

DEVELOPMENT OF PROBIOTIC JUICE FROM SPINACH USING BIFIDO BACTERIUM

Submitted in partial fulfillment of the requirements for
the award of Bachelor of Technology degree in
Biotechnology.

By

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**DEPARMENT OF BIOTECHNOLOGY
SCHOOL OF BIO AND CHEMICAL ENGINEERING.**

SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEMEED TO BE UNIVERSITY)**

Accredited with Grade “ A ” by NACC | 12B Status by UGC | Approved by AICTE

JEPPIAAR NAGAR, RAJIV GANDHI SALAI, CHENNAI – 600119

MARCH – 2021



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DEPARTMENT OF BIOTECHNOLOGY

BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of D. Subahan (37230016), who carried out the project entitled “Development of probiotic juice using spinach from spinach using bifido bacterium” under my supervision from December, 2020 to March 2021.

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DECLARATION

I D. SUBAHAN hereby declare that the Project Report entitled “DEVELOPMENT OF PROBIOTIC JUICE USING SPINACH FROM BIFIDO BACTERIUM” done by us under the guidance of Dr. Usha Nandhini and Dr. Savithiri Shivakumar at the Aaranya Biosciences Private Limited, Chennai is submitted in partial fulfillment of the requirements for the award of bachelor of Engineering / Technology degree in Biotechnology.

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ACKNOWLEDGEMENT

I am pleased to acknowledge my sincere thanks to Board of Management of SATHYABAMA for their kind encouragement in doing this project and for completing it successfully. I am grateful to them.

I convey my thanks to **Dr. Wilson Aruni, (Pro vice-chancellor), Dr.V. Ramesh Kumar, Mr. Narendra Kumar.G, Head of the Department**, Dept. of Biotechnology for providing me necessary support and details at the right time during the progressive reviews.

I would like to express my sincere and deep sense of gratitude to my Project Guide **Dr. Usha Nandhini** for her valuable guidance, suggestions and constant encouragement paved way for the successful completion of my project work.

I wish to express my thanks to all Teaching and Non-teaching staff members of the Department of Biotechnology who were helpful in many ways for the completion of the project.

ABSTRACT

The study is planned to prepare non-dairy based probiotic drink using spinach juice. Bifidobacterium was used as probiotic. phytochemical activity has been analysed; microencapsulation of Bifidobacterium beads is used for bacterial enumeration. Aqueous extract of spinach sample was assessed through gcms analysis.

Key words: - Encapsulation, Enumeration, gcms analysis.

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LIST OF ABBREVIATIONS

KCL	Potassium Chloride
NaOH	Sodium hydroxide
H ₂ SO ₄	Sulfuric acid
BHI	Brain-heart infusion
CaCl ₂	Calcium Chloride
GC-MS	Gas chromatography-Mass Spectrometry

CHAPTER 1

INTRODUCTION

The word probiotic (from the Latin pro and the Greek bios literally meaning “for life”) was introduced by the German scientist Werner Kollath in 1953 to designate “active substances that are essential for a healthy development of life.” In 1965, this term was used by Lilly and Stillwell in a different context to represent “substances secreted by one organism which stimulate the growth of another.” More specifically, Fuller in 1992 defined probiotics as “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance.”

The modern history of probiotics starts at the beginning of 1900s with the pioneering studies of the future Nobel laureate Elie Metchnikoff, a Russian scientist working at the Pasteur Institute in Paris. Louis Pasteur identified the microorganisms responsible for the process of fermentation, whereas Metchnikoff tried first to find out the possible effect of these microbes on human health. (Giovanni Gasbarrini, MD, PhD, Fiorenza Bonvicini, MD, and Annagiulia Gramenzi, MD)

Perhaps the most significant and nutritious vegetable that can be eaten fresh or cooked is spinach (*Spinacia oleracea*). It gives excellent measure of nutrients B6, riboflavin, folate, niacin, dissolvable dietary fiber, omega 3-unsaturated fat and minerals. It is additionally wealthy in iron which forestalls a portion of the infections like osteoporosis, weakness and so forth Spinach is high in beta carotene (master nutrient A), nutrient C, calcium, iron phosphorous, sodium and potassium. Spinach leaves, containing a few dynamic parts, including flavonoids, show antioxidative, antiproliferative, and mitigating properties in organic frameworks. Flavonoids and other phenolic constituents go about as cancer prevention agents by the free-radical scavenging properties of their hydroxyl groups. Broad formation across the flavonoid structure and various hydroxyl group improves their antioxidative properties, permitting them to go about as diminishing specialists, hydrogen-or electron-giving specialists, or singlet-oxygen foragers.

1.1Antioxidant Agents in Spinach: Flavonoids and phenolic compounds have antioxidative activity when spinach is eaten these all parts together controls the impact of free-radicals.

To decrease their harmful impact. All mixtures have antioxidant impact.

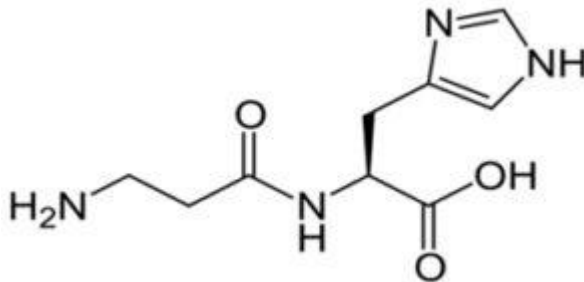
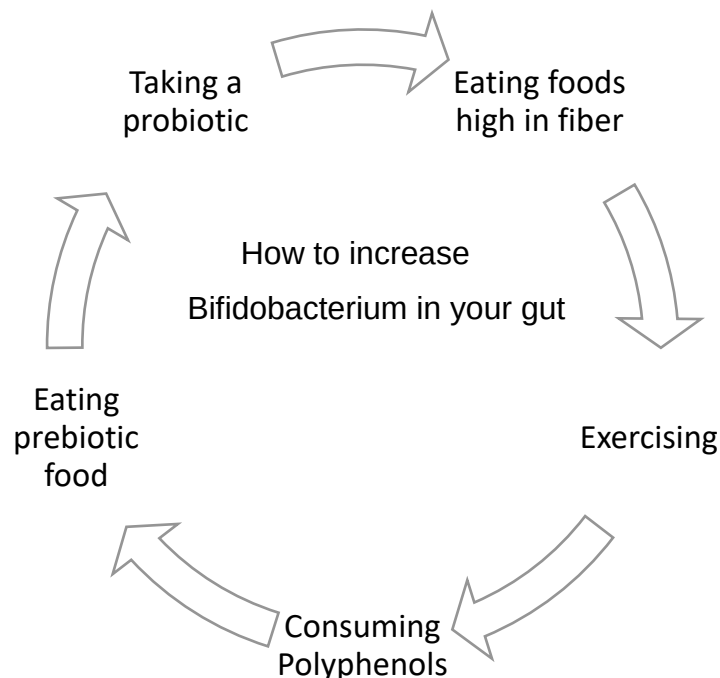


Figure.1.1: CHEMICAL STRUCTURE OF ANTIOXIDANT EFFECTS IN SPINACH.

1.2PROBIOTICS: It is characterized as "live micro-organism" which gives a medical advantage on the host when directed in adequate quantity. The most important source of probiotics for people is dairy-based food sources like milk, yogurt, and so on and non-dairy based food varieties like pineapple juice, beetroot juice and so on Which contains intestinal species like Lactobacillus or Bifidobacterium. Bifidobacterium longum is a probiotic bacterium which is in present in human gut. It can withstand gastric acid and bile. It is a gram-positive microorganism.



1.3. Benefits of Bifidobacterium in the gut: It stops the locking of awful microscopic organisms, it helps control the pH levels of your gut, it keeps awful microorganisms from developing, boosts our immune, It Fights against contaminations, Reduces inflammations in our body, and etc.

1.4. Phytochemical Analysis: Phytochemicals, for example, carotenoids and phenolic compounds, are trivial supplements 102 universally present all through the plant realm, a large number of which are found in spinach. Spinach likewise contains striking measures of phenolic intensifies that are delivered as auxiliary metabolites because of different stressors like bright radiation, low temperatures, microorganisms, and supplement lack. Phenolic intensifies work as cell reinforcements, flagging atoms, antimicrobial, antiviral, and antifungal specialists. Among ordinarily devoured verdant green vegetables, spinach contains ~40% and ~70% a larger number of phenols than cabbage and lettuce, individually. Notwithstanding being an astounding wellspring of phenolics, spinach just contributes roughly 0.8 mg gallic corrosive counterparts (GAE)/day per individual because of low day by day admission (2.5 g spinach/day).

CHAPTER 2

LITERATURE SURVEY

Plants are effectively responding to a variety of environmental stimuli, including gravity, light, temperature, physical stress, water and nutrient availability, plants are also responded to a variety chemical stimulus produced by soil and plant-associated microbes.

Spinach (*Spinacia oleracea*) is good food. It has a good nutrient in a low-calorie. Green leaf's like spinach significant for skin, hair, and bone health. They will give nutrient like protein, iron, nutrients, and minerals. The health benefits of eating spinach it can improving blood glucose control in individuals with diabetes, bringing down the danger of malignancy, and improving bone health, as well as providing minerals and nutrients that can give a scope of various Spinach has been utilized by different societies all through history, strikingly in Mediterranean, Middle-Eastern, and South-East-Asian foods.

Phytochemicals are commonly present in plants and vegetables such as flavonoids, alkaloids, saponins, tannins, phenolic compounds, terpenoids, glycosides, steroids, which are useful for medical uses.

Probiotic has as diary product and non-dairy product, Probiotic are live micro-organism which are helpful for health benefits. *Lactobacillus* and *Bifidobacterium* *Streptococcus*, *Lactococcus* and some species of *Enterococcus* and *Escherichia coli*, are widely used as probiotics. *Saccharomyces* are the most common type of microbe used as probiotic.

Probiotic either taken as eating food high in fiber, exercising, consuming polyphenols, eating prebiotic food, taking a probiotic food.

Microencapsulation is a technique by which little beads or particles of fluid or strong material are coated or covered with a continuous film of polymeric material.

The primary reason for microencapsulation is found to be either for sustained or prolonged drug release. This technique has been widely used for masking taste and

odour of many drugs to improve patient compliance. This technique can be used for converting liquid drugs in a free-flowing powder. The drugs, which are sensitive to oxygen, moisture or light, can be stabilized by microencapsulation. Incompatibility among the drugs can be prevented by microencapsulation. Vaporization of many volatile drugs e.g., methyl salicylate and peppermint oil can be prevented by microencapsulation. Many drugs have been microencapsulated to reduce toxicity and GI irritation including ferrous sulphate and KCl. Alteration in site of absorption can also be achieved by microencapsulation. Toxic chemicals such as insecticides may be microencapsulated to reduce the possibility of sensitization of factorial person. (K. Shekhar*, M. Naga Madhu, B. Pradeep, David Banji 2010).

CHAPTER 3

AIM AND SCOPE

3.1. AIM

To Encapsulate the *Bifidobacterium longum* as a probiotic in spinach juice.

Phytochemical analysis of aqueous extract.

Bacterial Enumeration of beads.

To analyse compounds, present in spinach through gcms analysis.

3.2. SCOPE

The field of probiotics continuous evolve and progress from dairy based product to non-dairy products.

Consuming of probiotic juice improves digestion.

CHAPTER 4

MATERIALS AND METHADODOLOGY

4.1. PHYTOCHEMICAL ANALYSIS OF AQUEOUS EXTRACT

4.1.1. Qualitative analysis of phytochemicals: phytochemicals have great antioxidants. Phytochemicals are seen in fruits and vegetables.

4.1.2. Sample preparation: 100gm/ml of spinach leafs are taken and soaked in hot water, dried, and make it as a juice. Then the juice is filtered using Whatman filter paper and filtrate is used for further tests.

The main phytochemicals are flavonoids, alkaloids, saponins, Tannin, phenolic compounds, terpenoids, glycosides, steroids

4.2. Flavonoids:

It is a group of natural substances with variable phenolic structures, found in fruits, vegetables etc. it has anti-oxidative, anti-inflammatory, anti-carcinogenic properties coupled with their capacity to modulate key cellular enzyme function.

4.2.1 Test for flavonoids:

4.2.1.1. Alkaline reagent test:

2ml of sample(juice) was mixed with 2ml of 20% NaOH; concentrated yellow colour is observed, which become colourless when we add 2 drops of 70% diluted HCl. Disappearance of yellow colour indicates presence of flavonoids.

4.2.1.2Test for alkaloids:

Hager's test: 1ml of sample was mixed with few drops of Hager's reagent. Appearance of yellow precipitate indicates presence of alkaloids.

4.2.1.3Test for saponins:

2ml of sample was mixed with 6ml of H₂O and vigorously shaken. formation of bubbles indicates presence of saponins.

4.2.1.4. Test for tannins:

2ml of sample was mixed with 0.5ml of chloroform and few drops of concentrated H_2SO_4 . brownish blue or black colour is observed

4.2.1.5. Test for phenolic compounds:

2ml of sample was mixed with 2ml of 5% ferric chloride. Blue colour is observed

4.2.1.6 Test for terpenoids:

2ml of sample was mixed with 2ml of 10% alcoholic ferric chloride. Brownish blue or black colour is observed.

4.2.1.7. Test for glycosides:

1ml of sample was mixed with 0.5ml glacial acetic acid and 1% of aqueous ferric chloride. Brown ring formation is observed.

4.2.1.8. Test for steroids:

2ml of sample was mixed with 2ml of chloroform and 2ml of concentrated H_2SO_4 is added. Chloroform layer appears red where acid layer shows fluorescence.

4.3. Quantitative analysis:

4.3.1. Test for flavonoids:

5ml of sample is mixed with 50ml of 80% aqueous methanol at room temperature. Then whole solution was filtered through Whatman filter paper. The filtrate was evaporated and weighed.

4.3.1.2. Test for saponin:

10ml of sample is mixed with 50ml of 20% aqueous ethanol. Then the whole is heated at 55°C on water bath for 4 hours with continuous stirring. Then the solution was filtered and mixed with 100 ml of 20% ethanol and it is reduced to 20 ml in hot water bath at 90°C . Then the concentrated extract was transferred to 250 ml separating funnel and 10 ml of diethyl ether is added and shaken. Ether layer was discarded and aqueous layer is collected and 30ml of n-butanol is added. Then the solution was washed twice

with 5ml of 5% aqueous sodium chloride. Then the remaining solution was heated in water bath. After evaporation the sample was collected, dried in oven and weighed.

4.3.1.3. Test for tannins:

Take 6 test tubes and make the concentration of sample to 1, 2, 3, 4, 5, and 6mg/ml. Then add 1ml of d.H₂O to all the test tubes and 0.5ml of folin's-phenol reagent. Then add 5 ml of 35% of sodium carbonate and kept for room temperature for 5min. Observed for blue colour indicates presence of tannins. Take O.D values at 640nm and drawn a standard graph.

O.D of the standard/concentration of solution = absorbance of test/absorbance of standard x concentration of standard.

O.D of standard = $\frac{2}{4} \times 4 = 2\text{mg/ml}$.

4.3.1.4. Test for terpenoids:

0.1ml sample (Wi) was mixed with 9ml of ethanol and kept for 24 hours. Then the extract was filtered. To extracted sample, add 10ml of petroleum ether using separating funnel. The ether extract was separated in pre-weighed glass vials and waited for its complete drying (Wf). After evaporation the yield of total terpenoids contents was measured by the formula $(Wi - Wf / Wi \times 100)$.

$$\begin{aligned}\text{Total terpenoids (\%)} &= Wi - Wf / Wi \times 100 \\ &= 0.1 - 0.0122 / 0.1 \times 100 \\ &= 88\%.\end{aligned}$$

4.4. Bacterial Strain and Their Inoculation

The *Bifidobacterium longum* strain is collected from the lab and plated 0.1ml of strain into the BHI (Brain-heart infusion) Agar and incubated for 24hours at 37°C. After incubation prepare 10ml of BHI broth and take one loop full of bacterial strain and inoculated into broth and placed in incubate shaker for incubation(24hrs).

4.5. Encapsulation:

Bacteria were concentrated by centrifuging at 15,000 rpm for 30 min. After centrifuging the cells were washed with sterile saline for two times. 2% of Sodium Alginate is mixed with 25ml distilled water in a magnetic stirrer at 50°C. 1% of CaCl₂ is mixed with 50ml of distilled water. 2ml (1×10^{-9}) of cells were mixed with sodium alginate Using a Syringe beads were formed in CaCl₂ containing Petri plate After leaving beads for 30 mins in CaCl₂, beads were washed with sterile saline solution two times. And stored in peptone solution at 4°C.

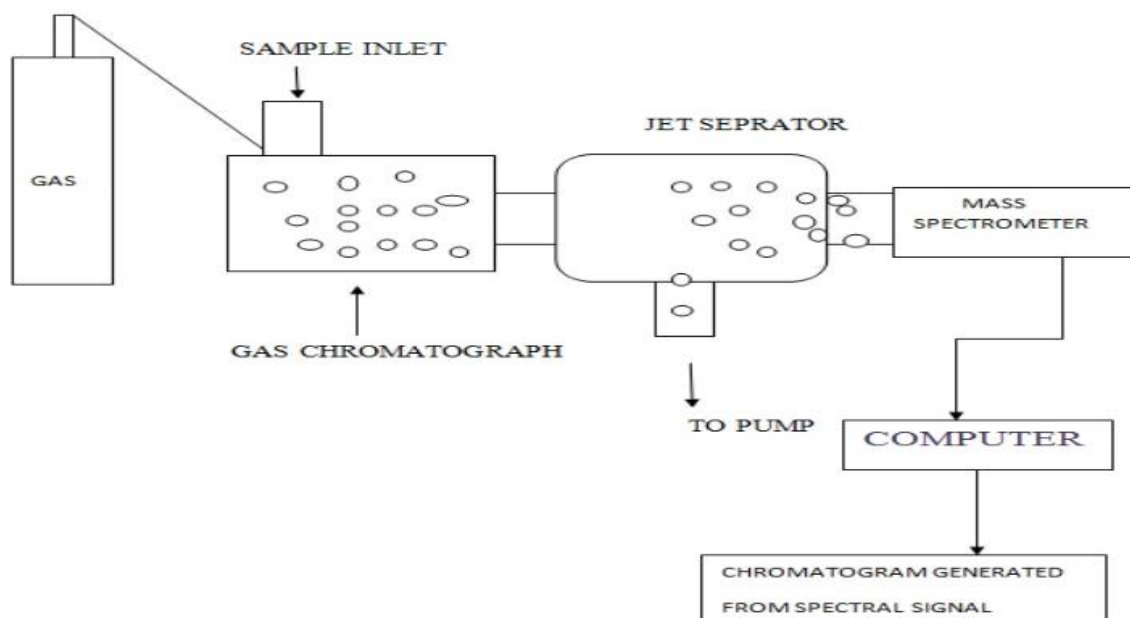
4.6. Bacterial Enumeration of Beads:

One gram of Alginate micro beads encapsulating probiotics were smashed using sterile glass rod in 9ml of 2%(w/v) sterile sodium citrate solution at pH 6.0. Take 1ml from this solution and add into 9ml of LB media and mix. Take 1ml of this into a new 9ml of LB, and continue. The ratio of LB media to cells will be ten times higher than the previous dilution in each new tube. (10^1 10^2 10^3 and so on). Colonies were enumerated following incubation at 37°C for 24hr & 36 hr under anaerobic conditions and comparison was made between the survivability in free and encapsulated cells. 16 colonies have been formed during bacterial enumeration.

Formula for cfu/ml calculation:

$$\text{cfu/ml} = \frac{\text{No. of colonies} \times \text{dilution}}{\text{volume of sample}}$$

4.7. GC-MS Analysis



4.8. Sample preparation: -

50gm/ml of spinach leaves were taken and soaked in hot water, dried, and make it as a juice. Then the juice is filtered using Whatman filter paper and filtrate. Filtered sample was transferred into petri dish for water bath until it dried. Dried powder was transferred into tube and weighed in analytical balance

Total weight of sample: -2.3055gm

CHAPTER 5

RESULT AND DISCUSSION

5.1. PHYTO CHEMICAL ANALYSIS OF AQUEOUS EXTRACT OF SPINACH.



Figure 5.1. Disappearance of yellow colour is not seen so, absence of flavonoids.

5.2. Hager's test for Alkaloids.

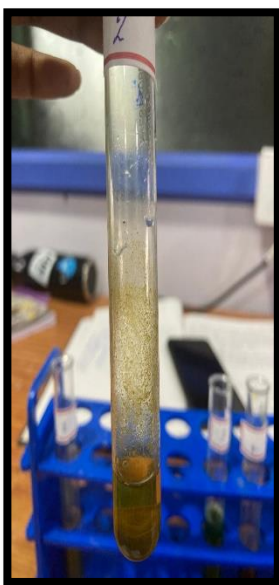


Figure 5.2. Appearance of yellow precipitate indicates presence of alkaloids.

5.3. Test for saponins.

formation of bubbles indicates presence of saponins.

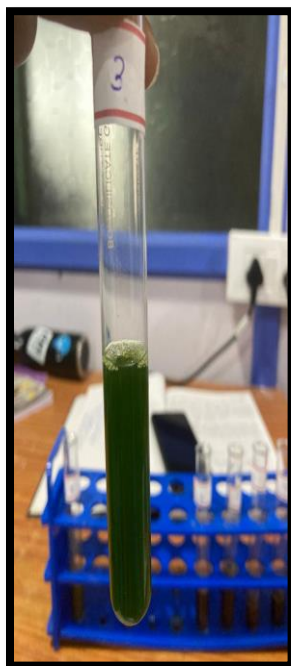


Figure 5.3. presence of saponins.

5.4. Test for tannins.

brownish blue or black colour is observed.



Figure 5.4. presence of tannins.

5.5. Test for phenolic compounds.

No Blue colour is observed



Figure 5.5. Absence of phenolic compounds.

5.6. Test for terpenoids.

Brownish blue or black colour is observed.

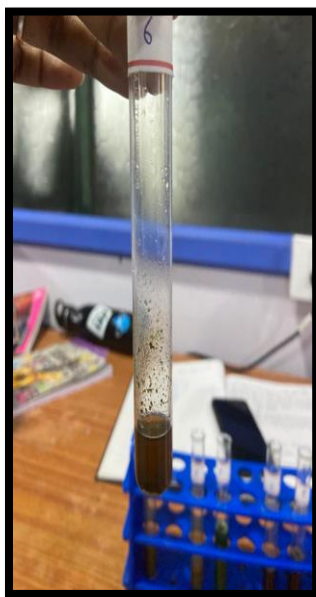


Figure 5.6. brownish blue is observed indicates presence of terpenoids.

5.7. Test for glycosides.

brown ring formation is not seen

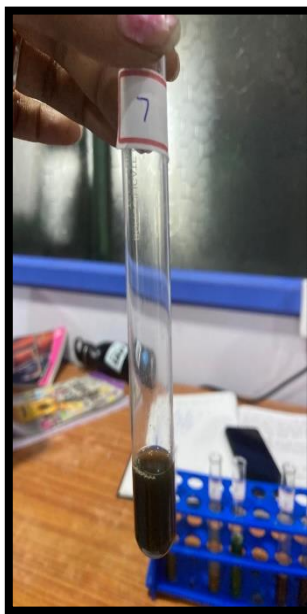


Figure 5.7. absence of glycosides.

5.8. Test for steroids.

Chloroform layer appears red where acid layer shows fluorescence.

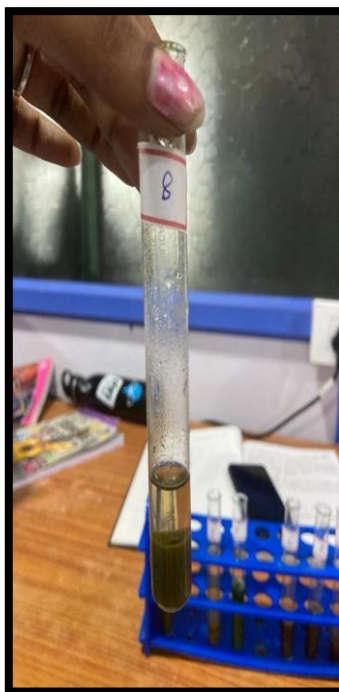


Figure 5.8. Presence of steroids.

5.9. Quantitative analysis of phytochemicals.

In 5ml of sample 0.1g of flavonoids are present.

5.10. Test for saponin.

Observed for blue colour indicates presence of tannins.

Take O.D values at 640nm and drawn a standard graph.

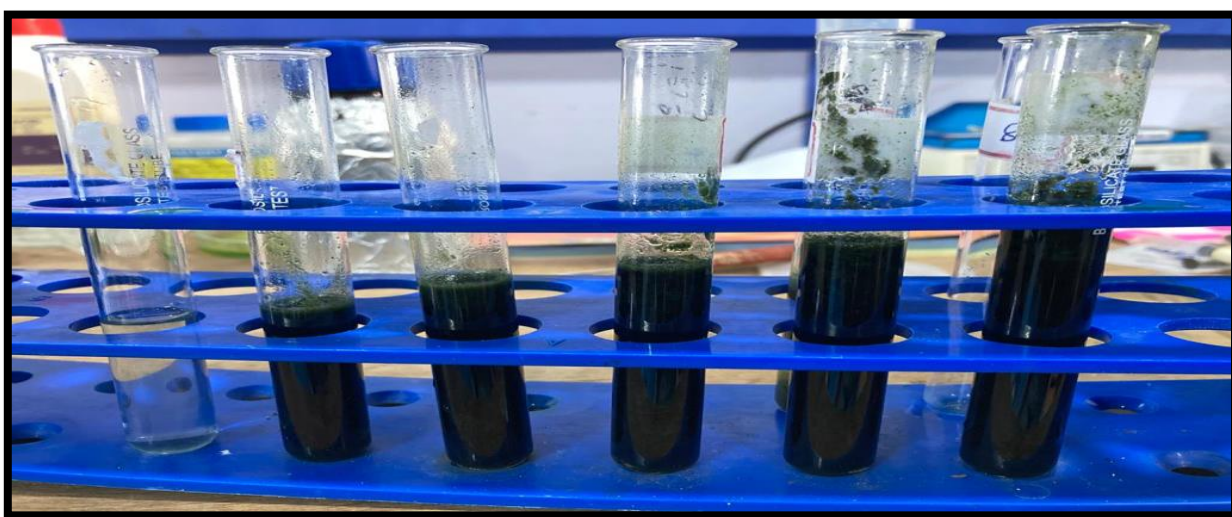
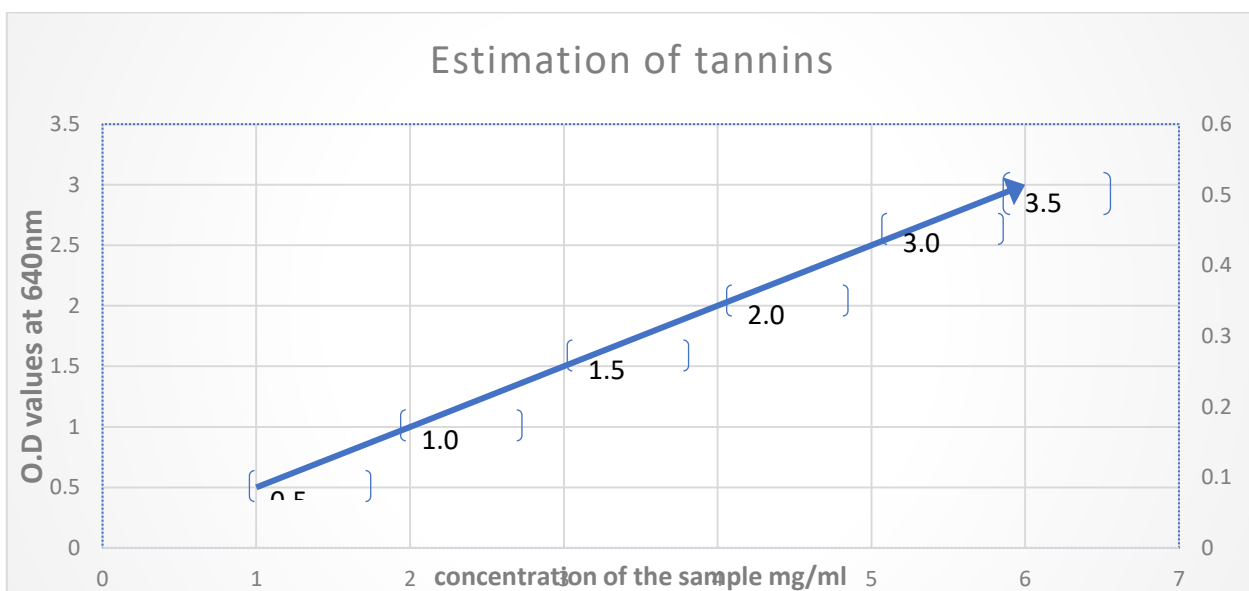


Figure 5.10. blue colour is formed.

5.11. Test for terpenoids.

In 0.1 ml of sample 88% of total terpenoids are present.

Total terpenoids (%) = $W_i - W_f / W_i \times 100$

$$= 0.1 - 0.0122 / 0.1 \times 100$$

$$= 88\%.$$



Figure 5.11. terpenoids are present.

5.12. Encapsulation of Bifidobacterium.

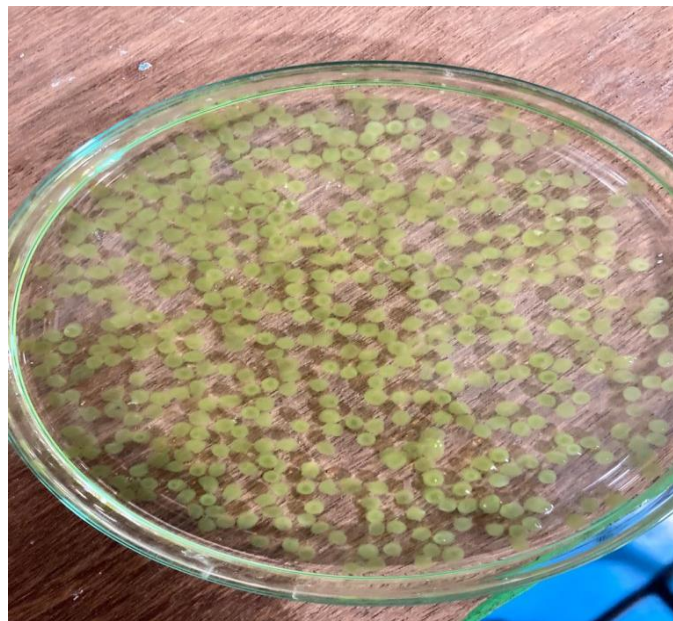


Figure 5.12. Encapsulation of Bifidobacterium.

5.13. Bacterial Enumeration Beads.

Formula for cfu/ml calculation:

$$\text{cfu/ml} = \frac{\text{No. of colonies} \times \text{dilution factor}}{\text{Volume of sample}}$$

$$= \frac{16 \times 10^1}{0.1}$$

$$= 1600 \text{ cfu/ml.}$$

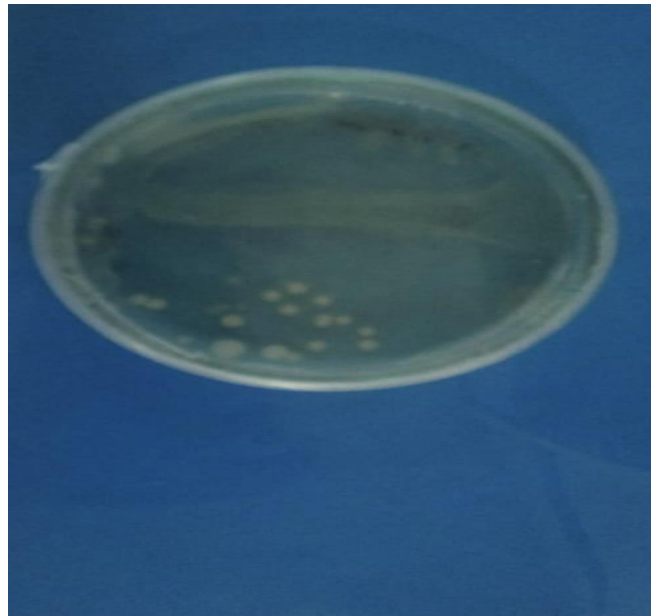


Figure 5.13. 16 colonies have been formed during bacterial enumeration.

5.14. GC-MS Analysis.

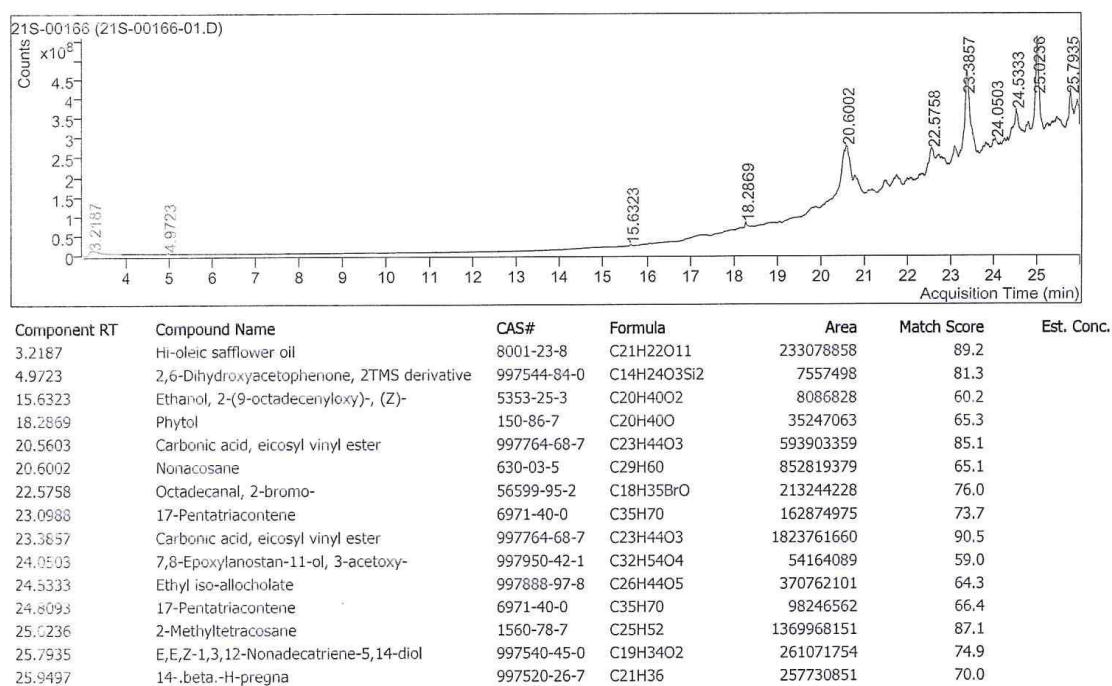


Figure 5.14. Results of a spinach aqueous extract sample.

CHAPTER 6

SUMMARY AND CONCLUSION

Due to the many benefits of probiotics in the absence of any risk, they have become a useful tool for the medical community. They not only boost the functioning of the immune system; they naturally aid in fighting bad bacteria by supplying the body with enough good to keep the body in symbiosis.

Consuming of a spinach includes improving blood glucose control in people with diabetes, lowering the risk of cancer and improve bone health.

The test of phytochemical analysis of aqueous extract of spinach sample which indicates presence in alkaloids, saponins, tannins, terpenoids, steroids, were flavonoids, phenolic compounds, glycosides, are absence in aqueous extract.

Microencapsulated probiotic bacteria were used for inoculation in juice sample.

Bacterial enumeration includes the bacterial cell count. Formula for cfu/ml calculation:

$$\text{cfu/ml} = \frac{\text{No. of colonies} \times \text{dilution factor}}{\text{Volume of sample}}$$

16 colonies have been formed during bacterial enumeration.

The aqueous extract of spinach sample has several peaks which presents different compounds shown in the total ion chromatogram by Gas Chromatography-Mass Spectrometry analysis. The peaks in the chromatogram were integrated and were compared with database of spectrum of known components stored in Gas Chromatography-Mass Spectrometry library.

Gas chromatography-Mass Spectrometry analysis of aqueous extract of *Spinacia oleracea* presence of different Hi-oleic safflower oil, carbonic acid, eicosyl vinyl ester, Phytol, are present.

Plants has a natural phytonutrient which are essential for to life in general. The phytochemical of aqueous extract of *Spinacia oleracea* tell by Gas Chromatography-Mass Spectrometry analysis its importance in pharmaceuticals and cosmetics.

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