Formulation of Effective Microbial Consortia and its Application in Sewage Treatment

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Thesis

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Dedicated

To my

Beloved Parents,

My Husband & Daughters

(Ayeza & Imrah)

Sher-e-Kashmir
University of Agricultural Sciences & Technology of Kashmir
Division of Basic Sciences & Humanities, Wadura Campus

Certificate – I

This is to certify that the thesis entitled, **“Formulation of Effective Microbial Consortia and its Application in Sewage Treatment”** submitted in partial fulfilment of the requirements for the award of the degree of **Doctor of Philosophy in Microbiology,** to the **Faculty of Horticulture, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir** is a record of bonafide research work carried out by **Ms. Gousia Qadir (Regd. No. 2015-583-D)** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

It is further certified that any help or information received during the course of investigation has duly been acknowledged.

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Certificate – II

We, the members of the Advisory Committee of **Ms. Gousia Qadir (Regd. No. 2015-583-D)**, a candidate for the degree of **Doctor of Philosophy in Microbiology** have gone through the manuscript of the thesis entitled, **"Formulation of Effective Microbial Consortia and its Application in Sewage Treatment"** and recommend that it may be submitted by the student in partial fulfilment of the requirements for the award of the degree.

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ABSTRACT

An experiment was carried out to isolate the most effective microorganisms for the degradation in sewage water. Various genera of lactic acid bacteria, *Pseudomonas,* yeasts, fermenting fungi and actinomycetes were isolated from some natural sources and screened to identify the most effective microbes for degradation. Three consortia (EM1, EM2and EM3) containing different concentrations of efficient and effective isolates of *Lactobacillus, Pseudomonas*, *Saccharomyces cerevisiae, Aspergillus niger* and *Streptomyces* were prepared and tested for *in vitro* degradation of sewage water with their respective controls. Effect of different parameters like pH, molasses concentration and incubation period on growth of microorganisms was observed and best pH, temperature, molasses concentration and incubation period for optimum microbial growth was found that was suitable for EM to flourish at maximum possible rate which was further employed in formulated effective microbial consortia to find out its effect on various parameters in sewage water. During *in vitro* decomposition of wastes using effective microbes, the consortium of EM1 exhibited faster decomposition followed by EM3 and EM2. A significant decrease in C, N, P, K, BOD, COD and Heavy metals like (Cd, Pb, Ni) was also observed from 32.6, 6.2, 95.1, 1.85, 57.6, 71.4, 50, 7, 240 to 16.3, 0.3, 0.6, 0.8, 26.3, 29.0, 0.0, 1.0, 74.4 in EM treated sewage water from untreated sewage water. Chemical characteristics of EM treated sewage water were found better and well within the WHO permissible limits as compared to untreated sewage water. The population of viable bacteria, fungi, phosphorus solubilizing bacteria were found in high range in EM treated Sewage water as compared to untreated sewage water.

Key words: Sewage water, Degradation, Effective microbes, Molasses concentration

Signature of Student
Dated : ___________

Signature of Major Advisor
Dated: ________________

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Place: Wadura

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

Chapter 1

INTRODUCTION

World generates about 20 billion tonnes of waste per year, of which 17 billion tonnes are industrial waste and 2.6 billion tonnes are municipal solid waste (Bhide and Sundersan, 1983). In India, the total quantity of solid waste has been reported as 960 million tonnes per year, of which about 48 million tonnes are municipal solid waste (Central Pollution Control Board, 2004). The Energy and Resources Institute (TERI) has also estimated that in India, waste generation will exceed 260 billion tonnes per year by 2047—more than five times the present level. In Srinagar city, the total quantity of municipal solid waste (MSW) has been reported as 428 t/day, and the average generation rate of MSW has been estimated at 0.48 kg/capita/day (Srinagar Municipal Corporation, 2008).

Wastewater is a complex mixture of natural, inorganic and organic material mixed with man-made substances. It contains everything discharged to the sewer, including materials washed from roads and roofs. It is this complex mixture that ends up at the wastewater treatment plant for purification. Wastewater may be categorized into domestic (sanitary) wastewater also known as sewage, industrial (trade) wastewaters and municipal wastewater which is a mixture of the two. Sewage is correctly the subset of wastewater that is contaminated with faecal matter and urine. The strength and composition of sewage changes on an hourly, daily and seasonal basis, with the average strength dependent on per capita water usage, infiltration, surface run-off as well as local habits and diet. Sewage is 99.9% water with the material that require to be removed amounting to just 0.1% by volume. This solid material is a mixture of faeces, food particles, grease, oils, soaps, salts, metals, detergent, plastics, sand and grit. The organic fraction is composed of proteins, carbohydrates and fats which reflects the diet of the community served by the treatment system (Metcalf and Eddy, 1991). Waste characteristics vary according to the extent of urbanization, the income level of the area and the degree of its industrialization and commercialization. The composition of municipal waste depends to a large extent on the affluence of the population contributing to the waste stream. It is essential to know the composition of waste, both at the source and at discharge, to assess the most suitable option for disposal and recovery. For example, the feasibility of composting is determined by a combination of the quantities of waste generated and the proportion of organic waste, amongst other factors.

Waste usually can be processed for disposal or recycling by one or more steps. The first step is the preliminary and primary treatment which is a physico-chemical treatment. Because of the objectionable properties of the effluent, the secondary treatment, which is biological treatment, is employed. The operation involves the biological degradation of organics, both dissolved and suspended materials by microorganisms under controlled conditions. Biological treatment can be accomplished in a number of ways, but the basic characteristic of the system is the use of mixed microbial culture: bacteria, fungi and/or algae, for the conversion of pollutants. In most cases, organic materials are converted to oxidized products, mostly carbon dioxide and new microbial cells (the sludge). The organic materials serve as an energy and carbon sources for cell growth (Chui *et al.,* 2006). A major problem facing municipalities throughout the world is the treatment, disposal and/or recycling of sewage sludge. Generally, sludge from municipal waste mainly consists of biodegradable organic materials with a significant amount of inorganic matter. However, sludge exhibits wide variations in the physical, chemical and biological properties (Javaid, 2006).

Wastewater treatment plant is a combination of separate treatment processes or units, designed to produce an effluent of specified quality from a wastewater (influent) of known composition and flow rate. Aims of wastewater treatment are to convert the waste materials present in wastewater into stable oxidized end products that can be safely disposed off to inland waters without any adverse effects on ecology and public health (Behera *et al.,* 2007) The sludge disposed during the various water treatment processes can be a major concern for water treatment plants. Most of the water treatment plants discharge the sludge into the rivers without treatment. The discharging of sludge into water body leads to a cumulative rise of aluminum concentrations in water, aquatic organisms and human bodies. Some researchers have linked aluminum's contributory influence to occurrence of Alzheimer's disease, children's mental retardation and the common effects of heavy metals accumulation (Prakhar, 1998). Consequently, stringent standards of effluent discharge are coming into effect and thus proper management of the sludge becomes inevitable. The use of water treatment sludge in various industrial and commercial manufacturing processes has been reported in UK, USA, Taiwan and other parts of the world. Successful pilot and full-scale trials have been undertaken in brick manufacture, cement manufacture and commercial land application. The mineralogical composition of the "water treatment sludge" is particularly close to that of clay and shale. This fact encourages the use of water treatment sludge in brick manufacture (Zhao *et al.,* 2006).

A new technology has been introduced in which a consortium of naturally occurring microorganisms is developed according to the principles of EM-technology (Effective Microorganisms) and it is mainly used for foul odour elimination and organic waste composting. The concept behind beneficial and effective microorganisms (EM) technology was developed by Professor Teruo Higa, at the University of Ryukyus, Okinawa, Japan (Sangakkara, 2002). EM contains various aerobic and anaerobic microbial species of photosynthetic bacteria, lactic acid bacteria, yeasts, actinomycetes and fermenting fungi and their mode of action improves biodegradation of organic wastes and deodorization of foul odour. EM is a natural, probiotic technology operational for over 25 years around the world. It is based on beneficial and effective microorganisms. The microbes in EM are non-harmful, non-pathogenic, not-genetically-engineered or modified and not-chemically-synthesized (Higa, 2002).

Studies have suggested that EM may have a number of applications in agriculture, livestock, gardening and landscaping, composting, bioremediation, cleaning septic tanks, algal control and household uses (Khaliq *et al.,* 2006). EM is a mixture of various groups of microorganisms that has a reviving action on humans, animals and the natural environment.

Jochen *et al.* (2008) have also described EM as a multi-culture of coexisting anaerobic and aerobic beneficial microorganisms. The main species involved in EM include: Lactic acid bacteria: *Lactobacillus plantarum, Lactobacillus casei, Streptoccus lactis*; Photosynthetic bacteria: *Rhodopseudomonas palustrus, Rhodobacter spaeroides*; Yeasts: *Saccharomyces cerevisiae*, *Candida utilis*, Actinomycetes: *Streptomyces albus, Streptomyces griseus,* fermenting fungi *Aspergillus niger,Fusarium oxysporum.*

The basis for using these EM species of microorganisms is that they produce various substances, like presence of lactic acid bacteria, which secrete organic acids, enzymes, antioxidants, and metallic chelates (Xu *et al.,* 2009). The creation of an antioxidant environment by EM assists in the enhancement of the solid-liquid separation, which is the foundation for cleaning water (Higa and Chinen, 1998). One of the major benefits of the use of EM is the reduction in sludge volume. Theoretically, the beneficial organisms present in EM should decompose the organic matter by converting it to carbon dioxide (CO_2), methane (CH_4) or use it for growth and reproduction. Studies have suggested that this is the case for both wastewater treatment plants and also septic tanks. Freitag (2011) suggests that introducing EM into the anaerobic treatment facilities help to reduce the unpleasant by-products of this decomposition and also reduce the production of residual sludge. These factors tend to suggest that theoretically EM should assist in the treatment of wastewater by improving the quality of water discharged and reducing the volume of sewage sludge produced. (Jochen *et al.,* 2008).

EM is eco-friendly, safe and organic. The EM fermented garbage is supplemented with useful microorganisms which makes the compost imminently suitable for agricultural use. EM is effective in all conditions. The turning process requires 20 to 22 days with higher C: N ratio due to presence of microbes. Just spraying on garbage heap is sufficient. With easy application, EM is safe for human health. It can treat the leachate coming out form the garbage as well and remove the foul smell from decomposed garbage. Menace of flies and mosquitoes is suppressed to the minimal by application of this technology. EM technology is not only environmental friendly but goes a step further to actually protect the environment. It suppresses harmful gases generated from garbage. It is very economical. EM provides healthy environment to the workers. All these mean lower cost of operations, easy application and at the same time protection of the environment (Higa and Chinen*,* 1998).

To adopt this technology, various studies have been conducted to develop a microbial consortium to recycle various types of biodegradable wastes as well as to safeguard the environment. However, no such work has been carried out for treatment of sewage in Jammu and Kashmir as yet. Keeping in view the increasing quantity of sewage in our state, its impact on environment and the possibility of recycling through indigenously isolated effective microorganisms, the present study was undertaken with the following objectives:

- Isolation and characterization of effective microbes from Sewage and respective sources.
- Identification of effective microbes based on morphological & biochemical characteristics.
- Development of low cost, eco-friendly and effective microbial consortia with biodegradable property for sewage treatment.
- Effect of Effective microbes on Sewage Effluent.

Chapter 2

REVIEW OF LITERATURE

Human activities related to agriculture including livestock, urban and industrial development sector generate large quantities of wastewater that have to be treated prior to their discharge in water bodies or land. Agricultural and urban wastewater accumulate large quantities of pollutants, such as organic nitrogen and phosphorus compounds in surface waters, underground waters and soils. These pollutants are responsible for eutrophication and are nutrients for a variety of microbial species. If they are not treated properly, their pollutant load can cause serious environmental deterioration with direct impact on human health. The most commonly applied wastewater treatment application is the biological process i.e. the use and exploitation of bacterial species for removing pollutants (Vanwonterghem *et al.*, 2014)/

Discharges of wastewater from municipal and industrial treatment plants have been recognised as one of the major factors of aquatic pollution around the world (Reemtsma *et al.,* 2006). In many developing countries, the bulk of domestic and industrial wastewater is directly discharged into water streams without any treatment processes or after primary treatment only (Dhote *et al.,* 2012). The discharge of untreated wastewater to the water bodies without any treatment processes will lead to several environmental problems such as:

1. Untreated wastewater which contains a large amount of organic matter will consume the dissolved oxygen for satisfying the biochemical oxygen demand (BOD) of wastewater and thus deplete the dissolved oxygen of the water stream required by the aquatic lives
2. Untreated wastewater usually contains a large amount of pathogenic or disease causing microorganisms and toxic compounds that can dwell in the human intestinal tract thus threatening the human health
3. Wastewater may also contain certain amount of nutrients which can

stimulate the growth of aquatic plants and algal blooms, thus, leading to eutrophication of the lakes and streams

4. Decomposition of the organic compounds present in wastewater can lead to the production of large quantities of malodorous gases (Topare *et al.,* 2011).

Literature pertaining to various aspects of "Formulation of Effective Microbial Consortia and its Application in Sewage Treatment". is reviewed under the following headings :

2.1 Sewage treatment plant Hazratbal

2.2 Isolation of microorganisms from various sources

  2.2.1 Isolation of Lactic acid bacteria

  2.2.2 Isolation of *Pseudomonas*

  2.2.3 Isolation of Yeasts

  2.2.4 Isolation of Actinomycetes

  2.2.5 Isolation of Fermenting fungi

2.3 Screening of effective microorganisms

2.4 Compatibility amongst microbial isolates

2.5 Formulation of effective microbial consortium

2.6 Treatment of sewage water using effective microbial consortium

2.1 Sewage treatment plant Hazratbal

Sewage treatment plant, Hazratbal, Srinagar is situated between 34°9′N, 74°91′E geographical coordinates. Disposal site of treated sewage is Dal lake which is a multi-basined, open drainage type lake (Zutshi and Khan, 1978). Catchment area of the STP includes University of Kashmir, National Institute of Technology, Hazratbal, Naseem-Bagh, Mirza-Bagh, Kanitar, Saderbal, Mahivar.

The treatment in the STP is carried out in three distinct stages. Pre-treatment which comprises of screening and grit removal; Biological treatment comprising of fluidized aerobic bioreactors(FAB), followed by clarification, and tertiary treatment comprising of chemical addition and precipitation to remove phosphates and addition of chlorine to remove the *E. coli*.

2.2 Isolation of microorganisms from various sources

The microorganisms that are most effective for biodegradation of wastes could be isolated and identified on the basis of their action on wastes. Such microbes could later on be used for sewage water treatment so that the environment in our surroundings is clean and free from pollution. There are various microorganisms involved in biodegradation of liquid waste. However, a few groups are compatible with each other and form an effective combination or consortium. Some of these are reviewed as follows:

2.2.1 Isolation of Lactic acid bacteria:

Lactic acid bacteria produce lactic acid from sugars and other carbohydrates, developed by photosynthetic bacteria and yeasts when grown together in a consortium. Therefore, some foods and drinks such as yoghurt and pickles have been made with lactic acid bacteria for decades. However, lactic acid is a strong sterilizing compound which suppresses harmful microorganisms and enhances decomposition of organic matter. Moreover, lactic acid bacteria promote the decomposition of material such as lignin and cellulose and ferment these materials, thereby removing undesirable effects of un-decomposed organic matter. Lactic acid bacteria have the ability to suppress disease inducing microorganisms such as *Fusarium.*

Kantachote *et al.* (2005) isolated lactic acid bacteria from fermented vegetables, fish and milk.

Kandler and Weiss (1986) identified the lactic acid bacterial isolates following the methods in Bergey's Manual of Systematic Bacteriology, vol 2.

They also conducted the carbohydrate fermentation in MRS fermentation broth with 0.004% bromocresol purple but without glucose and containing 2% of each of the treated sugars. However, Magnusson *et al.* (2003) isolated lactic acid bacterial strains which were primarily screened for anti-yeast activity using a dual culture overlay assay.

Lactobacilli were enumerated in MRS agar medium (Oxide) under anaerobic conditions (Gas Pak System, Becton Dickinson) at 30 °C for 3 days by Deveriese *et al.* (1987). They also counted Lactococci in M17 agar medium (Oxide) after incubation for 2 days at 30°C. Cell shape, cell arrangements, gram-staining, catalase activity, gas production from 1% glucose, temperature requirement (15, 40 and 45°C), NaCl tolerance (4 and 6.5% NaCl) and growth at pH 3.9 and 9.6 were assessed in M17 or MRS broth.

Jimenez *et al.* (1993) performed the identification of Lactobacilli with the Gas Pak System at 30°C for 48 hours. They also recommended its storage at 4°C in sterile reconstituted skimmed milk (10%) or at -20°C in MRS broth supplemented with 20% glycerol. Isolates were examined for their antagonistic activity against organisms by the direct method (Fleming *et al.,* 1975) or by well diffusion method (Barefoot and Klaenhammer, 1983). However, Fleming *et al.* (1975) tested Lactobacilli in MRS agar under anaerobic conditions (Gas Pak System, Becton Dickinson) at 30°C for 18 hours.

2.2.2 Isoaltion of *Pseudomonas*

The Pseudomonads are a group of independent, self-supporting microbes. These bacteria synthesize useful substances from secretions of roots, organic matter and/or harmful gases (e.g. hydrogen sulphide). Useful substances produced by these microbes include amino acids, nucleic acids, bioactive substances and sugars, all of which promote plant growth and development. The metabolites developed by these microorganisms are absorbed directly into plants and act as substrates for increasing beneficial populations (Higa, 2002).

Biodegradation by microorganisms represents one of the primary mechanisms by which petroleum and other hydrocarbon pollutants can be removed from the environment (Okoh, 2006). Some fungi, such as *Fusarium oxysporum*, and Bacteria *Pseudomonas cepacia*, *Pseudomonas putida, Pseudomonas aeruginosa* are used to degrade these pollutants (Kumar *et al.*, 1992), but genus *Pseudomonas* is capable of using different substrates, such as glycerol, mannitol, fructose, glucose, n-paraffins and vegetable oils, to produce Rhamnolipid-type biosurfactants (Kretschmer *et al.,* 1982). Isolation of *Pseudomonas* sp. was also reported from hydrocarbon contaminated region and its growth on crude oil associated with the production of surfactants (Desai and Banat, 1997).

Ashok (2007) isolated four bacterial strains which were able to degrade naphthalene, anthracene or mixture of both from the soil of oil refinery. Out of four isolates two of them were identified *Micrococcus* and other two were identified as *Pseudomonas* sp. and *Alcaligenes* respectively. These species degrade at the rate of 89, 67.5 and 92.1% of high molecular weight plasmid DNA.

2.2.3 Isolation of Yeasts

Yeasts synthesize antimicrobial and other useful substances required for plant growth from amino acids and sugars secreted by photosynthetic bacteria, organic matter and plant roots. The bioactive substances such as hormones and enzymes produced by yeasts promote active cell and root division. These secretions are also useful substrates for effective microbes such as lactic acid bacteria and actinomycetes. Yeasts have been isolated from natural substrates like leaves, flowers, sweet fruits like grapes, grains, fleshy fungi, exudates of trees, insects, dung and soil (Spencer & Spencer, 1997).

Yeasts have been isolated on Potato Dextrose Agar (PDA) after incubating at $30^{o}C$ for 48-72 hours (Deak and Beuchat, 1996). Identification of the isolated cultures was done up to genus level using cell shape, colony morphology, production of pigment, spores and biochemical tests. Sugar assimilation was examined in yeast nitrogen base medium containing 5% of maltose, galactose, sucrose, lactose, raffinose and insulin, whereas sugar fermentation was also investigated using 6% of maltose, trehalose, xylose, cellobiose, starch, raffinose, lactose, sucrose, galactose and glucose in a basal medium.

Rose & Harrison (1993) showed the ability of yeast to grow in YM (yeast and malt extract) medium with the addition of a compound such as 0.01 or 0.10% cycloheximide, 10 or 16% NaCl. Utilization of ethanol, methanol, urea and citrate by yeast were also conducted by Kurtzman and Fell (1999).

2.2.4 Isolation of Actinomycetes

Actinomycetes play a special role in creating humus and also produce antibiotics which kill the bacteria when they move in. They like moderate temperatures for their growth and are capable of degrading solid wastes. The actinomycetes give the pile a pleasing earthy smell, as a result of special enzymes they excrete. Actinomycetes are involved in the decomposition of complex organic compounds such as phospholipids. The majority of actinomycetes are free living saprophytes, found widely distributed in soil, water and colonizing plants. According to Alexander (1961), 70-90% of the actinomycetes in virgin and cultivated soils are *Streptomyces* sp. Since the discovery of actinomycin, the first antibiotic from an actinomycetes, many commercially important bioactive compounds have been produced using actinomycetes (Tanaka and Omura, 1990).Actinomycetes have been successfully isolated on Starch Caesin Agar medium (Nakeeb and Lechevalier, 1963). The antimicrobial activity has been studied primarily by spot inoculating the isolates on agar medium (Shomurat *et al.,* 1979). They also studied the zone of inhibition of the test microorganisms after incubation.

Gramer (1976) studied the antimicrobial activity of actinomycete isolates in broth culture. He also determined the bioactivity by measuring the diameter of inhibitory zones (mm) of test microorganisms around the wells after incubation. Locci (1989) characterized the actinomycete isolates morphologically to the genus level by comparing the morphology of spore bearing hyphae with entire spore chain as described in Bergey's manual. Williams and Cross (1971) observed the morphological details of *Streptomyces* sp, using 100x magnification. Holding and Cole (1971) studied the growth of actinomycetes on media under four different concentrations of NaCl (2, 5, 7, and 9%), three different pH (5, 8 and 9), five different temperatures (15, 25, 37, 42 and 50 ^{o}C) and utilization of different sugars and biochemical properties.

2.2.5 Isolation of Fermenting fungi

Inoculation with fungi eases the process of decomposition of lingo-cellulosic residues and improves the quality of finished compost. Thermophilic fungi have been isolated from soil, wheat straw compost and farm yard manure. Isolations have also been made from paddy straw enriched with each of these supplements separately, moistened with Reese minimal medium and incubated for 15 days at 50 ^{o}C (Reese and Mandel, 1963). However, Rautela and Cowling (1966) screened the fungal cultures qualitatively for the production of cellulase, xylanase, laccase and lignin peroxidase at 50 ^{0} C. The quantitative estimation of enzymes produced by fungal isolates involved the release of reducing sugars from filter paper, carboxy methyl cellulose, cellobiose and xylanose (Ghose, 1987; Bailey *et al.,* 1992). Reese minimal broth supplemented with 1% cellulose, xylose and paddy straw separately as the sole carbon source was also used for assaying reducing sugars, carboxy methyl cellulose, cellobiose and xylanose.

2.3 Screening of effective microorganisms

Screening of microorganisms is an important preliminary step in the selection of desired and suitable isolates. Besides, a good plate screening method essentially plays a vital role in the selection of appropriate isolate. Herrero (1983) studied the crude lactic acid bacterial cultures which inhibited some psychrophillic bacteria in milk and ground beef. A large number of lactic acid bacteria, singly or in combination, have also been shown to display varying degrees of antimicrobial activity against pathogenic microorganisms. Isolated lactic acid bacterial strains were also primarily screened for anti-yeast activity using a dual culture overlay assay (Magnusson *et al.,* 2003). However Muorad *et al.* (2005) isolated twenty two isolates of lactic acid bacteria from the spontaneous fermentation of olives and were identified on the basis of the phenotypic criteria. Eleven of them were identified as *Lactobacillus plantarum* which was followed by 4 *Lactococcus lactis* sub sp. *lactis* and 7 *Enterococcus* sp. Cell free supernatant of *Lactobacillus plantarum* OL9 was active against *Enterococcus*, *Propionibacterium* and *Erwinia.* The antibacterial activity was attributed to bacteriocin like substances. Buranakarl *et al.* (1988) isolated 226 photosynthetic bacteria in Thailand and were point inoculated on a modified Ormerod's agar medium containing 1% raw or cooked cassava starch and grown at 40 ^{o}C under light anaerobic conditions for 6 days. Potassium iodide solution was the testing reagent. Colonies showing a clear zone of greater than 2 cm diameter were selected for degradation of organic wastes. Various isolates of *Streptomyces* sp, obtained from different localities of Baghdad soils were tested for their antimicrobial activities (Rabah *et al.,* 2006). Five isolates exhibited the highest antimicrobial activities in the culture broth. The isolate AR1 was the most active one and on the basis of 16S ribosomal DNA amplification for phylogenetic study it was identified as *Streptomyces tendae*. This new strain was capable of producing antimicrobial agent(s), which was active under *in vitro* conditions against gram+ve and gram-ve bacteria (*Bacillus cereus,Bacillus subtilis, Staphylococcus aureus, Micrococcus luteus*, *Escherichia coli* and *Pseudomonas aeruginosa*), yeasts (*Candida albicans, Candida pseudotropicalis* and *Rhodotorula minuta*) Debananda *et al.* (2009) had worked out for the isolated actinomycetes from several niche habitats in Manipur, India, on selective media such as Chitin agar with or without antibiotics.

2.4 Compatibility amongst microbial isolates

The most common method to study the compatibility has been to study the antimicrobial activities of all the probable partners against one another by streaking on a suitable medium and incubating them at 28 °C for 48 to 96 hours. Lack of microbial growth (zone of inhibition) at the intersections was indicative of the antagonism of the cultures (Oskay, 2009) but the cultures growing in the close proximity were compatible to each other. Manjula *et al.* (2004) reported *in vitro* compatibility of *Pseudomonas fluorescens* and *Trichoderma* sp. in dual culture and found that *Pseudomonas fluorescens* had no effect on growth of *Trichoderma sp*. or vice versa. Dhoke and Kurundkar (2005) studied combined efficacy of efficient *Trichoderma viride* isolate with promising fluorescent *Pseudomonas* isolates. Combined dual culture of bacterial isolates and *Trichoderma viride* significantly influenced growth of *Fusarium oxysporum* sp, *Fusarium ciceri* and *Macrophomina phaseolina* thus suggested compatibility of these two antagonistic organisms, but the cultures growing in the close proximity were compatible to each other.

2.5 Development of microbial consortium

Consortium involves a micro-ecological system in which two or more microorganisms reach the advantage of the combined effects through common culture interaction and mutual influence and would ultimately enhance plant growth (Seneviratne, 2003). Consortia can perform complicated functions that individual populations cannot and can survive in more changeable environments than can uniform populations and can be more robust to environmental fluctuations. It has been suggested that development of plant growth promoting consortium, could be a feasible strategy for increased activity and better viability of plant growth promoting rhizobacteria (PGPR).

Shenoy and Kalagudi (2003) reported that when the strains are made into an inoculum consortium, each of the constituent strains of the consortium may compete with the others for rhizospheric establishments, or complement functionally for plant growth promotion.

Brenner *et al.* (2008) reported that the overall output of the consortium depends on a combination of tasks performed by constituent microbes. Mandalaywala and Trivedi (2016) formulated an effective microbial consortium of bacteria isolated from hydrocarbon polluted soils of Gujarat. Fulekar *et al.* (2017) studied bioremediation of toluene using microbial consortium obtained from industrial effluent. Piakong and Zaida (2018) worked on effectiveness of single and microbial consortium of locally isolated beneficial microorganisms in bio augmentation of oil sludge contaminated soil and reported that at different concentrations, bacterial consortium was better than that of single culture. Mixed populations can perform functions that are difficult or even impossible for individual strains or species. Application of microbes in a consortium may improve efficacy, reliability and consistency of the microbes under diverse soil and environmental conditions (Stockwell *et al.,* 2011). Population of yeast increased when applied as mixture rather than single application (Guetsky *et al.,* 2002).

2.6 Treatment of sewage water using effective microbial consortium

Bacteria, fungi and actinomycetes are helpful in decomposition of sewage water. They can be anaerobes, aerobes or facultative anaerobes. This microbial diversity enables the biodegradation process to continue despite the constantly changing environmental and nutritional conditions within. The microorganisms, responsible for biodegradation, degrade a broad range of compounds from amino acids and simple sugars to complex proteins and carbohydrates. This results in a thorough degradation of the compost material. Temperature levels and available food supply generally have the greatest influence in determining what class and species of organisms make up the microbial population at a particular time. Sherbiney *et al.* (2012) determined the maximum aerobic biodegradability of the domestic wastewater. They found that the minimum aerobic effluent COD concentration of rural areas was almost similar to the effluent standards for COD, while the minimum aerobic effluent COD concentration of urban areas was significantly lower than that of the Egyptian effluent standards for COD.

Ibrahim (2011) evaluated different technologies for domestic wastewater treatment in rural areas of Egypt. He also found that the effluent of these systems did not comply with Egyptian effluent standards for problems related to whole-cell metabolism of pesticides. The first report on bacterial utilization of dimethoate was reported by Liu *et al.* (2009). They isolated a strain of *Pseudomonas stutzeri* from water that was obtained in fields with frequent application of orthophosphates. 71.82% degradation was reported at 35 ^{0} C with shaking for 72 hrs. Thus, microbial degradation by fungi or bacteria is the means of disappearance of dimethoate from water as a source of P.

Abdel-Megeed and El-Nakieb (2009) evaluated the efficiency of EM on the degradation of dimethoate from contaminated water. The inherent abilities of microorganisms are suitable for the removal of metals from solutions (Beveridge and Murray, 1976; Langley *et al.,* 1999). These abilities have been identified as passive or active for accumulation and biosorption respectively (Brandl and Faramarzi, 2006). Indigenous strains are more suitable to overcome the challenges such as high concentration of metals, acidic conditions as they had adapted to conditions in situ. *Bacillus* strains have been widely used in the removal of metals (Pb, Cd, Cu, Ni, Co, Mn, Cr, Zn) from wastewaters (Philip *et al.,* 2001; Srinath *et al.,* 2003; Kim *et al.,* 2007). The high cost and large consumption of chemical agents have made chemical methods unattractive. To overcome these problems, heavy metals in sewage sludge are removed through bioleaching process, which emerged recently and is economical easy in operation, and non-hazardous to products (Babel and del Mundo Dacera, 2006; Pathak *et al.,* 2009). The experimental batch system studies by Xiang *et al.* (2000) showed that the isolated indigenous iron-oxidising bacteria have more ability in reducing the heavy metals from anaerobically digested sewage sludge from the Yuen, Long wastewater treatment plant, Hong Kong. In this study, to maintain low pH and to accelerate the solubilisation of Cr, Cu, Zn, Ni and Pb, FeSo4 is added along with the isolated indigenous microorganisms. After 16 days of bioleaching, more than 80% of Cu, Zn, Ni and Cd are removed as compared with that of the control. Similarly, studies of Wen *et al.* (2013) on leaching kinetics show that solubilisation of heavy metals or removal efficiency is more by the indigenous iron-oxidising bacteria than without using indigenous microorganisms in the control ones which were collected from the sewage water plant. Solid concentration, pH, inoculum concentration, and $FeSO_4$ effectively influence the bioleaching of metals. Effectiveness depends upon the metal species because of their different nature of binding of sludge; removal of Zn from the sludge was dominated by chemical leaching, while the removal of Cu, Pb and Cr was dominated by bioleaching (Wen *et al.,* 2013). The two species *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans* have a great significance in bioleaching process (Tyagi *et al.,* 1993; Chan *et al.,* 2003).

Iron-based bioleaching is considered to be superior to sulphur-based bioleaching due to sludge acidification and heavy metal solubilisation in sulphur-based bioleaching (Wang *et al.,* 2008). To leach successfully, ammonium ferrous sulphate and ferrous sulphate are used to enrich the indigenous iron-oxidising microorganisms in sludge with a neutral pH (Pathak *et al.,* 2009). Metal removal from dewatered metal plating sludge using *Acidophillous ferrooxidans* indicates that pH, oxidation–reduction potential (ORP), sulphate production, pulp density and agitation time were all important parameters in bioleaching (Bayat and Sari, 2010).

Chapter – 3

MATERIALS AND METHODS

The present investigation was carried out in the Division of Basic Sciences and Humanities, Faculty of Agriculture, Wadura Sopore and the laboratory work was conducted in the Division of Plant Pathology, Faculty of Horticulture, Shalimar, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir.

The materials used, experimental procedures followed and methods adopted during the present investigation "Formulation of Effective Microbial consortia and its application in Sewage Treatment" are described below:

3.1 Sampling

Sewage water sample was collected from Sewage treatment plant (STP) Hazratbal. The samples were taken from outlet component of STP Hazratbal in plastic bottles, between 8:00 a.m. and 10:00 a.m. The samples were then brought to the laboratory for detailed analysis. Standard methods as given in American public health association (APHA, 1998) were used for the analysis. The isolation, screening and most of the laboratory studies including *in vitro* biodegradation of sewage water was carried out in the Division of Plant Pathology SKUAST-K Shalimar.

3.1.1 Description of Sewage Treatment Plant Hazratbal

During the present investigation Sewage water was collected from Sewage treatment plant, Hazratbal, Srinagar situated between 34°9´N, 74°91´E geographical coordinates. Disposal site of treated sewage is Dal lake which is a multi-basined, open drainage type lake (Zutshi and Khan, 1978). Catchment area of the STP includes Kashmir University, National Institute of Technology, Hazratbal, Naseem-Bagh, Mirza-Bagh, Kanitar, Saderbal, Mahivar.

Effluent Discharge Million litre per day(MLD)	Targeted Sewer lines (m)	Sewer Lines completed (m)	Targeted laterals (m)	Laterals completed (m)
7.5	7909	7652.50	20187	12557

(Source: Lakes and Waterways Development Authority, Jammu and Kashmir, India)

3.2 Analysis of Sewage water to detect various chemical parameters

Prior analysis of Sewage water was done before application of Microbial Consortium employing various methods to detect various elements like N, P, K, C, BOD, COD and heavy metals. The detailed procedures employed for detection of various elements are given in section 3.12.

3.3 Isolation of most effective microorganisms

Following groups of microorganisms which constituted the effective microbial consortium were isolated:

1. Isolation of Lactic Acid Bacteria
2. Isolation of *Pseudomonas* species
3. Isolation of Yeasts
4. Isolation of Actinomycetes
5. Isolation of Fungi

3.3.1 Isolation of Lactic Acid bacteria

Lactic acid bacteria were isolated from curd samples which were obtained from different sources like Khyber, Zum Zum, Milkman, Snow Cap and Amul etc. The samples were collected and serial dilution was done. Culture suspension (0.1 ml) was inoculated by spread plate technique on de Man Rogosa and Sharpe agar medium (MRS). After incubation at 30^oC for 24 hours, single pure colonies were transferred to a new MRS plate and further 20 were purified. Working cultures were kept on MRS agar slants at 4^{0}C. The lactic acid bacteria were identified following the methods in Bergey's Manual of Systematic Bacteriology, vol. 2 (Kandler and Weiss, 1986). Isolates were characterized on the basis of their morphology, catalase, oxidase, IMVIC, citrate production, hydrogen sulphide production and on the basis of fermentation of various carbohydrates like galactose, glucose, lactose, maltose, mannitol etc.

3.3.2 Isolation of *Pseudomonas* species

Soil samples were collected from several areas of garages, containing spilled oil soils, randomly 15-20 cm beneath the surface using sterile spatula and were placed in sterile screw capped vials. The soil samples were placed in sterilized plastic bags and stored on ice during transfer from site to the laboratory. Soil samples were resuspended in 10 ml of sterile water and vigorously shaken for 5 min. Spread plate technique was used for culture isolation and enumeration. The cultures plates were then incubated at 24^{o}C for five days. Isolates exhibiting distinct colonial morphologies were purified and isolated by repeated sub culturing into basal medium (King's B) until purified strains were obtained. Isolates were characterized on the basis of their morphology, pigment production, biochemical tests like catalase, urease, IMViC, citrate production, starch hydrolysis, gram staining and cell size.

3.3.3 Isolation of Yeasts

Dry yeast granules were used for isolation of yeasts. One gram of yeast granules was suspended in 9 ml of distilled water and 0.1ml of culture suspension was withdrawn and spread on Potato Dextrose Agar medium and the culture plates were incubated at 27 ^{0}C for 48 hrs. After incubation single morphologically well-formed colonies were picked up. The appropriate ones were re-cultivated several times for purification. The ability of isolates to utilize glucose, sucrose, maltose, galactose, xylose, starch and lactose as carbon sources was determined in Yeast and Peptone medium containing the respective sugar and the pH indicator.

3.3.4 Isolation of Actinomycetes

One gram from the thoroughly mixed soil sample taken from decomposing pile of garbage was suspended in 100 ml sterile distilled water and incubated in an orbital shaking incubator at 28^0C with shaking at 140 rpm for 30 min. Sediments were allowed to settle down and serial dilutions upto 10^{-4} were prepared. From 10^{-4} dilution and aliquot of 0.1ml was pour plated (in triplicate) on Starch Casein Agar medium and incubated at 28^0C for 10 days. Individual colonies with characteristics of actinomycetes were picked up and pure cultures of the respective isolates were obtained by repeated streaking on Starch Casein Agar plates. The pure isolates were transferred to Starch Casein Agar slants and preserved at 4^0C. These isolates were evaluated for their antimicrobial activity (El-Nakeeb and Lechevalier, 1963; Kuster and Williams, 1964). Actinomycete isolates were characterized morphologically to the genus level by comparing the morphology of spore bearing hyphae with entire spore chain as per Bergey's manual.

3.3.5 Isolation of Fermenting fungi

The samples of onion, garlic, banana peel, saffron corms and rice were taken and suspension was made in distilled water and 0.1 ml of suspension was taken and inoculated on czapek's dox medium at 27^0C for 48 hrs. The fungal species thus obtained, were sub cultured and maintained in a refrigerated condition. The fungal species were identified by morphological and physiological tests like spore type, LCB staining and amylase production.

3.4 Characterization of the microbial isolates

3.4.1 Morphological characterization

All the Effective microorganisms were examined for the colony morphology, cell shape, gram reaction and spore forming ability as per the standard procedures given by Anonymous (1957) and Barthalomew and Mittewer

(1950). Colony morphology was studied with the help of magnifying lens and cell shape of the isolates under microscope

3.4.1.1 Gram Staining (Gram, 1884)

This technique is used to differentiate between the two major categories of bacteria i.e. Gram positive and Gram negative. The heat-fixed smear of bacterial culture was flooded with the primary stain Crystal Violet for two minutes. Slide was washed and then 1-2 drops of Gram's Iodine was put on the slide as mordant. Again slide was washed under tap water and then 2-3 drops of 95% alcohol or acetone was used for 05 seconds as decolourizer. Again the slide was washed and few drops of Safranin dye as a counter stain was put on the slide for half a minute. Then the slide was again washed for 2-3 seconds and air-dried and seen under 100 x oil immersion lens by using cedar wood oil. Violet coloured cells were taken as gram positive and pink coloured cells as gram negative.

3.4.1.2 Lacto Phenol cotton blue staining

LPCB is a stain used for making semi-permanent microscopic preparation of fungi. The LPCB stain has following three components:

Phenol: Is used to kill any live organism.

Lactic acid: Is used to Preserve fungal structures.

Cotton blue: Is used to stain the chitin and cellulose of the fungal cell wall intensely blue.

Procedure: A drop of 70% ethanol was placed on a clean microscopic glass slide. Then the specimen was immersed in a drop of alcohol and one or two drops of the LPCB was added before the alcohol dried out. Holding the cover slip between the index finger and thumb, one edge of the drop of mountant was touched with a cover slip edge and lowered gently avoiding air bubbles and it was examined for spores and other structures under microscope.

3.4.2 Biochemical characterization

The Biochemical characterization of the isolates was carried out as per the procedures given by Cappuccino and Sherman (2009). The tests which were carried out are outlined as under:

3.4.2.1 Catalase test (Blazevic and Ederer, 1975)

Nutrient agar slants were inoculated with test organisms and then incubated at 30 ^{0}C for 24 hours. After incubation the tubes were flooded with one ml of three per cent hydrogen peroxide (3% H_2O_2) and observed for production of gas bubbles. The occurrence of gas bubbles within 5 to 10 seconds was considered positive for catalase activity.

3.4.2.2 Urease test (James and Sherman, 2009)

Bacterial isolates were tested for urease activity by inoculating the test tubes containing five ml of pre-sterilized urea broth with phenol red as pH indicator and incubated at 30 ^{0}C for 24 to 48 hours. The formation of dark pink colour was taken as positive for urease activity.

Composition of Urease Agar

Peptic digest	1.0 g
Dextrose	1.0 g
Nacl	5.0 g
Disodium phosphate	1.2 g
Mono potassium phosphate	0.800 g
Phenol red	0.012 g
Agar	15 g
pH	6.8
Distilled water	1000 ml

3.4.2.3 Oxidase test (Cappuccino and Sherman, 2009)

The sterile cotton buds were taken and each one was wetted with about 4 inoculating loop fulls of de-ionized water. A large mass of overnight pure culture of the test isolate was transferred aseptically to the disk with the help of a loop. After inoculation, two to three drops of tetramethyl phenylene diamine dihydrochloride (TMPD) was added to the surface of the growth of test organism and observation was made for up to 3 minutes. The colour change of the area of inoculation from dark blue to maroon to almost black was taken as oxidase positive.

3.4.2.4 Methyl red test (Seeley and Van Demark, 1981)

The sterilized MR-VP broth was inoculated with the test cultures. The tubes were incubated at 28 (±2) ^{0}C for 48 hours. After incubation five drops of methyl red indicator was added to each tube and gently shaken. The production of red colour was taken as positive for the test and production of yellow colour was taken as negative for the test.

3.4.2.5 Voges-Proskauer test (Seeley and VanDemark, 1981)

The test cultures were inoculated to the pre-sterilized tubes containing MR-VP broth. The tubes were incubated for 48 hours at 37 ^{0}C. After incubation ten drops of Barritt's reagent A (6 grams of α-napthylamine in 100 ml of 95% alcohol) was added and gently shaken followed by addition of ten drops of Baritt's reagent B (16 grams of KOH in 100 ml of Distilled water). The development of rosy colour in the broth was taken as positive for the test.

Composition of MR-VP medium (Glucose phosphate broth)

Dipotassium hydrogen phosphate	5 g
Peptone	5 g
Glucose	5 g

Distilled water 1000 ml

3.4.2.6 Starch hydrolysis (Eckford, 1927)

Petri-plates, containing starch agar, were inoculated with test cultures and incubated at 30 ^{0}C for three days. After incubation, the plates were flooded with Lugol's iodine solution and allowed to stand for 15-20 minutes. The clear zone around the colony was considered as positive for the test.

3.4.2.7 Casein hydrolysis (Seeley and Van Demark, 1970)

Plates containing skim milk agar were streaked with test cultures and incubated at 30 ^{0}C for seven days in inverted position. The clear zones around the colonies against a black background after incubation were taken as positive for casein hydrolysis.

3.4.2.8 Citrate utilization test (Simmons, 1976)

Simmon's citrate medium was prepared, dispensed in test tubes and sterilized at 121 ^{0}C for 15 minutes, then the slants were allowed to set. The cells of test cultures were rinsed in distilled water and then transferred to Simmon's citrate agar slants. The slants were incubated for 96 hours at 28 ^{0}C. A positive test showed a blue colour on the streak of growth. Retention of original green colour and no growth on the line of streak indicated a negative reaction.

Composition of Simmon's Agar

NaCl (sodium chloride)	5.0 g
Sodium citrate	2.0 g
Ammonium Dihydrogen phosphate	1.0 g
Dipotassium phosphate	1.0 g
Magnesium sulphate	0.2 g
Bromothymol blue	0.08 g
Agar	15.0 g
Distilled water	1000 ml

3.4.2.9 Hydrogen sulphide production (Cowan and Steel, 1970)

Hydrogen sulphide (H_2S) production test is used mainly to assist in the identification of members of family Enterobacteriaceae and occasionally to differentiate other bacteria such as *Bacteroides* sp. and *Brucella* sp. H_2S is produced when sulphur – containing amino acids are decomposed. This test can be performed in many ways using different biochemicals.

Composition of Sulfite Indole Agar

HM Peptone B	3.000 g
Peptone	30.000 g
Peptonized iron	0.200 g
Sodium thiosulphate	0.025 g
Agar	3.000 g
Final pH (at 25°C)	7.3±0.2
Distilled water	1000ml

Bacterial isolates were inoculated in test tubes containing 5 ml of Sulfite indole motility agar medium (SIM) and incubated at 28 ^{0}C for three days. This medium contains ferrous ammonium sulfate and sodium thiosulfate, which together serve as indicators for the production of hydrogen sulfide. Hydrogen sulfide production was detected when ferrous sulfide, a black precipitate ring was produced as a result of ferrorus ammonium sulfate reacting with H_2S gas.

3.4.2.10 Carbohydrate fermentation (Neyra *et al.*, 1977)

The isolates were examined for their ability to ferment various carbohydrates by utilizing different carbon sources *viz.,* sucrose, maltose, glycerol, galactose, mannitol, lactose, raffinose, cellobiose, glucose and citrate. The carbon sources were added at the concentration of two per cent to the agar medium and 24 hour old cultures were streaked on the surface of agar medium and incubated at 28±2 ^{0}C for 24 hours. The extent of growth on the media containing different carbon sources was observed visually and growth was scored as no growth (-) and growth (+).

3.5 Qualitative screening of fungal isolates for their relative amylolytic activity

All the collected fungal isolates were primarily screened for their amylolytic activity on Yeast Peptone Soluble Starch medium (YPSS) by observing zone of clearance. The composition of YPSS medium was as follows:

Soluble starch	:	15.00 g
Yeast extract	:	4.00 g
K_2HPO_4	:	1.00 g
$MgSO_4.7H_2O$	:	0.50 g
Agar	:	20.00 g
pH	:	7.00 g
Distilled water	:	1000.00 ml

The YPSS medium was sterilized at 15 lbs per square inch pressure for 20 minutes. The isolated microorganisms were inoculated on the plates of YPSS medium. After three days of incubation at 30^0 C, petri-dishes were flooded with Gram's iodine solution and kept for 30 minutes. A clear zone around the growth in blue background indicates amylolytic activity and visible difference in extent of zone of clearing was recorded for relative amylolytic activity (Harrigan and McCance, 1966).

3.6 Phosphate solubilization

All the isolated cultures of *Pseudomonas* were grown in Tryptic soya Agar (TSA) broth. Log phase growing cells of each culture (15 µl) were spotted on Pikovskaya's medium plates. These plates were incubated at 28 ^{0}C for 3-4 days. Zone of solubilisation and colony size were measured using a measuring scale and these values were used to calculate solubilisation index as per the following formula (Edi-Premono *et al.,* 1996).

$$\text{Phosphate solubilisation index } = \text{PSI } \frac{\text{Colony diameter + Halo zone diameter}}{\text{Colony diameter}}$$

3.7 Chitinase assay

The chitinase activity of *Pseudomonas* isolates was estimated as per Reissig *et al.* (1995) and for preparation of colloidal chitin the method of Berger and Reynolds (1958) was adopted. For colloidal chitin preparation 10gm of powdered chitin was digested overnight with concentrated hydrochloric acid (500ml) at 4 ^{0}C. Then contents were centrifuged (10,000 rpm) for 20 minutes and supernatant removed by filtration. The collected contents were washed three times to remove all traces of acids till a pH of 4 was achieved. The pH was adjusted by using 2N NaOH and 1 N HCL. The collected contents were oven dried at 45 ^{0}C for 20 hours and later added to minimal media. All the selected cultures were grown on Luria bertani (LB) broth and spotted on already prepared minimal media. Petri plates amended with 0.3% colloidal chitin were incubated at 30 ^{0}C for 7 days, then iodine was added to these plates. Development of halo zone around the colony after addition of iodine was considered as positive for chitinase enzyme production. The halo zone "CZ" (the clear visible zone around bacterial colonies indicating chitin hydrolysis) and colony diameter/colony size (CS) of the isolates was measured with a measuring tape, Then the ratio of both the parameters was calculated (CS:CZ).

3.8 Antimicrobial activity

In vitro antimicrobial activity of the isolates against *Fusarium oxysporum, Fusarium moniliforme, Bacillus subtilis* and *E. coli* on potato dextrose agar (PDA) plates and nutrient agar plates was examined by dual inoculation technique (Sakthivel *et al.*, 1986). The fungal pathogen was inoculated on the plates containing potato dextrose agar medium and bacterial pathogen on nutrient agar medium and incubated at 28±2 ^{0}C for 72 hours. With the help of sterile cork borer, the disc of fungal growth from this plate was taken out and placed at the centre of the fresh potato dextrose agar medium containing plate. 24 hours old growth of each bacterial isolate was then streaked on either side of the disc and kept for incubation at 28±2 ^{0}C for 72 hours. After the incubation for 72 hours, the plates were visually observed for the inhibition of fungal pathogen by comparing with the control PDA plate inoculated with only fungal pathogen. Radial growth of the fungus was measured and same was performed with bacterial isolates and inhibition percentage was calculated by the formula:

$$\text{Per cent inhibition} = \frac{(C\text{-}T)}{C} \times 100$$

Where,

C = Colony diameter (mm) of the control

T = Colony diameter (mm) of the test plate

3.9 Compatibility

To study the antagonistic properties, a single bacterial strain was streaked as a straight line in the centre of nutrient agar and fungal strains on Potato Dextrose Agar plates. Cultures to be tested were streaked perpendicularly across the initial culture and incubated at 28 ^{0}C for 48 to 96 hours. Lack of microbial growth (zone of inhibition) at the intersections was indicative of the antagonism of the cultures (Oskay, 2009) but the cultures growing in the close proximity were compatible to each other. Then these compatible cultures were used in formulation of three different consortia.

3.10 Formulation of Effective microbial consortium

Microbial consortium was prepared by mixing 3 g of molasses in 100 ml of distilled water. The pH of the broth was adjusted to 7 with 0.1 N HCl. 1 ml of *Pseudomonas* bacterial culture was added to 100 ml of the molasses and incubated at 30 ^{0}C at 120 rpm for two days. In the second step, 1 ml of Actinomycetes culture was added to the broth containing *Pseudomonas* bacteria. This mixture was continuously shaken at 120 rpm at 30 ^{0}C for another two days. The final step was to add 1 ml each of the cultures of lactic acid bacteria, yeast and fermenting fungi to broth containing other two species and shaken at 120 rpm at 30 ^{0}C for another two days. The microbial consortia designated as Effective Microorganisms (EM) were stored for further studies.

3.11 Sewage water Management using Effective microbial consortium

Sewage water sample from STP Hazratbal was collected in sterile bottles and 250 ml of sewage sample was treated with 10 ml of Effective microbial consortium and different parameters like N, P, K, C, BOD, COD and Heavy metals were assessed at different intervals of time, at different pH, temperature and molasses concentration.

***In vitro* biodegradation :** Treatment of sewage water with:

i. EM1

ii. EM2

iii. EM3

iv. Sewage water without any treatment (Control)

Replications	:	3
Design	:	CRD

3.12 Effect of temperature, pH, molasses concentration and incubation period on growth of effective microorganisms

Growth of various constituent microorganisms in various effective microbial consortia was checked by growing these microbial consortia at different temperatures, pH, molasses concentration and incubation period.

3.12.1 Effect of Temperature, pH, molasses concentration and incubation period on total N, P, K, C, BOD, COD and heavy metals using various consortia

All the three different effective microbial consortia were grown at three different temperatures (20, 25 and 30^{0}C), different pH 6, 7, 8 different molasses concentration of 1, 3 and 10% and incubation period of 8, 18 and 28 days and various elements in sewage water with effective microbial consortia were estimated like N, P, K, C, BOD, COD and heavy metals and was observed for steep decrease in values from original to permissible values so that sewage water can be reused in agricultural activities.

3.13 Analysis of treated sewage

3.13.1 Estimation of organic carbon

Organic carbon was estimated by rapid titration method of Walkley and Black (1965). The sewage water (10ml) was put in 500 ml conical flask followed by the addition of 20 ml 0.1 N potassium dichromate and 30 ml of concentrated sulphuric acid. The mixture was kept undisturbed for 30 minutes. After that 250 ml water and 10 ml of 85 per cent orthophosphoric acid was added. The mixture was titrated against 0.5 N ferrous ammonium sulphate (FAS) using diphenylamine indicator. Simultaneously a blank was run without the sample for final calculation.

$$\text{Organic carbon (\%)} = \frac{(\text{B-S} \times 0{,}0006/\text{m})}{100}$$

Where, B is volume of ferrous solution used in blank

S is volume of ferrous solution used in sample

m is mass of sample in grams

3.13.2 Estimation of total nitrogen

Sewage water (10 ml) was treated with EM consortia and was taken in Kjeldhal's digestion flask and 25 ml of concentrated H_2SO_4, 10 g of digestion mixture (K_2SO_4 + $CuSO_4$ + $FeSO_4$ in the ratio of 10:0.5:1) and 1 g of selenium powder was added. The samples were digested till the solution became clear. Just after cooling, volume was made up to 100 ml with distilled water. 10 ml of aliquot was transferred into micro-Kjeldhal's distillation flask and 10 ml of 40 per cent

NaOH solution was added to it. The outlet was dipped into 4 per cent boric acid solution containing bromocresol green and methyl red indicator. After completion of distillation, boric acid was titrated against 0.005 N H_2SO_4 to the original shade (pink). Simultaneously blank was also run (Jackson, 1958).

Calculation:

$$\%N = \frac{[(VHCL \times NHCL) - (VBK \times NNaOH) - (VNaoH \times NNaOH)]/1.4007 \times W \times lab\ DM/100}{Weight\ of\ sample}$$

Where, VHCL= volume of acid used to titrate sample

VNaOH= volume of base used to titrate sample

NHCL= Normality of acid

NNaOH= Normality of base

VBK=ml of standard base needed to titrate 1ml of standard acid minus B

B= ml standard NaoH needed to titrate reagent blank carried through method and distilled into 1ml standard HCL

1.4007= milliequivalent weight of nitrogen × 100

3.13.3 Estimation of total phosphorus

Procedure: The method of Fiske and Subba Rao (1925) was adopted for the estimation of total phosphorus.5 ml of sewage water was taken in 10 ml of graduated test tube and 1 ml of 2.5% molybdic acid reagent was carefully added followed by the addition of 0.4ml of 1-amino-2 napthol-4-sulphuric acid. The colour of the solution turned blue and the volume was made upto 10 ml. The solution was shaken for 5 min for maximum colour development and transferred to a colorimetric tube. The intensity of the colour was read at 620 nm on spectrophotometer and blank was run simultaneously.

Calculation:

$$P = ppm \times dilution\ factor\ reading\ sample\ (g)$$

3.13.4 Estimation of total potassium

Procedure: The sewage water (10ml) was digested with 20 ml of tri-acid mixture (HNO_3 : $HClO_4$: H_2SO_4 in the ratio of 9:4:1) till the mixture became colourless. Flame photometer was set and standard curve was prepared by making standard solutions (0, 5, 10 ml per litre). The full scale (100) of flame photometer was set with the solution of highest concentration (10 ml/l). Readings of other standard solutions were also taken and curve was plotted. The digestion mixture was diluted up to the concentration laid between 0 to 5 ml/l. The samples were then read in flame photometer at 548 nm wave length (Jackson, 1967).

3.13.5 Estimation of heavy metals

Procedure: Atomic Absorption Spectrometry (AAS) is a very common and reliable technique for detecting metals and metalloids in environmental samples (Baron, 1990; Dauvaltermic, 1998). 5ml of Sewage water sample was diluted to 50 ml with double distilled water (DDW) and Heavy metals like Cd, Ni, Pb were detected using (AAS) Atomic Absorption Spectrometre (GBS Scientific Equipment Pvt. Ltd, Australia).The standard solution (MERCK manufacturers, Mumbai, India) for Cd, Ni, Pb was used for calibration. The heavy metal contents were directly calculated by the computer software AVANTA-2.0 version preinstalled in computer connected to AAS.

3.13.6 Estimation of BOD (Biological oxygen demand).

Procedure: 1 litre of sewage water was taken in a flask. To that 3 ml of EM consortia was added.

- Then 4 ml of calcium chloride solution was added and also 4 ml of phosphate buffer, 4 ml of magnesium sulphate and 4 ml of ferric chloride as a nutrient for survival of microorganisms was added.
- Mixture was mixed thoroughly.
- Then the prepared mixture was filled in two BOD bottles. One bottle

was used for initial Dissolved oxygen (DO) analysis and another bottle was used for final DO analysis after incubation of 5 days.

- For calculating the initial DO 1ml of the sample was taken from the BOD bottle and volume of 1 litre was made with the dilution water.
- The mixture was mixed and filled again in two bottles until the sample did not overflow and recapped again. Overflow was done so as to remove any air bubble or any gas present in it.
- After taken into bottles 2 ml of manganese sulphate, 2 ml of sodium azide solution was added and lid was closed and mixed thoroughly.
- After that solution was allowed to withstand for 2-3 min so as to settle down. Sodium azide solution eliminates disturbance due to nitrate which is the most common disturbance in naturally handled effluents.
- Then 2 ml of concentrated sulfuric acid was added mixed thoroughly until clear yellow solution was formed. From the above solution 200 ml of solution was taken and to that 2 ml of starch solution was added and then titrated with 0.025M sodium thiosulphate until colorless.
- The titration value was noted.
- Same procedure was followed for the sample after 5 days of incubation

Calculation

$$\text{BOD (mg/l)} = \frac{(T_1 - T_2) - (B_1 - B_2) \times 1000}{\text{Volume of sample taken per litre}}$$

Where, T_1 = initial D.O in the sample

T_2 = D.O after 5 days of incubation in sample

B_1 = Initial D.O in the blank and B_2 = D.O after 5 days of incubation in the blank.

3.13.7 Estimation of COD (Chemical oxygen demand)

Procedure

- 10 ml of sewage sample was taken in a round bottom reflux flask.
- Some glass beads were added to the solution to prevent from bumping while heating.
- Then 1 ml of Mercury sulfate solution was added by shaking and also 5 ml of potassium dichromate was added.
- Then 15 ml of silver sulfate sulfuric solution was added slowly and carefully.
- Then the reflux condenser was connected and the contents were digested on hot plate for 2 hrs.
- After digestion the flask was cooled and condenser was rinsed with 25 ml distilled water collected in same flask.
- Then 2-3 drops of ferroin indicator was added to flask and titrated with 0.025 M ferrous ammonium sulfate solution.
- Blank was prepared in same manner using distilled water

Calculation

$$\text{COD} = \frac{8\times1000\times\text{DF}\times\text{M}\times(V_B-V_S)}{\text{Volume of sample (in ml)}}$$

Where, DF= dilution factor (if applicable)

M= molarity of standardized Ferrous Ammonium sulfate sol

V_B = Volume consumed in titration for blank preparation

V_S = Volume consumed in titration for sample preparation

3.14 Determination of microbial population in sewage water

To determine microbial population in sewage water, 1 ml from each treatment was suspended in 10 ml of sterilized distilled water in a test tube and serial dilutions of the suspension were prepared by further dilutions. The total viable population of bacteria, fungi and actinomycetes were determined as per the following procedures.

3.14.1 Total viable bacteria

In order to determine the bacterial count in sewage water in each treatment, nutrient agar medium was used. Nutrient agar medium was autoclaved and poured into sterilized petri-plates. One ml of appropriate dilutions was evenly spread over cooled medium.

3.14.2 Total viable fungi

For the determination of total viable fungal population, potato dextrose agar (PDA) medium was used and the procedure followed. The plates were incubated at 28±2 ^{0}C for 2-3 days and fungal colonies developed on the medium were counted (Philipp *et al.,* 2001).

3.14.3 Actinomycetes

Individual colonies with characteristics of actinomycetes were isolated and pure cultures of the respective isolates were obtained by repeated streaking on Starch Casein Agar plates. The pure isolates were transferred to Starch Casein Agar slants and preserved at 4 ^{0}C (Nakeeb and Lechevalier, 1963; Kuster and Williams, 1964).

3.15 Statistical analysis

The data recorded in triplicate for various parameters was subjected to ANOVA (analysis of variance) in accordance with completely randomized block design using MINITAB statistical package to quantify and evaluate the sources of variation (Gomez and Gomez, 1984).

Chapter – 4

EXPERIMENTAL FINDINGS

Results pertaining to the present investigation entitled "Formulation of effective microbial Consortia and its application in sewage treatment" are presented in this chapter:

Sewage water was analyzed before treating it with effective microbial consortia and following parameters were observed in the pretreated sewage water which are given in Table 1.

Table 1: Characterization of Pretreated Sewage water from STP Hazratbal

S. No.	Parameters	Amount/concentration (mg/l)
1.	Carbon	1.85
2.	Total nitrogen	32.6
3.	Total phosphorus	6.2
4.	Total potassium	95.1
5.	BOD	95.6
6.	COD	157
Heavy Metals And Micro Elements		
7.	Mg	50
8.	Cd	0.0 (ppm)
9.	Ni	0.0 (ppm)
10.	Pb	0.0 (ppm)
11.	Zn	7.0
12.	Fe	7.0
13.	Ca	240

In Table 2 the permissible limits in sewage water for agricultural reuse are given which makes sewage water suitable as recommended by the World Health Organization (WHO).

Table 2: Permissible limits of sewage water for agricultural reuse as per World Health Organization (WHO)

S. No.	Parameters	Amount/Concentration (mg/l)
1.	Carbon	0.87
2.	Total Nitrogen	37.0
3.	Total Phosphorus	8.6
4.	Total Potassium	75.0
5.	BOD	80.0
6.	COD	150
Heavy Metals And Micro Elements		
7.	Mg	100
8.	Pb, Cd, Ni	840, 85, 420 (ppm)
9.	Fe	5.0
10.	Zn	5.0
11.	Ca	230

4.1 Isolation and characterization of most effective microorganisms

4.1.1 Isolation of lactic acid bacteria

4.1.1.1 Morphological characteristics of lactic acid bacteria isolated from curd sample

20 isolates of Lactic acid bacteria were isolated from the curd samples obtained from various sources like Khyber, Amul, Zum Zum, Milkman and Snow Cap. Morphological characteristics of isolates grown on MRS media showed that some of the isolates were low concave creamy, raised, with entire margin and some were faint white, irregular, undulate and some were transducent and non-pigmented. 16 isolates were short rods, 4 were spherical and all the isolates were gram positive as given in Table 3.

4.1.1.2 Biochemical characteristics of lactic acid bacteria isolated from curd samples

Various biochemical tests were performed with the isolates of lactic acid bacteria and it was observed that some of the isolates were negative (-) and some were positive (+) for pigment production, catalase, oxidase, IMViC and H_2S production (Table 4).

4.1.1.3 Carbohydrate fermentation by the lactic acid bacterial (LAB) isolates

Carbohydrate fermentation of all the 20 isolates was carried out and some of the isolates were able to utilize cellobiose, glucose, lactose and maltose and some isolates were able to ferment galactose, mannitol, raffinose and arabinose and on the basis of all these characters it was concluded that the isolated microorganisms probably belonged to the genus *Lactobacillus* and *streptococcus.* The isolated bacteria were observed by phase contrast microscope. It is clear that the bacteria were gram positive, rod shaped occurring single or in chains forms. The gram staining results indicated that the isolated bacteria could be identified as *Lactobacillus* and *streptococcus.* The catalase test is one of the most useful diagnostic tests for the recognition of bacteria due to their simplicity. Thus, the results obtained coincided with *Lactobacillus and streptococcus* strain characteristics Table 5.

Table 3: **Morphological characteristics of Lactic acid bacteria isolated from curd sample**

Isolate	Curd Sample	Colony Morphology	Cell shape	Gram reaction
SL1	Khyber	Creamy, raised, entire medium sized	Cocci	Positive
SL2	Zum zum	Creamy, raised, irregular medium sized	Short rods	Positive
SL3	Snow cap	Creamy, minute, lightly raised, irregular	Short rods	Positive
SL4	Milk man	Creamy white, entire raised	long rods	Positive
SL5	Amul	Faint white, irregular, raised undulate	Short rods	Positive
SL6	Khyber	Irregular, faint white, raised, non-pigmented	long rods	Positive
SL7	Zum zum	White, raised, entire spherical, small size	Cocci	Positive
SL8	Snow cap	Irregular, faint, white raised medium sized	Short rods	Positive
SL9	Milk man	Small, faint white, irregular raised	Long rods	Positive
SL10	Amul	Transducent, non-pigmented, irregular, medium	Long rods	Positive
SL11	Khyber	Creamy, minute, lightly raised, irregular	Short rods	Positive
SL12	Zum zum	Creamy white, entire raised	long rods	Positive
SL13	Snow cap	Faint white, irregular, raised undulate	Short rods	Positive
SL14	Milk man	Irregular, faint white, raised, non-pigmented	long rods	Positive
SL15	Amul	White, raised, entire spherical, small size	Cocci	Positive
SL16	Khyber	Irregular, faint, white raised medium sized	Short rods	Positive
SL17	Zum zum	Small, faint white, irregular raised	Long rods	Positive
SL18	Snow cap	Transducent, non-pigmented, irregular, medium size	Long rods	Positive
SL19	Milk man	Creamy, raised, entire medium sized	Cocci	Positive
SL20	Amul	Creamy, raised, irregular medium sized	Short rods	Positive

Table 4: Biochemical characteristics of lactic acid bacteria isolated from curd samples

Isolate	Pigment production	Catalase	Oxidase	Indole	Methyl red	Voges proskauer	Citrate production	H_2S production
SL1	+	-	-	-	-	+	+	-
SL2	-	+	-	-	-	-	+	-
SL3	+	-	-	+	+	-	-	+
SL4	+	-	-	-	+	-	+	-
SL5	-	-	+	+	-	+	-	+
SL6	-	-	+	-	+	+	-	-
SL7	+	-	-	-	+	-	-	-
SL8	+	+	+	-	+	+	+	-
SL9	-	-	+	+	-	+	+	+
SL10	-	-	+	+	+	+	-	+
SL11	+	+	-	-	+	-	+	-
SL12	-	+	+	-	-	+	+	-
SL13	+	+	+	+	+	+	+	+
SL14	-	-	-	-	-	-	-	-
SL15	-	+	+	-	-	+	+	-
SL16	-	-	-	-	-	-	-	-
SL17	+	-	-	+	+	-	-	+
SL18	+	+	-	+	+	-	+	+
SL19	-	+	+	-	-	+	+	-
SL20	-	-	+	-	+	+	-	-

SL (Shalimar-Lactic acid bacteria), Negative = (-), Positive = (+)

Table 5: Carbohydrate fermentation by the lactic acid bacterial isolates

Isolate	**Carbohydrates Fermentation**								**Probable genus**
	Cellobiose	**Galactose**	**Glucose**	**Lactose**	**Maltose**	**Mannitol**	**Raffinose**	**Arabinose**	
SL1	+	+	+	+	+	+	-	+	*Lactobacillus*
SL2	+	-	+	+	+	-	+	-	*Streptococcus*
SL3	+	+	+	+	+	+	-	+	*Lactobacillus*
SL4	+	+	+	+	+	+	-	+	*Lactobacillus*
SL5	+	-	+	+	+	-	+	-	*Streptococcus*
SL6	+	-	+	+	+	-	+	-	*Lactobacillus*
SL7	+	+	+	+	+	+	-	+	*Streptococcus*
SL8	+	+	+	+	+	+	-	+	*Lactobacillus*
SL9	+	+	+	+	+	+	-	+	*Lactobacillus*
SL10	+	-	+	+	+	-	+	-	*Streptococcus*
SL11	+	+	+	+	+	+	+	+	*Lactobacillus*
SL12	-	+	-	+	-	-	+	+	*Lactobacillus*
SL13	+	+	+	+	+	+	+	+	*Streptococcus*
SL14	+	+	+	+	+	+	+	+	*Streptococcus*
SL15	-	+	-	+	-	-	+	+	*Streptococcus*
SL16	-	+	-	+	-	-	+	+	*Lactobacillus*
SL17	+	+	+	+	+	+	+	+	*Streptococcus*
SL18	+	+	+	+	+	+	+	+	*Streptococcus*
SL19	+	+	+	+	+	+	+	+	*Lactobacillus*
SL20	-	+	-	+	-	-	+	+	*Streptococcus*

Negative (-), Positive (+)

4.1.2 Isolation of *Pseudomonas* species

4.1.2.1 Morphological characteristics of microorganisms isolated from oil spilled soil samples of garages

20 isolates were isolated from oil spilled soils of garages of various locations of Srinagar city. Colony morphology of the isolates showed that some isolates were small, raised entire, orange, circular, opaque. Some were yellowish pale, entire, flat, undulate, clear and some were pinkish, raised, circular, entire, opaque and creamy, raised, elevated, circular, opaque. Light green, circular, shining, slimy, irregular. Some of the isolates appeared as short rods and some as long rods but all the isolates were gram negative (Table 6).

4.1.2.2 Biochemical characteristics of microorganisms isolated from oil spilled soil samples from garage areas

The biochemical characterization of all the isolates was done up to genus level (Table 7). Pigment production, catalase, indole and methyl red, oxidase and voges proskauer, citrate production, starch hydrolysis and H_2S production was done and it was observed on the basis of all these biochemical characteristics. All the 20 isolates belonged to genus *Pseudomonas as per* Bergey's Manual of Systematic Bacteriology and were designated as Shalimar *Pseudomonas* sp. (Table 7).

4.1.2.3 Phosphate Solubilization activity of isolated bacterial isolates of *Pseudomonas* sp.

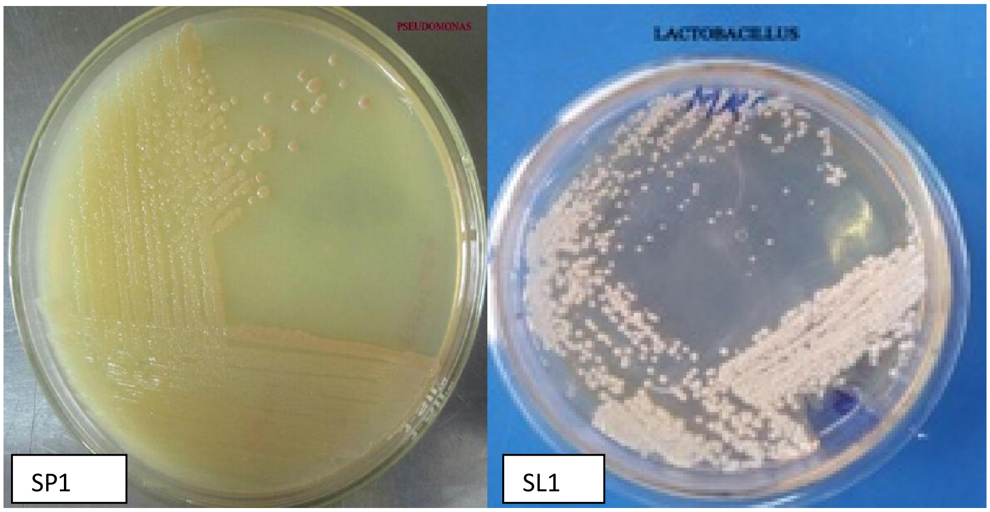
Phosphate solubilization activity of isolates of *Pseudomonas sp* was assessed and it was observed that out of 20 only 10 isolates were able to solubilize phosphate but isolate SP6 showed the maximum zone of clearance of 8.03 mm followed by SP5 (7.11 mm) and SP1 (7.05 mm). Solubilization index was maximum with respect to SP3 (2.40) followed by SP2 (2.30) and SP6 (2.20). Similarly release of P was maximum in SP6 (107.44 mg/ml), followed by SP5 (105.8 mg/ml) and SP1 (101.5 mg/ml) (Table 8).

Table 6: Morphological characteristics of *Pseudomonas* species isolated from oil spilled soil samples of garages

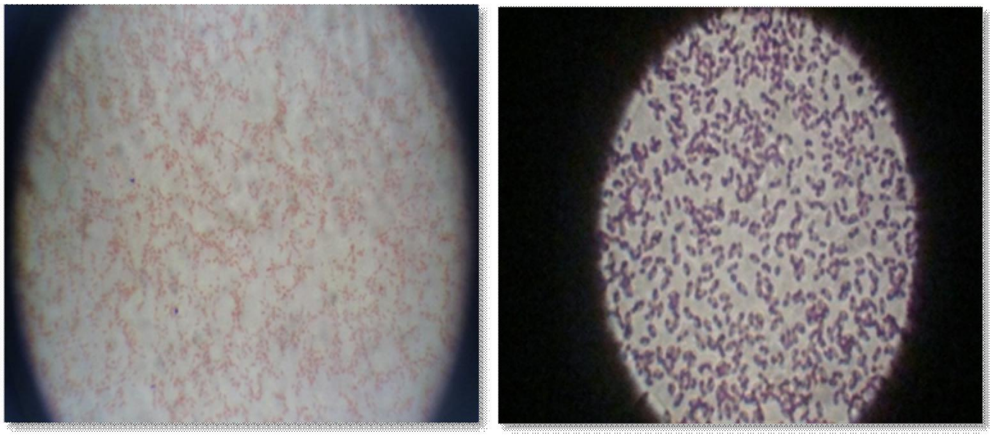
Isolate	Colony Characters	Cell shape	Gram reaction
SP1	Small, raised entire, Orange, circular, Opaque	Short rods	Negative
SP2	Yellowish pale, entire, flat, undulate, clear	Short rods	Negative
SP3	Pinkish, raised, circular, entire, opaque	Long rods	Negative
SP4	Yellowish, elevated, raised, opaque	Short rods	Negative
SP5	Light green, circular, shining, slimy, irregular	Short rods	Negative
SP6	Irregular opaque, flat, bluish, greenish	Short rods	Negative
SP7	Creamy, entire, opaque, flat, small	Long rods	Negative
SP8	Creamy, flat, elevated, opaque	Short rods	Negative
SP9	Creamy, small, flat, entire, opaque	Short rods	Negative
SP10	Whitish, entire, flat opaque, big	Short rods	Negative
SP11	Creamy, raised, elevated, circular, opaque	Short rods	Negative
SP12	Pinkish, circular, small, raised, opaque	Long rods	Negative
SP13	Yellowish, elevated, raised, opaque	Short rods	Negative
SP14	Light green, circular, shining, slimy, irregular	Long rods	Negative
SP15	Creamy, small, flat, entire, opaque, reddish brown	Short rods	Negative
SP16	Whitish, entire, flat opaque, big	Short rods	Negative
SP17	Creamy, raised, elevated, circular, opaque	Short rods	Negative
SP18	Pinkish, circular, small, raised, opaque	Long rods	Negative
SP19	Yellowish, elevated, raised, opaque	Short rods	Negative
SP20	Light green, circular, shining, slimy, irregular	Long rods	Negative

Table 7: Biochemical characteristics of microorganisms isolated from oil spilled soil samples from garage area

Isolates	Pigment productin	Catalae	Urease	Oxidase	Indole	Methyl red	Voges proskauer	Cittrate production	H_2S production	Starch Hydrolysis	Probable genus
SP1	+	+	+	-	+	+	-	-	-	+	*Pseudomonas*
SP2	+	+	-	-	+	+	-	-	-	+	*Pseudomonas*
SP3	+	+	+	+	+	+	-	-	-	+	*Pseudomonas*
SP4	+	+	+	+	+	-	-	-	-	+	*Pseudomonas*
SP5	-	+	-	+	+	+	+	+	+	-	*Pseudomonas*
SP6	+	+	-	+	+	+	+	+	+	-	*Pseudomonas*
SP7	-	+	+	_	+	-	-	-	-	+	*Pseudomonas*
SP8	-	+	+	-	-	+	-	-	-	+	*Pseudomonas*
SP9	+	+	+	-	+	+	-	-	-	+	*Pseudomonas*
SP10	+	+	-	-	+	+	-	-	-	+	*Pseudomonas*
SP11	-	-	+	-	+	-	-	+	-	-	*Pseudomonas*
SP12	+	-	+	-	-	+	+	+	+	+	*Pseudomonas*
SP13	-	+	-	+	-	+	+	-	+	+	*Pseudomonas*
SP14	-	-	-	+	+	-	-	-	-	-	*Pseudomonas*
SP15	+	+	+	-	-	+	+	+	+	+	*Pseudomonas*
SP16	+	+	-	-	+	+	+	+	+	+	*Pseudomonas*
SP17	+	-	+	-	+	+	+	+	+	+	*Pseudomonas*
SP18	+	-	+	-	-	-	-	+	-	-	*Pseudomonas*
SP19	+	+	-	-	-	+	+	+	+	+	*Pseudomonas*
SP20	-	+	-	+	+	+	+	-	+	+	*Pseudomonas*



Isolated colonies of *Pseudomonas* isolate SP1 **Isolated colonies of *Lactobacilluss* isolate SL1**



Gram staining of *Pseudomonas* **Gram staining of *Lactobacillus***

Plate 1: Isolation and staining of *Pseudomonas and Lactobacillus*

Table 8: **Phosphate solubilization activity of isolated bacterial isolates of *Pseudomonas***

Isolate	**Solubilization zone(mm)**	**Solubilization Index**	**P-released (mg/ml)**
SP1	7.057	1.837	101.510
SP2	5.117	2.303	96.503
SP3	6.057	2.403	92.333
SP4	4.090	2.103	88.543
SP5	7.110	2.203	105.833
SP6	8.037	1.960	107.443
SP7	4.220	1.807	65.320
SP8	3.630	1.187	47.307
SP9	2.147	1.500	55.823
SP10	3.570	1.277	62.203
C.D ($p \leq 0.05$)	**0.079**	**0.119**	**0.204**

4.1.2.4 Chitinase activity exhibited by isolates of *Pseudomonas* sp.

Chitinase activity of all the isolates was assessed and it was observed that out of 20 only 10 isolates exhibited chitinase activity but three isolates SP5, SP6 and SP1 showed maximum activity of 33.34, 25.12 and 22.18 units/ml respectively. Similarly CS:CZ ratio was maximum in SP5 (4.08) followed by SP1(3.67) and SP9 (3.45) respectively and were used in formulation of effective microbial consortia as shown in Table 9.

Table 9: Chitinase activity exhibited by isolates of *Pseudomonas*

Isolate	Chitinase activity *(units/ml)	** CS:CZ (Ratio)
SP1	22.18	3.67
SP2	11.05	1.63
SP3	15.33	2.85
SP4	9.41	2.09
SP5	33.34	4.08
SP6	25.12	3.06
SP7	13.82	3.11
SP8	12.73	3.05
SP9	11.21	1.63
SP10	14.69	3.45
C.D($p \leq 0.05$)	**0.07**	**0.17**

** C:Z depicts ratio of colony size to the zone of clearance

* One unit of enzyme activity (IU) is the amount of enzyme required for the formation of one micromole of product (N-acetyl glucosamine per minute) under the assay condition

4.1.2.5 Antifungal activity of *Pseudomonas* isolates

Out of all the isolates *of Pseudomonas* only 10 isolates showed antifungal activity against *Fusarium oxysporum* and maximum percentage of inhibition was exhibited by SP6, SP5 and SP1 which was 73.27, 61.15 and 56.63%. The Percentage of inhibition ranged from 10.40 to 73.27% Others also exhibited inhibition against the pathogen which was lesser as compared to the above mentioned isolates (Table 10).

Table 10: Antifungal activity of *Pseudomonas* isolates

Isolates	***Fusarium oxysporum* (inhibition%)**
SP1	56.63
SP2	49.66
SP3	39.56
SP4	28.63
SP5	61.15
SP6	73.27
SP7	38.76
SP8	56.16
SP10	10.40
C.D ($p \leq 0.05$)	**0.29**

Phosphate solubilization by *Pseudomonas* isolate SP5

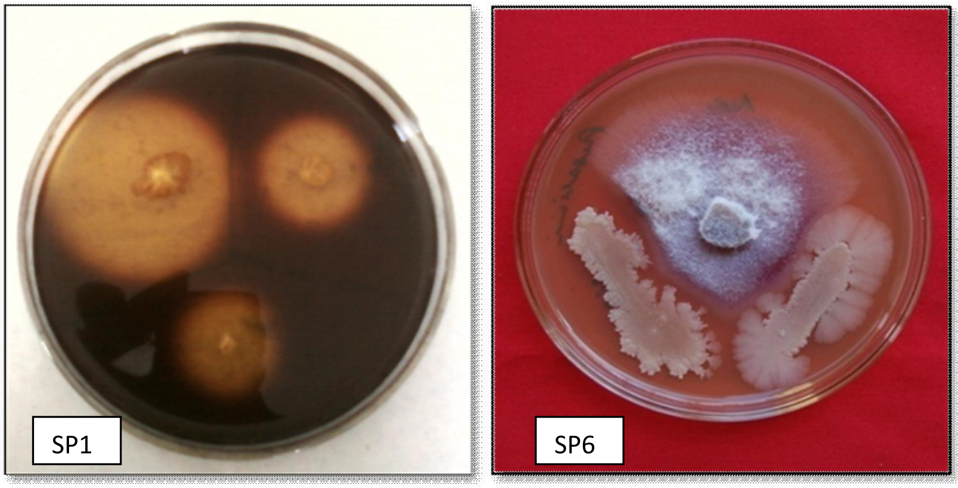


Plate 2: Chitinase activity by *Pseudomonas* isolate SP1and antifungal activity by *Pseudomonas* SP6.

4.1.3 Isolation of Yeasts

4.1.3.1 Morphological characterization of yeasts isolated from dry yeast granules

Morphological characterization of 20 yeast isolates showed that some isolates were mucoid, creamish, white or off white oval, fluffy. Some were mucoid convex with flat margins and some were spore forming but all isolates were gram positive (Table 11).

4.1. 3.2 Carbohydrate Utilization by isolated Yeasts

All the isolates were tested for carbohydrate utilization and it was observed that most of the isolates were able to utilize maltose, galactose, lactose, xylose and starch but none of them was able to metabolise cellobiose. Cream colonies are the characteristics of yeast especially *Saccharomyces* sp. *Saccharomyces* can be identified due to its ability to ferment sucrose, maltose, fructose, glucose, galactose, and raffinose but not lactose as the strain lacks lactase or β-galactosidase system. On the basis of characteristics it was concluded that the isolated species probably belonged to genus *Sacchromyce* (Table 12).

4.1.3.3 Anti-bacterial activity of yeast

Antibacterial activity of 10 isolated yeasts was tested against test organisms *Bacillus* sp. and *E. coli* and it was observed that all the 10 isolates were inhibiting the growth of test microorganisms. However, three isolates SS7, SS4 and SS1exhibited maximum zone of inhibition of 17.33, 15.0 and 14.0 mm against *Bacillus* sp. respectively and 17.66, 15.00 and 12.6 mm against *E. coli* respectively and were thus used in formulation of Effective microbial consortia (Table 13).

Table 11: **Morphological characterization of yeasts isolated from dry yeast granules**

Isolate	Colony morphology	Cell shape	Gram reaction	Spore
SS1	Creamy to white colour, fluffy and smooth margin	Oval	Positive	+
SS2	Mucoid, creamish irregular with filamentous margin	Filamentous	Positive	+
SS3	Colonies were either white or off white	Oval	Positive	-
SS4	Colonies were either white or off white	Filamentous	Positive	-
SS5	Mucoid, creamish irregular with filamentous margin	Oval	Positive	+
SS6	Mucoid, creamish irregular with filamentous margin	Oval	Positive	-
SS7	Creamy to white color, fluffy, and smooth margin	Oval	Positive	+
SS8	Colonies were either white or off white	Oval	Positive	-
SS9	Mucoid creamish in few cases it was convex and flat	Oval	Positive	+
SS10	Mucoid creamish irregular with filamentous margin	Filamentous	Positive	+
SS11	Mucoid creamish irregular with filamentous margin	Oval	Positive	+
SS12	Colonies were either white or off white	Oval	Positive	-
SS13	Creamy to white color, fluffy, and smooth margin	Filamentous	Positive	-
SS14	colonies were either white or off white	Oval	Positive	+
SS15	Irregular with filamentous margin was wavy	Filamentous	Positive	-
SS16	Colonies were either white or off white	Oval	Positive	+
SS17	Irregular with filamentous margin was wavy	Filamentous	Positive	-
SS18	Irregular with filamentous margin was wavy	Oval	Positive	+
SS19	Irregular with filamentous margin was wavy	Filamentous	Positive	+
SS20	Colonies were either white or off white	Oval	Positive	+

Table 12: Carbohydrate Utilization by isolated Yeasts

Isolates	**Carbohydrate Utilization**								**Probable genus**
	Raffinose	**Maltose**	**Glucose**	**Galactose**	**Sucrose**	**Lactose**	**Starch**	**Cellobiose**	
SS1	+	+	+	+	+	-	+	-	*Sacchromyces*
SS2	-	-	+	-	-	-	-	-	*Sacchromyces*
SS3	-	+	-	+	-	+	+	-	*Sacchromyces*
SS4	+	+	+	+	+	-	+	-	*Sacchromyces*
SS5	-	-	+	-	-	-	-	-	*Sacchromyces*
SS6	+	+	-	+	-	+	+	-	*Sacchromyces*
SS7	+	+	+	+	+	-	+	-	*Sacchromyces*
SS8	-	-	-	-	-	-	-	-	*Sacchromyces*
SS9	-	+	-	+	+	+	+	-	*Sacchromyces*
SS11	-	+	+	-	+	-	+	-	*Sacchromyces*
SS12	+	-	+	-	+	-	+	-	*Sacchromyces*
SS13	-	-	-	-	-	-	-	-	*Sacchromyces*
SS14	+	+	-	+	-	+	-	-	*Sacchromyces*
SS15	+	-	+	-	+	-	+	-	*Sacchromyces*
SS16	-	-	-	-	-	-	-	-	*Sacchromyces*
SS17	-	-	-	-	-	-	-	-	*Sacchromyces*
SS18	-	-	-	-	-	-	-	-	*Sacchromyces*
SS19	-	+	-	+	-	+	-	-	*Sacchromyces*
SS20	+	_	+		+		+	-	*Sacchromyces*

Positive = (+), Negative = (-) (SS)= Shalimar *Sacchromyces*

Table 13: Antibacterial activity of yeast isolates

Isolates	**Test organisms zone of inhibition (mm)**	
	Bacillus	***E. coli***
SS1	14.00	12.66
SS2	7.66	7.66
SS3	14.00	11.00
SS4	15.00	15.00
SS5	10.00	9.00
SS6	8.00	7.33
SS7	17.33	17.66
SS8	6.00	7.00
SS9	2.00	2.66
SS10	1.33	1.33
C.D($p \leq 0.5$)	**2.48**	**2.17**

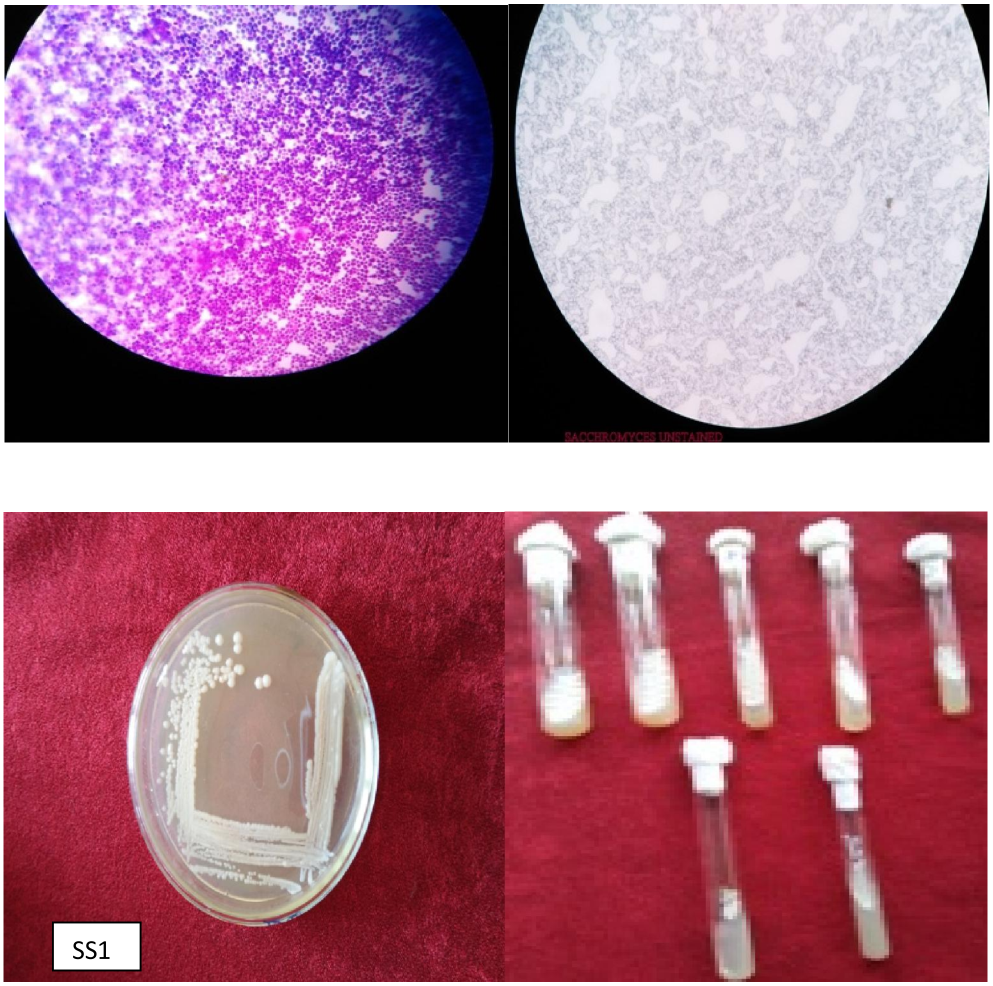


Plate 3: Isolated, purified, stained and unstained colonies of *saccharomyces* SS1,SS3 and SS4

4.1.4 Isolation of Actinomycetes

4.1.4.1 Morphological characteristics of Actinomycetes isolated from decomposing pile

From the soil samples of decomposing piles 20 isolates of actinomycetes were picked up.

Some isolates were having mycelial, filmentous creamish white growth. Some were spiral brownish, irregular with filamentous margin spore chain and creamish white colonies with powdery consistency (Table 14).

4.1.4.2 Carbohydrate utilization by isolates of Actinomycetes

Studies on sugar utilization of the isolates showed that all the isolates were able to utilize glucose, arabinose, mannitol, sucrose and starch as carbohydrate source and some were able to utilize galactose, lactose, fructose. xylose and raffinose. On the basis of all these morphological characteristics and carbohydrate utilization it was concluded that the isolated species probably belonged to genus *Streptomyces* (Table 15).

4.1.4.3 Antimicrobial activity of isolates of Actinomycetes

Out of 20 isolates of actinomycetes10 actinomycetes isolates showed antimicrobial activity against test organisms *Bacillus subtilis* and *Fusarium moniliforme* and it was observed that all the isolates inhibited the test organisms but three isolates were showing zone of inhibition greater than 10 mm which were SA5, SA3 and SA1 with 17.66 , 15.00 and 12.66 mm against *Bacillus subtilis* and 17.00, 15.00 and 14.00 mm against *Fusarium moniliforme* respectively and thus were used in formulation of Microbial consortia (Table 16).

Table 14: Morphological characteristics of Actinomycetes isolated from decomposing pile

Isolate	Colony morphology	Spore	Cell shape
SA1	Mycelial, filamentous creamish white	+	Short filamentous
SA2	Mycelial spiral, creamish white	+	Long rods
SA3	Mycelial spiral browinish	+	Short filamentous
SA4	Mycelial, spiral browinish	+	Long rods
SA5	Mycelia spiral, creamish white	+	Long rods
SA6	Mycelia spiral, creamish white	+	Short filamentous
SA7	Mycelial, spiral browinish	+	Long rods
SA8	irregular with filamentous margin	+	Long rods
SA9	Mycelia spiral, creamish white	+	Short filamentous
SA11	Mycelia spiral, creamish white	+	Long rods
SA12	irregular with filamentous margin	+	Short filamentous
SA13	Mycelial, filamentous creamish white	+	Long rods
SA14	Mycelia,l spiral, creamish white	+	Short filamentous
SA15	Mycelial spiral browinish	+	Long rods
SA16	Mycelial, spiral browinish	+	Long rods
SA17	irregular with filamentous margin	+	Short filamentous
SA18	Mycelia spiral, creamish white	+	Long rods
SA19	Mycelia spiral, creamish white	+	Long rods
SA20	irregular with filamentous margin	+	Short filamentous

Table 15: Carbohydrate utilization by isolates of Actinomycetes

Isolate	Carbohydrate utilization										Probable genus
	Glucose	Lactose	Fructose	Galactose	Arabinose	Mannitol	Xylose	Sucrose	Raffinose	Starch	
SA1	-	+	-	+	+	-	-	+	-	+	*Streptomyces*
SA2	-	-	+	+	+	+	-	+	-	-	*Streptomyces*
SA3	-	-	-	-	+	+	-	-	-	+	*Streptomyces*
SA4	-	-	-	+	+	-	-	+	-	+	*Streptomyces*
SA5	-	-	+	-	+	+	-	-	-	-	*Streptomyces*
SA6	-	+	-	+	+	+	-	-	-	+	*Streptomyces*
SA7	-	-	-	+	+	-	-	-	-	+	*Streptomyces*
SA8	-	-	-	+	+	+	-	-	-	-	*Streptomyces*
SA9	-	-	-	-	+	+	-	-	-	+	*Streptomyces*
SA10	-	+	-	+	+	-	-	+	-	+	*Streptomyces*
SA11	-	+	+	+	+	+	-	+	-	+	*Streptomyces*
SA12	-	-	+	+	+	+	-	+	-	-	*Streptomyces*
SA13	-	+	-	-	+	-	-	-	-	-	*Streptomyces*
SA14	-	-	+	+	+	+	-	+	-	+	*Streptomyces*
SA15	-	+	-	-	+	-	-	-	-	-	*Streptomyces*
SA16	-	+	+	+	+	+	-	+	-	-	*Streptomyces*
SA17	-	+	+	+	+	+	-	+	-	-	*Streptomyces*
SA18	-	-	+	+	+	+	-	+	-	-	*Streptomyces*
SA19	-	-	-	-	-	+	-	-	-	-	*Streptomyces*
SA20	-	+	-	-	-	+	-	-	-	-	*Streptomyces*

Positive= (+), Negative (-), (SA) = Shalimar Actinomycete

Table 16: Antimicrobial activity of Isolates Actinomycetes

Isolates	**Test organisms/Zone of inhibition (mm)**	
	Bacillus subtilis	***Fusarium moniliforme***
SA1	12.66	14.00
SA2	7.66	7.66
SA3	15.001	15.00
SA4	10.00	14.00
SA5	17.66	17.00
SA6	7.33	8.00
SA7	9.00	10.33
SA8	7.00	6.00
SA9	2.66	2.00
SA10	1.33	1.33
C.D($p \leq 0.5$)	**2.17**	**2.48**

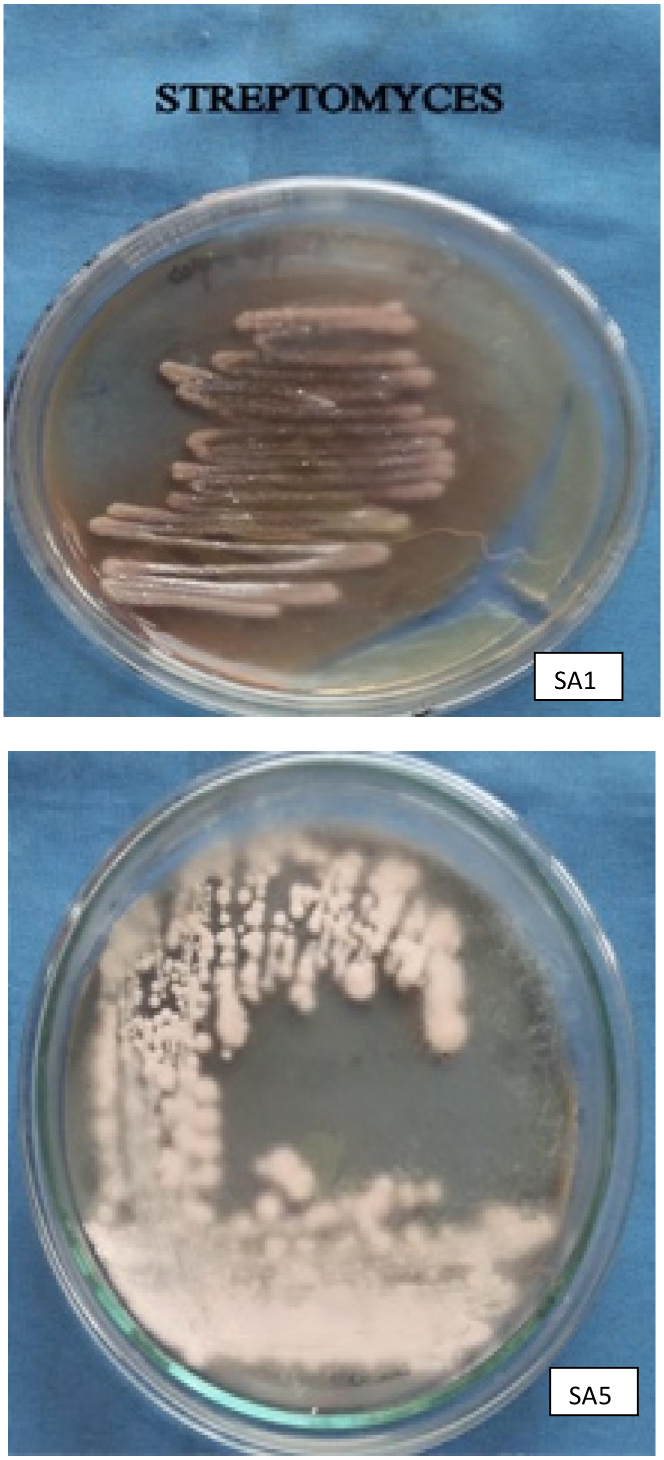


Plate 4: Isolated colonies of *Actinomycetes* isolate SA1 and SA5

4.1.5 Isolation of Fermenting Fungi

4.1.5.1 Morphological characteristics of fungi isolated from Garlic, Onion, Rice, Saffron corms and Banana peel

Fungal species were isolated from different sources like banana peel, garlic peel, onion peel, rice and saffron corms and 20 isolates were characterized morphologically. Some of the isolates showed carbon black or very dark brown spores, colourless conidiophores and spores and some were showing hyaline and septate hyphae and on microscopy all the isolates were bearing conidiospore (Table 17). The isolates resembled morphologically with *Aspergillus* sp.

4.1.5.2 Amylase production by *Aspergillus* isolates

The 10 isolates of *Aspergillus* were assessed for amylase production and it was observed that all the isolates were able to produce amylase. However, three isolates SF16, SF1 and SF6, were able to produce maximum of 57.66, 56.0, and 53.66 IU/ml/min respectively. These isolates were used in formulation of effective microbial consortia (Table 18).

Table 17: Morphological characteristics of fungi isolated from Garlic, Onion, Rice, Saffron corms and Banana peel

Isolate	Source	Colony morphology	Reverse colour
SF1	Garlic	Carbon black or very dark brown spores, colourless conidiophores and spores	Colorless to light yellow
SF2	Garlic	A hyphae that is hyaline and septate,	Pale to yellow
SF3	Saffron corm	Carbon black or very dark brown spores colourless conidiophores and spores	Colorless to light yellow
SF4	Saffron corm	Carbon black or very dark brown spores colourless conidiophores and spores	Pale to yellow
SF5	Banana peel	A hyphae that is hyaline and septate,	Pale to yellow
SF6	Banana peel	A hyphae that is hyaline and septate,	Colorless to light yellow
SF7	Onion peel	Carbon black or very dark brown spores colourless conidiophores and spores	Pale to yellow
SF8	Onion peel	Carbon black or very dark brown spores colourless conidiophores and spores	Colorless to light yellow
SF9	Rice	Carbon black or very dark brown spores colourless conidiophores and spores	Pale to yellow
SF10	Rice	Carbon black or very dark brown spores colourless conidiophores and spores	Colorless to light yellow
SF11	Garlic	Carbon black or very dark brown spores colourless conidiophores and spores	Colorless to light yellow
SF12	Garlic	A hyphae that is hyaline and septate,	Pale to yellow
SF13	Saffron corm	A hyphae that is hyaline and septate,	Pale to yellow
SF14	Saffron corm	Carbon black or very dark brown spores colourless conidiophores and spores	Colorless to light yellow
SF15	Banana peel	Carbon black or very dark brown spores colourless conidiophores and spores	Colorless to light yellow
SF16	Banana peel	Carbon black or very dark brown spores colourless conidiophores and spores	Pale to yellow
SF17	Onion peel	A hyphae that is hyaline and septate	Colorless to light yellow
SF18	Onion peel	A hyphae that is hyaline and septate	Colorless to light yellow
SF19	Rice	Carbon black or very dark brown spores colourless conidiophores and spores	Pale to yellow
SF20	Rice	Carbon black or very dark brown spores colourless conidiophores and spores	Colourless to light yellow

Table 18: Amylase production by *Aspergillus* isolates

Isolates	**Enzyme activity (IU/ml/min)**
SF1	56.000
SF3	41.333
SF6	53.667
SF8	45.333
SF9	30.000
SF12	53.333
SF14	18.000
SF16	57.667
SF17	34.000
SF20	15.667
C.D ($p \leq 0.5$)	**4.881**

IU/ml/min (international unit per mili litre per minute) (SF)= Shalimar Fungi

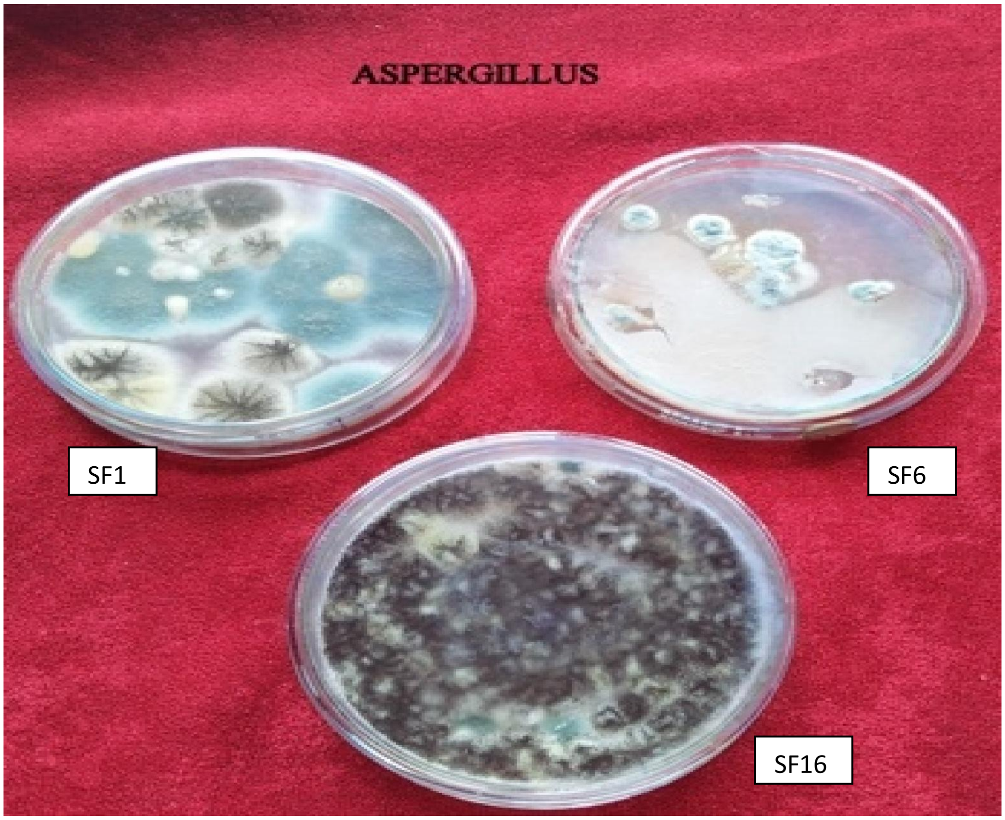


Plate 5: Isolation of *Aspergillus* isolates SF6,SF1 and SF16

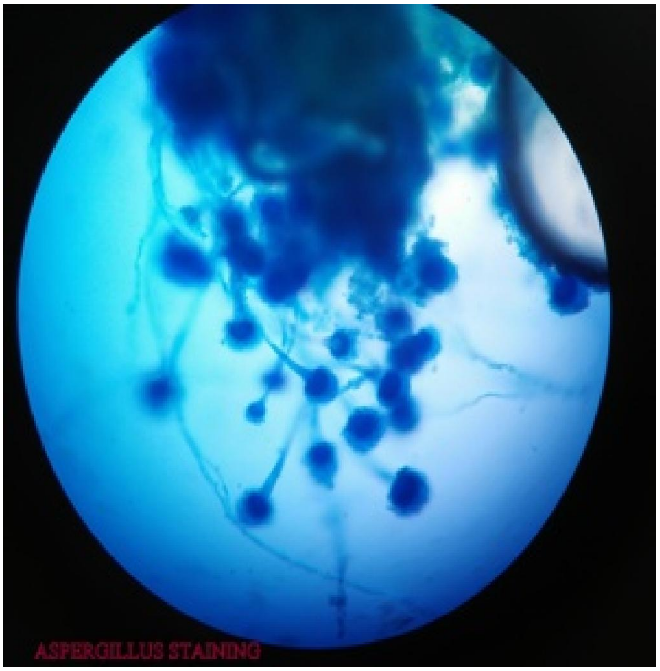


Plate 6;Staining of*Aspergillus* sp.

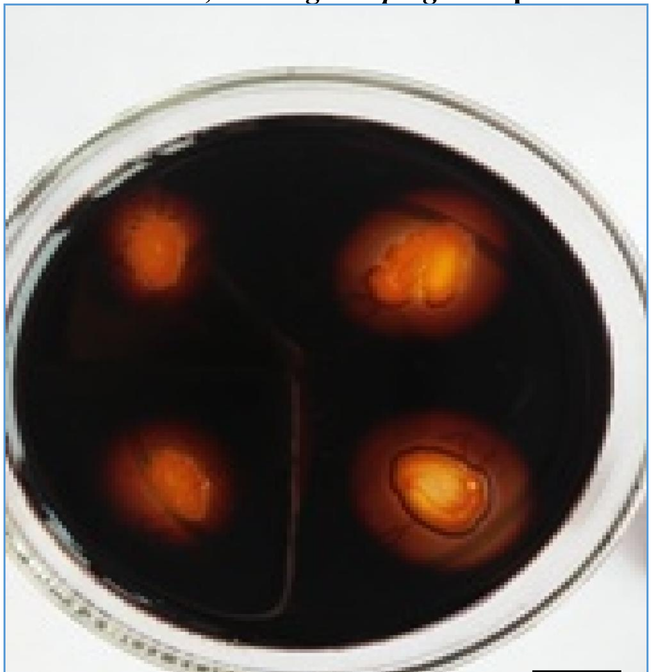


Plate 7;Amylase production by*Aspergillus* isolate SF1.

4.2 Compatibility between various microorganisms

Compatibility was assessed among the most efficient isolates from all the groups so as to find any synergistic or antagonistic activity. It was observed that *Lactobacillus* isolate SL1 was showing compatibility with *Pseudomonas* isolate SP1, *Sacchromyces* isolate SS1, actinomycets isolate SA1 and fermenting fungi isolate SF6.Similarly *Lactobacillus* isolate SL4 exhibited compatibility with *Pseudomonas* isolate SP5, *Sacchromyces* isolate SS4, actinomycets isolate SA3 and fermenting fungi isolate SF1. *Lactobacillus* isolate SL9 was compatible with *Pseudomonas* isolate SP6, *Sacchromyces* isolate SS7, actinomycets isolate SA5 and fermenting fungi isolate SF16 and hence three different Effective microbial consortia were formulated on the basis of compatibility (Table 19).

Table 19: Compatibility between various organisms

Lactobacillus	***Pseudomonas***	***Sacchromyces***	**Actinomycetes**	**Fermenting fungi**
SL1	SP1	SS1	SA1	SF6
SL4	SP5	SS4	SA3	SF1
SL9	SP6	SS7	SA5	SF16

4.3 Formulation of microbial consortia

Three microbial consortia were formulated by randomly selecting the microorganisms on the basis of various screening procedures like antimicrobial activity, phosphate solubilization chitinase activity, amylase production, carbohydrate fermentation and most effective among them were used for formulation of three different microbial consortia each containing five effective isolates and the three consortia were designated as Effective microbial cosortia EM1, EM2 and EM3 (Table 20).

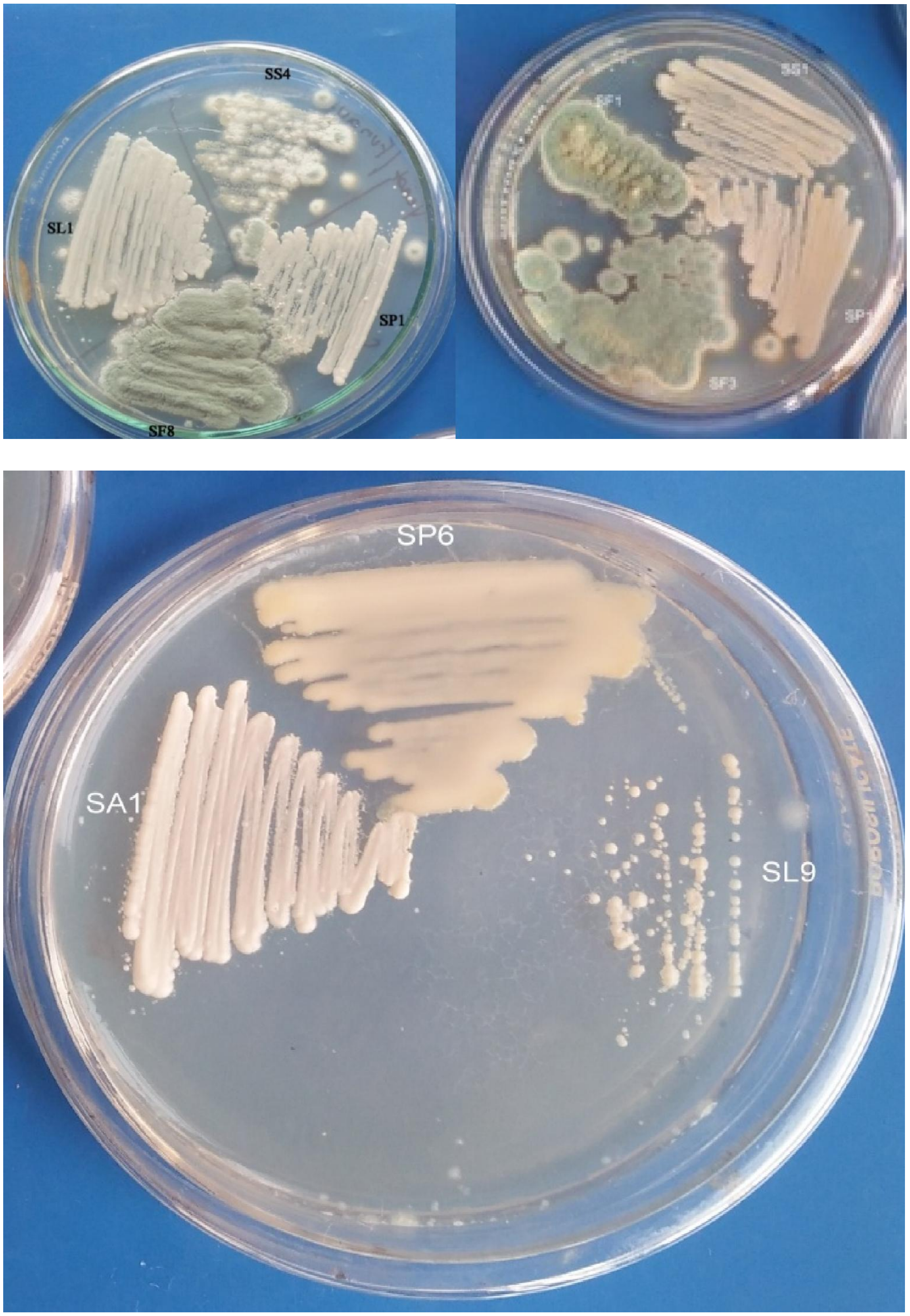


Plate 8: Compatibility of various isolates

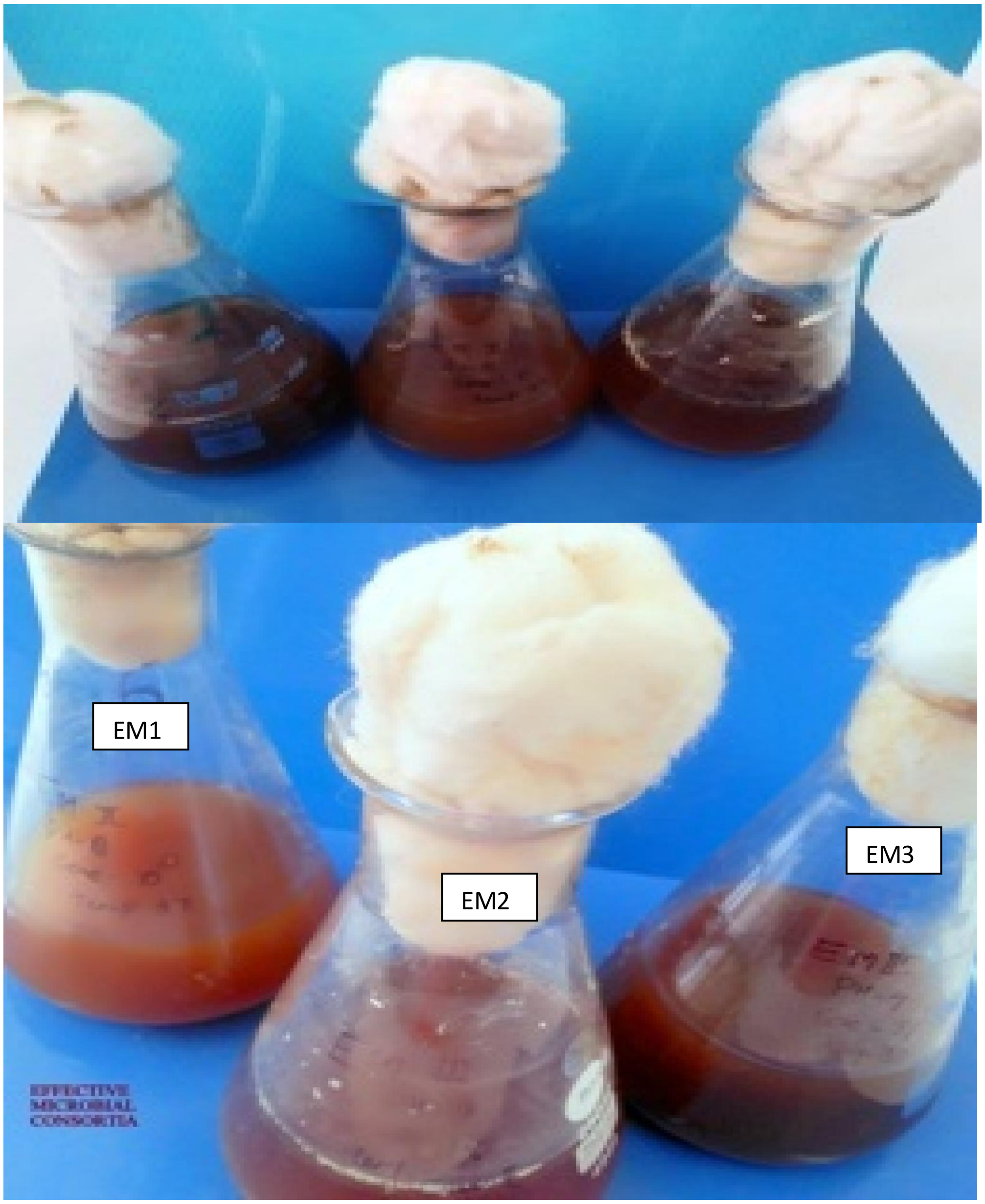


Plate 9: Effective microbial consortia

Table 20: Formulation of Effective Microbial Consortia

Type of consortia	**Isolates**
EM1	SL1
	SP1
	SS1
	SA1
	SF6
EM2	SL4
	SP5
	SS4
	SA3
	SF1
EM3	SL9
	SP6
	SS7
	SA5
	SF16

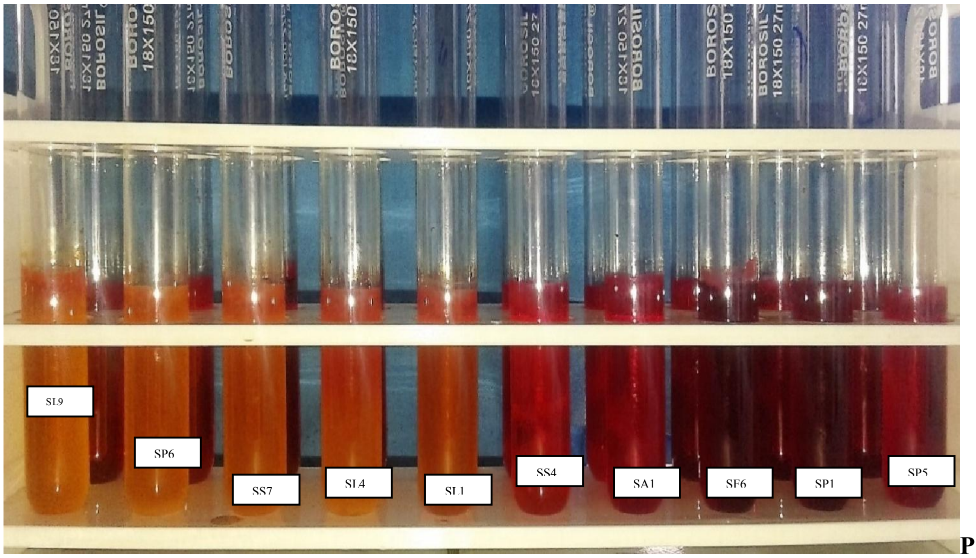


Plate 10: Sucrose metabolism by *lactobacillus* isolates (SL9,SL4) *Pseudomonas* isolates (SP6,SP1,SP5) *Sacchromyces* isolate (SS7,SS4) Actinomycetes isolate (SA1) and *Aspergillus* isolate (SF6)

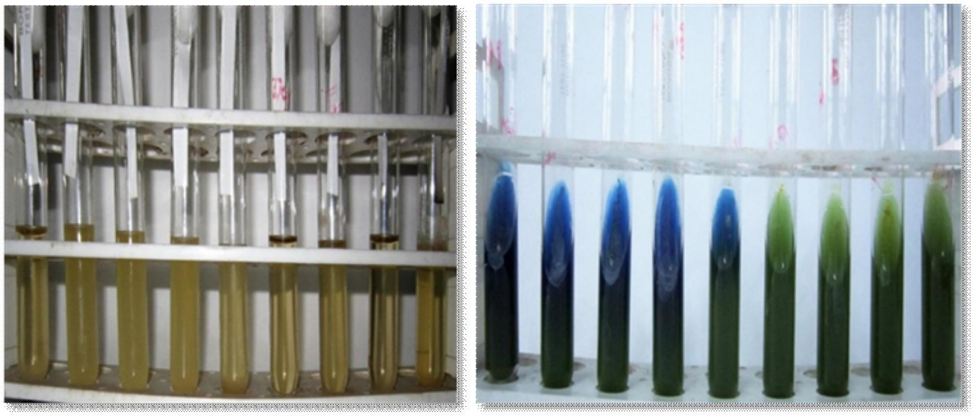


Plate 11:Hydrogen sulphide production and Citrate Utilization

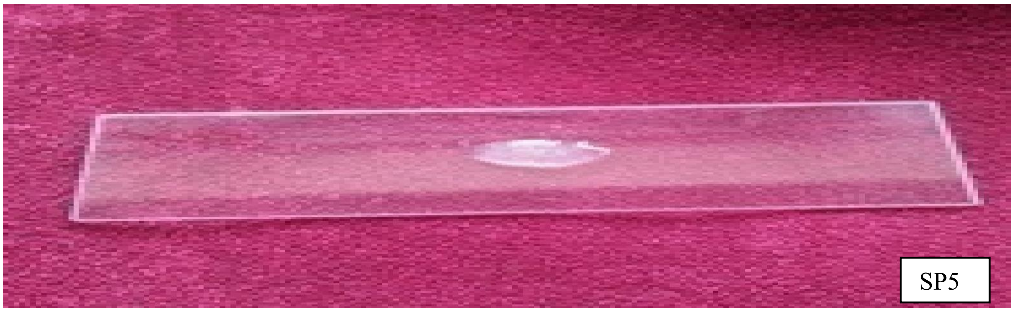


Plate 12:Catalase test

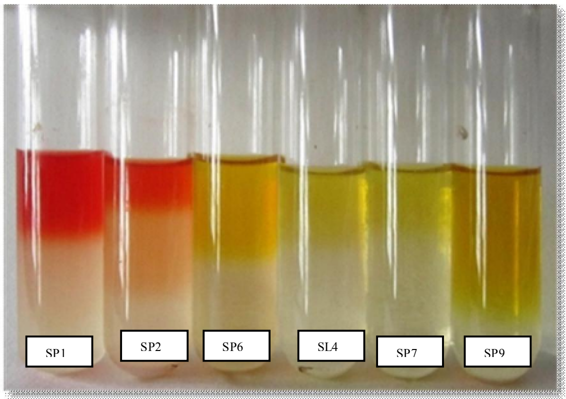


Plate 13: Methyl red test by *Pseudomonas* isolates (SP1,SP2,SP6,SP9) and *Lactobicullu*s isolate SL4

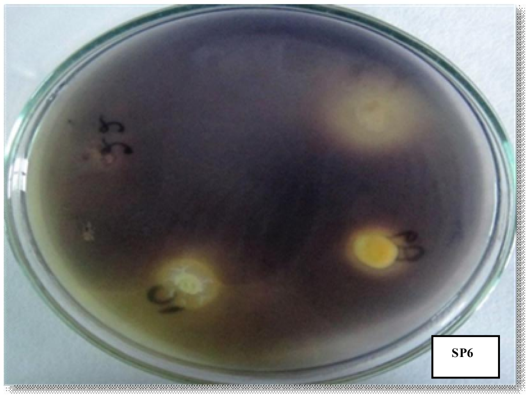


Plate 14: Starch hydrolysis

Plate 15: Oxidase test

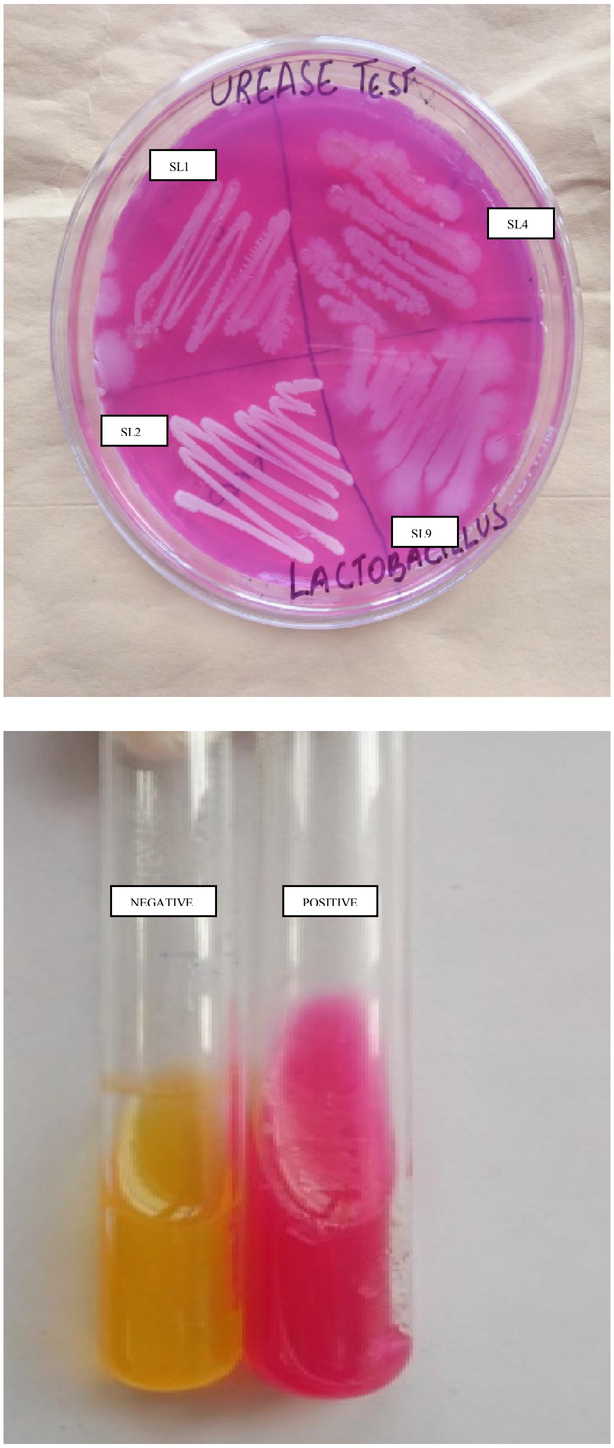


Plate 16: Urease test by *Lactobacillus* isolates (SL1,SL2, SL4 & SL9) With Negative and Positive control

4.4 Effect of temperature on growth of microbes in various consortia

Perusal of the data presented in Table 21 shows that all the consortia were showing maximum microbial population at 25°C and minimum growth of the constituent microbes was observed at 20°C. Hence it was concluded that the optimum temperature for all the constituent microbes was 25°C (Table 21).

4.5 Effect of pH on growth of microbes in various consortia

The data presented in Table 22 shows that all the consortia were showing maximum individual microbial population at pH 7 and minimum growth of all the constituents was observed at pH 8. And it was concluded that the optimum pH for all the constituent microbes was pH 7 (Table 22).

4.6 Effect of Molasses concentration on growth of microbes in various consortia

Data given in Table 23 shows that all the effective microbial constituents grew on 3 different molasses concentration of 1, 3 and 10% respectively and it was observed that all the constituents in three different consortia showed maximum microbial activity at molasses concentration of 3% and minimum growth of all the constituent microbes was observed at 10% concentration of molasses. Hence 3% was the optimal concentration required for growth by all constituents individually (Table 23).

4.7 Effect of Incubation period on growth of microbes in various consortia

Data given in Table 24 shows that all the three effective microbial constituents in three consortia i.e. EM1, EM2, EM3 were grown at different incubation period of 2-30 days. However, it was observed that all the constituent microbes were showing maximum microbial growth on 8th day of incubation. Hence optimum incubation period was 8 days for all microorganisms and minimum growth of all constituent microbes was found on 30th day of incubation (Table 24).

Table 21: Effect of temperature on growth of microbes in various consortia

Type of consortium	Isolate	Microbial population : (cfu/mL) ×10^4		
		20°C	25°C	30°C
EM1	SL1	5.6	6.7	6.6
	SP1	6.3	5.1	5.6
	SS1	6.5	6.5	4.4
	SA1	6.7	7.1	6.8
	SF6	7.6	8.0	7.6
EM2	SL4	5.8	7.6	5.4
	SP5	7.8	8.5	6.2
	SS4	7.0	9.8	7.7
	SA3	6.6	7.2	6.7
	SF1	6.2	8.7	6.0
EM3	SL9	5.6	7.2	7.0
	SP6	6.5	7.1	6.6
	SS7	8.2	8.6	5.5
	SA5	8.0	8.1	7.7
	SF16	6.6	6.9	6.5
Mean temperatures		**6.4**	**7.1**	**7.0**
Consortia Mean		**EM1=6.7**	**EM2=7.5**	**EM3=6.4**

C.D($p \leq 0.5$)

Temperature: NS (Non-significant)

Types of consortia: 0.6938 (significant)

Table 22: Effect of pH on growth of microbes in various consortia

Type of consortium	Isolate	Microbial population : (cfu/mL) $\times 10^4$		
		pH 6	**pH 7**	**pH 8**
EM1	SL1	6.5	7.8	5.6
	SP1	5.4	7.7	6.6
	SS1	7.6	8.7	6.2
	SA1	6.7	7.1	5.7
	SF6	7.7	8.9	6.6
EM2	SL4	7.7	6.8	6.5
	SP5	7.5	8.8	6.8
	SS4	9.8	7.8	5.4
	SA3	7.6	8.2	6.8
	SF1	7.8	8.1	6.0
EM3	SL9	6.8	8.2	5.0
	SP6	6.5	7.1	6.8
	SS7	8.2	8.6	7.0
	SA5	7.0	9.1	6.6
	SF16	7.6	8.9	6.5
Mean pH		**6.9**	**7.4**	**7.3**
Consortia Mean		**EM1=7.3**	**EM2=8.1**	**EM3=6.2**

C.D($p \leq 0.5$)

pH: NS (Non-significant)

Types of consortia: 0.5637 (significant)

Table 23: **Effect of Molasses concentration on growth of microbes in various consortia**

Type of consortium	Isolate	Molasses concentration		
		1%	**3%**	**10%**
		Microbial population : (cfu/mL) ×10^4		
EM1	SL1	7.6	9.8	6.7
	SP1	7.7	8.4	6.5
	SS1	8.5	9.7	5.5
	SA1	7.7	8.1	5.5
	SF6	6.6	8.9	6.5
EM2	SL4	8.7	8.8	6.5
	SP5	6.5	8.8	6.7
	SS4	7.9	9.8	5.6
	SA3	8.6	9.2	6.6
	SF1	7.8	8.2	6.0
EM3	SL9	7.7	9.2	5.0
	SP6	7.5	9.1	7.0
	SS7	8.2	8.5	6.1
	SA5	6.9	9.1	6.7
	SF16	8.6	8.9	6.4
Mean Molasses concentration		**7.5**	**7.7**	**7.6**
Consortia Mean		**EM1=7.7**	**EM2=8.9**	**EM3=6.2**

C.D($p \leq 0.5$)

Molasses concentration : NS (Non-significant)

Types of consortia: 0.4822 (significant)

Table 24: Effect of Incubation period on growth of microbes in various consortia

Type of consortium	Isolate	Microbial population : (cfu/mL) $\times 10^4$								
		2 Day	5 Day	8 Days	12 Days	15 Days	18 Days	21 Days	25 Days	30 Days
EM1	SL1	7.8	6.6	9.2	5.7	5.7	5.1	4.7	4.2	3.1
	SP1	7.7	7.5	9.3	6.3	6.6	5.2	5.0	4.0	3.8
	SS1	8.8	7.5	8.3	7.0	6.6	6.0	5.1	4.8	4.0
	SA1	7.1	6.7	7.8	6.8	5.8	5.7	4.8	4.6	3.8
	SF6	7.9	7.7	9.7	6.2	6.6	5.6	4.8	4.5	4.0
EM2	SL4	6.7	6.5	9.2	6.0	6.5	5.1	5.1	4.5	3.8
	SP5	8.5	7.5	8.8	6.3	5.4	5.6	5.3	4.7	3.8
	SS4	8.9	7.7	9.7	7.6	6.6	5.9	5.6	5.3	4.6
	SA3	8.2	9.1	9.6	5.2	6.7	5.9	5.2	4.6	3.8
	SF1	8.2	7.7	8.6	4.5	7.1	5.3	5.2	4.0	3.6
EM3	SL9	6.8	8.2	9.3	7.5	5.0	6.0	7.2	4.8	4.0
	SP6	6.6	7.1	9.1	8.0	6.8	6.5	5.4	4.6	4.5
	SS7	8.2	8.8	9.6	7.7	7.1	6.3	5.8	4.2	4.1
	SA5	9.1	6.7	9.6	8.0	6.6	5.4	4.3	3.7	3.1
	SF16	8.9	7.5	9.0	8.4	6.0	5.8	6.1	4.0	3.3
Mean incubation period		**8.0**	**7.3**	**8.3**	**6.7**	**6.6**	**6.0**	**5.9**	**4.6**	**4.6**
Consortia Mean		**EM1=6.6, EM2=6.0, EM3=5**								

C.D($p \leq 0.5$)

Incubation period : 0.6956 (significant)

Types of consortia : 0.4016 (significant)

4.8 Effect of temperature on total N, P, K, C, BOD, COD, heavy metals using various consortia

Perusal of the data presented in Table 25 indicates that there was significant reduction in macronutrient concentration of the sewage after treatment with EM1, EM2 and EM 3. Similar trend was observed with respect to micro nutrients, heavy metals, BOD and COD. The final concentration of all the nutrients in sewage water after treatment at 25^{o}C was within the permissible limits as defined by the WHO. Maximum reduction in the concentration of nutrients BOD, COD and heavy metals was recorded in the sewage water treated with EM1 followed by EM2 and EM3. However all the three consortia could be recommended as the parameters studied were within the WHO standards. Minimum reduction in the concentration of nutrients was observed at 20^{o}C (Table 25 and Fig. 1).

4.9 Effect of pH on Total N, P, K, C, BOD, COD, heavy metals using various consortia

Perusal of the data presented in Table 26 indicates that there was significant reduction in macronutrient concentration of the sewage water after treatment with EM1,EM2 and EM3 at different pH and maximum reduction was observed at pH 7.similar trend was observed with respect to micro nutrients, BOD,COD and heavy metals. The final concentration of all the nutrients in sewage water after treatment at pH 7 was within the permissible limits as defined by WHO. Maximum reduction in the concentration of nutrients, BOD, COD and heavy metals was recorded in the sewage water treated with EM3 followed by EM2 and EM1.However all the three consortia could be recommended as the parameters studied were within the WHO standards. Minimum reduction was observed at pH 6 (Table 26 and Fig. 2).

Table 25: Effect of temperature on Total N, P, K, C, BOD, COD, heavy metals using various consortia

Type of consortium	Parameters	Before treatment values(mg/l)	Temperature (^oC)			Mean	C.D ($p \leq 0.5$)	WHO Permissible Limits(mg/l)
			20	25	30			
EM1	N	32.6	20.3	**16.3**	18.3	18.3	0.119	**37.0**
	P	6.2	0.4	**0.3**	0.4	0.3	0.11	**8.6**
	K	95.1	68.0	**45.0**	61.0	59.6	9.67	**75.0**
	C	1.85	0.8	**0.6**	0.7	0.7	0.06	**0.87**
	BOD	57.6	51.2	**26.3**	39.5	39.0	0.61	**80.0**
	COD	71.4	47.0	**29.0**	31.3	35.8	0.39	**150**
	Mg	50	35.1	**30.5**	41.7	35.7	0.106	**100**
	Pb, Cd, Ni	0.00	0.0	**0.0**	0.0	0.0	-	**840,85,420ppm**
	Fe	7.0	2.1	**1.0**	1.0	1.4	0.09	**5.0**
	Zn	7.0	1.9	**0.9**	1.2	1.3	0.09	**5.0**
	Ca	240	235	**160**	175	190	10.22	**230**
EM2	N	32.6	20.3	**18.3**	22.3	20.3	0.11	**37.0**
	P	6.2	0.5	**0.3**	0.6	0.4	0.07	**8.6**
	K	95.1	50.2	**48.2**	54.1	50.8	0.09	**75,0**
	C	1.85	0.6	**0.8**	0.7	0.7	0.07	**50.0**
	BOD	57.6	39.1	**47.2**	41.2	42.5	0.10	**80.0**
	COD	71.4	51.1	**66.3**	57.2	58.2	0.39	**150**
	Mg	50	47.1	**39.6**	42.2	42.9	0.38	**100**
	Pb, Cd, Ni	0.00	0.0	**0.0**	0.0	0.0	-	**840,85,420ppm**
	Fe	7.0	1.2	**1.0**	1.0	1.1	0.08	**5.0**
	Zn	7.0	1.4	**0.8**	1.5	1.2	0.49	**5.0**
	Ca	240	135	**120**	161	499	10.90	**230**

Contd…

Table 25 contd…

Type of consortium	Parameters	Before treatment values(mg/l)	Temperature (^oC)			Mean	C.D ($p \leq 0.5$)	WHO Permissible Limits(mg/l)
			20	**25**	**30**			
EM3	N	32.6	26.5	**24.3**	28.5	26.5	0.53	**37.0**
	P	6.2	1.0	**0.7**	0.7	0.8	0.07	**8.6**
	K	95.1	67.6	**53.0**	62.3	60.9	10.69	**75,0**
	C	1.85	30.5	**25.2**	32.5	19.8	0.55	**50.0**
	BOD	57.6	42.3	**33.5**	39.3	38.4	0.62	**80.0**
	COD	71.4	60.4	**51.5**	57.6	56.5	0.66	**150**
	Mg	50	45.4	**36.8**	40.3	40.8	0.50	**100**
	Pb, Cd, Ni	0.00	0.0	**0.0**	0.0	0.0	-	**840,85,420ppm**
	Fe	7.0	8.3	**1.2**	1.2	3.6	0.46	**5.0**
	Zn	7.0	91.6	**1.5**	1.1	31.4	6.70	**5.0**
	Ca	240	176	**135**	151	154	25.96	**230**

Mean: temperature: 44.59 31.67 38.35

Consortia : EM1:35.82, EM2:35.10 , EM3:43.68

Parameters:N:21.5,P:0.38,K:50.63,C:10.07.BOD:39.73,COD:49.42,Mg:39.65,Fe:1.69,Zn:11.09,Ca:157.85

C.D($p \leq 0.05$)Temperature: 0.3438 (Significant)

Consortia 0.3438 (Significant)

Parameters: 0.6276 (Significant)

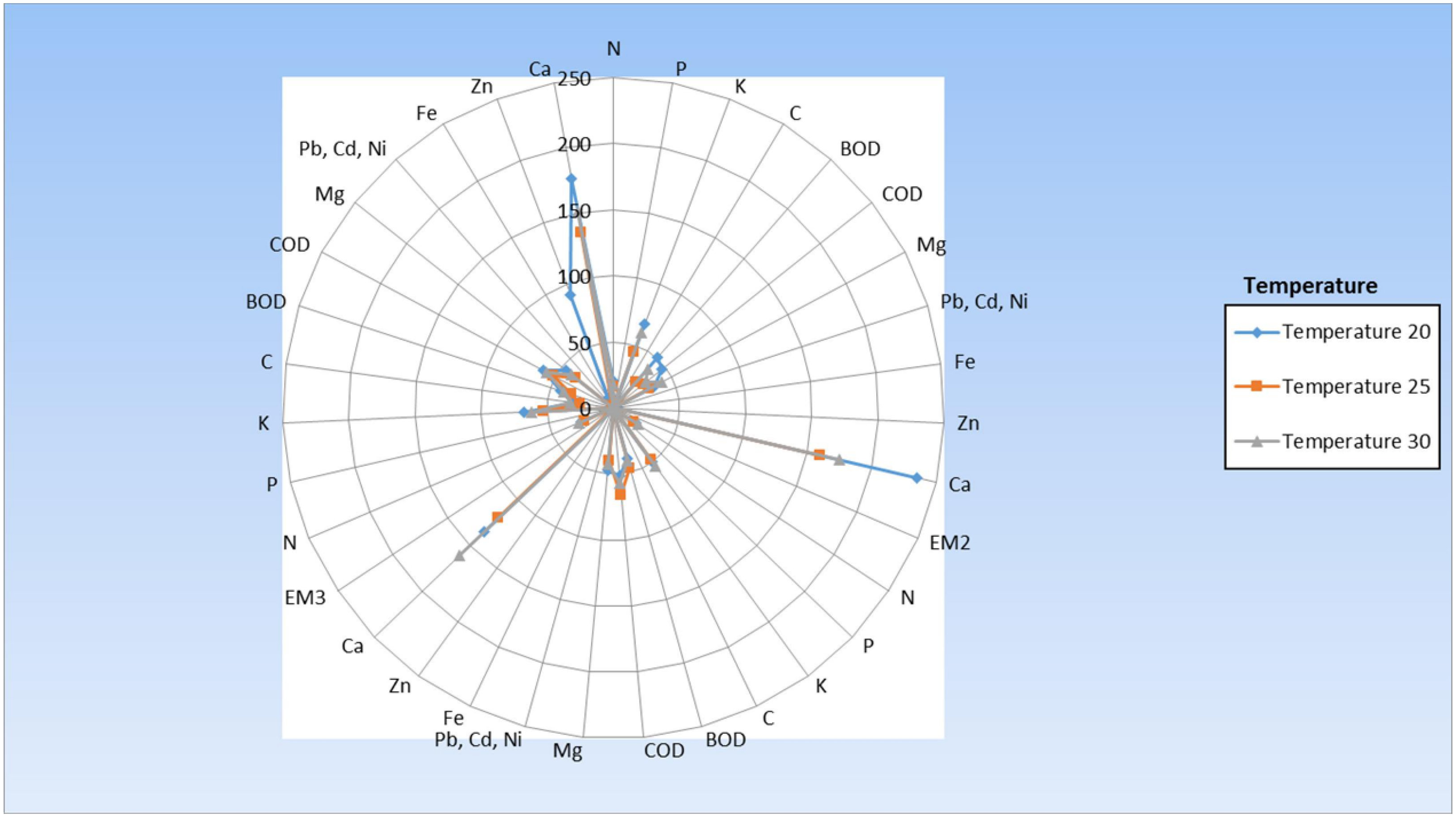


Fig. 1: Effect of temperature on Total N, P, K, C, BOD, COD, Heavy metals using various consortia

Table 26: Effect of pH on total N, P, K, C, BOD, COD, heavy metals using various consortia

Type of consortium	Parameters (ppm)	Before treatment values (mg/l)	pH			Mean	C.D ($p \leq 0.5$)	WHO Permissible Limits (mg/l)
			pH 6	pH 7	pH 8			
EM1	N	32.6	32.5	**25.5**	28.5	28.8	0.126	**37.0**
	P	6.2	2.1	**1.2**	1.2	1.5	0.069	**8.6**
	K	95.1	96.0	**86.0**	96.6	92.8	4.605	**75.0**
	C	1.85	0.3	**0.30**	0.4	0.3	0.029	**50.0**
	BOD	57.6	54.8	**37.2**	45.5	45.9	0.184	**80.0**
	COD	71.4	47.0	**29.0**	31.5	35.8	0.492	**150**
	Mg	50	33.8	**24.4**	27.3	28.5	0.58	**100**
	Pb, Cd, Ni	0.00	0.0	**0.0**	0.0	0.0	-	**840, 85, 420 ppm**
	Fe	7.0	2.1	**2.1**	2.1	2.1	-	**5.0**
	Zn	7.0	1.2	**0.8**	1.1	1.0	0.073	**5.0**
	Ca	240	235	**155**	335	241	5.95	**230**
EM2	N	32.6	26.3	**22.3**	24.3	24.3	0.093	**37.0**
	P	6.2	1.0	**0.7**	0.7	0.8	0.076	**8.6**
	K	95.1	81	**56**	71	69.3	5.88	**75,0**
	C	1.85	0.4	**0.3**	0.4	0.4	0.069	**50.0**
	BOD	57.6	51.1	**26.5**	39.1	38.9	0.488	**80.0**
	COD	71.4	39.5	**23.3**	31.7	31.5	0.519	**150**
	Mg	50	35.1	**30.5**	41.7	35.7	0.106	**100**
	Pb, Cd, Ni	0.00	0.0	**0.0**	0.0	0.0	-	**840, 85, 420 ppm**
	Fe	7.0	0.8	**0.3**	0.5	0.6	0.052	**5.0**
	Zn	7.0	1.1	**0.6**	0.4	0.7	0.574	**5.0**
	Ca	240	156	**74.4**	125	118	7.139	**230**

Contd…

Table 26 contd…

Type of consortium	**Parameters (ppm)**	**Before treatment values(mg/l)**	**pH**			**Mean**	**C.D (p≤0.5)**	**WHO Permissible Limits(mg/l)**
			pH 6	**pH 7**	**pH 8**			
EM3	N	6.2	0.4	**0.3**	0.4	0.4	0.105	**8.6**
	P	95.1	72.0	**57.0**	68.0	65.6	7.344	**75,0**
	K	1.85	45.0	**43.0**	56.0	48.0	8.773	**50.0**
	C	57.6	57.2	**42.5**	50.3	50.0	0.48	**80.0**
	BOD	71.4	148	**98.2**	105	117	7.217	**150**
	COD	50	42.5	**36.4**	40.1	39.6	0.572	**100**
	Mg	0.00	0.0	**0.0**	0.0	0.0	-	**840, 85, 420 ppm**
	Pb, Cd, Ni	7.0	2.1	**0.4**	1.	1.2	0.568	**5.0**
	Fe	7.0	0.6	**0.4**	0.5	0.5	0.082	**5.0**
	Zn	240	336	**274**	294	301	8.878	**230**
	Ca	240	176	**135**	151	154	25.96	**230**

Mean:
pH: 53.27 38.17 50.60
Consortia : EM1:47.15, EM2:31.55, EM3:63.33
Parameters:N:23.65P:0.70,K:73.82,C:15.49BOD:44.75,COD:60.38,Mg:34.45,Fe:1.69,Zn:0.55,Ca:218.61

C.D($p \leq 0.05$)
pH: 0.3375 (Significant)
Consortia: 0.3375 (Significant)
Parameters: 0.6162 (Significant)

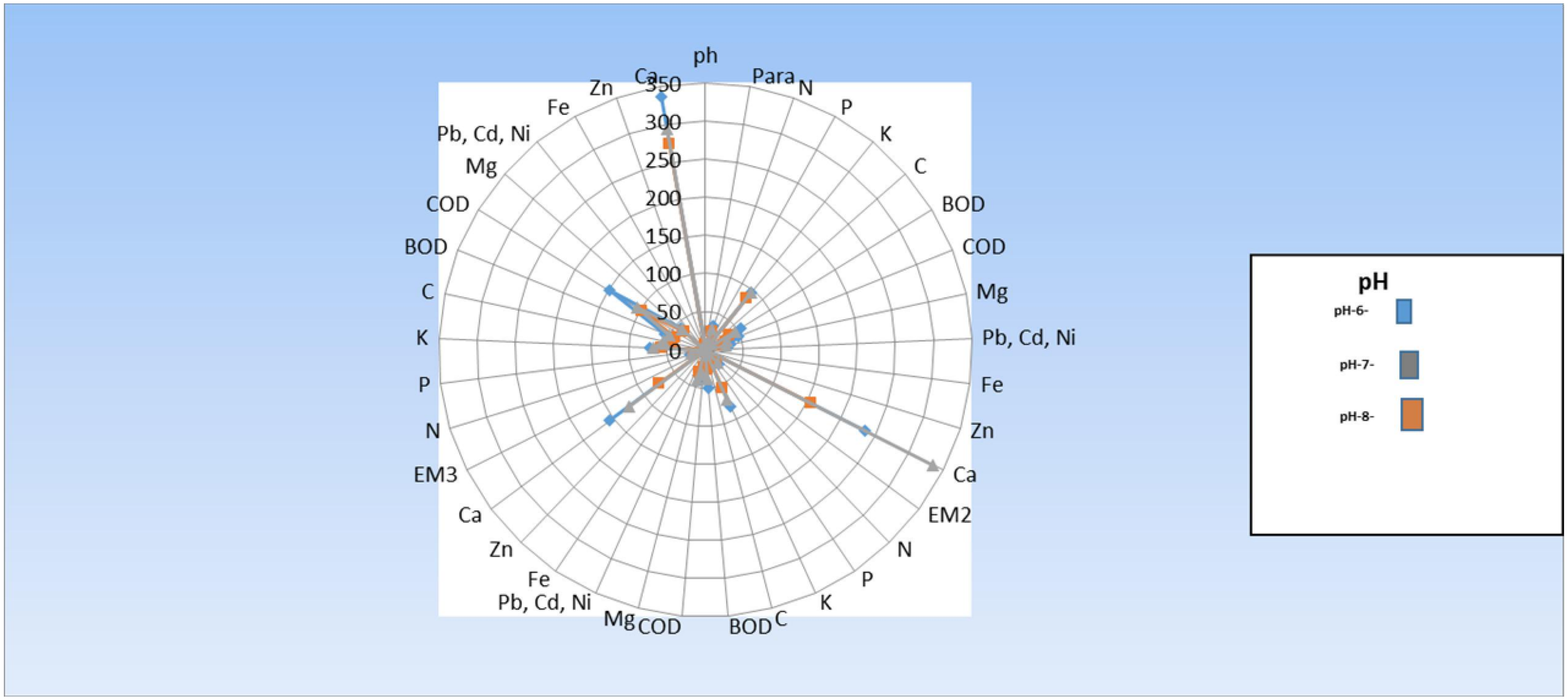


Fig. 2: Effect of pH on total N, P, K, C, BOD, COD, heavy metals in various consortia

4.10 Effect of Molasses concentration on total N, P, K, C, BOD, COD, heavy metals using various consortia

Perusal of the data presented in the Table 27 indicates that there was significant reduction in the macro nutrient concentration of the sewage water after treatment with EM1, EM2 and EM3. Similar trend was observed with respect to micro nutrients, heavy metals, BOD and COD. The final concentration of all the nutrients in sewage water after treatment at different molasses concentration (1, 3 and 10%) was within the permissible limits as defined by WHO. Maximum reduction in the concentration of all nutrients, BOD, COD and heavy metals was recorded at molasses concentration of 3% in the sewage water treated with EM3 followed by EM2 and EM1. However, all the three consortia could be recommended as the parameters studied were within WHO standards. However, minimum reduction in all the parameters was observed at molasses concentration of 1% in all the three consortia (Table 27, Fig. 3).

4.11 Effect of incubation period on Total N, P, K, C, BOD, COD, heavy metals using various consortia

Perusal of the data presented in table 28 indicates that there was significant reduction in macronutrient concentration of the sewage water after treatment with EM1, EM2 and EM3 similar trend was observed with respect to micro nutrients, heavy metals, BOD and COD. The final concentration of all the nutrients in sewage water treated at different incubation period of 8, 18, 28 days was within the permissible limits as defined by WHO. Maximum reduction in the concentration of nutrients, BOD, COD and heavy metals was recorded in sewage water treated with EM3 followed by EM2 and EM1 on 8th day of incubation. However, all the three consortia could be recommended as the parameters studied were within the WHO standards. Minimum reduction of all the parameters in all the three consortia was observed on 28th day of incubation (Table 28 and Fig. 4).

Table 27: Effect of molasses concentration on total N, P, K, C, BOD, COD, heavy metals using various consortia

Type of consortium	Parameters	Before treatment values(mg/l)	Molasses concentration (%)			Mean	C.D (p≤0.5)	WHO Permissible Limits (mg/l)
			1	3	10			
EM1	N	32.6	35.3	**25.4**	30.4	30.4	0.33	**37.0**
	P	6.2	1.3	**1.2**	1.4	1.3	0.076	**8.6**
	K	95.1	**55.0**	**39.0**	51.0	48.3	6.47	**75.0**
	C	1.85	45.6	**33.3**	35.6	38.2	9.33	**50.0**
	BOD	57.6	51.2	**26.3**	39.2	38.9	0.42	**80.0**
	COD	71.4	39.5	**23.4**	31.8	31.6	0.47	**150**
	Mg	50	45.3	**36.3**	40.2	40.6	0.42	**100**
	Pb, Cd, Ni	0.00	0.0	**0.0**	0.0	0.0	-	**840, 85, 420 ppm**
	Fe	7.0	1.2	**89.5**	1.1	30.6	0.50	**5.0**
	Zn	7.0	1.3	**91.6**	1.0	31.3	5.92	**5.0**
	Ca	240	174	**134**	147	151.6	20.60	**230**
EM2	N	32.6	32.3	**25.3**	28.3	28.6	0.16	**37.0**
	P	6.2	1.3	**1.2**	1.4	1.3	0.07	**8.6**
	K	95.1	49.0	**42.6**	58.6	50.1	9.88	**75,0**
	C	1.85	49.6	**35.6**	55.3	46.8	7.88	**50.0**
	BOD	57.6	57.3	**35.0**	43.1	45.1	16.34	**80.0**
	COD	71.4	149	**98.34**	107	118.1	7.97	**150**
	Mg	50	45.3	**36.3**	40.1	40.56	0.40	**100**
	Pb, Cd, Ni	0.00	0.0	**0.0**	0.0	0.0	-	**840, 85, 420 ppm**
	Fe	7.0	1.2	**89.2**	1.1	30.5	0.22	**5.0**
	Zn	7.0	1.3	**89.3**	1.0	30.5	2.96	**5.0**
	Ca	240	169	**128**	142	146	10.29	**230**

Contd…

Table 27 contd…

Type of consortium	**Parameters**	**Before treatment values(mg/l)**	**Molasses concentration (%)**			**Mean**	**C.D (p≤0.5)**	**WHO Permissible Limits(mg/l)**
			1	**3**	**10**			
EM3	N	32.6	26.3	**22.3**	24.3	24.3	0.17	**37.0**
	P	6.2	1.2	**0.5**	0.9	0.9	0.16	**8.6**
	K	95.1	50.0	**37.3**	50.3	45.8	9.74	**75,0**
	C	1.85	40.3	**32.6**	**46.3**	39.7	9.90	**50.0**
	BOD	57.6	51.2	**26.3**	39.1	38.9	0.38	**80.0**
	COD	71.4	39.5	**23.4**	31.7	31.5	0.50	**150**
	Mg	50	36.3	**30.4**	33.3	33.3	0.79	**100**
	Pb, Cd, Ni	0.00	0.0	**0.0**	0.0	0.0	NA	**840, 85, 420 ppm**
	Fe	7.0	2.1	**1.0**	1.0	1.4	0.13	**5.0**
	Zn	7.0	1.9	**0.9**	1.2	1.3	0.12	**5.0**
	Ca	240	235	**162**	176	191	12.08	**230**

Mean:

Molasses concentration: 48.88 46.86 41.77

Consortia : EM1:43.94, EM2:53.20, EM3:40.37

Parameters:N:27.6,P:0.90,K:47.03,C:41.37.BOD:40.84,COD:59.70,Mg:37.27,Fe:21.97,Zn:20.80,Ca:160.85

C.D(p≤0.05)

Molasses concentration: 0.8355 (Significant)

Consortia: 0.8355 (Significant)

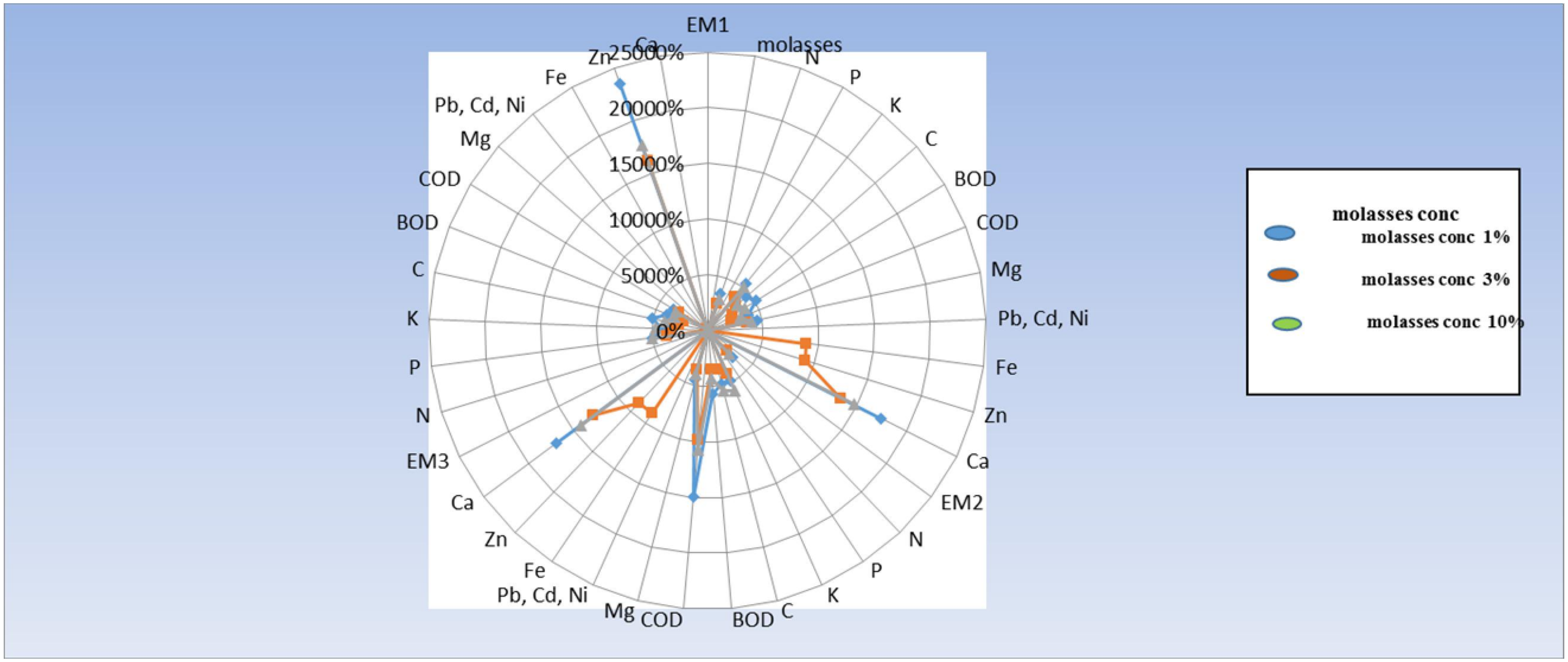


Fig. 3: Effect of Molasses concentration on total N, P, K, C, BOD, COD, Heavy metals using various consortia

Table 28: Effect of incubation period on Total N, P, K, C, BOD, COD, heavy metals using various consortia

Type of consortium	Parameters	Before treatment values(mg/l)	No of days			Mean	C.D (p≤0.5)	WHO Permissible Limits (mg/l)
			8th	18th	28th			
EM1	N	32.6	**30.3**	36.4	32.4	33.0	0.381	**37.0**
	P	6.2	**1.2**	1.3	1.4	1.3	0.07	**8.6**
	K	95.1	**65.0**	75.0	85.0	75.0	8.28	**75.0**
	C	1.85	**25.2**	27.5	29.3	27.3	0.42	**50**
	BOD	57.6	**32.3**	45.4	40.4	39.4	0.60	**80**
	COD	71.4	**50.6**	60.4	56.2	55.8	0.60	**150**
	Mg	50	**11.5**	27.4	19.6	19.5	0.30	**100**
	Pb, Cd, Ni	0.00	**0.0**	0.0	0.0	0.0	-	**840,85,420ppm**
	Fe	7.0	**0.0**	0.0	0.0	0.0	-	**5.0**
	Zn	7.0	**0.0**	0.0	0.0	0.0	-	**5.0**
	Ca	240	**110**	176	165	150	15.3	**230**
EM2	N	32.6	**26.3**	30.3	28.4	28.3	0.2	**37.0**
	P	6.2	**1.1**	1.3	1.4	1.3	0.1	**8.6**
	K	95.1	**46.0.**	59.0	53.0	52.6	7.6	**75.0**
	C	1.85	**23.7**	25.8	28.5	26.0	0.3	**50**
	BOD	57.6	**27.3**	39.2	30.4	32.3	0.3	**80**
	COD	71.4	**36.2**	43.3	40.5	40.0	0.2	**150**
	Mg	50	**11.5**	27.4	19.6	19.55	0.30	**100**
	Pb, Cd, Ni	0.00	**0.0**	0.0	0.0	0.00	-	**840,85,420ppm**
	Fe	7.0	**0.0**	0.0	0.0	0.0	-	**5.0**
	Zn	7.0	**00.0**	0.0	0.0	0.00	-	**5.0**
	Ca	240	**110.0**	176.4	165.0	150.4	15.30	**230**

Contd…

Table 28 contd…

Type of consortium	Parameters	Before treatment values(mg/l)	No of days			Mean	C.D (p≤0.5)	WHO Permissible Limits(mg/l)
			8th	18th	28th			
EM3	N	32.6	**25.4**	28.3	32.3	28.7	0.27	**37.0**
	P	6.2	**0.7**	0.9	1.1	0.9	0.17	**8.6**
	K	95.1	**48.0**	54.6	42.6	48.4	8.69	**75.0**
	C	1.85	**15.6**	21.6	30.0	22.4	6.82	**50**
	BOD	57.6	**37.3**	41.2	40.3	39.6	0.32	**80**
	COD	71.4	**50.4**	56.4	65.5	57.4	0.35	**150**
	Mg	50	**36.7**	45.3	40.3	40.7	0.348	**100**
	Pb, Cd, Ni	0.00	**0.0**	0.0	0.0	0.0	-	**840,85,420ppm**
	Fe	7.0	**0.0**	0.0	0.0	0.0	-	**5.0**
	Zn	7.0	**92.0**	1.4	1.3	31.6	7.35	**5.0**
	Ca	240	**134**	175	147	152	20.01	**230**

Mean:

Incubation period: 38.07 50.32 43.91

Consortia : EM1:44.23, EM2:41.82 , EM3:46.24

Parameters: N:39.84, P:0.98, K:57.15, C:24.91, BOD:36.94, COD:5088, Mg:25.97, Zn:11.35, Ca:148.88

C.D(p≤0.05)

Incubation period: 5.406 (Significant)

Consortia: (Non-significant)

Parameters: 9.3635 (Significant)

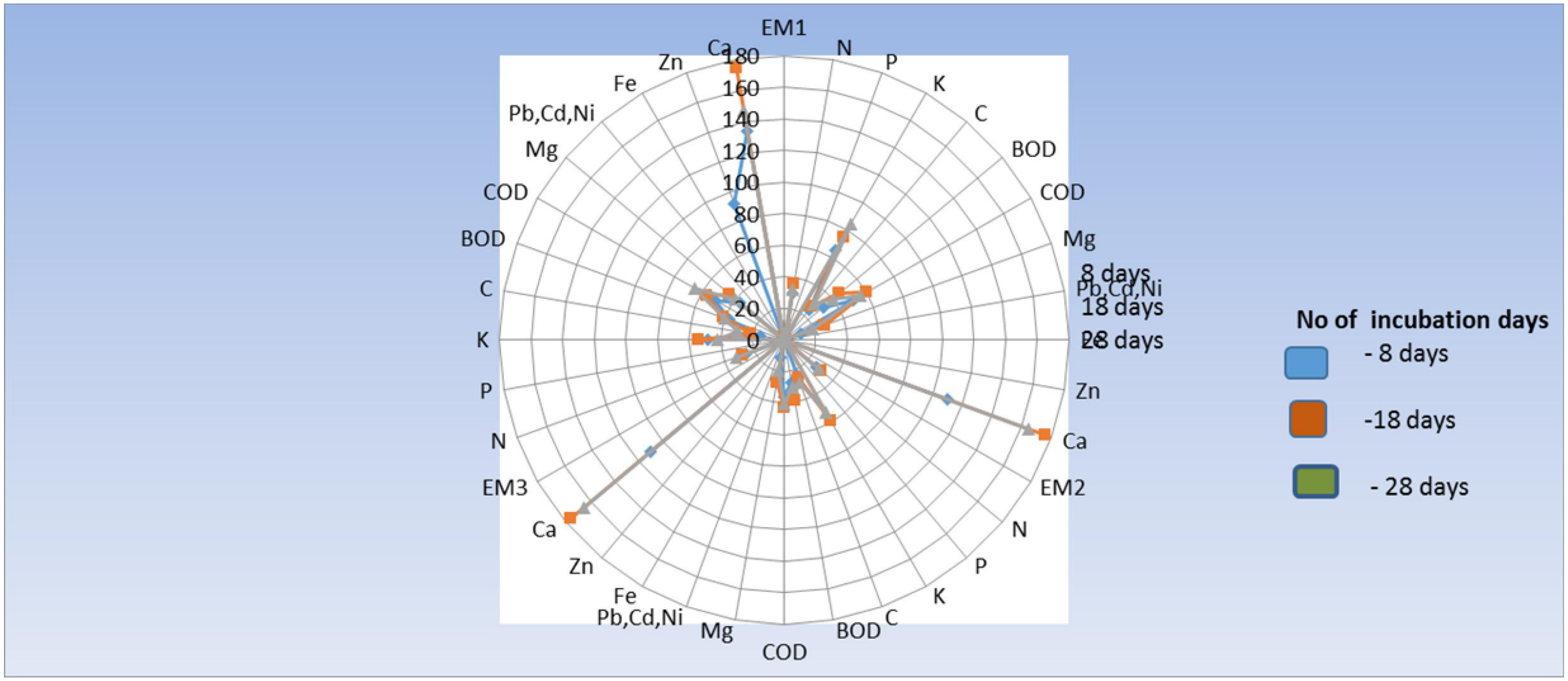


Fig. 4: Effect of incubation period on total N, P, K, C, BOD, COD, heavy metals using various consortia

Chapter – 5

DISCUSSION

The experimental findings obtained during the present investigations are discussed under the following headings:

5.1 Isolation of effective microorganisms

5.2 Development of effective microbial consortium

5.3 Biodegradation of sewage water

5.4 Analysis of EM treated sewage water

5.1 Isolation of effective microorganisms

The results obtained regarding the isolation of effective microorganisms are discussed as under:

5.1.1 Isolation of Lactic acid bacteria

In this study, we isolated and identified most promising isolates of lactic acid bacteria belonging to genus *Lactobacillus.* On the basis of biochemical tests and sugar fermentation by various isolates of lactic acid bacteria, isolates SL1, SL4 and SL9 showed maximum positive results for sugar fermentation and hence were selected for the formulation of microbial consortia. Our results corroborate with that of Savard *et al.* (2002) who found *Lactobacillus* sp. most promising for sugar fermentation. Several researchers have reported that *Lactobacillus* has an ability to control fungi, produce more lactic acid and is effective for cellulose degradation (Pitt and Hocking, 1997; Strom *et al.,* 2002).

5.1.2 Isolation of *Pseudomonas*

Out of 20 isolates of *Pseudomonas* which were isolated from different oil spilled soils of garages three isolates SP6, SP5, and SP1 showed the maximum phosphate solubilization index of 8.03, 7.11 and 7.05mm respectively. Isolate SP5, SP6 and SP1 exhibited chitinase activity of 33.34, 25.12,and 22.18 units/ml and produced 973.27,61.15 and 56.63mm zones of inhibition against fungal isolate *Fusarium oxysprum* as shown in (Table 8,9 and 10)and thus were used in effective microbial consortia. Decrease in pH indicates the production of acids, which is considered to be responsible for P-solubilization (Rashid *et al.,* 2004). The clear or halo zone was formed due to the solubilization of insoluble phosphates by acidification either by proton extrusion or organic acid secretion (Darmwall *et al.,* 1989). Among phosphate solubilizing microorganisms the most efficient ones belong to the genera *Pseudomonas* and *Bacillus* (Dave and Patel, 1999). Fungus and bacteria in combination were also used by some researchers to increase the degradation rate. The fungal-treated wastewater was again treated with the bacteria for the biodegradation process. Chuphal *et al.* (2005) used *Paecilomyces* sp. and *Pseudomonas syringae* to report significant reduction in colour (88.5%), lignin (79.5%), COD (87.2%) and phenol (87.7%) in two steps. Several rhizobacterial antagonists belonging to the fluorescent *Pseudomonas* and *Bacillus* spp. are found to produce chitinase, which degrades the chitin present in the fungal and nematode cell wall. Saima and Roohi (2013) reported the maximum amount of chitinase production by HS4 and HS6.

5.1.3 Isolation of Yeasts

On the basis of fermentation of sugar, *Saccharomyces* was the yeast isolated from dry yeast granules and on the basis of antibacterial activity against test organisms *Bacillus* and *E. coli* isolate SS7, SS4 and SS1 exhibited maximum zone of inhibition (Table 13) and were used in formulation of effective microbial consortia. Adams and Moss (2002) also reported the isolation of *Pichia* sp and *Saccharomyces* sp. from a number of tropical fermented products, where *Saccharomyces* was the most frequently encountered yeast in fermented beverages and foods based on fruits and vegetables. Naturally occuring yeasts or their intentional addition as part of starter cultures, apparently contributes to fermentation by breaking down of lipids and proteins, by forming specific metabolic products, as well as the depletion of oxygen (Metiva *et al.,* 1986). On the basis of zone of inhibition SS7, SS4 and SS1 was used for the preparation of effective microbial consortium for the degradation. Similar results were also reported by Savard *et al.* (2002) and Kantachote *et al.* (2005).

5.1.4 Isolation of Actinomycetes

Actinomycetes have been frequently isolated from soil, water and among 20 isolates which were isolated from decomposing pile (SA1-SA20), SA5 exhibited the maximum zone of inhibition against the test organisms *Bacillus subtillis* and *Fusarium monoliforme* of 17.66 and 17.00 mm followed by SA-3, SA1 of 15.00, 15.00 and 12.66 and 14.00 mm respectively as shown in Table 16. It could utilize a wide range of carbon sources and was thus included in effective microbial consortium. Similar findings had also been reported by Weyland (1981) and Smith *et al.* (1999).

5.1.5 Isolation of fermenting fungi

On the basis of amylase activity of fermenting fungi, isolate SF16, SF1 and SF6 exhibited the maximum production of amylase activity of 57.66, 56.00 and 53.66 IU/ml/min respectively (Table 18) and were identified as *Aspergillus niger*. The fungal isolates were compared with one another on the basis of amylase production. Minty *et al.* (2013) designed synthetic fungal-bacterial consortia for production of bioethanol from cellulosic biomass. The fungus *Trichoderma reesei*, which secretes cellulase enzymes to hydrolyze lignocellulosic biomass into soluble saccharides. SF16, SF1, SF6 produced the maximum enzyme and were thus selected for the preparation of effective microbial consortium.

5.2 Development of effective microbial consortium

A microbial consortium is a group of different species of microorganisms that act together as a community. They are more resistant to environmental shock and can better compete and survive in the complex environment than single microorganism. Microbial consortia are capable of degrading a wide variety of complex wastes. Various natural examples of microbial consortia are found in activated sludge basins, biofilms on trickling filters and in various soil ecosystems. In a microbial consortium, the organisms work together in a complex system where all are benefited from the activities of others in a community. The advantages of employing mixed cultures as opposed to pure cultures in bioremediation have been widely demonstrated. It could be attributed to the effects of synergistic interactions among members of the association. It is possible that one species removes the toxic metabolites that otherwise may hinder microbial activities of the species preceding it. It is also possible that the second species are able to degrade compounds that are partially degraded by the first (Kumar *et al.,* 1992). Various reports are available on the study of microbial consortia for their biotechnological application. Sarunyou and Nelson (2010) showed degradation of lignocellulosic agro-industrial residues by means of complex microbial community and had shown it as a promising approach providing efficient biomass decomposition for subsequent conversion to value added products. In a study, Kumar *et al.* (1992) showed degradation of oil by individual bacteria and consortia and concluded that mixed bacterial consortium showed more growth and degradation than did individual strains. Perumal *et al.* (2012) used single bacteria and mixed bacterial consortia for dye decolourization and showed that bacterial consortia can decolourize dye more efficiently than single bacteria. John *et al.* (2011) reported that compost derived microbial consortia were excellent in degradation of cellulose and hemicelluloses in the production of biofuel.

5.2.1 Effective microbial consortium

After isolation, identification and screening of microorganisms, the already effective isolates of various microbial groups were mixed in different proportions to prepare microbial consortia with the names of EM1, EM2 and EM3 and their microbial and physico-chemical characteristics were noted accordingly. The presence of large proportion of lactic acid bacteria help in decreasing pH, making conditions conducive for cellulose degradation. At the same time, aerobic microbes were suppressed while *Lactobacillus* sp. and anaerobic microbes increased. This problem was overcome by the presence *Pseudomonas* bacteria which metabolized the organic acid and then maintained the number of microbes. However, the presence of *Streptomyces* sp. helps to check harmful species and the presence of *Aspergillus* sp. helps in the degradation of cellulosic materials. These observations were also in line with the observations of Higa (1994).

5.3 Biodegradation of sewage water

5.3.1 *In vitro* biodegradation

5.3.1.1 Effect of Temperature on reduction of various parameters in sewage water

Significant differences were observed in EM1, EM2 and EM3 applications with respect to the temperature in reduction of various nutrients, BOD, COD and heavy metals in sewage water after treatment with consortia at different temperatures of 20-30°C. Maximum reduction in all the parameters was observed at temperature of 25°C and was found maximum in EM1treated sewage water. Daiz *et al.* (1993) also worked on wastes and reported similar observations. Temperature influences growth, metabolism, and nutrition, enzymes, biomass and cell permeability (Hayase *et al.,* 1984). The contribution in maximizing reduction process at the temperature of 25°C by EM1 could also be due to high concentration of nutrients available for the optimal growth of microorganisms in the form of lactic acid bacteria, *Pseudomonas*, actinomycetes, fermenting fungi and yeast as compared to EM2 and EM3. Minimum reduction in all parameters was observed at 30°C in all the effective microbial consortia treated sewage water. EM1 was recommended for further use (Table 25).

5.3.1.2 Effect of pH on reduction of various parameters in sewage water

Significant differences were observed with respect to application of EM1, EM2 and EM3 in reduction of the various macro and micro nutrients, BOD, COD and heavy metals with respect to change in pH from 6-8. Maximum reduction in all the studied parameters was observed at pH 7 in EM3 treated sewage water followed by EM2 and EM1. Minimum reduction in all the parameters was observed at pH 6. Hayase *et al.* (1984) while studying the degradation of melanoidins by hydrogen peroxide at different pH (3.0–13.0) found that melanoidin decolourization in alkaline pH proceeds more rapidly than in acidic and neutral pH and it reached 94% at pH 10 in waste waters. Mohana *et al.* (2007) have reported the highest decolourization (67%) at pH 7.0 as the solubility of melanoidins depends on pH i.e. it is less soluble in acidic pH than in alkaline pH and pH more or less than pH 7.0 led to decrease in decolourization activity as well as the growth of microbes. The best decolourization (80%) and highest COD removal (75%) at on initial pH of 5 (Benito *et al.,* 1997). The *Pseudomonas* may grow in a wide pH range of 4-10 but the optimal condition was pH 7 (Murad *et al.,* 2007). Praveen and Jain (2007) stated that *Streptomyces* shows its growth at pH of 5-10 (Table 26).

5.3.1.3 Effect of Molasses concentration on reduction of various chemical parameters in sewage water

Significant difference in reduction of various parameters was observed on application of various consortia to sewage water at molasses concentration of 1%, 3% and 10%. The lowest concentration of molasses facilitated the growth of EM and the increased concentration inhibited the growth and survival of effective microbes and hence resulted in reduction of nutrients, BOD, COD and heavy metals. It was observed that 3% of molasses was favourable for constituent microbes as maximum reduction of all studied parameters was observed at 3% of molasses concentration in EM3 followed by EM2 and EM1.Minimum reduction was observed at 1% of molasses concentration. Lee *et al.* (2000) investigated the dye-decolourizing peroxidase by cultivating *Geotrichum candidum Dec*1 using molasses as a carbon source. Components in the molasses medium stimulated the production of decolourizing peroxidase but inhibited the decolourizing activity of the purified enzyme. It was found that the inhibitory effect of molasses can be eliminated at dilution ratios of more than 25. Two flocculant strains of yeast, *Hansenula fabianii* and *Hansenula anomala* was used for the treatment of wastewater from beet molasses-spirits production and achieved 25.9 and 28.5% removal of BOD and COD respectively from wastewater without dilution (Moriyal, 1990).

5.3.1.4 Effect of incubation period on reduction of various parameters in sewage water

The incubation period has greatest effect on microbial consortia formulation. At longer incubation period, the growth of microorganisms was inhibited due to depletion of nutrients, accumulation of toxic end products and change in pH. Significant differences were observed in EM1, EM2 and EM3 applications with respect to reduction of nutrients, BOD, COD and heavy metals at different incubation periods of 8 to 28 days. Maximum reduction of all the parameters was observed on 8th day of incubation in EM3 followed by EM2 and EM1 treated sewage water and minimum reduction of all the parameters was observed on 18th day of incubation in all the consortia treated sewage water. Mansur (1997) obtained a maximum reduction of N, P, K and BOD,COD of around 60% on day 8 after inoculating with fungus *Trametes* sp. A similar biphasic treatment of the effluent was carried out in a constructed wetland with *Bacillus thuringienesis* and *Typha angustata* by Chandra *et al.* (2008) which resulted in 98- 99% BOD, COD and color reduction after 7 days (Table 28).

5.4 Analysis of Effective Microbial treated sewage water

5.4.1 Biological oxygen demand

The effective microbial consortia reduced the BOD of sewage water treated with EM1, EM2 and EM3 from 57.6 to 26.3 mg/l (Table 25 to table 28). Ohmomo (1987) screened fungal strains mainly of genus *Aspergillus* and observed the decrease of about 51% in Biological oxygen demand (BOD) and 56% removal of the total organic carbon (TOC) in initial solution. Banerjee (2004) designed a semi continuous batch digester to investigate biomethanation of distillery waste in mesophilic and thermophilic range of temperatures. The study revealed that there was an important effect of the temperature of digestion and of substrate concentration in terms of BOD and COD loading on the yield of biogas as well as its methane content. Maximum BOD reduction (86.01%), total gas production and methane production (73.23%) occurred at a BOD loading rate of 2.74 kg m^{-3} at 50°C digestion temperature. The microbial strains reduced the BOD by 58.5-82.2% (Sirianuntapiboon *et al.,* 2004). Dharmsthiti *et al.* (2002) formulated bacterial consortium including *Pseudomonas*, *Bacillus* and *Acinetobacter* using molasses for treating lipid rich waste water and the consortium reduced BOD from 448 to 72 mg/l. Kumar *et al.* (1992) used the bacterial consortium of *Pseudomonas aeruginosa*, *Bacillus megaterium* and *Stenotrophomonas maltophilia* for treating paper and pulp mill effluent and observed BOD reduction from 87 to 89%.

5.4.2 Chemical oxygen demand

There was a significant decrease in COD (Chemical oxygen demand) by effective microbial consortia treated sewage water from 71.4 to 29.0 mg/l (Table 25-28). The effective microbial consortia reduced the COD effectively while treated at concentration of 3 ml/l for 8 days. The EM reduced the COD of waste water from Nestle and Trebor companies to 76% in 11 days at concentration of 1 ml/l (Gede Ngurah Wididana, 1994). The bacterial strain BP103 reduced the COD by 35.5-71.2% (Sirianuntapiboon *et al.,* 2004). Stanley (1979) reported that whey disposed from cheese manufacturing industry was treated using *Kluyveromyces fragilis* which reduced the COD by 29 and 37% in 16 and 20 hours, respectively after the growth of culture. Kumar *et al.* (2007) used the bacterial consortium of *Pseudomonas aeruginosa*, *Bacillus megaterium* and *Stenotrophomonas maltophilia* for treating paper and pulp mill effluent and observed COD reduction from 67 to 71%. The consortium of five white-rot fungi, *Phanerochaete chrysosporium, Pleurotus ostreatus, Lentinus edodes*, *Trametes versicolor* and S22 removed 71% of lignin content and 48% of COD from wastewater (Juan *et al.,* 2007). By using sulphate reducing bacteria, Hao and Man (2006) showed

COD removal of up to 70-75% after 3 weeks which increased to 82-88% by subsequent aerobic treatment for 48h.

5.4.3 Variation in N, P, K, C Heavy Metals and micro elements

The effective microbial consortia reduced the macro nutrients, heavy metals like (Cd, Pb, Ni) and micro elements like (Ca, Fe, Zn) of sewage water from 32.6, 6.2, 95.1, 1.85, 50, 7, 240 mg/l to 16.3, 0.3, 0.6, 0.8, 0.0, 1.0, 74.4 mg/l (Table 25-28). Wang *et al.* (2010) reported a decrease in nitrogen (83% N as NH_4^+) and phosphorus (90% P as PO_4^{3-}) in municipal wastewater by *Chlorella* sp. They suggested that the nutrient removal rates were independent of the optimal N/P ratio but that the concentrations of these nutrients were important for the algal growth systems. In another study, Samori *et al.* (2013) clearly demonstrated the high potential of using an isolated algal strain from an artificial freshwater pond, *Desmodesmus communis*, to remove contaminants from primary wastewater. The consortium with combination of *Bacillus* sp. RS-1, *Pseudomonas* sp. YLW-7 and *Enterobacter* sp KLW-2 was able effectively to remove the phosphate in the synthetic medium when compared to individual strains. The phosphate removal was observed to be maximum of 92.5% in mineral salts medium (MSM) at pH 7and 5, and 63.4% in synthetic phosphate solution at neutral pH with lactose as a carbon source by the consortium after 72 h (Usharani Krishnaswamy *et al.,* 2011). *Pseudomonas* and *Bacillus* are the commonly known bacteria for brewery waste treatments. A study conducted by De Souza *et al.* (1998) have reported that bacteria from different genera can work together in an environment and survive through the metabolites interaction because a mixed culture has more competence and has a higher tolerance to toxic metabolites. Studies by Kanhaiya and Vaishya (2017) using three Consortia 3 showed better degradation with 93.78% ability in reducing zinc when incubated for 72 hours and 86.16% when incubated for 24 hours. The lead reduction was found to be 84.33% by Consortia 1(A3 + B4) when incubated at 37°C for 72 hours incubation. The chromium was reduced by Consortia 2(C6 + D7) with 87.61% ability when incubated for 72 hours.

Chapter – 6

SUMMARY AND CONCLUSION

Development of technology of Effective Microorganisms (EM) for the treatment of waste waters is a significant step to safeguard our environment which attracted much scientific attention worldwide and this was also the prime focus of our study. Therefore, the present investigation entitled "Formulation of effective microbial consortia and its application in sewage treatment" was undertaken with the following objectives:

1. Isolation and characterization of effective microbes from sewage and respective sources.
2. Identification of effective microbes based on morphological & biochemical characteristics.
3. Development of low cost, ecofriendly and effective microbial consortia with biodegradable property for sewage treatment.
4. Effect of Effective microbes on Sewage Effluent.

In order to achieve the above objectives, various groups of microorganisms which formed partners of the consortium, were indigenously isolated, identified and characterized on the basis of some important relevant properties. Various microorganisms belonging to the Lactic acid bacteria, *Pseudomonas* sp., yeasts, actinomycetes and fermenting fungi were isolated. The most effective microorganisms selected for the consortium were identified as *Lactobacillus, Pseudomonas, Saccharomyces, Streptomyces* and *Aspergillus niger.*

Various tests which included antibiotic activities against number of test microorganisms were conducted to select the most effective isolates from each compatibility. One isolate from each group was selected and were mixed to form liquid consortium and latter given the name as Effective Microorganisms (EM).

The effect of EM on the reduction of various parameters of Sewage waste water is summed up as follows:

- Among the three effective microbial consortia (EM1, EM2 and EM3) used during *in vitro* biodegradation, EM3 was the most promising one for *in vitro* biodegradation on the basis of reduction of macro nutrients like N, P, K and micro-nutrients like Ca, Zn, BOD, COD and heavy metals like Pb, Ni in sewage water treated with effective microbial consortia and the effect of various parameters like temperature, pH, molasses concentration and incubation period on reduction of nutrients was assessed.
- Effective microbial consortia contained constituent microbes like *Lactobacillus, Pseudomonas, Saccharomyces, Streptomyces* and *Aspergillus.*
- Chemical characteristics of effective microbial treated sewage water like BOD and COD was found better as compared to untreated sewage water.
- Nitrogen content showed reduction from 32.6 mg/l in untreated sewage water to 25, 24, 22, 18 and finally 16.3 mg/l in treated sewage water which was within the permissible limit as per WHO. Phosphorus and potassium also showed the reduction. Total phosphorus showed reduction from 6.2 mg/l in untreated sewage to 3, 2, 1, 0.7, 0.5 mg/l in treated sewage water. Total potassium also showed reduction from 68 to 39 mg/l in EM treated sewage water.
- Ca^{2+} content reduction varied from 235 to 119 mg/l in EM treated sewage water to untreated water. Mg^{2+} reduction ranged from 45.3 to 11.5 in EM treated sewage water to untreated sewage water, followed by Iron which ranged from 3.9 to 0.00 mg/l in EM treated sewage water to untreated sewage water. Similar trend in reduction of BOD

and COD was also observed from untreated sewage water to treated one.

- The population of viable bacteria, fungi, phosphorus solubilizing bacteria were found in high range in EM treated sewage water compared to untreated sewage water. The sewage water was found absolutely free from total coliform bacteria on and beyond 8th day of incubation.

CONCLUSION

On the basis of results obtained during the present investigation, following conclusions could be drawn:

- For the development of microbial consortium, the most effective and suitable species isolated, identified and screened were *Pseudomonas*, *Lactobacillus*,*Saccharomyces*, *Streptomyces* and *Aspergillus.*
- Among the three treatments of liquid consortia (EM1, EM2 and EM3) which were used during *in vitro* degradation of waste, EM3 was found most prominent in reduction of the studied nutrients at various parameters.
- Nutritional composition in terms of N, P, K, Ca, Mg, Fe and Zn were observed minimum in effective microbial consortia treated sewage water than untreated sewage and also reduction in BOD, COD and heavy metals was observed in treated sewage water than untreated.
- The population of total viable bacteria, fungi and phosphorus solubilizing bacteria was also found maximum in effective microbial consortia than treated sewage water.

RECOMMENDATION

On the basis of success of treatment of sewage water using effective microorganisms, following recommendation is drawn:

Effective Microbial technology, using indigenous microorganisms *Lactobacillus, Pseudomonas, Sacchromyces, Streptomyces* and *Aspergillus* are recommended for treatment of Sewage water to reduce the quantity of various elements, BOD, COD, N, P, K, Ca, heavy metals etc., offensive odours and end up into safe to use water for agricultural purposes.

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CERTIFICATE

Certified that all the corrections/amendments as suggested by External Examiner Dr. Harvinder Singh, Professor & Head, Department of Microbiology, Guru Nanak Dev University of Amritsar during Viva-Voce examination held on 03-05-2019 have been incorporated in the manuscript entitled **"Formulation of Effective Microbial Consortia and its Application in Sewage Treatment"** submitted by **Ms. Gousia Qadir (Regd. No. 2015-583-D).**

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