ISOLATION AND CHARACTERIZATION OF PIGMENT PRODUCING BACTERIA AND ITS POSSIBLE ADVANTAGE AS BIO COLOURS

MUNEEFA K. I

(19MPBCF002)

M.Phil Biochemistry

A Thesis submitted in Partial Fulfilment of the
Degree of Master of Philosophy
(M.Phil)

Department of Biochemistry,
Biotechnology and Bioinformatics.

Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore-641043

January 2021

CERTIFICATE

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29/1/2021

Signature of the Head of the Department | **Signature of the Supervisor**

CERTIFICATE FROM THE SUPERVISOR

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I certify that the dissertation entitled **Isolation and Characterization of pigmented bacteria and its possible advantage as bio colours** submitted for the degree of **Master of Philosophy (M.Phil.)** is the record of research work carried out by her during the period from **July, 2019** to **January, 2021** under my guidance and supervision, and that this work has not formed the basis for the award of any Degree, Diploma, Associateship, Fellowship or other Titles in this University or any other University or other institution of Higher Learning.

P. Aruna

Signature of the Supervisor with designation

ASSOCIATE PROFESSOR

DECLARATION

DECLARATION

I declare that the dissertation entitled **Isolation and Characterization of pigmented bacteria and its possible advantage as bio colours** submitted by me for the degree of **Master of Philosophy (M.Phil.)** is the record of work carried out by me during the period from **July, 2019** to **January, 2021** under the guidance of **Dr.T.Angayarkanni** and has not formed the basis for the award of any Degree, Diploma, Associateship, Fellowship, Titles in this University or any other University or other similar institution of Higher Learning.

Signature of the Candidate

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INTRODUCTION

1.0 INTRODUCTION

Microorganisms produce variety of pigments, so they can be used as natural colorants instead of synthetic colorants. Microorganisms found in different environments produce various pigments. These pigments are carotenoids, flavins, chlorophyll, quinines, prodigiosins. These natural pigments can be used in various commercial fields of pharmaceutical, food, textile and food industry. β – Carotene, riboflavin and lycopene are food grade pigments which are used in food industries (Mali *et al.*, 2017).

Microorganisms are the most effective way to create a range of molecules like pigments, antibiotics and enzymes in biotechnology (Selvi and Iyer, 2018). Many of the synthetic dyes used has never been screened for their toxic potential to the environmental and health because of their corrosive potential (Sajjad *et al.*, 2020). Dyes are used in lots of products now a days and it contains lots of harmful chemicals. It can easily fermented in a cheap culture and can produced in a lots of colours (Sharma, 2019). Most beautiful function of the metabolites are its ability to produce colours and it mostly occurs inside the cytoplasm (Pagano and Dhar, 2015).

Microbial sources like bacteria and some fungi can produce pigments that give the bacteria colourful appearance. There are several researches about bacteria producing different colour pigments. *Staphylococcus aureus*, *Micrococcus luteus* and *Malleomyces mallei* produce yellow pigments. *Mycobacteria* strains, *Prevotella melaninogenicus*, *Serratia marcescens*, *Chromobacterium violaceum* and *Planococcus citreus* produce orange, black, red, purple and lemon-yellow pigments, respectively. *Pseudomonas aeruginosa* is known as green, blue and fluorescence pigments producing bacteria. Some pigments are produced by bacteria as extracellular and intracellular. Intracellular pigments do not change the colour of the medium as they do not get soluble in water. So the colour is limited to the bacterial colony. Extracellular produced pigments can easily soluble in water, so they give their colours on media. It can be obtained from different processes such as extraction, isolation or synthesis (Simsek *et al.*, 2018).

Some of the microbial colorants not only function as natural colourants, but also possess anticancer, antioxidant, anti-inflammatory and antimicrobial properties. Hence, natural colorants have huge importance due to the available technology for fermentation and the stability of pigment production, because microbial colours are available in numerous shades of colours. These colors are eco-friendly and biocompatible (Sharma, 2020).

In food industry, colours are used because it gives a vibrant beautiful appearance to the food which will make the food very desirable. But chemically synthesized colorants give lots of harmful to human body if we consume it. So natural colorants are used to give colour to foods. It can also achieved by modifying the genes in a bacteria to produce desirable colours. So scientist can create a pigments with no toxicity (Jensen *et al*., 2011).

Lactobacillus sp. are a major group of rod shaped Gram-positive, anaerobic and lactic acid bacteria. They can ferment carbohydrates, catalase-negative and oxidase-negative (Bratcher, 2018). These organism have lots of functional and desirable characteristics. Studies also have been published that they also associated with cognitive ability and have enhance physiological function. (Wang *et al*., 2020). Several articles also reported about the effect of *Lactobacillus* sp. on oxidative stress (Aabed *et al*., 2019).

The genus *Exiguobacterium* sp. can be isolated from different temperature varying between 12 ^{0}C to 55 ^{0}C. This genus is habitat to moderate thermophilic, mesophilic and psychrotrophic organism and straints with agriculture, bioremediation, industrial and biotechnological properties. This species produce astaxanthin, which can contribute lots of advantages in pharmaceutical industry. The value of integrating various molecular methods to effectively grasp the bacterial tolerance to cold was demonstrated in recent studies using *Exiguobacterium antarcticum.* In cold environment this organism shows a possible change in the fatty acid metabolic pathway (Barauna *et al*., 2017).

Pigment are the important organic constituents of bacterial protoplasm. Some of the pigments are prodigiosin, violacein, iodinin, indigoidine and melanin are metabolic by-products formed under special circumstances. Natural pigments have lots of demands in the food industry. When colours are derived from natural sources like microrganism, plants and animals they considered as natural colorants. So lots of industries now able to produce some microbial pigments for applications in food, cosmetics and textiles industries are . In nature, colour rich and pigment producing microorganisms like fungi, yeasts and bacteria are quite common.

The pigments was isolated from species such as *Serratia plymuthica*, *Serratia rubidaea*, *Hahella chejuensis* and *Vibrio gazogenes* . These pigment have been reported to have antifungal, antibacterial, algicidal, antiprotozoal, antimalarial activities, immunosuppressive and anticancer activities. Carotenoids is a group of bioactive compounds which are responsible for yellow, orange and red pigments. Carotenoids are abundantly plants, microorganisms and animals and are widely distributed in the nature (Pratik *et al.*, 2019)**.**

Prodigiosin is a red colour pigment. In bacteria we can found that this colorants was formed in the growth stages. It is named after the microorganism *Bacillus prodigious.* (Sajjad *et al*., 2020).

Melanin is a dark color pigment which is not soluble in water and organic solvents. It mostly present in low temperature. This pigment act as a protective layer to microorganisms because of its well absorption to UV radiation.

Violacein, is named after the pigment color which is purple or violet. It is found to be not soluble in aqueous solvent. It shows antileishmanial activity. It is used in the production of biofilms (Marizcurrena *et al*., 2019).

Indigoidine is a very rare blue color pigment only found in few microbes. It is soluble in aqueous solution. The main role and function of this pigment is still unknown. But some studies shows that it have antimicrobial activity and act against oxidative stress (Cude *et al*., 2012).

Carotene and xanthophylls are two different type of carotenoids. Provitamin A is another category of carotenoid. It is lipophilic in nature. So the extraction of this pigment is very easy. Carotenoids act against disease causing bacteria. So many species are not able to grow in the presence of this pigment because of its antibacterial activity (Ravikumar *et al*., 2016).

Oxygen is one of the prominent molecule present in carotenoids. It arrest the cell cycle which cause cancer. It decrease the inflammation in the cells and eliminate superoxide anion which will result in decrease in cardiovascular diseases by increasing the cell mechanism (Pietro *et al*., 2016).

Some microorganism can infect the cornea. Deficiency of vitamins can cause night blindness. Eating carotenoid containing food can help to reduce ophthalmic infections (Bhatt and Phatel, 2020). Our lifestyle and the fast food intake are the main reason for obesity and hyperglycemia. It also can reduced by carotenoid intake. There is lots of multidrug resistant bacteria are present in the environment. Carotenoids are proved to fight against those pathogens (Kumar *et al*., 2020).

Depending upon the concentration of pigments, the production could visibly provide coloration to show such as green due to chlorophylls, various yellow shades due to xantophylls, and orange to red due to carotenoids (Anesio *et al*., 2017).

This suggests that the pigments produced by microorganisms will very soon dominate the organic market and the pigment industry (Ramesh *et al*., 2019). Natural pigments can substitute environmental friendly colorants for synthetic colorants in the textile industry. Pigments derived from microorganisms are safe to the environment and considered ideal for the textile industry (Chadni *et al*., 2017).

Violacein derived from bacterial sources has been used in food industries. Canthaxanthin is used in food products such as drinks, snacks, meat, vegetables, fish, sweets, and cheese. Natural colorants are used in food industries. Many pigments obtained from microorganisms are approved and used for several purposes in foods (Dufosse, 2018).

Several forms of studies have been carried out on microbial pigments as anticancer agents. Novel red pigment from *Athrobacter* sp. Shows anticancer potential against the line of oesophageal cancer cell lines (Afra *et al*., 2017).

Nearly one million tons of dyes are developed and used by the textile industry, almost all of them synthetically produced. Synthetic colorants have certain limitations like hazardous chemicals are needed in their production process, concern for the safety of workers, hazardous waste can be generated and these colorants are not ecological-friendly. In recent years, the biosynthesis of natural dyes for textile applications has sparked growing interest. In several papers, the textile-dyeing potential of micro-organisms has been investigated. It indicates that when applied to cotton, silk and wool pigmented micro-organism gave an excellent colour (Usman *et al*., 2017).

Most of the humans are died due to infectious disease now a days. These infections are caused by pathogenic microbes. Natural pigments have the ability to fight against these types of pathogens. Phenazine can used as medication against these infection causing microbes. It also used in pharmaceutical industry. Flexirubin is a red colorant which can scavenge the free radical from our body. Treatment for cancer is still a major challenge now a days due to its severity and side effects. Research shows that these natural pigments have lots of ability to cure cancer but still it is in a research level (Sajjad *et al*., 2020).

Natural colours not only boost the product's marketability, but they also have other qualities, such as anticancer and antioxidant properties. These microbial pigments are commonly used in the food industry, in the pharmaceutical industry and in the textile industry. (Sharma, 2020).

With this above background the present study entitled "**Isolation and characterization of pigment producing bacteria and its possible advantage as biocolours"** is framed with the following objectives:

- Isolation of pigment producing bacteria from various sources.
- Identification of isolated pigment producing bacteria
- Extraction of pigments from pigmented bacteria
- Characterization of extracted pigments
- Strategic manipulation of the growth media for maximum pigment production by bacterial isolates
- Antioxidant potential
- Antibiotic susceptibilty test of selected isolated bacteria
- Textile application of extracted pigments.

REVIEW OF LITERATURE

2.0 REVIEW OF LITERATURE

The review of literature pertaining to the present study entitled "**Isolation and characterization of pigment producing bacteria from and its possible advantage as bio colours"** is discussed under the following headings:

2.1 Synthetic colorants

2.2 Modified Natural dyes

2.3 Microbial colorants

2.4 Microorganisms as natural colorants

2.5 Advantages of bacterial pigments

2.6 Classification of Pigments

- 2.6.1 Prodigiosin
- 2.6.2 Melanin
- 2.6.3 Violacein
- 2.6.4 Indigoidine
- 2.6.5 Scytonemin
- 2.6.6 Carotenoids

2.7 Properties of carotenoids

- 2.7.1 Antibacterial and Anti-viral activity
- 2.7.2 Anti-Inflammatory activity
- 2.7.3 Anti-cancer activity
- 2.7.4 Cardiovascular diseases
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- 2.7.8 UV Radiation Protection
- 2.7.9 Anti-Tuberculosis
- 2.7.10 Regenerative liver

2.8 Role in food Industries

2.9 Role in Pharmaceutical industries

2.10 Role in Dying Industry

2.1 Synthetic colorants

Colorants are used in lots of products include cosmetics, food and textiles. These colorants are synthetic and very harmful for humans. It is very harmful to environment because it contain toxic compounds and non-biodegradable. Several synthetic colorants contains dioxins. Dioxin can cause disorders in digestive, immune and nervous system. These dioxin containing synthetic dyes when disposed contaminate the environment and accumulates in living organisms which is very harmful for our eco-system. So now a days there is a growing demand for natural dyes which can be biodegradable and less harmful for the humans and our environment (Devyani *et al*., 2017).

2.2 Modified Natural dyes

Natural dyes are dyes or colorants derived from plants, invertebrates, or minerals. The majority of natural dyes are vegetable dyes from plant sources—roots, berries, bark, leaves, and wood and other biological sources such as fungi and lichens. Since the dawn of synthetic dyes, focus has completely shifted away from the application of natural dyes in textile dyeing However, due to the stringent environmental standards imposed by many countries in response to the hazardous effluent generated during synthesis of synthetic dyes, the textile researchers have once again been enthralled by natural dyes. Today, many are rediscovering the joy of achieving colour through the use of renewable, non-toxic, natural sources by using natural dyes as available or by modifying them by various methods (Shahid *et al*., 2013). Since coupling with metal ions also reduced the naturality of the so obtained dyes, only the work involving dye modification without metal ion coupling has been considered. Availability of very limited data on the use of modified natural dyes is facilitated by the fact that this method is relatively very new and no commercial scale progress of this process has been observed. Nevertheless, research on this method still continues based on the advantages offered by this process and its future prospects (Sharma, 2019).

2.3 Microbial colorants

Cultivation of microorganisms can be accomplished through solid state and submerged fermentation on natural raw material or industrial organic waste. Microbes can grow easily and at a very fast rate in the cheap culture medium and their growth is independent of the weather conditions. Many of the microbial pigments not only act as colorants, but also possess anticancer, antioxidant, anti-inflammatory, antimicrobial properties. Hence, microbial pigments are of great interest owing to the stability of the pigments produced and the availability of cultivation technology. Microbial colours are available in different shades. These

Colours are biodegradable and environment friendly. Only limited research studies are available on exploration of microorganisms for colour/pigment production especially in the Indian scenario, which really points towards exploring microbial pigments in more detail (Sharma, 2019).

2.4 Microorganisms as natural colorants

Among these metabolites, pigments are charismatic traits of micro-organisms that are studied to exploit for several industrial applications. Pigments production mostly occurs within cytoplasm. It is a responsive agent to hostile ecological conditions presenting several ecological functions (Pagano and Dhar, 2015). Various natural products from macro algae metabolites plays an important role in pigment productions. Algae has been widely used as bioactive source metabolites such as proteins, lipids, mineral salts, polyphenols and polysaccharides. They are very useful in various indusries like food, cosmetics and pharmacy. Red algae has an extraordinary potential like pharmaceutical, nutraceutical and cosmeceutical prospects (Lunggani *et al*., 2020).

2.5 Advantages of bacterial pigments

Pigments produced by bacteria are of traditional use in oriental countries and have been a subject of intense research in the present decades because of its potential for applications. Bacterial pigments offer the following benefits and advantages as follows:

- Increasingly attractive to science because of broad ranging activities.
- Easy propagation and wide strain selection.
- High versatile and productive over other sources.
- Fermentation is inherently faster and more productive compared to any other chemical process.
- Easy to manipulate genes.
- Simple and fast culturing techniques allowing continuous bioreactor operation. Structural complexity suits for industrial needs.
- Cheap substrates used for bulk production (Usman *et al*., 2017).

2.6 Classification of Pigments

2.6.1 Prodigiosin

Prodigiosin is a red linear tripyrrole pigment primarily reported from *Serratia marcescens.* Prodigiosin was named after its extraction from *Bacillus prodigious* later given the name of *S. marcescens* Prodigiosin produces only in later growth stages of bacteria. Biosynthesis of prodigiosin is controlled by quorum sensing (Bhatt and patel, 2020). Prodigiosin was extracted from psychrotrophic bacterial strain *Janthinobacterium lividum* isolated from Alaskan soil (Schloss *et al*. 2010). Genes in Antarctic volcanic island sediments responsible for the biosynthesis of 3-oxoacyl-[acyl-carrying protein] reductase (K00059) enzyme that belongs to the fatty acid synthesis pathway type II associated to the production of prodigiosin (Centurion *et al*., 2019).

2.6.2 Melanin

Melanin is dark in color (brown to dark green, or fully black) and higher molecular weight biological pigment found in hair, feather, skin, eyes, scales, and some interior membranes. Melanin is chemically a polymerized product of phenolic and/or indolic compounds (Tarangini and Mishra, 2014). Melanin is further classified into three groups based on structure and colour (i) pheomelanins (red or yellow), (ii) eumelanins (black-brown), and (iii) allomelanins (black to dark brown). Due to variations in the occurrence and structure of melanin, its biosynthesis is not from a single route. Melanin synthesis has been associated in providing resistance against UV- and visible light-irradiations, confrontation the attack of cell wall enzymes, fortification against oxidizing and reducing agents, and acts as an antiviral agent to enhanced the competitive and survival abilities in environmental stresses (Solano, 2014).

Melanin is a strong absorber of UV radiations and provides strong protective functions to microbes that’s why extreme low temperature ecosystems are suitable habitats for microbial biosynthesis of melanin (Gessler *et al*., 2014).

In general, melanin pigment is insoluble both in organic and aqueous solvents, however, isolated bacteria strain *Lysobacter oligotrophicus* from the Antarctic environment that produced water-soluble heteropolymer (Lo-melanin). Lo-melanin protects against UV radiation and scavenges ROS (Kimura *et al.*, 2015). In the Antarctic rocky deserts, rock-inhabiting fungi produce melanin pigments that protect the cells from extreme cold and heat, polychromatic UV radiations, extreme pH and osmotic conditions, and provides tolerance towards potentially toxic metals (Selbmann *et al*., 2015).

2.6.3 Violacein

Violacein is violet or purple bisindole water-insoluble pigment and first reported from *Chromobacterium violaceum* from the Amazon River in Brazil. In nature, violacein protects cells from UV radiations (Füller *et al*., 2016). Several studies have reported the antibacterial, antiviral, anticancer, antiulcerogenic, antileishmanial, and enzymatic modulation activities of violacein pigments (Soliev *et al*., 2011).

In nature, violacein is associated with biofilm production and its production is regulated by quorum sensing thus acts as a marker of quorum sensing molecules (Burt *et al*., 2014). Violacein plays a vital role in protecting bacterial cells from predation (Choi *et al*., 2015). Violacein producing psychrophilic *Janthinobacterium svalbardensis* bacterium was isolated from Glacier, Spirsbergen, Arctic region (Ambrožič *et al*., 2013).

Violacein producing psychrophilic bacterium *Janthinobacterium* sp. Inhibited the growth of *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* (Kim *et al*., 2012) . At low doses, the same pigment showed activity against methicillin-resistant and multiple drug-resistant clinical strains of *Staphylococcus aureus* (Huang *et al*., 2012).

2.6.4 Indigoidine

Indigoidine is a brilliant blue, water-soluble pigment synthesized by very few microorganisms namely *Erwinia chrysanthemi* , *Phaeobacter* sp., *Streptomyces chromofuscus* and *Vogesella indigofera* , and the biological and environmental role of indigoidine is unclear, however, it has been described that this pigment could protect against oxidative stress(Bhatt and patel, 2020). Indigoidine also possesses antimicrobial activities as stated in *Leisingera* isolates (Gromek *et al*., 2016). Consequently, microbes producing indigoidine could have advantage of competition in the environment due to the antibiotic and antioxidant properties of indigoidine. Furthermore, indigoidine acts as intracellular signaling molecules related to motility (Cude *et al*., 2012).

Additionally, indigoidine provides adaptability to microbial cells such as *Vogesella* sp. reported from Andean Patagonia in iron-rich environments (Day *et al*., 2017). Several isolates of Antarctic genus *Arthrobacter* were reported to produce indigoidine (Sutthiwong *et al*., 2014).

2.6.5 Scytonemin

Scytonemin and its methoxylated and methylated derivatives are mostly reported in the upper portion of the microbial mats; that could protect the cells from extreme environments, which makes this pigment a potential biomarker molecule for studying the presumed exobiology habitats. Exposure of cyanobacteria to UV radiation produces scytonemin along with several other protective metabolites (Bhatt and patel, 2020).

2.6.6 Carotenoids

Carotenoids are classified into two groups,

- Carotens are the compound having single long carbon chain
- Xanthophyll is the compound having oxygen atom in its structure.

These carotenoids are further divided into the category of having provitamin A activity. They are able to characterize further into vitamin A. They have lipophilic and hydrophobic nature. So they can be easily extracted from natural sources like green vegetables, flowers, fruits and from microorganisms. The Methods used to extract carotenoids are Supercritical fluid extraction and Solvent extraction (Mezzomo and Ferreira, 2016).

2.7 Properties of carotenoids

2.7.1 Antibacterial and Anti-viral activity

In all over the world billions of bacteria are present and many of them can able to cause chronic diseases and various kind of infections. Some of the natural organic pigments can able to combat against those pathogenic bacteria and hundreds of research experiments show that antibacterial property. According to that the turbidity observations of minimal bactericidal concentration assay shows the lowest concentration of bacterial colonies on nutrient media. This is used for comparison and cross checking. The Minimal inhibitory concentration assay carried out and 95% similarity observed. This study carried out through halobacterial carotenoids against antibiotic resistant microbes which are *Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus epidermis, Pseudomonas aeruginosa* and

Streptococcus pneumonia. The results observed were terrifying as some species were not able to grow under the presence of carotenoids. So we can observe that some bacterial pigments can have antibacterial activity (Ravikumar *et al.,* 2016).

Carotenoids can act as anti-viral agent. Carotenoids has been shown that it helps to fight against viruses. In an experiment on Herpes Simplex Virus Type 1 it was found that *D. salina* extract and *H. pluvialis* extract can reduce its activity ranging from 50 to 85%. Purified microbial pigments contain antimicrobial substances and it have the ability to inhibit the growth of human pathogens (Kamla *et al*, 2016).

2.7.2 Anti-Inflammatory activity

Carotenoids having oxygen in structure like fucoxanthin and astaxanthin. These are proved to suppress the expression of cytokines IL-6, TNF-α and IL-1β and act as pro and anti-inflammatory compounds. Carotenoids scavenges the oxygen radicle, it will not further able to interact with NF-κB which results into macrophage foam cells and decrease in TNF-α (Bhatt and Patel, 2020).

2.7.3 Anti-cancer activity

Several experiments show the activity of carotenoids as anti-cancer agents. Most of cases that carotenoids arrest the cell cycle which is associated with down regulation of cyclin D1, cyclin D2, CDK4 and CDK6 expression. Consequently, it also up regulates GADD45 α, which inhibits the entry of cell into S phase (Almeida *et al*., 2020). Compounds like crocin and crocetin extracted from saffron showed the anti-metastasis properties like anti-migration, antiinvasive and anti non adhesive effects in combination on 4T1 cell line in breast cancer (Azri *et al*., 2020). Carotenoids like β-cryptoxanthin and lycopene are found suppressing the NF-κB signalling pathway. This pathway is effective against lung cancer and prostate cancer (Lim *et al*., 2020). β-carotene also has found to have anti angiogenic activity that is it helps to halt the process of developing new blood vessels which is often seen in tumours cells (Bodade *et al*., 2020).

2.7.4 Cardiovascular diseases

The various experiments which are occurring inside of body and at lab facility have appeared that carotenoids reduced the inflammation and oxidative stress by promoting normal mechanism of cell. Many other scientific studies also show that by including carotenoid rich food helps in reducing cardiovascular disease in patients (Abdalla., 2003) Carotenoids works by evacuating superoxide anion (O2−), in receptive oxygen species (ROS) generation, also have appeared to re-establish nitric oxide (NO) endothelial bioavailability. So they might be viewed as a potential source of oxidant modulators of endothelial reaction to pro-oxidant/inflammatory stimuli (Pietro *et al*., 2016).

2.7.5 Ophthalmic Infections

Staphylococcus aureus can infect cornea (keratitis) or the inner chambers of the eye (endophthalmitis) (Callaghan *et al.,* 2018), *Escherichia coli* in Conjunctivitis , and *Streptococcus pyogenes* and *Pseudomonas aeruginosa* in blepharitis (Teweldemedhin *et al.,* 2017). Deficiency of vitamins can cause night blindness and this can be prevented by intaking of carotenoids in our diet (Bhatt and Patel, 2020).

2.7.6 Neurogenerative diseases

The ability to cross the blood brain barrier, and cell mitochondrial membrane with stability along with antioxidant property carotenoid, astaxanthin can be able to reduce the risk of diseases related to the nervous system. Astaxanthin can combat neurodegenerative diseases by different properties such as anti-apoptosis, reduction in cerebral infarction in brain tissue, lowers ischemia by induced apoptosis, reduction of glutamate release and reduce free radical damage (Kowsalya *et al*., 2019).

2.7.7 Anti-Hyperglycemia

The lifestyle and food habits are the main important reason for hyperglycemia. Due to hypertension oxidative stress is induced in our body which results in obesity, diabetes, dyslipidaemia and hyperhomocysteinemia. Fatty acid radicles and reactive oxygen species play vital role in increasing the GR, GPx and other hormones in our body, which leads to diseases. Carotenoids by scavenging this fatty acid radicle and brings regulatory signals back to normal and reduce 40 to 79% of diseases (Nascimento *et al*., 2020).

2.7.8 UV Radiation Protection

Carotenoids like Beta-carotene and canthaxanthin have explicit photo defensive properties. The reactive oxygen species and other reactive free radicals helps alongside in oxygen quenching, which respond in blood and skin erythropoietic protoporphyria patients. Moreover, erythropoietic protoporphyria patients have shown decreased level of β carotene in their serum. So they need to take β carotene as dietary supplement (Callaghan *et al*., 2018)

2.7.9 Anti-Tuberculosis

Nowadays world is fighting against Multidrug resistant bacteria like *Mycobacterium tuberculosis.* With a lots of experiments on biomass of *C. vulgaris*, this come up with the new therapeutic application of carotenoids as anti-Tuberculosis agent (Kumar *et al*., 2020).

2.7.10 Regenerative liver

Carotenoids are found to be connected with adipose tissue and multiple serum in humans for different metabolism such as insulin sensitivity in liver and adipose tissue (Harari *et al*., 2020). In Most of the cases liver damage happens from the increased level of cholesterol. Astaxanthin can able to function as protection for cells, fats and other membrane proteins towards oxidative damage. The intestinal cells partially allow Astaxanthin enter into chylomicrons. The Astaxanthin dissolved in chylomicrons through lipid enzymes are secreted from lymph to liver. In chylomicrons ROS will be quickly evacuated from different tissues. Astaxanthin have the ability to fight and cure liver (Kowsalya *et al*., 2019).

2.8 Role in food Industries

The development of foods with an attractive appearance is an important goal in the food industry. Increasingly, food producers are turning to natural food colors, since certain artificial color additives have demonstrated negative health issues following their consumption. Due to the lack of availability of natural food colorants, its demand is much especially in food industry. This demand can be fueled by research to offer a more natural healthy way of coloring foods and provide a clean label declaration (Aberoumand, 2011). It is therefore, essential to explore various natural sources of food grade colorants and their potentials. Though many natural colors are available, bacterial colorants play a significant role as food coloring agent, because of its production and easy down-streaming process. 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 (Kamala *et al*., 2016).

Bacteria could be genetically modify 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 colorants 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 (Bener *et al*., 2010).

Scientists have isolated food grade pigments from bacteria and blue pigment from cultured soil bacteria and could offer a natural color with an excellent stability and toxicology profile for food. The researchers from the East China University of Science and Technology reported that the blue pigment taps into the trend for edible natural pigments (Jensen *et al*., 2011). Bacterial colors were already used in fish industry to enhance the pink color of farmed salmon. 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 colors and probiotic health benefits in food products (Venkatasubramanian *et al*., 2011). They are considered safe and approved by FDA. The successful marketing of pigments derived from bacteria, both as a food colour and a nutritional supplement depend on consumer safety and freshness of the products (Soliev, 2011).

2.9 Role in Pharmaceutical industries

Most studies investigated that bacteria have shown the efficacy and the potential in clinical applications and their pigment has been used in treating several diseases and they also have certain properties like anti-biotic, anticancer, and immunosuppressive compounds. Significant progress has been achieved in this field, and investigations of bioactive compounds produced by these microbes are rapidly increasing. As such, the number of compounds isolated from bacteria is increasing faster when compared with artificial counterpart (Soliev, 2011).

Anthocyanins are involved in a wide range of biological activities. Decrease the risk of cancer. Reduce inflammatory insult and modulate immune response. The genus, *Serratia* can produce a red substance called prodigiosin, these substances have been known to have an antibiotic and antimalarial effect and immunosuppressing activity. These researches showed the presence of prodigiosin and metacycloprodigiosin in culture broth of *Serratia* and observed selective inhibition of polyclonal proliferation of T-cells as compared to that of B-cells. Besides that, the cytotoxic potency of prodigiosin has also been investigated in the standard 60 cell line panels of human tumor cells derived from lung, colon, renal, ovarian, brain cancers, melanoma and leukemia. Inhibition of cell proliferation as well as induction of cell death has been observed in these cell lines (Usman *et al*., 2017).

In vitro anticancer activity has also been reported for prodigiosin analogs and synthetic derivative of prodigiosin. The anti-proliferative and cytotoxic effects of prodigiosin have been observed not only in cultured tumor cell lines but also in human primary cancer cells from B-cell chronic lymphocytic leukemia patients. The use of prodigiosin for treating diabetes mellitus has also been reported where prodigiosin was found to be an active component for preventing and treating diabetes mellitus. 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* specie and flexirubin from *Flavobacterium* specie 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 (Usman *et al*., 2017).

2.10 Role in Dying Industry

The textile industry produces and uses approximately 1.3 million tons of dyes, pigments and dye precursors, valued at around U$23 billion, almost all of which is manufactured synthetically. However, synthetic dyes have some limitations, primarily, their production process requires hazardous chemicals, creating worker safety concerns, they may generate hazardous wastes, and these dyes are not environment friendly (Chidambaram, 2013).

Biosynthesis of colorants (natural dyes) for textile applications has attracted increased interests in recent years.The pigments called prodigiosin from marine *Serratia sp*. for application as dye in the textile industry. The results of the study indicated that pigment could be used as natural dye for imparting red color to various grades of textile materials. The color was observed to be stable after wash performance studies. Characterized the bright red pigment prodigiosin from *Vibrio spp*. and suggested that it could be used to dye many fibers including wool, nylon and silk.The capability of using pigment from *Serratia marcescens* to color five types of fabric namely acrylic, polyester microfiber, polyester, silk and cotton using tamarind as mordant. Similar textile-dyeing ability was also reported for *Janthinobacterium lividum* gave good color tone when applied on silk, cotton and wool and nylon and vinylon (dark blue, both synthetic fibers) (Usman *et al*., 2017).

Characterized 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. The potentiality of prodigiosin and violacein in batik making. Kumar et al. [38] reported that red pigment prodigiosin from *Vibrio sp*. *Serrtia sp*. violet pigment from *Chromobacterium violaceum*, anthraquinone from Dermocybe sanguine, pink pigments from *Roseomonas fauriae* and *Fusarium oxysporum, Trichoderma sp.* and *Alternaria sp.* are suitable for in textile industry for dyeing of all fibers including cotton, wool, silk, nylon and acrylic fibers (Ahmad *et al*., 2012).

Among the natural Sources, pigment producing microorganisms hold a promising potential to meet present day challenges. Furthermore, natural colours not only improve the marketability of the product but also add extra features like anti-oxidant, anti-cancer properties etc. These microbial pigments have broad area of application, mainly in food industries, pharmaceutical industries and textile industries. Food grade pigments such as β-carotene, Arpink Red, Riboflavin lycopene and Monascus pigments are used in food industry. In pharmaceutical industry pigments like Anthocyanin, Prodigiosin and Violacein are widely used to treat diseases. Several microbial Pigments are also used in textile industry (Sajjad *et al*., 2020).

2.11 Biomedical applications

2.11.1 Antimicrobial activities

Infectious diseases are the second major reason for global human deaths and third in developed countries after noncontagious diseases (WHO, 2018). In the recent few decades, new drug entry in the market has been decreased and microbial resistance against antibiotics is increasing. This increasing trend of microbial antibiotic resistance amplified the demand for novel antimicrobial agents. As an alternative to antibiotics, several microbial pigments have been successfully evaluated for antimicrobial activities.

Pigments extracted from *Micrococcus luteus* that exhibited inhibitory potential against wound pathogens such as *Pseudomonas* sp. *Klebsiella* sp. and *Staphylococcus* sp. (Umadevi and Krishnaveni, 2013). Carotenoids extracted from *Holomonas* sp. exhibited antimicrobial potential against antibiotic- resistant *S. aureus, Klebsiella* sp., and *Pseudomonas aeruginosa,* and ophthalmic *S. aureus, Streptococcus pyogens,* and *E. coli* (Ravikumar *et al*., 2016). Prodigiosin obtained from *Vibrio ruber* DSM 14,379 exhibited bacteriostatic activity against *E. coli* and *Bacillus subtilis* (Danevčič *et al*., 2016).

Prodigiosin revealed promising antibacterial activity against pathogens such as *S. aureus*, *S. pyogenes*, *P. aeruginosa* and *Klebsiella pneumoniae* (Nwankwo *et al*., 2017). Studies shows that antimicrobial activity of prodigiosin against *E. coli*, *S. aureus*, and *Candida albicans* is strong (Suryawanshi *et al*., 2017). Similarly, several prodiginine compounds are reported having fungicidal activities against fungi such as *Penicillium*, *Aspergillus*, *Candida*, and *Cryptococcus* sp. (Stankovic *et al*., 2014).

Throughout human history, several viral outbreaks occurred and still occurring such as the Western African Ebola virus epidemic and the recent Coronavirus pandemic COVID-19 accompanied with moderate to high mortality rate (Sajjad *et al*., 2020). The outcome proposed potential antiviral activities of prodigiosin pigment against all tested viruses excluding HCV, where no binding interactions with active sites were reported. Moreover, compounds of quinone such as naphthoquinones, anthraquinones, and benzoquinones possess strong antiviral potential (Gessler *et al*., 2013).

Phenazine compounds extracted from *Streptomyces* and *Pseudomonas* species have been demonstrated promising antiviral activities (Schneemann *et al*., 2011). Therefore, microbial pigments are potential agents to administered as a novel source of medications against pathogens.

2.11.2 Antioxidant activity

The rise of free radicals inside the body increases the risks of chronic diseases such as diabetes, autoimmune disorders, and cancer (Phaniendra *et al*., 2015). Antioxidants are obtained either from natural or synthetic sources, however, synthetic antioxidants are losing demand due to possible side effects on the human body (Ahmed *et al*., 2013). Therefore, microbial- based antioxidants are gaining ground in the pharmaceutical industry. Pigments from microorganisms such as carotenoid, xanthomonadin, and naphthaquinone showed antioxidant potential (Tuli *et al*., 2015).

Carotenoids extracted from Antarctic bacterium *Pedobacter* exhibited solid antioxidant activity with protection against oxidative harm. Similarly, flexirubin (red carotenoid), obtained from *Fontibacter flavus* showed antioxidant activity against 2, 2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH), nitric oxide, hydroxyl radical, and inhibition of lipid peroxidation. Additionally, melanin reported from fungal isolate *Streptomyces glaucescens* and anthraquinones obtained from endophytic *Stemphylium lycopersici* were characterized as antioxidants. Violacein can also safeguard the cellular lipid membranes from peroxidation due to hydroxyl radicals. Several studies reported melanin obtained from *Pseudomonas* sp, *S. glaucescens* NEAE-H, and *Bifidobacterium infantis* were found antioxidant agents. These reports recommend that pigments obtained from microorganisms could be used as antioxidant agents to prevent several chronic diseases (Sajjad *et al*., 2020).

2.11.3 Anticancer activity

Cancer is one of the most lethal diseases in human history. Till now, several anticancer medicines have been designed and are in the stage of the clinical trial. However, success limitations, adverse effects, and resistance towards treatments are the major challenges in cancer treatment (Foo and Michor, 2014). Therefore, search for novel and effective anticancer agents with least or no side effects is of great interest. Several kinds of research conducted on microbial pigments as anticancer agents exhibited promising results. One such study on novel red pigment extracted from *Athrobacter* sp. G20 exhibited anticancer potential against the oesophageal cancer cell line (KYSE30) (Afra *et al*., 2017). A yellow pigment reported from *Streptomyces griseoaurantiacus* demonstrated strong cytotoxic activity against cervical cancer cells (HeLa) and HepG2 and resulted in a lower number of viable cells (Prashanthi *et al*., 2015).

Carotenoid extracted from *Kocuria* sp. QWT-12 revealed anticancer potential against breast cancer cell lines and lung cancer cells (Rezaeeyan *et al*., 2017). Similarly, carotenoids from *Haloferax volcanii* killed 53.52% of human liver carcinoma cell lines (Sikkandar *et al*., 2013). Black melanin extracted from *S. glaucescens* exhibited significant cytotoxic activity against the HFB4 skin cancer cell line (El-Naggar *et al*., 2017). Dihydroxyphenylalanine melanin obtained from *Streptomyces* sp. MVCS6 exhibited dose–response anticancer activity against the cervical cancer cell line (Sivaperumal *et al*., 2015).

Prodigiosin obtained from *Pseudoalteromonas* sp. having a cytotoxic effect against U937 leukemia cells. Strong anticancer activity of prodigiosin obtained from *S. marcescens* is reported against human cervical cancer cells and laryngeal cancer cells. The influence of prodigiosin reported from *Vibrio* sp. against human oral squamous carcinoma cells (OSCC) and found that the prodigiosin arrests their cell cycle. Recently, prodigiosin showed a reduction of the intracellular signalling pathway during the cell cycle and induced apoptosis in lung cancer cells and also showed in vivo tumoricidal activity. Anthraquinone derivatives from fungus *Alternaria* sp. have been studied for anticancer activity against human breast cancer cell lines (Sajjad *et al*., 2020). In light of the above studies, the microbial pigment could be potential chemotherapeutic agents for cancer treatment.

2.12 Bio‑indicators

Bioindicator bacteria are bacteria that can monitor environmental health and reveal the qualitative status of the environment by changing their behavior and specific physiological

characteristics (Parmar *et al*., 2016). The existence of carotenoid in *Lecanoraceae lichens* has been confirmed to depend upon the pollution level in the atmosphere of the surrounding environment, where they reside by analyzing the carotenoid content of fungi acting as bioindicator (Ibarrondo *et al*., 2016). Using the gene clusters encoding prodigiosin biosynthesis
in *S. marcescens* and violacein biosynthesis from *C. violaceum* demonstrate the implementation of reporter systems for the signal of biosynthetic gene expression (Domröse
et al., 2017). The participation of melanin pigments in protection from environmental stress like UV radiation and potentially toxic metals is regarded as a bioindicator due to its overproduction in adverse conditions (Egorova *et al*., 2011). Similarly, cyanobacteria naturally present in water
sources could act as excellent bioindicators for heavy metals, since in the presence of heavy metals, the carotenoids content in these cyanobacteria reduces (Wong and Teo, 2014).

2.13 Miscellaneous applications

Pigment-producing microbes in cryospheric environments could act as a potential source of electrons and may be employed for developing dye-sensitized solar cells (DSSC) that would be a potential alternative of conventional photovoltaic- silicon cells based. Consequently, DSSC might signify a remarkable alternative that could somewhat solve the energy requirement at Antarctic regions. For instance, the orange-xanthophyll pigment extracted from UVCresistant *Hymenobacter* sp. was exploited for DSSC development. Similarly the eumelanin and graphene integration and observed improved electrical conductivity which shows the scope of eumelanin in bioelectronics.

Bacterial pigments can be used as biodegradable ink on plastic materials. The hue and chroma values are observed in red and violet color suggestive of prodigiosin and violacein pigments isolated from *S. marcescens* and *C. violaceum,* respectively. Astaxanthin from radioresistant *Deinococcus* sp. exhibiting radio-protective and antioxidant activities can be incorporated in cosmetics including sunblock and sunscreen (Sajjad *et al*., 2020).

The bacterial pigments prodigiosin and violacein exhibiting antioxidant and antimicrobial activities represent a new paradigm for sunscreens that utilize substances of biological origin (Suryawanshi *et al*., 2015). These pigments can be a potential ingredient in a range of commercial sunscreen products. Indigoidine can be used as an organic semiconductor with numerous applications in carbon dioxide capture devices, electrochemical cells, super capacitors, batteries, etc. (Yumusak *et al*., 2019).

2.14 Current limitations and future perspectives

Bacterial pigments are one of the pleasing fields of research in life sciences to demonstrate their applications in various industries. Certain limitations such like high cost investment required for large scale production, lower stability, variations in shades due to some operational parameters, less percentage in terms of annual production, specifications in terms of bacteria capable of producing some colors and technological imperfection lead to delay in progress for industrial production of pigment from bacteria and extraction of pure and concentrated forms are drawing back the effort of researchers to replace synthetic pigments with bacterial pigments produced from biological origin. Studies should be concern especially on finding the easiest method for harvesting bacterial pigments in order to increase their industrials applications. Also there is a need to look on various operational parameters that may cause a variation due to change and develop a new low cost process for the production of bacterial pigments by using agrowaste as substrate in the future. Future investigation on various technologies that would reduce the cost and increase yields for large scale production (Usman *et aI.*, 2017).

METHODOLOGY

3.0 MATERIALS AND METHODS

The experimental procedure pertaining to the present study entitled "**Isolation and characterization of pigment producing bacteria and its possible advantage as bio colours"** is discussed under the following headings:

3.1 Collection of samples

3.2 Isolation of pigment producing bacteria

3. 3 Identification of bacteria

    3. 3.1 Morphological Characteristics

    3. 3.2 Biochemical Characteristics

3.4 Sequencing of isolated bacteria by RT-PCR

3.5 Screening of pigment producing bacteria in Nutrient broth

3.6 Extraction of pigments

3.7 Test for carotenoids by UV-Visible spectroscopy

3.8 Confirmation test for carotenoids

3.9 Characterization of extracted pigments by FT-IR Spectroscopy

3.10 Strategic manipulation of the growth media for maximum pigment production by bacterial isolates

    3.10.1 Effect of Temperature on the pigment production

    3.10.2 Effect of pH on the pigment production

    3.10. 3 Effect of NaCl on the pigment production

3.11 Antioxidant potential of isolated bacteria

3.12 Antibiotic susceptibility test

3.13 Pigment extract on textile application

3.1 Collection of Sample

Samples were collected from soil, vegetables and fruit wastes. From this, pigment producing bacteria was isolated and used for the present study.

3.2 Isolation of pigment producing bacteria

1 g of each sample was mixed with 9 ml of saline (0.85% NaCl w/v). The mixture was vortexed for uniform suspension. From this 100 µl was inoculated in nutrient agar plate using spread plate technique. The inoculated plate were incubated at 370C for 24 hours. From this yellow pigment producing bacteria was selected and repeatedly sub cultured for axenic culture (Suman *et al,* 2013).

3.3 Identification of bacteria

Identification of bacterial isolate was performed by morphological characteristics and biochemical tests.

3.3.1 Morphological Characteristics

Colony characterization of pigment producing bacteria from nutrient agar plate was done by colony size, colour, shape, margin, opacity, consistency, elevation and Gram staining (Harley and Prescott, 2007).

a) Grams staining

Gram staining method is important technique for bacterial identification and taxonomic division. Differential staining procedure divide most bacteria into two groups on the basis of cell wall composition. Gram-positive bacteria *(*thick layer of peptidoglycan-90% of cell wall) give purple colour and Gram-negative bacteria *(*thin layer of peptidoglycan-10% of cell wall and high lipid content*)* give red /pink colour after staining.

Procedure:

The bacteria was air-dried, heat-fixed for 1 minute with crystal violet staining reagent. Please note that the quality of the smear (too heavy or too light cell concentration) will affect the Staining results. Wash the slide in a gentle and indirect stream of tap water for 2 seconds and add mordant, Gram's iodine**.** Wait for 1 minute. Again wash slide in a gentle and indirect stream of tap water for 2 seconds. After that flood slide with decolorizing agent (Acetone-alcohol decolorizer**)**. Wait for 10-15 seconds or add drop by drop to slide until decolorizing agent running from the slide runs clear. Then add slide with a counterstain, safranin. Wait for 30 seconds to 1 minute. Again wash the slide in a gentle and indirect stream of tap water until no colour appears in the effluent and then air dry or blot dry with absorbent paper. Observe the results under oil immersion (100x) using a Bright field microscope.

3.3.2 Biochemical test

Biochemical tests performed were Indole test, Methyl Red (MR), Voges Proskauer (VP), Simmon's citrates test, Oxidase test, Catalase test, TSI test, Urease test and nitrate reduction tests recommended in the Bergey' Manual of Determinative Bacteriology and the procedure was described in Appendix I.

3.4 Sequencing of isolated bacteria by RT-PCR

The isolated orange and yellow pigmented bacteria was subjected to RT-PCR to find the bacterial genus which was described in Appendix II.

3.5 Screening of pigment production in Nutrient broth

The colour pigment producing bacteria was taken from the nutrient agar for pigment production. A loop full of culture was inoculated in to sterile 100 ml Nutrient broth mixed with 2% glycerol and incubated at 37 ^{o}C for 2 days in a rotatory shaker (Megha and Shabib, 2018).

3.6 Extraction of pigments

The pigment producing bacteria was centrifugation at 2000rpm for 20 mins. The supernatants were discarded and then the pellets were resuspended in acidified ethanol. The mixture was vortexed and the suspension was centrifuged at 2000 rpm for 10 mins and the supernatant was collected and pigment extract were filtered through Whatmann filter paper. (Waghela and Khan, 2019).

3.7 Test for carotenoids

The bacterial cell isolates were grown in Nutrient broth and the pigments were extracted from the organisms. Carotenoid pigments were identified by using of UV- Visible spectroscopy ranging from 450 nm to 600 nm (Balraj *et al*, 2014).

3.8 Confirmation test for carotenoids

The extracts of yellow and orange pigments was added with 1 ml of sulphuric acid in 9 ml of water. The appearance of blue colour confirm the presence of carotenoids (Jiang *et al*, 2005).

3.9 Characterization of extracted pigments by FT-IR Spectroscopy

The extracted orange and yellow pigment was studied by FT-IR spectrum. The prepared sample was recorded in the range of 4000 -400 cm-1 at a resolution of 4 cm-1 using KBr pellet method.

3.10 Strategic manipulation of the growth media for maximum pigment production by bacterial isolates (Suman *et al*, 2013)

3.10.1 Effect of Temperature on the pigment production

Petriplates containing nutrient agar was prepared in four sets (I, II, III, IV) and inoculated with bacterial isolates and incubated for 24 hours at different temperature. The petriplate belonging set I, II, III, IV were incubated at 250C , 300C, 350C and 400C respectively to study the effect of temperature on the pigment production.

3.10.2 Effect of pH on the pigment production

Petriplates containing nutrient agar was prepared in four sets (I, II, III, IV). Prior to autoclaving the pH of the petriplate belonging set I, II, III, IV was adjusted to 6, 7, 8 and 9 respectively and inoculated with bacterial isolates and incubated for 24 hours at 370C to study the effect of pH on the pigment production.

3.10.3 Effect of NaCl on the pigment production

Petriplates containing nutrient agar was prepared in four sets (I, II, III, IV). Prior to autoclaving the NaCl concentration of the petriplates belonging set I, II, III, IV were used are 2% , 4%, 6% and 9% respectively and inoculated with bacterial isolates and incubated for 24 hours at 370C to study the effect salt concentration on the pigment production.

3.11 Antioxidant potential

The antioxidant activity of the extracted pigment was calculated by using the DPPH assay.200 µl of pigment extract was mixed with 2 ml of 0.02 % DPPH and incubated in dark for 30 minutes. The absorbance was measured at 517 nm using methanol as blank. The ability to scavenge DPPH radical was calculated by using the equation

DPPH scavenging effect (%) = [(A0-A1/A0] x 100;

Where,

A0 is absorbance of control reaction

A1 absorbance of sample

3.12 Antibiotic susceptibility test

The plate was inoculated with 0.3 ml of bacterial culture and spread evenly by using spread plate technique. The plate was kept in refrigerator for 10 minutes for pre-diffusion of culture. An antibiotic disc of Hi-Media containing 12 standard antibiotics in specified concentration was placed on the medium and incubated at 370 C for 24 hours. After incubation the plate was observed for zone of inhibition and the diameter of the zone was calculated (Pratik *et al*, 2019).

3.13 Pigments extract on textile application

The bacterial extract yellow and orange pigment extract was mixed with alum potassium aluminium sulphate (6%). The cotton fabric and thread was kept immersed in the solution for about 5 minutes and kept for drying. Dried cotton fabric was soaked in detergent solution for 20 minutes and washed using tap water. The fabric was dried for 30 minutes (Mukherjee and Mitra, 2012).

RESULTS AND DISCUSSION

4.0 RESULTS AND DISCUSSION

Synthetic colorants which used in textile, food and cosmetic industries are very harmful to environment. This synthetic colorants contains dioxins which can cause disorders in nervous, immune and digestive system (Devyani *et al.,* 2017). So we can use natural colorants which derived from plants, animals and microorganisms. Today, many textile industries are enthralled by the use of non-toxic, renewable natural colorants (Shahid *et* al., 2013).

Bacteria can used as bio colorants because it has several advantages. The genes of microorganisms are very easy to manipulate, easy propagation, wide strain collection and fermentation was inheritantly faster. Cheap substrate can used for bulk production (Usman *et al*., 2017).

Carotenoids can be derived from flowers, fruits, microorganisms and vegetables. Carotenoids are lipophilic and hydrophobic in nature. In fact, carotenoids are lipophilic and hydrophobic (Mezzomo and Ferreira, 2016). Carotenoids have anti-bacterial (Kamla *et al*., 2016), anti-viral activity (Ravikumar *at al.,* 2016), cardiovascular activity (Pietro *et al*., 2016) and anti-inflammatory activity (Bhatt and patel, 2020).

The results of the present study entitled "**Isolation and characterization of pigment producing bacteria and its possible advantage as bio colours"** were discussed as follows.

3.1 Collection of samples

Various samples like soil, fruits and vegetable wastes were taken for the isolation of bacteria and inoculated in petriplate containing nutrient agar.

3.2 Isolation of Pigment producing bacteria

Bacterial colonies formed on the nutrient agar, yellow and orange colour colonies are produced were taken. From these, yellow and orange colour colonies are repeatedly sub cultured in nutrient agar to produce pure cultures (Plate 1).

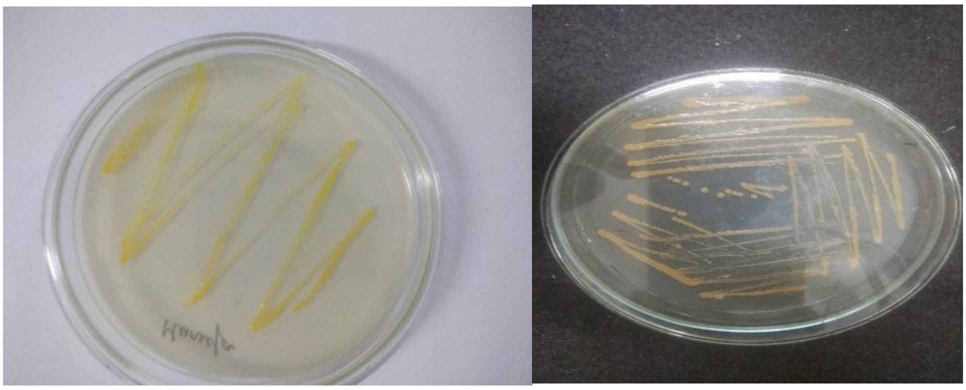


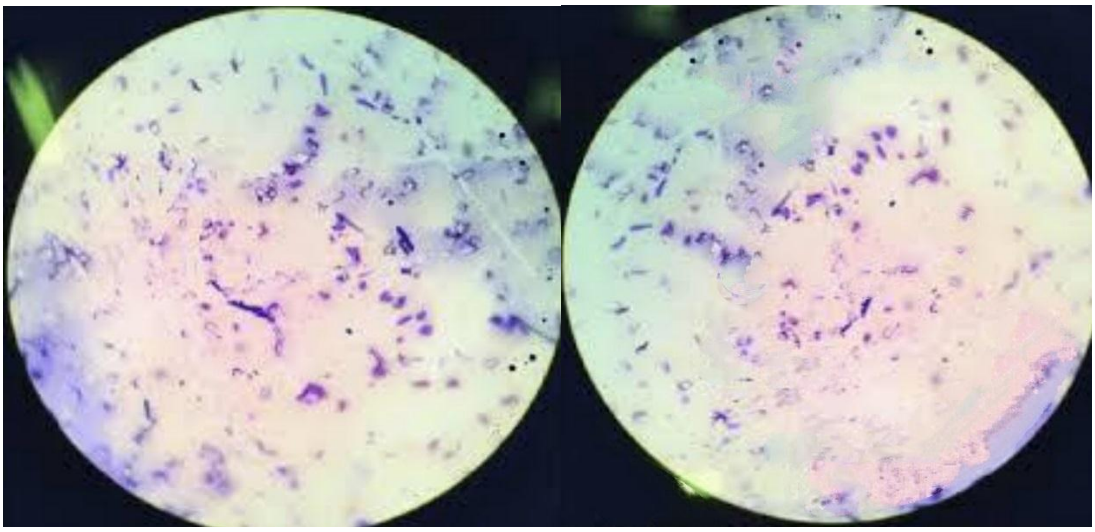
Plate 1 : Nutrient agar containing Yellow and Orange colour colonies

3. 3 Identification of bacteria

Yellow and orange colour bacterial isolates are grown on nutrient agar . Identification of bacteria were done by performing morphological and biochemical test.

3.2.1 Morphological Charaterization

Characterization of Yellow pigment producing bacteria from nutrient agar plate was done by colony shape, colour, margin, opacity , consistency, elevation and gram staining are mentioned in Table 1. The yellow and orange colour isolated colonies are found to be Gram Positive, rod shaped bacteria (Fig. 1)



Yellow colour isolate Orange colour isolate

Fig. 1: Gram positive rod shaped bacteria of yellow and orange colour isolates

Table 1: Morphological characterization of isolates

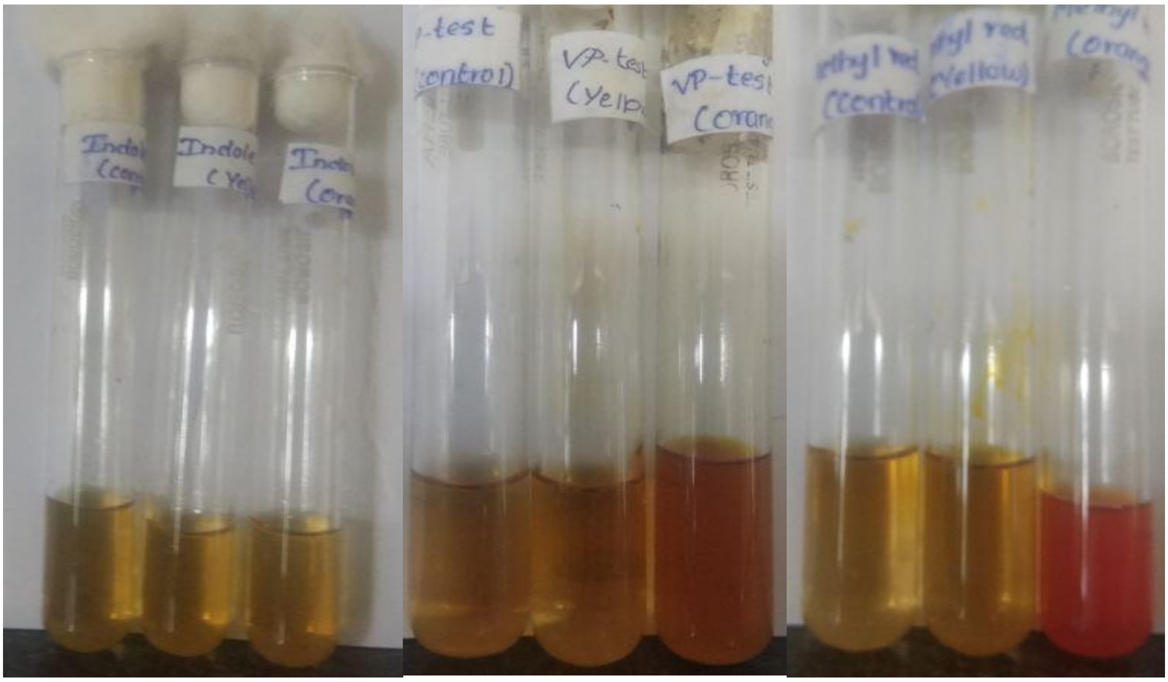
S.No	CHARACTERS	OBSERVATION (Yellow colour colonies)	OBSERVATION (Orange colour colonies)
1	Color	Yellow	Orange
2	Shape	Rod	Rod
3	Margin	Entire	Entire
4	Elevation	Convex	Convex
5	Opacity	Non-Opaque	Non-Opaque
6	Consistency	Sticky	Non-sticky
7	Gram Staining	Gram Positive	Gram Positive

3.2.2 Biochemical Characteristics

Yellow colour isolated bacteria shows positive result in TSI test and gave negative results in other tests. Orange colour isolated bacteria shows Positive results in Vogues proskaur, Methyl red, Catalase, Oxidase and TSI test, gave negative results for Indole, citrate and Urease test which shows in Table 2 and Fig. 2.

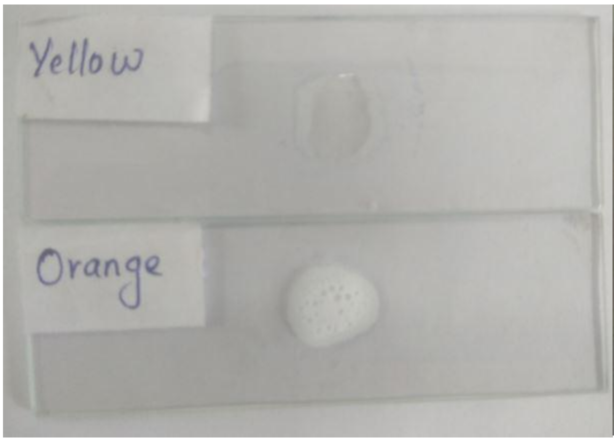
Table 2: Biochemical characterization of isolates

TEST	OBSERVATION (Yellow colour colonies)	OBSERVATION (Orange colour colonies)
Indole test	Negative	Negative
Voges-proskauer tsest	Negative	Positive
Methyl red test	Negaive	Positive
Citrate utilization test	Negative	Negative
Oxidase tset	Negative	Positive
Catalae tset	Negative	Positive
TSI tset	Positive	Positive
Urease test	Negative	Negative



Indole test Vogues proskauer test Methyl red test

Citrate utilization test TSI test Urease test



Catalase test Oxidase test

Fig. 2: Biochemical Characterization of yellow and Orange isolates

From the above observation the yellow pigment isolates was found to be *Lactobacillus* sp. and orange pigment isolates was found to be *Exiguobacterium* sp.

3.4 Sequencing of bacteria by RT-PCR

The 16S rDNA gene sequences of yellow and orange color isolated strains were compared with the entire 16S rDNA gene sequence strains to establish the relationship of strains at the genetic level, sequenced from each isolate. The phylogenetic trees showed that the isolates of the colonies of yellow and orange color were closely associated with *Lactobacillus* sp. and *Exiguobacterium aurantiacum*.

3.5 Screening of pigment producing bacteria in Nutrient agar

Isolated pigment were grown in nutrient agar broth for 2 days at 37 ^{0} C for screening of pigment producing bacteria (Fig 3). The Nutrient broth colour changed to yellow and orange after incubation.

3.6 Extraction of pigments from pigment producing bacteria

Yellow and Orange colour pigment was extracted from nutrient broth using different technique like centrifugation and filteration and addition of acidified ethanol so the cells get lysed (Fig. 4). The extracted pigments were used for further experiments.

Orange Pigment Yellow Pigment

Fig 3: Visual Observation of cultural of isolated pigment producing organism

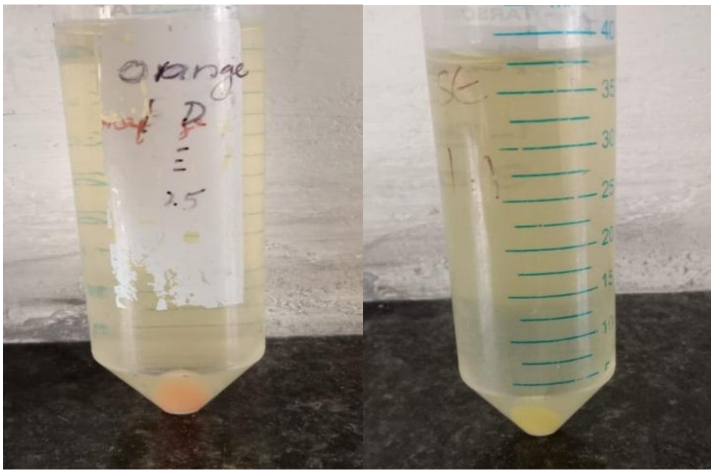


Fig 4: Extracted pigments of orange and yellow

3.7 Test for carotenoids by UV-Visibe Spectroscopy

Carotenoid pigments were identified by using of UV- Visible spectroscopy ranging from 450 nm to 600 nm in extracts of yellow and orange pigment. Both pigment shows peak between 450 nm to 600 nm (fig. 5), it shows the presence of carotenoid pigments.

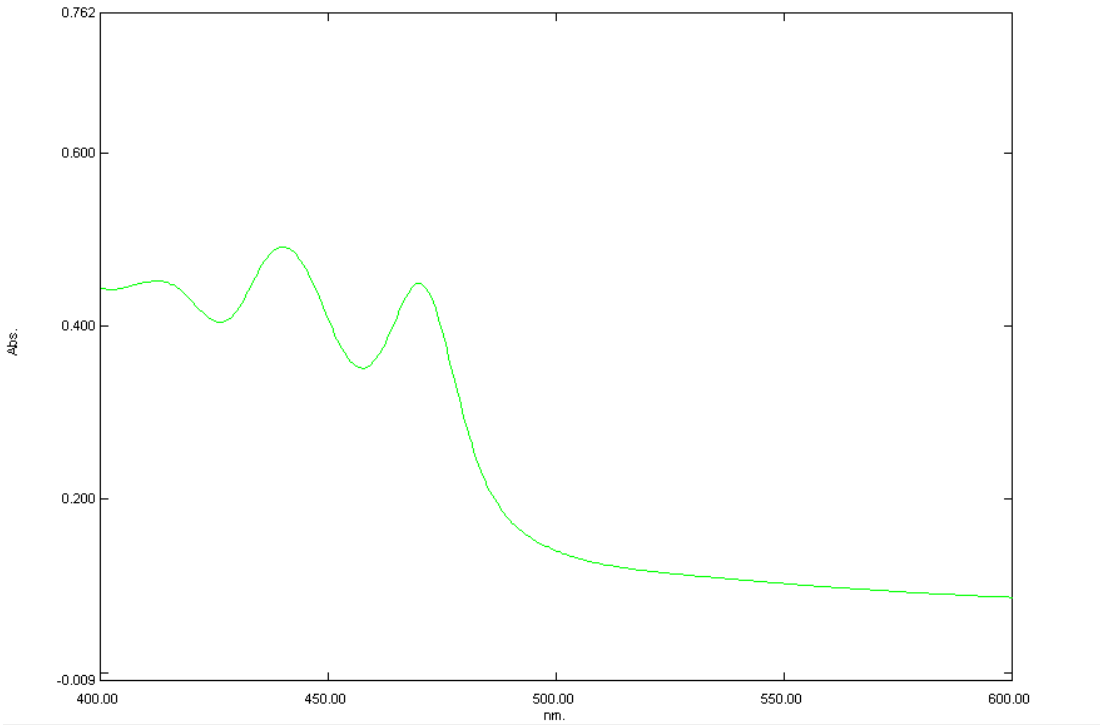


Fig. 5a : UV-Visible Spectroscopy of yellow pigment

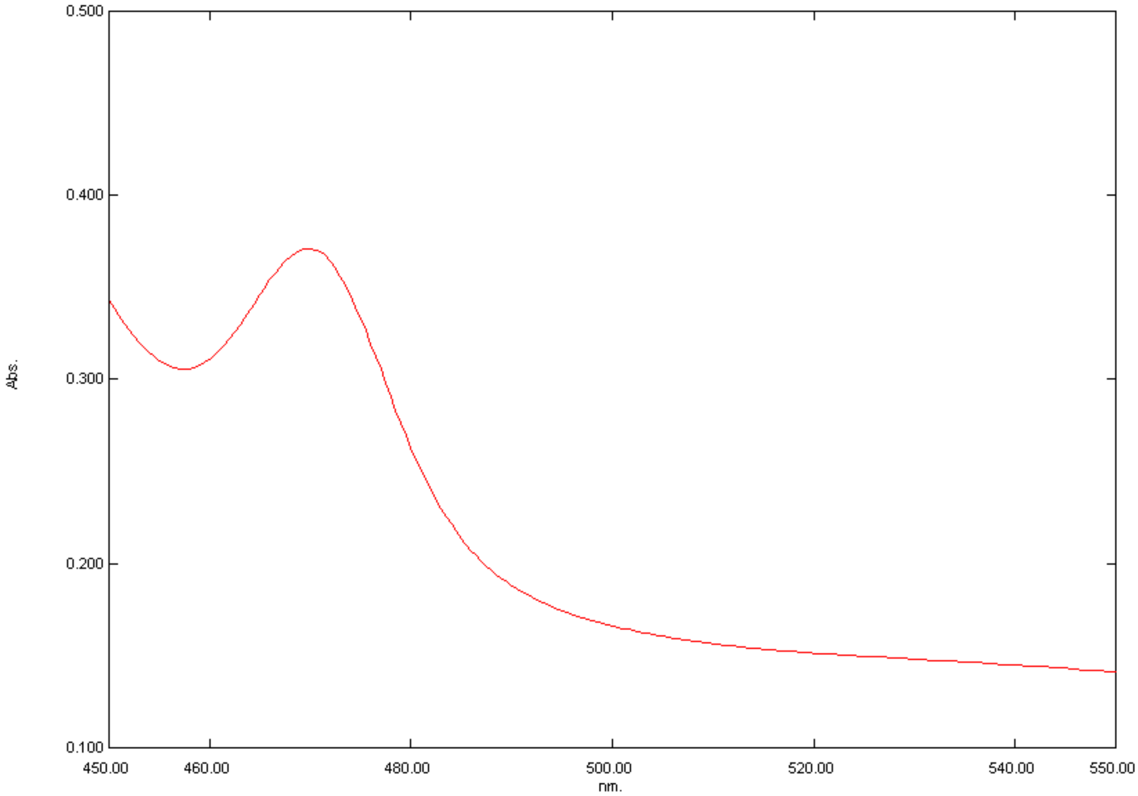


Fig. 5b: UV-Visible Spectroscopy of yellow pigment

3.8 Confirmation test for carotenoids

The extracts of yellow and Orange pigments were added with 1 ml of sulphuric acid in 9 ml of water. The appearance of blue colour confirm the presence of carotenoids. Selvi and Iyer (2018) reported that yellow pigments from *Xanthomonas* sp. And orange pigments extracted from *Sarcina* sp. also gave blue colour in the presence of sulphuric acid confirm the presence of carotenoids.

3.9 Characterization of extracted pigments by FT-IR

The yellow and orange colour pigments were characterized by FT-IR. The FT-IR spectra of yellow pigment showed that it has several degrees of similarities to the spectra of carotenoids. It shows in table 3 and Figure 6.

Table 3 : FT-IR Spectra of yellow pigment

S.no	Wave Number (cm^{-1})	Functional Group
1	3356.14	N-H
2	1643. 35	Aromatic C-H
3	2978.09	Streching of methyl grp
4	1381.03	C=C
5	1273.02	C-O

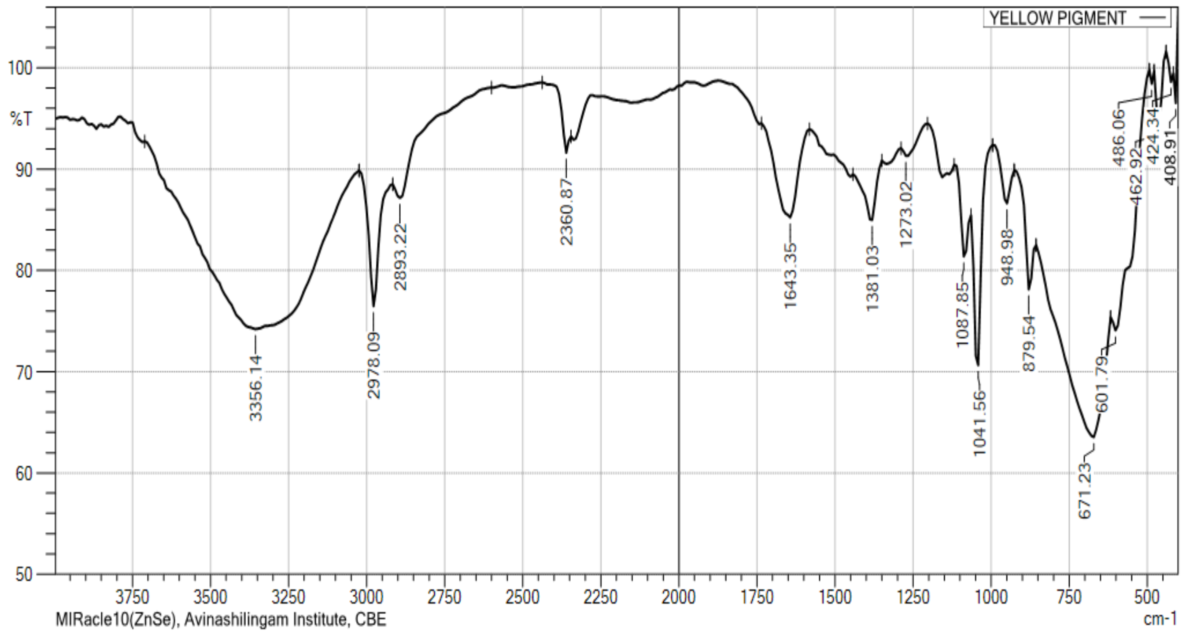


Fig. 6 : FT-IR Spectra of yellow pigment

The FT-IR spectra of Orange pigment also showed that it has several degrees of similarities to the spectra of carotenoids. It shows in table 4 and Figure 7.

Table. 4 : FT-IR Spectra of Orange pigment

S.no	Wave Number (cm^{-1})	Functional Group
1	3325.28	N-H
2	1643. 35	Aromatic C-H
3	2978.09	Streching of methyl grp
4	1381.03	C=C
5	1265.30	C-O

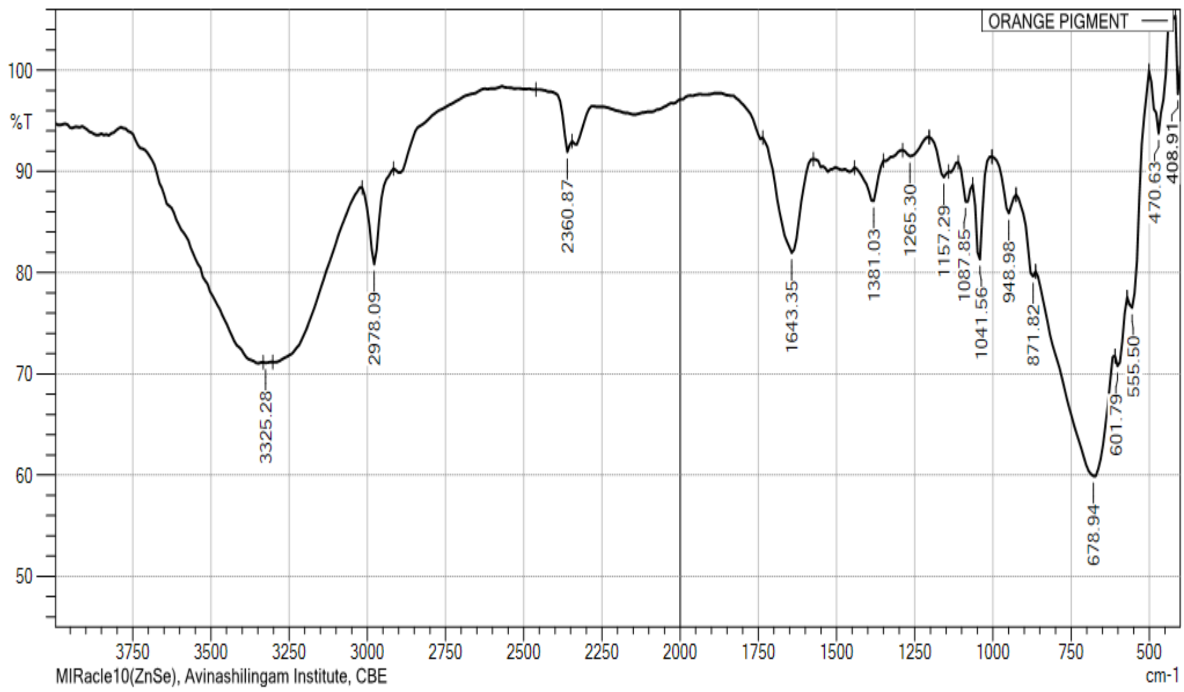


Fig. 7: FT-IR Spectra of Orange pigment

3.10 Strategic manipulation of the growth media for optimum pigment production

3.10.1 The effect of temperature on the production of pigments

The findings in Table 5, Plate 2 show that the development of yellow pigment was found to be at least 25 ^{0}C relative to 30 ^{0}C ,35 ^{0}C and 40 ^{0}C . Thus, an increase in temperature indicated an increase in bacterial growth and the production potential of the pigment. But the maximum development of orange color pigments was found at 25 ^{0}C, 30 ^{0}C ,35 ^{0}C and 40 ^{0}C respectively. Depending on the form of inoculated microorganism, the development of microbial pigments is significantly impacted by the incubated temperature (Goswani *et al.*, 1998). Different temperature ranges for the production of pigments by the isolated bacteria showed that there was a steady increase in growth and production of pigments with temperature increases up to 40 ^{0}C.

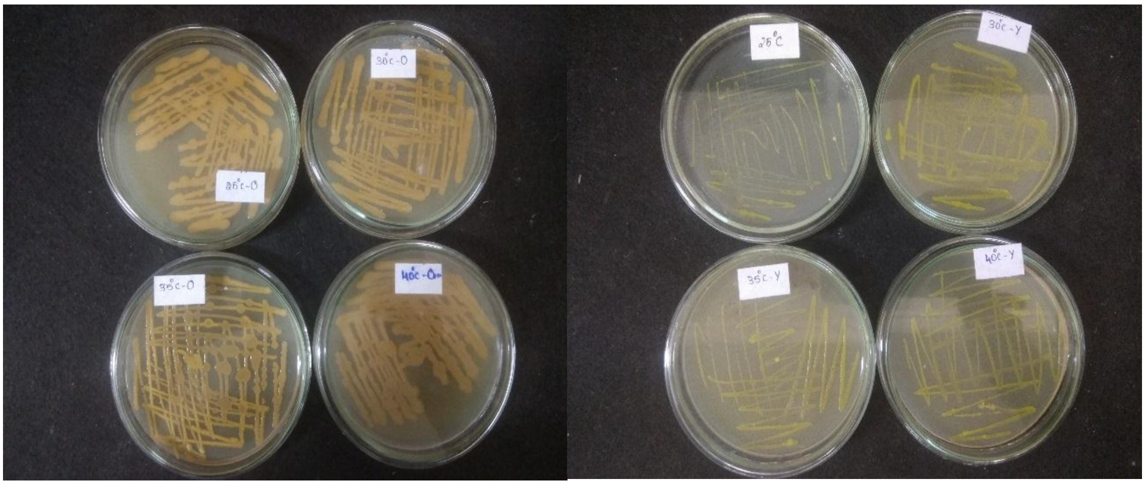


Plate 2 : The effect of temperature on the production of pigments

Table 5: The effect of temperature on the production of pigments

TEMPERATURE	YELLOW	ORANGE
25 ^{0}C	++	+++
30 ^{0}C	+++	+++
35 ^{0}C	+++	+++
40 ^{0}C	+++	+++

Where; +++ (excellent); ++ (good); + (Present) - (nil)

3.10.2 The effect of pH on the production of pigments

The findings concerning the effect of pH on the development of pigments are shown3 in Table 6 and Figure 3. The production of pigments increased with the rise in pH. At pH 9, maximum pigment production was observed. At pH 6, yellow pigment production was found to be minimum but Orange pigment production was found to be none. The results showed that the increase in pH favors the development of pigments. The effect of pH on pigment production in the microorganism *Micrococcus flavus* with different pH ranges (6, 7and 7.5). was studied by Deb and Madhugiri (2012). They observed that pH 7.5 was found to be optimum for maximum pigment production. The result showed that the neutral to alkaline pH favors pigment production.

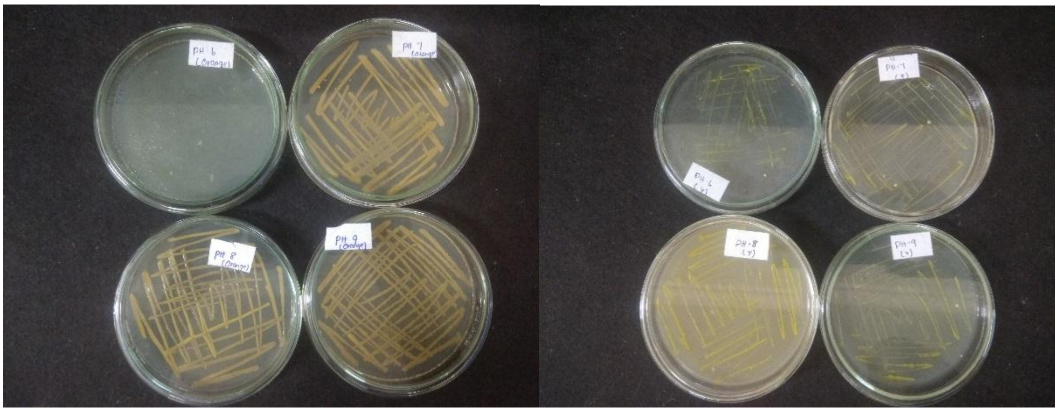


Plate 3 : The effect of pH on the production of pigments

Table 6: The effect of pH on the production of pigments

pH	YELLOW	ORANGE
6	+	-
7	++	+++
8	+++	+++
9	+++	+++

Where; +++ (excellent); ++ (good); + (Present) - (nil)

3.10. 3 The Effect of NaCl on the production of pigments

The results of the effects of different NaCl concentrations are presented in Table 7 and in Plate 4. With an increase in salt concentration from 4% to 8%, there was a gradual decrease in pigment development, but maximum pigment production was observed at 2% salt concentration. Orange colour pigment production was found to be present in 2%,4% and 6% NaCl but absent in 8% Nacl Concentration. In yellow colour pigment production was found to be present only in 2%, minimum in 4% and 6% and absent in 8% NaCl concentration.

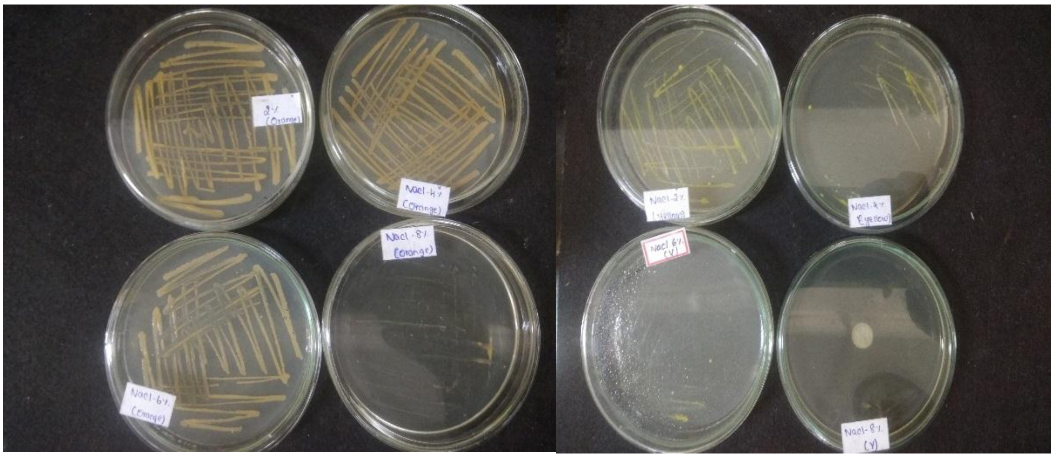


Plate 4 : The Effect of NaCl on the production of pigments

Table 7: The Effect of NaCl on the production of pigments

NaCl CONCENTRATION	YELLOW	ORANGE
2%	++	+++
4%	+	+++
6%	-	++
8%	-	+

Where; +++ (excellent); ++ (good); + (Present) - (nil)

3.11 Antioxidant potential study by DPPH assay :

Percent radical scavenging activity calculated for the extract of yellow and orange pigment. Ascorbic acid was used as a standard (fig. 8). The yellow colour extracts shows 28.5 percent inhibition. Orange colour extracts shows 42.8 percent Inhibition. Compared these two pigmented isolates, orange colour extracts have more antioxidant potential than yellow colour extracts.

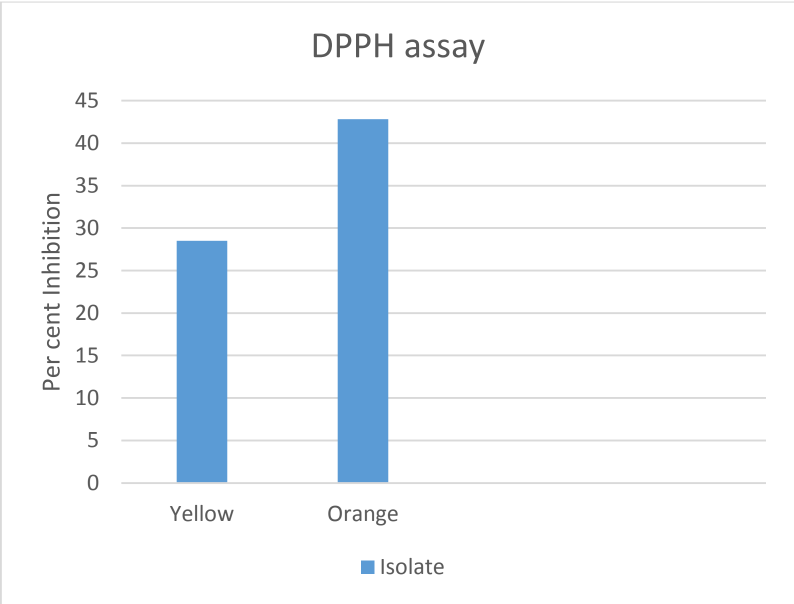


Fig. 8: Antioxidant potential study by DPPH assay

3.12 Antibiotic susceptibility test:

Disc containing antibiotics are from the various families like Polymyxin-B, Bacitracin-B, Cephoaxime, Ampicillin and Pencillin-G. The *Lactobacillus* Sp. shows susceptibility against Polymyxin-B and Bacitracin but shows resistant against Cephoaxime, Ampicillin and Pencillin-G (Table 8). The *Exiguobacterium auranticum* shows susceptibility all the antibiotics disc present (Table 9). Zone of inhibition was observed (Plate 5). It conclude that the isolate which is susceptible to used antibiotics and if any causes happens due to this species it can be treat with the help of this antibiotics.

Table 8: Antibiotic susceptibility test of *Lactobacillus* Sp.

S.No	Name of Antibiotic	Potency (mg/unit)	Zone of Inhibition (mm)	Resistance/Susceptible (*Lactobacillus* Sp.)
1	Polymyxin-B	300	0.1	Susceptible
2	Bacitracin-B	10	0.1	Susceptible
3	Cephoaxime	30	-	Resistance
4	Ampicillin	10	-	Resistance
5	Pencillin-G	10	-	Resistance

Table 9: Antibiotic susceptibility test of *Exiguobacterium auranticum*

S.No	Name of Antibiotic	Potency (mg/unit)	Zone of Inhibition (mm)	Resistance/Susceptible (*Exiguobacterium auranticum*)
1	Polymyxin-B	300	0. 3	Susceptible
2	Bacitracin-B	10	0.4	Susceptible
3	Cephoaxime	30	0.1	Susceptible
4	Ampicillin	10	0.5	Susceptible
5	Pencillin-G	10	0.1	Susceptible

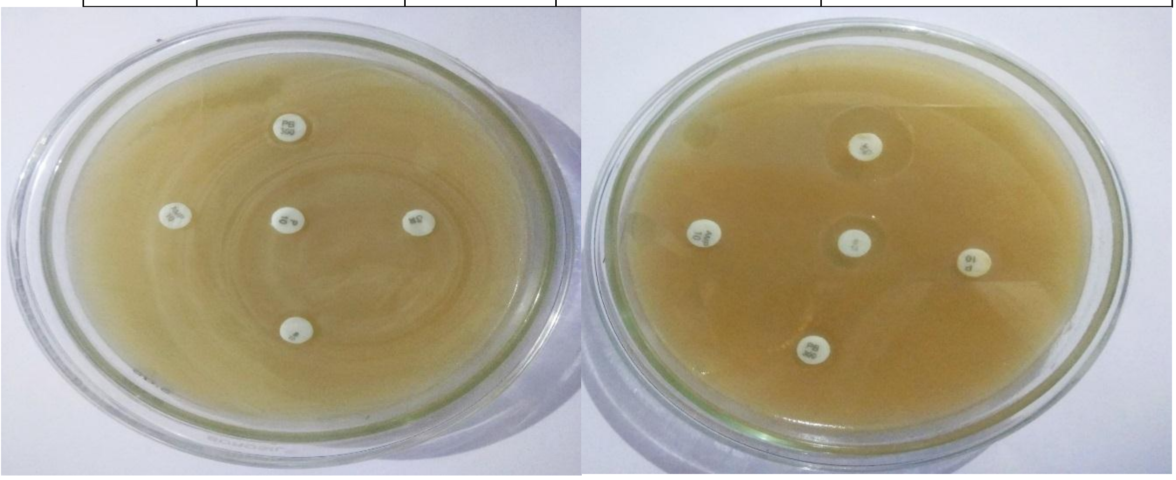


Plate5: Antibiotic susceptibility test of *Lactobacillus* Sp. and *Exiguobacterium auranti*

3.13 Extracted pigment application on cotton fabric

The isolated bacterial pigments were mixed with alum potassium aluminium sulphate to dye the cotton fabric and kept drying for 5 minutes. The fabrics sustained the yellow and orange colour after washing (Fig 6). Such pigments can be used instead of synthetic colorants in the textile industry because they are more eco-friendly.

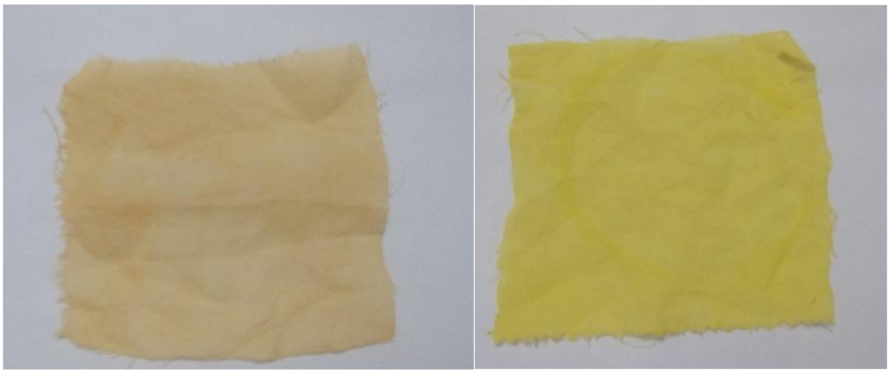


Fig. 9: Colourization done in cotton cloth using yellow and orange pigments

An outline of the findings of the present study and conclusion drawn thereof presented in the following chapters.

SUMMARY AND CONCLUSION

5.0 SUMMARY AND CONCLUSION

Colorants are used in lots of products include cosmetics, food and textiles. These colorants are synthetic and very harmful for humans. It is very harmful to environment because it contain toxic compounds and non-biodegradable. Several synthetic colorants contains dioxins. Dioxin can cause disorders in digestive, immune and nervous system. These dioxin containing synthetic dyes when disposed contaminate the environment and accumulates in living organisms which is very harmful for our eco-system. So now a days there is a growing demand for natural dyes which can be biodegradable and less harmful for the humans and our environment. It can grow rapidly which give high yeild throughout the year. The pigments produced from higher organism like fungi, plant and animals are less accessible for exploration because the pigment – bearing tissue structure was more complex and the pigments are produced only at the particle point.

The present study was carried out to investigate the isolation of pigment producing bacteria from various sources and Identification of isolated pigment producing bacteria by morphological and biochemical characteristics. The pigments were extracted from the bacteria and the pigments were characterized. The tactical manipulation of the pigment production by orange yellow isolated bacteria was carried out by different temperature, pH abd NaCl concentration. Antioxidant potential of pigment producing bacteria was done by using DPPH assay. Antibiotic susceptibility test of selected isolated bacteria was done by using antibiotic disc. The extracted pigmented bacteria were applied on cotton cloth to evaluate its use in textile industry. The result of the present study summarized below.

- ❖ Various samples like soil, spoiled fruits and vegetables were taken. From yellow and orange colour colonies are produced in nutrient agar. Morphological and biochemical characterization of the isolated pigmented bacteria shows that the yellow colour isolated bacteria was found to be *Lactobacillus* Sp. and Orange colour pigmented bacteria was found to be *Exiguobacteruim auranticum*.
- ❖ The pigments were extracted by centrifugation and adding acidified ethanol. The yellow and orange pigmented extract was used for further characterization
- ❖ The FT-IR spectra of yellow and orange pigment shows that it have several similarities to the spectra of carotenoids.

- The effect of temperature on the development of pigments shows that the production of yellow pigments was found to be least at 25 ^{0}C compared to 30 ^{0}C, 35 ^{0}C and 40 ^{0}C. Thus, an increase in temperature indicated an increase in bacterial growth and the production potential of the pigment. The maximum development of orange coloured pigments was found at 25 ^{0}C, 30 ^{0}C, 35 ^{0}C and 40 ^{0}C respectively.
- The production of pigments increased with the rise in pH. At pH 9, maximum pigment production was observed. Yellow pigment production was found to be limited at pH 6, but there was no orange pigment production. The results showed that increase in pH favors the development of pigments.
- With an increase in salt concentration from 4 % to 8 %, there was a gradual reduction in pigment production, but maximum pigment production was observed at 2 % salt concentration. Orange colour pigment production was found to be present in 2%, 4% and 6% NaCl but absent in 8% NaCl Concentration. In yellow colour pigment production was found to be present only in 2%, minimum in 4% and 6% and absent in 8% NaCl concentration.
- Antioxidant potential by DPPH assay shows that the yellow colour extracts shows 28.5 percent inhibition and orange colour extracts shows 42.8 percent Inhibition. So orange colour extracts have more antioxidant potential than yellow colour extracts.
- The *Lactobacillus* Sp. shows susceptibility against Polymyxin-B and Bacitracin but shows resistant against Cephoaxime, Ampicillin and Pencillin-G. The *Exiguobacterium auranticum* shows susceptibility all the antibiotics disc present.
- The extracted pigments were used for dying cotton cloth to analyse its possible use in textile industries.

Future recommendation:

- Anticancer study can be carried out by using the extracted pigments.
- Potential bacterial isolates will be studied for their use in cosmetic, textile and food industry.

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6.0 BIBLIOGRAPHY

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APPENDICES

7.0 APPENDIX I

BIOCHEMICAL CHARACTERISTICS

(Sundarajan, 1995)

A) Catalase test

Use a loop to transfer a small amount of colony to the surface of op of a clean and dry glass slide. To this place a drop of Hydrogen peroxide on to the medium and observe for the air bubbles. Presence of bubbles indicates positive results.

B) Oxidase test

The ability of a micro-organism to produce the cytochrome C oxidase can be determined by using the reagent tetramethyl-p-phenylenediamine dihydrochloride impregnated in filter disk. This reagent serves as an artificial substrate donating electrons and thereby becoming oxidized to a deep purple compound in the presence of the enzyme oxidase and free Oxygen. Development of pink, then maroon and finally dark purple colour after rubbing the organism in the oxidase disc containing the reagent indicates positive result. No colour change is indicative of the negative test result.

Procedure:

Take a commercially available oxidase disc containing the reagent and pick the isolated colony to be to be tested and rub in the disc. Observe for colour change in the disc within 10 seconds.

C) Urease test

Urea is the product of decarboxylation of amino acids and Hydrolysis of urea produces ammonia and CO2. The pH shift is detected by the colour change of phenol red from light orange to magenta or pink. Rapid urease-positive organisms turn the entire medium pink within 24 hours and weakly positive organisms may take seven days or more, and negative organisms produce no colour change or yellow as a result of acid production.

$$(NH_2)_2CO + 2H_2O \longrightarrow CO_2 + H_2O + 2NH_3$$

Procedure:

Streak the surface of a urea agar slant with a well-isolated colony. Leave the cap on loosely and incubate the tube at 37°C for 48 hours to 7 days. Examine for the development of a pink colour for as long as 7 days. This test can be used as part of the identification of several species of *Enterobacteriaceae* , including *Proteus, Klebsiella, Yersinia* , *Citrobacter* , and *Corynebacterium* species.

D) TSI test

Triple Sugar Iron Agar test has three sugar (lactose, sucrose, and glucose) and also iron; and it contains agar as solidifying agent (TSI is a semi-solid media having slant and butt).

Procedure:

With a sterilized straight inoculation needle touch the top of a well-isolated colony and inoculate TSI agar by first stabbing through the center of the medium to the bottom of the tube and then streaking on the surface of the agar slant. Leave the cap on loosely and incubate the tube at 37°C for 18 to 24 hours.

- Alkaline slant/no change in butt (K/NC) i.e Red/Red = glucose, lactose and sucrose non- fermenter
- Alkaline slant/Alkaline butt (K/K) i.e Red/Red = glucose, lactose and sucrose non- fermenter
- Alkaline slant/acidic butt (K/A); Red/Yellow = glucose fermentation only, gas (+ or -), H2S (+ or -)
- Acidic slant/acidic butt (A/A); Yellow/Yellow = glucose, lactose and/or sucrose fermenter gas (+ or -), H2S (+ or -).

E) Nitrate Reduction test

Nitrate Reduction test was used to detect nitrate reduction in micro-organism.

Proedure:

Inoculate the nitrate broths with bacterial suspension and incubate the tubes at the optimal temperature 37°C for 24 hours. After incubation look for nitrogrn gas first before adding reagents. Then add 6-8 drops of nitrite reagent A and nitrite reagent B. Observe for the reaction (color development) within a minute or less. If no colour develops add zinc powder. Observe for at least 3 minutes for a red colour to develop after addition of zinc.

F) IMVIC test:

Indole test:

It is performed on tryptophan broth. Organism was inoculated and incubated at the optimal temperature 37°C for 24 hours. After incubation result is read after adding Kovac's reagent. The positive result is indicated by the red layer at the top of the tube after the addition of Kovács reagent. A negative result is indicated by the lack of colour change at the top of the tube after the addition of Kovács reagent.

Methyl Red (MR) test:

Positive methyl red test are indicated by the development of red colour after the addition of methyl red reagent. A negative methyl red test is indicated by no colour change after the addition of methyl red reagent

Voges-Proskauer (VP) test:

Negative test is indicated by lack of colour change after the addition of Barritt's A and Barritt's B reagents. A positive Voges-Proskauer test is indicated by the development of red-brown colour after the addition of Barritt's A and Barritt's B reagents.

G) Citrate Utilisation test

The test is performed on Simmons citrate agar, A negative citrate utilization test is indicated by the lack of growth and colour change in the tube A positive citrate result as indicated by growth and a blue colour change.

APPENDIX II

SEQUENCING OF ISOLATED BACTERIA BY RT-PCR

The details of the steps involved in the Sequencing of 16S Ribosomal RNA is discussed below.

Materials Required

1. Bacterial culture
2. TE Buffer
3. Lysozyme solution
4. SDS (10% and 1%)
5. Proteinase K

6. Phenol
7. Chloroform
8. Isoamyl alcohol
9. Sodium acetate
10. Absolute ethanol
11. Micropipettes
12. Water bath
13. Centrifuge

Procedure:

Extraction of Dna was done by Centrifuge the bacterial culture at 5000 rpm for 10 minutes. After centrifugation, add 5.6ml of TE buffer into the pellet. Add 100ul of lysozyme solution into the pellet and keep it in the water bath for 1.5 hours at 37^0C. Again add 1000ul of 10% SDS and 2.5ul of Proteinase K to the tube with pellet solution and in water bath for 1hour at 37^0C. Then add 3.5 ml of phenol to the microfuge tube. Centrifuge at 1000 rpm for 15 minutes. After centrifugation, 2.5ml of phenol, 2.4ml of Chloroform and 100ul of isoamyl alcohol is added to the new tube containing the supernatant. Centrifuge at 1000 rpm for 15 minutes. After centrifugation, add 4.8ml of chloroform and 200ul of isoamyl alcohol to the new tube containing the supernatant. Centrifuge at 1000 rpm for 10 minutes. After centrifugation, add 100ul of sodium acetate and 1000ul of absolute ethanol to the new tube containing supernatant solution. Transfer the thread like DNA structures to a new sterile microfuge tube. Add 50 ul of TE buffer to the DNA solution to store it for future purposes. Polymerase Chain Reaction was done to amplified the DNA. Then Agarose Gel Electrophoresis and elution of DNA was done. Visualize the low melting point agarose gel with DNA bands under a UV transilluminator and locate the desired DNA band to cut. Carefully cut around the desired DNA band using a scalpel blade. Transfer the gel piece into a microfuge tube. Add elution buffer into the microfuge tube until the level of buffer is just above the level of gel slice. Heat the gel slice at 65^oC until it melts. Freeze the melted gel with DNA by placing in a -70oC freezer for10 minutes. After freezing, centrifuge for 10minutes and transfer the supernatant into a new microfuge tube. Again add half amount of elution buffer that you added in the previous step into the pellet. Heat at 65^oC until the agarose melts. Freeze the melted gel with DNA by placing in a -70^oC freezer for10minuts. Centrifuge the tube again for 10 minutes and transfer (pool) the supernatant into the previous tube with supernatant. Discard the tube with pellets. Add an equal volume of n-Butanol to the supernatant and mix the contents well. Vortex the tube for 15 minutes in order to remove the

Ethidium bromide. Discard the upper phase of butanol and repeat the process by adding n-butanol again for one or more times. Add 2 times volume of 95% ethanol and mix thoroughly. Keep for precipitation in -70^{o}C freezer for 30minutes to overnight. After precipitation, centrifuge for 15 minutes. Discard the supernatant into a waste beaker and add 200µl of 70% ethanol to the pellets. Centrifuge for 5minutes and discard the supernatant again. Allow the pellets to dry well. Suspend the pellets in 20µl of TE buffer. (If you want to confirm the recovered DNA, run (1µl) it on a gel. The recovered DNA can be now used for further process of cloning otherwise can stored in -20^{o}C freezer. Radiolabeling Technique was used and Restriction was done. Southern blotting technique was done and observe the band corresponding to the 16S Ribosomal RNA sequence of the test organism.