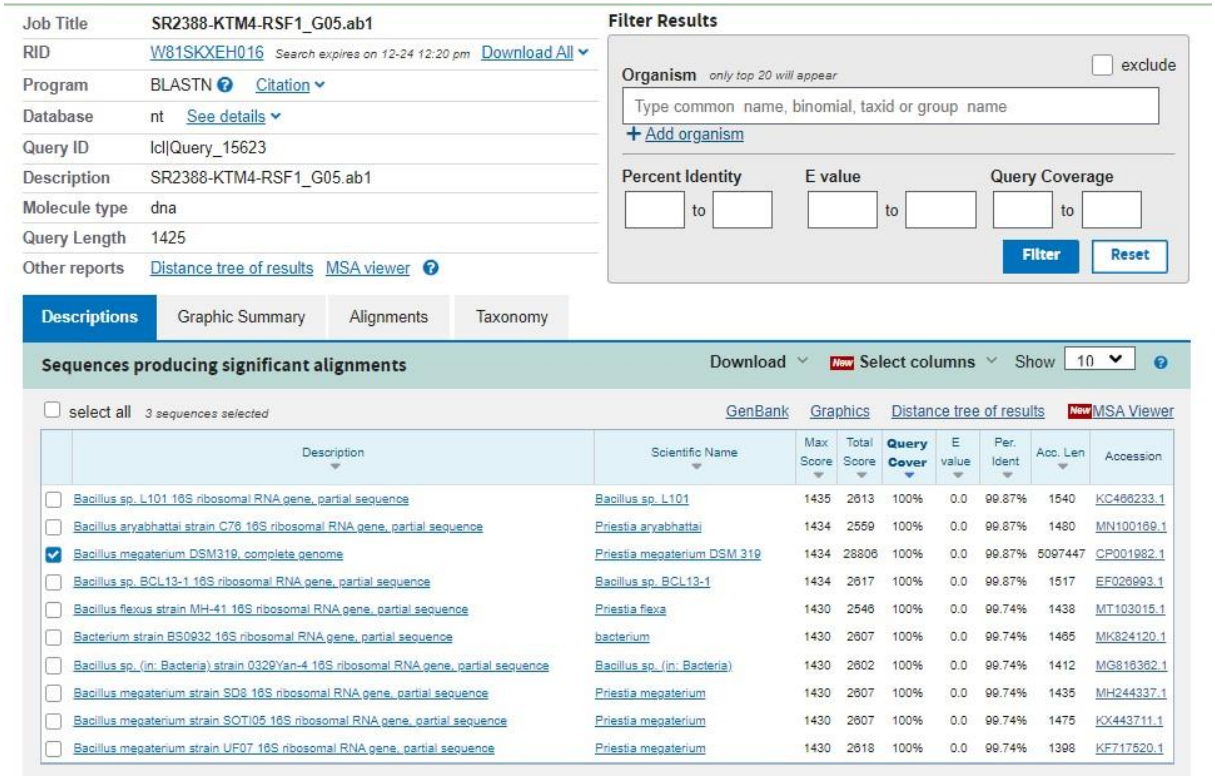
Klebsiella pneumoniae strain SCPM-O-B-8923 (3TKP/19g) chromosome, complete genome	Klebsiella pneu...	1467	20844	100%	0.0	99.87%	5351820	CP086671.1
Klebsiella pneumoniae strain SCPM-O-B-8912 (11PKP/19a) chromosome, complete genome	Klebsiella pneu...	1467	20872	100%	0.0	99.87%	5351360	CP086664.1
Klebsiella pneumoniae strain TH12852 chromosome, complete genome	Klebsiella pneu...	1467	20872	100%	0.0	99.87%	5531835	CP087122.1
Klebsiella pneumoniae strain C11 chromosome, complete genome	Klebsiella pneu...	1467	20866	100%	0.0	99.87%	5171985	CP084103.1
Klebsiella pneumoniae strain KP 16S ribosomal RNA gene, partial sequence	Klebsiella pneu...	1467	2615	100%	0.0	99.87%	1501	OK394046.1
Klebsiella pneumoniae strain NRZ-28775 chromosome	Klebsiella pneu...	1467	20910	100%	0.0	99.87%	5196801	CP084531.1
Klebsiella pneumoniae strain NRZ-33224 chromosome	Klebsiella pneu...	1467	18959	100%	0.0	99.87%	5330753	CP084479.1
Klebsiella pneumoniae strain B17602 chromosome, complete genome	Klebsiella pneu...	1467	20888	100%	0.0	99.87%	5189356	CP084168.1
Klebsiella pneumoniae subsp. pneumoniae strain WRC12_S471M chromosome, complete genome	Klebsiella pneu...	1467	20949	100%	0.0	99.87%	5379175	CP084044.1

d)Priestia megaterium KTM4



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Filter Results

Organism *only top 20 will appear* exclude

Type common name, binomial, taxid or group name

+ Add organism

Percent Identity to

E value to

Query Coverage to

Filter Reset

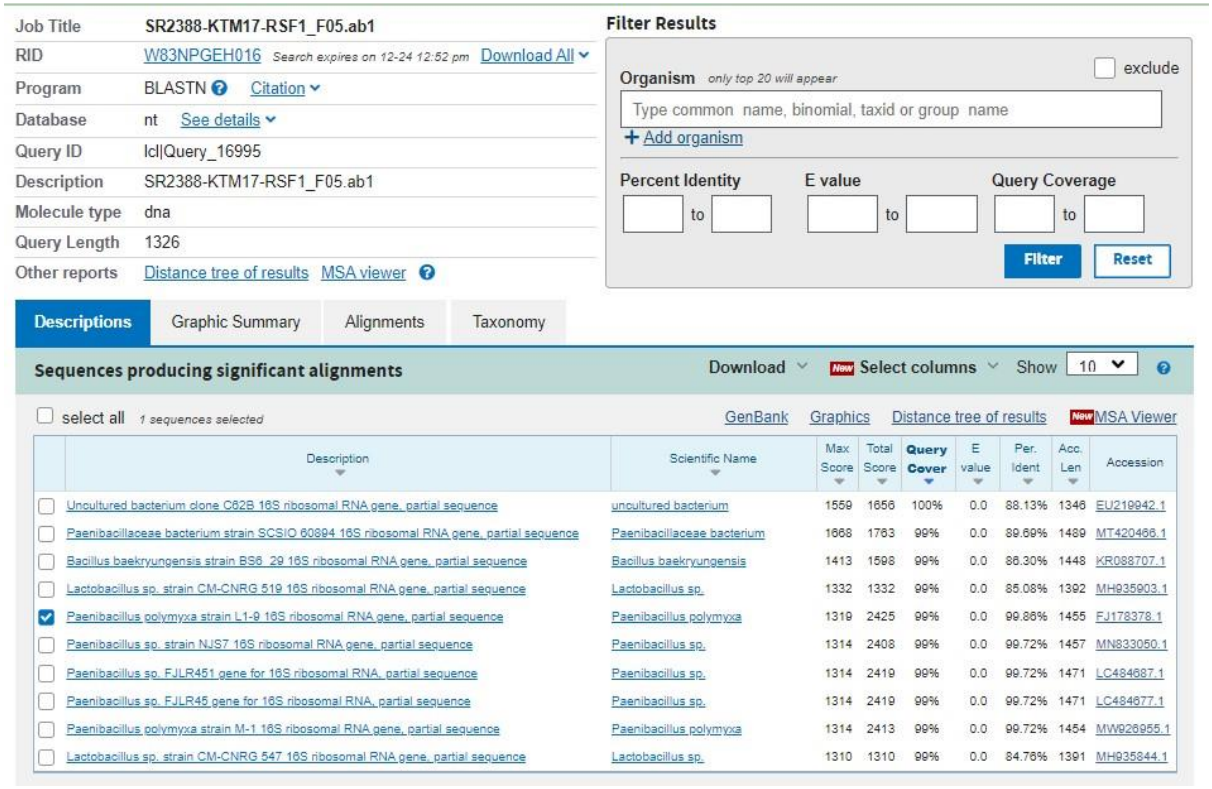
Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments Download New Select columns Show 10

select all *3 sequences selected* GenBank Graphics Distance tree of results New MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bacillus sp. L101 16S ribosomal RNA gene, partial sequence	Bacillus sp. L101	1435	2613	100%	0.0	99.87%	1540	KC466233.1
Bacillus aryabhattai strain C76 16S ribosomal RNA gene, partial sequence	Priestia aryabhattai	1434	2559	100%	0.0	99.87%	1480	MN100169.1
Bacillus megaterium DSM319, complete genome	Priestia megaterium DSM 319	1434	28806	100%	0.0	99.87%	5097447	CP001982.1
Bacillus sp. BCL13-1 16S ribosomal RNA gene, partial sequence	Bacillus sp. BCL13-1	1434	2617	100%	0.0	99.87%	1517	EF026993.1
Bacillus flexus strain MH-41 16S ribosomal RNA gene, partial sequence	Priestia flexa	1430	2546	100%	0.0	99.74%	1438	MT103015.1
Bacterium strain BS0932 16S ribosomal RNA gene, partial sequence	bacterium	1430	2607	100%	0.0	99.74%	1465	MK824120.1
Bacillus sp. (in: Bacteria) strain 0329Yan-4 16S ribosomal RNA gene, partial sequence	Bacillus sp. (in: Bacteria)	1430	2602	100%	0.0	99.74%	1412	MG816362.1
Bacillus megaterium strain SD8 16S ribosomal RNA gene, partial sequence	Priestia megaterium	1430	2607	100%	0.0	99.74%	1435	MH244337.1
Bacillus megaterium strain SOTI05 16S ribosomal RNA gene, partial sequence	Priestia megaterium	1430	2607	100%	0.0	99.74%	1475	KX443711.1
Bacillus megaterium strain UF07 16S ribosomal RNA gene, partial sequence	Priestia megaterium	1430	2618	100%	0.0	99.74%	1398	KF717520.1

a) *Paenibacillus polymyxa* KTM17



Job Title	SR2388-KTM17-RSF1_F05.ab1
RID	W83NPGEH016 Search expires on 12-24 12:52 pm Download All
Program	BLASTN Citation
Database	nt See details
Query ID	lcl|Query_16995
Description	SR2388-KTM17-RSF1_F05.ab1
Molecule type	dna
Query Length	1326
Other reports	Distance tree of results MSA viewer

Filter Results

Organism only top 20 will appear — exclude

Type common name, binomial, taxid or group name

+ Add organism

Percent Identity: to — E value: to — Query Coverage: to

Filter Reset

Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments — Download — New Select columns — Show 10

select all 1 sequences selected — GenBank Graphics Distance tree of results New MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Uncultured bacterium clone C62B 16S ribosomal RNA gene, partial sequence	uncultured bacterium	1559	1656	100%	0.0	88.13%	1346	EU219942.1
Paenibacillaceae bacterium strain SCSIO 60894 16S ribosomal RNA gene, partial sequence	Paenibacillaceae bacterium	1668	1763	99%	0.0	89.69%	1489	MT420466.1
Bacillus baekryungensis strain BS6_29 16S ribosomal RNA gene, partial sequence	Bacillus baekryungensis	1413	1598	99%	0.0	86.30%	1448	KR088707.1
Lactobacillus sp. strain CM-CNRG 519 16S ribosomal RNA gene, partial sequence	Lactobacillus sp.	1332	1332	99%	0.0	85.08%	1392	MH935903.1
Paenibacillus polymyxa strain L1-9 16S ribosomal RNA gene, partial sequence	Paenibacillus polymyxa	1319	2425	99%	0.0	99.86%	1455	FJ178378.1
Paenibacillus sp. strain NJS7 16S ribosomal RNA gene, partial sequence	Paenibacillus sp.	1314	2408	99%	0.0	99.72%	1457	MN833050.1
Paenibacillus sp. FJLR451 gene for 16S ribosomal RNA, partial sequence	Paenibacillus sp.	1314	2419	99%	0.0	99.72%	1471	LC484687.1
Paenibacillus sp. FJLR45 gene for 16S ribosomal RNA, partial sequence	Paenibacillus sp.	1314	2419	99%	0.0	99.72%	1471	LC484677.1
Paenibacillus polymyxa strain M-1 16S ribosomal RNA gene, partial sequence	Paenibacillus polymyxa	1314	2413	99%	0.0	99.72%	1454	MW926955.1
Lactobacillus sp. strain CM-CNRG 547 16S ribosomal RNA gene, partial sequence	Lactobacillus sp.	1310	1310	99%	0.0	84.76%	1391	MH935844.1

ABSTRACT

EXOPOLYSACCHARIDE PRODUCING BACTERIA FROM SOIL BASED NESTING STRUCTURES OF INSECTS

by

SRUTHI SURESH

(2019-11-236)

ABSTRACT

Submitted in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of agriculture

Kerala Agricultural University

DEPARTMENT OF AGRICULTURAL MICROBIOLOGY

COLLEGE OF AGRICULTURE,VELLAYANI

THIRUVANANTHAPURAM-695 522

KERALA, INDIA

2022

ABSTRACT

The study entitled ―Exoploysaccharide producing bacteria from soil based nesting structures of insects‖ was conducted during the year 2019-2021 in the Department of Agricultural Microbiology, College of Agriculture, Vellayani, Thiruvananthapuram with the objective to isolate and characterize exopolysaccharide producing bacteria from mud wasp and termitaria and the ability of the best two isolates in soil aggregate stability study.

Thirty three bacterial isolates were obtained from different termitaria and mud dauber wasp nest. Among them colonies of fifteen isolates had slimy mucoidal appearance and were identified as exopolysaccharide producers. The exopolysaccharide production of the isolates ranged from 25.78 µg mL^{-1}(*Klebsiella pneumoniae* KWP23) to 2.58 µg mL^{-1}. The total carbohydrate content in exopolysaccharide was maximum in bacterial isolate *Paenibacillus polymyxa* KTM17 (14.48 µg mL^{-1}).

The best five isolates were selected for molecular characterization by 16s rRNA sequencing and identified as *Priestia aryabhattai* TWP12, *Priestia aryabhattai* TWP13, *Klebsiella pneumoniae* KWP23, *Priestia megaterium* KTM4, *Paenibacillus polymyxa* KTM17. They were screened for phosphate, potassium and silicate solubilization. *Klebsiella pneumoniae* KWP23 showed solubilization zone for phosphate (4 mm) and potassium (1 mm). None of the isolates chosen were silicate solubilizers.

Two bacterial isolates with maximum EPS production *viz. Paenibacillus polymyxa* KTM17 and *Klebsiella pneumoniae* KWP23 were selected for the further studies. Effect of carbon source and carbon: nitrogen ratio on exopolysaccharide production was studied. Glucose was found to be the best carbon source for EPS production in *Klebsiella pneumoniae* KWP23 at C: N ratio of 10:1. In *Paenibacillus polymyxa* KTM17, highest EPS

production by *Klebsiella pneumoniae* KWP23 was (23.00 μg mL^{-1}) at 50°C and *Paenibacillus polymyxa* KTM17 at 30°C (31.50 μg mL^{-1}). The effect of salinity on EPS production by *Klebsiella pneumoniae* KWP23 and *Paenibacillus polymyxa* KTM17 isolates was also assessed. For *Klebsiella pneumoniae* KWP23, the EPS production was maximum without NaCl (18.0 μg mL^{-1}) and for *Paenibacillus polymyxa* KTM17, the EPS production was maximum at 6% (1 M) NaCl (26.25 μg mL^{-1}).

Klebsiella pneumoniae KWP23 and *Paenibacillus polymyxa* KTM17 were further subjected to dual culture plate assay to study the biocontrol potential of the isolates. *Paenibacillus polymyxa* KTM17 showed a clear inhibition zone on dual culture assay with *Rhizoctonia solani,* but *Klebsiella pneumoniae* KWP23 did not inhibit the growth of the fungus. Both *Klebsiella pneumoniae* KWP23 and *Paenibacillus polymyxa* KTM17 inhibited the growth of *Helminthosporium* sp.

The potential of the isolates to form soil aggregates and its aggregate stability was studied. The mean weight diameter of dry aggregates obtained was highest for the soil amended with *Paenibacillus polymyxa* KTM17 (1.068 mm). The study on wet aggregate stability on water immersed revealed that the best soil aggregate stability was given by the isolate *Paenibacillus polymyxa* KTM17. The stability of the aggregate lasted for more than 24 hours when immersed in water whereas that in the aggregate kept as control disintegrated immediately on pouring water. The effect of agitation and water immersion on the aggregate stability was also analyzed. On oscillation of the aggregates in water the aggregates in control soil showed maximum turbidity (0.254 nm). The dry weight of the disintegrated soil was minimum in the soil aggregate amended with *Paenibacillus polymyxa* KTM17 (0.014 g).

Based on the results of the present study, it can be concluded that among the selected isolates *Klebsiella pneumoniae* KWP23 had maximum production of EPS. Both *Klebsiella pneumoniae* KWP23 and *Paenibacillus polymyxa* KTM17 has biocontrol potential against plant pathogenic fungi. *Paenibacillus polymyxa* KTM17 is a potential isolate which can effect soil aggregate stability in dry and wet condition.

സംഗ്രഹം

'ഷഡ്പദങ്ങളുടെ മൺകൂടുകളിലെ എക്സോപ്ലോസാക്കറൈഡ് ഉൽപാദിപ്പിക്കുന്ന ബാക്ടീരിയകൾ' എന്ന പഠനം 2019-2021 വർഷ കാലയളവിൽ വെള്ളായണി കാർഷിക കോളേജിലെ അഗ്രികൾച്ചറൽ മൈക്രോബയോളജി വിഭാഗത്തിൽ നടത്തുകയുണ്ടായി. വേട്ടാളൻ കടന്നലുകളുടെ കൂട്ടിൽനിന്നും ചിതൽ പുറ്റുകളിൽ നിന്നും ക്സോപോളിസാക്കറൈഡ് ഉൽപ്പാദിപ്പിക്കുന്ന ബാക്ടീരിയകളെ വേർതിരിച്ചു അവയുടെ സവിശേഷതകൾ പഠനവിധേയമാക്കി മെച്ചപ്പെട്ട ഇനങ്ങൾ ഉപയോഗിച്ച് മണ്ണിന്റെ അഗ്രിഗേറ്റ് സ്റ്റബിലിറ്റി മെച്ചപ്പെടുത്താനുള്ള സാധ്യത പഠിക്കുക എന്ന ലക്ഷ്യത്തോടു കൂടെയാണ് ഈ ഗവേഷണം നടത്തിയത്.

വ്യത്യസ്ത ചിതൽ പുറ്റുകളിൽ നിന്നും കടന്നൽകൂടുകളിൽ നിന്നും മുപ്പത്തിമൂന്ന് ബാക്ടീരിയൽ ഐസൊലേറ്റുകൾ ലഭിച്ചു. അവയിൽ പതിനഞ്ച് എണ്ണം മ്യൂക്കോയ്ഡൽ കോളനികൾക്ക് ഉണ്ടാക്കിയതിനാൽ അവ എക്സോപോളിസാക്കറൈഡ് (ഇ.പി.എസ്.) ഉത്പാദകരാണെന്ന് തിരിച്ചറിഞ്ഞു. ഈ ബാക്ടീരിയകൾക്ക് 25.78 $\mu g\ mL^{-1}$ മുതൽ 2.58 $\mu g\ mL^{-1}$ വരെ ഇ.പി.എസ്. ഉൽപാദിപ്പിക്കാനുള്ള ശേഷിയുണ്ട്.

മികച്ച അഞ്ച് ഐസൊലേറ്റുകളെ മോളിക്യുലാർ പഠനത്തിനായി തിരഞ്ഞെടുത്തു. 16s rRNA ജീൻ ശ്രേണീകരണത്തിന്റെ അടിസ്ഥാനത്തിൽ അവയെ പ്രീസ്റ്റിയ ആര്യഭട്ടെ TWP12, പ്രീസ്റ്റിയ ആര്യഭട്ടെ TWP13, ക്ലെബ്സിയെല്ല ന്യുമോണിയ KWP23, പ്രീസ്റ്റിയ മെഗാറ്റീരിയം KTM4, പെയ്നിബാസിലസ് പോളിമിക്സ KTM17 എന്ന് തിരിച്ചറിഞ്ഞു. ഈ ഐസൊലേറ്റുകളുടെ ഫോസ്ഫേറ്റ്, പൊട്ടാസ്യം, സിലിക്കേറ്റ് ലയനശേഷി പരിശോധിച്ചു. ക്ലെബ്സിയെല്ല ന്യുമോണിയ KWP23 ഫോസ്ഫേറ്റ് ലയനശേഷിയും (4 മില്ലിമീറ്റർ), പൊട്ടാസ്യം ലയനശേഷിയും (1 മില്ലിമീറ്റർ) പ്രകടിപ്പിച്ചു. തിരഞ്ഞെടുത്ത

ഐസൊലേറ്റുകളൊന്നും സിലിക്കേറ്റ് ലയനശേഷിയുള്ളവ ആയിരുന്നില്ല.

കൂടുതൽ ഇ.പി.എസ്. ഉൽപ്പാദനശേഷിയുള്ള രണ്ട് ബാക്ടീരിയൽ ഐസൊലേറ്റുകളെ - പെയ്നിബാസിലസ് പോളിമിക്സ KTM17, ക്ലെബ്സിയെല്ല ന്യുമോണിയ KWP23 - തുടർപഠനത്തിനായി തിരഞ്ഞെടുത്തു. എക്സോപോളിസാക്കറൈഡ് ഉൽപ്പാദനത്തിൽ കാർബൺ സോർസിന്റെയും C: N അനുപാതത്തിന്റെയും സ്വധീനം പഠികുകയുണ്ടായി. 10:1 എന്ന C: N അനുപാതത്തിലെ ഗ്ലൂകോസിന്റെ ഉപയോഗം ക്ലെബ്സിയെല്ല ന്യുമോണിയ KWP23-ൽ എക്സോപോളിസാക്കറൈഡ് ഉൽപ്പാദനാം മെച്ചപ്പെടുത്തുന്നതായി കണ്ടെത്തി. പെയ്നിബാസിലസ് പോളിമിക്സ KTM17 -യിൽ 50:1 എന്ന C:N അനുപാതത്തിൽ സുക്രോസ് കാർബൺ സ്രോതസ്സായി ഉപയോഗിച്ചപ്പോൾ ഏറ്റവും ഉയർന്ന എക്സോപോളിസാക്കറൈഡ് ഉൽപ്പാദനം രേഖപ്പെടുത്തി. ബാക്ടീരിയയുടെ വളർച്ച താപനിലയുടെ ഇ.പി.എസ്. ഉൽപാദനത്തിലെ സ്വധീനതെകുറിച്ചു പഠികുകയുണ്ടായി. ഇൻകുബേഷൻ താപനിലയിലെ വർദ്ധനവ്, ക്ലെബ്സിയെല്ല ന്യുമോണിയ KWP23-ലെ എക്സോപോളിസാക്കറൈഡ് ഉൽപാദനത്തിൽ സമാനമായ വർദ്ധനവിന് കാരണമായി. ക്ലെബ്സിയെല്ല ന്യുമോണിയ KWP23-ഇൽ 50°C-ലും പെയ്നിബാസിലസ് പോളിമിക്സ KTM17 -ൽ 30°C-ലും കൂടുതൽ ഇ.പി.എസ്. ഉൽപാദനം കണ്ടു. ക്ലെബ്സിയെല്ല ന്യുമോണിയ KWP23-ലെയും, പെയ്നിബാസിലസ് പോളിമിക്സ KTM17-ലെയും ഇ.പി.എസ്. ഉൽപ്പാദനത്തിൽ ലവണാംശത്തിന്റെ പ്രഭാവം വിലയിരുത്തി. ക്ലെബ്സിയെല്ല ന്യുമോണിയ KWP23-യിൽ സോഡിയം ക്ലോറൈഡ് ഇല്ലാതെയും പെയ്നിബാസിലസ് പോളിമിക്സ KTM17-ൽ 6% സോഡിയം ക്ലോറിഡിലും പരമാവധി എക്സോപോളിസാക്കറൈഡ് ഉത്പാദനം കാണിച്ചു.

ക്ലെബ്സിയെല്ല ന്യുമോണിയ KWP23-യുടെയും, പെയ്നിബാസിലസ് പോളിമിക്സ KTM17-ന്റെയും രോഗനിയന്ത്രണശേഷി പഠിക്കാൻ ഡ്യൂവൽ കൾച്ചർ പ്ലേറ്റ് പരിശോധനയ്ക്ക് വിധേയമാക്കി. പെയ്നിബാസിലസ് പോളിമിക്സ KTM17 ഡ്യൂവൽ കൾച്ചർ പരിശോധനയിൽ റൈസോക്ടോണിയ സോളാനിക്കെതിരെ വ്യക്തമായ ഇൻഹിബിഷൻ സോൺ കാണിച്ചു. പക്ഷേ ക്ലെബ്സിയെല്ല ന്യുമോണിയ KWP23 ഈ കുമിളിന്റെ വളർച്ചയെ തടഞ്ഞില്ല. ക്ലെബ്സിയെല്ല ന്യുമോണിയ KWP23-യും, പെയ്നിബാസിലസ് പോളിമിക്സ KTM17-ഉം ഹെൽമിൻതോസ്പോറിയം കുമിളിന്റെ വളർച്ചയെ തടഞ്ഞു.

മണ്ണിന്റെ അഗ്രിഗേറ്റ് സ്റ്റബിലിറ്റി മെച്ചപ്പെടുത്താനുള്ള ഈ ഐസൊലേറ്റുകളുടെ കഴിവും പഠനവിധേയമാക്കി. പെയ്നിബാസിലസ് പോളിമിക്സ KTM17 ചേർത്ത മണ്ണിന്റെ മീൻ വെയ്റ്റ് ഡയമീറ്റർ ഉയർന്നതായും അഗ്രഗേറ്റു സ്ഥിരത കൂടുന്നതായും കാണപ്പെട്ടു. പെയ്നിബാസിലസ് പോളിമിക്സ KTM17-ന്റെ ഉപയോഗം വെള്ളത്തിൽ മൂക്കുംപോളുള്ള അഗ്രഗേറ്റിന്റെ സ്ഥിരതയും മെച്ചപ്പെടുത്തി. വെള്ളത്തിൽ മുക്കിയപ്പോൾ 24 മണിക്കൂറിലധികം മണ്ണ് പൊടിയാതെ നിന്നു.

ഈ പഠനത്തിന്റെ അടിസ്ഥാനത്തിൽ ക്ലെബ്സിയെല്ല ന്യുമോണിയ KWP23-ക്കു പരമാവധി എക്സോപോളിസാക്കറൈഡ് ഉത്പാദനശേഷി ഉള്ളതിയായി കണ്ടെത്തി. ക്ലെബ്സിയെല്ല ന്യുമോണിയ KWP23-ക്കും പെയ്നിബാസിലസ് പോളിമിക്സ KTM17-നും രോഗകാരികളായ കുമിളുകൾക്കെതിരെ ജൈവനിയന്ത്രണ ശേഷിയുണ്ടെന്നും കണ്ടെത്തി. പെയ്നിബാസിലസ് പോളിമിക്സ KTM17-നു വരണ്ടതും നനവുള്ളതുമായ മണ്ണിന്റെ അഗ്രഗേറ്റ സ്ഥിരത മെച്ചപ്പെടുത്താൻ കഴിവുണ്ടെന്ന് കണ്ടെത്തി.