

THE USE OF EXO-POLYSACCHARIDE PRODUCING LACTOBACILLUS INOCULANT FROM OLIVE BRINE TO GROW WHEATGRASS USING SEA SAND, SEA WATER AND THEIR EFFECTS ON NUTRIENT COMPOSITION AND SILAGE MICROBIOLOGY

Submitted in partial fulfilment of the requirements for the award of
Bachelor of Science degree in Microbiology

by

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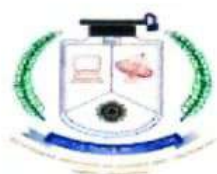


**DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIO AND CHEMICAL ENGINEERING
SATHYABAMA
INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

Accredited with Grade "A" by NAAC

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SATHYABAMA

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of AKSHAYA P (40300002) who carried out the project entitled "**THE USE OF EXO-POLYSACCHARIDE PRODUCING LACTOBACILLUS INOCULANT FROM OLIVE BRINE TO GROW WHEATGRASS USING SEA SAND, SEA WATER AND THEIR EFFECTS ON NUTRIENT COMPOSITION AND SILAGE MICROBIOLOGY**" under our supervision from Oct 2022 to Mar 2023.


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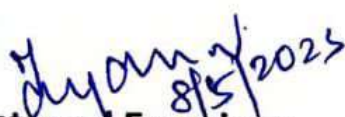

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ABSTRACT

In light of the current environmental crisis, which encompasses various issues such as soil erosion and loss of fertility, traditional farming soil is becoming increasingly unsuitable for growing crops. As a viable alternative, sea sand is being utilized for agricultural purposes with the aid of sea water and lactobacillus to enhance growth and overcome the challenge of water scarcity. The process involves isolating a lactobacillus bacterial strain with exopolysaccharide activity from olive brine solution using MRS agar and EPS medium. The lactobacillus strain with exopolysaccharide activity is then combined with desalinated seawater to provide moisture content for growing wheat grass in sea sand. The growth of the wheat grass in sea sand with lactobacillus mixed desalinated water is compared to two control models - one using sea sand and normal desalinated water without lactobacillus and the other utilizing normal fertile farm soil with fresh water. The study aims to prove that sea sand is a viable alternative for plant growth over traditional farming soil. Nutrient composition analysis of the wheat grass is conducted by comparing the nutrients present in the control (sea sand and sea water without lactobacillus) with the grass grown using lactobacillus and sea water in sea sand. Furthermore, microbiological analysis of the silage produced from the wheat grass is conducted. Microorganisms are commonly utilized as bio-fertilizers to stimulate plant growth, increase yields, and reduce the application of inorganic nitrogen. The survival and proliferation of these foreign microorganisms in soil are essential for promoting plant growth.

KEYWORDS: Exopolysaccharide producing lactobacillus, Lactobacillus, Sea sand, Sea water, Nutritional Analysis, Silage Microbiology, Wheat grass, Olive Brine Solution

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LIST OF ABBREVIATION

NSLAB	Non-Starter Lactic Acid Bacteria
LAB	Lactic Acid Bacteria
EPS	Exo-Polysaccharide
ESM	Exo-Polysaccharide Selective Medium
pH	Potential of Hydrogen
sp.	Species
MRS	De Mann, Rogosa, Sharpe agar
MSA	Mannitol Salt Agar
EMB	Eosin Methylene Blue Agar
MaC	MacConkey agar
MYP	Phenol red egg yolk polymyxin agar base
SDA	Sabouraud Dextrose Agar
AOAC	Association of Official Agricultural Chemists
PCR	Polymerase Chain Reaction
DNA	Deoxy-Ribonucleic Acid
RO	Reverse Osmosis
DGGE	Denaturing Gradient Gel Electrophoresis
PFGE	Pulsed Field Gel Electrophoresis
UV	Ultra Violet
NAA	Naphthalene Acetic Acid
IOOC	International Olive Oil Council
Conc.	Concentration
MR	Methyl Red
VP	Voges – Proskauer
TSI	Triple Sugar Iron
RNA	RiboNucleic Acid
rRNA	ribosomal RNA
Taq	Thermus Aquaticus
dNTP	deoxynucleotide Triphosphate
HCL	HydroChloric Acid
EDTA	Ethylene Diamine Tetra Acetic Acid
IS	Indian Standards
Mg	MilliGram

DM	Dry Matter
IMVIC	Indole, Methyl Red, Voges – proskauer, citrate
NCBI	National Centre for Biotechnology Information
RL	Ringer Lactate solution
IgA	Immuno- Globulin A

CHAPTER 1

INTRODUCTION

1.1 LACTOBACILLUS

Lactobacillus is a genus of gram positive, aero-tolerant anaerobe or microaerophilic, rod shaped, non spore forming bacteria. Lactobacillus species are a significant compound of human and animal microbiota at a number of body sites, such as the digestive system and female genital system. Lactobacillus form biofilms in the vaginal and gut microbiota allowing them to persist during the harsh environmental conditions and maintain an ample population. Lactobacillus exhibits a mutual relationship with the human body, as it protects the host against potential invasion by pathogens, and in return, the host provides a source of nutrients. Lactobacilli are among the most common probiotics found in food such as yogurt, and it is diverse in its application to maintain human well-being, as it can help treat diarrhoea, vaginal infections, and skin disorders such as eczema. Lactobacillus species constitute a significant component of the human and animal microbiota at a number of body sites, such as the digestive system, and the female genital system, Lactobacillus, are normally a major part of the vaginal microbiota. Lactobacillus forms biofilms in the vaginal and gut microbiota. (Credits; Wikipedia)

1.1.1 METABOLISM

Lactobacilli are homofermentative i.e, hexoses are metabolised by glycolysis to produce lactate as a major end product, or heterofermentative i.e, hexoses are metabolised by the phosphoketolase pathway to lactate, CO₂ and acetate or ethanol as a major end products. Most lactobacillus is aero-tolerant and some species respire if heme and menaquinone are present in the growth medium. Aero-tolerance of lactobacillus is manganese-dependent and has been explored

in *Lactobacillus plantarum*. Lactobacilli generally do not require iron content for growth. Traditionally, the genus is split into three groups based on the carbohydrate fermentation pathways: obligate homofermentative, facultative heterofermentative, and obligate heterofermentative lactobacilli. The two most obvious beneficial roles of lactobacilli are as starter cultures (to produce acid rapidly) and as probiotic cultures. Lactobacilli are one of the few contaminant bacteria that are able to grow in cheese after manufacture (as nonstarter lactic acid bacteria (NSLAB)). Lactobacilli as starters and as NSLAB may play different roles in the metabolism of lactose, lactate, and citrate, and in proteolysis and lipolysis, which are considered the primary events during cheese ripening.

1.1.2 PROBIOTIC

Fermentative bacteria like LAB produce H_2O_2 which protects them from oxygen toxicity. The accumulation of H_2O_2 in growth media, and in the antagonistic effect of *Staphylococcus Aureus* and *Pseudomonas*. The probiotic cells treat IBS by returning haemostasis when the gut microbiota experiences unusual high levels of opportunistic bacteria. Lactobacilli can be administered as probiotics during cases of infection by ulcer causing bacterium *Helicobacter pylori*. Lactobacilli with other probiotic organisms in ripened milk and yogurt aided development of immunity in the mucous intestine in humans by raising the number of IgA. (Beijerinck MW, 1901)

1.1.3 INTERACTION WITH PATHOGENS

Lactobacillus produces hydrogen peroxide which inhibits the growth and the virulence of the fungal pathogens *Candida albicans* in vitro and in vivo. In vitro studies show that lactobacilli reduce the pathogenicity *Candida albicans* through the production of organic acid and certain metabolites.

1.1.4 GENOMES

Genomes of lactobacilli are highly variable, ranging in sizes from 1.2 to 4.9 mb (mega bases). Accordingly, the number of protein coding genes ranges from 1267 to about 4758 genes (fruits lactobacillus sanfranciscensis and lentil lactobacillus parakefri, respectively). Even within a single species there can be substantial variation. Lactobacillus contains a wealth of compound microsatellites in the coding region of the genome, which are imperfect and have variant motives. Many lactobacilli also contain multiple plasmids. A recent study has revealed that plasmids encode the genes which are required for adaptation of lactobacilli to the given environment.

1.1.5 FOOD PRODUCTION

In many traditional pickling processes vegetables are submerged in brine and salt tolerant lactobacilli feed on natural sugars found in the vegetables. In the resulting mix of salt and lactic acid is a hostile environment for other microbes such as fungi. Lactobacilli comprise most of the food fermenting LAB and used as starter cultures in industries for controlled fermentation in the production of wine, yogurt, cheese.

1.2 EXOPOLYSACCHARIDE PRODUCING LACTOBACILLUS

EPS are biological polymers secreted by microorganisms including LAB to cope with harsh environmental conditions. It has components which will form an extracellular biofilm matrix to protect microorganisms from factors such as temperature, PH, antibiotics, host immune defences etc.

1.2.1 THE PHYSIOLOGICAL FUNCTIONS OF EPSs

EPS has many contributions to human health such as prebiotic, anti-coagulant, anti-oxidant, anti-inflammatory, anti-viral, cholesterol lowering effects and even anti-cancer activity.

EPS have an essential role in blood coagulation prevention. The strong anticoagulant activity of EPSs in sulphate derivatives has been demonstrated. Heparin cofactor II is a potent inhibitor of thrombin in the coagulation pathway and the sulphated EPS gives an acidic medium to facilitate the inhibitory effect of heparin cofactor II. *L. plantarum* 47FE and *Lactiplantibacillus pentosus* 68FE, respectively, exhibit strong anticoagulant and fibrinolytic activity.

Prebiotic effects were also observed at LAB's EPSs. EPSs from LAB can be used by probiotic strains and have the capacity to stimulate the growth of probiotic bacteria and maintain balance of intestinal microflora. Prebiotic potential of LAB's EPSs has been demonstrated in studies. *L. plantarum* is low digested by artificial gastric juice and shows non-probiotic bacteria off growing Enterobacteriaceae. (Zheng, et al, 2022)

1.2.2 IMPACT OF ENVIRONMENTAL STRESSES ON EPS PRODUCTION IN LAB

Under environmental stresses, LAB have different adaptations mechanisms which involve the accumulation of compatible solutes and the energy storage compounds; regulation of energy production pathways as well as modulation of cell envelope i.e., membrane cell wall surface layers and energy storage compounds; regulation of energy productions, pathways as well as the modulation of cell envelope i.e., membrane, cell wall, surface layers, EPSs.

1.2.3 NUTRIENT STRESS FACTORS

Composition of nutrients is one of the factors which affect the growth and metabolism of cells. EPS synthesis is also influenced by culture medium compositions. The starvation or over supply of nutrients such as nitrogen, sugars, carbon dioxide etc. may change EPS synthesis. Effects of nutritional stress on EPS synthesis in LAB have been proved by prior studies. *Lactobacillus delbrueckii* ssp. *Bulgaricus* recorded increased EPS production in additional nitrogen enriched.

Lactobacillus confusus TISTR 1498 increased sucrose concentration in MRS medium for EPS over production. It yields the highest EPSs when growing on fermentation medium comprising 20% sucrose has carbon source.

In some LAB strains CO₂ can be used as a carbon source for growth because it is a substrate in carbamoyl phosphate synthesis and other metabolic reactions in LAB.

The possible explanations for the increased EPS synthesis under stress of high sugar concentration are osmosis, unlimited supply of sugar building blocks and high energy availability.

1.3 USAGE OF SEA SAND AND SEA WATER

As depletion of agricultural sand and water in this project we are using sea sand and sea water.

1.3.1 PROCESSING OF SEA SAND

The sea sand is first collected then the large and small stones are separated. Then the sea sand is sieved. Small amount of water is added to the sea sand and makes it firm for future cultivation.

1.3.2 PROCESSING OF SEA WATER

The sea water is first treated with potassium alum powder for a day without shaking and then the sea water is filtered using a normal filter to get purified treated sea water. Then the filtered water is autoclaved at 121 degree Celsius for 15 minutes. First the lactobacillus is grown in the MRS agar medium. For selecting the EPS producing lactobacillus the cultured grown from the MRS is swabbed in ESM. After incubating for one day under anaerobic condition the grown culture is inoculated into the processed sea water.

1.3.3 WHY TO USE SEA SAND?

As the sea sand is rich in content of quartz or silicon dioxide, pure sea sand cannot provide good space for water and oxygen movement. Some salt tolerant and xerophytes can be grown in the areas that have a significant proportion of sea sand in composition of soil.

Mulching - mulching is a process of spreading out nutrients over bare soil or covering the ground around plants with organic matter to make soil more fertile.

Plants and vegetables that can be well grown in sea sand-select plants according to the specific conditions in your ecological zone. Cultivating salt tolerant varieties is a good approach to protect seeds, seedling and plants from salt damage. Seeds can be planted in any tray and one month old seedlings can be transplanted to the flower beds or pots. In this way, the plants can tolerate salt stress and will reduce healthy yield.

- Root vegetables give a high yield when grown in well managed beach soils (such as carrot, beetroot).
- Squash, tomatoes, melons and peppers can grow well in sea sand.

- Potatoes can be scab free when grown in sea sand.

1.3.4 WHY TO USE SEA WATER?

Sea water may be used in plant irrigation especially in arid and semi-arid areas which fall under bio-saline agriculture. In coastal areas, sea water farming addresses the severe lack of fresh water and undesirable soil conditions for agricultural activities.

Sea water or saline in general is used in toxicity from excessive uptake of salt substances such as sodium, water stress and reduction in uptake of essential nutrients particularly potassium. Salinity effects extend causing cracks that spread across the surface of the soil.

Smaller and darker leaves than the normal are the earlier signs of plants salinity. It is concluded that usage of sea water in agricultural sector should fill two requirements to be cost effective.

1. It should produce useful crops at yields high enough to justify the expenses of pumping irrigation water from the sea.
2. Researchers should develop agro-economic techniques for growing sea water in a sustainable manner, one that does not damage the environment.

1.4 WHEAT GRASS (R.Chatrath, et al,2016)

Wheat grass is sprouted in the common wheat plant in the first leaf. They are served freeze dried or fresh, and so it differs from wheat malt, which is convectively dried wheat grass that is allowed to grow longer and taller than

wheat malt. Wheat grass contains minerals, vitamins, enzymes, amino acids, and chlorophyll.

1.4.1 CULTIVATION

1.4.1.1 *Indoor cultivation*

Wheat grass can be grown indoors or outdoors. A common method for sprout production indoors is often on trays in a growing medium such as potting mix. Leaves are harvested when they develop a split as another leaf emerges. These can then be cut-off with the scissors and allow a second crop of shoots to form. Sometimes third cutting is possible, but may be tougher and have fewer sugars than the first.

1.4.1.2 *Outdoor cultivation*

Schnabel's research was conducted with wheatgrass grown outdoors in Kansas. His wheatgrass needed a slow growth period of 200 days through the winter and yearly spring, when it was harvested at jointing stage he claimed that at this stage the plant reached its peak nutritional value; after jointing, concentrations of chlorophyll, proteins, and vitamins, declined sharply. Wheatgrass is harvested, freeze-dried, and then sold in tablet and powdered concentrates for human and animal consumption. Indoor- grown wheat grass is used to make wheatgrass juice powder. (R.Chatrath, et al, 2016)

1.4.2 NUTRITIONAL CONTENT

Wheatgrass is the source of potassium, dietary fibre, vitamin A, vitamin C, vitamin E, vitamin K, vitamin B6, iron, zinc, copper, magnesium. It is also a good source of protein; with up to 8 grams per ounce if consumed in powder

form is around 1 gram in a shot of juice. This protein content consists of at least 17 forms of amino acids. (Chatrath, etal, 2016)

1.4.3 FUNGAL DISEASES (Richard.W,2007)

Most wheat diseases caused by root-, crown- and lower culm infecting fungi are not yet efficiently managed by genetic resistance or by application of fungicide or biological control agent. The best management strategy for many of these diseases continues to depend upon changing the soil environment.

TABLE 1.1 – Types of fungal diseases in plants or crops.

<i>Diseases</i>	<i>Principal pathogen(s)</i>	<i>Primary infection site</i>	<i>Primary symptoms</i>	<i>Conditions leading to highest disease severity</i>	<i>Practices that generally minimize crop damage</i>	<i>Efficacy of seed-applied fungicides</i>	<i>Efficacy of genetic resistance</i>
Common root rot	<i>Bipolaris sorokiniana</i>	Stem base, crown, sub crown internode	Lesion on sub crown internode; stunting; white heads	Cultivation; planting wheat annually; drought	Crop rotation; summer fallow; shallow sowing; delay autumn planting date; plant resistance cultivar	Poor to fair	Poor to fair
fusarium crown rot	<i>Fusarium culmorum</i> , <i>F. pseudograminae</i>	Stem base, crown, sub crown internode	Browning of lower leaf sheath; lesions on sub	No-till or minimum tillage; host crops annually or 2-year	Destroy infested crop residue; crop rotation; judicious application of N; delay	Poor; more effective for spring than autumn planting	Poor to fair; currently not widely deployed

			crown internode; dry rot of crown and roots; whiteheads	rotations ; planting winter wheat too early; high N fertility; drought	autumn planting date; plant resistant cultivar; shallow sowing	gs	
Pythium root rot	Pythium ultimum, P. irregular, and others	Seed, roots	Seed rot; damping-off; root rot, stunting; delayed maturity	Cool, wet soil; planting host crops annually ; planting winter wheat too late or spring wheat too early	Plant when soil temperature favour's rapid seed germination; band a starter fertilizer below seed; control green bridge; plant fresh seed	Poor to fair; more effective for spring than autumn plantings	Not available
Rhizoctonia root rot	Rhizoctonia salami AG- 8, R. oryzae	roots	Root rot; stunting; delayed maturity	No-till; planting host crops annually ; planting winter wheat too late or spring wheat too early	Control green bridge ; disruption of seed row at planting; pre- plant tillage; band a starter fertilizer below seed; summer fallow	Poor for wheat planted in autumn; poor to fair for spring wheat	Not available
Take-all	Gaeumannomyces graminis var. tritici, G.	roots	Root rot ; stunting; blackened stems	Planting host crops annually ; planting winter	Crop rotation; control green- bridge; manage form of N	Poor to fair; more effective for spring than	Not available

	<i>graminis</i> var. <i>avenae</i>		base in wet or humid sites; whiteheads	wheat too; major increase in PH after lime application	absorbed by roots; delay autumn planting date	autumn plantings	
Cephalosporium	<i>Cephalosporium graminum</i>	Roots and crown	Long leaf stripes merging into dark veins in leaf sheath; stunting; whiteheads	No-till or minimum tillage; acid soil: host crops annually or 2 year rotations; planting winter wheat too early; freeze/thaw cycles during winter	Crop rotation; plant resistant; winter wheat; plant spring wheat; destroy infested crop residue; lime good acid delay autumn planting	Not effective	Fair to good
Eye spot	<i>Oculimaculaya alluriae</i> O. <i>acutiformis</i>	Stem base	Elliptical lesions on lower culm; plant lodging; whiteheads	Host crops annually; planting winter wheat too early	Plant resistant winter wheat; delay autumn planting date; plant spring wheat; crop rotation; apply foliar fungicide	Fair to good; not necessary for spring plantings	good

1.5 OLIVES

The Olive is known as the oldest cultivated tree in the world (Ozbek 1975). The olive tree grows in a subtropical climate of a traditional main crop in meridian countries. Olive oil is a liquid fat obtained from olives (the fruit of *Olea European*; family *Oleaceae*), a traditional tree crop of the Mediterranean Basin. The oil is produced by pressing whole olives. It is commonly used in cooking, whether for frying or as a salad dressing. It is also used in cosmetics, pharmaceuticals, and soaps, and as a fuel for traditional oil lamps, and has additional uses in some religions. The olive is one of three core food plants in Mediterranean cuisine; the other two are wheat and grapes, Olive oil. (Vitaly Borisenko,1999)

Olive oil is composed mainly of the mixed triglyceride esters of oleic acid and palmitic acid and of other fatty acids, along with traces of squalene (up to 0.7%) and sterols (about 0.2% phytosterol and tocosterols). The composition varies by cultivar, region, altitude, time of harvest, and extraction process.

The composition of olive oil is primarily triacylglycerol (~99%) and secondarily free fatty acids, mono- and diacylglycerols, and an array of lipids such as hydrocarbons, sterols, aliphatic alcohols, tocopherols, and pigments. A plethora of phenolic and volatile compounds are also present. Some of these compounds contribute to the unique character of the oil. Fatty acids present in olive oil are palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3). Myristic (C14:0), heptadecanoic, and eicosanoic acids are found.

Data on physical properties of agro-food materials are valuable because: they are needed as input to models predicting the quality and behaviour of produce in pre-harvest, harvest and post-harvest operations such as cleaning, conveying and storage; they aid the understanding of food processing; they are difficult to acquire. New specialized product specific and rapid

measurement techniques are often needed to be developed. Replacing measurement by prediction is also difficult for most materials which are inhomogeneous and have a complex structure (Nesvadba et al., 2004).

Recently, scientists have made great efforts in evaluating the basic physical properties of agricultural materials and pointed out their practical and control engineering (Baryeh.K 2002 utility in machine and structural design (Amin et al., 2004). Recent scientific developments have improved the handling and processing of bio-materials through mechanical, thermal, electrical, optical, diffusion and other techniques, but little is known about the basic physical characteristics of bio-materials. Such basic information is important not only to engineers but also to food scientists, processors, plant breeders and other scientists who may find new uses (Amin et al, 2004; Nesvadba et al 2004)

1.6 SILAGE

Ensiling is a method of preserving a moist crop. A moist crop can support the growth of a wide range of microorganisms, most of which will degrade the nutrient value to livestock. However, ensiling generally controls microbial activity by a combination of an anaerobic environment and a natural fermentation of sugars by lactic acid bacteria on the crop. This fermentation and the resulting low pH primarily suppress the growth of other anaerobic microorganisms. The fermentation can also inhibit yeasts, molds and aerobic bacteria, but the anaerobic environment is essential to preventing most of the spoilage microorganisms from growing. Inoculants have become the dominant additives for making silage.

Homofermentative strains help guarantee a rapid suppression of anaerobic stains early in storage, increase dry matter recovery and have improved animal performance by means that we do not fully understand. Inoculants containing *Lactobacillus buchneri*, a heterofermentative species capable of fermenting lactic acid to acetic acid, are recent additives. The added acetic acid inhibits

yeast and mold growth, increasing aerobic stability of silages at feeding.(Condon.s,2000)

1.6.1 INTRODUCTION

A moist crop can support the growth of a wide range of microorganisms, most of which will degrade the crop's nutrient value to livestock. However, ensiling generally controls microbial activity by a combination of an anaerobic environment and a natural fermentation of sugars by lactic acid bacteria on the crop. It is important to understand both the microorganisms that are present on the crop at ensiling and how ensiling preserves the crop, inhibiting detrimental microorganisms. Today, there is also a wide range of microbial additives to aid in preservation. It is equally important to know how they alter silage fermentation, preservation, and utilization by livestock. This paper will review these areas briefly.

Silage microbiology -

The crop at ensiling contains both aerobic and anaerobic microorganisms and a range of both bacteria and fungi that affect silage quality. Typical classes of microorganisms and the range of their populations at ensiling are shown in Table 1.2. As would be expected, the dominant populations are aerobic microorganisms or facultative aerobes. Often the lactic acid bacteria that we depend upon to preserve the crop are orders of magnitude lower in population than other groups of microorganisms on the crop at ensiling.

TABLE 1.2 -
Typical populations of bacterial and fungal
groups on plants prior to ensiling (Pahlow et al., 2003)

Group	Population (Colony-forming units/g crop)
Total aerobic bacteria	>10,000,000
Lactic acid bacteria	10 – 1,000,000
Enterobacteria	1000 – 1,000,000
Yeasts and yeast-like fungi	1000 – 100,000
Molds	1000 – 10,000
Clostridia (endospores)	100 – 1,000
Bacilli (endospores)	100 – 1,000
Acetic acid bacteria	100 – 1,000
Propionic acid bacteria	10 – 1,000

1.6.2 LACTIC ACID BACTERIA

The term, lactic acid bacteria, covers bacteria from a number of genres (Lactobacillus, Pediococcus, Lactococcus, Enterococcus, Streptococcus and Leuconostoc) that are found in silage (Pahlow et al., 2003). They all produce lactic acid as their principal product from fermenting sugars, but other products, particularly acetic acid, ethanol and carbon dioxide, are common. They have been commonly grouped into homofermenters and heterofermenters by the products of fermenting glucose. The homofermenters produce 2 moles of lactic acid from one mole of glucose. The heterofermenters produce one mole of Lactic acid, one mole of carbon dioxide and either one mole of ethanol or one mole of acetic acid from glucose. Today, there are three groups of lactic acid

bacteria: obligate homofermenters that are unable to ferment pentoses because they lack phosphoketolase, facultative heterofermenters that ferment hexoses like the obligate homofermenters but are able to ferment pentoses, and obligate heterofermenters that ferment hexoses to a range of products. Most lactic acid bacteria in silage fall into the latter two categories. The lactic acid bacteria generally need various amino acids and vitamins for growth (Pahlow et al., 2003). Yet in spite of their complex requirements, they dominate the fermentation of crops in the silo once anaerobic conditions are established. How exactly this occurs is not understood, but lactic acid bacteria have several mechanisms that might explain their dominance. First, many can grow in aerobic conditions and a common end product of their activity under aerobic conditions is hydrogen peroxide (Condon, 1987), which can kill other microorganisms and in some cases accumulate sufficiently to inhibit the lactic acid bacteria too. Various strains of lactic acid bacteria produce bacteriocins that can inhibit other microorganisms (Gollop, et al., 2005). However, their dominance may not be due to either of these factors. For example, various lactic acid bacterial inoculants do not or are not known to produce but lactic acid bacteria have several mechanisms that might explain their dominance. First, many can grow in aerobic conditions and a common end product of their activity under aerobic conditions is hydrogen peroxide (Condon, 1987), which can kill other microorganisms and in some cases accumulate sufficiently to inhibit the lactic acid bacteria too. Various strains of lactic acid bacteria produce bacteriocins that can inhibit other microorganisms (Gollop, et al., 2005). However, their dominance may not be due to either of these factors. For example, various lactic acid bacterial inoculants do not or are not known to produce bacteriocins (Gollop et al., 2005) and yet are effective in dominating fermentation. So their dominance may only depend on rapid growth under anaerobic conditions.

1.6.3 YEAST

Yeasts are perhaps the most significant aerobic microorganisms on the crop relative to silage quality. Yeasts grow on soluble substrates, sugars and lactic acid being the most important relative to silage. In most circumstances, yeasts are the first group of microorganisms to develop once oxygen comes in contact with silage, either during storage or during feed out. The reason for this is that many types of yeast are capable of growing at pH 3.5, well below the pH of most silage. Acetic acid bacteria and molds are capable of growing under such acidic conditions, but acetic acid bacteria are rarely present and molds grow much more slowly than yeasts. As yeast species that can utilize lactic acid aerobically develop, pH increases in the silage. This opens the way for the growth of other spoilage (aerobic) microorganisms, particularly once pH is above 4.5. Yeasts are also important because some species can grow anaerobically, fermenting sugars to ethanol. When silages have substantial levels of sugars remaining after the lactic acid bacteria are inhibited by low pH, yeasts may develop and are the presumed cause of most high ethanol silages (>20 g/kg dry matter [DM]). Certainly this would be a concern in your sugarcane silages. Some of the fermentative yeasts are active aerobically whereas others grow but slowly. So high ethanol silage can be highly variable in terms of aerobic stability.

1.6.4 MOLDS

Molds are the filamentous fungi present on the crop. These microorganisms are strictly aerobic. By comparison with other microorganisms in silage, they are on average the slowest growers. While they can grow on a wide variety of compounds, they rarely are apparent or at sufficient population to affect gross measures of silage quality until the silage have undergone substantial aerobic deterioration by yeasts and various aerobic bacteria. So their visual presence is an indication of silage that is of considerably lower quality than the crop at ensiling. Molds are also of concern because of their production of mycotoxins. An adequate discussion of mycotoxins could constitute a separate review on its own. Mycotoxins are generally produced under stress conditions for the molds,

and the environmental stressors that initiate mycotoxin production vary widely across species. Mycotoxins are not a serious issue relative to the preservation of the crop in the silo, but are of concern relative to the health of livestock eating the contaminated silage. With very good management, mycotoxin concentrations in silage are generally similar to those present on the crop at ensiling. The development of mycotoxins in the silo is an indication of excessive oxygen exposure.

1.6.5 BACILLI

There are two major groups of bacteria that can be Active aerobically and anaerobically, bacilli and Enterobacteria. Some bacilli can ferment sugars and organic acids in the silo. However, their activity under anaerobic conditions is considered to be of relatively rare importance. Their more significant activity is in advancing the spoilage of silage when exposed to oxygen. After yeasts or acetic acid bacteria have raised pH (approximately 4.5 or above), and temperature has increased modestly (40°C), a second wave of heating usually occurs with temperatures rising to 50°C or higher. This often is the activity of bacilli (Muck & Pitt, 1994), producing a silage that has a slimy feel.

1.6.6 ENTEROBACTERIA

In contrast to the bacilli, the primary effects of the Enterobacteria, facultative anaerobes, on silage come under anaerobic conditions. Various species of Enterobacteriaceae can use nitrate as an electron acceptor in place of oxygen, reducing nitrate to nitrite or nitrogen oxide. These bacteria are the principal source of silo gas (a mixture of various NO_x gases) emanating from silos. Enterobacteria are also the principal competitors of the lactic acid bacteria for the sugars in the crop. Their principal fermentation product is acetic acid, not lactic. Other fermentation products in silage that are signs of their presence are

succinic acid and 2, 3-butanediol. As a consequence, their fermentation is less desirable than that of lactic acid bacteria.

1.6.7 CLOSTRIDIA

Clostridia are obligate anaerobes and their effects on Silage quality usually occurs long after the lactic acid bacteria have stopped actively growing in the silo. The most important silage species are divided into three groups by the principal compounds they ferment: proteolytic clostridium that ferment amino acids primarily, the *Clostridium butyric* group that ferment carbohydrates and *C. tyrobutyricum* that ferments some sugars but more importantly ferments lactic acid. The proteolytic clostridia produce various compounds in catabolizing amino acids; the most significant are ammonia, amines and carbon dioxide. The primary products of the *C. butyric* group and *C. tyrobutyricum* are butyric acid, acetic acid, hydrogen and carbon dioxide. Clostridia activity is undesirable for several reasons. First and foremost is the reduced intake by livestock of silages with evidence of substantial clostridia activity (i.e., butyric acid at > 5 g/kg DM). The cause of the reduced intake is uncertain. It does not appear to be butyric acid, a common acid in the rumen that ruminants routinely taste as they chew their cud. Amines have been associated with reduced intake in small ruminants but not necessarily in cattle. Whatever the cause, the practical realities are that farmers have to reduce the proportion of clostridia silage in a ration as butyric acid increases during storage in order to keep intakes constant. Secondly, lactating dairy cows, particularly those in transition, may be more susceptible to ketosis from the added butyric acid from consuming clostridia silage. Thirdly, the fermentation of lactic acid to butyric acid is one of the most wasteful anaerobic fermentations in silage, producing a 51% loss of DM and 18% loss of gross energy (McDonald et al., 1991).

CHAPTER 2

LITERATURE SURVEY

Isolation and identification of LAB in olive probiotics is studied, role of LAB on table olive and can be used as. LAB detection and identification is done. Despite the fact that the olive industry is perceived to be highly traditional, new identification and typing techniques for LAB are commonly developed and implemented. Fluorescence in situ hybridisation (FISH) has been used with specific probes to assess the presence of members of the *L. plantarum* group (Ercolini et al., 2006). DGGE analyses (Abriouel et al., 2011a).

And various Rep-PCR (De Bellis et al., 2010) have been used to obtain a great diversity of LAB on natural and treated green olives, respectively. PFGE has been used to follow starter species during treated green olives inoculation (Saravanos et al., 2008).

It has been possible also to discriminate between isolates and to distinguish closely related strains by applying an electrophoretic method based on cell-wall protein fingerprinting (Lonigro et al., 2009).

GTG5 Rep-PCR has been used to type strains (Panagou et al., 2008), and to follow starter strains during controlled fermentations (Hurtado et al., 2010).

This technique has also been used, combined with a simplified DNA extraction (Ruiz-Barba et al., 2005), as a fast method to discriminate between numerous isolates from the closely related species *L. plantarum*, *L. pentosus* and *Lactobacillus. Para plant arum* (Hurtado et al., 2011a).

Almost all studies dealing with the identification of microorganisms in fermented table olives have focused on brine analysis and assumed that the nutrients in the olive flow to the brine because of the osmotic gradient. However, various studies have reported that yeast and LAB can adhere to the

olive skin surface and may be able to develop its metabolism there (Nychas et al., 2002; Lavermicocca et al., 2005; Arroyo-López et al., 2008b).

LABS are able to form biofilms, which have very complex polysaccharide-based matrices, enabling them to adhere not only to olive surfaces, but also to industrial equipment. (Albert. 2012).

Isolation and preparation of selective media of Exopolysaccharide producing lactobacillus. The composition of EPS selective medium is 9% skimmed milk powder, 0.35% bacto peptone, 0.35% yeast extract, 1% glucose. LAB plays a critical role in many traditional food fermentations, and they are responsible for important characteristics such as flavour, texture, and shelf life. (Dick.1993)

The authors describe the different methods used for isolating and screening LAB, including selective media and physiological tests. They also discuss the identification of LAB using both classical and molecular techniques. The paper concludes with a discussion of the potential applications of LAB in food biotechnology, including the production of starter cultures, probiotics, and functional foods. (Dick.1993).

Found major growth factors in exopolysaccharide producing lactobacillus. So the yield will increase. Olive oils are highly important due to the presence of mono-unsaturated. They have low content of poly-unsaturated fatty acids. EPS are polymers produced by some LAB strains that can contribute to the texture, viscosity, and stability of fermented foods.

The authors discuss the methods used for EPS extraction, purification, and characterization, as well as their technological properties such as emulsifying, gelling, and water-holding capacities. They also review the biological activity of EPS, including their potential prebiotic, immunomodulatory, and anti-carcinogenic effects. The paper concludes with a discussion of the potential applications of *L. plantarum* EPS in the food industry, including as a functional

ingredient, a bio-preservative, and a starter culture for fermented foods. (Liliane. 2019).

LAB has many factors like heavy metal removal, many advantages such as decomposing site, agro ecosystem. LAB and other bacillus based bio-fertilizers have been validated with established microbes in agriculture. LAB have been widely used as probiotics in human and animal health, but they also have potential applications in agriculture due to their ability to improve soil health, promote plant growth, and protect crops from pathogens. The authors discuss the different mechanisms by which LAB can benefit plants, including nutrient cycling, production of growth hormones and enzymes, and induction of systemic resistance. They also review the challenges and limitations of using LAB in agriculture, such as variability in effectiveness, compatibility with other agricultural inputs, and regulatory issues. The paper concludes with a discussion of the potential future directions for research on the application of LAB in sustainable agriculture (Jegadeesh. 2022).

Salt tolerant lines that can tolerate soil pH up to 8.5 and exchangeable sodium percentage around 15% could be useful alternative. The authors discuss the various biotic and abiotic stresses that affect wheat production, including diseases, pests, water stress, and soil fertility. They also examine the socio-economic factors that impact wheat production, such as labour availability, market infrastructure, and government policies. The paper concludes with a discussion of the potential strategies for addressing these challenges, including the use of improved crop varieties, integrated pest management, conservation agriculture, and public-private partnerships. (R. Chatrath, 2007).

The growth of wheat has several fungal Diseases. When growing it in a sea sand which Salinity and inhibits fungal growth. Salinity is a major abiotic stress that affects the growth and survival of fungi in many environments. The authors discuss the different mechanisms by which salinity affects fungal physiology, including osmotic stress, ion toxicity, and oxidative stress. They also examine the ways in which fungi have adapted to saline environments, such as by

producing compatible solutes, adjusting cell wall composition, and modulating gene expression. The paper concludes with a discussion of the potential applications of fungi in saline environments, such as bioremediation, biocontrol, and bioprospecting for novel enzymes and bioactive compounds. (Boualem, 2022).

Most of these diseases are not yet effectively managed by genetic resistance, fungicides, or biological agents. Optimal disease management generally requires changing the soil environment to reduce survival of the pathogens between susceptible host crops. Diseases are caused root-, crown- and lower culm-infecting fungi. The authors discuss the symptoms, causal agents, and epidemiology of these diseases, as well as the cultural and genetic management strategies that can be used to mitigate their impact. They also highlight the importance of integrated disease management approaches that combine multiple tactics for effective disease control. (Richard w, 2016).

The paper contains complex methods such as Reverse osmosis (ro), harsh chemicals for treating the water which can have many side effects. We found a new method for processing which is easy and chemical free. Despite the fact that the olive industry is perceived to be highly traditional, new identification and typing techniques for LAB are commonly developed and implemented. Fluorescence in situ hybridisation (FISH) has been used with specific probes to assess the presence of members of the *L. plantarum* group (Ercolini et al., 2006). DGGE analyses (Abriouel et al., 2011a) and various Rep-PCR (De Bellis et al., 2010) have been used to obtain a great diversity of LAB on natural and treated green olives, respectively. PFGE has been used to follow starter species during treated green olives inoculation (Saravanos et al., 2008).

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This technique has also been used, combined with a simplified DNA extraction (Ruiz-Barba et al., 2005), as a fast method to discriminate between numerous isolates from the closely related species *L. plantarum*, *L. pentosus* and *Lactobacillus Para plantarum* (Hurtado et al., 2011a).

Almost all studies dealing with the identification of microorganisms in fermented table olives have focused on brine analysis and assumed that the nutrients in the olive flow to the brine because of the osmotic gradient. However, various studies have reported that yeast and LAB can adhere to the olive skin surface and may be able to develop its metabolism there (Nychas et al., 2002; Lavermicocca et al., 2005; Arroyo-López et al., 2008b). V. Martinez. 2015.

Using olives as a sample for inoculation. They have growth regulators such as acid profile, Peroxide value (PV), Iodine value (IV), Thiobarbituric acid (TBA). This paper investigates the effect of growth regulators on the physicochemical properties of olive oil extracted from treated olive fruits. The authors describe the different growth regulators used in the study, including cytokinins, gibberellins, and auxins, and discuss their potential impact on olive fruit quality and oil yield. They also present the results of experiments conducted to measure the physicochemical properties of the extracted olive oil, such as acidity, peroxide value, and UV absorbance. The paper concludes with a discussion of the potential applications of growth regulators in olive oil production, as well as the need for further research in this area to optimize the use of these substances.

- Physical properties
- Refractive index:

Refractive index of olive oil extracted from Manzanillo olive fruits treated by GA3 and NAA growth regulators estimated using Carl Zeiss Refractometer,

and the obtained results expressed at 25°C according to the method described by the A.O.A.C., (2005).

- Colour:

Colour of all the oil tested samples was determined by a Lovibond tintometer using three colour scales (yellow, red and blue) in a 5.25 inch cell. These analytical methods were carried out according to the methods described by (A.O.A.C., 2005).

- Specific extinction coefficients:

The specific extinction coefficients K₂₃₂ and K₂₇₀ absorption at 232 and 270 nm (1 cm path length) of a 1% (w/v) solution of oil in cyclohexane was measured using a Beckman DU 640 UV spectrophotometer (Beckman, Fullerton, CA) (EEC 1995).

- Induction period (stability test):

The induction periods, as the oxidative stability index, of the tested samples were measured by an automated Rancimat (Metrohm Ltd. CH-9100 Herisau, Switzerland, model 679), comprises the control unit and the wet section containing 6 reaction vessels, according to the method described by (Mendez et al., (1996).

- Chemical properties:

Chemical composition of Manzanillo olive fruits:

Moisture, Lipid, crude protein, fibre and ash were determined according to A.O.A.C., (2005); Total carbohydrates were calculated by difference. % Total carbohydrates = 100 - (% moisture + % crude protein + % fat + % ash).

Chemical properties of olive oil:

Free fatty acid (F.F.A) (as % Oleic acid); Peroxide value (meq. active O₂/kg); Iodine value (g I₂/100 g oil) (measured according to the procedure of Hannes

method); TBA value (as mg malonaldehyde/kg) and unsaponifiable matter (%) were determined according to the procedure of A.O.A.C., (2005).

Total phenol compounds:

Total phenol compounds were isolated by extraction of a solution of oil in hexane, three times, with a water/methanol mixture (60:40). Folin-Ciocalteu reagent and sodium molybdate, 5% in 50% ethanol (Merck), were added to a suitable aliquot of the combined extracts and the absorbance of the solution at 725 nm were measured. Values were given as mg of Gallic acid per kg of oil (Gut finger, 1981 and Vazquez et al., 1973).

Chlorophyll and Carotene contents:

Chlorophyll and carotenoid compounds (mg/ Kg) were determined at wavelength of 670 nm and 472 nm, respectively, in cyclohexane using the specific extinction values, by the method of (Minguez Mosquera et al.,1991).

Fatty acids composition of the oil:

The fatty acid composition of the tested oil samples were determined by gas liquid chromatography according to the method described by International Olive Oil Council IOOC., (1996). (Hamid, 2015)

Wheat grass can be used for dunes so this also prevents soil erosion. Permanent control of active sand dunes can be accomplished only by establishing on them a vegetative cover, either by natural succession or by seeding. Mechanical structures stop sand movement only temporarily. Grasses have a major role in the fixation of sand dunes, but only a few grasses are widespread and important on these sandy areas. For example, on the Pacific and Atlantic coasts and on the shore. (Charles J,1948)

CHAPTER 3

AIM AND SCOPE OF THE PRESENT INVESTIGATION

Gene level identification of lactobacillus using molecular techniques will be basics to know about its biological activities. Our target is to investigate nutritional analysis of wheat grass in sea sand and lactobacillus treated sea water.

1. To isolate lactobacillus strain from olive brine solution (green olive).
2. To isolate EPS producing lactobacillus strains, from the streaked culture of lactobacillus in MRS agar from olive brine solution.
3. To grow wheatgrass in processed sea sand and desalinated sea water.
4. To analyze the Nutritional composition of wheat grass.
5. To isolate the bacteria and to study the microbiology of wheat grass silage.
6. To eradicate soil erosion related problems, processed sea sand is used as an alternative for normal agricultural soil.
7. To eradicate water scarcity and water contamination related problems, desalinated sea water is used as an alternative for fresh water.

CHAPTER 4

MATERIALS AND METHODS

4.1 SAMPLE COLLECTION

Olive brine solution samples were collected from a nearby supermarket on JANUARY 2023. Samples are collected in a sterile glass jar. Physical parameters were determined. After collection they are stored in the laboratory refrigerator at 4°C.

Sea sand and sea water were collected from KALPAKKAM TOWNSHIP beach for further cultivation.

Wheat seeds were collected from farms near KALPAKKAM Lat 12.509194° Long 80.161652° On JANUARY 2023. Samples were stored at normal room temperature.

4.2 MEDIA PREPARATION

MRS agar is prepared for isolation of Lactobacillus for 100 ml of sterile water by adding it with 0.65 grams of MRS agar. Then sterilizing by wrapping the top of the conical flask with newspaper and sterilizing the media using an autoclave by heating it at 121°C. At 15 lbs for 15 to 20 mins.

EPS selective medium is prepared for 100 ml of sterile water with a composition of 9% skimmed milk powder, 0.35% Yeast extract, 0.35% Bacto peptone and 1% glucose. Then it is autoclaved and used for cultivation.

4.3 ISOLATION OF LACTOBACILLUS FROM OLIVE BRINE SOLUTION

For isolation of lactobacillus from olive brine solution MRS (de man Rogosa Sharpe) agar is used as a selective medium for lactobacillus isolation. Olive brine is streaked on mrs agar and incubated at 37°C for 24 hours at anaerobic condition. Colonies isolated after 24 to 48 hours showed lactobacillus species growth and these species for 24 hours. After the period of incubation the specific isolated colonies were grown. The isolated colonies formed were identified using gram staining, biochemical tests. The identification was performed according to Bergey's manual of determinants of bacteriology. The culture was kept in Mrs agar slant and stored at 4°C for long term storage.

4.4 ANTIBIOTIC SUSCEPTIBILITY ASSAY AND BIOCHEMICAL CHARACTERIZATION OF THE ISOLATED BACTERIAL STRAIN

Identification of the isolated bacteria as Lactobacillus species was performed according to their morphological, cultural, and physiological and biochemical characteristics by the procedures as described in Bergey's Manual of Systematic Bacteriology. The tests carried out were Gram staining, Capsule staining motility test, production of catalase, Indole, Methyl Red, Voges-Proskauer, Citrate, Starch Hydrolysis, endospore test, milk coagulation activities and NaCl and phenol tolerance test. Tolerance of NACL and Phenol Observing the tolerance of NaCl of the culture of the 1% fresh one night culture of the bacteria incubate into MRS broth with 4% NaCl Conc for 24 hours and then observe their turbidity. Similar experiments were performed using 0.4% phenol as an inhibitory substance.

4.4.1 ANTIBIOTIC SUSCEPTIBILITY ASSAY

The Antibiotic Susceptibility was performed by Disk diffusion method. The isolated bacterial samples were spread in the MRS Agar plate. 30µg concentration Ampicillin, Tetracycline and Azithromycin are used for this experiment.

4.4.2 IMVIC TEST

Methods from Bergey's Manual of Systematic bacteriology were used for the biochemical identification of the isolates.

4.4.2.1 INDOLE TEST

Indole production test was done to determine the ability of microorganisms to degrade the amino acid tryptophan by the enzyme tryptophanase.

- For the indole test each indole broth containing 6 ml of peptone, sodium chloride was taken.
- Using sterile technique, small amount of the experimental bacteria from fresh culture was inoculated into the tubes by means of loop inoculation method with an inoculating loop
- The tubes were then incubated for 24 hours at 37°C.
- In order to detect the indole production, 10 drops of Kovacs reagent was added to all the tubes.
- If a red reagent layer develops then it indicates indole positive and absence of red colour indicates that the substrate tryptophan was not hydrolysed and it indicates indole negative reaction. (Cappuccino & Sherman, 2005)

4.4.2.2 METHYL RED TEST

Methyl red test was done to determine the ability of the bacteria to oxidize glucose with the production and stabilization of high concentration of acid end products.

- For methyl red test each MR broth containing 5 ml of di-peptone, dextrose and potassium phosphate was taken.
- Using sterile technique, each tube was inoculated by fresh culture of experimental bacteria by means of loop inoculation method.
- The tubes were then incubated for 48 hours at 37°C.
- After 48 hours, 5 drops of methyl red indicator were added to each tube and the colour of the tubes was observed.
- If red colour develops then it indicates that the organism was capable of fermenting glucose with the production of high concentration of acid.
- If orange or yellow colour develops then it indicates methyl red negative result (Cappuccino & Sherman, 2005).

4.4.2.3 VOGES-PROSKAUER (VP) TEST

The Voges-Proskauer (VP) test was done to determine if an organism produces acetyl methyl carbinol from glucose fermentation.

- For the Voges-Proskauer test each VP broth containing di-peptone, dextrose and potassium phosphate was taken.
- Using sterile technique, each tube was inoculated by fresh culture of experimental bacteria by means of loop inoculation method.
- The tubes were then incubated for 48 hours at 37°C.
- After 48 hours, 10 drops of Barritt's reagent A was added to each tube and the tubes were shaken. Then immediately 10 drops of Barritt's reagent B was added and the tubes were shaken.

- The colour was observed after 15-30 minutes of the reagent addition.
- If red colour developed then it indicates that the organism was capable of fermenting glucose with ultimate production of acetyl methyl carbinol and it indicates positive result.
- If no colour developed then it indicates voges- proskauer negative result. (Cappuccino & Sherman, 2005)

4.4.2.4 CITRATE UTILIZATION TEST

Citrate utilization test was done to differentiate among enteric organisms on the basis of their ability to ferment citrate as a sole source of carbon by the enzyme citrase.

- For citrate utilization test each vial containing 2.5 ml of Simmons citrate agar was taken.
- Using sterile technique, a small amount of the experimental bacteria from 24-hours fresh culture was inoculated into the vials by means of a streak inoculation method with an inoculating loop.
- The vials were then incubated at 37°C for 24-48 hours.
- After 48 hours incubation, if the Prussian blue colour developed then it indicates the citrate positive result which means the organism was capable of fermenting citrate as a sole source of carbon.
- If there was no colour change then it indicates a negative citrate result.

4.4.2.5 TRIPLE SUGAR-IRON (TSI) AGAR TEST

Triple sugar iron agar test was done to differentiate between Gram negative enteric bacilli based on their ability to ferment carbohydrate and reduce hydrogen sulphide.

- For the TSI test each tube containing TSI agar was taken.

- Using sterile technique, a small amount of the experimental bacteria from fresh culture was inoculated into the tubes by means of stab inoculation method with an inoculating needle.
- The tubes were then incubated at 37°C for 24-48 hours.
- After 24-48 hours the colour of both the butt and slant of agar slant cultures were observed.

4.5 CATALASE TEST

The differentiation of bacteria that produce the enzyme catalase from non-catalase producers is achieved using this test. Catalase acts as a catalyst in the breaking down of hydrogen peroxide to Oxygen and water, two to three ml of 3% hydrogen peroxide solution was poured into a test tube. A 24 hour culture of the test organism from the nutrient agar was emulsified in the hydrogen peroxide solution. The release of bubbles immediately indicated a positive test while it was negative when no bubble was formed.

4.6 OXIDASE TEST

The oxidase test detects the presence of a cytochrome oxidase system that will catalyse the transport of electrons between electron donors in the bacteria and a redox dye- tetramethyl-p-phenylene-diamine. The dye is reduced to a deep purple colour. Place the overnight grown culture on the oxidase disc using a sterile swab or tooth picker. Especially the oxidase discs should not be exposed to the sunlight. Then wait for the change of colour into purple if the microorganism is oxidase positive otherwise it is oxidase negative.

4.7 GRAM STAINING

Gram staining:

- Primary stain as crystal violet
- Mordant as iodine
- Decolouring agent as ethyl alcohol
- Counterstain as safranin

4.7.1 PROCEDURE

- One clean glass slide was taken
- Smear was air dried and heat fixed and flooded with crystal violet for 1 min.
- Gently washed with tap water.
- Decolourized with 95% ethyl alcohol reagent. It was added drop by drop until no further violet colour came out and gently washed with tap water.
- Counterstained with safranin for 45 seconds.
- Gently washed with tap water. It was dried with bibulous paper and examined under oil immersion.

It helps to differentiate between Gram-positive and Gram-negative organisms. While simultaneously helping to know about cellular morphology and arrangement.

4.8 BACTERIAL IDENTIFICATION BY 16s rRNA SEQUENCING

4.8.1 METHODOLOGY

Genomic DNA isolation: DNA isolation from Microbial samples was done using the Biobee Spin EXpire Microbial DNA isolation kit developed by Bogar Bio Bee stores Pvt Ltd.

4.8.2 PROTOCOL

1. **Lysis/homogenization:** Cells grown in monolayer should be lysed by suspending 1-3 colonies aseptically and mixed with 500 µl of lysis buffer in a 2 ml micro centrifuge tube and lyse the cells by repeated pipetting.
2. Add 4 µl of RNase and 500 µl of neutralization buffer into it.
3. Vortex the content and incubate the tubes for 30 minutes at 65°C in a water bath. To minimize shearing the DNA molecules, mix DNA solutions by inversion.
4. Centrifuge the tubes for 10 minutes at 10,000 rpm.
5. Following centrifugation, transfer the resulting viscous supernatant into a fresh 2 ml microcentrifuge tube without disturbing the pellet.
6. Add 600 µl of Chloroform Isoamyl Alcohol and do hand mixing vigorously.
7. Centrifuge the tubes for 10 minutes at 10,000 rpm. Carefully, transfer 600 µl of aqueous phase into a fresh 2 ml microcentrifuge tube.

8. Binding: Add 600 μ l of binding buffer to the content and mix thoroughly by pipetting and incubating the content at room temperature for 5 minutes.
9. Transfer 600 μ l of the contents to a spin column placed in a 2 ml collection tube.
10. Centrifuge for 2 minutes at 10,000 rpm and discard flow-through.
11. Reassemble the spin column and the collection tube then transfer the remaining 600 μ l of the lysate.
12. Centrifuge for 2 minutes at 10,000 rpm and discard flow-through.
13. **Washing:** Add 500 μ l washing buffer I to the spin column. Centrifuge at 10,000 rpm for 2 mins and discard flow- through
14. Reassemble the spin column and add 500 μ l washing buffer II and Centrifuge at 10,000 rpm for 2mins and discard flow-through. Dry spin the tube for 5 minutes at 10,000 rpm.
15. Transfer the spin column to a sterile 1.5-ml microcentrifuge tube.
16. **Elution:** Add 100 μ l of Elution buffer at the middle of the spin column. Care should be taken to avoid touch with the filtrate.
17. Incubate the tubes for 2 minutes at room temperature and Centrifuge at 10,000 rpm for 2 minutes. The buffer in the micro centrifuge tube contains the DNA.
18. DNA concentrations were measured by Qubit fluorometer 3.0 or 1% Agarose Gel Electrophoresis.

PCR Protocol Polymerase Chain Reaction (PCR) is a process that uses primers to amplify specific cloned or genomic DNA sequences with the help of a very unique enzyme. PCR uses the enzyme DNA polymerase that directs the synthesis of DNA from deoxy nucleotide substrates on a single stranded DNA template. DNA polymerase adds nucleotides to the 3` end of a custom-designed oligonucleotide when it is annealed to a longer template DNA. Thus, if a synthetic oligonucleotide is annealed to a single-stranded template that contains a region complementary to the oligonucleotide, DNA polymerase can use the oligonucleotide as a primer and elongate its 3` end to generate an extended region of double stranded DNA.

Composition of the Taq Master Mix

Taq DNA polymerase is supplied in 2X Taq buffer

- 0.4mM dNTPs,
- 3.2mM MgCl₂ and
- 0.02% bromophenol blue.

4.8.3 PRIMER DETAILS

TABLE 4.1 – Primer details used for GENOME SEQUENCING.

PRIMER NAME	SEQUENCE_DETAILS	NUMBER OF BASE
27F	5' AGAGTTTGATCCTGGCTCAG 3'	20
1492R	5' GGTTACCTTGTTACGACTT 3'	20

Add 5 µl of isolated DNA in 25 µl of PCR reaction solution (1.5 µl of Forward Primer and Reverse Primer, 5 µl of deionized water, and 12 µl of Taq Master Mix).

Perform PCR using the following thermal cycling conditions.

1. Denaturation the DNA template is heated to 95°C. This breaks the weak hydrogen bonds that hold DNA strands together in a helix, allowing the strands to separate creating single stranded DNA.
2. Annealing the mixture is cooled to anywhere from 55°C. This allows the primers to bind (anneal) to their complementary sequence in the template DNA.
3. Extension The reaction is then heated to 72° C, the optimal temperature for DNA polymerase to act. DNA polymerase extends the primers, adding nucleotides onto the primer in a sequential manner, using the target DNA as a template.

TABLE 4.2 – Different conditions and their TIME, TEMPERATURE DURING PCR

STAGES	TEMPERATURE	TIME	25 cycles
Initial Denaturation	95°C	2 min	
Denaturation	95°C	30 sec	
Annealing	50°C	30 sec	
Extension	72°C	2 min	
Final extension	72°C	10 min	
Hold	4°C	∞	

4.8.4 PURIFICATION OF PCR PRODUCTION

Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore). The PCR product was sequenced using the primers. Sequencing reactions were performed using an ABI PRISM® BigDye™ Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

4.8.4.1 SEQUENCING PROTOCOL

Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labelled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were re-suspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

4.8.4.2 BIOINFORMATICS PROTOCOL

1. The 16s rRNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of query sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment.
2. The program MUSCLE was used for multiple alignments of sequences (Edgar 2004). The resulting aligned sequences were cured using the program Gblocks 0.91b. This Gblocks eliminates poorly aligned positions and divergent regions (removes alignment noise) (Talavera and Castresana 2007). Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as a Substitution model.

3. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. The program MEGAX Software was used for tree rendering.

4.9 DETERMINATION OF NUTRIENTS IN WHEAT GRASS JUICE

4.9.1 PHOSPHORUS

- Phosphorus is one of the major plant nutrients in the soil. It is a constituent of plant cells, essential for cell division and development of the growing tip of the plant. For this reason it is vital for seedlings and young plants.

4.9.1.1 Scope

This standard operating procedure describes a method for the determination of Phosphorus in dairy and dairy products.

4.9.1.2 Apparatus

- UV spectrophotometer.
- Volumetric flasks.
- Pipettes

4.9.1.3 Reagents and Chemicals

- Molybdovanadate reagent: Dissolve 4 g ammonium Molybdate in 40 ml hot H₂O and cool. Dissolve 0.2 g ammonium metavanadate in 25 ml hot H₂O and cool; add 25 ml Conc. Nitric acid. Gradually add Molybdate solution to vanadate solution with stirring, and dilute to 200ml.
- Phosphorus standard solutions: (1) Stock solution - 2 mg P/ml. Dissolve 0.878 g KH₂PO₄ in H₂O and dilute to 100ml. (2) Working solution - 0.1 mg P/ml. Dilute 5 ml stock solution to 100ml.
- Hydrochloric acid: (1:1) solution
- Concentrated Nitric acid

4.9.1.4 Procedure

Preparation of Standard Curve

- Transfer aliquots of working standard solution containing 1.0, 2.0, 3.0, 4.0 and 5.0 ppm Phosphorus to 100 ml volumetric flasks.
- Add 20 mL Molybdovanadate reagent, dilute to volume with H₂O, and mix well. Let stand for 10 min; then read absorbance at 400 nm at absorbance.
- Prepare a standard curve by plotting Phosphorus (Concentration) against Absorbance.

DETERMINATION

- Ash 2 g sample, in silica crucible at 550°C for 4 hours. Cool, and add 5 ml (1:1) HCl and several drops conc. HNO₃, and bring to boiling.
- Cool, transfer to 250 ml volumetric flask, and dilute to volume with H₂O. Filter, and pipette out 10 ml of aliquot to 100 ml volumetric flask.

- Add 20 ml molybdovanadate reagent, dilute to volume with H₂O, and mix well. Let stand for 10 min, and then read absorbance at 400 nm.
- Determine Phosphorus (Concentration) from the standard curve.

4.9.1.5 Calculation

Phosphorus content (in ppm) =

$$\frac{\text{Abs sample} \times \text{Std concentration} \times \text{Dilution factor}}{\text{Abs}_{\text{std}} \times \text{Weight of sample}}$$

Conversion factor of PO₄ = P X 3.065

P₂O₅ = P X 2.291

Na₂HPO₄ = P X 4.583

4.9.2 CALCIUM AND MAGNESIUM

4.9.2.1 Scope

This standard prescribes the methods for the volumetric determination of calcium and magnesium using ethylene diamine tetra-acetic Acid (EDTA).

NOTE - These methods do not cover analysis of metals where limits for various interfering elements and/or radicals are prescribed.

4.9.2.2 References

TABLE 4.3 - The Indian Standards listed below are the necessary adjuncts to this standard:

IS No.	TITLE
1070 : 1977	Specification for water for general laboratory use (second revision)
4285 : 1967	Method for volumetric determination of calcium

4.9.2.3 Quality of Reagents

- Unless specified otherwise, pure chemicals and distilled water (see IS 1070: 1977) shall be used in tests.

NOTE -'Pure chemicals' shall mean chemicals that do not contain impurities which affect the result of analysis.

4.9.2.4 Reagents

Patton and Reeder's Indicator (P and R Indicator)

1 Percent (m/v) Mix 1.0 g of solid indicator (2 hydroxyl - 1 - (2 hydroxy - 4 - sulpho - 1 - naphthylazo) - 3 - naphthoic acid - (HHSNNA)] with 99 g of pure sodium chloride or sodium sulphate anhydrous in a mortar and pestle and grind well to about 250 to 150 micron, mixing it intimately so as to make the diluted reagent powder homogeneous. Preserve in a tightly stoppered amber-coloured bottle.

Eriochrome Black T Indicator (EBT Indicator)

Dissolve 0.5 g of EBT indicator and approximately 4.5 g of hydroxylamine hydrochloride in 100 ml of rectified spirit (ethyl alcohol or ~methyl alcohol).

Standard Zinc Solution, 0.01 M

Dissolve 0.6538 g of pure zinc dust or granules of 99.9 percent purity in 20 ml of approximately 1:1 hydrochloric acid, warm if necessary. Cool and make up to 1 litre exactly in a volumetric flask. Preserve this solution in a tightly closed glass bottle.

Buffer Solution

Dissolve 70 g of ammonium chloride and 570 ml of 30 percent ammonia solution (relative density 0.88 - 0.90) in water and make up to 1 litre.

Standard Ethylene Diamine Tetra acetic

Acid (EDTA) Solution - (0.01 M) Dissolve 3.75 g of disodium ethylene diamine tetraacetate dihydrate in water and make up to 1 litre in a volumetric flask. Standardize this with a standard zinc solution. Pipette out 25 ml of standard zinc solution in a 250 ml conical flask. Adjust the OH to approximately 10 with buffer solution. Dilute to about 100 ml and add 3 to 4 drops of EBT indicator solution. This will give a red colour. Titrate with 0.01 M EDTA solution to a clear blue end

point free from violet tinge. This solution will be slightly stronger than 0.01 M. Dilute the solution to exactly 0.01 M by adding a calculated amount of water and recheck the strength by titrating 25 ml of standard zinc solution in exactly the same manner as given above. This should consume exactly 25.0 ml of standard EDTA solution.

Sodium Hydroxide Solution, 20 percent (m/v)

NOTE - Preserve in a polyethylene bottle.

Triethanolamine Solution, 10 percent (m/v)

Potassium Cyanide Solution, 10 percent (m/v)

NOTE - Preserve in a polyethylene bottle.

Hydroxylamine Hydrochloride Solution, 10 percent m/v

Magnesium EDTA Complex Solution

Dissolve 2.465 g of pure magnesium sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) in 300 to 400 ml of water. Add to this 3.72 g of sodium EDTA solid. Dissolve and adjust pH to 9.5 to 10. Add 8 to 10 drops of EBT indicator solution. The colour should be pink. Titrate this with 0.01 M EDTA solution till the colour changes to pure blue free from violet tinge. Dilute this to exactly 1 litre in a volumetric flask.

4.9.2.5 Determination of Calcium

4.9.2.5.1 Procedure

Take a suitable aliquot of the sample solution prepared as prescribed in the material specification and expected to contain approximately 5 to 20 mg of

calcium as CaO, in a 500 ml conical flask, Add 25 ml of triethanolamine solution, 10 ml of hydroxylamine hydrochloride solution and 2 ml of potassium cyanide solution. Dilute to about 150 to 200 ml with water and adjust the OH to 12.5 to 13 with 20 percent sodium hydroxide solution (4.6) using a PH indicator paper or preferably a PH meter. Add approximately 0.1 g of Patton and Reeder's indicator mixture and stir to dissolve. Titrate with 0.01 M EDTA solution from a burette, till the colour changes from red to pure blue free from any violet tinge. Towards the end point, the colour turns a violet which on further dropwise addition gives the pure blue end point. It is easier to note the end point by comparing the colour with that of a previously titrated solution having a pure blue end point, where a slight excess of EDTA has been added.

NOTE-Where the solution containing calcium and magnesium contains ammonium salts, that is, in minerals analysis, the amount of sodium hydroxide solution required to bring the OH to 12.5 to 13 will be more. It is important that OH must be brought to 12.5 to 13 as otherwise all magnesium will not precipitate, leading to higher titration reading.

4.9.2.5.2 Calculation

Calcium (Ca) percent by mass = $V_1 \times 0.04008$ divided by M

Where

V_1 = volume in ml of EDTX solution consumed in titration; and

M = mass in g of the sample in the solution taken for the test.

4.9.2.6 Determination of Magnesium

4.9.2.6.1 Procedure

Take a suitable aliquot of the solution of the material exactly the same as under 5.1, expected to contain approximately 10 to 30 mg of calcium and magnesium

combined oxides in a 500 ml conical flask. Add 10 ml of hydroxylamine hydrochloride solution, 2 ml of potassium cyanide solution and 25 ml of triethanolamine solution. Dilute to 150 to 200 ml and add sufficient quantity of buffer solution to bring the pH to 9.5 to 10. Add 3 to 4 drops of EBT indicator solution and titrate with 0.01 M EDTA solution till the red colour changes to pure blue end point free from violet tinge. It is easier to note the end point by comparing the colour with that of previously titrated solution having a pure blue end point, where a slight excess of EDTA solution has been added. NOTE-In case magnesium is not present; add 5 ml of magnesium EDTA complex solution in order to get the proper end point. This should be added before titration.

4.9.2.6.2 Calculation

Magnesium (as Mg), percent by mass = $0.0243 \times (V_2 - V_1)$ divided by M

Where

V₂ = volume in ml of EDTA solution consumed in titration;

V₁ = volume in ml of EDTA consumed in titration for calcium ~determination, in the same aliquot of solution of sample; and

M = mass in g of the sample taken for the test.

4.9.3 SODIUM AND POTASSIUM

4.9.3.1 Scope

This standard prescribes methods for the estimation of sodium and potassium by flame photometer.

4.9.3.2 Quality of reagents

Unless specified otherwise, pure chemicals and distilled water (IW IS: 1070-1977*) shall be employed in tests.

NOTE - 'Pure chemicals' shall mean chemicals that do not contain impurities which affect the results of analysis.

4.9.3.3 Outline of the methods

The material is powdered and homogenized so as to pass through 150 micron IS Sieve. A known mass of the air dried sample is suitably decomposed in an acid mixture, filtered and diluted to a definite volume. This test solution or its suitable dilutions are then aspirated in the flame photometer.

From the galvanometer reading the concentration of sodium or potassium can be obtained by referring to a calibration curve. Ground water sample or its suitable dilution can be directly aspirated and alkali metals determined.

4.9.3.4 Apparatus

Flame Photometer

4.9.3.5 Reagents

1. Concentrated Hydrochloric Acid -See IS : 265-19767.
2. Concentrated Nitric Acid - See IS: 264-1976%.
3. Hydrofluoric Acid
4. Potassium Chloride
5. Sodium Chloride

4.9.3.6 Procedure

1. Preparation of Standard Sodium Solution - Weigh accurately 2.5422 g of sodium chloride previously dried at 120°C to constant mass and cooled to room

temperature in a desiccator over silica gel. Transfer the weighed salt with water to a 250 ml beaker, dissolve in about 100 ml of water and add 10 ml of concentrated hydrochloric acid. Transfer the solution quantitatively to one-litre one-mark volumetric flask and dilute to the mark. Mix well and store in a polyethylene bottle. This solution contains 1 mg/ml of sodium.

1.1 Preparation of the Standard Matching Solutions - Into a 100 ml volumetric flask take 10 ml of the standard solution (set 6.1), add 2 ml concentrated hydrochloric acid and make up the volume. This solution will contain 100 ug/ml of sodium. Dilute 1, 2, 3, 4 and 5 ml of this Number these flasks solution to 100 ml in 100-ml volumetric flasks. As 1,2,3,4 and 5 respectively. The strength of these solutions shall be as given in Table 4.4. Store these solutions in polyethylene bottles.

TABLE 4.4 - STRENGTH OF SODIUM SOLUTIONS FOR CALIBRATION

Number of solution	Volume of standard solution (ml)	Quantity of sodium (ug/ml)
(1)	(2)	(3)
1	1.0	1.0
2	2.0	2.0
3	3.0	3.0
4	4.0	4.0
5	5.0	5.0

1. Preparation of Standard Potassium Solution - Weigh accurately 1.9068 g of potassium chloride dried at 400°C and cooled in a desiccator. Transfer to a 250 ml beaker with washing. Dissolve in about 100 ml of water. Add 10 ml of concentrated hydrochloric acid.

Transfer to 1 litre volumetric flask and dilute to mark. Mix well and store in a polyethylene bottle. The solution contains 1 mg \ml of potassium.

TABLE 4.5 - STRENGTH OF POTASSIUM SOLUTIONS FOR CALIBRATION

NUMBER OF SOLUTIONS	VOLUME OF STANDARD SOLUTION (mg)	QUANTITY OF POTASSIUM (ug/ml)
(1)	(2)	(3)
1	2.0	2.0
2	4.0	4.0
3	6.0	6.0
4	8.0	8.0
5	10.0	10.0

Preparation of Calibration Chart

1. Switch on the flame photometer in advance to allow sufficient time for its stabilization. Put sodium/potassium filter in position, adjust sensitivity of the apparatus and galvanometer should read zero while atomizing water with 2 percent hydrochloric acid.
2. Atomize the standard matching solution (5 µl /ml for sodium and 100 µl /ml for potassium) into the flame and adjust sensitivity to read 100 points on digital or full scale on galvanometer. Check zero and 100. Atomize the rest of standard solutions (see 6.1.1 and 6.2.1) and measure the intensities of emitted radiation from galvanometer readings.

Note- Quantity of solution atomized in the flame should be kept constant per unit of time throughout preparation of calibration curve, as well as sample analysis, by keeping the pressure constant.

3. Plot a curve having the number of micrograms of sodium and potassium contained in the standard matching solutions on the abscissa and the corresponding values of the intensities of radiation from galvanometer reading on the ordinate.

NOTE - For complex materials standard matching solutions should also contain equivalent amounts of major interfering constituents.

Determination

1. Preparation of Test Solution - Powder the material so that it passes 150 micron IS Sieve [see IS: 460 (Part 1) - 1978* and IS: 460 (Part II) - 1978

1.1. Weigh accurately about 5 g of the powdered material, dry in an air oven at 110°C for an hour, cool in a desiccator and weigh to constant mass. The difference will give the moisture content of the sample. Decompose a suitable mass of the test sample to bring sodium and potassium into solution as chloride/nitrate. For ore sand minerals proceed as follows.

1.2 Weigh accurately 0.2 g of as received mineral sample and transfer to a 100-ml beaker. Add 5 ml of concentrated hydrochloric acid, 5 ml of concentrated nitric acid and 10 ml of water. Boil for 30 to 40 minutes till the reaction is complete. Evaporate to syrupy consistency, extract the residue with hydrochloric acid (2 percent), filter if necessary, transfer quantitatively to a 100-ml volumetric flask, transfer the washings and make up the volume to the mark.

1.3 Decompose the silicates (0.2g) with hydrochloric acid (3 ml) and nitric acid (10 ml) in a 50-ml platinum dish and evaporate just to dryness or to syrupy consistency. Repeat the process thrice for complete decomposition. Add 10 ml of nitric acid and evaporate again. Transfer quantitatively into a 100 ml beaker

with minimum water. Add 10 ml of hydrochloric acid. Evaporate to syrupy consistency. Take up with 2 ml of concentrated hydrochloric acid and 10 ml of water, Transfer solution and washings quantitatively to a 100 ml volumetric flask and make up to the mark.

1.4 For determination of alkali metals in organic materials incinerate at 400 to 450°C the sample, previously dried and pulverised to 150 micron IS Sieve, till carbon is completely burnt. Then proceed as in 1.3.

2. Aspirate the solution. If galvanometer readings exceed maximum value in the standard curve (see 6.3), dilute it suitably, add 2 ml of hydrochloric acid per 100 ml and aspirate. A reagent blank should be run throughout the estimation. Check the standard curve (6.3.3) before or while aspirating the test solution.

4.9.3.7 CALCULATION

Concentration of alkali metal, percent by mass

$$= \frac{A \times D}{10\,000}$$

Where

A = concentration in µg/ml of alkali metal in the sample

Solution, and D = dilution factor for the sample taken.

4.10 SILAGE MICROBIOLOGY

For the silage making process the grass is first fermented in anaerobic condition by the process of wrapping the wheat grasses using a paper and they are kept in a zip-lock cover and left undisturbed for about 2 to 3 weeks. Then the wheat grasses are checked for the dried or fermented stage under anaerobic condition. Then the wheat grass silage is soaked in Ringer Lactate solution and left for 2 nights or 36 hours.

4.10.1 COMPOSITION OF RINGER LACTATE SOLUTION

The composition of Ringer lactate solution is 600 mg of sodium chloride, 310 mg of sodium lactate, 30 mg of potassium chloride, and 20 mg of calcium chloride per 500ml of solution.

4.10.2 PROCEDURE

1. The wheat grass silage is made.
2. Then the wheat grass silage is soaked in 500ml of Ringer Lactate solution and left for 2 nights or 36 hours.
3. Different selective mediums are used here for culturing microorganisms grown from different selective media.
4. The selective media used are :-
 - (i) LACTOBACILLUS MRS AGAR
 - (ii) MACONKEY AGAR
 - (iii) SABOURAUD DEXTROSE AGAR
 - (iv) EOSIN METHYLENE BLUE AGAR
 - (v) MANNITOL SALT AGAR

(vi) PHENOL RED EGG YOLK POLYMYXIN AGAR BASE

5. Then 10ul of Ringer lactate solution soaked with wheat grass silage is poured into each agar plate containing a different selective medium using a micropipette.
6. Then spread plate technique is used in which the RL solution poured over the media is spread using sterile glass L – ROD.
7. Then the plates are incubated in an incubator at 37 degree Celsius for 24 hours.
8. Then the results are observed.

4.10.3 HOW ENSILING PRESERVES A CROP

Most silage is made at dry matter content between 200 to 500 g/kg. Within this DM range, many enzymes in the plant are still active at ensiling. Also the wide variety of bacteria, yeasts and molds described above can all grow within this range. So it is a substantial challenge to bring all of this biological activity under control, but that is what a well-managed ensiling process does. There are two key processes that preserve the crop the silo: the creation of an anaerobic environment and the fermentation of sugars by lactic acid bacteria to lactic acid and other products. Both processes are important to good preservation, and one cannot substitute for the other. The main process that we associate with ensiling is the fermentation of sugars by lactic acid bacteria. Once the crop is anaerobic, lactic acid bacteria grow rapidly and quickly become, in most cases, the dominant microorganisms on the crop. They ferment sugars mainly to lactic acid, acetic acid, upon the species. Typical fermentation reactions are shown in Table 2. Assuming a mix of hetero- and homofermentative lactic acid bacteria, lactic acid will be the dominant product on a molar basis followed in descending order by carbon dioxide, acetic acid, and ethanol. The acids lower crop pH to

between 4.0 and 5.0 in alfalfa and grass silages and below 4.0 in corn silage. Both the low pH and the acids are beneficial in preserving the crop. The principal bacterial competitors of the lactic acid bacteria under anaerobic conditions (enterobacteria, clostridia and bacilli) are all inhibited by achieving sufficiently low pH. Once pH drops below 4.5 to 5.0 enterobacteria and bacilli will be inhibited, and enterobacteria populations will usually drop below detectable levels within a few days after the pH is below 5.0. Counts of bacilli do not drop as quickly due to their ability to form spores, and standard enumeration techniques for bacilli in silage only measure spores. The exact pH to inhibit these groups does vary by crop and DM content in addition to the bacterial strains present. However, fermentation by the lactic acid bacteria usually takes silage pH to a level that inhibits these bacteria. So often we forget about the two groups unless we have high acetic acid silage that is feeding poorly. Clostridia can grow at lower pH values than the enterobacteria and bacilli, making them more difficult to control. As a consequence, it is of greater importance to understand the interaction of pH, DM content and crop. Figure 1 shows the critical pH to inhibit *C. tyrobutyricum*, one of the principal fermenters of lactic acid to butyric acid. Ensiling a wet crop requires a lower pH to prevent clostridium growth. Grasses including corn have a higher water activity at a given DM content compared with legumes like alfalfa so that fermentation to a lower pH is required in grasses to prevent clostridium growth. Because it is easy to obtain a silage pH below 4.0 in corn, clostridia silage is uncommon in corn silage. In alfalfa and grass silages, it is more difficult to achieve a sufficiently low pH to inhibit clostridia. Thus in these crops the producer may need to wilt the crop to a high DM content (reducing the amount of fermentation needed to inhibit the clostridia) or use an additive to achieve a lower pH than is possible by natural fermentation. The lactic and acetic acids together with low pH are inhibitors of various aerobic microorganisms. Low pH is normally thought to inhibit *Listeria monocytogenes*, the cause of listeriosis.

TABLE 4.6 - Typical lactic acid bacterial fermentations

Group	Fermentation
Homofermenter /Fac. Hetero.	1 6-C Sugar → 2 Lactic acid
Facultative Heterofermenters	1 5-C Sugar → 1 lactic acid + 1 acetic acid
Heterofermenters	1 6-C Sugar → 1 lactic acid + 1 Acetic acid + CO ₂
Ethanol + CO ₂	1 6-C Sugar → 1 Lactic acid + 1 Acetic acid
Acetic acid	1 5-C Sugar → 1 Lactic acid + 1 Acetic acid + CO ₂
	1 Lactic acid → 1 Acetic acid + CO ₂

However, Donald et al. (1995) observed listeria at pH 4.2 in grass silage with restricted fermentation, suggesting that the fermentation acids in addition to low pH are critical to reducing their population. Lactic acid is an inhibitor of acetic acid bacteria, which can begin the spoilage of corn silage (Courtin & Spoelstra, 1990). It is possible that the reason acetic acid bacteria have only been reported in corn silage is that whole-plant corn is low in buffering capacity so that relatively little lactic acid is required to lower pH to a stable value below 4.0. The volatile fatty acids are good inhibitors of yeasts and molds (Moon, 1983). Acetic acid is not as good an inhibitor as the longer chain fatty acids, but more effective than lactic acid. The effect of acetic acid on fungal growth is related to the dissociated concentration in solution. Thus a given concentration of acetic acid becomes inhibitory to yeasts and molds as silage pH decreases. Unfortunately, lactic acid bacterial fermentation rarely lowers pH sufficiently and produces enough acetic acid to prevent yeasts and molds from growing in silage. Many yeasts and molds as well as acetic acid bacteria will grow at pH 3.5, well below normal silage Ph. Once oxygen is present, yeasts, molds and

acetic acid bacteria can begin to grow on silage, using fermentation products and residual sugars in the silage and producing carbon dioxide, water and heat. As fermentation products are used up, silage pH rises. Once the pH is above 4.5, a wide variety of other aerobic microorganisms can grow, spoiling the silage more and causing even greater heating of the silage. All of these losses are of the most digestible parts of the silage. This spoilage loss can only be prevented by keeping oxygen out of the silo and minimizing silage exposure to oxygen while emptying the silo. In summary, good preservation and stabilization of the crop in the silo is due to the combination of an anaerobic environment and the growth of lactic acid bacteria. The lactic acid bacterial fermentation produces acids that lower pH and stop the growth of detrimental anaerobic microorganisms. The low pH and acids help slow, but usually do not stop, the growth of aerobic spoilage microorganisms. The anaerobic environment promotes the growth of lactic acid bacteria. More importantly, anaerobic conditions are the only way to prevent the growth of yeasts, molds and aerobic bacteria that spoil and heat silage.

4.10.4 CONCLUSION FOR SILAGE MICROBIOLOGY

Ensiling preserves a crop by controlling microbial activity through a combination of an anaerobic environment and a natural fermentation of sugars by lactic acid bacteria on the crop. Two fermentation products (lactic and acetic acids) and the resulting low pH primarily suppress the growth of other anaerobic microorganisms. The fermentation can also inhibit yeasts, molds and aerobic bacteria, but the anaerobic environment is essential to preventing most of the spoilage microorganisms from growing. Inoculants, products supplementing the crop with selected strains of lactic acid bacteria, have become the dominant additives for making silage. Homofermentative strains help guarantee a rapid suppression of other anaerobic microorganisms early in storage, increase dry matter recovery and have improved animal performance by means that we do not fully understand. These inoculants may lower pH sufficiently to prevent clostridia growth in limited cases where the epiphytic lactic acid bacterial

fermentation falls just short of suppression. Inoculants containing *Lactobacillus buchneri*, a heterofermentative species capable of fermenting lactic acid to acetic acid, are recent additives. The added acetic acid inhibits yeast and mold growth, increasing aerobic stability of silages at feeding. Acetic acid production does result in additional CO₂ loss, reducing dry matter recovery approximately 1 percentage point on average. Overall, inoculants are additives that can be used to improve silage quality when used appropriately together with good silage management.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 RESULTS

The olive brine solution is streaked using quadrant streaking method on the MRS agar and the *Lactobacillus* colonies isolated are shiny, thick, opaque, milky white, round, mucoid colonies were observed.

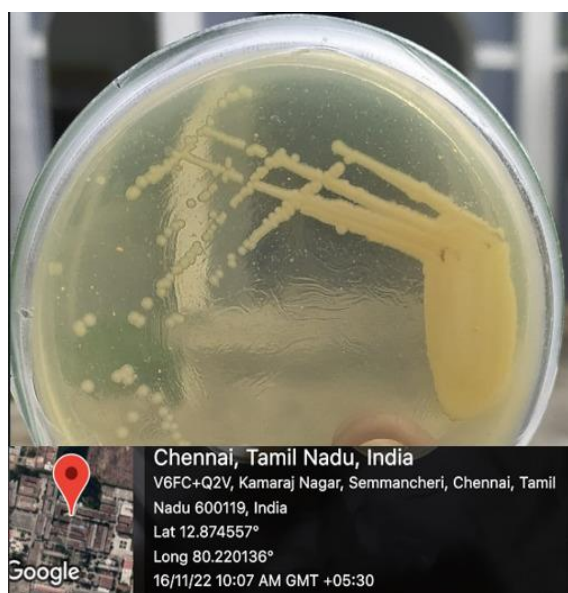


FIG. 5.1 *Lactobacillus* culture grown on *Lactobacillus* MRS agar.

The result for gram staining of lactobacillus bacteria isolated from LACTOBACILLUS MRS AGAR is GRAM POSITIVE RODS (BACILLI)

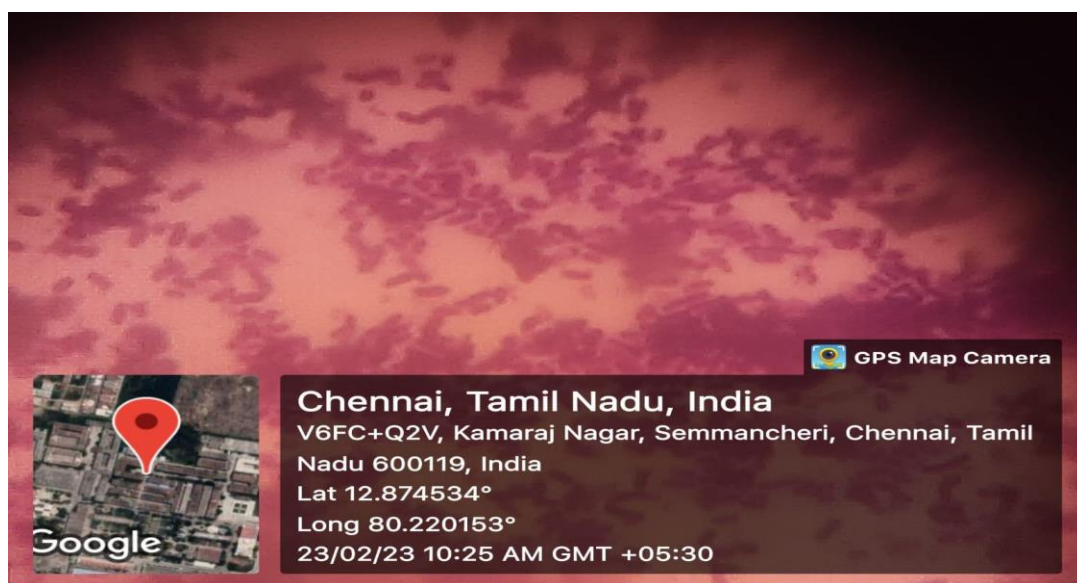


FIG. 5.2 Gram staining results for *Lactobacillus* grown on MRS agar (Gram positive).

S.NO	Morphology of the colony	Image of a colony	Gram staining +/-
1	Shape: round Edge: slippery Elevation: thick Color: milky white Medium size		 Gram: Positive Cell shape: <i>Bacillus</i>

FIG 5.3 Colony morphology and gram staining results of previous review paper.

Credits - (Resti Fevria, Indra Hartanto, 2019). Flat colonies were observed in their project, while we noticed mucoid colonies in our isolated samples. Although we isolated *Lactobacillus* from olive brine solution, a previous review paper reported isolating the *Lactobacillus* from sauerkraut.

The results for gram staining of EXO-POLYSACCHARIDE PRODUCING LACTOBACILLUS is GRAM POSITIVE RODS (BACILLI)



FIG. 5.4 *EPS Lactobacillus* grown on ESM agar.



FIG. 5.5 Gram staining results for *EPS producing Lactobacillus* (Gram positive).

(Dominika Jurášková, Susana C. Ribeiro and Celia C. G. Silva 2021) The slimy phenotype is characterized by mucilaginous colonies and they are white .



(a)



(b)

FIG 5.6 – Characteristics produced by EPS LAB a) Muroid colonies—slimy phenotype; (b) Ropy phenotype.

The figure 5.6 above illustrates the colony morphology of EPS *Lactobacillus* as isolated in the previous study. However, our isolated strain displayed distinct characteristics, such as mucoid, curdy, shiny, and dark yellow colonies, indicating differences between the two strains.

BIOCHEMICAL ANALYSIS

RESULTS OF IMVIC TEST FROM LACTOBACILLUS BACTERIA GROWN ON MRS AGAR

TABLE 5.1 – Results of biochemical (IMVIC) analysis of lactobacillus growth on MRS AGAR.

TEST	RESULT
1. CITRATE UTILIZATION	NEGATIVE
2. INDOLE	NEGATIVE
MOTILITY(tested with indole)	POSITIVE
3. TSI	YELLOW COLOUR (FERMENTATION +VE)
4. MR	POSITIVE
5. VP	POSITIVE
6. SULPHUR REDUCTION	NEGATIVE



FIG. 5.7 Biochemical (IMVIC) analysis for Lactobacillus.

Biochemical characterization	Result
Indole	-
Methyl red	-
Voges proskauer	-
Citrate utilization	-
Urease	-
Catalase	-
Oxidase	-

FIG 5.8 Biochemical result of previous review paper for MRS Lactobacillus.

Credits - (P. Kavitha*, D. Sindhuja and M. Banumathi 2016)

The results presented here are from the review paper and show negative results. However, in our project, we observed positive results for MR, VP and TSI agar fermentation tests, as well as positive results for catalase and oxidase tests, indicating differences between our findings and those reported in the review paper.

RESULTS OF IMVIC TEST OF EPS PRODUCING LACTOBACILLUS

TABLE 5.2 – Results of biochemical (IMVIC) analysis of EPS producing lactobacillus.

TEST	RESULT
1. INDOLE	NEGATIVE
MOTILITY(tested with indole)	NEGATIVE
2. MR	POSITIVE
VP(MR and VP are tested with same tube)	POSITIVE

3. CITRATE UTILIZATION	NEGATIVE
4. TSI	YELLOW COLOUR (FERMENTATION +VE)
5. SULPHUR REDUCTION	NEGATIVE



FIG. 5.9 Biochemical (IMVIC) analysis for EPS producing *Lactobacillus*.

Biochemical IMVIC analysis was not conducted for EPS *Lactobacillus* in any of the review papers. As a result, we concluded that the MR and VP tests were positive and that TSI agar fermentation was positive based on our own experiments with the strain.



FIG. 5.10 Control for biochemical (IMVIC) tests.

OXIDASE TEST

The EXO-POLYSACCHARIDE producing LACTOBACILLUS is tested for the OXIDASE PRODUCTION using OXIDASE DISC is NEGATIVE (OXIDASE NEGATIVE)



FIG. 5.11 Oxidase test for EPS producing Lactobacillus (oxidase negative).

The LACTOBACILLUS grown in MRS AGAR is tested for the OXIDASE PRODUCTION using OXIDASE DISC is POSITIVE (OXIDASE POSITIVE)



FIG. 5.12 Oxidase test for *Lactobacillus* (oxidase positive).

Credits - (P. Kavitha*, D. Sindhuja and M. Banumathi, 2016)

The results reported in the review paper showed that MRS *Lactobacillus* is oxidase negative. However, in our own experiments, we observed that MRS *Lactobacillus* was oxidase positive while EPS *Lactobacillus* was oxidase negative.

CATALASE TEST

The EXO-POLYSACCHARIDE producing LACTOBACILLUS is tested for the CATALASE PRODUCTION using HYDROGEN PEROXIDE is POSITIVE (CATALASE POSITIVE)



FIG. 5.13 Catalase test for *Lactobacillus* (catalase positive).

The LACTOBACILLUS grown in MRS AGAR is tested for the CATALASE PRODUCTION using HYDROGEN PEROXIDE is POSITIVE (CATALASE POSITIVE)



FIG. 5.14 Catalase test for EPS producing *Lactobacillus* (catalase positive).

Credits - (P. Kavitha*, D. Sindhuja and M. Banumathi, 2016). The results reported in the review paper showed that MRS Lactobacillus is catalase negative. However, Our experiments showed that both MRS Lactobacillus and EPS Lactobacillus were positive for catalase.

ANTIBIOTICS DISK DIFFUSION TEST LACTOBACILLUS

MRS LACTOBACILLUS

MULLER HINTON AGAR IS PREPARED, LACTOBACILLUS IS SWABBED IN THAT PLATE AND DISC ARE PLACED TO PERFORM ANTIBIOTIC DISK DIFFUSION TEST



FIG. 5.15 Antibiotics disk diffusion test for *Lactobacillus*.

TABLE 5.3 – Antibiotic disk diffusion test results for MRS- *lactobacillus*.

Antibiotic disc	measurement
1. E 15 erythromycin	10 mm
2. C 30 chloramphenicol	14 mm

3. TE 30 tetracycline	6 mm
4. Amx 10 amoxicillin	9 mm

EPS PRODUCING LACTOBACILLUS –

MULLER HINTON AGAR IS PREPARED, EXOPOLYSACCHARIDE PRODUCING LACTOBACILLUS IS SWABBED IN THAT PLATE AND DISC ARE PLACED TO PERFORM ANTIBIOTIC DISK DIFFUSION TEST.



FIG. 5.16 Antibiotics disk diffusion test for EPS producing *Lactobacillus*.

TABLE 5.4 – Antibiotic disk diffusion test results for EPS producing *lactobacillus*.

Antibiotic disc	measurement
1. E 15 erythromycin	10 mm
2. C 30 chloramphenicol	5 mm

3. TE 30 tetracycline	1 mm
4. Amx 10 amoxicillin	0 mm

The primary objective of conducting the antibiotic disk diffusion test was to evaluate the resistance of MRS Lactobacillus and EPS Lactobacillus to antibiotics in the context of silage microbiology.

ANOVA AND STATISTICAL ANALYSIS OF ANTIBIOTIC DISK DIFFUSION TEST

ANOVA

TABLE 5.5 – ANOVA FOR ANTIBIOTIC DISK DIFFUSION TEST

	E 15 ERYTHROMYCIN (unit- mm)	C 30 CHLORAMPHENICOL (unit – mm)	TE 30 TETRACYCLINE (unit – mm)	AMX 10 AMOXICILLIN (unit – mm)
MRS LACTOBACILLUS	10	14	6	9
EPS LACTOBACILLUS	10	5	1	0

Anova: Single Factor

SUMMARY

<i>Groups</i>	<i>Count</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
E 15 ERYTHROMYCIN	2	20	10	0
C 30 CHLORAMPHENICOL	2	19	9.5	40.5

TE 30 TETRACYCLINE	2	7	3.5	12.5
AMX 10 AMOXICILLIN	2	9	4.5	40.5

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
			22.45833333	0.96078431	0.4926	6.59138
Between Groups	67.37	3	3	4	9	2
Within Groups	93.5	4	23.375			

Total	160.8	7
-------	-------	---

STATISTICAL ANALYSIS

TABLE 5.6 STATISTICAL ANALYSIS FOR ANTIBIOTIC DISK DIFFUSION TEST

ANTIBIOTICS	MRS LACTOBACILLUS	EPS LACTOBACILLUS
E 15 ERYTHROMYCIN	10	10
C 30 CHLORAMPHENICOL	14	5
TE 30 TETRACYCLINE	6	1
AMX 10 AMOXICILLIN	9	0
MEAN	9.75	4
MEDIAN	9.5	3
MODE	#N/A	#N/A

STANDARD DEVIATION	4.793969426	
MINIMUM	0	
MAXIMUM	14	
RANGE	14	
VARIANCE	22.98214286	
COVARIANCE	5	
CORRELATION	0.443842105	

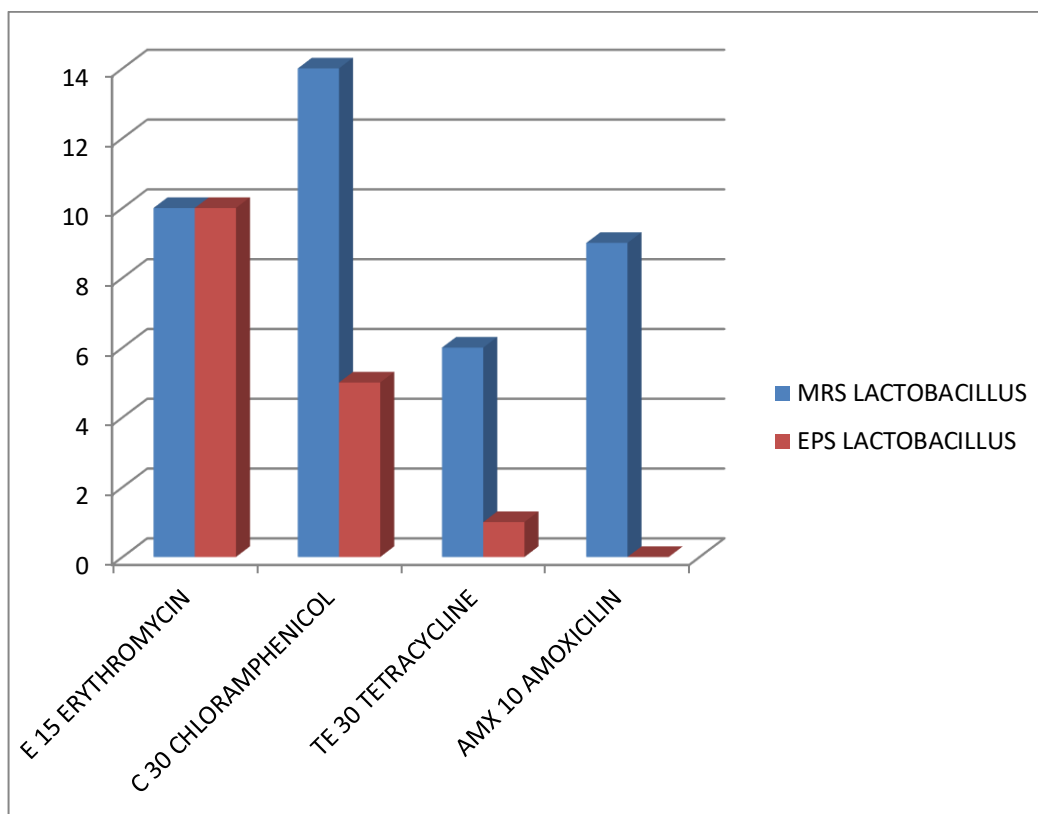


FIG. 5.17 Bar graphical representation for results of antibiotic disk diffusion test.

GENOME SEQUENCING

The strain of lactobacillus which was identified to produce exopolysaccharide activity is *Lactobacillus plantarum*.

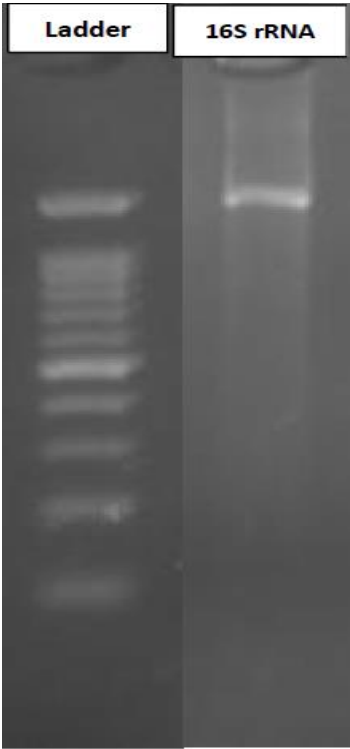


FIG. 5.18 16S rRNA Gene in 1% of Agarose Gel Electrophoresis after PCR Amplification.

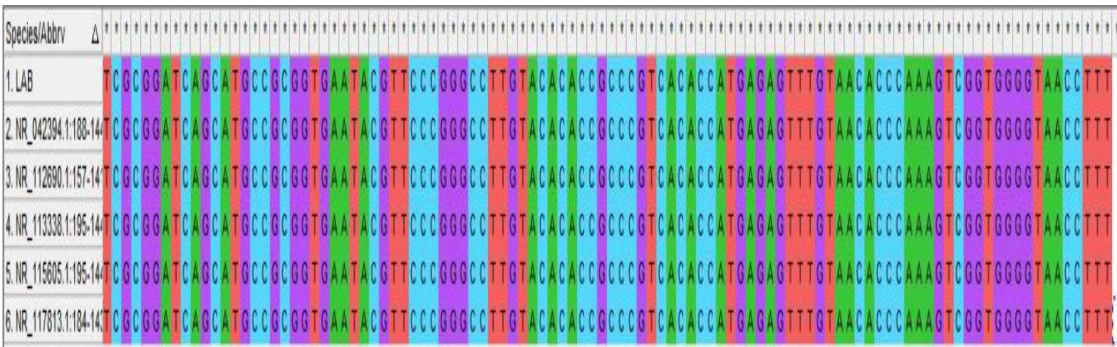


Fig. 5.19 Multiple Sequence Alignment of 16S rRNA Gene of bacterial isolate from Olive Brine Solution. The following parameters used to build the sequence alignment (gapopen: 400.00, gapextend: 0.00, maxmb: 2048, maxiters: 16, cluster1: upgma, cluster2: upgma, maxiters: 16, seqtype: DNA

Sequence of LAB sample:

>LAB_Lactiplantibacillus plantarum (Olive Brine Solution)_FORWARD

GGCTTCGGCNNTCACTTTGGACGGCGTATTAGCTAGATGGTGGGTAACGG
CTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACAT
TGGGACTGAGACACGGCCCAAACCTCCTACGGGAGGCAGCAGTAGGGAAT
CTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAA
GGGTTTCGGCTCGTAAAACTCTGTTGTAAAGAAGAACATATCTGAGAGTA
ACTGTTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTG
CCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGG
GCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGG
CTCAACCGAAGAAGTGCATCGGAAACTGGGAACTTGAGTGCAGAAGAGG
ACAGTGGAACCTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAAC
ACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACCTGACGCTGAGGCTCGA
AAGTATGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCATACCGTAAAC
GATGAATGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAAC
GCATTAAGCATTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAG
GAATTGACGGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCTGAAG
CTACGCGAAGAACCTTACCAGGTCTTGACATACTATGCAAATCTAAGAGAT
TAGACGTTCCCTTCGGGGACATGGATACAGGTGGTGCATGGTTGTCGTCA
GCTCGTGTCGTGAGATGTTGGGTAAAGTCCCGCAACGAGCGCAACCCTTA
TTATCAGTTGCCAGCATTAAAGTTGGGCACTCTGGTGAGACTGCCGGTGAC
AAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCTTATGACC
TGGGCTACACACGTGCTACAATGGATGGTACAACGAGTTGCGAACTCGCG
AGAGTAAGCTAATCTCTTAAAGCCATTCTCAGTTCGGATTGTAGGCTGCAA
CTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCCGC
GGTGAATACGTTCCCGGGCCTTGACACACCGCCCGTCACACCATGAGAG
TTTGTAACACCCAAAGTCGGTGGGGTAACCTTT

Sequence of LAB sample:

>LAB_Lactiplantibacillus plantarum (Olive Brine Solution) _REVERSE

AAAGGTTACCCACCGACTTTGGGTGTTACAACTCTCATGGTGTGACGG
GCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCG
CGATTACTAGCGATTCCGACTTCATGTAGGCGAGTTGCAGCCTACAATCCG
AACTGAGAATGGCTTTAAGAGATTAGCTTACTCTCGCGAGTTCGCAACTCG
TTGTACCATCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGGCATGAT
GATTTGACGTCATCCCCACCTTCCTCCGGTTTGTACCGGCAGTCTCACCA
GAGTGCCCAACTTAATGCTGGCAACTGATAATAAGGGTTGCGCTCGTTGC
GGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCA
CCTGTATCCATGTCCCCGAAGGGAACGTCTAATCTCTTAGATTTGCATAGT
ATGTCAAGACCTGGTAAGGTTCTTCGCGTAGCTTCGAATTAAACCACATGC
TCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGCCTTGCG
GCCGTACTCCCCAGGCGGAATGCTTAATGCGTTAGCTGCAGCACTGAAGG
GCGGAAACCCTCCAACACTTAGCATTTCATCGTTTACGGTATGGACTACCAG
GGTATCTAATCCTGTTTGCTACCCATACTTTTCGAGCCTCAGCGTCAGTTAC
AGACCAGACAGCCGCCTTCGCCACTGGTGTTCTTCCATATATCTACGCATT
TCACCGCTACACATGGAGTTCCACTGTCCTCTTCTGCACTCAAGTTTCCCA
GTTTCCGATGCACTTCTTCGGTTGAGCCGAAGGCTTTCACATCAGACTTAA
AAAACCGCCTGCGCTCGCTTTACGCCCAATAAATCCGGACAACGCTTGCC
ACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGG
TTAAATACCGTCAATACCTGAACAGTTACTCTCAGATATGTTCTTCTTTAAC
AACAGAGTTTTACGAGCCGAAACCCTTCTTCACTCACGCGGCGTTGCTCCA
TCAGACTTTTCGTCCATTGTGGAAGATTCCCTACTGCTGCCTCCCGTAGGAG
TTTGGGCCGTGTCTCAGTCCCAATGTGGCCGATTACCCTCTCAGGTCGGC
TACGTATCATTGCCATGGTGAGCCGTTACCCACCATCTAGCTAATACGCCG
TCCAAAGTGANNGCCGAAGCC

Job title: Contig_MRS

RID [7WW1GHHJ01R](#) (Expires on 04-21 14:09 pm)

Query ID Id|Query_135669
Description Contig_MRS
Molecule type dna
Query Length 1251

Database Name nt
Description Nucleotide collection (nt)
Program BLASTN 2.11.0+

Graphic Summary



FIG 5.20 Distribution of the top 140 Blast Hits on 100 Subject sequences.

BLASTn ANALYSIS OF LAB ISOLATE IN NCBI

The 16S rRNA gene sequence obtained from LAB isolate was analysed by BLASTn in NCBI. The sequence shared 99.92% sequence identity with *Lactobacillus plantarum* strain (NR115605.1) 16S ribosomal RNA, partial sequence with accession number OQ119542 which showed maximum and total score of 2278 with 98% query coverage and E – value of 0.0. The obtained species from the homology search for 16S rRNA gene of LAB sample against nr/nt databases is *Lactobacillus plantarum*.

Lactiplantibacillus plantarum strain LAB 16S ribosomal RNA gene, partial sequence

GenBank: OQ119542.1

[FASTA](#) [Graphics](#)

[Go to:](#) 

LOCUS OQ119542 1236 bp DNA linear BCT 29-DEC-2022

DEFINITION Lactiplantibacillus plantarum strain LAB 16S ribosomal RNA gene, partial sequence.

ACCESSION [OQ119542](#) REGION: complement(1..1236)

VERSION OQ119542.1

KEYWORDS .

SOURCE Lactiplantibacillus plantarum

ORGANISM [Lactiplantibacillus plantarum](#)
Bacteria; Bacillota; Bacilli; Lactobacillales; Lactobacillaceae; Lactiplantibacillus.

REFERENCE 1 (bases 1 to 1236)

AUTHORS Akshaya,P., Sai Vishnu,K., Sudha,S. and Usha Nandhini,S.

TITLE Isolation of Lactiplantibacillus plantarum from Olive Brine Solution

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 1236)

AUTHORS Akshaya,P., Sai Vishnu,K., Sudha,S. and Usha Nandhini,S.

TITLE Direct Submission

JOURNAL Submitted (24-DEC-2022) Department of Biotechnology, Sathyabama Institute of Science and Technology, Kamaraj Nagar, Semmancheri, Chennai, Tamil Nadu 600119, India

COMMENT ##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##

FEATURES Location/Qualifiers

source 1..1236
/organism="Lactiplantibacillus plantarum"
/mol_type="genomic DNA"
/strain="LAB"
/isolation_source="Olive Brine Solution"

FIG 5.21 Lactobacillus plantarum strain LAB 16S ribosomal RNA gene sequence was submitted in NCBI.

Sequence was submitted in NCBI and the Accession Number was obtained as OQ119542 for *Lactiplantibacillus plantarum*. The FASTA file was opened and multiple sequence alignments were saved in *.meg format and loaded in MEGA v7.0 for the construction of phylogenetic trees.

PHYLOGENETIC TREE

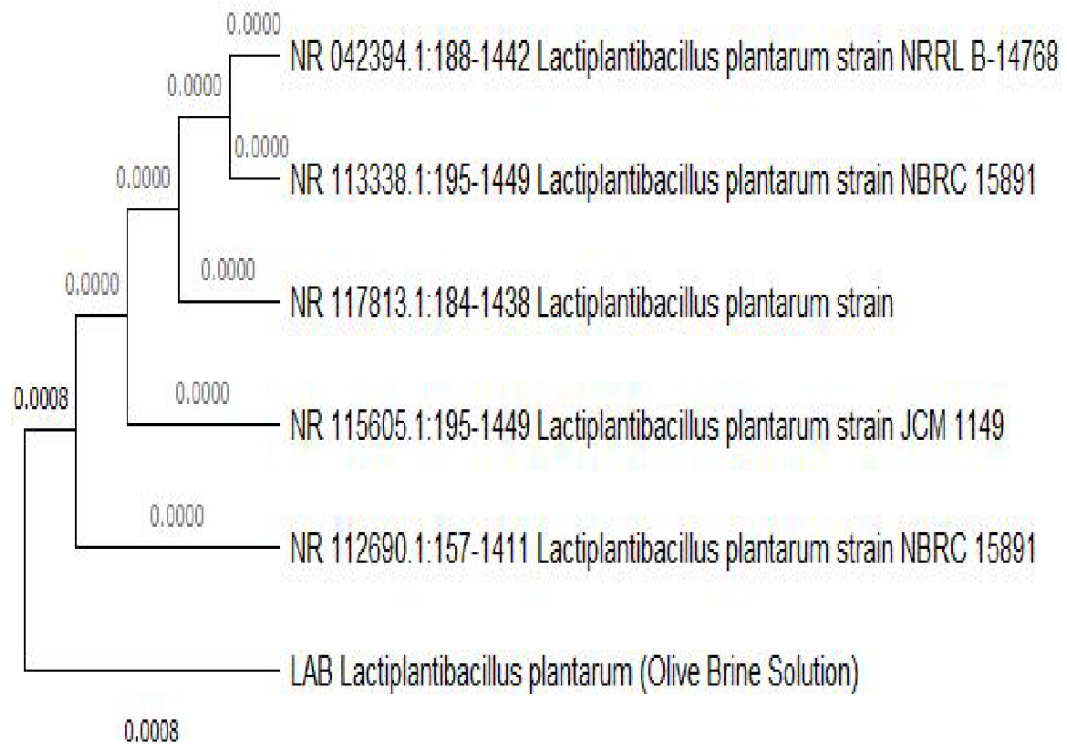


Fig. 5.22 Phylogenetic Tree was constructed to determine the relationship of Bacterial isolate (*Lactobacillus plantarum*) from Olive Brine Solution. The MEGAX Software was utilized to build a phylogenetic tree.

HARVESTING AND YIELD ANALYSIS OF WHEAT GRASS

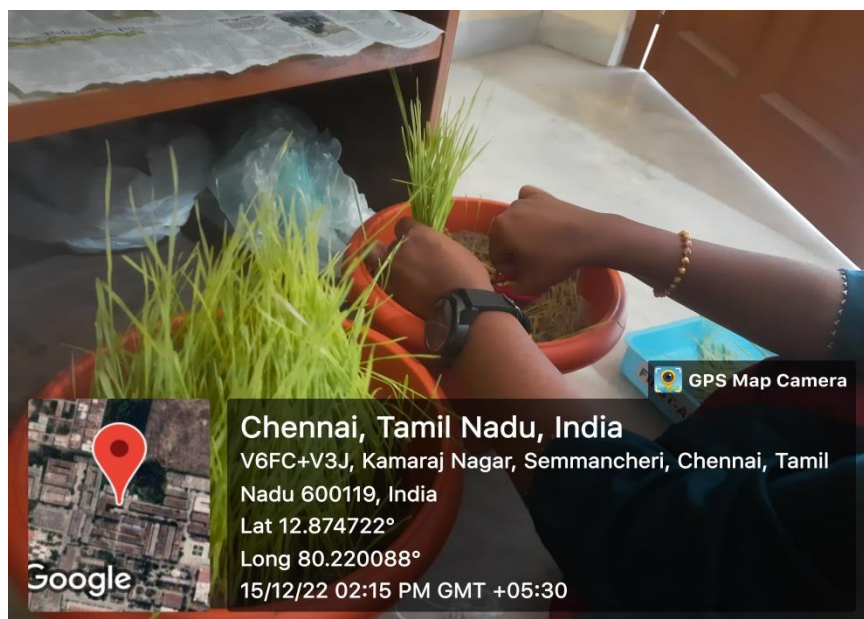


FIG 5.23 Harvesting of wheat grass.

The wheatgrass is harvested from the pots to conduct yield analysis, nutritional analysis, and silage microbiology.

YIELD ANALYSIS

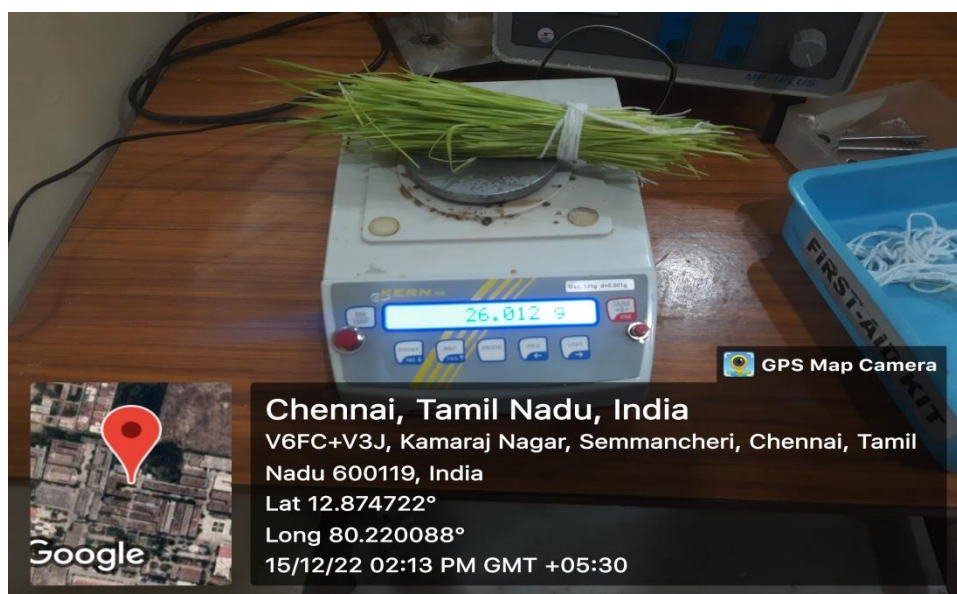


FIG 5.24 Measurement of yield for wheat grass grown in sea sand using EPS Lactobacillus mixed sea water.

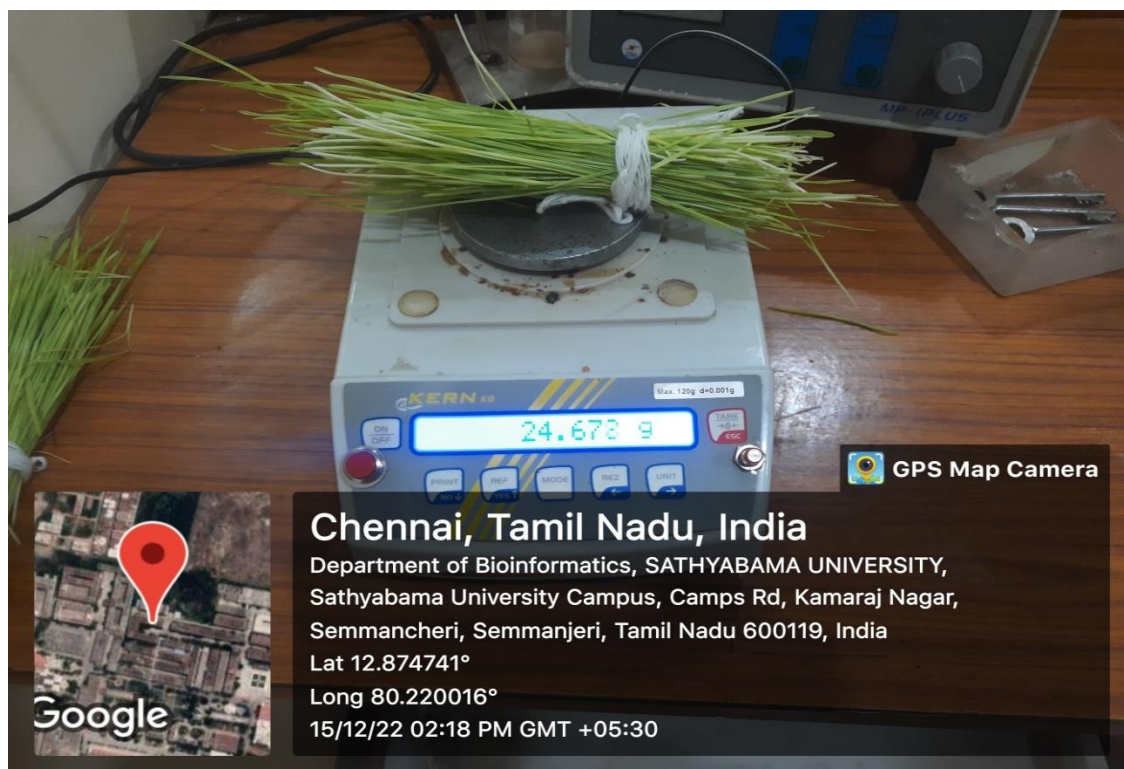


FIG 5.25 Measurement of yield for wheat grass grown in sea sand using sea water.

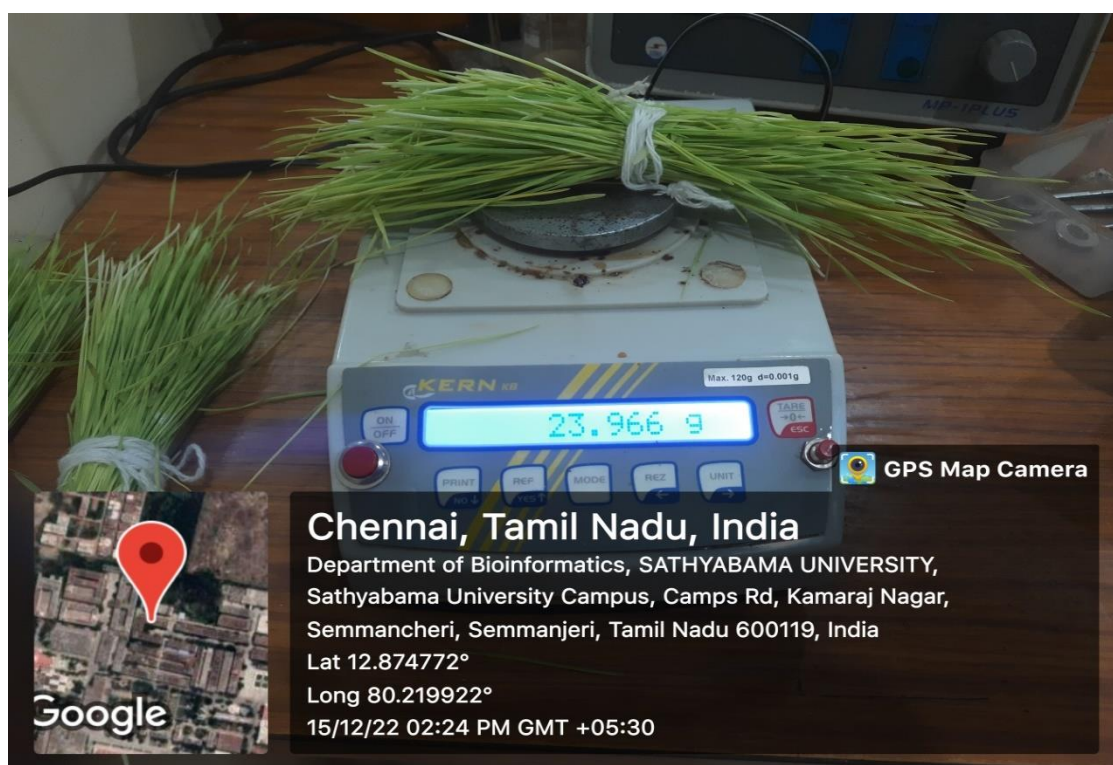


FIG 5.26 Measurement of yield of wheat grass grown in farm soil using fresh water.

According to the figure 5.23 above, the weight of wheat grass grown in sea sand using EPS Lactobacillus was 26.012 grams, which is greater than the yield in figure 5.24 (24.678 grams) and figure 5.25 (23.966 grams). This indicates that the use of EPS has a positive effect on the yield of wheat grass.

TABLE 5.7 - results of different wheat grass yield.

Type of wheat grass	Weight (grams)
Lactobacillus wheat grass	26.012
Sea sand Wheat grass	24.678
Control wheat grass (farm soil)	23.966

NUTRITIONAL ANALYSIS

The nutritional analysis was performed on control wheatgrass grown in farm soil. The wheatgrass was harvested and processed into juice by grinding it and mixing it with sterile water. The juice was then analyzed for the nutritional composition of five different nutrients.

TABLE 5.8 – Nutritional analysis results for control wheat grass.

S.NO	PARAMETERS	TEST METHOD	UNIT	CONTROL WHEAT GRASS
1	POTASSIUM	AOAC 20th Edn 2016, 969.23	Mg/100g	42
2	IRON	ALPL/FD/S OP/068	Mg/100g	0.66
3	PHOSPHORUS	IS:7874(Part -2)1975 (RA:2009)	Mg/100g	21

4	MAGNESIUM	IS:5949:1990 (RA:2015)	Mg/100g	8
5	CALCIUM	IS:5949:1990 (RA:2015)	Mg/100g	7.2

The nutritional analysis was performed on wheatgrass grown in sea sand without the presence of Lactobacillus. The wheatgrass was harvested and processed into juice by grinding it and mixing it with sterile water. The juice was then analysed for the nutritional composition of five different nutrients.

TABLE 5.9 – Nutritional analysis results for sea sand wheat grass.

S.NO	PARAMETERS	TEST METHOD	UNIT	SEA SAND WHEAT GRASS
1	POTASSIUM	AOAC 20th Edn 2016, 969.23	Mg/100g	150
2	IRON	ALPL/FD/SO P/068	Mg/100g	3.02
3	PHOSPHORUS	IS:7874(Part-2)1975 (RA:2009)	Mg/100g	70.8

4	MAGNESIUM	IS:5949:1990 (RA:2015)	Mg/100g	20.4
5	CALCIUM	IS:5949:1990 (RA:2015)	Mg/100g	28.2

The nutritional analysis was performed on wheatgrass grown in sea sand and mixed with Lactobacillus in the processed sea water. The wheatgrass was harvested and processed into juice by grinding it and mixing it with sterile water. The juice was then analysed for the nutritional composition of five different nutrients.

TABLE 5.10 – Nutritional analysis results for EPS lactobacillus wheat grass.

S.NO	PARAMETERS	TEST METHO D	UNIT	EXOPOLYSACCHA RIDE LACTOBACILLUS WHEAT GRASS
1	POTASSIUM	AOAC 20th Edn 2016, 969.23	Mg/100 g	180.2
2	IRON	ALPL/FD /SOP/06 8	Mg/100 g	3.44

3	PHOSPHORUS	IS:7874(Part-2)1975 (RA:2009)	Mg/100g	78.4
4	MAGNESIUM	IS:5949:1990 (RA:2015)	Mg/100g	25.2
5	CALCIUM	IS:5949:1990 (RA:2015)	Mg/100g	31.01

The nutritional analysis results indicate that the utilization of EPS Lactobacillus as an inoculant not only increases the yield, but also significantly improves the nutritional composition of wheatgrass compared to farm soil.

OBSERVATION VALUES

LACTOBACILLUS WHEAT GRASS

TABLE 5.11 – Observation values of nutritional analysis for lactobacillus wheat grass

<i>POTASSIUM</i>	<i>CONC.</i>	<i>100g</i>	<i>20</i>	<i>100</i>	<i>SAMPLE WEIGHT</i>	<i>RESULT (mg/kg)</i>	<i>RESULT (mg/100g)</i>
	39	100	50	100	4.3284	1802.1	180.21

<i>CALCIUM</i>	<i>SAMPLE WEIGHT</i>	<i>TV</i>	<i>FACTOR</i>	<i>RESULT (g/100g)</i>	<i>RESULT (mg/100g)</i>
	4.1359	3.2	0.04008	0.03101	31.01

IRON	SAMPLE ABSORB- ANCE	STD CONC.	SAMPLE WEIGHT	SAMPLE VOLUME	STD ABSORB- ANCE	RESULT mg/100g
	0.0254	0.1	1.3126	25	0.0225	3.440

MAGNESIUM	SAMPLE WEIGHT	TV	FACTOR	RESULT (g/100g)	RESULT (mg/100g)
	4.1359	4.3	0.0243	0.0253	25.26

PHOSPHOR US	SAMPLE ABSORB ANCE	STD CONC.	SAMPL E WEIGH T	SAM PLE VOL UME	STD. ABSOR BANCE	RESUL T mg/100 g
	0.4215	0.3	1.26	25	0.0512	78.404

CONTROL WHEAT GRASS

Table 5.12 – Observation values of nutritional analysis for control wheat grass.

POTASSIU M	CONC .	100 g	2 0	10 0	SAMPL E WEIGH T	RESUL T (mg/kg)	RESULT (mg/100g)
	34	100	5 0	10 0	4.5324	1500.3	150.03

CALCIU M	SAMPLE WEIGHT	TV	FACTOR	RESULT (g/100g)	RESULT (mg/100g)
	5.8251	4.1	0.04008	0.02821	28.21

IRO N	SAMPLE ABSORBA NCE	STD. CON C	SAMPL E WEIGH T	SAMPL E VOLUM E	STD. ABSORBAN CE	RESU LT (mg/100g)
	0.0232	0.1	1.3657	25	0.0225	3.020

MAGNESIUM	SAMPLE WEIGHT	TV	FACTOR	RESULT (g/100g)	RESULT (mg/100g)
	5.4794	4.6	0.0243	0.0204	20.40

PHOSPHORUS	SAMPLE ABSORBANCE	STD. CONC	SAMPLE WEIGHT	SAMPLE VOLUME	STD. ABSORBANCE	RESULT (mg/100g)
	0.3514	0.3	1.1632	25	0.0512	70.804

ANOVA AND STATISTICAL ANALYSIS OF NUTRITIONAL ANALYSIS

ANOVA

TABLE 5.13 – ANOVA

PARAMETERS	CONTROL WHEAT GRASS	SEA SAND WHEAT GRASS	LACTOBACILLUS WHEAT GRASS
POTASSIUM	42	150	180.2
IRON	0.66	3.02	3.44
PHOSPHORUS	21	70.8	78.4
MAGNESIUM	8	20.4	25.2
CALCIUM	7.2	28.2	31.01

ANOVA : SINGLE FACTOR

SUMMARY

Groups	Count	Sum	Average	Variance
CONTROL WHEAT GRASS	5	272.42	54.484	3472.65728
SEA SAND WHEAT GRASS	5	318.25	63.65	4992.6203
LACTOBACILLUS WHEAT GRASS	5	78.86	15.772	269.37392

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	6458.228973	2	3229.114487	1.109070403	0.361435	3.885294
Within Groups	34938.606	12	2911.5505			
Total	41396.83497	14				

STATISTICAL ANALYSIS

TABLE 5.14 – STATISTICAL ANALYSIS

PARAMETERS	CONTROL WHEAT GRASS	SEA SAND WHEAT GRASS	LACTOBACILLUS WHEAT GRASS
POTASSIUM	42	150	180.2
IRON	0.66	3.02	3.44
PHOSPHORUS	21	70.8	78.4
MAGNESIUM	8	20.4	25.2
CALCIUM	7.2	28.2	31.01
Mean =	15.772	54.484	63.65
Median =	8	28.2	31.01
Mode =	#N/A	#N/A	#N/A

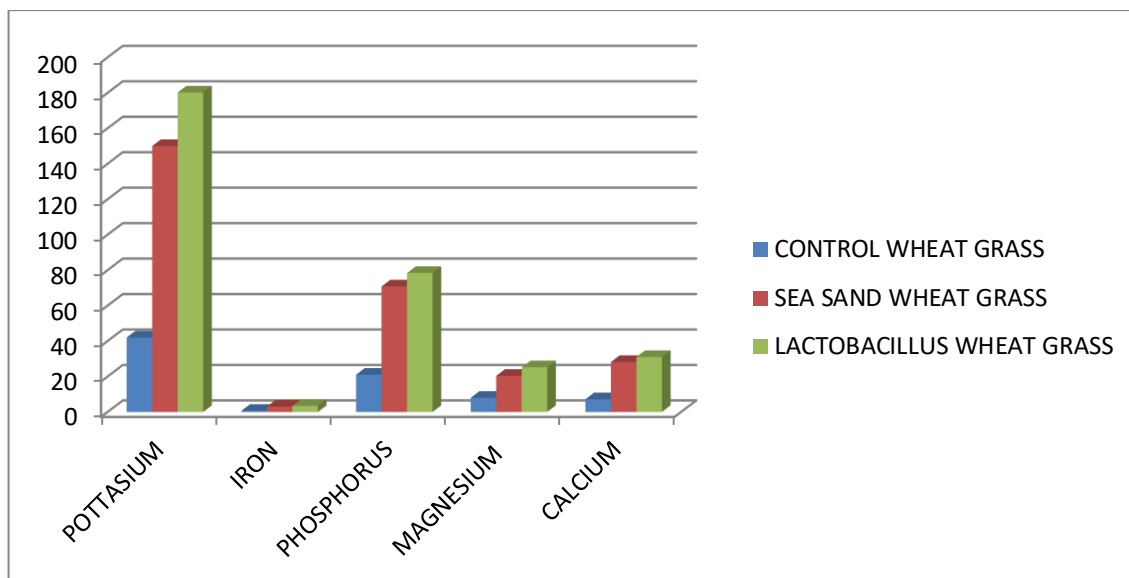


FIG. 5.27 Bar graphical representation for results of nutritional composition.

SILAGE MICROBIOLOGY

The wheatgrass is harvested from sea sand and then kept in anaerobic condition for the fermentation and to make the wheat grass as silage.



FIG. 5.28 Fermented wheat grass silage.

Then the Ringer Lactate solution is made ready and then wheat grass silage is soaked in the Ringer Lactate solution.

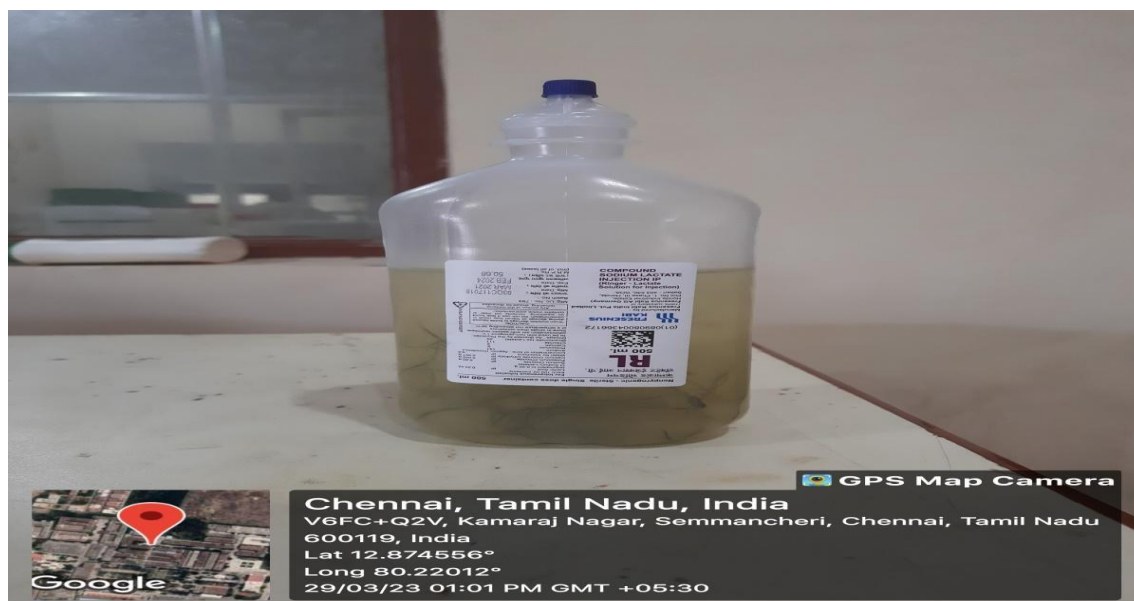


FIG. 5.29 Wheat grass silage soaked in Ringer lactate solution.

Microorganisms grown from wheat grass silage soaked with Ringer Lactate solution are cultured using spread plate technique in different selective media.



FIG. 5.30 Control media for wheat grass silage soaked with Ringer lactate solution.



FIG. 5.31 Culture grown in selective media.

1. **MacConkey agar** – Lactose fermenting lactobacillus is grown with pink, shiny, transparent, round colonies observed.
2. **Sabouraud Dextrose Agar** – Creamy white, opaque colonies are observed as growth of YEAST.
3. **Phenol Red Egg Yolk Polymyxin Agar Base** – white, shiny colonies are observed and purple colonies are not observed which indicates absence of *B. Cereus*.
4. **Eosin Methylene Blue Agar** – Shiny, transparent colonies are observed and absence of Metallic sheen colour so that it is confirmed that absence of *E.coli*
5. **Lactobacillus MRS Agar** - shiny, thick, opaque, round colonies were observed with presence of *Lactobacillus* in it.
6. **Mannitol Salt Agar** – light reddish or pinkish colour colonies as the same colour of the mannitol salt agar is observed as Coagulase-Negative *Staphylococcus Aureus*.

- . The Ringer Lactate solution, containing lactate, is effective in preserving the microorganisms present in wheatgrass silage during anaerobic fermentation.
- Therefore, the microorganisms isolated from the silage, including the coagulase-negative *S. aureus* and gut health improving *Lactobacillus*, are not harmful.
- The main goal of producing silage from wheatgrass is to provide a nutritious feed for cattle.
- Yeasts, which promote the growth of beneficial bacteria that break down plant cellulose in the cattle's first stomach, are also not harmful.
- These bacteria are essential for proper digestion of feed, making wheatgrass silage a highly beneficial feed for cattle due to the microorganisms obtained through anaerobic fermentation.

CHAPTER 6

SUMMARY AND CONCLUSIONS

The main objective of this research is to isolate the Exopolysaccharide producing lactobacillus (EPS) which is a lactic acid bacteria (LAB) from olive brine solution and then growing wheatgrass in sea sand with partially desalinated sea water as the moisturizer and then using the EPS producing lactobacillus as an inoculant for enhancing the growth of the crop. The research hypothesis is to eradicate the soil erosion and drought related problems by using sea sand as an alternative and then to eradicate water scarcity and water pollution related problems using sea water by partially desalination it as a cost effective method. Wheat seeds and olive brine solution are the main source of sample for this research work. The good quality wheat seeds are collected from nearby farms and they are soaked in water for 24 hours or until partial germination. By the way, processing of sea sand and sea water takes place where we process the sea sand by sieving and the sea water by adding potash alum powder in it for overnight purification and then autoclaved for the removal of remaining salt content. The wheatgrass are grown in positive and negative control.. The wheat grass took 15 days to grow at a certain good height and then the wheat grasses are harvested separately and measured for their weight on a weighing scale in order to analyse the yield among the three. The wheat grass grown using EPS lactobacillus inoculant gave more yield compared to the other 2 types of pot. Even the yield is higher for wheat grass is EPS lactobacillus compared to the wheat grass grown with sea sand in another pot without the EPS lactobacillus inoculant which proves that the EPS producing lactobacillus enhances the growth of the wheat grass. The salinity inhibits the growth of the yeast . So no yeast growth was present. The wheat grasses are further made as a wheat grass juice by grinding it and mixing it with sterile water for the Nutritional analysis. The nutritional analysis of wheat grass juice is analysed for 5 parameters such as potassium, magnesium, iron, calcium and phosphorus. We got the results again that the EPS producing lactobacillus as an inoculant in sea sand enhances the nutrients more compared to other 2 types of wheat grasses. After all these analyses of wheat grass we come upon

to confirm the type of bacteria and the morphology and characteristics and their strain of lactobacillus which is producing EPS activity. So certain tests were carried out such as Gram staining, Catalase test, Oxidase test, Biochemical (IMVIC) analysis, antibiotics disk diffusion test and finally Genome sequencing in which we found the LAB producing EPS activity to be *Lactiplantibacillus plantarum*. We compared the LAB grown on both MRS agar and ESM agar for those particular tests mentioned above and their results are represented in chapter 5. The wheat grass is made as silage under anaerobic condition by folding it in a paper for fermenting it under anaerobic condition. The fermented silage looks like dried light blackish brown colour grass. Then it is soaked in Ringer's lactate solution for 2 nights in which the lactate present in the solution preserves the microorganisms present or grown during anaerobic fermentation. Then after 2 nights the Ringer lactate solution is used for culturing in various selective media such as EMB, MSA, MRS, SDA, MYP and MaC. The main aim of silage making is to bring the concept of giving the wheat grass silage as a feed for the cattle. The Lactobacillus and EPS producing lactobacillus are tested for the Antibiotic disk diffusion test using 4 types of antibiotics such as Erythromycin, Chloramphenicol, Tetracycline and Amoxicillin. This test is taken forward with the cause of silage microbiology, while fermenting the wheat grass silage.

In conclusion, our project has provided evidence to support the hypothesis that wheat grass can be successfully grown in sea sand using sea water, provided that an EPS-producing lactobacillus is used as an inoculant to enhance growth and nutrient composition. We isolated the LAB from olive brine solution due to the availability of this source and the longer lifespan of olive trees compared to other sources. The use of LAB as an inoculant was beneficial due to its resistance and tolerance to unfavourable conditions, such as the salinity and pH of sea sand. Additionally, the EPS produced by the LAB further enhanced its resistance to these conditions. This alternative method of using sea sand and sea water as a substitute for farm soil and freshwater, respectively, is promising due to the abundance of these resources on Earth, in contrast to the limited supply of freshwater. The resulting wheat grass silage can serve as a healthy

feed for cattle, promoting gut health through the presence of beneficial microorganisms.

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