ISOLATION AND MOLECULAR CHARACTERIZATION OF PIGMENT PRODUCING BACTERIA FROM DIFFERENT SOIL SAMPLES

A DISSERTATION SUBMITTED TO ST. TERESA'S COLLEGE (AUTONOMOUS), ERNAKULAM IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF

DEGREE OF MASTER OF SCIENCE IN ZOOLOGY

SUBMITTED BY,

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2020-2022

CERTIFICATE

This is to certify that the dissertation entitled "**Isolation and Molecular Characterization of Pigment producing Bacteria from different Soil samples"** is an authentic record of original project work carried out by **GRACY ANU K. F.** (Reg. No: SM20ZOO003), during the Academic year **2020-2022**, under my guidance in partial fulfillment of the requirement of the Degree of Master of Science in Zoology from St. Teresa's College (Autonomous), Ernakulam.

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DECLARATION

I hereby declare that the dissertation entitled "**Isolation and Molecular Characterization of Pigment producing Bacteria from different Soil samples"** submitted to St. Teresa's College (Autonomous), Ernakulam in partial fulfillment of the requirements, for the award ofthe Degree of Master of Science in Zoology is a record of original research work done by me under the supervision and guidance of Ms. Tiya K. J., Assistant Professor, Department of Zoology, St. Teresa's College (Autonomous), Ernakulam and to the best of my knowledge and belief, this project contains no material previously published or written by another person, except where due reference is made.

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ACKNOWLEDGMENT

I hereby express my deepest gratitude to God Almighty for blessing me in successfully completing this project. I am indebted to my parents and my sister with respect, without their blessing and support I could not have been able to complete this work.

I convey my heartfelt respect and gratitude to Ms. Tiya K. j., Assistant Professor, Department of Zoology, St. Teresa's College (Autonomous), Ernakulam, my supervising teacher whose consistent support and encouragement have helped me throughout the work for making its end meet success.

My sincere gratitude to Dr. Soja Louis, Head, Department of Zoology, St. Teresa's College (Autonomous), Ernakulam for her immense support during the course of work. I am extremely thankful to Ms. Cynthia Mathew Thaliath, Assistant Professor, and Ms. Indu Vasudevan, Assistant Professor, Department of Zoology, St. Teresa's College (Autonomous), Ernakulam for their constant guidance, support and valuable suggestions for the successful completion of my project work.

Special thanks to Dr. Helvin Vincent, Assistant Professor, Department of Zoology, St. Teresa's College (Autonomous) Ernakulam, and Dr. Jasmin C., Director, Enfys Lifesciences Pvt. Ltd. for their support and valuable suggestions during the study. Also, I am very much obliged to my friends and the non-teaching staff, Department of Zoology, St Teresa's College Ernakulam for their help and support during the course of my project work.

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LIST OF ABBREVIATIONS

1.	%	Percentage
2.	/	Per
3.	°C	Degree Celsius
4.	16S rDNA	16 S ribosomal deoxy ribonucleic acid
5.	1X	1 time higher
6.	50X	50 times higher
7.	6 X	6 times higher
8.	BLAST	Basic local alignment search tool
9.	Blastn	Blast Nucleotide
10.	BOD	Biological oxygendemand
11.	bp	Base pair
12.	c.m.	Centimetre
13.	D-Galactose	Dextro galactose
14.	dNTP	Deoxy ribonucleotide triphosphate
15.	DBPs	Disinfection Byproducts
16.	*E.coli*	*Escherichia coli*
17.	E.g.	Example
18.	ESI-MS	Electrospray ionization mass spectroscopy
19.	Et al.,	And other
20.	Etc.	Exectra
21.	FCF	For coloring Food
22.	FDA	Food and Drug Asministration
23.	Fig	Figure

24.	FTIR	Fourier transform infrared spectroscopy
25.	G	Gram
26.	HPLC	High performance liquid Chromatography
27.	Hr	Hour
28.	i.e.	That is
29.	K	Potassium
30.	K.M.	Kilometre
31.	Kb	Kilo base pair
32.	KM2	Square kilometre
33.	L-Galactose	Leavo galactose
34.	M	Molarity
35.	$MgCl_2$	Magnesium chloride
36.	Min	Minute
37.	Ml	Milli liter
38.	mM	Milli meter
39.	NCBI	National Centre for Biotechnology information
40.	N	Nitrogen
41.	NCBI	National Centre for Biotechnology information
42.	nM	Nano meter
43.	NMR	Nuclear Magnetic Resonance
44.	PCR	Polymerase chain reaction
45.	pH	Potential hydrogen
46.	RNA	Ribonucleic acid
46.	rpm	Revolution per minute
48.	SDS	Sodium docedyl sulphate

49.	Sec	Second
50.	TAE	Trips acetate EDTA
51.	Taq	Thermus aquaticus
52.	TE buffer	Tris EDTA buffer
53.	TLC	Thin layer chromatography
54.	ul	Micro litre
55.	µm	micrometre
56.	UV	Ultra Violet

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ABSTRACT

Colours and their attractive appeal have been an integral part of human lives. Microorganisms are considered to be one of the most promising niches for production and application of bioactive compounds like bio-pigments and of biotechnological interest. Bacteria offer certain distinctive advantages due to their short life cycle, their low sensitivity to seasonal and climatic changes, their easy scaling as well as their ability to produce pigments of various colours and shades. As natural primary producers, microbes with their extensive diversity, ease of all-season production and peculiar bioactivities are attractive entities for industrial production of these marketable natural colorants.

In the study, "Isolation and molecular characterization of Pigment producing bacteria from different soil samples", test samples were collected from different locations such as mangrove, garden soil, Canal soil, rotten vegetable compost pit, waste soil, poultry farm soil and agricultural land. These soils were separately undergone serial dilution and pour plate method. It was found that most of the desired bacterial colonies were found to be in the dilution concentrations such as 10^{-2}, 10^{-3} and 10^{-4}. Further, the bacteria having pigment producing capacity with dissimilar morphological characteristics were selected for molecular characterization. Out of the nine pigmented bacterial colonies observed, four distinct strains namely ZA1, ZA2, ZA3 and ZA4 exhibited different pigments such as orange, yellow, pink and red. These cultures were subjected to DNA isolation followed by molecular identification.

The 16S rDNA was sequenced and the obtained sequence were analyzed with bioinformatics tool, specifically, NCBI BLAST. The homology search using NCBI BLAST from the obtained sequence of ZA1 showed 99.89% similarity with *Rossellomorea aquimaris,* ZA2 showed 100% similarity with *Stenotrophomonas maltophilia,* ZA3 showed 99.88% similarity with *Bacillus paramycoides* and ZA4 showed 100% similarity with *Serratia marcescens* respectively.

Considering the toxic and carcinogenic properties of synthetic or artificial colourants, the use of bacterial pigments should be doubled in the present decade because it offers various advantages over those harmful chemical pigments. It is estimated that only 1% of the microbial world has been explored so far. On the contrary, synthetic pigments are produced by the use of toxic chemicals that are harmful for the humans and the natural environment around us. To overcome this situation, isolation of bacterial strains from novel environments and studies regarding them are necessary for production of pigments from bacteria. Due to its fast growth, easy propagation and culturing, easy growth on different media sources, simple culture techniques make this technique attractive. Bacterial pigments also have applications in food and textile industries as colorants, additives and dyes. Future studies, research and developments in this field is substantial. If done so, there is no doubt, bacterial pigments can be a potential commercial interest.

INTRODUCTION

Colour pervades all aspects of our lives, influencing our moods and emotions and generally enhancing the way in which we enjoy our environment. Natural colours are all around us, in the earth, the sky, the sea, animals and birds and in the vegetation, for example in the trees, leaves, grass and flowers. These colours can play important roles in the natural world, for example as sources of attraction and in defence mechanisms associated with camouflage. Plant pigments, especially chlorophyll, the dominant natural green pigment, play a vital role in photosynthesis in plants, and thus may be considered as vital to our existence. Synthetic colours often serve a purely decorative or aesthetic purpose in the clothes we wear, in paints, plastic articles, in a wide range of multicoloured printed materials. Colour is introduced into these materials and applications using substances known as dyes and pigments, or collectively as colorants. The essential difference between these two colorant types is that dyes are soluble coloured compounds which are applied mainly to textile materials from solution in water, whereas pigments are insoluble compounds incorporated by a dispersion process into products such as paints, printing inks and plastics (Christie *et al.*, 2014).

Dyes or pigments has been a part of human lives for centuries and has been an integral component to increase the desirability of any product. Apart from multiple cultural meanings, colour has enormous market potential in food, agriculture, cosmetic, textile, pharmaceutical, and nutraceutical sectors. The molecules capable of bestowing colour, owing to their ability to absorb light in the visible range are referred as pigments. Nevertheless, by chemical definitions, pigments are insoluble coloured substances while colorants are soluble coloured substances (Rana, *et al.*, 2021).

Pigments can be classified by their origin as natural, synthetic, or inorganic. Natural pigments are produced by living organisms such as plants, animals, fungi, and microorganisms. Synthetic pigments are obtained from laboratories. Natural and synthetic pigments are organic compounds. Inorganic pigments can be found in nature or reproduced by synthesis. Also, pigments can be classified by taking into account the chromophore chemical structure.

Chromophores with conjugated systems are carotenoids, anthocyanins, betalains, caramel, synthetic pigments, and lakes. Metal-coordinated porphyrins can be myoglobin, chlorophyll, and their derivatives. Considering the pigments as food additives, their classification by the FDA is of two types, (1) Certifiable pigments are manmade and subdivided as synthetic pigments and lakes. (2) Exempt from certification. This group includes pigments derived from natural sources such as vegetables, minerals, or animals, and manmade counterparts of natural derivatives (Delgado-Vargas *et al.,* 2000).

Natural products were the beginning and main source of treatments for hundreds of years, however, the application of bioactive natural metabolites in traditional medicine, and the discovery of new drugs continue to be active and constant. Microorganisms are an abundant source of novel bioactive compounds since, unlike higher organisms, they are a source of easily renewable resources that give rise to production with a potentially greater yield. Among the bioactive compounds of microbial origin, natural pigments have attracted the attention of industry due to an increasing interest in the generation of new safe, easily degradable, ecologically friendly products with no adverse effects (Celedón, *et al.*, 2021).

With the advent of synthetic dyes, such as lesser availability of dye producing materials due to difficulty in collection or lack of farming of the dye-plants, poor colour yield, complexity of dying process, non-reproducibility of shades, limited number of dyes, these problems encountered with natural dyes, and the development of synthetic dyes came in to existence. The development of synthetic dyes at the beginning of the twentieth century led to a more complete level of quality and more reproducible techniques of application. Synthetic dyes are readily available at low cost (Affat *et al.*, 2021).

Though synthetic Petro-derived colorants have dominated the industries due to low cost and high yield, the rising concerns about their non-biodegradability, carcinogenicity, and environmental toxicity has enthused both industry and researchers to find natural and safe alternatives. In this context, natural and eco-friendly bio pigments have sparked great interest due to their non-toxicity, biodegradability, non-carcinogenicity, and non-allergenicity which increases their consumer acceptance, prevents occupational health hazards while negating environmental concerns (Pailliè, *et al.*, 2020).

The textile industry is one of the important industries that generates a large amount of industrial effluents. Use of synthetic dyes has an adverse effect on all forms of life. Presence of sulphur, naphthol, vat dyes, nitrates, acetic acid, soaps, enzymes chromium compounds, and heavy metals like copper, arsenic, lead, cadmium, mercury, nickel, and cobalt and certain auxiliary chemicals all collectively make the textile effluent highly toxic. These organic materials react with many disinfectants, especially chlorine, and form by-products (DBPs) that are often carcinogenic and therefore undesirable. This effluent, if allowed to flow in the fields, clogs the pores of the soil resulting in loss of soil productivity. The effect of synthetic pigments and dyes can also be visible in human health. The most widely recognized peril of responsive colours is respiratory issues because of the inward breath of colour particles. Respiratory sharpening, tingling, watery eyes, sniffling and indications of asthma etc. can occur (Manzoor *et al.*, 2020).

More than 200,000 tons of dye effluents are generated annually, which cause persistent pollution and pose health hazards. To counter the perilous environmental challenges, health risks, allergenicity poised by synthetic dyes, the use of microbial pigments as safe colorants have generated a fervent interest in the textile sector. Further, the added advantage of incorporating antimicrobial properties to develop protective clothing will help to reduce hospital-acquired infections as well as increase consumer acceptance, especially in the post-pandemic era (Grewal, *et al.*, 2022).

Soil is the outer covering of the earth, which consists of loosely arranged layers of materials composed of inorganic and organic compounds in different stages of organization. It is a natural medium in which microbes live, multiply and die. Microbial diversity in the soil is a critical environmental topic that concerns people from all walks of life (Benizri, *et al.*, 2002).

Microbes, being an important component of the natural soil environment, not only play a very critical role in creating and maintaining this biosphere but also serve as a source of biotechnologically valuable and important products. They are able to recycle nutrients produce and consume gases that affect global climate, destroy pollutants, treat anthropogenic wastes and can also be used for biological control of plant and animal pests. Microorganisms from soil environments such as mangrove, wetlands, wastes dumping areas are a major source of antimicrobial agents and also produce a wide range of important medicinal compounds,

including enzymes, antitumor agents, insecticides, vitamins, immunosuppressant, and immune modulators (Newton C.M, *et al.* 2011).

Most soil bacteria prefer well oxygenated soils and are called aerobic bacteria and use the oxygen to decompose most carbon compounds. Anaerobic bacteria prefer and some require an environment without oxygen. Anaerobic bacteria are generally found in compacted soil, deep inside soil particles (microsites), and hydric soils where oxygen is limiting. Many pathogenic bacteria prefer anaerobic soil conditions and are known to outcompete or kill off aerobic bacteria in the soil. Many anaerobic bacteria are found in the intestines of animals andare associated with manure and bad smells (Lowenfels, *et al.*, 2006).

Among natural sources, microbial pigments are an attractive target as compared to plant or animal sources due to their fast growth, all-season availability and ease of regulating microbial cell factories for high production yields. Therefore, microbial pigment production by fermentative technology is a dynamic toolkit to produce a plethora of stable and safe pigments. Nonetheless, economic and marketing difficulties remain the key concern for microbial production at a commercial scale (Ramesh, *et al.*, 2019).

The microbial growth medium is one of the vital parameters contributing to fermentative production cost. Therefore, the use of inexpensive waste substrates as a growth medium can translate to the low cost of pigment production. In this context, the agro-industrial residues have the potential to serve as ideal substrates for microbial pigment production. These residues left unutilized add to waste generation which is expected to reach 3.40 billion metric tons by 2050 (Usmani, *et al.*, 2020).

Isolation is essential for taxonomic and experimental work on micro-organisms. In the microbiological sense, it is the process of separating a single species of microorganism from its natural habitat and growing it by itself, without interference from other organisms, on a sterile substratum, i.e., in pure culture. The micro-organism can then be distinguished from other species by its individual characters and propagated to provide experimental material. Many microorganisms like bacteria can live for years in culture if competitors are excluded, and if the nutrient medium is renewed periodically. Such cultures can be used for comparison

with freshly isolated and unidentified micro-organisms, although some diagnostic characters may be lost and sporulation may cease after long periods in culture (Frankland, *et al.,* 1995).

Pigments are compounds with characteristics of importance to many industries, such as in the food industry they are used as additives, colour intensifiers, antioxidants, etc. Nature produces many bio colourants from various resources including plants and microorganisms, which are possible alternatives to synthetic dyes and pigments. colorants or dyes derived from flora and fauna are believed to be safe because of non–toxic, non–carcinogenic and biodegradable in nature. Industrial production of natural food colorants by microbial fermentation has several advantages such as cheaper production, easier extraction, higher yields through strain improvement, no lack of raw materials and no seasonal variations. Bacteria could be genetically modified by inserting genes coding for the colorants even colorants not naturally produced by bacteria. These pigments are looked upon for their safe use as a natural food colorant and will not only benefit human health but also preserved the biodiversity, as harmful chemicals released into the environment while producing synthetic colorants could be stopped. Further, some natural food colorants have commercial potential for use as antioxidants. Thus, bacterial colorants in addition to being environment friendly, can also serve the dual need for visually appealing colours and probiotic health benefits in food products. They are consideredsafe and approved by FDA. The successful marketing of pigments derived from bacteria, bothas a food colour and a nutritional supplement depend on consumer safety and freshness of theproducts (Usman *et al.,* 2017).

An extraordinary range of microbial pigments in various environments has been described, such as carotenoids, flavins, phenazines, quinones, monascines, violaceins, indigoidines, melanins etc. Several studies grant these natural pigments a variety of beneficial properties such as anticancer activity, pro-vitamin A, and desirable characteristics such as high photostability, thermal stability, pH, and a great contribution to the preservation of biodiversitywhile reducing the release of harmful chemicals into the environment as a result of the manufacture of synthetic dyes. Of all the microorganisms capable of producing natural pigments, bacteria, yeasts, and fungi are the most relevant in the area (Celedón, *et al.,* 2021).

The present study, entitled, “Isolation and Molecular Characterization of Pigment Producing Bacteria from Different Soil Samples”, comprehensively encompasses utilization of soil samples for pigment production. The samples were exclusively from soil which have been collected from different locations such as mangrove, agricultural areas, garden soil and waste soils like poultry farm waste soil, rotten vegetable compost soil, Canal lining soil and local waste dumping sites. From these soil samples pigment producing bacterial colonies were isolated and cultured separately. Further, the isolated colonies were morphologically characterized and the organisms were identified by Molecular methods. The versatile applications of microbial pigments are also encompassed. Overall, the illustration of all these diverse aspects will help to contribute to the development of cost-effective bioprocesses for the production of natural colorants from natural environment of soil, waste soil feedstocks with its positive societal, industrial, and environmental implications.

AIM

Isolation, identification and molecular characterization of Pigment producing bacteria from different soil samples.

OBJECTIVES

- ✓ Isolation of Bacteria from different soil samples like mangrove, agricultural land, poultry farm soil, waste soil etc.
- ✓ Screening of obtained bacterial isolates depending on their ability to produce pigments.
- ✓ Morphological characterization of pigment producing bacterial colonies on Nutrient agar medium.
- ✓ Molecular characterization of pigment producing bacteria.

REVIEW OF LITERATURE

A pigment is defined as any substance capable of absorbing light, so it could be considered pigments in almost all substances. Usually, substances absorb very specific wavelengths, and emit a characteristic electromagnetic signal that allows us to identify them. Colour is the property of electromagnetic radiation with a wavelength between 300 - 400 and 700 - 800 nm. The colour of individual chemical substances derived from their interaction with white light. If the total radiation fallingon a chemical compound passes through or is reflected from it, the substance has a white colour. If white light on contact with a substance is completely absorbed, appears black. Most often chemical compounds selectively absorb within certain radiation ranges and the colour of a given compound is complementary to the absorbed radiation (Herrera, *et al.,* 2015).

Over the years, man has used colouring matters, which are known as dyes and pigments, for their aesthetic qualities and used them to embellish various articles and the world in which he lived. Indigo, the oldest known dye, was discovered in India; Tyrian purple (or Royal Purple) was discovered in the ancient city of Tyre; Alizarin was discovered among the Turks; and Cochineal was discovered among European and Mexican dyers. Indigenous dye-yielding plants have been discovered in almost every area of the world. The first synthetic dyes were found in the early twentieth century. Parenteral administration was not formulated until the 1930s: methylene blues and methyl violet, for example, were used to treat leprosy and filariasis, respectively. Following World War II, the use of intravenous dyes for medicinal purposes decreased rapidly. Just a few dyes, such as patent blue V or fluorescein, are still used as diagnostic drugs today. A dye is nothing but a coloured substance that has an affinity to the substrate to which it is being applied. The dye is applied in an aqueous solution and needs a mordant to boost the fastness of the dye on the textile fibre. The pigment may be a material that modifies the colour of mirrored or transmitted light as the result of wavelength-selective absorption. Pigments are used for colouring paint, ink, plastic, fabric, cosmetics, food and other materials (Kumar, *et al.,* 2021).

The use of synthetic pigments goes back to the 1850s when these were put in trend for the first time due to their supercilious colouring properties, lower prices, and easy production strategies, the significance of which remains empirically the same to this day. The importance of artificial or synthetic colouring agents is still based on the fact that the appearance of food items influences consumer's emotions, attitudes, and preferences. Thus, need for "synthetic pigments" cannot be overseen if client orientation is to be fulfilled. Cancers of skin, liver, and bladder have been found positively related to the use of artificial pigments because of their high azo-dye and heavy metal compositions. Furthermore, the precursors involved and the waste generated through their production process is environmentally hazardous as well. The outcry against the use of synthetic colorants in many health-conscious countries has already caused the ban of several artificial colorants, including Blue NO 1, Blue NO 2, Blue FCF, and Yellow NO 6 (Ali Nawaz, *et al.,* 2020).

Industries are the greatest polluters, with the textile industry generating high liquid effluent pollutants due to the large quantities of water used in fabric processing. In this industry, wastewaters differing in composition are produced, from which coloured water released during the dyeing of fabrics may be the most problematic since even a trace of dye can remain highly visible. Other industries such as paper and pulp mills, dyestuff, distilleries, and tanneries are also producing highly colouredwastewaters. It is in the textile industry that the largest quantities of aqueous wastes and dye effluents are discharged from the dyeing process, with both strong persistent colour and a high biological oxygen demand (BOD), both of which are aesthetically and environmentally unacceptable. Synthetic dyes are undesirable because they can affect living creatures in the water discharged as effluent into the environment. Industrial effluents containing synthetic dyes reduce light penetration in rivers and thus affect the photosynthetic activities of aquatic flora, thereby severely affecting the food source of aquatic organisms. The thin layer of discharged dyes that can formover the surfaces of the receiving waters also decrease the amount of dissolved oxygen, thereby affecting the aquatic fauna (Pereira, *et al.,* 2012).

One of the main consequences of these synthetic additives is cell damage due to oxidation, which can lead to immunosuppression in the human being which, in the worst-case scenario, can involve carcinogenesis. Pigments of natural origin play an important role in the physiology and molecular processes of microorganisms because they act as a method of adaptation to various extreme environments, have a protective function against solar radiation, and are also involved in functional processes like photosynthesis. Additionally, being influenced by environmental factors, microorganisms give rise to a variety of pigments with unique characteristics mainly related to the connection between the microorganism and the ecosystem. (Celedón, *et al.*, 2021).

Synthetic dyes tend to remain quite stable to common oxidation and reduction processes as per their designing and so are very difficult to remove from textile industry effluents; natural dyes are biodegradable without the use of any oxidant or reductant. Synthetic dyes, if at all degraded, are full of by-products that are directly or indirectly proven health hazards; such hazardous compounds have so far not been detected in the natural dye degraded by-products. It is possible that natural dyes completely degrade under natural conditions. Synthetic dye-based effluents can causea serious hazard to the water stream and environment due to their synthetic origin and complex molecular structures, which decrease their ability to biodegrade. Many carcinogenic and allergic synthetic dyes are banned now. Many dyes, though not banned yet, may not be completely safe. Most synthetic dyes are not biodegradable; they accumulate on lands and in river causing ecological problem (Affat, *et al.,* 2021).

Bio-pigments are eco-friendly and proved additionally propitious as antitoxic, antitumor, antioxidant, anticancer, and antimicrobial agents. Other advantages include fast and economic extraction techniques, higher yield, and time- and cost- efficient production. Moreover, the production of microbial pigments can also be made more convenient by the optimization capacity of their growth parameters. Keeping the capacity of bio-pigments into consideration, many biotech industries are now developing protocols for efficient extraction of natural pigments as a

replacement to synthetic counterparts. For instance, natural pigments such as zeaxanthin, saproxanthin, myxol and many others which illicit antioxidant activities are being instigated against artificial antioxidants such as butylated hydroxyl toluene and butylated hydroxyl acids. (Ali Nawaz, *et al.*, 2020).

Soil, in particular, is a complex matrix characterized by distinguishable layers, some of which are capable of supporting rooted plants. The overall properties of a soil fluctuate with time due to changing weather patterns and plant growth cycles. For this reason, pH, soluble salts, organic mass, flora, fauna, temperature, moisture, and the number and types of microorganisms all change with the seasons and overextended periods of time. Some naturally occurring organisms can be pathogenic to animals and humans. Appropriate sampling methods for soil are thus needed to help determine where, how, and to what extent soils might have been contaminated following a tularaemia event. (Silvestri, *et al.,* 2016).

Mangroves are highly productive marine ecosystem where bacteria actively take part in bio-mineralization and biotransformation of minerals. The distribution of microbial activities in estuarine systems are clearly complex and variable. Leaves and wood provided by mangrove plants to the soil are degraded primarily by large varietyof microbes which actively participate in the heterotrophic food chain. Major products of general recycling of organic matter are detritus which is rich in enzymes and proteins and contains large microbial population. Thus mangrove-derived detritus constitutes a large reservoir of carbon and energy potentially available to the estuarine food web and Bacteria are the major participants in the carbon, sulfur, nitrogen and phosphorous cycles in mangrove forest. Roughly 30% to 50% of the organic matters in mangrove leaves are leachable water-soluble compounds such as tannins and sugars (B.C.Behera, *et al.,* 2013).

Patterns of microbial colonization are influenced by nutrient fluxes in litter. Nutrient release from rapidly decaying litter fractions stimulates decomposition of adjacent recalcitrant litter mixtures. The interaction between litter types can alter decomposer communities and rates of nutrient release from single species litter (Giri, *et al*., 2005*)*.

Pour plate technique method is used to count the number of microorganisms in a mixed sample, which is added to a molten agar medium prior to its solidification. Molten agar should be cooled to 44 before plating otherwise it may lead to death of the desired organism. The process results in colonies uniformly distributed throughout the solid medium when the appropriate sample dilution is plated. This technique is used to perform viable plate counts, in which the total number of colonies forming units within the agar and on surface of the agar on a single plate is enumerated. Viable plate counts provide scientists a standardized means to generate growth curves, to calculate the concentration of cells in the tube from which the sample was plated, and to investigate the effect of various environments or growth conditions on bacterial cell survival or growth rate. This method is advantageous when our organism is environment bacteria and the prevalence is less. Streaking is used for obtaining pure culture from the mixed culture. Quadrant streaking is done in the petri plate in such way that all four corners are used for isolating a single bacterialcolony. (Kumar, *et al.,* 2019).

The streak-plate procedure is designed to isolate pure cultures of bacteria, or colonies, from mixed populations by simple mechanical separation. Single colonies are comprised of millions of cells growing in a cluster on or within an agar plate. A colony, unlike a single cell, is visible to the naked eye. In theory, all the cells in a colony are derived from a single bacterium initially deposited on the plate and thus are referred to as a clone, or cluster of genetically identical cells. Bacteria exist in a variety of shapes and sizes. For example, individual Escherichia coli cells are rod- shaped with an average length of 2 μm and width of 0.5 μm while Streptococcus cellsare spherical with an average diameter of 1 μm. Some bacteria (such as E. coli) exist as single cells while others form distinct patterns of association. Streptococcus, for instance, grow in pairs or form chains or clusters of cells. It is generally assumed that a single colony arises from a single cell undergoing binary fission; however, this assumption is not true for those bacteria that naturally exist as pairs, chains, or clusters or that divide by other mechanisms. Alternatively, if too many bacteria are plated, then overlap of cells may occur and increase the probability of two or more bacteria giving rise to what appears to be a single colony. To avoid these complications when describing or enumerating

bacterial cultures growing on a solid medium, colonies are referred to as colony forming units (cfu). (Sanders, *et al.*, 2012).

Increasing attention is being drawn to microorganisms because the fertility of soil depends not only on its chemical composition, but also on the qualitative and quantitative nature of microorganisms inhabiting it. Maintenance of viable, diverse populations and functioning microbial communities in the soil is essential for sustainable agriculture (Beare, *et al.*, 1995).

The pigmented bacteria can be sourced from various environmental sources which can be cultured and purified. Various growth media can be used to isolate different types of bacteria producing pigments. However, due to the high cost of using synthetic medium, there is a need to develop new low-cost process for the production of pigments. The use of agro-industrial residues would provide a profitable means of reducing substrate cost. Pigment produced by bacteria can be separated using solvent extraction and further characterized using various instrumental based analytical techniques such as TLC, UV–vis, FTIR, ESI-MS, NMR, HPLC and Gel Permeation Chromatography. Extraction of bacterial pigments in relatively pure and concentrated forms is the main technological challenge. Bacteria produce two types of pigments: those that predominantly remain bound to the bacterial mycelia and those that are secreted into the fermentation broth. Whereas pigments from the former class can be conveniently recovered by disrupting the filtered mycelia with acetone, secreted natural products are typically recovered by extracting the aqueous broth with large quantities of organic solvents such as ethyl acetate. To mitigate environmental and health concerns associated with solvent use, alternative separationtechnologies such as spray-drying (widespread in the food and feed industry) and solid-phase extraction (commonplace in the fine chemical industry) may be appropriate (Venil, *et al.*, 2013).

The fact that only a small fraction of the microorganisms present in a microbial community can be cultured and the behaviour of microorganisms as pure cultures is different from their behaviour in a microbial community has caused a shift from single-organism studies to whole community studies. Molecular fingerprinting and

high-throughput sequencing techniques are used to characterize these microbial communities. These techniques use a top-down approach and target microbial communities as a whole. Metagenomics, meta transcriptomics and meta proteomics give information on the taxonomic and functional diversity, the population structure, the presence of genes, as well as their levels of expression and translation into proteins. A drawback is the complex post-processing of the big amount of data obtained by these high-throughput techniques. Even with the most advanced bioinformatics tools and sequencing technology, it is almost impossible to assign the(expressed) genes and proteins, and thus the functionality, to specific species. Furthermore, it is not possible to fully map and understand the microbial interactions,which are often the driving force of a community. (De Roy, *et al.*, 2014).

Soil extraction protocols are generally classified as direct and indirect DNA extraction procedures. Direct DNA isolation is based on cell lyses within the sample matrix and subsequent separation of DNA from the matrix and cell debris. While the indirect approach involves the extraction of cells from the environmental material prior to the lytic release of DNA, direct DNA extraction protocol involves soft and harsh lysis methods. Soft lysis method is based on the disruption of microorganism solely by enzymatic and chemical means, whereas harsh lysis approach involves the mechanical cell disruption by bead beating, sonication, freeze-thawing and grinding. Because the composition of different habitats varies with respect to their matrix, organic and inorganic compounds and biotic factors, standardization of total DNA extraction technique is desirable. Improved DNA extraction techniques could help to ensure analyses that adequately represent the entire community's genome without inhibitory substances (Titio, *et al.*, 2015).

During a molecular investigation of an environment, the lysis of all microorganisms within the given habitat is important and has to comply with two requirements: the effective lysis of cells, and the removal of any possible inhibitors for further analysis (e.g., Humic acids). Furthermore, the resulting DNA should be intact and not sheared since strongly fragmented nucleic acids are sources of artifacts in PCR amplification and may contribute to the formation of chimeric PCR products. Taking all these

requirements into account, every PCR-based method will most likely be biased by an insufficient or preferential disruption of cells. This results in an underestimation of the biodiversity present, and thus the choice of the optimal extraction method is critical (Leuko, *et al.*, 2008).

Agarose gel electrophoresis is the most effective way of separating DNA fragments of varying sizes ranging from 100 bp to 25 kb. Agarose is isolated from the seaweed genera *Gelidium* and *Gracilaria*, and consists of repeated agarobiose (L- and D-galactose) subunits. During gelation, agarose polymers associate non-covalently and form a network of bundles whose pore sizes determine a gel's molecular sieving properties. The use of agarose gel electrophoresis revolutionized the separation of DNA. Prior to the adoption of agarose gels, DNA was primarily separated using sucrose density gradient centrifugation, which only provided an approximation of size. To separate DNA using agarose gel electrophoresis, the DNA is loaded into pre-cast wells in the gel and a current applied. The phosphate backbone of the DNA (and RNA) molecule is negatively charged, therefore when placed in an electric field, DNA fragments will migrate to the positively charged anode. Because DNA has a uniform mass/charge ratio, DNA molecules are separated by size within an agarose gel in a pattern such that the distance travelled is inversely proportional to the log of its molecular weight. The leading model for DNA movement through an agarose gel is "biased reptation", whereby the leading edge moves forward and pulls the rest of the molecule along. The rate of migration of a DNA molecule through a gel is determined by the following: 1) size of DNA molecule; 2) agarose concentration; 3) DNA conformation; 4) voltage applied, 5) presence of ethidium bromide, 6) type of agarose and 7) electrophoresis buffer. After separation, the DNA molecules can be visualized under Ultra Violet light after staining with an appropriate dye. (Lee, *et al.*, 2012).

The production of bio-pigments from bacterial species is being conducted globally with soaring interest under the research of microbial autecology. A massive array of these compounds, also referred to as “bioactive pigmented molecules”, can be derived from both Gram-positive and Gram-negative bacterial species. By isolating

specific bacterial colonies and close examination could deliver the fact that individual bacterial cells can coordinate with others in their colony to carry out constitutive functions especially involving the secretion of numerous specific chemical compounds. These compounds can help them with survival, competence, bioluminescence, biofilm formation, and even sporulation, etc. Bio-pigments can be produced by triggering regulatory mechanisms of these species and can be extensively used in various bio-medical and bio-industrial sectors, including textiles, food, pharmaceutical, and cosmetic industries, owing to their beneficial attributes and biological activities. These are moreover convenient to harvest in large volumes through utilizing simple gene manipulating strategies. The rising consumer concerns regarding safety and quality of industrial products holds a significant ground as to why scientists are shifting their focus towards naturally derived, non-toxic, and eco-friendly pigment alternatives (Ali Nawaz, *et al.,* 2020).

Beneficial microbes produce enzymes that degrade organic matter and mitigate the effects of harmful pathogens. The enzyme-producing capabilities of bacteria are an important research topic, owing to the efficiency of these enzymes in catalysing chemical reactions in living organisms. More specifically, bacteria-produced enzymes break down organic material into a stable material through various biochemical processes; thus, they are essential tools for waste conversion. Moreover, food waste consists predominately of carbohydrates, proteins, and fats. Therefore, theenzymes produced by bacteria can break the bonds between macromolecules and are beneficial in the waste conversion process. Despite the importance of bacteria- produced enzymes in the waste conversion process, relatively little is known about the relationship between food waste and the nature of microbial species of different origins and processes (Kim, *et al.,* 2021).

The environmental safety, conservation and awareness have switched the trend towards the natural sources for colouring agents. Non-toxicity, biodegradability and non-carcinogenicity of the natural dyes and colorants derived from natural flora and fauna make them an attractive source for human use. Anthraquinones and flavonoids are natural products that are traditionally used as colouring agents which are

commonly derived from animals and plants. Currently, as the trend is shifting towards the natural sources for the eco-friendly compounds, natural colorants are leading the market demand over the passage of time. These natural colorants and pigments are derived from many different sources such as insects, ores microbes and plants. Among these sources, microbes and bacteria in particular have the capacity to synthesize a wide of range secondary metabolites and pigments. Pigments extracted from bacteria have a diverse range of commercial applications. It is an emerging fieldand is in the stage of infancy. Efforts are needed to develop a cheap media for bacterial growth to reduce its cost and make it feasible for commercial production (Numan *et al.*, 2018).

Bacterial genus, *Serratia* can produce a red substance called prodigiosin, these substances have been known to have an antibiotic and antimalarial effect and immunosuppressing activity. Violacein is produced by several bacterial species, including the Gram–negative species *Chromobacterium violaceum, Janthinobacterium lividum* and *Pseudoalteromonas luteoviolacea*. Violacein was reported to have antiprotozoan, Anticancer, Antiviral, Antibacterial, and antioxidant activities. The antimycobacterial activity of two pigments, violacein from *Janthinobacterium* species and flexirubin from *Flavobacterium* species might be valuable compounds for chemotherapy of tuberculosis. These characteristics provide the possible applications of violacein for therapeutic purposes. Thus, pigments from bacteria offer the wide range of biologically active properties and continue to provide promising avenues to end enormous challenge of antibiotic resistant. The red pigment prodigiosin from *Serratia marcescens* and violet pigment violacein from *Chromobacterium violaceum* and tested its dyeing efficiency in different fabrics i.e., pure cotton, pure silk, pure rayon, silk satin and polyester. Their results suggested that prodigiosin could be used to dye acrylic and for violacein intense colorations was observed in pure rayon and silk satin. The red pigment prodigiosin from *Vibrio sp. Serrtia sp*. violet pigment from *Chromobacterium violaceum*, anthraquinone from *Dermocybe anguine*, pink pigments from *Roseomonas fauriae* and *Fusarium oxysporum, Trichoderma* sp. and Alternaria sp. are suitable for in textile industry for dyeing of all fibres including cotton, wool, silk, nylon and acrylic fibres (Usman *et al.*, 2017).

MATERIALS AND METHODS

I. STUDY AREAS

Soil samples were collected from the following locations and desired bacteria were isolated from them.

A) SITE 1: AROOR

Aroor is a census town at the northern end of Alappuzha district and the southern suburb of the city of Kochi in the state of Kerala, India. It is a seafood related industrial area of Alleppey district, and acts as the Southern entry point into Kochi city. Seafood export is a major industry of the area, given that Aroor is near Vembanad lake, a part of Kerala backwaters. This ecosystem helps large-scale prawn and shrimp farming to be an alternative to the low-lying paddy fields, which have the interlocked water system connected to the Kerala backwaters. The abundance of marine wealth and logistical advantages have helped the seafood export to grow, which provide employment to a number of people. The sample was taken from Veluthully viewpoint, Aroor, one of the major tourist attractive places in Kochi blessed with large areas of mangrove filled forests.

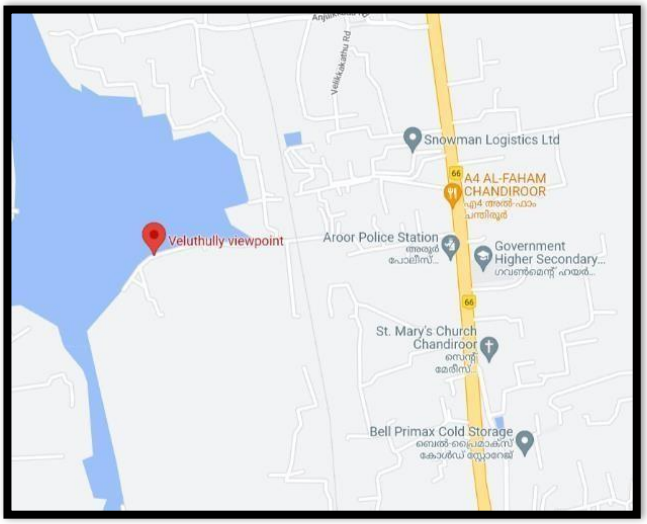

Figure 1: Map showing Velluthully Viewpoint, Aroor.

B) SITE 2: MARAKADAVU

Marakadavu is a small village near the sub locality of Kappalandimukku, Mattancherry falls in Ernakulam district situated in Kerala. The size of the area is about 0.61 square kilometre. The soil composition is mainly of recent sediments such as Alluvium, Teri's, Brown sands etc. Hydromorphic saline soils are also found in the areas surrounding the backwaters. The place locates in the western part of Cochin corporation, which was once a bustling centre of trade, particularly in spices. Invasions from across the sea was frequent and the invaders left indelible marks on the landscape, culture, art and social history of the place. The major livelihood of the local people is business which have a high number of small- scale enterprise like shops such as grocery, bakeries, and other retails. Another major area of income contains agricultural and poultry sectors. Soil sample was collected from a poultry farm in Marakadavu. The farm is raising high quality poultry birds like chickens, ducks, turkey, emu and their eggs too.

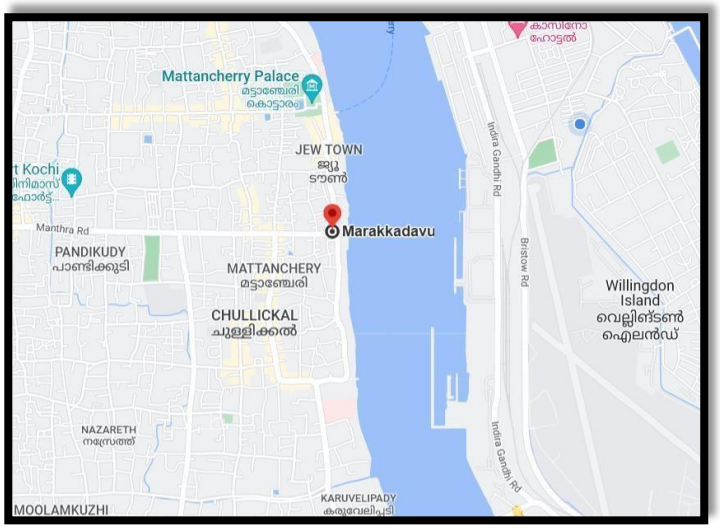

Figure 2: Map showing Marakadavu.

C) SITE 3: WILLINGDON ISLAND, KOCHI

Willingdon Island is the largest artificial island in India, which forms part of the city of Kochi, in the state of Kerala. Willingdon Island has tropical monsoon climate with temperatures ranging between 20°C and 35°C. Maximum temperature recorded in summers is about 38°C and minimum in winters is 17°C. The South - west monsoons are from June to September where Kochi experiences heavy rainfall and light showers from October to December due to north – west monsoon. The average annual rainfall is about 350 cm with approximate 132 days of yearly rainfall. Along the sea belts and backwaters the soil type is mostly hydromorphic saline soil. Much of the present Willingdon Island was claimed from the Lake of Kochi, filling in dredged soil around a previously existing, but tiny, natural island. Willingdon Island is significant as the home for the Port of Kochi as well as the Kochi Naval Base, the Southern Naval Command of the Indian Navy, Plant Quarantine station, Custom House Cochin and Central Institute of Fisheries Technology, a constituent unit of Indian Council of Agricultural Research. Two soil samples were collected from Willingdon Island, one from soil lining from a local Canal and another sample from rotten vegetable compost pit.

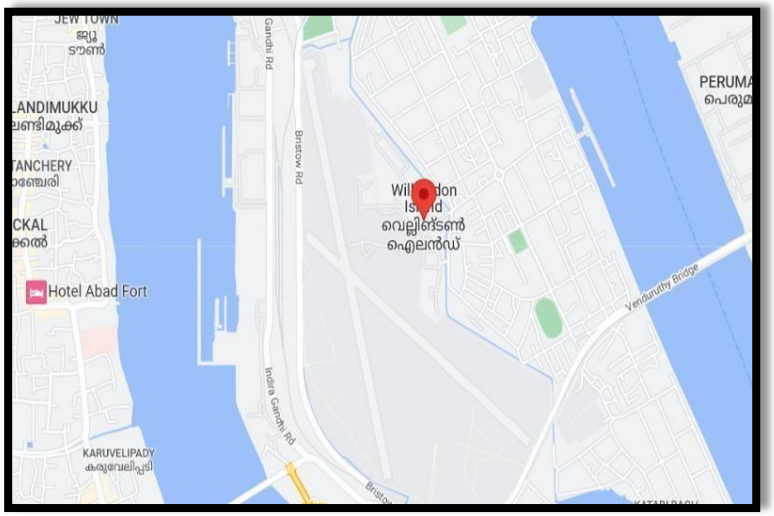

Figure 3: Map showing Willingdon Island, Kochi.

D) SITE 4: ST TERESA'S COLLEGE, ERNAKULAM

St. Teresa's College is an autonomous women's college located at Kochi, Ernakulam, Kerala. Ernakulam lies between North latitudes 09° 47' 13" and 10°10'44" and East longitudes 76°10' 05" and 77° 05' 24. Kochi known as the Queen of Arabian Sea is the headquarters of Ernakulam district. It is a major port city on the west coast of India. Ernakulam district has wet monsoon type of climate. The district experiences heavy rainfall during southwest monsoon season followed by northeast monsoon. The mean monthly maximum temperature ranges from 28.1 to 31.4°C and the minimum ranges from 3.2 to 26 °C. The soils of the district are classified as Lateritic, Hydromorphic saline, Brown hydromorphic, Riverine alluvium and Coastal alluvium soil out of which lateritic soil is the most predominant soil type. These soils are well drained, low in organic matter and plant nutrients. The soil sample was collected from the garden area of St. Teresa's College, Science Block.

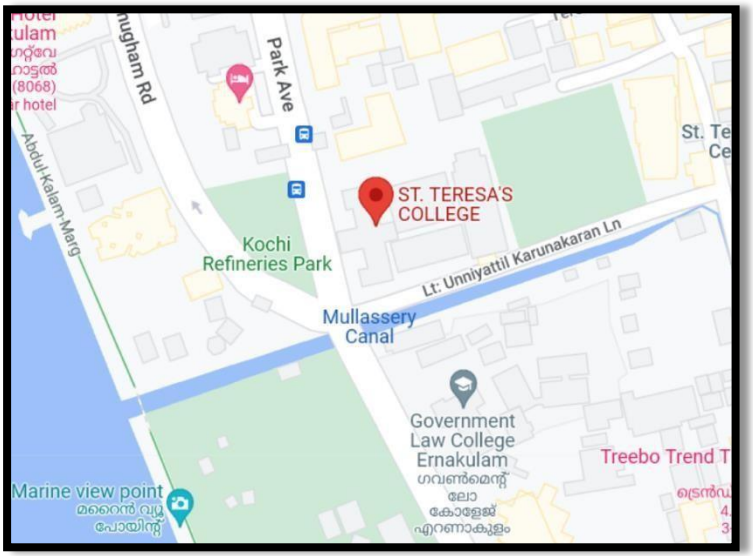

Figure 4: Map showing St. Teresa's College (Autonomous), Ernakulam.

E) SITE 5: KAPPADU, KANJIRAPPALLY

Kappadu is a small Village in Kanjirappally Block in Kottayam District of Kerala. The place has major number of rainy days in Kerala. The district is naturally divided into high land, midland and lowland, the bulk being constituted by the midland regions. Kappadu and Kanjirappally have high land and midland areas and have a mix of laterite soil, as well as Alluvial soil. The district has no coastal area. Kanjirappally receives the highest amount of summer rains, winter rains and northeast monsoons, making it one of the very few places in India enjoying equatorial rainforest type climate, with no distinct dry season. The well-distributed rainfall pattern of Kanjirappally, which is the nearby major city, the primary reason for the phenomenon of high yield of latex from Rubber plantations in and around the town. A part of the total population describes their work as permanent employment while most of the people are engaged in agriculture as cultivators (owner or co-owner) and some are agricultural labourer. Soil sample was collected from a local waste dumping area which contained mixture of household, garbage as well as kitchen wastes.

Figure 5: Map showing Kappadu.

F) SITE 6: VARAPUZHA

Varapuzha, is a northern suburb of the city of Kochi. It is a census town in Paravur Taluk, Ernakulam district in the Indian state of Kerala. It covers an area of 7.74 km². Weather is 26 °C and had a humidity rate of 92%. Its specialty is that considerably large-scale paddy cultivating area is situating western part of Varapuzha which is called Devaswompadam, specialised with Pokkali paddy cultivation and interim crop as fish cultivation locally called 'Kettu'. The common work of the natives is fishing and agriculture. The soil sample was obtained from a local area which is composed of agricultural wastes.

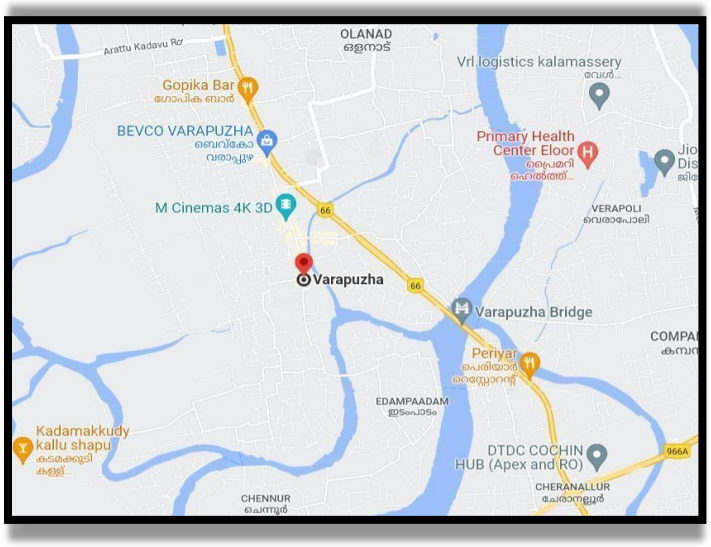

Figure 6: Map showing Varapuzha.

II. SOIL SAMPLE COLLECTION

Materials required: Sterile plastic bags, spatula, Gloves, pH papers etc.

Soil samples were collected from specific locations like mangrove, poultry farm soil, Canal lining soil, rotten vegetables compost, garden soil, agricultural land and waste soils. Stainless steel spoons were used and soil was collected from below ground surface where conditions are generally soft and non-indurated. Samples were collected from 10 cm depth using standard soil collection protocols in a sterile polyethylene bag and stored for further procedure.

III. SERIAL DILUTION OF SOIL SAMPLE

Materials required: Soil sample, distilled water, test tubes, 1ml pipette, 1 ml sterile tips etc.

Ten clean sterile test tubes were filled with 9 mL of sterile water. In to the first test tube containing 9 ml of sterile water, 1gram of sample is dissolved. The soil must be thoroughly mixed. It provide an initial dilution of 10^{-1} . Then 1 ml from the stock is transferred to the next tube and it is named as 10^{-2} . Repeat the same for the remaining test tubes, until it reaches 10^{-10}. The dilution for the soil sample in the last test tube will be 10^{-10} (1 in 1,00,00,00,000).

IV. POUR PLATE CULTURE

Materials Required: Serially Diluted soil samples, distilled water, Nutrient Agar, I ml pipette, tips etc.

Using pour plate technique protocol, Nutrient agar plates were prepared. The agar medium is pre-sterilized in an autoclave. Once the agar reaches a little low temperature, 20-25 ml of media is poured into a petri plate and 1 ml diluted sample is added. The same step is repeated for rest of the dilutions. The plates were kept for 24 hours incubation at 37˚C in the bacteriological incubator.

V. SCREENING OF PIGEMENTED BACTERIAL ISOLATES

After incubation period, the petri plates were examined for bacterial growth. Different bacterial colonies were observed, out of which pigmented bacteria with dissimilar morphological features were selected for further DNA isolation and molecular characterization.

VI. PURE CULTURE PREPARATION (Katz, 2008)

Materials required: Sterile loop, bacterial isolates, nutrient agar, petri-plates, test tubes, refrigerator.

In order to obtain well-isolated pure discrete colonies, quadrant streak technique is used. For this one loop full of enrichment culture from the pour plate is streaked on nutrient agar plate. The streaked plates were placed in a bacteriological incubator at 37˚C for 24 hours. The separated pure colonies were sub-streaked and were maintained at 4°C for bacterial characterization as slant cultures. The cultures were named as ZA1, ZA2, ZA3 and ZA4.

VII. MORPHOLOGICAL CHARACTERIZATION OF BACTERIAL ISOLATES

Morphological features like size, shape, colour and texture were observed. Culture characteristics on agar plate including colony shape, margin, and elevation was determined and recorded as colony morphological characteristics.

VIII. GENOMIC DNA ISOLATION (Ausubel *et al*., 1987)

Materials required: Bacterial isolates, nutrient broth, incubator, microfiche tubes, centrifuge, TE buffer, proteinase K, water bath, 10% SDS, Phenol chloroform Isoamyl alcohol, chloroform, sodium acetate, ice cold ethanol, 70% alcohol, refrigerator, sterile tips and pipettes.

The bacterial colonies that exhibit pigmentation were further subjected for DNA isolation. The selected colonies are inoculated in the Nutrient broth and incubated at 37°C for 24 hours.

About 2 ml of bacterial culture was transferred into the microfuge tube and centrifuged for 10 minutes at 8000 rpm. The pellet obtained after the centrifugation was re-suspended in 875 µl of TE buffer and 5 µl of proteinase K was added to the same. The mixture was kept in water bath at 37°C for 1 hour after the addition of 100 µl of 10% SDS. It was centrifuged at 8000 rpm for 5 minutes after equal volumes of Phenol-Chloroform- Isoamyl alcohol mixture was added in the ratio of 25:24:1. The upper layer was collected after the centrifugation and equal volume of Chloroform was added. This mixture was centrifuged for 3 minutes at 8000 rpm. Tothe upper layer 0.1 volume of sodium acetate and the double volume of ice-cold ethanol was

added and centrifuged at 8000 rpm for 10 minutes. Supernatant was decanted and 70% alcohol was added, followed by centrifugation and the decantation of alcohol. The pellet thus obtained was air dried. The air-dried pellet was dissolved in 100 of µl TE buffer and stored at -20°C.

IX. AGAROSE GEL ELECTROPHORESIS (Sambrook *et al*., 2000)

Materials required: Agarose, 1X TAE buffer, hit palate, Ethidium bromide, conical flask, gloves, sterile tips, pipettes, gel casting tray, gel combs, gel tank, DNA samples, gel loading dye, parafilm, power package, gel documentation system and Clingfilm.

The agarose gel is prepared by mixing 0.5 g agarose in 50 ml 1X TAE and the solution is heated to dissolve the agarose. The dissolved gel was cooled to room temperature followed by addition of 10 µlof the Ethidium bromide dye with proper mixing. The gel is poured into the gel casting tray followed by placing of the comb (to create wells) and allowed to stand for 1 hour undisturbed. The comb was removed from the set gel and the gel is placed in the gel tank filled with 1X TAE.DNA samples are loaded into the wells in the gel. The DNA sample to be loaded was mixed with gel loading dye on a parafilm. The well is loaded with the DNA samples alone with the gel loading dye and the power is switched on. After the samples have reached to end of the gel, the power is switched off and bands are visualized using Gel documentation system.

X. POLYMERASE CHAIN REACTION

Materials required: PCR Thermal cycler, template DNA, 16 Sr DNA forward primer, Reverse primer, 5 X assay buffer, dNTP mix, MgCl2, Taq-polymerase (5 µl), sterile water, PCR tubes and Micro tips.

PRIMER DETAILS

PRIMER	SEQUENCE
16 S Forward	5`GAGTTTGATCCTGGCTCAG 3'
16 S Reverse	5`ACGGCTACCTTGTTACGACTT 3`

Table 1: Primers used to amplify 16SrDNA

PREPARATION OF REACTION MIXTURE FOR PCR

Serial number	Ingredients	Volume in µl
1	Molecular biology grade water	28.25
2	5X assay buffer	10
3	Template DNA	2.5
4	Forward primer	2.5
5	Reverse primer	2.5
6	25mM Mgcl2	3
7	Wpm dNTP mix	1
8	Taq Polymerase	0.25
	Total	**50**

Table 2: PCR Reaction mixture preparation

The contents are mixed thoroughly followed by the addition of 25 µl of mineral oil. The above mixture is placed in thermo cycler block for the amplification to occur.

PCR PROGRAM

STEP	TEMPERATURE	TIME	
Initial Denaturation	95	2 minutes	35 Cycles
Denaturation Annealing Extension	95 56 72	30 seconds 30 seconds 2 minutes	
Final Extension	72	5 minutes	

Table 3: CYCLES FOR PCR

The amplicon was then electrophoresed and visualized using Gel documentation system and was sent for sequencing.

XI. SEQUENCING AND BIOINFORMATICS ANALYSIS

The amplified 16S rDNA was sequenced by Sangers Dideoxy method at Enfys Lifesciences Lab Pvt. Ltd, Kochi, Kerala. The identity of the sequence was determined by comparing with the sequence in the NCBI database using the online tool BLAST (Altschul, *et al.*, 1990). For this, the sequences in the fasta format were copied on to the NCBI BLAST page (http://blast.ncbi.nom.nih.gov) and BLAST was carried out and the sequences showing maximum identity was recorded.

RESULT

1. SAMPLE COLLECTION

The soil samples were collected from two districts of Kerala such as Ernakulam and Kottayam. Study areas included mangrove, agricultural land, Canal soil, rotten vegetable compost, garden area, poultry farm and waste soil. The samples were named as ZAS <number>, according to the number which the soil collected.

2. ISOLATION OF SOIL BACTERIA

The soil samples collected from different locations were serially diluted for obtaining bacterial colonies. Following, the diluted samples were subjected to pour plate method to isolate desired colonies.

Figure 7: Serial Dilution of collected soil samples.

3. SCREENING OF PIGMENTED BACTERIAL COLONIES

Primary screening of obtained bacteria was done on the basis of production of pigmentation. Further, phenotypic characteristics of the pigmented bacteria was observed carefully. Bacteria having pigment producing capacity were chosen. Among them, morphologically dissimilar strains were selected for further study.

Out of the seven samples taken for serial dilution followed by pour plating, five soil samples exhibited the growth of pigmented bacteria in pour plate (Shown in the table 4 and figure 8). Those pigmented colonies were separately isolated and cultured by Quadrant Streaking.

Sl No.	Soil type (location)	Presence of pigemented bacteria	Number of pigmented colonies (colour)	Colonies selected for DNA analysis
1.	Mangrove (Aroor).	YES	Two (orange)	1
2.	Poultry farm (Marakadavu)	YES	One (yellow)	1
3.	Canal Lining soil (Willingdon Island).	NO	Zero	0
4.	Rotten Vegetable pit (Willingdon Island).	YES	One (yellow)	0
5.	Garden Soil (St. Teresa's College)	NO	Zero	0
6.	Waste Soil (Kappadu).	YES	Three (one pink, two yellow)	1 (pink)
7.	Agricultural land (Varapuzha)	YES	Two (red, orange)	1 (red).

Table 4: Screening of pigmented colonies.

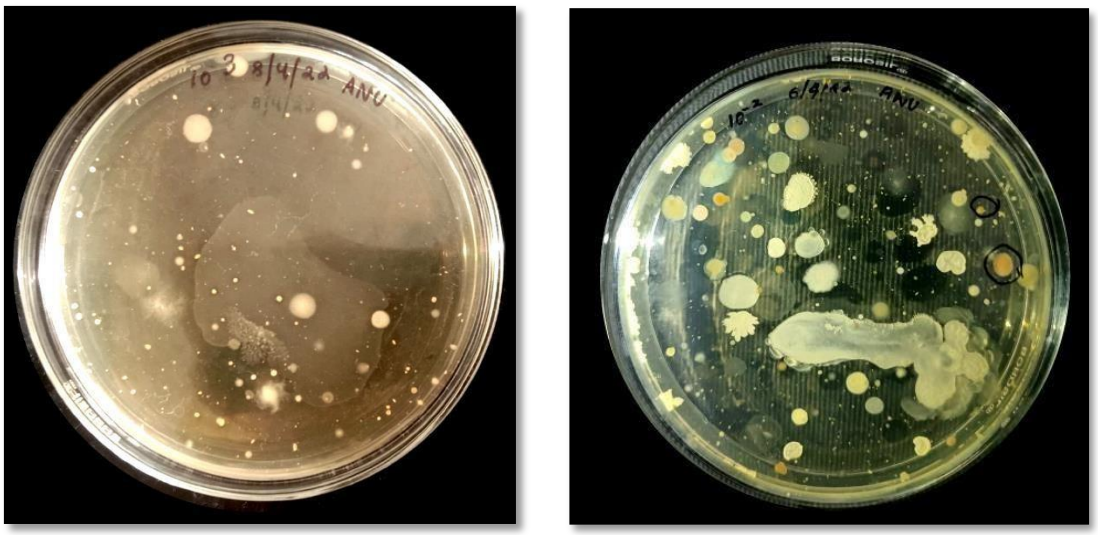

Figure 8: Bacterial isolates obtained by Pour plate method

4. PURE CULTURE PREPARATION

To obtain pure culture of the selected samples, pigmented bacterial colonies were separately cultured on Nutrient agar medium by Streak Plate technique. The selected bacteria samples were labelled as ZA <number> according to which they were isolated.

Figure 9: Pure culture of ZA1

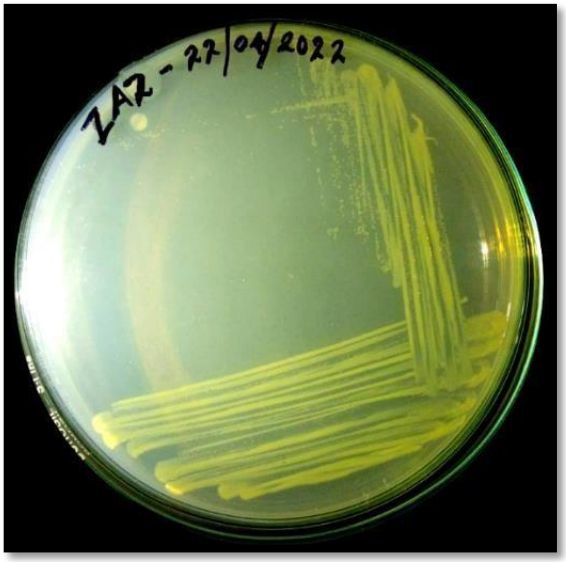

Figure 10: Pure culture of ZA2

Figure 11: Pure culture of ZA3

Figure 12: Pure culture of ZA4

5. MORPHOLOGICAL TEST

On Nutrient agar plate

The bacterial colonies showed different characters on nutrient agar and were determined using basic identification techniques. The characters like colour of the colony, form, margin, relative size, texture and elevation were accounted (Table 4).

Bacteria	Colour	Colony Form	Margin	Size	Texture	Elevation
ZA1	Orange	Circular	Entire	Small	Glistening	Raised
ZA2	Yellow	Punctiform	Entire	Punctiform	Smooth	Raised
ZA3	Pink	Circular	Entire	Large	Rough	Flat
ZA4	Red	Circular	Entire	Small	Glistening	Raised

Table 5: Morphology characteristics of bacterial colony

6. GENOMIC DNA ISOLATION FOR IDENTIFICATION OF THEBACTERIAL STRAINS

Genomic DNA isolated from the four pigmented bacterial samples such as ZA1, ZA2, ZA3 and ZA4 were visualized by Agarose gel electrophoresis and compared with 100bp DNA ladder (Figure). For each bacterium a single band was visible on the gel and size was compared with DNA ladder.

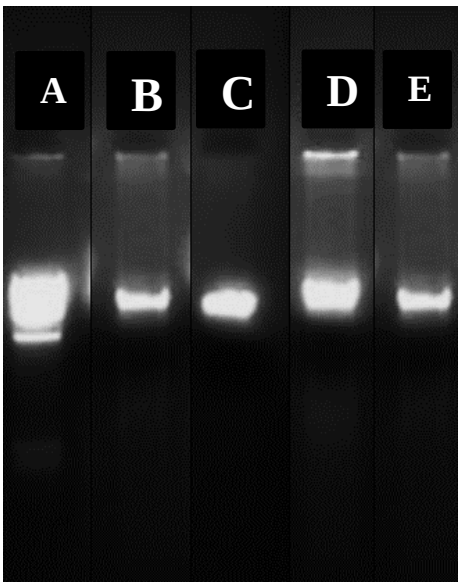

Figure 13: Agarose gel showing genomic DNA of bacterial samples, ZA1, ZA2, ZA3 and ZA4.

1. **Lane A:**100 bp DNA ladder
2. **Lane B:** ZA1 DNA
3. **Lane C:** ZA2 DNA
4. **Lane D:** ZA3 DNA
5. **Lane E:** ZA4 DNA

7. PCR AMPLIFICATION OF 16S rDNA

The isolated genomic DNA was amplified using PCR, followed by visualization of the PCR product by Agarose gel electrophoresis in a gel documentation system (Figure 14). The size of the samples was compared using the standard DNA marker (100 bp ladder).

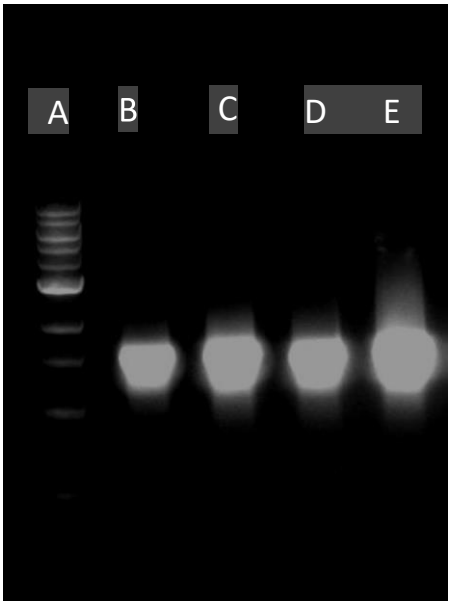

Figure 14: Agarose gel showing PCR product of ZA1, ZA2, ZA3 and ZA4

1. **Lane A**:100bp DNA ladder.
2. **Lane B**:16S rDNA product of ZA1.
3. **Lane C**:16S rDNA product of ZA2.
4. **Lane D**: 16s rDNA product of ZA3.
5. **Lane E**: 16s rDNA product of ZA4.

8. SEQUENCE CHARACTERIZATION AND BIOINFORMATICSANALYSIS

The amplified PCR product was sequenced for its 16S rDNA by Sanger's Dideoxy method for identification of the bacteria. The sequencing was done by Enfys Lab Pvt. Ltd Kochi. The homology search using NCBI BLAST from the obtained sequence of ZA1 showed 99.89% similarity with *Rossellomorea aquimaris (*Figure 15), ZA2 showed 100% similarity with *Stenotrophomonas maltophilia* (Figure 16), ZA3 showed 99.88% similarity with *Bacillus paramycoides* (Figure 17) and ZA4 showed 100% similarity with *Serratia marcescens* (Figure 18).

>A1_PET.Forward_33424-3_P4796, Trimmed Sequence (886 bp)

ACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAG
TGCCGTTCGAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACG
TGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAG
CGCGCGCAGGTGGTTCCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCA
TTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCAAGTGTAGCGGTGAA
ATGCGTAGATATTTGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACA
CTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAA
ACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAG
CACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGC
ACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGA
CATCCTCTGACAACCCTAGAGATAGGGCTTTCCCCTTCGGGGGACAGAGTGACAGGTGGT
GCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAAC
CCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGATGACTGCCGGTGACAAACC
GGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTG
CTACAATGGGACGGTACAAAGGGCAGCGAGACCGCGAGGTTTAGCC

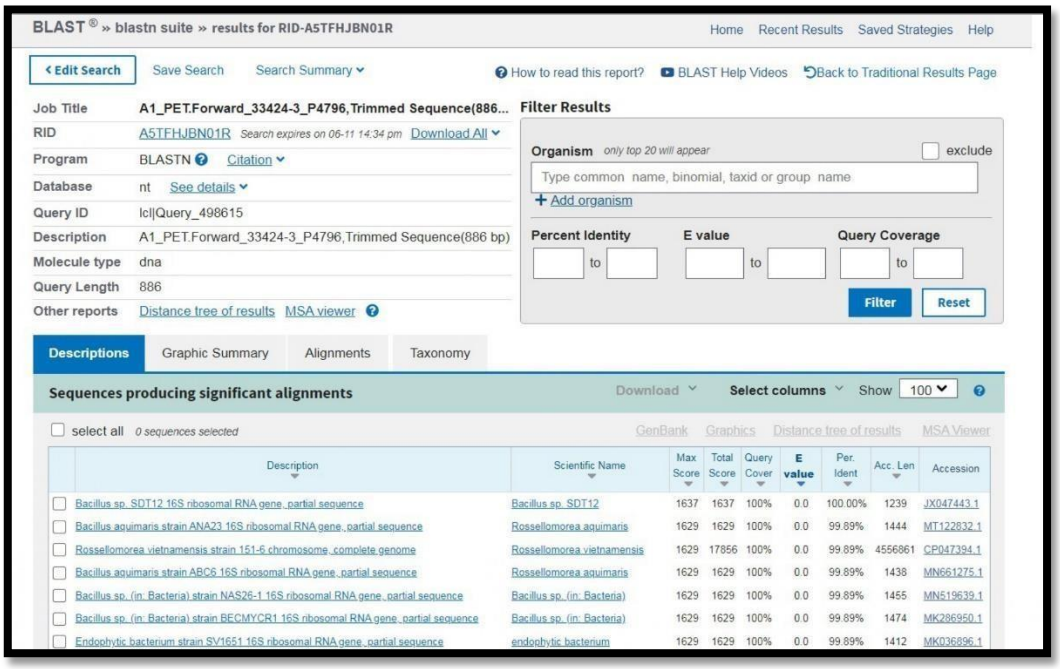

Figure 15: NCBI BLAST list depicting sequence similarity of ZA1

>A2_PET.Forward_33424-3_P4796, Trimmed Sequence (571 bp)

AAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGG
GTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTCG
TTTAAGTCCGTTGTGAAAGCCCTGGGCTCAACCTGGGAACTGCAGTGGAT
ACTGGGCGACTAGAATGTGGTAGAGGGTAGCGGAATTCCTGGTGTAGCAG
TGAAATGCGTAGAGATCAGGAGGAACATCCATGGCGAAGGCAGCTACCTG
GACCAACATTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAG
ATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGGATGTTGGGTGCA
ATTTGGCACGCAGTATCGAAGCTAACGCGTTAAGTTCGCCGCCTGGGGAG
TACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGC
GGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGCC
TTGACATGTCGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCG
AACACAGGTGCTGCATGGCTG

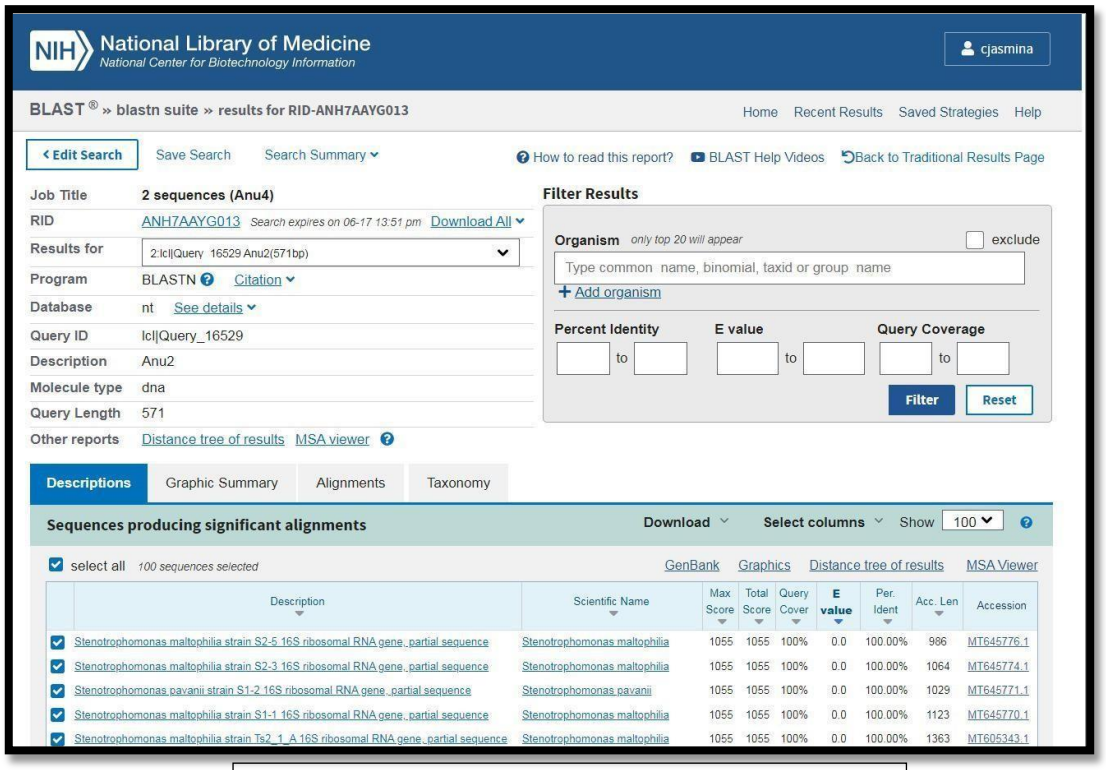

Figure 16: NCBI BLAST list depicting sequence similarity of ZA2

>A3_PET.Forward_33424-4_P4796, Trimmed Sequence (831 bp)

ACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAG
TGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACG
TGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAG
CGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCA
TTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAA
ATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACA
CTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAA
ACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAG
CACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGC
ACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGA
CATCCTCTGACAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGC
ATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
TTGATCTTAGTTGCCATCATTTAGTTGGGCACTCTAAAGTGACTGCCGGTGACAAACCGG
AGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTAC

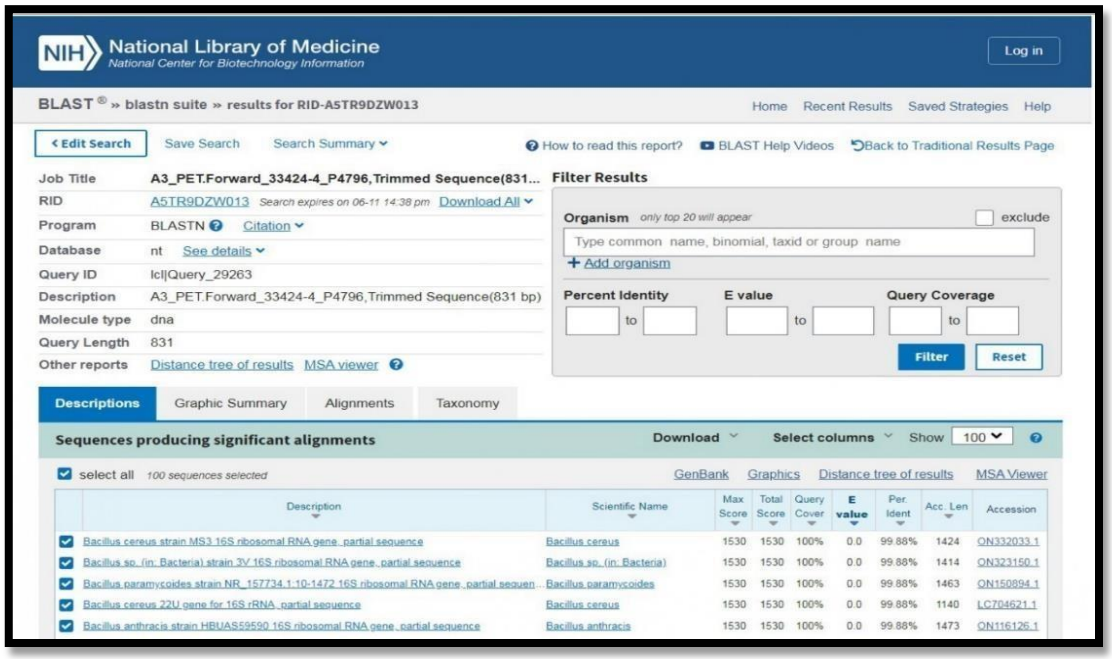

Figure 17: NCBI BLAST list depicting sequence similarity of ZA3

≥A4_PET.Forward_33424-4_P4796, Trimmed Sequence (776 bp)

AGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGGTGGTGAG
CTTAATACGCTCATCAATTGACGTTACTCGCAGAAGAAGCACCGGCTAAC
TCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAAT
TACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATC
CCCGGGCTCAACCTGGGAACTGCATTTGAAACTGGCAAGCTAGAGTCTCG
TAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGG
AGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGG
TGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCT
GTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAG
CTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACT
CAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT
CGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTAGC
AGAGATGCTTTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTG
TCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAA
CCCTTATCCTTTGTTGCCAGCGGTTCGGCCGGGAACTCAAAGGAGACTGC
CAGTGATAAACTGGAGGAAGGTGGGG

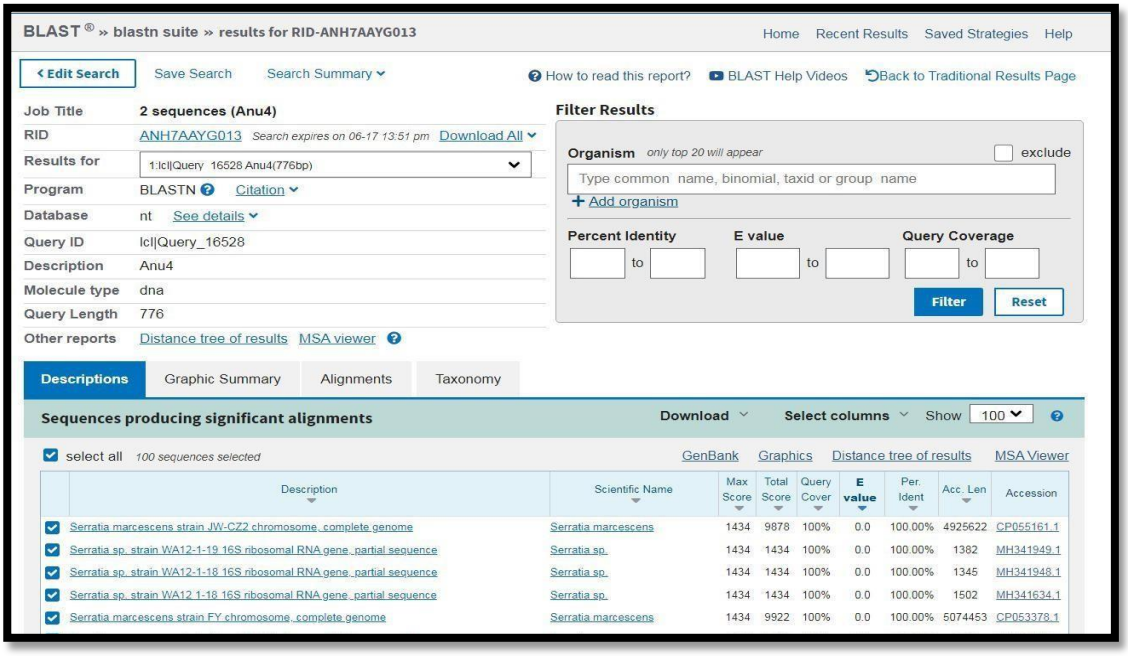

Figure 18: NCBI BLAST list depicting sequence similarity of ZA4

Bacteria	Identification	Percentage similarity	Accession number
ZA1	*Rossellomorea aquimaris*	99.89%	MT122832.1
ZA2	*Stenotrophomonas maltophilia*	100%	MT645776.1
ZA3	*Bacillus paramycoides*	99.88%	ON150894.1
ZA4	*Serratia marcescens*	100%	CP055161.1

Table 6: Identification of bacteria by sequencing

DISSCUSSION

In the current study, the primary aim was to isolate pigment producing bacteria, for that, diverse location sites were chosen as study areas. Soil samples were collected from different areas including mangrove, agricultural land, poultry farm garden soil, compost pit, Canal and waste dumping area. The sample from mangrove was collected from the soil neighbouring to the roots of mangrove bushes. This was to obtain microorganisms that may found to live along with the roots of mangroves. Another study was conducted by McPherson in the year 2018, aimed at learning which microbes are exclusively inside the root and how they differ from microbes immediately outside the root in the rhizosphere. The amplicon sequencing used in thisstudy identifies the microbial taxa found in the DNA sample and allows investigators to determine how the communities change depending on sample type or treatment. Thestudy was done by collection of samples exclusively from roots of the plant. Desired plant was selected and obtained the sample by shaking the roots to remove soil or usea spade or a hand-held tiller to remove soil from the roots. After shaking, the bulk of the soil is collected in a wash pan and then mixed the soil in the wash pan and crushedany soil clods with a hand-held tiller for further studies.

In the present study, soil samples were collected in sterile, polythene bags from specific study areas from 4 cm to 10 cm deep into the surface ground, so that the soil is well obtained in its natural condition with most of the microorganisms present and is uninterpreted from any external disturbances. Later these samples were subjected to serial dilution to form up to 10^{-10} concentration and used for further study.A similar study was done by Burh in the year 2011. Samples were collected from top 4 cm soil profile where most of the microbial activity takes place, and thus where mostof the bacterial population is concentrated. Soil samples were collected in clean, dry and sterile polythene bags using sterilized spatula, and were carried to the laboratory for further analysis. Then the sample was serially diluted by adding I gram of soil to 9ml of sterile water. This is repeated up to three dilutions (10-1 up to 10-3).

In the present study, Sterile water was used as the diluent for the Serial dilution technique. The samples were diluted to a concentration of 10^{-10} dilutions. According to a study conducted by McLaughlin in the year 2010, sterile water, PBS, or saline would be used as simple diluents, while a growth medium (e.g., tryptic soy broth, peptone water) could be used to enrich a bacterial population or subpopulation. Soil is normally added at a rate of 10 g per 95 ml diluent. This essentially provides an initial 10-fold dilution based on the moist soil (10 g) in the suspension.

In the present study, following serial dilution, pour plate technique was carried out in order to obtain bacterial colonies. Individual sample dilution concentrations were poured into petri plates having enrichment media for the optimum growth of bacteria. In th study, Nutrient agar medium was used as growth media and after pouring the diluted samples, the plates were maintained at 37ºC for 24 hours. A similar study was conducted by Kumar in the year 2019. According the mentioned experiment, pour plate method is used to count the number of microorganisms in a mixed sample, which is added to a molten agar medium prior to its solidification. Molten agar should be cooled to 44 before plating otherwise it may lead to death of the desired organism. The process results in colonies uniformly distributed throughout the solid medium when the appropriate sample dilution is plated. This technique is used to perform viable plate counts, in which the total number of colonies forming units within the agar and on surface of the agar on a single plate is enumerated. Viable plate counts provide a standardized means to generate growth curves, to calculate the concentration of cells in the tube from which the sample was plated, and to investigate the effect of various environments or growth conditions on bacterial cell survival or growth rate. This method is advantageous when our organism is environment bacteria and the prevalence is less.

In the present experiment, the screening of desired bacterial colonies was done by examination of the pour plates after 24 hours on incubation at 37ºC. Colonies showing unique pigments with dissimilar morphological features were chosen for pure culture preparation and further molecular characterization. A similar study conducted by Taragini in the year 2014, states that the screening of pigment producing soil bacteria

using morphological characters can done between two to ten days after incubation. In the mentioned work, various colonies were seen after 2 days, whereas orange pigmented colonies were observed after 10 days of incubation. Those colonies which showed colour were picked and sub-cultured for further experimentation.

Identification of bacteria includes phenotypic and molecular characterization. Phenotypic characterization of any microorganisms is the first step towards its identification and taxonomic classification. However, many microbes from the same genus share overlapping phenotypic characters and therefore, they are difficult to distinguish only on the basis of phenotypic tests (Rai and Mukherjee 2010).

In the current work, the soil from mangrove confers the presence of orange pigmented bacterial colony. In a similar study conducted in a suburban mangrove forest in Bangladesh by Saha *et al.,* in the year 2017 reveals that among the 30 isolates, yellow (30%) and orange colour (30%) pigments dominated over the others. Various types of pigments were noticed among the isolated bacteria and it might help to survive the bacteria in the saline environment. By culture these bacteria in different growth medium, they found that, among them LB was found to be the most suitable for all the bacterial isolates to produce better pigment. The bacterial isolates were cultured in various range of, temperatures and salt concentrations to find out the optimum pH, temperature and salt concentration for maximum bacterial growth and pigment production. It was found that optimum pH, temperature and salt concentration were 6.5, 37°C and 2 - 14%, respectively both for culture and pigment production.

In the current experiment, yellow pigmented bacterial colony was isolated from poultry farm soil. The bacteria exhibited smooth translucent colonies. A similar studywas conducted by Indra Arulselvi *et al.,* in the year 2017. They sourced samples fromfertile land, wasteland and sewage areas from different regions. From the 41 soil samples, 24 yellow pigmented colonies were isolated. Microscopic observation showsthat they are arranged in clusters. It was found that carotenoid production of the organism was mainly dependent on the environmental conditions and the compositionof the culture medium and solvent. The findings emphasized that predominant numbers of the yellow pigmented colonies were obtained from the soil samples of fertile land and hill stations.

where the climate is slightly colder. Preliminary morphological observations revealed that the colonies were circular, convex and yellow in colour. Gram staining showed that the bacterium was gram positive, cocci.

In the present study, pink coloured bacteria were isolated from waste soil sample. The colony showed complete light pink shade by 24 hours of incubation at 37ºC in Nutrient agar medium. According to the observation of current experiment, the morphology is large circular, entire, flat and rough colonies. In a study conducted by Kumar *et al.*, 2009 sources samples from leaves of a Neem tree. They found that out of the fifteen leaf imprints, twenty (20) PPFM bacterial isolates were obtained in this study. Pink colonies were observed by the 6^{th} to 7^{th} day of incubation. Other bacterial and fungal colonies also appeared during the one-week observation period. The colonial morphology of the isolates possessed round, raised, smooth margined and translucent colonies.

In the present study, red pigmented bacteria was isolated from agricultural land soil. Red pigmentation was visible by more than 24 hours of incubation in Nutrient agar medium at 37ºC. The morphology showed circular, entire glistening colonies. A similar study was conducted by Anzum *et al.,* in the year 2022. A total of ten soil samples were randomly collected from ten different quadrates of a swamp Forest. They found difference in bacterial count in different samples collected and suggested that it might be due to the difference between the physical and chemical properties of soil and biotic-abiotic stresses that might lead to pigment production of the bacteria present at that particular habitat as their defence mechanism. The indigenous organisms ecologically adapted to a particular habitat may be the cause of differences between isolated and referred organisms. In course of this adaptation their characteristics couldbe modified gradually. The results revealed that pigment producing bacteria are randomly distributed in the soil in the swamp. During their study, 71 pigment- producing bacteria were isolated from the different samples and finally 11 isolates were selected for conventional identification. Two Gram-negative isolates were identified as *Serratia marcescens* and *Erwinia stewartii.* Which is reported by red and light orange pigment production.

In the present study pigment producing microbes ZA1, ZA2, ZA3, and ZA4 were identified by molecular methods with the help of universal primers and sequenced. The gene sequence was subjected to GenBank BLAST search analysis.

In the present study, using molecular methods, ZA1 was identified as *Rossellomorea aquimaris.* A similar study was conducted by Gupta *et al.,* in the year 2020. They found that *R.aquamaris* is a rod-shaped, moderately halophilic or halotolerant aerobic bacteria. They exhibit Gram-positive or Gram-variable staining response. The bacteria form endospores and are motile by means of single polar flagella or peritrichous flagella. The bacterial colonies generally show yellow-orange pigmentation. Most species are catalase-positive and oxidase- negative. Mainly isolated from marsh lands and marine environments. Optimum growth of the bacteria occurs in the range 30–37°C at pH 6.0–8.0 and generally in the presence of 2–5% (w/v) NaCl. Members of this genus form a monophyletic clade in phylogenetic trees based on concatenated sequences for several large datasets of proteins. *Rossellomorea aquamaris* named for the Spanish microbiologist Prof. Ramon RosselloMóra, Universitat de les illes Balears, Spain, for his work on microbial diversity and systematics in diverse habitats and his contributions to these fields as editor of the journal of Systematic and Applied Microbiology.

The bacterial sample ZA2 was identified as *Stenotrophomonas maltophilia,* a non-fermenting gram-negative rod. A similar study conducted by Said *et al.,* in the year 2021 confers that *S. maltophilia* is frequently isolated in the environment, particularly from water bodies like rivers, wells, and lakes, also bottled water, sewage, swine/chicken feces, soil, plants, salads, frozen fish, and raw milk. It has also been isolated from animals, especially aquatic species, with some of those animal isolates found to be within the same genogroups as the human strains, suggesting the possibility of strain or gene exchange with the human infection-causing isolates. Specific isolates are being utilized for the biosynthesis of organic compounds or their degradation, plant growth promotion against agricultural fungal pathogens, and bioremediation of soil or water. The first noteworthy virulence factor is its ability to form a biofilm, which consist of a polymeric matrix of polysaccharides, proteins,

lipids, nucleic acids, and minimally active bacteria, which can become mature very rapidly, colonizing new surfaces within less than 24 hours. The process starts with adherence to the surface, followed by irreversible attachment, then final maturation, and is mediated through its motile flagella, fimbriae/pili, adhesins, and the outer membrane lipopolysaccharide positively charged surface, quorum sensing by diffuse single molecules, and extracellular polymeric substances. Often, the biofilms are polymicrobial and contain other organisms that benefit from the same advantages. The *spgM* gene was recognized to promote high biofilm production by isolates, along with several other genes. *Stenotrophomonas maltophilia* in biofilms was also shown to tolerate a wide range of pH, nutrient scarcity, and exposure to free radicals.

The bacterial sample named ZA3 was identified as *Bacillus paramycoides*. These are Gram-stain-positive, facultatively anaerobic, non- motile, rod-shaped bacteria. Endospores are not observed. The bacterial colonies are waxy, circular, non-translucent and it was found that colony were having 2–3 mm in diameter after incubation at 32ºC for 48 h on LB medium. *B. sp.* shows whit to pink pigmentation. Catalase and oxidase tests are positive. Growth occurs at 15–39ºC (optimum 30 C), at pH 5–9 (optimum pH 7) and with 0–5 % (w/v) NaCl (optimum 0.5 %). Hydrolyses starch, skimmed milk and casein.

The fourth bacterial sample ZA4, was identified as *Serratia marcescens*, a gram-negative bacillus that occurs naturally in soil and water and produces a red pigment at room temperature. It is a species of rod-shaped bacteria. A study conducted by Roy *et al.*, in the year 2014 suggested that these bacteria are motile, aerobic, non-lactose fermenting which belongs to the family of Enterobacteriaceae, both assortment of pigmented and non-pigmented form exits depending on various habitats including main soil, water, plant also vertebrate and invertebrate hosts. The organism is widespread in the environment but not a commonly recognized component of the human fecal microbiota; thus, most infections appear to be acquired exogenously. Pigmented biotypes of Serratia marcescens are mostly recovered from natural environments, whereas the non-pigmented biotypes are prevalent in the hospital. Non-pigmented strains of Serratia marcescens are generally more resistant to antibiotics

than pigmented strains because they often harbour resistance plasmids. Infections caused by the organism may be difficult to treat because of resistance to a variety of antibiotics, including ampicillin and first and second generation cephalosporins. Recently, cytotoxin production was detected in non-pigmented isolates of *S*erratia *marcescens*, and this characteristic has been considered an important virulence factor in several species of bacteria. However, the occurrence of cytotoxin in pigmented Serratia marcescens remains to be demonstrated. Therefore, due to the presence of drug-resistant plasmids and cytotoxin production, the nonpigmented strains have emerged as significant pathogens of nosocomial infections.

CONCLUSION

Microorganisms have been used for a long time for production of molecules as diverse as antibiotics, enzymes, vitamins, texturizing agents, and so on. In nature, pigmented bacteria, yeasts, or fungi are quite common, and the main functions of pigments in these living organisms are related to light. Concerning natural pigments, microorganisms have been shown to be a valuable niche for acquiring harmless pigments, with various biotechnological applications that can be easily produced and scaled. This is where, of all existing microorganisms, bacteria must be considered an inexhaustible source of new natural pigments, which have been shown throughout this review to have an endless number of beneficial properties that can be applied as biomedical treatments.

The present study entitled, "Isolation and molecular characterization of pigment producing bacteria from different soil samples" encompasses on diverse nature of soil from different locations and about the presence of pigmented bacteria in specific soil samples. Study areas included two districts of Kerala, namely Ernakulam and Kottayam. Test samples were sourced from different regions like mangrove, garden soil, Canal soil, poultry farm soil, waste soil and agricultural land. These were undergone serial dilution to obtain the sample in different concentrations and further subjected to pour plate method. About nine pigmented colonies were observed, out of which morphologically dissimilar colonies were separately grown for obtaining pure culture. This was done by Quadrant Streak technique.

The selected bacterial strains namely, ZA1, ZA2, ZA3 and ZA4 exhibited pigments such as orange, yellow, pink and red. These pure cultures were undergone morphological test based on the characteristics on Nutrient agar plate including colony shape, margin, texture and elevation. Further, the selected strains were subjected to DNA isolation molecular characterization. The genomic DNA isolation, its PCR amplification and sequencing of 16S rRNA reveals that the isolated strains may be the following: ZA1- *Rossellomorea aquimaris,* ZA2- *Stenotrophomonas maltophilia,* ZA3- *Bacillus paramycoides and* ZA4- *Serratia marcescens.*

Bacterial pigment production is now one of the emerging fields of research and the isolated strains in the present study demonstrate its potential for various industrial applications.

Many synthetic dyes contain toxins that are harmful to humans and animals. Though utilization of by-products is a sustainable strategy for minimizing environmental contamination and concomitant pigment production, it has major bottlenecks. One of the major challenges encountered is the limited potential of natural pigment producers, which have been majorly used for production from agro-industrial wastes. Therefore, the use of genetic, metabolic or protein engineering could be a viable strategy for developing improved strains for pigment production. Efforts in finding new microbial sources for pigment production and decrease in production cost through optimization, strain improvement and genetic engineering have to be carried out to eradicate toxic synthetic dyes. The task of discovering pigments suitable for being used as food colorants is a difficult one, as pigments must not only produce a desired colour, but also be safe for human consumption.

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APPENDIX -I (MICROBIOGY MEDIA)

Nutrient agar

Peptone	5.0 g
NaCl	5.0 g
Yeast extract	3.0 g
Agar	15 g
Distilled water	1000 ml
Adjust pH to 6.8	

Nutrient broth

Peptone	5.0 g
NaCl	5.0 g
Yeast extract	3.0 g
Distilled water	1000 ml
Adjust pH to 6.8	

APPENDIX II (MOLECULAR BIOLOGY)

Genomic DNA Extraction from bacteria

1. Materials Required 0.85 % (W/V) NACL Solution

NaCl	0.85 g
Distilled water	90 ml
Add NaCl to distilled water and make up to 100 ml, Autoclave and store at 4 °C	

2. 0.5 M EDTA

Na2EDTA.2H2O	4.65 g
Distilled water	20 ml
Adjust the pH to 8 using 1 M NaOH and make up to final volume to 25 ml, Autoclave and store at 4°C.	

3. 1M Tris (pH 8.0)

Tris - Cl	3.93 g
Distilled water	20 ml
Adjust the pH to 8.0 using HCl and make up to the volume to 25 ml, Autoclave and store at 4° C.	

4. 20% SDS

SDS	5.0 g
Distilled water	20 ml
Make up to final volume to 25 ml and store at 4 °C	

5. **0.1 M Tris HCl (pH 8.0)**

1M Tris HCl	15 ml
Distilled water	135ml

6. **Chloroform: Isoamyl alcohol (24:1)**

Chloroform	48 ml
Isoamyl alcohol	2 ml
Both of the components are mixed properly by inversion	

7. **3 M Sodium acetate (pH 5.2)**

Sodium citrate	6.2 g
Distilled water	20 ml
Adjust the pH to 5.2 using glacial acetic acid and make up to the final volume to 25 ml , Autoclave and store at 4 ℃	

8. **TE Buffer (DNA dissolving solution)**

10 mM Tris	20 ul from 1 M Tris stock
1mM EDTA	4 ul from 0.5 M EDTA stock
Make up to 2 ml and store at 4 ℃	

9. **70% Isopropanol**
10. **Nutrient broth**

Bacto Tryptone / peptone	1 g
Yeast extract	0.5 g
Sodium chloride	1 g
Distilled water	100 ml

Add the ingredients to 90 ml of Deionised water, stir for complete solubility. Adjust the pH to 7.0 with 5M NaOH and make up to the volume to 100 ml using deionised water. Sterilize by autoclaving for 20 min at 15 lbs / sq. in.

11. Lysis buffer

Final conc.	**Stock**	**Required stock volume**
50 mM EDTA	0.5 M EDTA (pH 8.0)	5 ml
100 mM Tris	1 M Tris (pH 8.0)	5 ml
3% SDS	20% SDS	100 ml
	Distilled water	32.5 ml
	Total volume	50 ml
The buffer should be stored at 4ºC		

12. Proteinase K

Protienase K	20 mg
Sterile distilled water	1 ml
Dissolve proteinase K in 1 ml of autoclaved distilled water and store at 20°C	

AGAROSE GEL ELECTIPHORESIS

Composition of 0. 08 % Agarose

Agarose	0.24 g
1X TBE Buffer	30 ml
Prepare 1X TBE Buffer from the 10 X TBE, and dissolve the 0.24 g of Agarose in 1X TBE Buffer by boiling.	

Composition of 1.5 % Agarose

Agarose	0.45 g
1X TBE Buffer	30 ml
Prepare 1X TBE Buffer from the 10 X TBE, and dissolve the 0.45 g of Agarose in 1X TBE Buffer by boiling	

Composition of 5X TBE Buffer (pH 8.0) for 1 Litre

Tris	54 g
Boric acid	27.5 g
0.5 M EDTA	20 ml (0.5 M EDTA)
Autoclave and store at 4°C	

Composition of loading dye (Bromophenol blue)

0.5 %Bromophenol blue	100 ul
Glycerol	300 ul
1X TBE Buffer	600 ul

Standard DNA markers

Commercially available DNA markers were used as standard molecular weight DNA markers to determine the weight of DNA /PCR amplification. Standard 100 bp markers and Lambda DNA /EcoR1 HindIII double digest markers were used.