Development of probiotic *Psidium guajava* beverage by using *Pediococcus pentosaceus* VNK-1 and its bioactive potential

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Submitted by

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CERTIFICATE

This is to certify that the thesis entitled "**Development of probiotic *Psidium guajava* beverage by using *Pediococcus pentosaceus* VNK-1 and its bioactive potential**" submitted by **Ms. NAVJOT KAUR** (Reg. Number: 602004014), a postgraduate student of **Department of Biotechnology** in partial fulfillment of the requirement for the award of degree of **Master of Technology** in **Biotechnology** at **Thapar Institute of Engineering & Technology, Patiala**, is a record of student's own work carried out under my supervision and guidance. This report has not been submitted for the award of any other degree or certificate in this institute or any other university or institute.

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DECLARATION

I (**NAVJOT KAUR**) hereby declare that the work that has been presented in the thesis entitled "**Development of probiotic *Psidium guajava* beverage by using *Pediococcus pentosaceus* VNK-1 and its bioactive potential**" submitted by me in partial fulfilment of the requirement for the award of the degree of **Master of Technology** in **Biotechnology, Thapar Institute of Engineering and Technology, Patiala**, is an original record of my own work done during the period from August 2021 to June 2022, carried out under the guidance of **Dr. M. Vasundhara**. This dissertation report has not been submitted in part or full to any other university or institute for the award of any degree.

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In life to achieve a success, one should strive for it. Success never comes to us but this is one's hard work to achieve one's goals and touches the height of success. The secret of key to success when one comes out from comfort zone and one's heart, mind and soul collectively focusing on the goals, avoiding and not focusing the obstacles. Behind every successful individual there are several hands that help, encourage and support to achieve one's goal.

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ABBREVIATIONS

%	Percent
°B	Degree Brix
°C	Degree Celsius
µg	Microgram
µl	Microliter
16S rRNA	16 S (Svedberg unit) ribosomal Ribonucleic Acid
3-D	Three-Dimensional
$CaCO_3$	Calcium Carbonate
CCD	Central Composite Design
CFU/ml	Colony Forming Units/ml
CVD	Cardio-vascular Disease
D.W.	Distilled Water
DNA	Deoxyribonucleic Acid
DNSA	3,5-Dinitrosalicylic Acid
DPPH	2,2-Diphenyl-1-picryl-hydrazyl
ELISA	Enzyme Linked Immune Sorbent Assay
EtBr	Ethidium Bromide
G	Gram
GAE	Gallic Acid Equivalent
GYP	Glucose, Yeast extract and Peptone
Kg	Kilogram
LA	Lactic Acid
LAB	Lactic Acid Bacteria
mg	Milligram

Continued…

MHA	Mueller Hinton Agar
ml	Milliliter
MRS	DeMan-Rogosa-Sharpe
NaOH	Sodium Hydroxide
P: C: I	Phenol: Chloroform: Isoamyl-alcohol
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
QE	Quercetin Equivalent
RNA	Ribonucleic Acid
RSM	Response Surface Methodology
SDS	Sodium Dodecyl Sulfate
TFC	Total Flavonoid Content
TPC	Total Phenolic Content

ABSTRACT

Nowadays, the demand for probiotics and their fortification in vegetables and fruit juices have been extensively studied because of wide acceptability by the consumers. Probiotic based non-dairy products have become boon to the current food industries for the development of fortified and functional food products. The consumption of such probioticated products provides essential nutrients that manifest health benefits to the consumers and prevent them from milk-allergies, lactose-intolerance and regulates cholesterol levels in the body. Exploitation of lactic acid bacteria (LAB) for fermenting fresh fruit juices is a sustainable and inexpensive fermentation technology which aims towards increasing the nutritional values and enhancing organoleptic features as well as extending the shelf life of fermented products. Guava is nutritionally rich fruit, containing carotenoids, polyphenols and other antioxidants acting as free radical scavengers and rich source of ascorbic acid and organic acids such as malic and tartaric acid. In this study, a prominent lactic acid bacterium; *Pediococcus pentosaceus* has been isolated from the epicarp of guava fruit that was used for the fermentation of guava fruit juice for the aim of developing probiotic guava beverage. With the aid of morphological, molecular studies and evolutionary studies, the isolated LAB was identified as *Pediococcus pentosaceus* VNK-1 (GenBank Id: ON810480). Fortification of guava juice was done with efficient LAB aiming for enhancing its physicochemical properties. Response surface optimization (RSM) technique was used for optimization of lactic acid (LA) fermentation of guava juice to formulate probiotic beverage. On 4^{th} day, maximum LA production for sample A1 was found as 561.75 ± 1.44 µg/ml, and the microbial cell viability in the juice medium was 3.77×10^9 CFU/ml (9.58 log CFU/ml). While pH, °Brix and concentration of reducing sugar decreased throughout the fermentation period. Thus, natural and fresh guava juice have proven as suitable substrate for the growth of *Pediococcus pentosaceus* VNK-1 for the development of probiotic beverage at 24°C with enhanced phenolics (855.96 ± 12.37 µg GAE/ml), flavonoid content (2001.44 ± 10.59 µg QE/ml) and antioxidant activity (69.79 ± 0.04 %) as compared to the juice samples fermented at 37°C. Additionally, probiotic beverage has been able to diminish the growth of the test bacteria namely *Bacillus megaterium, Staphylococcus aureus* and *Bacillus subtilis* when juice was inoculated with 12% inoculum, incubated at 24°C and agitation provided with 60 rpm. Thus, above properties have shown potential to develop a novel probiotic guava beverage using *Pediococcus pentosaceus* VNK-1 that would be able to exert benefits to the host in terms of gastrointestinal health and increasing the shelf life of the product.

Keywords: Guava; *Pediococcus pentosaceus* VNK-1; Lactic acid fermentation; Lactic acid bacteria; Probiotics; Beverage; Antibacterial; Antioxidant; Phenolics; Flavonoids.

CHAPTER 1

INTRODUCTION

INTRODUCTION

In the present scenario, consumers focus on natural products that are free from any additives/chemicals concerning to their health. The demand for the fruits and vegetable-based products have been rising extensively worldwide which has increased the focus of food industries to develop the innovative functional food products containing probiotics/ prebiotics that prevent the consumers from various health-related disorders or lifestyle diseases conferring the health benefits. Probiotics have proven to exert the potent benefits for improvement of gut health, protection against pathogenic bacteria that cause colonization/ infection in gut mucosa by lowering pH of the gut, modulating and strengthening of immune system, production of bioactive peptides such as bacteriocin that is harmful for various food-borne pathogens and production of short chain fatty acids during microbial fermentation conferring benefits by prevention against inflammation and reduce the risk of onset of colorectal cancer (Hidalgo-Cantabrana et al., 2017; Kechagia et al., 2013). The most important and conventional sources of probiotics are the dairy or milk-based products such as milk, butter, cheese, yogurt, sour milk, whey and casein. But in the last few years, the consumers have shifted their demand towards the food products containing probiotics due to some disadvantages of dairy-based products exerting negative impact on the health due to lactose intolerance, cholesterol levels in the blood and milk-protein-based allergies (Kandylis et al., 2016; Plessas, 2022). Thus, these drawbacks have been tackled by developing additional food products based on the vegetables, fruits and cereals that can be consumed in fermented or unfermented form and act as suitable carriers for the probiotics or substrate for the growth of good bacteria (Ranadheera et al., 2017).

In lactic acid fermentation (lacto-fermentation), lactic acid producing bacteria (LAB) such as *Lactobacillus* species, *Pediococcus* species, *Lactococci*, *Leuconostocs* and *Streptococci thermophilus* have been widely exploited in terms of preserving perishable food products, dairy based products, vegetables and meat by inhibiting the growth of harmful bacterial species leading to increase in the shelf life of the products (Malo & Urquhart, 2016). In Asian countries, LAB has been extensively used to ferment the fruits and vegetables delivering the probiotic function such as *kimchi*, the Korean fermented product investigated with *Leuconostoc mesenteroides* exerting positive effects on gut microbiota leading to improvement of digestion and preventing stomach related disorders (Rhee et al., 2011).

Fruit juice are rich source of nutrients, minerals, vitamins and antioxidant compounds providing the health benefits by reducing risk of onset of disease due to oxidative stress, acting as a preventive measure for type 1 and type 2 diabetes and coronary heart diseases (Ruxton & Myers, 2021). Various studies have shown the lactic acid fermentation of perishable fruits using different types of lactic acid bacteria resulting in enhancement of sensory characteristics, improved phenolic content and antioxidant profile. *Lactobacillus plantarum* have been used for the fermentation of pomegranate juice resulting in the enhancement of organoleptic properties, total phenolics and antioxidant activity and increased production of volatile compounds (Mantzourani et al., 2018). In apple juice fermentation, increased production of phenolic compounds reduces the oxidative stress in mouse macrophages when *Lactobacillus plantarum* ATCC14917 used as starter culture for fermentation (Li et al., 2018).

Poor man's apple of tropics, a captivating name for Guava (*Psidium guajava* Linn.) fruit, belonging of Myrtaceae family, widely cultivated and commercialized in tropical and subtropical regions due to its known bioactive potential and medicinal properties. Guava's epicarp is of yellow or greenish in color and can be broadly classified into two varieties on the basis of the pigments of the flesh/pulp that develop during the ripening of the fruit; white or pink fleshed cultivars (Bhat et al., 2015). Guavas are nutritionally rich source of dietary fibers and high antioxidant profile due to presence of carotenoids, polyphenols, vitamins and minerals (magnesium and phosphorus). In addition to this, fermentable sugars are present in higher amounts particularly glucose, fructose and sucrose (Jiménez-Escrig et al., 2001). Higher amounts of pectin and lower sweetening, tart and sour taste make it unpleasant to consume the raw guavas directly. Shelf life of the guava is compromised due to damage occurred in chilling conditions, mechanical injuries and/ or spoilage occur due to decomposition by bacteria or fungal species. Thus, for reducing post-harvest losses and in order to extend the shelf life and for retaining its nutritional and economic values, various products have been developed from guava fruit. Some of these products are guava juice, concentrated purees, jams, syrups, pastes, soft drinks, gummy jellies, ice-creams and fermentation based products such as wine prepared using fermentation of guava juice and currently lactic acid fermentation based products (Bhat et al., 2015; Jain & Asati, 2004; Palachum et al., 2020).

Bacteria have been extensively used for the guava fruit fermentation in order to develop probiotic drinks. *Lactobacillus plantarum* has been used for the development of guava juice based probiotic

drink with enhanced antioxidant potential and increased cytotoxic activity against prostate and breast cancer cell lines (Dipjyoti et al., 2015). Various *Lactobacilli* species such as *Lactobacillus paracasei*, *Lactobacillus plantarum, Lactobacillus fermentum* and *Lactobacillus casei* have been exploited for studying the effects of sub-merged fermentation leading to development of fermented product. These fermented product have enhanced physicochemical properties including phenolics and flavonoid content and enhanced antioxidant activity in acerola (*Malpighia emarginata* D. C.) and guava (*Psidium guajava* L.) fruits (de Oliveira et al., 2020).

Particularly, *Pediococcus* species are mostly used LAB nowadays, in food industries for development of fermented products. This, gram positive bacteria produces a bioactive peptide pediocin that can be used against food-borne pathogens (Porto et al., 2017). Fruit juices are considered to be the efficient carriers for the probiotics in order to develop the functional foods that are safe for the consumption. Recent study reported the used of Mango juice, which was pro-bioticated with *Pediococcus pentosaceus* and *Pediococcus acidilactici* with enhanced antagonistic activity against test pathogenic bacteria. In addition to this improved lactic acid production and vitamin C content was also reported in the study (Adebayo-Tayo et al., 2021).

Optimum experimental conditions are required for the fermentation in such a way that probiotic bacteria grow at its maximum pace for the development of fermented functional product possessing enhanced physicochemical characteristics. The experimental factors, parameters and their response must be validated in the form of experimental designs that can be accomplished using response surface methodology (RSM).

Hence, the aim of this research study is to develop a probiotic guava beverage using prominent *Pediococcus pentosaceus* VNK-1, a probiotic lactic acid bacterium, providing a novel functional product to cope up with human health related diseases and providing health benefits to the host. By optimizing the parameters for lactic acid fermentation using RSM- central composite design (CCD) in design expert software optimum conditions were obtained. Further evaluations were performed on the probiotic guava juice such as pH, total soluble solids, lactic acid production, antioxidant potential, total phenolic content, total flavonoid content, reducing sugar analysis, antimicrobial activity and sensory analysis for the better understanding of the formulated product which was fermented at optimized temperature (24°C) and its comparison with 37°C temperature for 4 days. This application based probiotic formulation with *Pediococcus pentosaceus* offers the

advantage of extending the shelf life of the guava juice by diminishing the growth of pathogenic microorganisms and also, beneficial for consumers facing intolerance to dairy- based products and a boon for the beverage industry in today's competitive market.

OBJECTIVES

The objectives of the research project are given as follows:

1. Isolation and characterization of microorganism for guava juice fermentation.
2. Optimization of parameters for lactic acid fermentation using RSM.
3. Evaluation of guava based probiotic beverage.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Functional food and beverages

Beverage is also called a drink in liquid form consumed by humans for satisfying thirst starting from the drinking water, fruit juice, milk, soft drinks, smoothies till the warm drinks such as tea, coffee, caffeinated drinks. In order to meet the fundamental need of human, which is hydration, the development of different varieties of beverages have been taken place over the period of many centuries. Due to increase in the demand of consumers for the drinks, beverage industries have become enormous and extensively diversified. Conventional drinks or fruit juice have been extended into ready-to-drink beverages or soft drinks. Carbonated beverages contain the basic constituents such as water, flavoring and coloring agents, sweeteners, acids and preservatives. Besides this, to provide the sparkling effect or fizziness sensation in mouth to make the drink pleasant, carbon dioxide is dissolved in a liquid under high pressure (Ranadheera et al., 2020).

There are different types of beverages given as follows (Galanakis, 2019):

2.1.1 Alcoholic beverage: This type of beverage has varying concentrations of alcohol along with its other constituents. Alcoholic beverages can be categorized into two types. Beverages that are prepared by fermentation which is biological and anaerobic process where carbon source such as sugars converts into ethanol and CO_2 by the yeast species mainly the *Saccharomyces cerevisiae*. Examples of alcoholic beverages are wine, beer, cider. The second category of alcoholic beverages is based on the distillation process where fermented liquid is distilled to form the distilling liquor, such as whiskey, vodka, brandy, tequila (Galanakis, 2019). Alcoholic drinks such as beer is highly popular among the population, as it has sensory and nutritional values and used for medicinal purpose. Studies have shown that low alcoholic content beverages have good impact on the health providing nutrients to the body such as anti-cancerous, protecting against cardiovascular diseases, stimulating the immune system, prevention from dementia and acting against osteoporosis.

On the other hand, excessive consumption results into the various health disorders such as obesity, diabetic, induction of mutations and increase risk of cancer (Sohrabvandi et al., 2012).

2.1.2 Non-alcoholic beverage: An alcohol-free beverage is a type of beverage that are free from alcohol.

2.1.3 Carbonated-cola: These drinks are mostly liked by the consumers all around the world. In 1863, A. Mariani, a French pharmacist developed the formula of cola drinks for the very first time and Vin Mariani brand of alcoholic coca-wine beverage constituting of wine and cocaine was developed. Now a days, cola-drinks are consisting of blending of carbonated water, flavoring substances caramel for the color, sugars or artificial sweeting agents along with various acidulants or acids such as carbonic, citric or phosphoric acid to give a sour, tangy or tart flavor in soft drinks as well as acting as preservatives to prevent or reduce the growth of bacteria and mold/fungi aiming to increase the shelf life of the soft drinks. Recent study have shown the strong and consistent relationship between the consumption of soft drinks and its impact on health (Vartanian et al., 2007). There is a direct association has been established in the soft drinks consumption that results in the increased energy intake and gaining of the body weight. Based on the fact that, soft drinks provide energy with less nutrients available to body, which may also result in health issues e.g., diabetes.

2.1.4 Dairy based beverages: Milk is rich source of minerals, vitamins, antioxidants, fatty acids, lactose and acting as a probiotic, consumed widely all around the world. Dairy beverages are enriched with calcium, potassium and magnesium and vitamin sources such as vitamin A, vitamin D and vitamin B12 that acting as a functional beverage providing the physiological benefits to the consumers. Dairy beverage supplementary with vitamins named as Dairy Land Milk-2-Go R (Canada) and milk beverage rich in minerals available in the Japan named as Meiji Love R, and India's Nestle Milo - Cocoa-Malt Milk Beverage, rich in vitamins & minerals (Grumezescu & Holban, 2019). Consumption of dairy products fortified with vitamin D reduces the risk of deterioration of bone structure that occurs due to deficiency of vitamin D, as vitamin D facilitates in increasing the absorption of calcium in intestine (Polzonetti et al., 2020).

2.1.5 Energy-based beverages: Energy drinks are functional beverages providing the physiological benefit to the consumers by enhancing strength and performance as well as improving focus and stamina. The basic constituents of such drinks are sugar, stimulants (caffeine), non -nutritive stimulants such as guarana acting as antioxidant agent, ginseng provide mental and physical benefits and stimulates immune system, yerba mate possess the anti- cancerous activity, taurine possess anti-inflammatory activity, and inositol has a

function of lowering cholesterol level, for cellular functioning and maintaining metabolism, vitamins and minerals are essentially required in these beverages (Grumezescu & Holban, 2019; Gunja & Brown, 2012; Heckman et al., 2010). Commercially available energy beverages are red bull (Austria), monster energy beverage originated from California United states.

2.1.6 **Sports beverages:** These are the drinks containing the basic components such as sugar (glucose, fructose, sucrose), vitamins and electrolytes (calcium, magnesium, sodium, potassium, chloride and phosphates). These drinks are consumed during physical exercise providing nutritional benefit, as well as to boost energy and prevent from dehydration (Grumezescu & Holban, 2019).

2.1.7 Functional beverage is the part of functional food that contain some specified bioactive compounds that provide health benefits beyond their basic nutritional values. Such as beverage with antioxidants, phenolics, flavonoids, dietary fibers, probiotics etc.

2.2 Intolerance to Dairy products lead to development of non-dairy probiotic products

Milk has disaccharide reducing sugar called lactose ($C_{12}H_{22}O_{11}$) composed of D-glucose and D-galactose linked by β-1,4-glycosidic linkage. In the infants and adults, the health risks related to consumption of milk-based products have been raised these days due to lactose malabsorption or lactose intolerance occurs when consumers are unable to digest lactose due to failure of its conversion into glucose and galactose as if inactive or low levels of lactase enzyme is present (Szilagyi & Ishayek, 2018).

Developing allergies to cows' milk proteins, 80% of which are caseins e.g., alpha S1-casein protein and whey protein contributing 20% in the milk such as alpha or beta-lactoglobulins that are recognized as foreign antigen by the immune system (Odedra, 2015), and excessive fat and cholesterol in milk that is responsible for onset of cardiovascular diseases particularly increasing cholesterol in blood plasma called as hypercholesterolemia (Vijaya Kumar et al., 2015).

Unacceptance of dairy products due to their intolerance and negative impact on health, have led to the development of non-dairy probiotic fermented products out of which fruits, vegetables, soy and cereal based fermented products have gained great importance on the basis of high dietary fibers, antioxidants and confers better digestion (Vijaya Kumar et al., 2015).

2.3 Probiotics and its benefits to human

The term probiotic has been derived from two Greek words “pro” and “bios” meaning “for life” first coined in 1907 by Metchnikoff (Moradi Moghaddam, 2011).

In 1954, this term described by Ferdinand Vergin in his article explaining comparison of benefits provided by useful bacteria and harmful effects of antibacterial agents and antibiotics on human body (Markowiak & Śliżewska, 2017).

In 1965, Lilley and Stillwell explained the word “probiotics” as the microorganisms secrete some useful substances that have positive effect on the growth of other microorganisms (Fuller, 1992). The United Nations Food and Agriculture Organization (FAO) and the World Health Organization (WHO) in 2001 derived the definition for probiotics which can be explained as living microorganisms provide health benefits to the host body once it is ingested in sufficient amounts. Probiotic bacteria are widely known as “good bacteria” that are friendly to the body because these confers various potential health benefits to the body and human intestine.

In last few decades, probiotic bacteria have extensively accepted on the basis of scientific evidences proving their positive effects on human body. Eventually, probiotic bacteria have been used extensively in the manufacturing of various food products and functional foods such as probiotic beverages, infants’ food and also find in milk-based products. In this era, probiotics have become boon to the food industries for providing health benefits such as preventing from intestine or bowel infections, improving the immunity, balancing of gut microbiota, control the levels of high cholesterol in the blood, lowers hypersensitivity (Markowiak & Śliżewska, 2017).

There are various strains of *Bifidobacterium* and *Lactobacillus* genera that have been proved to be safe for human consumption. Several other microorganisms such as *Aspergillus niger*, *Saccharomyces cerevisiae*, and some strains of bacillus genera considered as potential probiotics. Some commercially used bacillus strains as probiotics are *Bacillus coagulans*, *Bacillus licheniformis*, *Bacillus polyfermenticus*, *Bacillus pumilus*, and *Bacillus subtilis* as these strains possess the anti-cancerous, antimicrobial, antioxidant and production of vitamin in the body but safety concern rises when such probiotics produces enterotoxins, biogenic amines and can transfer genes involved in antibiotic resistance (Lee et al., 2019).

2.3.1 Anti-cancerous activity

In the past few years, cancer mortality rate is arising steadily and has become health issue worldwide. Among various types of cancers such as bladder carcinoma, breast, kidney, lung, colorectal cancer. Probiotics have been proved as anti-colorectal cancer in recent studies. In addition to this, lactic acid bacteria (LAB) and their metabolic by-products have been proved to combat the cancer disease by inhibiting mutagenic activity, inactivating enzymes involved in tumor stimulating pathways, and regulation of tumor suppressor genes.

Consumption of probiotics and functional food plays a crucial role against the gastro-intestinal cancer and reduces the occurrence of post-surgery inflammation (Stavropoulou & Bezirtzoglou, 2020). In vitro studies, *Bifidobacterium adolescentis* SPM0212 bacterial strain shown anti-proliferative activity against HT-29, SW 480, and Caco-2 human colon cancer cell lines (Kim et al., 2008). Inhibition of proliferation and apoptosis by *Lactobacillus paracasei* IMPC2.1 and *Lactobacillus rhamnosus* GG strains have been studied in human gastric and colonic cell lines respectively (Orlando et al., 2012). LAB is involved enhancing immune system response and release of antimutation and antitumor substances and degrade potential cancer-inducing agents (Hirayama & Rafter, 1999).

2.3.2 Probiotics as immunomodulatory

Recent studies have reported the role of probiotic bacteria in activation of the immune system when certain probiotics administered in the animal models. Early innate immunity was activated by strain of *Lactobacillus paracasei* interacting with the lining of epithelial cells that conferred the activation and recruitment of cytokine named as tumor necrosis factor-alpha (TNF-α) and phagocytic cells at the site of infection (Ashraf & Shah, 2014).

2.4 Fruits and Fruit Juices

Fruits and fruit juices are well known for their rich nutritional values as antioxidants, essential minerals such as sodium, potassium, magnesium and vitamins including vitamin A and vitamin C, carotenoids, dietary fibers, polyphenolics and other phytochemicals possessing bioactive potential, are present in appreciable quantity (Benton & Young, 2019; Slavin & Lloyd, 2012). In regards to

these nutritive qualities of fruits and fruit juices, U. S. Food and Drug Administration (FDA) a public health authority, has consistently increased the recommendation on consumption of such nutritionally abundant fruits and fruit juices in concern with the public health as studies have been reported for decreasing the onset of diabetes, cardiovascular diseases (CVD) and other metabolic disorders on increased intake of fruits and fruit juices (Agarwal et al., 2019; World Health Organization, 2009). On the contrary, low consumption of these would result in increased risk of onset of metabolic disorders, chronic disease leading to poor health. One of the study reported the lower risk of heart strokes by 20-24%, when pure fruit juices were consumed (Scheffers et al., 2019). Also, intake of 500 ml of fruit juice lowers the risk of heart diseases by improving vascular function, thus reducing blood pressure (Ruxton & Myers, 2021). In contrast with the consumption of whole fruit, studies recommended the 100% fruit juice consumption for possessing high nutritional value and improved diet quality excepting the higher fiber content as present in fruits itself (Agarwal et al., 2019). Consumers in all around the world have started preferring the fruit juices, fruit nectars with concentrated pulp and ready-to-drink beverages (Plessas, 2022) as these are the vital source of nutrition and well accepted in taste especially due to natural free sugars, with less or no additives and/or preservatives present. Therefore, the demand has shifted towards natural food and their products conferring the healthy lifestyle.

On the contrary, in previous studies, it has been reported that unpasteurized fruit juices are prone to contamination by the presence of various food associated pathogenic bacteria on the surface of the fruits such as *Escherichia coli* O157:H, *Salmonella* and *Shigella* species have ability to survive in lower pH around 3.3-4 in apple juice and tomato juices at lower temperatures. Presence of *Staphylococcus aureus* has also been investigated in fruit juices (Aneja et al., 2014). Numerous food borne outbreaks came into picture (1921-2010) due to consumption of contaminated fruit juices leading to global health issue. The major concern for the notable spoilage of food by various microbes such as thermally stable molds, *Pseudomonas*, *Aspergillus* species and *Alicyclobacillus* (Snyder & Worobo, 2018). Due to the rich nutritional content, fruit juices are the suitable medium for the growth of various pathogenic bacteria. Thus, fruit juices become susceptible to deterioration and spoilage resulting in the decreased shelf life to less than a week at lower temperatures (4-7°C). Contamination and spoilage of fruit juice reduce the economic value by affecting its cost (Ashurst, 2016). Thus, contamination in the fruit juices can be controlled by various methods such as addition of preservative/chemicals widely used are $C_6H_7KO_2$ (Potassium sorbate), $C_7H_5NaO_2$

(Sodium benzoate), $Na_2S_2O_5$ (Sodium metabisulfite) and KMS (potassium metabisulfite), thermal pasteurization, pulse electric field, ultrasound and hydrostatic pressure and irradiations (Aneja et al., 2014; Rojo et al., 2015).

As consumers nowadays, have focused on the chemical/preservative free food and food products that can be safely consumed providing benefit to the host body. The possible and cost-effective alternative for extending the shelf life of fruit juices and to meet the needs and preferences of consumers, is LA fermentation by probiotic LAB. Extensive research has been going on for the fermentation of different fruit juices by various genera of LAB.

In current scenario, presence of probiotics in fruit juices formulating it into functional fruit juices that provides nutritional benefits beyond their basic nutritional values. Such admirable attributes of the fermented fruit juices have seek the attention of consumers worldwide (Bujna et al., 2017). LA fermentation has proven to enhance the nutritional qualities of fruit juices by production of vitamins, enhancing polyphenols that acting as antioxidants after metabolic bioconversion into bioactive compound providing protection against CVD, colorectal cancer, cataract and boost immunity. In addition to this, probiotic bacteria possess ability to degrade large lipid molecules and lowers the cholesterol level in blood (Markowiak & Śliżewska, 2017; Sybesma & Hugenholz, 2004). The concept of fermentation of fruit juices by LAB has become the boon for today's food industries, concerning about the lifestyle-related issues due to lactose intolerance and allergies to dairy products in consumers. In leu of the usage of dairy-based products, the fruit juice fermentation has become pronounced concept of developing fermented fruit juice products. Thus, fruit juices are considered as appropriate probiotic "vehicles" due to-

(i) rich in nutritional values;
(ii) deliver positive effects for maintain mental and physical well-being;
(iii) widely accepted and consumed by consumers in all around the world for their natural flavor and nutritional attributes
(iv) acting as attractive and suitable substrate for the flourish growth of probiotics (Plessas, 2022).

The wide variety of fruit juices have been used for the development of probiotic fermented product by using potential LA bacteria (**Table 1**).

Table 1: Fruit juices fermented by potential microorganisms for probiotic products' development

S. No.	Fruit type	Scientific name	Microorganism used/Starter culture/Probiotic strains	Beneficial impact/ Positive activities	References
1.	Guava (pulp)	*Psidium guajava*	*Lactobacillus plantarum* WU-P19	Formulated gummy jelly enriched with vitamin B12 and conjugated linoleic acid.	(Palachum et al., 2020)
2.	Guava (Juice)	*Psidium guajava*	*Lactobacillus plantarum*	Formulated probiotic beverage developed possessing antibacterial, antioxidant potential and anti-cancerous activity against breast and prostate cancer cell lines.	(Dipjyoti et al., 2015)
3.	Guava (Fruit extract)	*Psidium guajava*	*Lactobacillus plantarum* NCIM 2912	Fermented product possessed enhanced antioxidant potential, high phenolic content and short and medium chain fatty acids.	(Bhat et al., 2015)
4.	Banana and red guava (Juice)	*Musa acuminata* and *Psidium guajava*	*Lactobacillus casei*	Increased the viability of LAB and antimicrobial activity against *E. coli*, *Klebsiella* species and *Salmonella* in both fermented juices.	(Dalu et al., 2019)

S. No.	Fruit type	Scientific name	Microorganism used/Starter culture/Probiotic strains	Beneficial impact/ Positive activities	References
5.	Pomegranate (Juice)	*Punica granatum* L.	*Lactobacillus plantarum, L. delbruekii, L. paracasei, L. acidophilus*	Probiotic pomegranate juice showed survivability of *L. plantarum* and *L. delbruekii* were capable during storage conditions at 4°C.	(Mousavi et al., 2011)
6.	Pomegranate (Juice)	*Punica granatum* L.	*Lactobacillus plantarum* PU1	Enhanced antioxidant activity and phenolic content by 40 % and 60 % respectively.	(Pontonio et al., 2019)
7.	Pomegranate (Juice)	*Punica granatum* L.	*Lactobacillus plantarum* ATCC 14917	Functional juice found with high levels of volatile compounds, antioxidant potential and increased TPC.	(Mantzourani et al., 2018)
8.	Blue berry and carrot juice blend	*Vaccinium sect. Cyanococcus* and *Daucus carota*	*Lactobacillus reuteri* LR92	Elevated levels of antioxidant activity with microbiological stability during storage period.	(Mauro et al., 2016)
9.	Fig (Juice)	*Ficus carica*	*Lactobacillus casei*, *Lactobacillus plantarum* and *Lactobacillus delbrueckii*	Enhanced taste, odor and organoleptic properties of juice and maintain the survivability of LAB during storage of two weeks.	(Khezri et al., 2016)

S. No.	Fruit type	Scientific name	Microorganism used/Starter culture/Probiotic strains	Beneficial impact/ Positive activities	References
10.	Carambola, guava, mango and pitaya	*Averrhoa carambola* L., *Psidium guajava*, *Mangifera indica* L. var. Tommy Atkins, *Hylocereus undatus*)	*Lactobacillus casei*, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*	Enhanced sensory characteristics and nutritional value.	(Maldonado et al., 2017)
11.	Pomegranate juice	*Punica granatum* L.	*Lactobacillus plantarum* POM1 and *Lactobacillus plantarum* C2	Elevated levels of antioxidant activity, antimicrobial and enhanced sensory properties of fermented juice.	(Filannino et al., 2013)
12.	Cantaloupe/ rockmelon juice	*Cucumis melo* var. cantalupensis	*Lactobacillus casei*	Probiotic fermented drink able to maintain survivability of LAB upto 42 days.	(Fonteles et al., 2012)
13.	Mango	*Mangifera indica*	*Levilactobacillus brevis* (MLB), *Lacticaseibacillus casei* (MLC), *Lacticaseibacillus rhamnosus* (MLR), *Lactiplantibacillus plantarum* subsp. *plantarum* (MLP), and *Pediococcus pentosaceus* (MPP)	Elevated levels of volatile compounds and enhanced sensory characteristics.	(Mandha et al., 2022)
14.	Melon and orange (Juice)	*Cucumis melon* and *Citrus sinensis*	*Lacticaseibacillus rhamnosus*	Elevated levels of aromatic compounds recovered and enhanced floral and fruity notes.	(Hadj Saadoun et al., 2021)

S. No.	Fruit type	Scientific name	Microorganism used/Starter culture/Probiotic strains	Beneficial impact/ Positive activities	References
15.	Sweet lemon juice	*Citrus limetta*	*Lactobacillus plantarum* LS5	Increased production of lactic acid and enhanced antioxidant profiles, antibacterial activity in fermented juice.	(Hashemi et al., 2017)
16.	Ginkgo kernel	*Ginkgo biloba* L.	*Lactobacillus acidophilus*, *Lactobacillus plantarum* and *Lactobacillus casei*	Lactic acid production increased, phenolic, terpenes and lactones were enhanced. TPC content enhanced by 9%, enhancement in antioxidant and antimicrobial activity.	(Wang et al., 2019)
17.	Sweet cherry	*Prunus avium* L.	*Pediococcus pentosaceus* SWE 5 and *Lactobacillus plantarum* FP3	Enhanced phenolics and antioxidants, improved color appearance and organoleptic attributes, microbial cell viability maintain upto during 60 days of storage.	(Di Cagno et al., 2011)
18.	Avocado fruit	*Persea americana* Mill.	*Lactobacillus plantarum* AVEF17	Increased antioxidant activity due to bioconversion of fatty acid.	(Filannino et al., 2020)
19.	Cherry and broccoli (Juice and puree respectively)	*Prunus avium, Brassica oleracea* var. italica	*Lactobacillus fermentum* FUA3165 and *Lactobacillus reuteri* FUA3168	Elevated levels of phenolic compounds.	(Filannino et al., 2015)

S. No.	Fruit type	Scientific name	Microorganism used/Starter culture/Probiotic strains	Beneficial impact/ Positive activities	References
20.	Apple (Juice)	*Malus pumila*	*Lactobacillus plantarum* 90, *Lactobacillus helveticus* 76, *Lactobacillus casei* 37, *Lactobacillus paracasei* 01, *Lactobacillus acidophilus* 85 and *Bifidobacterium lactis* 80	Better flavor due to volatile compounds and improved antioxidant activity.	(Wu et al., 2020)
21.	Mulberry (Juice)	*Morus alba*	*Lactobacillus plantarum, Lactobacillus acidophilus* and *Lactobacillus paracasei*	Improved phenolic profile and increased antioxidant activity.	(Kwaw et al., 2018)

2.5 Different strains of *Pediococcus pentosaceus* employed in nutritionally rich fruit juices' fermentation

Pediococcus pentosaceus is a promising gram-positive LAB that is currently known for their important characteristic features such as production of extracellular peptides that are acting against pathogenic bacteria, fermentation of fruits and vegetable juices for enhancing nutritional values. Therefore, *Pediococcus pentosaceus* is widely accepted by consumers as a probiotic bacterium and have become boon to the food industries for production of successive probiotic products increasing the economic value. Various strains of *Pediococcus pentosaceus* have been investigated for possessing anti-inflammatory potential, anti-cancerous activity, property of scavenging the free radicals thus, acting as antioxidants, detoxification of reactive oxygen and nitrogen species generated in the body due to metabolic by-products and lowers lipid content conferring health benefits to the host (Jiang et al., 2021).

Pediococcus pentosaceus and *Pediococcus acidilactici* have been used for formulation of functional mango juice product as incorporation of probiotics in fruits and vegetables have raised the demand among consumers by providing prevention from health-related diseases and easy for digesting such probiotic product to the ones facing dairy product related allergies. During fermentation hours, increased lactic acid production (17.75–19.10 mg/L and 19.28–20.54 mg/L) and content of vitamin C (41.35–48.77 and 44.59–48.59) for both of the strains was observed with enhanced iron, calcium and sodium concentration except for magnesium. Probiotic mango product has shown strong antagonistic activity against food borne pathogens named as *Escherichia coli*, *Bacillus cereus, Pseudomonas aeruginosa*, and *Klebsiella pneumonia* with enhancement in aroma, color and sensory attributes at 4°C under storage conditions (Adebayo-Tayo et al., 2021). Fruit extract of black raspberry, has been proved to another suitable medium for growth of *Pediococcus acidilactici* M76 leading to development of fermented product with enhanced lactic acid production. This product was able to elicit increased expression of cytokines conferring the effective immune response. In addition to this product was found with reduced levels of flavonoid content and anthocyanins, elevated levels of polyphenols, thus increasing free radical scavenging activity, ABTS scavenging activity and enhanced the flavoring compounds by lowering the flavors due to terpenes during the hours of fermentation (Song et al., 2021). LA fermentation was performed for the evaluation of sensory acceptability and production of volatile compounds in extract of watermelon. *Pediococcus pentosaceus* was used as starter culture for the LA fermentation where during fermentation days, samples have shown increased levels of acids alcohol, ketones, furans and monoterpenes with reduction in aldehydes and alkanes. The fermented fruit was found with 4-decanone and butanedione with enhanced sensory attributes of probiotic fermented watermelon juice as reported previously (Mandha et al., 2021). Another study reported the evidence that a potential probiotic *Pediococcus pentosaceus* was used as antimicrobial in different foods by diminishing the growth of foodborne pathogenic bacteria such as *Vibrio cholerae, E. coli* and *Staphylococcus aureus,* exhibiting features of tolerance to high salt concentration, survival at lower pH and resistance to bile salts. Thus, the report suggested the *Pediococcus pentosaceus* strains would be used as supplementation in the food and food products due to minimizing the negative impact of pathogenic bacterial growth via LA fermentation (Thao et al., 2021).

LA fermentation was carried out by *Pediococcus pentosaceus* and *Levilactobacillus brevis* for fermenting juice of black gamju giving innovative approach for the development of novel probiotic fermented beverage delivering high quality nutrition and health benefits. As these probiotics have proven to adhere to HT-29 cells conferring the enhancement of immune response and tolerance to higher gastric pH and bile salts. β-glucuronidase is absent in such probiotic bacteria proving their potency to provide health benefits as if this particular enzyme released in the body would convert into toxin production and resistance to broad spectrum antibiotics. Thus, probiotics shown enhanced antioxidant potential manifesting that probiotics may be extensively used in fruit juice fermentation for the development of functional products (Yang et al., 2020). Another study has shown the innovative approach where orange juice bubbles used as a vehicle for carrying probiotic *Pediococcus pentosaceus* ARG-MG12 by encapsulating the bacteria into the bubbles, aiming for the formulation of probiotic juice bubble as a functional product. On investigation, the functional bubbles were found with increased antioxidant activity and enhanced ascorbic acid content. Whereas, in probiotic cell survival studies, cells were found viable at lower pH and bile salts showing 83%-94% survivability respectively (Tanganurat, 2020). Another study reported the use of *Pediococcus pentosaceus* in fermentation of silage that was incorporated with *Medicago sativa* L. i.e., alfalfa, maize, soyabean residues, multivitamins as supplements and calcium carbonate and salts. The fermentation resulted in improvement of quality of silage fermentation with increased production of lactic acid and lower pH. Moreover, this probiotic bacteria was able to diminish the growth of *Monascus* during the 28 days of ensiling (Jiang et al., 2020). *Pediococcus pentosaceus* Ca-4 strain, in fermented cherry and white-fleshed pitaya juice have been shown the higher surviving ability (22.4 %) in lower pH and gastrointestinal juices including pepsin and HCl, making it a potential probiotic candidate for the application of food fermentations in food industries (Xu et al., 2018). *Pediococcus pentosaceus* strain LBF2 have also proven to be as a potential starter culture for the development of probiotic soursop juice enriched with nutritional and enhanced organoleptic attributes during hours of LA fermentation and storage conditions for three weeks at 4°C. 1621 - 2450 mg/l was the maximum lactic acid production during storage period. Along with this, the enhanced sensory attributes made the product better for consumption during 2nd week of storage (Akpeji et al., 2017). Pineapple juice was fermented with different strains of *Pediococcus pentosaceus* and *Lactobacillus rhamnosus* to develop a probiotic beverage. The fermented pineapple juice was found with enhanced vitamin C content and lactic acid

production also increased during the fermentation hours. In this study, cell viability of LAB (1.1 × 10^9 cfu/ml) was found to be constant during 4 weeks of storage at 4°C (AdebayoTayo & Akpeji, 2016). In another study, *Pediococcus damnosus* was exploited to ferment the two old varieties of grapes (Caino and Albarino) where malolactic fermentation was studied. Wines produced after fermentation were found with maximum lactic acid and improved sensory attributes. This *Pediococcus damnosus* bacteria did not produce any type of biogenic amines in the fermented wine, thus proved to be safe for consumption (Juega et al., 2014). *Pediococcus pentosaceus* and *Lactobacillus plantarum* were used for the fermentation of puree of sweet cherry. The fermented product showed the highest antioxidant activity to scavenge the free radicals (Di Cagno et al., 2011). Different strains of *Pediococcus pentosaceus, Lactobacillus plantarum* and *Leuconostoc mesenteroides* were used for the fermentation of marrows, carrot and French beans. Fermented products were found with higher firmness, vitamin C content and possessed antimicrobial activity (Di Cagno et al., 2008).

2.6 Bioactive potential of probiotic beverage

The various probiotic strains have potential to produce numerous bioactive compounds that provide therapeutic benefits to the body. These therapeutic compounds are the metabolic products which can be named as vitamins (B2, B6, B9 and B12), amino acids, enzymes (amylase), bacteriocins, short chain-fatty acids and immunomodulatory compounds (Indira et al., 2019). These metabolic products are produced by probiotic bacteria and known to have bioactivity providing health benefits to the host system. Various probiotic bacteria have been used to ferment fresh fruit juices and vegetables to formulate products with bioactive potential with enhanced phenolics, vitamins and minerals that boosts immunity and protect the cells in oxidative stresses (Paramithiotis et al., 2022). Some of the bioactive compounds such as biogenic amines produced during fermentation by LAB, can cause some allergies, headache and health related issues (Pessione & Cirrincione, 2016).

2.6.1 Antioxidant potential

In a recent study, Kiwi fruit (Guichang) was used to prepare pulp which was fermented with *Lactobacillus plantarum* for 28 hours at 37°C. The samples were withdrawn during the

fermentation hours and analyzed for antioxidant potential. It was observed that phenolics and flavonoid profile improved in fermented kiwi pulp and thus resulted in enhancement of the antioxidant activity (Zhou et al., 2020). Enhanced antioxidant activity and phenolic compounds were found in fermented juice samples when silverberry juice was inoculated with *Lactobacillus plantarum* and *Lactobacillus casei*. Fermentation was carried out for 72 hours at 35°C with agitation rate of 150 rpm (Lizardo et al., 2020) . The blueberry fruit juice was fermented with probiotic bacteria (*Lactobacillus brevis* and *Bacillus amyloliquefaciens*) and a yeast culture (*Starmerella bombicola*). The fermented product showed the improved antioxidant activity as compared to the control juice (Oh et al., 2017).

Another study reported the 40% increased radical scavenging activity and 60% rise in phenolic compounds when pomegranate juice was inoculated with prominent probiotic *Lactobacillus plantarum*. Balb3T3 fibroblast mouse cell lines were used to study the effect of oxidative stress caused by reactive oxygen species (ROS). In these cell lines, oxidative stress was induced by using hydrogen peroxide. After induction of oxidative stress in the cell lines, 60% of the cells survived. Pomegranate juice was fermented by *Lactobacillus plantarum*. The fermented samples showed the significant increase in the cell viability of these Balb3T3 by 40% (Pontonio et al., 2019).

In a previous study, a slight increase in the antioxidant activity of the fermented apricot juice as compared to the control juice was reported when extract of apricot fruit was inoculated with the cultures of different strains of *Lactobacillus* and *Bifidobacterium* (Bujna et al., 2017). The fermentation of guava juice with lactic acid bacteria, *Lactobacillus plantarum* showed the potential to scavenge the free radicals (DPPH). Control guava extract showed insignificant antioxidant activity, whereas fermented samples displayed a significant increase in the antioxidant potential (Dipjyoti et al., 2015). Another study reported the enhanced antioxidant activity and increased phenolic content in guava juice when it was fermented with *Lactobacillus plantarum* (Bhat et al., 2015).

Recently, one study reported the potential of *Lactobacillus plantarum* ATCC14917 to improve the antioxidant potential in fermented apple juice. Mouse macrophages (Raw264.7) were used to determine the antioxidant activity of fermented apple extract, where cell viability was increased when macrophages were treated with fermented samples (Li et al., 2018). Another study reported the increased antioxidant activity in fermented samples of mulberry juice. The fermentation of

mulberry juice with different strains of *Lactobacillus* also results in the increased flavonoid content (Kwaw et al., 2018).

2.6.2 Anti- cancerous activity

In a recent study, fermentation of blueberries (80% ethanolic extract) using probiotic *Lactobacillus plantarum* was performed where various phenolic compounds were produced such as gallic acid, chlorogenic acid and catechol. Such compounds enhanced the antioxidant activity of the fermented extract to scavenge the free radicals. In addition to this, anti-cancerous activity was reported where apoptotic pathways were induced by the fermented blueberry extracts in the HeLa cell lines (human cervical cancer cells) and inhibit the proliferation of these cervical cancer cells as compared to other human cancer cell lines such as Panc-1 (Pancreatic cell lines), AGS (gastric adenocarcinoma), HepG2 (carcinoma of hepatic cells) where no significant difference found in the anti-cancerous activity (Ryu et al., 2019).

In a previous study, anti- cancerous activity was reported in fermented guava juice. Guava juice was fermented with prominent lactic acid bacteria, *Lactobacillus plantarum.* 5-15 µl of the fermented juice sample volume were used to study anti-proliferative property on prostate and breast cancer cell lines. 15 µl of fermented guava juice inhibited the proliferation of the prostate cancer cells by 28%. On the contrary, breast cancer cells were killed by 12 % when 15 µl of fermented guava juice was used (Dipjyoti et al., 2015).

2.6.3 Enzyme inhibition

In the process of melanogenesis, melanin is produced by the action of enzyme tyrosinase. Thus, overexpression of the tyrosinase gene causes the accumulation of melanin in the skin. In a recent study, inhibition of tyrosinase enzyme was reported when silverberry was fermented with *L. casei* which showed significant inactivation of the enzyme as compared to the samples fermented with mixed cultures of *L. plantarum* and *L. casei* (Lizardo et al., 2020). The fermented samples of cherry silverberry also showed the anti α-Glucosidase activity. In a metabolic pathway, carbohydrates are metabolized into glucose and other simple molecules as the end products, which can enter into the blood via absorption through intestine. This results in the increased level of glucose in the blood. Consequently, individuals face the problem of diabetes or hyperglycemia. Thus, inhibiting such

enzymes by fermented products is a promising strategy for regulating the level of sugars in the blood (Lizardo et al., 2020).

2.6.4 Antimicrobial activity

The blueberry fruit juice was fermented with probiotic bacteria (*Lactobacillus brevis* and *Bacillus amyloliquefaciens*) and a yeast culture (*Starmerella bombicola*). The fermented product showed the antimicrobial activity against four different skin infection causing bacteria named as *Bacillus cereus, Brevibacterium linens, Propionibacterium acne* and *Staphylococcus epidermis* as compared to the control juice (Oh et al., 2017). Mango juice pro-bioticated with *Pediococcus pentosaceus* and *Pediococcus acidilactici* displayed the antimicrobial activity against test bacteria named as *Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa* and *Bacillus cereus* (Adebayo-Tayo et al., 2021). Guava juice when fermented with *Lactobacillus plantarum*, showed antimicrobial activity against *E. coli, Pseudomonas* and *S. aureus* (Dipjyoti et al., 2015).

2.6.5 Cytotoxic studies

Fibroblast cells lines (Balb3T3) derived from mouse embryo were used to determine the cytotoxicity of fermented pomegranate juice samples when fresh pomegranate juice was inoculated with *Lactobacillus plantarum.* 0.1 mg/ml of the fermented samples showed an insignificant cytotoxic effect. In contrast, the higher concentration of non-fermented pomegranate juice samples (5 mg/ml and 10 mg/ml) showed the highest cell cytotoxicity leading to a reduction in the viability of the cell lines (Pontonio et al., 2019). Fermented samples showed the highest cell viability/lower cytotoxicity. In another study, for assessing the cell cytotoxicity of blueberry fermented extracts, primary human dermal fibroblast cell lines (HDFs) were used by using resazurin assay. Among the 60 isolates, two probiotic bacteria (*Bacillus amyloliquefaciens* and *Lactobacillus brevis*) and yeast (*Starmerella bombicola*) culture was selected for the fermentation of blueberry juice on the basis of significant antioxidant and antibacterial activity. Three of the extracts showed less cytotoxicity at lower concentration (16 µg/ml and 32 µg/ml) whereas, at higher concentration i.e., 80 µg/ml, the extracts showed 10 % reduction of the viable cells after 24 and 72 hours of fermentation when all the samples were compared with control (Oh et al., 2017).

CHAPTER 3

MATERIALS AND METHODS

3.1 Collection of fresh guava fruit samples and laboratory preparation of guava (*Psidium guajava*) juice

For this study, 3 Kg of mature, fresh and raw guavas (pink-fleshed) were purchased from the Patiala market. Healthy and mature guavas were selected for the laboratory preparation of the juice. Overripened, spoiled/ rotten guavas were discarded. Guavas were washed with tap water, followed by rinsing with distilled water. Guavas were treated in 0.01 % potassium metabisulphite (KMS) solution for half an hour followed by washing with distilled water and then air-dried. Guavas were sliced into small pieces using sterile knife. 3 kg of guava pieces were added into 3000 ml of distilled water (1:1) and blending was performed using electric hand blender to obtain guava pulp. Muslin cloth was used to filter the guava puree in order to separate the thick fiber content, chunks of guava and seeds to obtain a homogenous mixture of the juice. Filtered guava juice was collected in autoclaved flasks followed by pasteurization of the juice at 80°C for 5 minutes. Juice was allowed to cool down at room temperature. Further, it was stored at 4°C until further use. Guava juice preparation was performed with slight modifications as discussed by Bhat et al., 2015. Initial pH and °B were analyzed by Cole-Parmer pH meter and Erma hand refractometer respectively.

3.2 Isolation of the bacteria for fermentation

For the isolation of lactic acid bacteria (LAB) required for the guava juice fermentation, initially LAB was isolated from the surface of the guava fruit (Sarker et al., 2018). Guavas were rinsed with distilled water and sliced into small pieces using sterile knife. The De Man-Rogosa-Sharpe (MRS) media (final pH= 6.5±0.2 at 25°C) was prepared by using the different components during the experiments as per the list given in **Table 2** (M369.Pdf, n.d.). Always freshly and autoclaved MRS media was used for the sufficient growth of LAB. Guava pieces were suspended in selective medium i.e., 50ml MRS broth in two separate flasks. Both the flasks were incubated for 48 hours at 28°C and 37°C under shaking conditions at 120rpm. Once the growth was observed, subsequent streaking was performed on MRS agar plates to obtain pure single colony. Plates were incubated at 28°C and 37°C for 24 hours. Isolated bacterium was sub-cultured on MRS agar plates once in two weeks and plates were stored at 4°C for further use. 50% sterile glycerol stock was prepared to preserve the bacterial culture at -80°C (Linares-Morales et al., 2020).

Table 2: MRS growth medium composition

S. No.	Components	Grams/Liter
1.	Proteose peptone	10
2.	HM Peptone B (Equivalent to beef extract)	10
3.	Yeast extract	5
4.	Dextrose (Glucose)	20
5.	Polysorbate 80 (Tween 80)	1
6.	Ammonium citrate	2
7.	Sodium acetate	5
8.	Magnesium sulphate	0.1
9.	Manganese sulphate	0.05
10.	Dipotassium hydrogen phosphate (or Potassium phosphate dibasic)	2
11.	Agar-Agar	2%

3.3 Characterization of the bacteria (LAB)

After the isolation of the bacteria (LAB) from the surface of the guava samples, bacteria were purified to obtain single colony. One single isolated colony (IS-1) was selected, picked up and inoculated into the flask containing 50 ml of the MRS broth. The flask was incubated at 37°C for 24 hours at 120 rpm. Similarly, single colony of IS-2 was inoculated into the flask containing 50 ml of the MRS broth. The flask was incubated at 28°C for 24 hours at 120 rpm. After obtaining the growth in both of the flasks, the respective LAB bacterial culture was subjected to the following tests.

3.3.1 Biochemical test (catalase test)

Overnight grown culture of LAB (IS-1 and IS-2) were used for performing catalase test. Microscopic slide was cleaned with 70% ethanol. 200µl of culture was placed on the slide followed by dropping 2-3 drops of 3 % hydrogen peroxide (H_2O_2). This biochemical test ensures that the bacteria that respire using oxygen, synthesize the catalase enzyme. This enzyme particularly breaks H_2O_2 into oxygen and water. Thus, the formation of oxygen bubbles ensures the breakdown of H_2O_2 into water and oxygen molecules by the action of catalase enzyme. The formation of bubbles in the test culture ensures that the microorganisms are catalase positive and absence of oxygen bubbles indicates that the bacterial culture is catalase negative (Ismail et al., 2018).

$$2\,(H_2O_2) \xrightarrow{\text{Catalase enzyme}} 2\,H_2O + O_2$$

3.3.2 Selective medium for Lactic-acid producing bacteria

The glucose, yeast extract and peptone (GYP) with $CaCO_3$ medium is selective for lactic acid producing bacteria and allows the detection of LAB members from other bacteria. The principle behind this concept is that, on production of lactic acid (LA) in the medium, purple colored bromocresol-purple dye gets converted into yellow color due to the reduction in the pH of the medium and the release of LA to the medium which reacts with $CaCO_3$ resulting in the formation of calcium lactate. This results in the formation of clear zone around the colony. The bacterial colony that doesn't produce lactic acid will be able to grow on the medium but not be able to form any zone on the medium. The composition of GYP selective medium having pH- 6.8 is given in the **Table 3**.

In order to confirm the production of lactic acid (LA) in the medium and glucose fermenting characteristic of IS-1 and IS-2 bacterial isolates, GYP medium was prepared as discussed by (Cai et al., 1999). Serial dilutions were prepared from 1 ml of overnight grown culture of IS-1 in 9 ml of 0.85% of sterile sodium chloride (NaCl) solution to make 10^{-1} dilution. Subsequent dilutions were prepared by withdrawing 1 ml of suspension from previous dilution and adding into the succeeding test tubes containing 9 ml of NaCl. Thus, 10^{-1} to 10^{-8} dilutions were prepared. Similarly, dilutions for IS-2 bacterial isolate were prepared. 50 µl of bacterial suspension was taken from 10^{-5}, 10^{-6} and 10^{-7} dilution and spreading was performed on GYP-agar plates and these plates were incubated at 37°C and the plates were observed after 24 hours.

3.3.3 Gas production test

In this test, to ensure the gas production after utilization of sugars by LAB, a method discussed by (Hayward, 1957) was followed with slight modifications. MRS media was prepared and 10 ml of the media was added into each of the test tubes. Durham tubes were put upside down into each of the test tubes containing media. These test tubes were autoclaved for 15 minutes at 15 psi at 120°C. After sterilization of the media, test tubes were inoculated with overnight grown culture of isolated microorganism and sealed with parafilm in order to prevent gas loss. Test tubes were incubated for 48 hours at 37°C.

Table 3: Composition of GYP selective medium for LAB

S. No.	Components	g/L of distilled water
1.	Glucose	10
2.	Yeast extract	5
3.	Peptone	5
4.	Sodium acetate	2
5.	Tween 80	0.25
6.	$MgSO_4.7H_2O$	0.2
7.	$MnSO_4.4H_2O$	0.01
8.	$FeSO_4.7H_2O$	0.01
9.	NaCl	5
10.	$CaCO_3$	5
11.	Bromocresol purple	0.04
12.	Agar-agar	2%

3.4 Identification of the isolated microorganism

IS-1 and IS-2 were characterized as LAB with catalase negative and homo-fermentive features.

3.4.1 Morphological identification/Phenotypic characteristics

For characterizing IS-1 and IS-2 morphologically, colony appearance was observed and gram staining was performed (Ismail et al., 2018). Overnight grown cultures of IS-1 and IS-2 were used for making smear on sterilized glass slide. Initially, microscopic slide was cleaned and sterilized using 70% ethanol, followed by heating the needle in lamp fire. After cooling aseptic needle, smear was formed and slide was heat fixed in Bunsen burner's flame. On the smear, crystal violet- a primary stain was dropped down using dropper and applied for one minute followed by removing the dye using distilled water. Then few drops of Gram's iodine, called as mordant stain used for fixing primary stain, were dropped on the smear and allowed the slide to stand for one minute. This was followed by gentle rinsing of the smear with distilled water. For decolorization, 50% acetone-alcohol solution was dropped on the fixed bacteria and applied for 30 seconds followed by removing decolorizer with few drops of distilled water. Smear was etched with safranin, a counterstain for thirty seconds. Slide was gently rinsed with distilled water and air dried the smear. Slides were then observed under microscope at 10X, 40X and 100X magnification. Bacteria are

considered as gram positive if cells appear as purple or violet in color due to crystal violet stain and gram-negative bacteria if stained as pink color due to counterstain-safranin.

3.4.2 Molecular identification of the isolated LAB strain

IS-1 bacterial isolate was subjected to genomic DNA isolation by using the organic method of DNA extraction.

3.4.2.1 Genomic DNA isolation procedure for lactic acid bacteria

For isolation of DNA, method described by Azcárate-Peril & Raya, 2001 was followed with slight modifications. For the isolation of genomic DNA, the required material was: Lysis buffer: 75 mM sodium chloride, 25 mM ethylenediamine tetra-acetic acid (EDTA), 20 mM Tris–HCl, pH 7.5, freshly prepared 10 mg/mL (1 ml) of lysozyme solution in 2 ml microfuge tube, 10% sodium dodecyl sulfate (SDS), 5 M sodium chloride, A 24 : 1 (v/v) mixture of chloroform and isoamyl alcohol, 100% isopropanol, 70% ethanol chilled to –20°C, Tris-EDTA (TE) buffer: Tris–HCl 10 mM, and EDTA 1 mM, pH 8.0., TE-buffer-saturated phenol, 3 M sodium acetate, adjusted to pH 5.2 with glacial acetic acid, 37°C water bath, 55°C water bath, refrigerated centrifuge set at temperature 4°C and pH Meter.

Procedure

1. 60 ml of the MRS broth was inoculated with 600 µl (1 % inoculum) of overnight grown LAB culture and incubated at 37°C in an incubator shaker at 120 rpm.
2. After about 20 hours, culture was centrifuged in two vials having 15 ml volume at 8000 rpm for 10 minutes at 4°C. After obtaining cell pellet supernatant was discarded and this step was repeated again.
3. Cell pellet was resuspended using 5 ml of lysis buffer by pipetting up and down. 5 ml of lysis buffer was used for each 30 ml culture media in each vial.
4. After dissolving the pellet, 400 µl of lysozyme (10 mg/ml) was added into each vial. Content was mixed by inverting the vials followed by incubating the vials at 37°C in water bath for about one and half hour. During incubation vials were occasionally shaken after every 20 minutes by inverting tubes (1-2 times).

5. 500 µl of 10 % SDS was added into the vials, content was mixed by inverting tubes and both the vials were incubated at 55 °C in water bath for one hour. Content was shaken occasionally.
6. 2 mL of 5 M NaCl was added into both tubes, contents were mixed followed by addition of 6 mL of chloroform: iso-amyl alcohol (C: I) in each vial. Total volume in the vials was approx.14 ml. Contents were mixed by inverting the vials for 2-3 times and vials were incubated at room temperature for 30 minutes.
7. After incubation vials were centrifuged at 12,000 rpm for 10 minutes at 4°C to eliminate the cellular debris. Three layers were obtained. Upper aqueous transparent phase, interphase white layer having proteins and cellular debris, and bottom organic phase containing lipids and remaining cellular contents. Upper aqueous layer was transferred to fresh vials.
8. RNase treatment was given by adding 3.5 µl of RNase into each vial containing aqueous layer and vials were incubated at 37°C in water bath for about 25 minutes.
9. After incubation, 6 ml of C: I was added into each vial and the contents were mixed by inverting the tubes. Centrifugation was performed at 12,000 rpm for 10 minutes at 4°C.
10. Two layers were obtained, upper phase was transferred to fresh tube. As upper layer obtained was 7 ml thus, 1 volume of 100 % isopropanol (chilled at -20°C) was added i.e., 7 ml of isopropanol was used to precipitate the nucleic acid.
11. Vials were centrifuged at 12,000 rpm for 10 minutes at 4°C to obtain the DNA pellet. Supernatant was removed carefully and pellet obtained was air dried for about 30 minutes by keeping vials at 45° angle.
12. Pellet was resuspended in 1 ml of Tris- EDTA buffer by tapping bottom of the vials. 200 µl of crude DNA was transferred into microfuge tubes.
13. Rest 800 µl of crude DNA was purified by adding 1 volume of TE-buffer-saturated- phenol i.e., 800 µl. Contents were mixed by inverting the tubes and contents were centrifuged at 12,000 rpm for 5 minutes. Upper phase was transferred to fresh tube and this step was repeated again by adding 800 µl of TE-buffer-saturated-phenol. Centrifugation was done at 12,000 rpm for 5 minutes at 4°C.
14. Upper phase was transferred to a fresh vial and 1 volume of C: I mixture (800 µl) was added into each vial followed by centrifugation at 12,000 rpm for 5 minutes at 4°C to remove phenol contamination.

15. Upper phase from both the vials was transferred to new vials carefully.
16. 50 µl of 3M sodium acetate (pH= 5.2) was added in each vial and 2 volumes (i.e., 2 mL) of chilled 100 % ethanol was added. Vials were incubated at -20°C overnight.
17. After incubation, centrifugation was done at 12,000 rpm for 10 minutes to obtain the DNA pellet.
18. Pellet was washed twice with 70 % ethanol chilled at -20°C and air-dried the pellet.
19. DNA pellet was resuspended in 100 µl of TE buffer and stored at 4°C.

3.4.2.2 Agarose gel electrophoresis of the isolated DNA

A method of agarose gel electrophoresis is molecular biology technique used for separation, identification and purification of nucleic acids (DNA and/or RNA). Deoxyribonucleic acid (DNA) and Ribonucleic acid (RNA) being negatively charged move towards anode (positive) through the agarose gel pores on the basis of size and charge, where small sized fragments as having less molecular mass move faster in the gel compared to large sized fragments that move slower due to large molecular mass. For separation of large sized molecules, concentration of agarose should be low (preferably, 0.8% for genomic DNA of ≥1kb size) as less concentrated agarose will have large pore size assisting in fluent migration of the high molecular-mass fragments and vice-versa.

Requirements during agarose gel electrophoresis were: Electrophoresis unit, power supply, gel tray, comb, ethanol (70%), ethidium bromide (EtBr), bromophenol blue, Tris-Borate-EDTA (TBE) running buffer, microtips, micropipettes, tissue and parafilm.

Procedure

1. Preparation of Agarose gel (0.8% of agarose):
   0.5 X TBE buffer was prepared by taking 50ml of 5X TBE in 450 mL of distilled water. Agarose gel was prepared in Erlenmeyer flask by adding 0.32 g of agarose in 40 ml of 0.5X TBE buffer. Agarose was dissolved in microwave by heating for 1 minute 30 seconds.
2. Agarose was allowed to cool to bearable temperature (40-45°C) followed by adding 4 µL of EtBr. Flask was swirled to mix the contents (avoid bubble formation).
3. Agarose gel tray was wiped with ethanol and the edges of the tray were sealed with tape. Comb was placed into gel tray. The molten agarose was poured into this mold and allowed to solidify at room temperature for about 25-30 minutes.

4. After solidification of the gel, comb was removed to create wells.
5. Gel tray was placed in the electrophoretic chamber and enough buffer was added to cover the surface of the gel.
6. On a parafilm/ tape, 4 µL of loading dye and 3 µL of DNA sample was mixed and loaded in the wells along with the 1 kb ladder. Power supply was turned on and gel was run at 70 V for about 45 minutes.
7. Once the running dye migrated to the bottom of the gel, power supply was turned off.
8. Agarose gel was removed from the gel tray and visualized in gel documentation system where gel was exposed to UV light to visualize the DNA bands.

3.4.2.3 Amplification of the gene

Polymerase chain reaction is used for the amplification of specific gene of DNA using forward and reverse primer to obtain millions of copies of the target DNA. Materials used during PCR amplification were PCR tubes, PCR reaction mixture, autoclaved and filter sterilized MQ water. To amplify the 16S rRNA region in bacterial DNA, PCR amplification was done and the following PCR reagents were used:

i. dNTPs: Acts as building blocks during synthesis of new strands
ii. Template DNA: Contains target sequence which has to be amplified
iii. Primers: Forward and reverse primers bind to complementary regions in template DNA and thus provide the site for polymerase binding and synthesis of new strands.
iv. *Taq* (*Thermus aquaticus*) DNA polymerase: Thermotolerant enzyme catalyzing the DNA synthesis.

The different components of PCR reaction mixture are listed in the **Table 4** and different dilutions of DNA used in PCR are given in **Table 5.**

Procedure

DNA amplification was performed using GeneAmp PCR System 9700 (Applied Biosystem, USA). PCR reaction mixture was prepared for 3 reactions to amplify undiluted template DNA, $1/20^{th}$ and $1/50^{th}$ time diluted DNA.

As faint bands were observed in undiluted and 1/50th dilution, so further 1/100th dilution of template was prepared to amplify the DNA.

Thus, PCR reaction mixture was prepared in 1/100th dilution for 7 reactions to pool up DNA as 1/100th dilution gave the sharp bands.

Table 4: PCR reaction mixture

S. No.	Components	1 reaction (µL)	3 reactions (µL)	7 reactions (µL)
1.	Taq buffer	2.5	7.5	17.5
2.	2 mM dNTPs	2	6	14
3.	Forward primer	1	3	7
4.	Reverse primer	1	3	7
5.	$MgCl_2$ -50mM	0.5	1.5	3.5
6.	Milli-Q water	15.7	47.1	109.9
7.	*Taq* polymerase	0.3	0.9	2.1
8.	Template/Diluted DNA	2	6	14
	Total volume	25	75	175

Table 5: Dilution of genomic DNA

S. No.	Dilution	DNA (µL)	MQ water (µL)
1.	--	2	--
2.	1/20th	2	38
3.	1/50th	2	98
4.	1/100th	2	198

3.4.2.4 PCR conditions

PCR was performed using thermal cycler, and using the conditions as described in **Table 6**.

Table 6: PCR conditions

Steps	Temperature (°C)	Time (minutes: seconds)	No. of cycles
Initial denaturation	95	5:00	-
Denaturation	95	1:00	35
Annealing	55	0:40	
Extension	72	2:00	
Final Extension	72	7	-
Cooling	4	∞	-

3.4.2.5 Purification of the PCR product

The amplified PCR product was verified by electrophoresis of aliquots of PCR products (4 µL) and 3 µL of loading dye on 0.8% agarose gel in 0.5X TBE buffer. Further, PCR product was purified using Thermo Scientific™ GeneJET PCR Purification Kit (#K0701, #K0702). PCR products were collected in 1.5 ml of microfuge tubes. In 100 µL of PCR product, 100 µL of binding buffer was added. 200 µL of the solution was transferred from 1.5 mL into GeneJET purification column followed by centrifugation for 60 seconds. Further, 200 µL of washing buffer (diluted with ethanol) was added in to the column and centrifugation was done for 1 minute 30 seconds. Flow through was discarded and purification column was placed back into the collection tube. Empty column was centrifuged to remove residual wash buffer if present, followed by transferring the column to clean 1.5mL of microfuge tube. 40 µL of MQ water (preferably lukewarm) was added in the center of the column and centrifugation was done for 1 minute 30 seconds. Eluted PCR product was stored at -20°C. For quantitative analysis, Nanodrop (Thermo scientific, Nanodrop) was used to assess the purified PCR product. MQ water was used as blank and 1 µL of the sample was used to measure the absorbance at 260 nm and 280 nm. The ratio of 260/280 was calculated for the isolated DNA to analyze the quality of purified DNA.

3.4.2.6 16S rRNA sequencing

The forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 3'-ACGGGCGGTGTGTTC-3' (Weisburg et al., 1991) were used for the amplification of 1.5 kb gene

of 16S rRNA. The amplified and purified PCR product was sent to Biokart India Pvt. Limited for 16sRNA sequencing.

3.4.2.7 Construction of Phylogenetic Tree

The sequence obtained was then searched for sequence similarity with non- redundant database maintained by NCBI. The homology in the sequence was obtained by comparing the 16S rRNA sequence of LAB using NCBI-BLAST National center for Biotechnology Information-Basic Local Alignment Search Tool) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and phylogenetic tree was constructed using Neighborhood joining method using Mega BLAST to find out the evolutionary relationship.

3.5 Optimization of the parameters for lactic acid fermentation using Response surface methodology (RSM)

Parameters like inoculum concentration, temperature and rate of agitation play an important role in the formulation of probiotic beverage. Optimization can be done by using central composite design (CCD) by employing RSM. The experimental design helps to find out the optimum parameters that influence the formulation. Thus, formulation of functional probiotic beverage was accomplished by inoculating the 250 ml of pasteurized guava juice in autoclaved glass bottles by providing inoculum concentration (%), temperature (°C) and agitation (RPM) as designed by CCD using Design-Expert® version 13 software.

In order to develop guava probiotic beverage, the three different independent factors such as inoculum concentration (%), temperature (°C) and agitation (RPM) were selected to study the interactive effects on lactic acid production in guava juice that was quantitatively analyzed and results were predicted using optimizing technique i.e., response surface methodology.

RSM is a multivariate statistical method that provides the design of experiments in the best possible combinations on the basis of different input variables, optimum operating conditions, analyzing its experimental data followed by relating the response on the basis of influencing variable in such a way that response reaches out to be at its maximum desired value or minimum desired value. To avoid redundancy in the experimental information, various no. of experiments can be performed rationally using RSM (M'hir et al., 2021).

For the probiotic beverage formulation, randomized central composite design consisted of 14 non-center points and 6 center points w.r.t three independent variables. 5 factor levels were assigned (-α, -1, 0, +1, + α) to the three different input variables as shown in **Table 7.**

The dependent variable or the response to be studied was lactic acid concentration (µg/ml). In this, 20 experiments were designed in the CCD out of which 8 points were factorial, 6 center points and 6 axial points (± α) **(Table 8)**.

Table 7: Experimental variables and factor levels used in RSM-CCD for developing probiotic beverage

Factors	**Independent variables**	**Units**	**Factor levels**					**Std. Dev.**
			-α (1.682)	**-1**	**0**	**+1**	**+ α (1.682)**	
A	Temperature	°C	15	18.4459	23.5	28.5541	32	4.28
B	Inoculum concentration (size)	%	5	6.41889	8.5	10.5811	12	1.76
C	Agitation	RPM	50	60.1349	75	89.8651	100	12.60

In order to understand the effects of different independent variables on the lactic acid concentration response, the polynomial equation (second-order) was used to fit the results (outputs) that were obtained from the RSM-CCD. The polynomial equation is given as follows:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3,$$

Where Y stands for the response that has to be predicted (maximum lactic acid concentration); b_0 = model constant (intercept); b_1, b_2, b_3 = A, B, C (linear effects of the three variables), b_{11}, b_{22}, b_{33} = (AA, BB, CC) squared effects of the three variables; b_{12}, b_{13}, b_{23} = (AB, AC, BC) interactive effects of the three variables; X_1, X_2, X_3 are the three independent variables i.e., A, B, C (M'hir et al., 2021).

Table 8: Experimental design matrix for three different independent variables using RSM

Standard	Run	Space Type	Factor 1	Factor 2	Factor 3
			A: Temperature	B: Inoculum concentration	C: Agitation
			°C	%	RPM
1	18	Factorial	18.4	6.4	60
2	13	Factorial	28.5	6.4	60
3	7	Factorial	18.4	10.5	60
4	4	Factorial	28.5	10.5	60
5	1	Factorial	18.4	6.4	89
6	15	Factorial	28.5	6.4	89
7	19	Factorial	18.4	10.5	89
8	10	Factorial	28.5	10.5	89
9	14	Axial	15	8.5	75
10	9	Axial	32	8.5	75
11	16	Axial	23.5	5	75
12	20	Axial	23.5	12	75
13	5	Axial	23.5	8.5	50
14	11	Axial	23.5	8.5	100
15	3	Center	23.5	8.5	75
16	6	Center	23.5	8.5	75
17	2	Center	23.5	8.5	75
18	12	Center	23.5	8.5	75
19	17	Center	23.5	8.5	75
20	8	Center	23.5	8.5	75

3.6 Preparation and evaluation of the probiotic beverage

Probiotic guava beverage was prepared by inoculation of IS-1 bacterial isolate that was identified as *Pediococcus pentosaceus* VNK-1, in 250 ml of the autoclaved guava juice and providing the parameters as developed by CCD-RSM. The overnight grown culture of LAB having starter culture concentration 12 %, temperature 24 °C and 60 rpm. The optimized fermentation period was 4 days to obtain the probiotic beverage. Using same parameters for agitation rate and inoculum size the fermentation was carried at 37°C for studying the effect of fermentation at 24°C and 37°C.

3.6.1 Coding of fermentation samples

The fermented samples were named as A1, A2, B1, B2. A1 stands for juice inoculated with overnight grown culture with 12% inoculum and A2 stands for juice inoculated with culture pellet that was collected by centrifugation of 12% inoculum at 8000 rpm for 10 min at 4°C. B1 stands for juice inoculated with overnight grown culture with 12% inoculum and B2 stands for juice inoculated with culture pellet that was collected by centrifugation of 12% inoculum, at 8000 rpm for 10 min at 4°C. Incubation temperature for A1, A2 was 24 °C and for B1, B2 was 37°C. CJ encoded for control guava juice in which fermentation was not performed.

3.6.2 Quantitative estimation of Lactic acid

For quantitative estimation of lactic acid in fermented juice samples a method was followed as described by (Pryce, 1969). This procedure was adopted after suitable modification for the guava juice fermentation samples.

In order to remove proteins, sugar and other dispensable substances that may interfere in the reaction, fermented samples must be treated with the precipitating reagent. Once the pretreatment is complete, lactic acid in the samples is oxidized and converted into acetaldehyde by concentrated sulphuric acid. After this reaction, on addition of p-hydroxydiphenyl (pPP), purple colored product forms which develops due to the reaction of pPP and acetaldehyde. For degradation of pPP, solution must be heated at boiling temperature and the intensity of the purple-colored product is measured at 570 nm. By comparing the optical density (O.D.) of the solution with standard, concentration of lactic acid in the sample was determined.

Precipitating reagent was prepared by dissolving 3g of sodium tungstate dihydrate (AR, ACS S.D. Fine chemical limited) in 100ml of distilled water, followed by adding 6.6ml of orthophosphoric acid and 1.5g of $CuSO_4$ (cupric sulphate pentahydrate 99\%, AR/ACS, LOBA Chemie Pvt. Ltd.). Volume of the solution was made up to 300 ml by adding 293.4 ml of distilled water. The colour developing reagent was prepared by dissolving 75 mg of pPP (98 % (for synthesis LOBA Chemie Pvt. Ltd.) in 5 ml of dimethylformamide.

Standard stock solution of lactic acid was prepared as 1mg/ml of concentration in distilled water. Different dilutions of lactic acid were prepared using distilled water ranging from 50-300 µg/ml.

Pretreatment of the samples was done by adding 4 ml of the precipitating agent. Further, test tubes were vortexed to mix the contents. Content of each test tube was centrifuged at 10,000 rpm for 10 minutes at 4°C. 1 ml of pre-treated solution (supernatant) was transferred into fresh test tubes followed by addition of 6 ml of H_2SO_4 (98 % analytical reagent grade) subsequently. Tubes were allowed to stand for 5 minutes at room temperature followed by vortexing the solution. Test tubes were allowed to cool with the aid of tap water for about 3 minutes. 50µl of p-hydroxybiphenyl-dimethylformamide solution was added into each test tube and mixed well using vortex. Further test tubes were allowed to stand for 10 minutes at room temperature. Test tubes were incubated in boiling water bath for 1 minute 30 seconds followed by cooling the test tubes in tap water. Contents of each of the test tube were centrifuged at 8,000 rpm for 10 minutes at 4°C. Absorbance was measured against blank at 570 nm. 50 µl of distilled water, unfermented and fermented guava juice samples were used as blank, negative control and test samples respectively for lactic acid estimation.

3.6.3 Analysis of pH and Total Soluble Solids (TSS)

The calibrated and digital pH meter (Cole-Parmer pH meter) was used to detect the initial and final pH of control and fermented juice samples during the fermentation days. The degree brix (°B) or TSS were determined using calibrated hand refractometer (ERMA 0-32% hand refractometer). All the analysis were performed in triplicates and their average mean was considered as final values for pH as well as °B.

3.6.4 Determination of Total Phenolic Content (TPC)

In control juice (CJ or non-fermented) and fermented samples, total phenolic content was estimated by following the Folin-Ciocalteu's (FC) reagent procedure (Castro-López et al., 2016) with slight modifications. In brief, 0.1mg/ml (2.5-30 µg/ml) of gallic acid standard was prepared to form the calibration curve. 100 µl (Dilution factor 100) of the fermented samples were used for TPC analysis, to which 250 µl of diluted (1:1) FC reagent was added. Solution was mixed well followed by addition of 250 µl of 7.5 % of sodium carbonate (Na_2CO_3). Test tubes were allowed to stand for 30 minutes at 40°C in a water bath. After incubation, 2 ml of distilled water was added into each tube subsequently. On completion of the reaction light blue color appeared in the samples and absorbance was observed at 750 nm. Calibration curve and sample values were analyzed in

triplicates and data was represented as the average and their standard deviation and the obtained concentrations of total phenols were expressed in gallic acid equivalent (GAE µg/ml).

3.6.5 Estimation of Total Flavonoid Content (TFC)

Bioactive plant polyphenols can be classified into low molecular weight compounds called flavonoids, majorly found in plants, fruits, cereals and vegetables, possessing the basic flavan nucleus structure consisting of a 15-carbon chain organized in two aromatic rings connected by three carbon atoms to form an oxygenated heterocyclic ring (C6–C3–C6). On the basis of structure, flavonoids have subclassification flavones, flavonols, flavonones, flavononols, isoflavones, flavanols, and anthocyanidins, having properties such as antimicrobial and antiviral, antiangiogenic, antimalarial, antioxidant, neuroprotective, antitumor, and anti-proliferative agents.

Flavonoid content estimation was performed as described in (Fernandes et al., 2015) using aluminium chloride spectrophotometric method, in which aluminium, chloride reacts with flavonoids resulting in the formation of stable complexes with either C-3 or C-5 hydroxyl group of flavones and flavonols or C-4 keto groups. In addition, it also forms acid labile complexes with the orthodihydroxyl groups in the A- or B-ring of flavonoid. NaOH is used to neutralize the reaction.

Stock solution of quercetin was prepared with 1mg/ml concentration using methanol as solvent. Serial dilutions were prepared in the range of 100-900 µg/ml followed by mixing the solution using vortex. 5% of $NaNO_2$ was prepared out of which 300 µl was used in each dilution of quercetin followed by mixing the solution. All the tubes were allowed to stand for 1-2 minutes at room temperature, further 300 µl of 10% $AlCl_3$ was added into each tube and the contents were mixed. After incubation for 6 minutes, 2 ml of 1M NaOH was added. Contents were mixed well. The O. D. was observed at 510 nm. 100 µl of the unfermented and fermented juice samples were used for flavonoid content estimation and expressed in µg of quercetin equivalent extract per ml of the juice. Flavonoid content was estimated in triplicates.

3.6.6 Analysis of Reducing sugars

Reducing sugars in fermented samples were assessed by following the 3,5-Dinitrosalicylic acid (DNSA, IUPAC name 2-hydroxy-3,5-dinitrobenzoic acid) method (Miller, 1959). DNSA, an

aromatic yellow colored compound when allowed to react under alkaline conditions with the solution containing reducing sugars, gets reduced to orange-red colored compound called 3-amino-5-nitrosalicylic acid. As reducing sugars possess free functional carbonyl groups (C=O) groups such as aldehyde group present in glucose and ketone group present in fructose, therefore, such sugars have capability to reduce the DNSA.

DNSA solution was prepared as 1g of DNSA was dissolved in 20ml of sodium hydroxide (2N NaOH solution was prepared by dissolving 4 g of NaOH in 20ml of distilled water) with the help of a magnetic stirrer. 30g of sodium potassium tartrate was dissolved in 50mL of distilled water. Finally, sodium potassium tartrate was added in DNSA and NaOH solution and volume was made up to 100 ml of distilled water.

Glucose standard stock solution of 1mg/ml was prepared. Distilled water was used for making dilutions in the range of 100-900 µg/ml for preparing glucose standard followed by mixing the contents using vortex. 3 ml of DNSA solution was added into each tube and contents were mixed well. Test tubes were allowed to stand for 15 minutes in boiling water bath. Solution in the tubes was allowed to cool down at room temperature. O. D. was taken at 540 nm against distilled water as blank. For analysis of the fermented juice samples 10 µl of sample was diluted with 990 µl of distilled water. All the experiments were performed in triplicates.

3.6.7 Determination of microbial cell count

The viability of the probiotic starter culture *Pediococcus pentosaceus* VNK-1 in the probiotic guava beverage was assessed using the method described by (Palachum et al., 2020) with slight modifications. Probiotic juice samples (A1, A2, B1 and B2) withdrawn from the jar bottles on 0^{th} day, 1^{st} day, 2^{nd} day, 3^{rd} day and 4^{th} day. Serial dilutions of each of the samples were prepared from 10^{-5} to 10^{-9} in 0.85 % of sterile sodium chloride (NaCl). 100 µl of the diluted sample was withdrawn from each of the 10^{-7} dilution of A1, A2, B1 and B2 and added on the sterile MRS agar plate. These aliquoted samples were appropriately spread on MRS agar's surface using sterile glass spreader. Plates were sealed with parafilm and incubated at 37°C in an incubator for 24 hours. All the plates were compared with control MRS agar plate in which 100 µl of the control juice (non-fermented) was spread. The colony obtained in each plate were counted and viability of the culture

in guava juice was calculated in colony forming units (CFU/ml) and expressed in $\log_{10}$ CFU/ml of the total 250ml of probiotic beverage.

$$\text{Colony forming unit (CFU/ml)} = \frac{(\text{No. of colony} \times \text{Total dilution factor})}{\text{Volume/amount of culture plated (ml)}}$$

3.6.8 Organoleptic studies

Sensory characteristics for the probiotic beverage was evaluated using 9-point hedonic scale (Sharma et al., 2020). The rating for 9 points were in decreasing order as follows; 9- Like extremely, 8- Like very much, 7- Like moderately, 6- Like slightly, 5- Neither like nor dislike, 4- Dislike slightly, 3- dislike moderately, 2- dislike very much, 1- dislike extremely. For sensory analysis, members of faculty, lab staff and students were randomly selected. Appearance/color, mouth-feel, aroma/odor, taste and overall acceptance of the beverage, were taken into consideration for rating from 9 to 1 point.

3.7 Bioactive potential of probiotic beverage

Fermented juice samples were subjected to the following tests for assessing their bioactive potential.

3.7.1 Antioxidant activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay is a colorimetric method where deep purple-colored DPPH is reduced by receiving hydrogen atom/ an electron from antioxidant molecules (therefore, acting as reducing agents and known as free radical scavengers) and thus, converted into yellow-colored/colorless product called as 2,2-Diphenyl-1-picrylhydrazine. DPPH acts as free radical molecule that has activity to oxidize the other molecules. DPPH acting as a free radical unstable molecule, can be neutralized and become stable at the end of the reaction. Thus, antioxidant activity of guava juice samples can be screened by free radical scavenging method using a free radical DPPH.

Antioxidant potential of the fermented juice samples were estimated by DPPH assay (Sharma et al., 2020). In brief, freshly prepared DPPH having concentration of 0.1mM was used to assess the

antioxidant activity of the beverage samples. 50 µl of the sample volume was allowed to mix with 150 µl of the methanolic solution of DPPH in 96-well plate followed by incubation for half an hour at room temperature in dark conditions. After incubation, plate was put in ELISA plate reader and absorbance was taken at 517 nm. For the standard, ascorbic acid was used. Percent of antioxidant activity was calculated using the given formula:

$$\text{Antioxidant activity (\%)} = \frac{\text{Absorbance of control – absorbance of sample}}{\text{Absorbance of control}} \times 100$$

3.7.2 Antibacterial activity

For antimicrobial susceptibility testing in clinical microbiological laboratory, 0.5 Mc-Farland standard is used to adjust the turbidity of the active bacterial culture which gives the O.D. of 0.1 at 625 nm which represents 1.5×10^8 cells/ml.

Antimicrobial assay for the beverage was performed by agar-well diffusion technique (Sourangshu C, 2015). At the end of the fermentation on 4^{th} day, samples were withdrawn from the guava beverage. Supernatants were collected by centrifugation of the fermented juice samples at 8000 rpm for 10 minutes at 4°C. Supernatants were kept at 4°C until further use. The antimicrobial activity of the samples was determined against three bacteria *Bacillus megaterium* (FH 1127), *Staphylococcus aureus* (MRSA, ATCC 33591) and *Bacillus subtilis* on Muller Hinton agar plates. Overnight grown test bacterial cultures having 0.1 O. D. at 625 nm (0.5 Mc-Farland standard used as reference) was used for this test. 100 µl of the active bacterial cultures were spread on MHA plates. After spreading, 8 mm borer was sterilized with 70 % ethanol and heated to red hot in flame. Further borer was allowed to cool down for two minutes and then, cooled borer was used to create the wells on the agar plates. 100 µl of the control juice (non-fermented) and fermented beverage was added into the wells using sterile tips. Plates were sealed with parafilm and incubated at 37°C for 24 hours and the results were observed on the next day. The diameter of zone of inhibition if present was measured in millimeters (mm) with the help of the scale.

CHAPTER 4

RESULTS & DISCUSSION

4.1 Laboratory preparation of guava (*Psidium guajava*) juice

Pink-fleshed guava fruit samples collected from the Patiala market were processed for preparing guava juice in the laboratory **(Figure 2)**. Healthy and fresh guava samples were selected and washed with tap water, followed by rinsing the surface of guavas with distilled water **(Figure 2 (a))**. Potassium metabisulphite (0.01%) was used to treat the surface of guava to reduce microbial load. Thereafter, guavas were rinsed with distilled water, allowed to air dry and slices of pink fleshed guavas were blended to form a homogenous mixture followed by filtration of the juice using muslin cloth **(Figure 2 (b & c))**. Guava juice was collected into the autoclaved flasks **(Figure 2 (d))**. Pasteurized flasks containing guava juice were kept under storage conditions (4°C). Initial pH and °B (Total soluble solids) of freshly prepared juice was found to be 4.24 and 4.2 respectively. A flow chart for the manual preparation of guava juice is given below **(Figure 1)**.

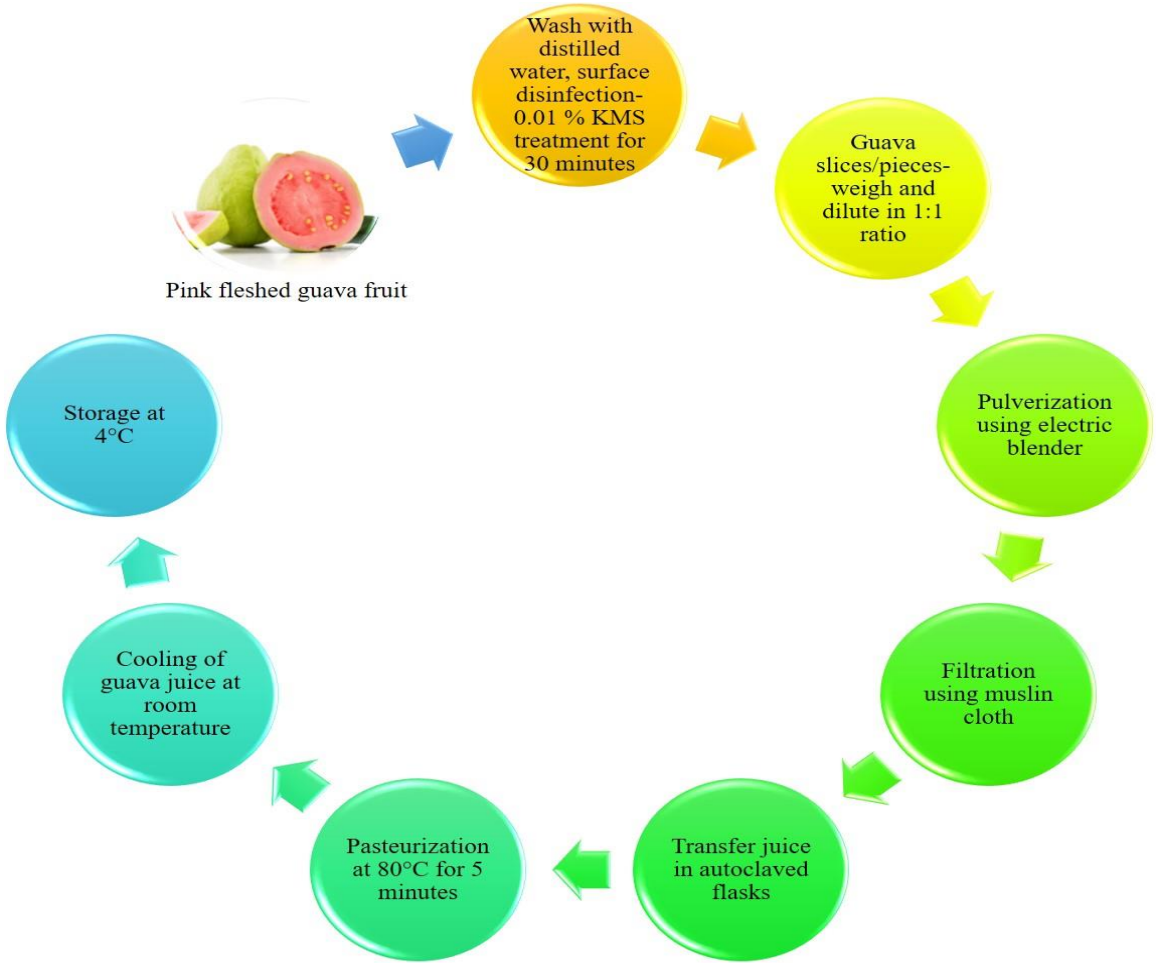


Figure 1: A flow chart for the laboratory preparation of guava puree

(a) (b) (c)

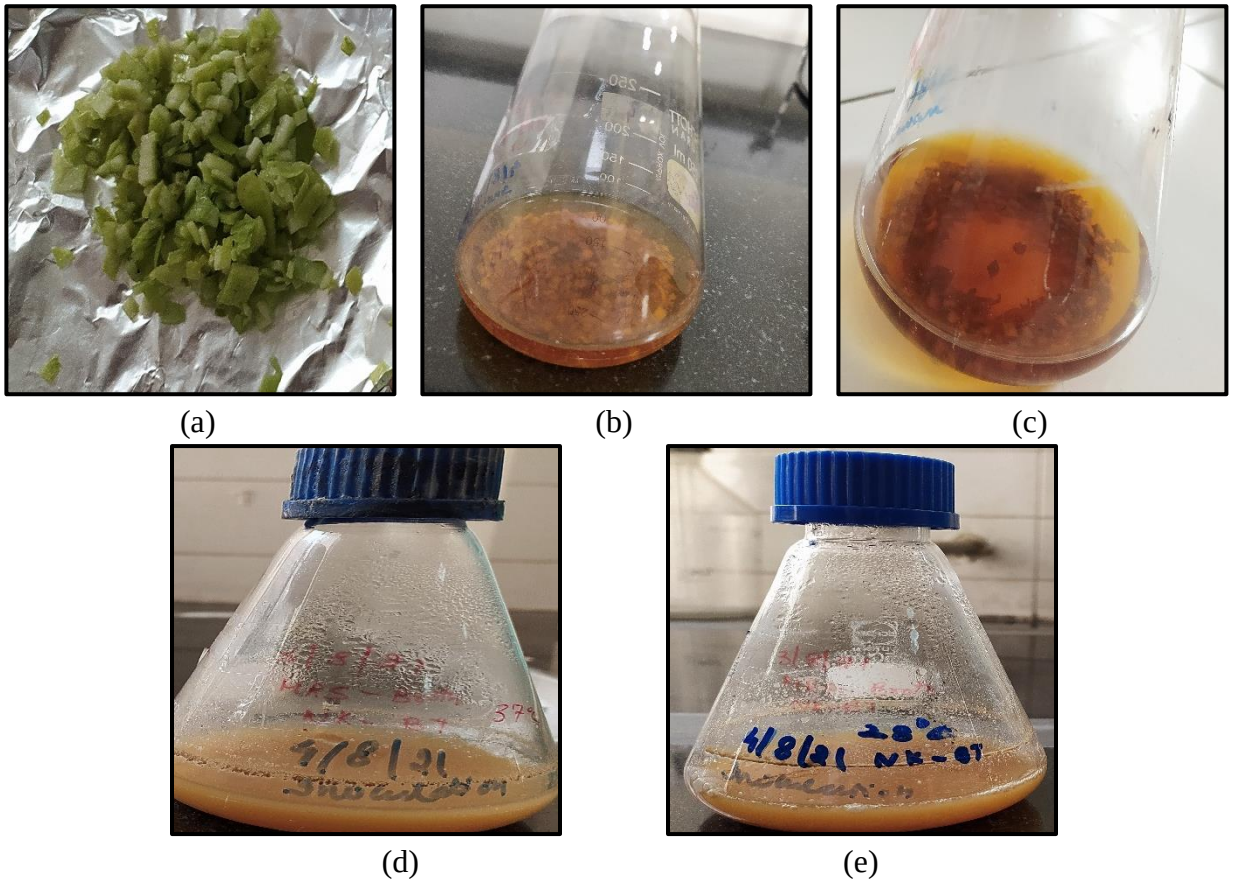
(d)

Figure 2: Steps for the preparation of guava juice (a-d)

4.2 Isolation of the bacteria for fermentation

For the isolation of bacteria from guava surface, chunks of fresh guava's epicarp were added into MRS media and the flasks were incubated at 28°C and 37°C at 120 rpm **(Figure 3 (a, b & c))**. After 48 hours, growth (turbidity) was observed in both of the flasks incubated at 28°C and 37°C at 120 rpm (**Figure 3 (d & e**). Samples were taken from the flasks and streaking was done on MRS agar plates and incubated at 28°C and 37°C for 24 hours. The bacterial colony obtained were again streaked on MRS agar plate to obtain a single colony. The bacterial culture isolate obtained at 37°C was named as isolate-1 (IS-1) **(Figure 4 (a))** and another culture obtained at 28°C was coded as isolate-2 (IS-2) **(Figure 4 (b))**. The visual appearance of the colony was tiny, creamy, silverish, convex and shiny in both of the plates **(Figure 4)**. Single colony was picked up and inoculated into MRS broth where further subculturing was performed. Isolated bacterium was sub-cultured on

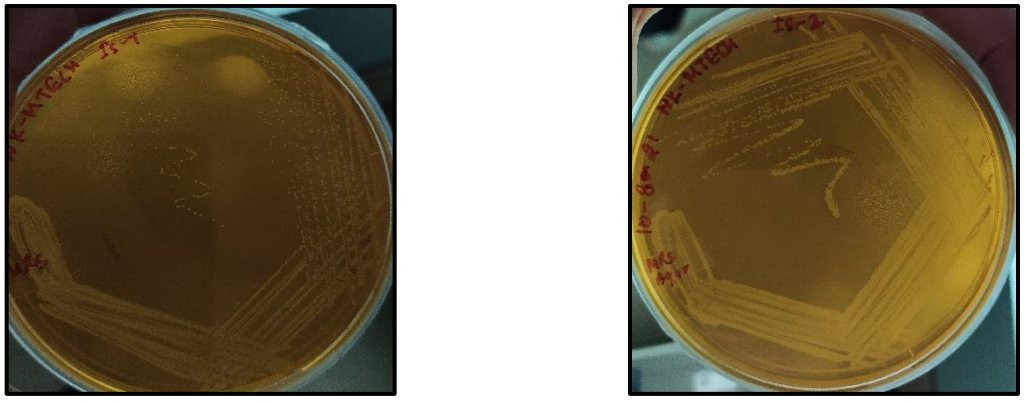
MRS agar plates once in two weeks and plates were stored at 4°C for further use. For preserving the isolated and pure bacterial culture, 50% sterile glycerol stock was prepared and microfuge tubes containing glycerol stock of the culture were stored at -80°C for further use.



(a) (b) (c)

(d) (e)

Figure 3: Steps of isolation of LAB from the guava's epicarp (a-e)



(a) (b)

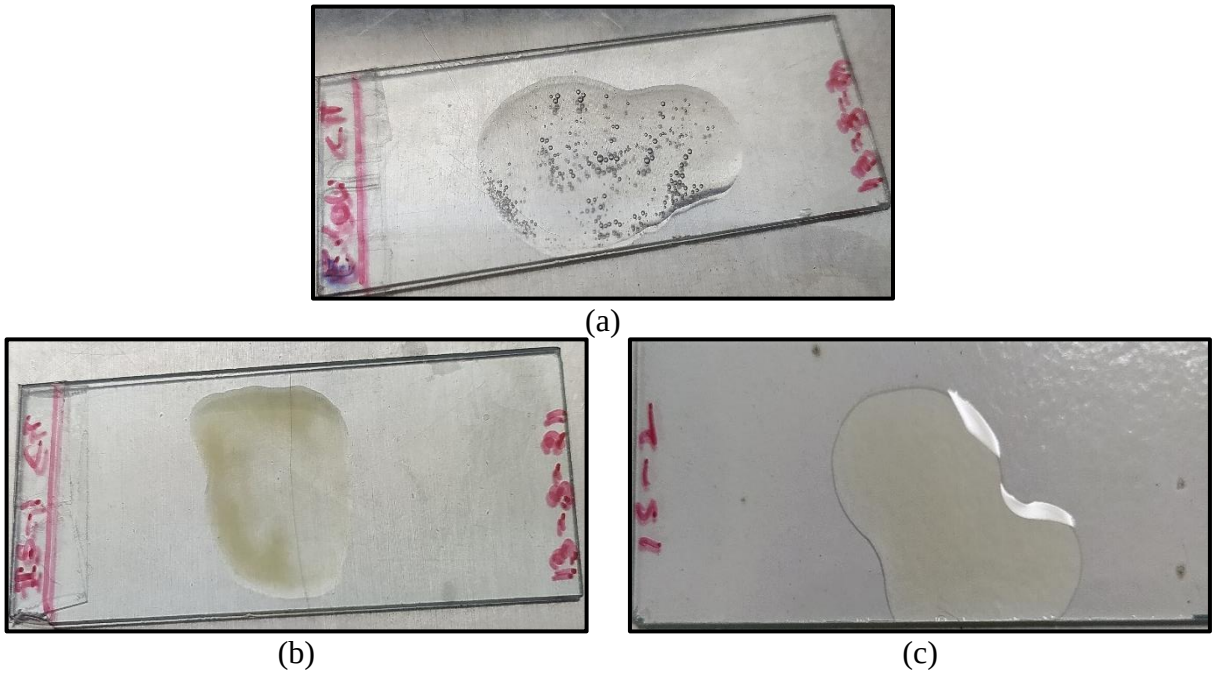
Figure 4: IS-1 (a) and IS-2 (b) on MRS agar plates

4.3 Characterization of the isolated LAB for fermentation

IS-1 and IS-2 isolated from the surface of guava fruit were characterized using following tests.

4.3.1 Catalase test

Single colony of IS-1 and IS-2 were picked up and inoculated in 50 ml of MRS broth. The flasks were incubated at 37°C and 28°C for 24 hours at 120 rpm. After the incubation period of 24 hours, 200 µl of the culture was withdrawn and tested using 3% H_2O_2 on a clean microscopic slide. IS-1 and IS-2 isolated bacterial culture were found to be catalase negative i.e., catalase enzyme was absent, because there was no bubble formation when 3% H_2O_2 was allowed to react with overnight grown bacterial culture **(Figure 5 (b and c))**. Thus, IS-1 and IS-2 both the isolates were considered as catalase negative. On the contrary, *Escherichia coli* DH5α which was used as positive control, showed the production of oxygen bubbles when H_2O_2 was allowed to react with it **(Figure 5 (a)).** Rod-shaped gram-negative bacteria, *Escherichia coli*, was able to produce enzyme which catalyzed the breakdown of H_2O_2 into water molecules and oxygen bubbles. Recent studies have shown that LAB are catalase- negative, gram-positive and facultative- anaerobes. These bacteria do not form any kind of spores and are safe to use (Linares-Morales et al., 2020).



(a)

(b) (c)

Figure 5: Catalase test for *Escherichia coli* DH5α (a); IS-1 (b) and IS-2 (c)

4.3.2. Confirmatory test for LAB on GYP medium

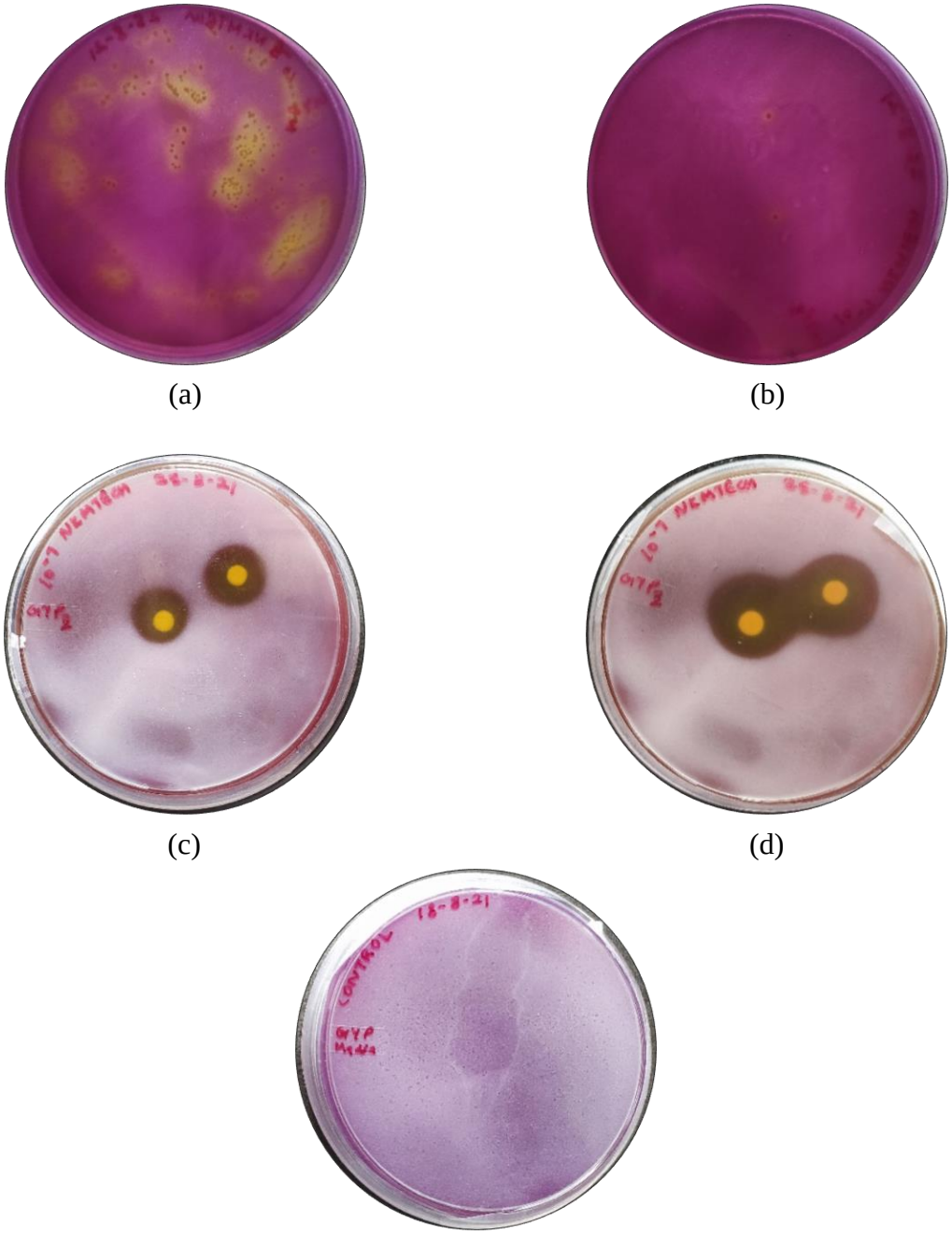
The glucose, yeast extract and peptone (GYP) with $CaCO_3$ medium was used as a selective for the lactic acid producing bacteria. This GYP medium allows the detection of LAB members from other types of bacteria. In order to confirm whether IS-1 and IS-2 were able to produce lactic acid, the overnight grown culture of IS-1 and IS-2 were used. Initially serial dilutions of IS-1 and IS-2 were prepared. 50 µl of the bacterial suspension was taken from each 10^{-5}, 10^{-6} and 10^{-7} dilution and spreading was performed on GYP-agar plates and these plates were incubated at 37°C and plates were observed after 24 hours. After incubation, approximately 208 yellow-colored tiny colony forming units appeared in 10^{-5} dilution **(Figure 6 (a))** and 2 colony forming units appeared in 10^{-7} dilution **(Figure 6 (b))**. Dissolved $CaCO_3$ appeared as wide clear zone after 48 hours of incubation **(Figure 6 (c)).** The size of the colony increased and zone became wider under storage conditions also **(Figure 6 (d))** and these plates were compared with GYP-medium control plate **(Figure6 (e))**.

After incubation period, it was observed that colony of LAB were possessing yellow color as characteristic feature that appeared on the GYP plates. Both the colony of IS-1 and IS-2 were found yellow in color and clear zone was seen around the colony. This zone of clearance occurred due to dissolved $CaCO_3$ when lactic acid was produced in the medium by IS-1 and IS-2 bacterial isolates. This production of an acid caused change in the pH of the medium, which reduced from 6.8 to lower pH where bromocresol purple, a pH indicator, changed into yellow colored dye. Clear zone around the colony was observed due to conversion of $CaCO_3$ into calcium lactate by lactic acid after 24 hours of incubation at 37 °C. Results indicated that the IS-1 and IS-2 bacterial isolates produced lactic acid in presence of glucose.

The above mentioned procedure was followed as discussed by (Cai et al., 1999), in which isolation and selection of *Pediococcus* species (LAB) from forage crops were carried out using GYP-$CaCO_3$ medium.

$$\mathbf{2\,(C_3H_6O_3) + CaCO_3 \rightarrow Ca(C_6H_{10}O_6) + H_2O + CO_2}$$

Lactic acid + Calcium carbonate → Calcium lactate + water + carbon dioxide



(a) (b) (c) (d) (e)

Figure 6: Clear zone around the IS-1 colony on selective GYP- $CaCO_3$ agar medium plates

4.3.3 Gas production test

Gas production test was performed on IS-1 and IS-2 following the method suggested by (Hayward, 1957) with slight modifications. Overnight grown culture of IS-1 and IS-2 were taken and

inoculated in test tubes containing MRS media and Durham tubes **(Figure 7)**. Test cultures were compared with control (uninoculated) media **(Figure 7 (a))**. After 48 hours of incubation, it was observed that there was no gas entrapment in the upper side of the Durham tubes. There was no significant difference between the Durham tubes as there was no visible gas bubbles in the durham tubes **(Figure 7 (b and c))**. Thus, it was interpreted that the bacterial isolate was homo-fermentive as it was only able to produce lactic acid but no CO_2 production was seen in the medium. Carbohydrate fermentation involves the production of gas and acids such as lactic acid, acetic acid, formic acid etc. From the above results it was confirmed that the isolated bacterial strain (IS-1 and IS-2) were homo-fermentive but not hetero-fermentive **(Figure 7).**

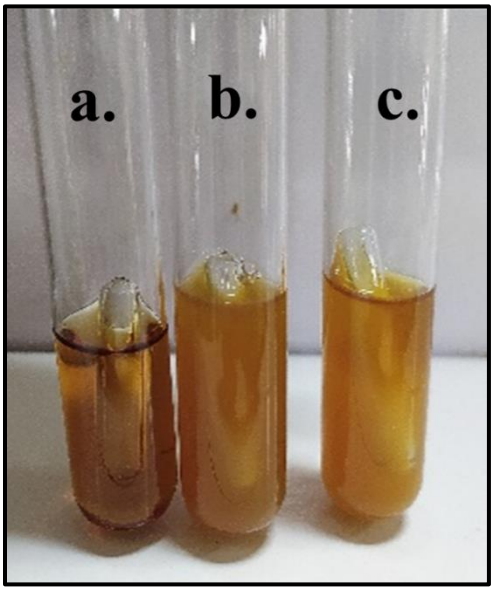


Figure 7: Gas production test on IS-1 and IS-2

4.4 Identification of the isolated microorganism

4.4.1 Morphological identification/Phenotypic characteristics

Gram staining was performed on both the isolates IS-1 and IS-2. Both the isolates were observed under microscope. Violet-colored spherical-shaped cells appeared in pairs (diplococcus or tetrads) of IS-1 under compound microscope at 40X **(Figure 8 (a))**, 100X **(Figure 8 (b))** magnification; cell morphology of IS-2 was also observed at 40X **(Figure 8 (c))**, 100X **(Figure 8 (d))**

magnification. Colony characteristics and morphology of cell using gram staining (Phenotypic characterization) confirmed that IS-1 and IS-2 bacterial isolates both were coccus shaped gram-positive lactic acid bacteria (LAB). Similar appearance of the colony characteristics and gram staining indicated that both the isolates IS-1 and IS-2 have same characteristic features. Therefore, IS-1 bacterial isolate was subjected for molecular identification and further analysis.

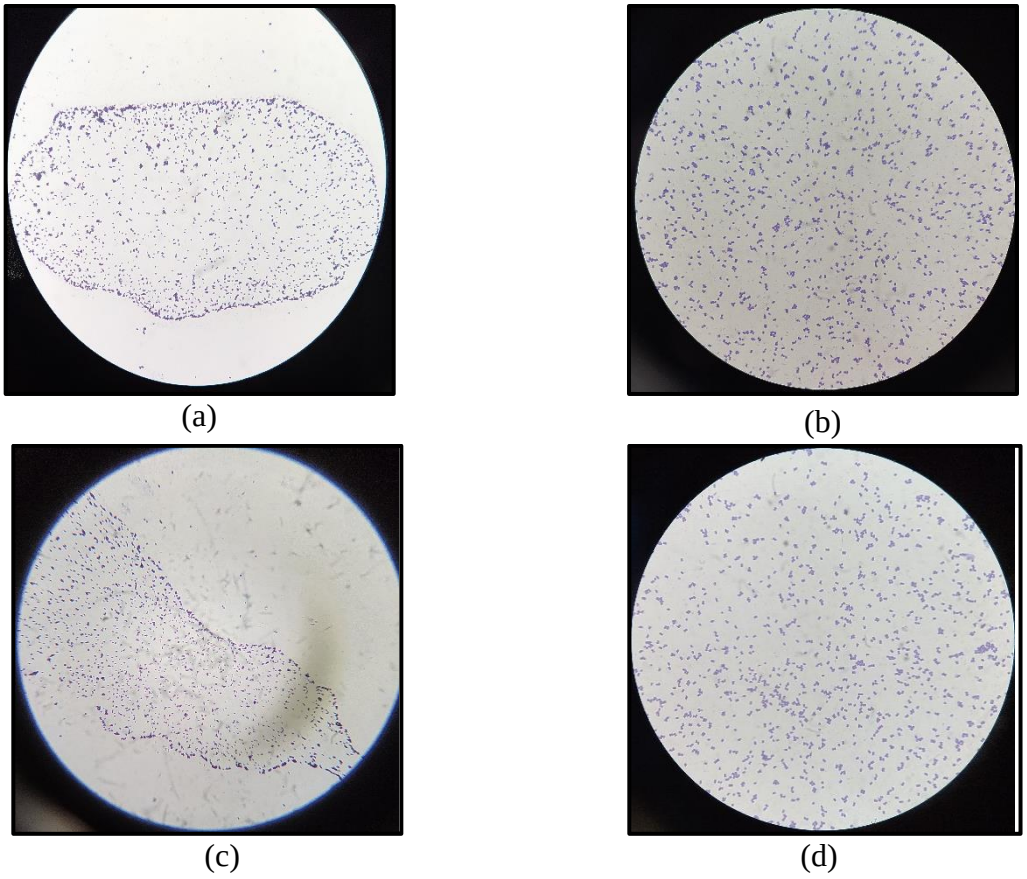


Figure 8: Pictures of gram staining of IS-1 and IS-2 at 40X (a and c) and 100X (b and d) magnification

4.4.2 Molecular Identification of isolated LAB strain (IS-1)

4.4.2.1 Genomic DNA isolation and agarose gel electrophoresis

For isolation of DNA, overnight grown culture of IS-1 was used and procedure for DNA isolation was followed as given by (Azcárate-Peril & Raya, 2001) with slight modifications. This method was phenol: chloroform: iso-amyl alcohol (PCI) method which is also known as organic extraction.

For separation and identification of genomic DNA agarose gel electrophoresis was performed. Molecular bands were visualized under UV light in gel documentation system where it was observed that genomic DNA has a size of more than 1 kb (>1000bp) **(Figure 9)**.

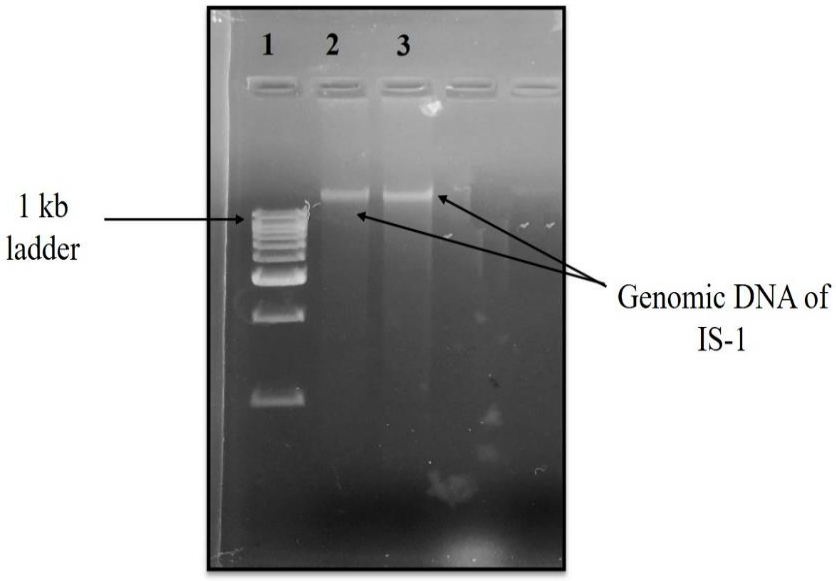


Figure 9: Genomic DNA of IS-1. Lane 1: - 1 kb DNA, Lane 2 and 3: - genomic DNA of IS-1

4.4.2.2 PCR-amplification of isolated DNA

Isolated DNA was PCR amplified with forward and reverse primers that were specific to 16S rRNA gene (conserved gene in bacteria) and purified **(Figure 10)**. The 260/280 ratio of purified DNA came out to be 1.83, thus purified DNA was free of any type of protein or RNA contamination and DNA was considered as pure DNA **(Table 9).**

Table 9: Absorbance of isolated, amplified and purified DNA

DNA	Absorbance
λ-230 nm	3.631
A-260 (10 mm path)	2.040
A-280 (10 mm path)	1.113
260/280	1.83
260/230	0.56
ng/µL	102.0

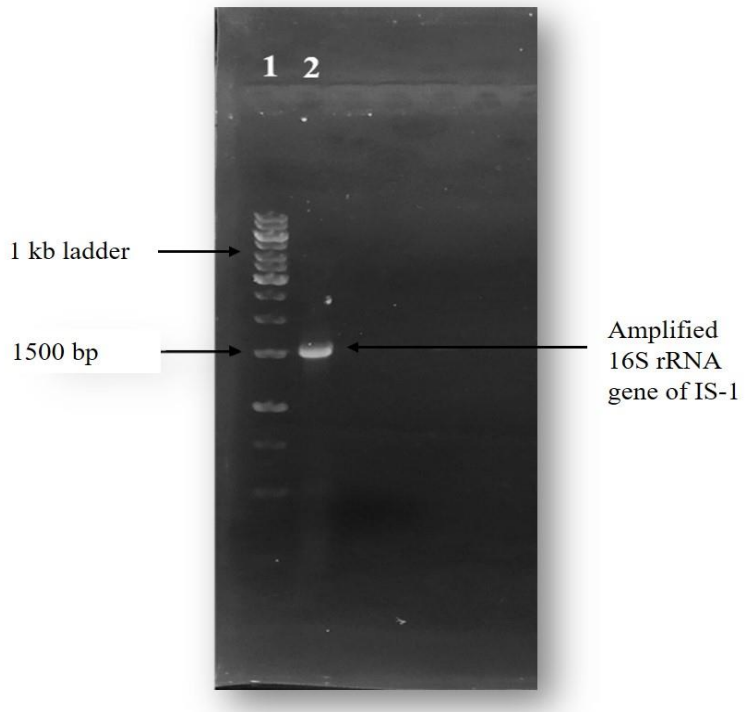


Figure 10: Purified PCR product of IS-1 after amplification of 16S rRNA gene

4.4.2.3 Construction of Phylogenetic Tree

Sanger dideoxy sequencing of purified PCR product of 16S rRNA gene provided the 16S rRNA sequence of isolated LAB. >1500 bases of 16S rRNA of IS-1 were determined. Using sequence similarity search tool (NCBI-BLAST), closely related strains of isolated LAB were obtained. The phylogenetic tree was constructed where IS-1 was found to be closely related to *Pediococcus pentosaceus* (MZ853323, ON000313, JX39793) as the highest sequence homology was found with IS-1 sequence having 99.40% identity. Bootstrap values in the phylogenetic tree were given for the branch point among different species of *Pediococcus.* Thus, it was confirmed that the isolated bacterium was *Pediococcus pentosaceus* which falls in the family of lactic acid producing bacteria. The sequence of *Pediococcus pentosaceus* was submitted on the NCBI-portal. The accession number/ID provided by NCBI-GenBanK of *Pediococcus pentosaceus* VNK-1 is ON810480 **(Figure 11)**. The taxonomy of *Pediococcus pentosaceus* VNK-1 is given in **Figure 12.**

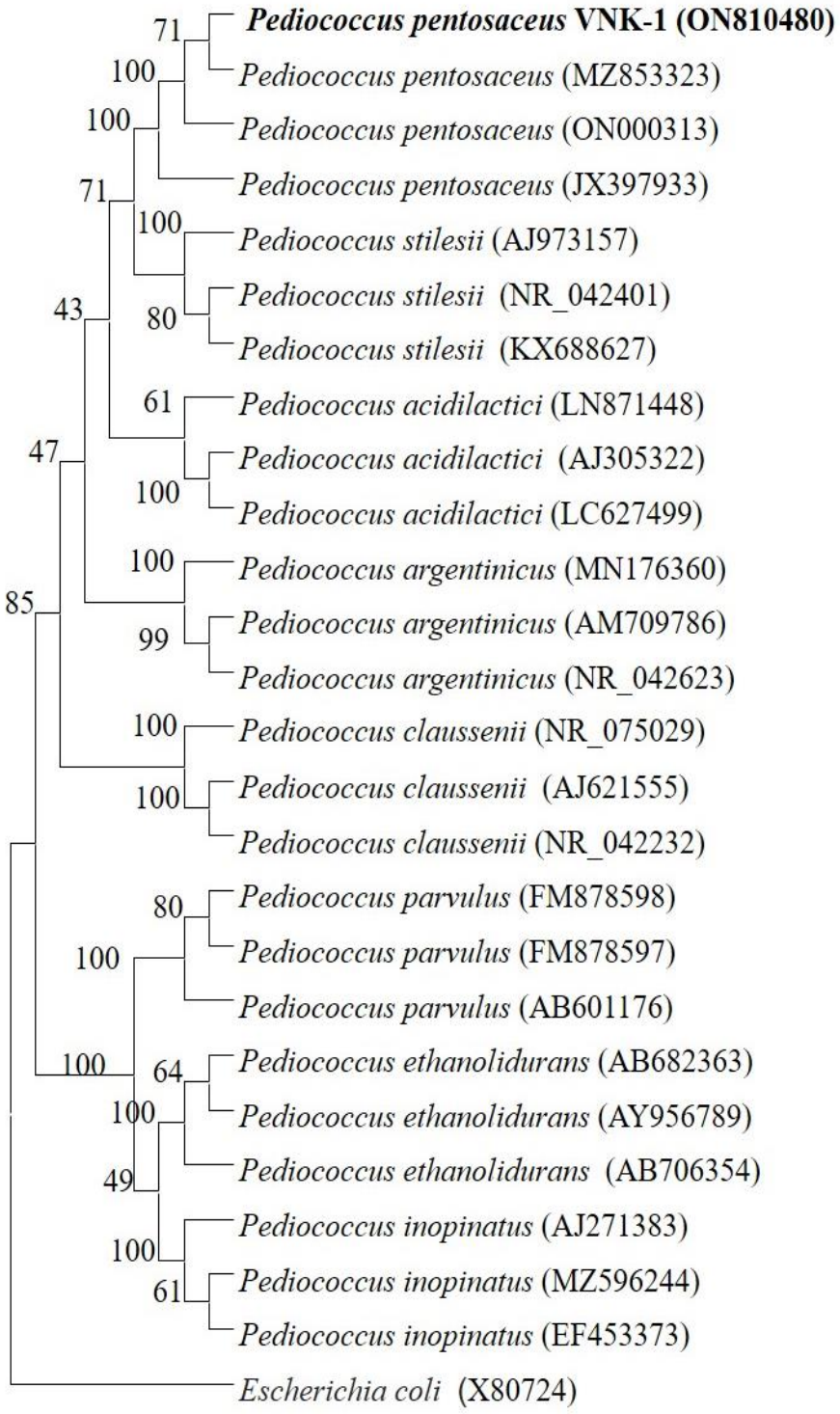


Figure 11: Phylogenetic tree derived by Neighbor-joining method

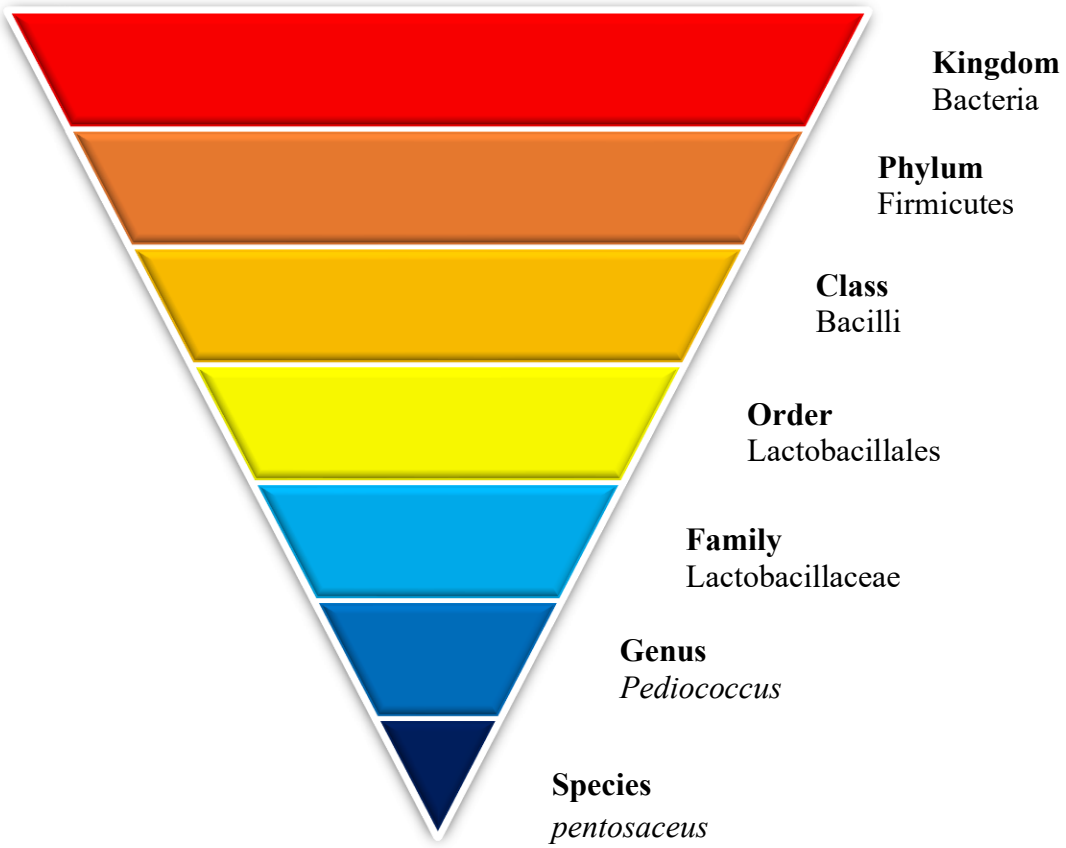


Figure 12: Taxonomy of *Pediococcus pentosaceus* VNK-1

4.5. Optimization of lactic acid fermentation using RSM

Lactic acid fermentation was performed according to the combinations provided by the RSM on the basis of three independent variables. Different concentrations of lactic acid that were obtained during experiments, are given in the matrix of CCD **(Table 10).**

The maximum lactic acid production (273.52 µg/ml) during lactic acid fermentation was at temperature 23.5 °C, inoculum concentration 12 % and RPM was 75 as shown in Table 1. Model was analyzed in CCD which was found to be quadratic model **(Table 10)**.

ANOVA was used to analyze the model and optimization of the parameters was done using RSM and the models were validated. Adequacy of the model, R^2, adjusted R^2, predicted R^2, lack of fit model was developed **(Table 11, Table 12 and Table 13)**. R^2 and adjusted R^2 for the experiments were found to be 0.9068 and 0.8230 respectively and the difference between both values was

0.0838 which was less than 0.1. Thus, model and the predicted responses were falling in acceptable range.

Table 10: Matrix of the three independent variables in CCD and the measured response (Lactic acid concentration)

		Factor 1	Factor 2	Factor 3	Response 1
Std	**Run**	**A: Temperature**	**B: Inoculum concentration**	**C: Agitation**	**Lactic acid**
		°C	**%**	**rpm**	**µg/ml**
1	18	18.5	6.6	60	176.28
2	13	28.5	6.5	60	150
3	7	18.5	10.5	60	260.92
4	4	28.5	10.5	60	206.52
5	1	18.5	6.5	89	82.83
6	15	28.5	6.5	89	166.12
7	19	18.5	10.5	89	116.76
8	10	28.5	10.5	89	183.88
9	14	15	8.5	75	146.1
10	9	32	8.5	75	150
11	16	23.5	5	75	110
12	**20**	**23.5**	**12**	**75**	**273.52**
13	5	23.5	8.5	50	177.5
14	11	23.5	8.5	100	85.5
15	3	23.5	8.5	75	124.97
16	6	23.5	8.5	75	170.47
17	2	23.5	8.5	75	120
18	12	23.5	8.5	75	118
19	17	23.5	8.5	75	118
20	8	23.5	8.5	75	120

Table 11: Analysis of model

Source	**Sequential p-value**	**Lack of Fit p-value**	**Adjusted R^2**	**Predicted R^2**	
Linear	0.0033	0.0643	0.4836	0.2789	
2FI	0.1049	0.0978	0.5972	0.2438	
Quadratic	**0.0101**	**0.4351**	**0.8230**	**0.5568**	**Suggested**
Cubic	0.3408	0.4682	0.8470	-0.2290	**Aliased**

Table 12: ANOVA for quadratic model

Source	Sum of Squares	Degree of freedom (Df)	Mean Square	F-value	p-value	
Model	45097.76	9	5010.86	10.82	0.0005	significant
A-Temperature	426.16	1	426.16	0.9199	0.3601	
B-Inoculum size	16027.85	1	16027.85	34.60	0.0002	
C-Agitation	11648.75	1	11648.75	25.14	0.0005	
AB	245.20	1	245.20	0.5293	0.4836	
AC	6675.32	1	6675.32	14.41	0.0035	
BC	1000.61	1	1000.61	2.16	0.1724	
A^2	1116.54	1	1116.54	2.41	0.1516	
B^2	8478.87	1	8478.87	18.30	0.0016	
C^2	125.48	1	125.48	0.2708	0.6141	
Residual	4632.91	10	463.29			
Lack of Fit	2494.00	5	498.80	1.17	0.4351	not significant
Pure Error	2138.91	5	427.78			
Cor Total	49730.67	19				

The model F-value of 10.82 implied that the model was significant. There is only a 0.05% chance that an F-value this large could occur due to noise. P-values less than 0.05 indicated model terms were significant. In this case B, C, AC, B^2 were significant model terms. Values greater than 0.1 indicated the model terms were not significant. The F-value of 1.17 implied the lack of fit was not significant relative to the pure error. There was a 43.51% chance that the F-value in lack of fit test this large could occur due to noise. According to the ANOVA of quadratic model lack of fit came out to be not-significant which means that model to be fitted and this model was significant and acceptable.

Table 13: Fit statistics of the predicted model

Std. Dev.	21.52	**R^2**	0.9068
Mean	152.87	**Adjusted R^2**	0.8230
C.V. %	14.08	**Predicted R^2**	0.5568
		Adequate Precision	12.8629

Adequate Precision measures the signal to noise ratio. ANOVA of predicted model gave the ratio greater than 4 i.e., 12.8629 which was found to be desirable which indicted that suggested model could be used for navigating design space. Results indicated the adequacy of fitted models because value of R^2 and adjusted R^2 were close to 1 **(Table 13)**. The results that were generated for lactic acid concentration (the measured response) in the CCD, were fitted in polynomial equation (**Table 14 and Table 15**).

Table 14: Final equation in terms of coded factors

Lactic Acid	=	
+128.28		
+5.59	A	B1
+34.26	B	B2
-29.21	C	B3
-5.54	AB	B12
+28.89	AC	B13
-11.18	BC	B23
+8.80	A^2	B11
+24.26	B^2	B22
+2.95	C^2	B33

The multiple coded equation is given as follows:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3,$$

$$\text{Lactic acid production} = +128.28 + 5.59\ X_1 + 34.26\ X_2 - 29.21\ X_3 + 8.80\ X_1^2 + 24.26\ X_2^2 + 2.95\ X_3^2 - 5.54\ X_1\ X_2 + 28.89\ X_1\ X_3 - 11.18\ X_2\ X_3$$

Table 15: Estimated coefficients in terms of coded factors

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High
Intercept	128.28	1	8.78	108.72	147.84
A-Temperature	5.59	1	5.82	-7.39	18.56
B-Inoculum size	34.26	1	5.82	21.28	47.24
C-Agitation	-29.21	1	5.82	-42.18	-16.23
AB	-5.54	1	7.61	-22.49	11.42
AC	28.89	1	7.61	11.93	45.84
BC	-11.18	1	7.61	-28.14	5.77
A^2	8.80	1	5.67	-3.83	21.44
B^2	24.26	1	5.67	11.62	36.89
C^2	2.95	1	5.67	-9.68	15.58

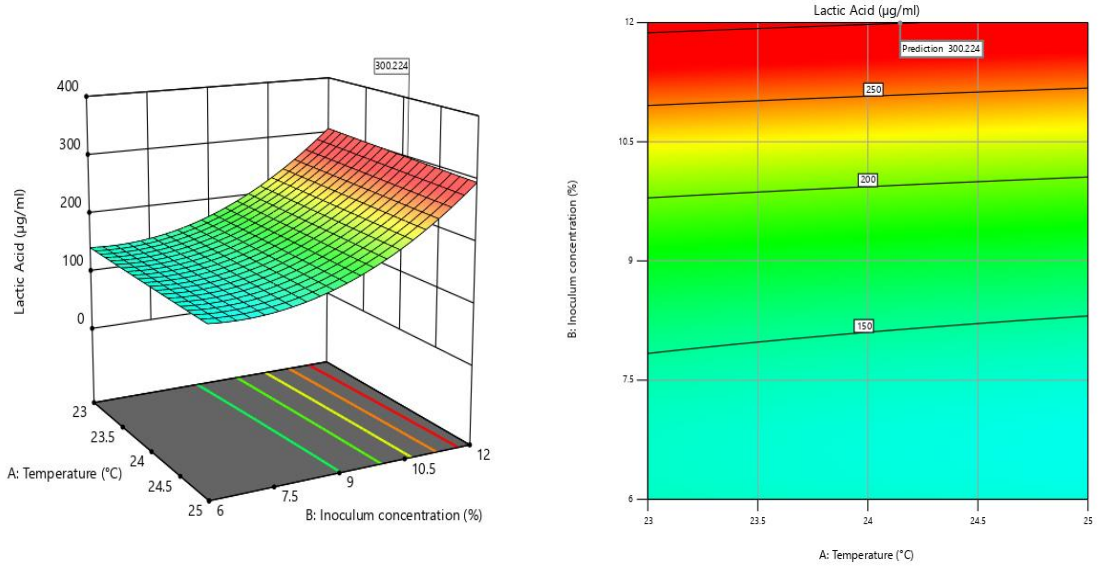
4.5.1 3D-Response surface and contour graphs

Three-dimensional surface plots have been analyzed using RSM based on three independent factors **(Figure 13)**. The optimum factors were found to be temperature 24°C, 12 % of the inoculum concentration of the starter culture and 60 rpm was the agitation rate and desirability came out to be 1 which was considered as optimal **(Figure 14)**. At these optimized parameters, maximum lactic acid concentration was predicted to be 300.224 µg/ml. Using these optimum conditions, the fermentation was performed to obtain the maximum lactic acid production and further analysis were performed.

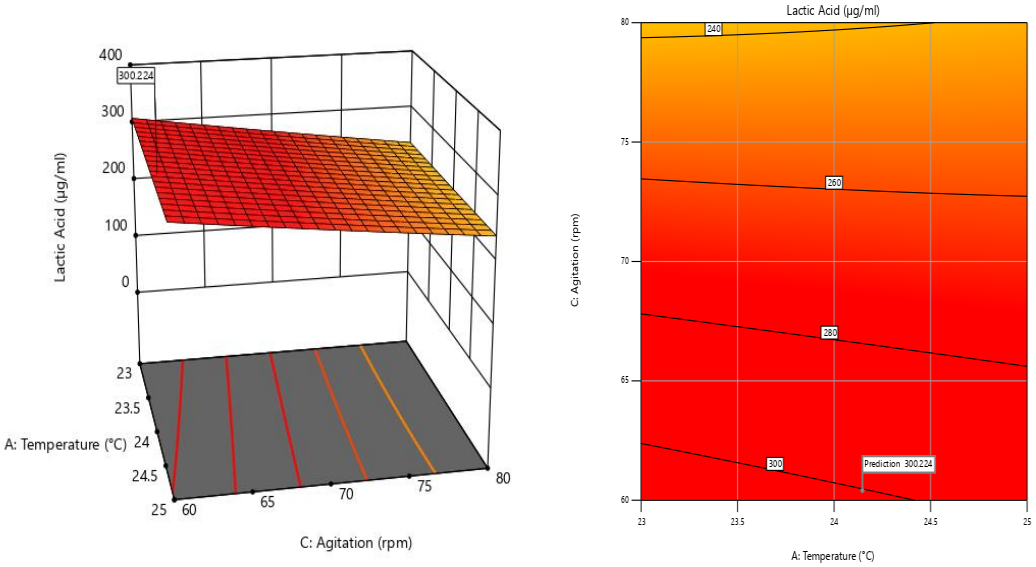
3-D graph of inoculum concentration (X1-axis) and temperature (X2-axis) represented that with increase in the inoculum concentration, the lactic acid production (Y-axis) was increasing whose concentration was found to be maximum at 12% inoculum size. Because increase in the inoculum concentration eventually cause the increase in number of LAB cell count.

The lactic acid production was found at maximum in relation with highest inoculum concentration and lower temperature i.e., 24 °C. The concentration of lactic acid beyond 24 °C temperature (upto 32 °C) was insignificant as there was least production of lactic acid above the optimal temperature 24 °C as per the result generated by RSM **(Figure 13 (a))**.

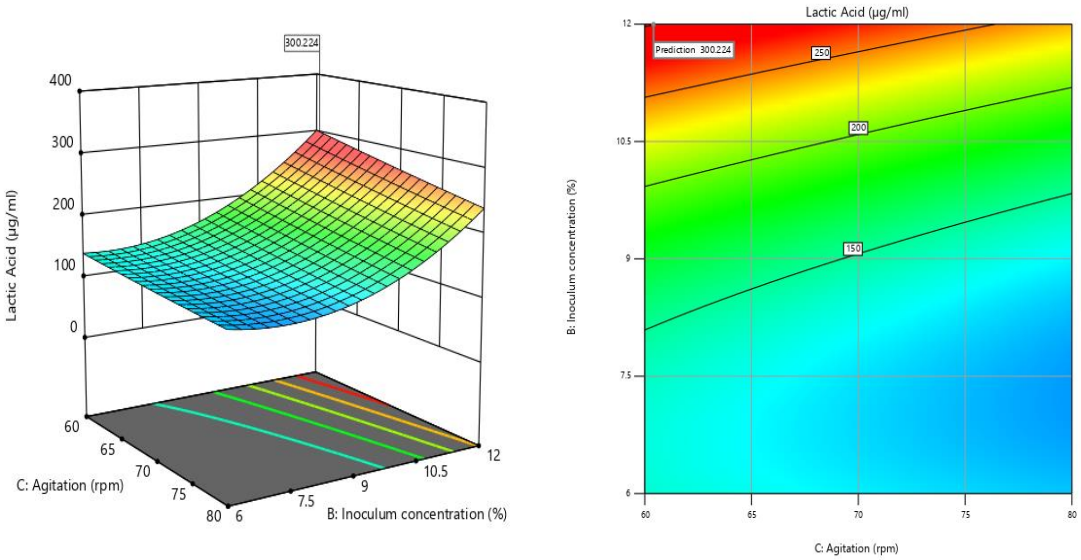
In second 3D graph of agitation and temperature **(Figure 13 (b))**, it was observed that at lower rpm i.e., 60 and temperature 24 °C, the maximum lactic acid production would occur, whereas with increase in the rpm it would result into decreased lactic acid production.



(a)



(b)



(C)

Figure 13: Three-dimensional response surfaces and contour graphs representing the effect of inoculum concentration, temperature and agitation rate on lactic acid production

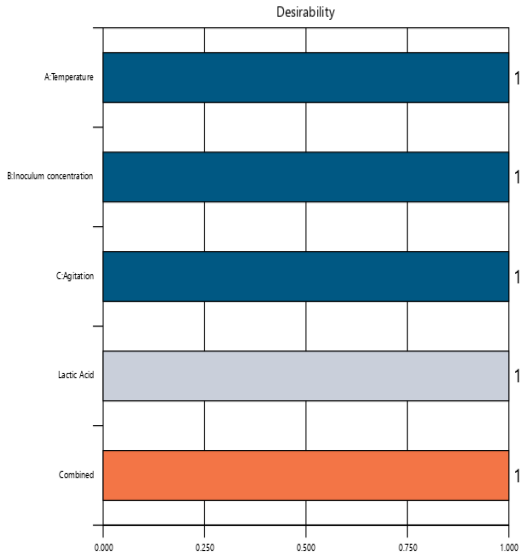


Figure 14: Bar graph of independent variables, lactic acid and their combined effect showing the desirability equal to 1

Another 3D graph represented the relationship between inoculum concentration and agitation where at lower inoculum concentration and lower rpm, the minimum lactic acid production was observed, whereas, with increase in the concentration of inoculum concentration (12%) and minimum agitation at 60 rpm, it was predicted to achieve the maximum lactic acid production around 300 µg/ml **(Figure 13 (c)).** Based on this analysis, the optimum conditions developed using CCD were selected to proceed for further experimentation.

4.6 Preparation and evaluation of the probiotic beverage

The guava juice samples were coded as given in the **Table 16.**

Table 16: Code for fermented samples

Code	Summary of the samples	Fermentation temperature	Figure No.
CJ	Control Juice	-	15 (a)
A1	Juice inoculated with 12% inoculum	24 °C	15 (b)
A2	Juice inoculated with culture pellet	24 °C	15 (c)
B1	Juice inoculated with 12% inoculum	37°C	15 (d)
B2	Juice inoculated with culture pellet	37°C	15 (e)

250 ml of the guava juice was pasteurized and used for lactic acid fermentation. The overnight grown culture of LAB having starter culture concentration 12 %, Temperature 24°C and 60 rpm. One set was A1 and A2, on the other hand, another set of B1 and B2 was prepared for 37°C keeping other parameters constant. Fermentation was carried out for 4 days. 0^{th} day, 1^{st} day, 2^{nd} day, 3^{rd} day and 4^{th} day samples were withdrawn from each jar and further analysis were performed in triplicates **(Figure 15)**.

4.6.1 Quantitative analysis of Lactic acid

A1, A2, B1 and B2 guava juice samples were evaluated for lactic acid concentration during fermentation days. Standard calibration curve prepared for lactic acid by colorimetric method where it was observed that with increase in the lactic acid concentration, intensity of the colored product increased gradually as shown in **Table 17** and **Figure 16**.

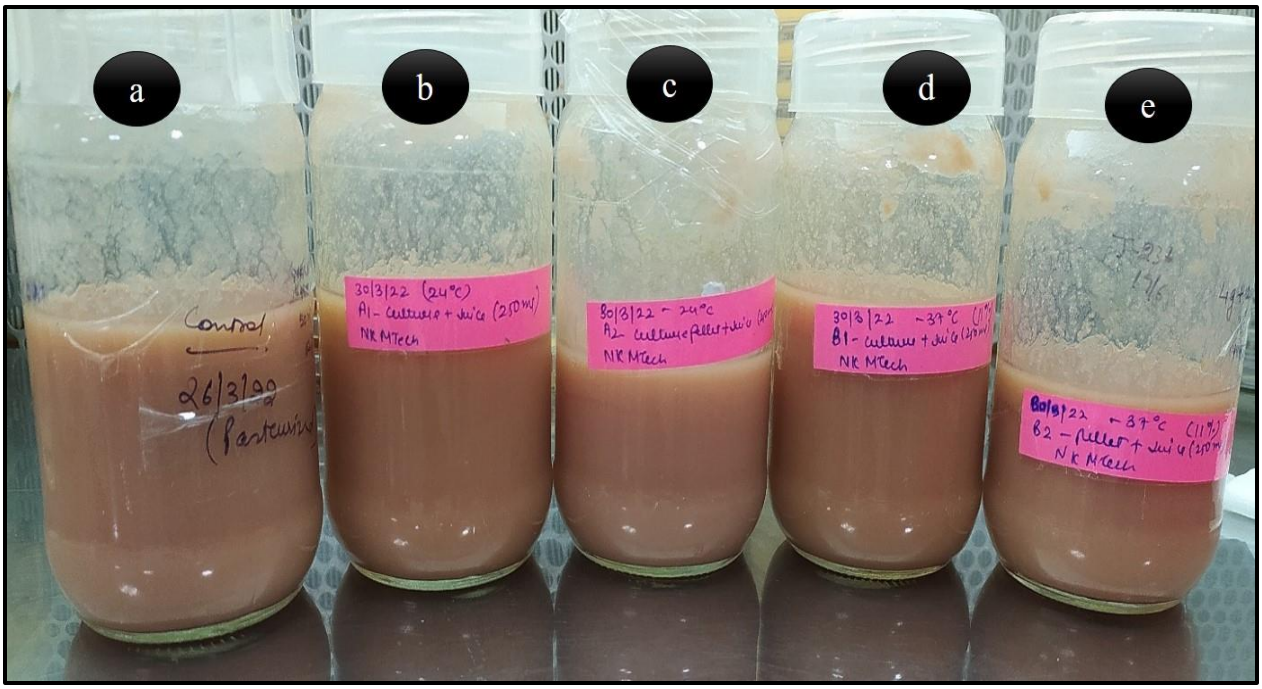


Figure 15: Control guava juice and probiotic beverage in glass jars

Table 17: Quantitative estimation of lactic acid standard

S. No.	Lactic acid (µg/ml)	O. D. at 570 nm
1	50	0.1705
2	75	0.2445
3	100	0.3536
4	125	0.4415
5	150	0.5742
6	175	0.6583
7	200	0.7765
8	225	0.8656
9	250	0.9654
10	275	1.1003
11	300	1.2252

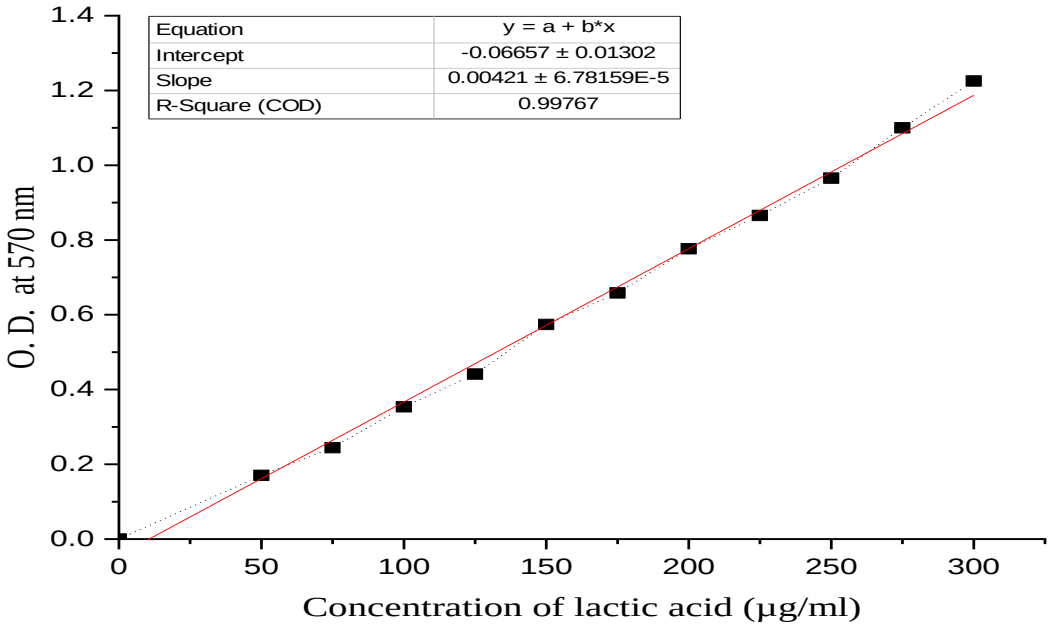


Figure 16: Standard curve of lactic acid

Fermented juice samples were analyzed for the presence of lactic acid during the fermentation period **(Table 18)**.

Table 18: Lactic acid concentration in fermented samples

Sample	L. A. (µg/ml)
CJ	0.000 ± 0.00
A1-0	175.86 ± 2.20
A1-1	393.97 ± 1.36
A1-2	431.07 ± 1.10
A1-3	481.31 ± 1.13
A1-4	561.75 ± 1.44
A2-0	70.00 ± 5.00
A2-1	123.30 ± 1.55
A2-2	195.40 ± 2.25
A2-3	267.66 ± 1.80
A2-4	356.26 ± 0.65

Values given in the table are mean± standard deviation

Sample	L. A. (µg/ml)
CJ	0.00 ± 0.00
B1-0	157.20 ± 3.61
B1-1	282.34 ± 0.73
B1-2	393.79 ± 1.01
B1-3	449.00 ± 9.64
B1-4	553.39 ± 1.21
B2-0	81.60 ± 2.09
B2-1	258.01 ± 3.42
B2-2	364.45 ± 0.91
B2-3	408.65 ± 3.33
B2-4	450.96 ± 1.41

Values given in the table are mean± standard deviation

During the quantitative analysis of the fermented samples of optimized parameters, in blank (0% guava juice) and control juice (non-fermented guava juice), there was no lactic acid detected. The fermented samples were detected with lactic acid confirmed by the presence of the purple color using colorimetric-assay. As the fermentation was performed upto 4 days, all the samples showed the increase in lactic acid production. There was significant increase in the lactic acid (L.A.) concentration due to consumption of available sugars (mainly glucose) by probiotic lactic acid producing bacteria. Sugars were further metabolized to produce lactic acid in the medium.

In A1 samples, lactic acid concentration in guava juice significantly increased from 175.86 ± 2.20 µg/ml on 0^{th} day to 561.75 ± 1.44 µg/ml on 4^{th} day **(Figure 17 (a))**. The A2 samples have shown less lactic acid production as compared to A1 samples. In A2 samples, lactic acid concentration increased from 70.00 ± 5.00 µg/ml on 0^{th} day to 356.26 ± 0.65 µg/ml on 4^{th} day **(Figure 17 (b))**. Similarly, in B1 samples lactic acid concentration increased from 157.20 ± 3.61 µg/ml on 0^{th} day to 553.39 ± 1.21 µg/ml on 4^{th} day **(Figure 17 (c))**. B2 samples shown increased lactic acid concentration from 81.60 ± 2.09 µg/ml on 0^{th} day to 450.96± 1.41 µg/ml on 4^{th} day **(Figure 17 (d))**. At 24° C, it was observed that A1 samples have maximum lactic acid concentration as compared to B1 samples of 37° C. Highest L.A. concentration was found in the fermented juice samples containing overnight grown cell culture as inoculum whereas less and slow L.A. production was found in juice samples containing cell culture pellet as starter inoculum **(Figure 17).** Also, 561.7507 ± 1.44806 µg/ml was the highest L.A. production at 24°C in comparison fermentation carried out at 37°C at 12% inoculum concentration and 60 rpm **(Table 18)**. Thus, it was interpreted that, aiming for maximum lactic acid production in guava juice may occur at 24°C having 12% inoculum concentration (culture broth) and 60 rpm.

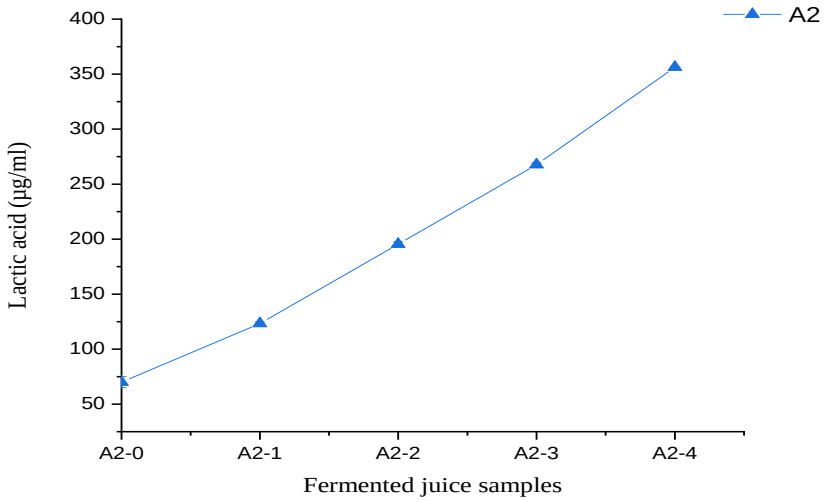
Recent studies reported that the lactic acid production during fermentation at 37°C was 6500 µg/ml when six LAB were used for fermenting apple juice (Wu et al., 2020). After fermentation of pineapple juice with different strains of *Pediococcus pentosaceus* and *Lactobacillus rhamnosus*, Lactic acid concentration was found in the range from 53.14 µg/ml to 91.88 µg/ml which further increased to 224.29-317 µg/ml when this probiotic juice was kept under storage conditions (AdebayoTayo & Akpeji, 2016).

One study reported that when *Pediococcus pentosaceus* was used as starter culture, lactic acid fermentation can be carried out at 24°C, that would improve the product texture. According to the

study, it has been shown that fermentation when performed between temperature 23–30 °C, consumers accepted the fermented product obtained with improved texture. Although at higher temperature (37°C) rapidly growing bacteria would able to formulate low quality product due to synthesis of biogenic amines in fermented product of silver carp sausages (Xu et al., 2010) .

Thus, guava juice can be potentially used as a natural substrate for the growth of LAB to develop a probiotic beverage. As discussed in a recent study that nutrient rich apricot juice can be used as natural substrate for the growth and lactic acid fermentation by LAB without addition of any kind of supplements (Bujna et al., 2017). Thus, according to the results obtained in this study, guava juice can be a good substrate/ medium for LAB that has influenced its growth in natural juice to form a probiotic beverage.

(a)



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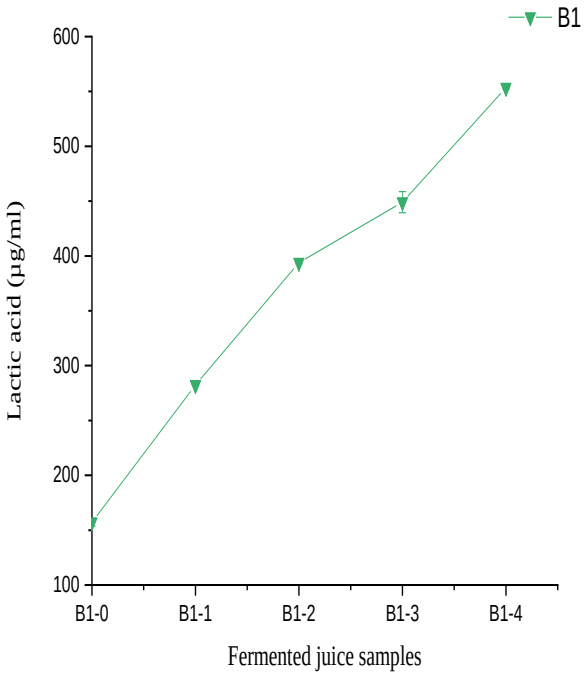

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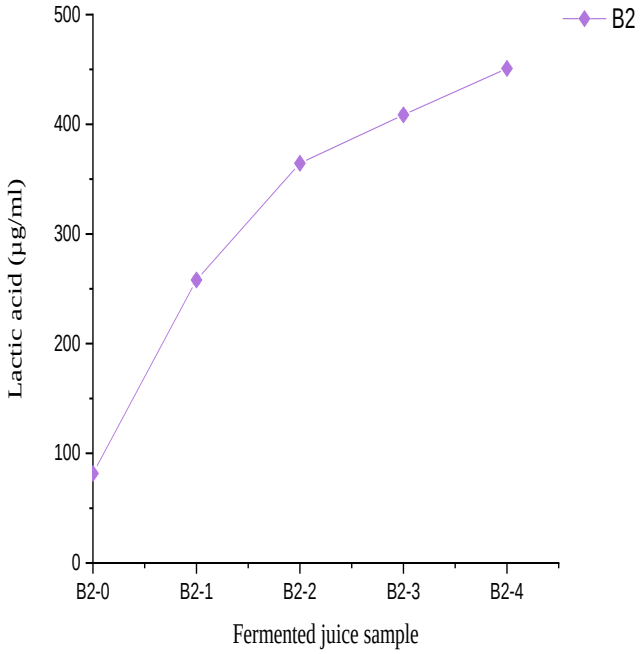

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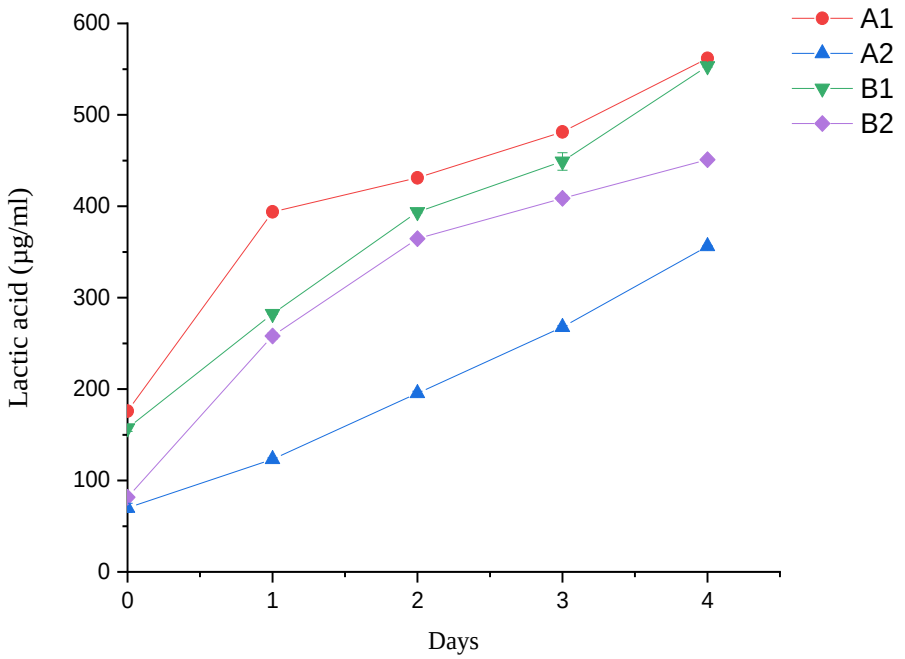


(e)

Figure 17: Lactic acid production in fermented juice samples (0^{th} to 4^{th} day)

4.6.2 Analysis of pH and Total soluble solids (TSS)

A1, A2, B1 and B2 fermented juice samples were analyzed for pH and TSS (°Brix) during the fermentation period of 4 days **(Table 19 and Table 20)** and represented in graphs in **Figure 18** and **Figure 19** for pH and TSS (°Brix) respectively.

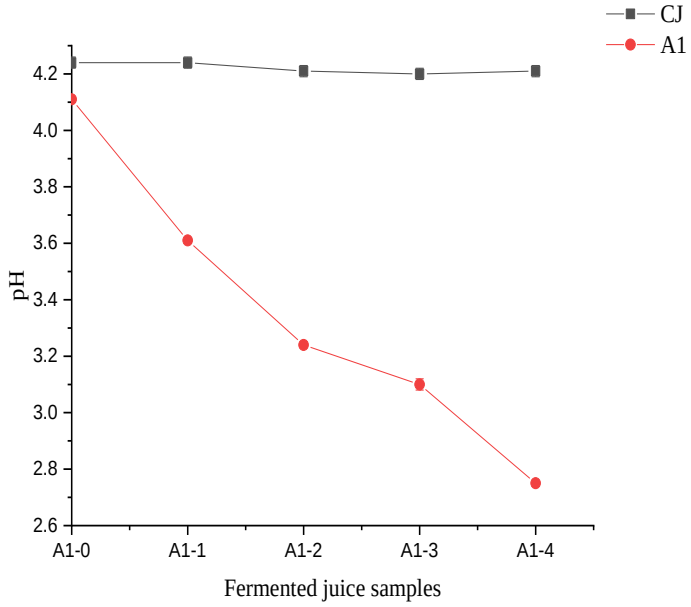
Table 19: pH of the fermented juice samples

Sample	pH
CJ	4.24± 0.02
A1-0	4.11 ± 0.01
A1-1	3.61 ± 0.01
A1-2	3.24 ± 0.01
A1-3	3.10 ± 0.02
A1-4	2.75 ± 0.01
A2-0	4.21 ± 0.03
A2-1	3.5 ± 0.01
A2-2	3.41 ± 0.01
A2-3	3.06 ± 0.01
A2-4	2.86 ± 0.02

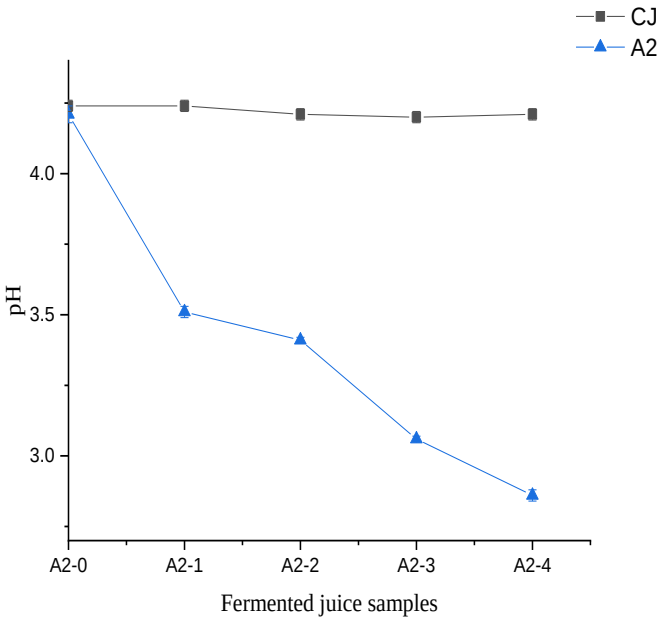
Values given in the table are mean± standard deviation

Sample	pH
CJ	4.24± 0.02
B1-0	4.12 ± 0.01
B1-1	3.71 ± 0.01
B1-2	3.51 ± 0.02
B1-3	3.23 ± 0.06
B1-4	2.83 ± 0.06
B2-0	4.22 ± 0.01
B2-1	3.81 ± 0.01
B2-2	3.41 ± 0.01
B2-3	3.41 ± 0.02
B2-4	3.18 ± 0.05

Values given in the table are mean± standard deviation



(a)



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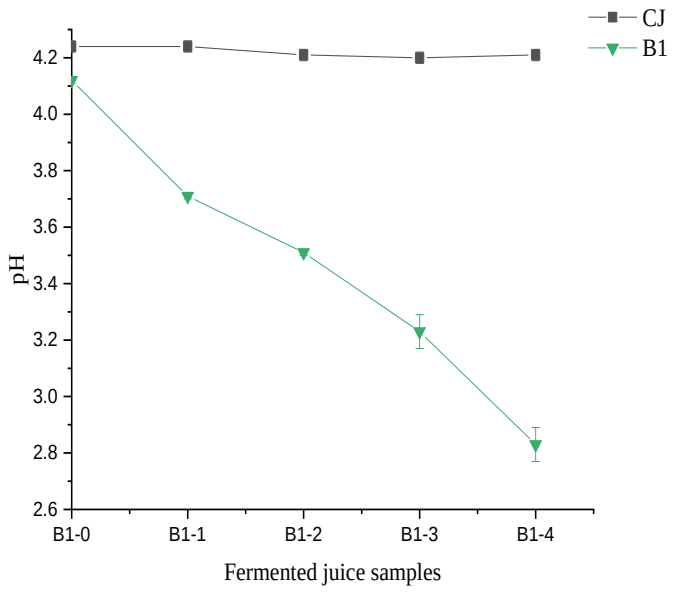

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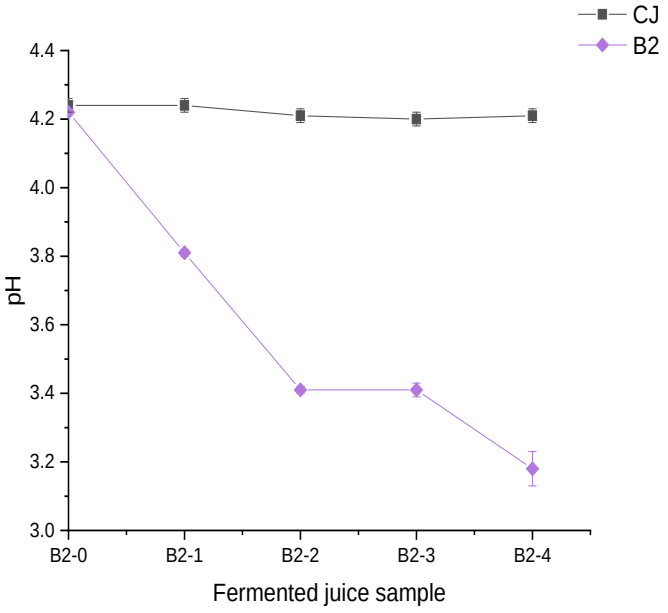

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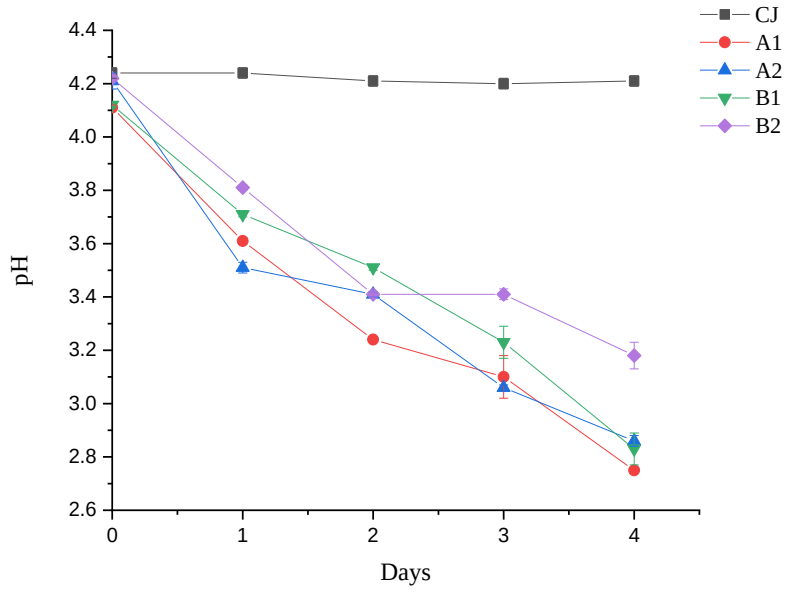


(e)

Figure 18: pH of fermented juice samples from 0th to 4th day

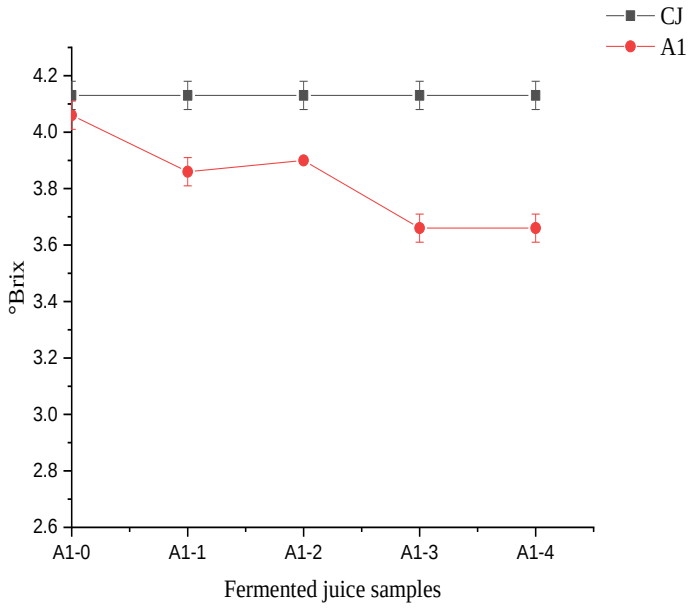
Table 20: °Brix of the fermented juice samples

Sample	°Brix
CJ	4.13 ± 0.05
A1-0	4.06 ± 0.05
A1-1	3.86 ± 0.05
A1-2	3.90 ± 0.01
A1-3	3.66 ± 0.05
A1-4	3.66 ± 0.05
A2-0	4.00 ± 0.01
A2-1	4.00 ± 0.01
A2-2	3.90 ± 0.01
A2-3	3.76 ± 0.06
A2-4	3.80 ± 0.01

Values given in the table are mean± standard deviation

Sample	°Brix
CJ	4.13 ± 0.05
B1-0	4.10 ± 0.01
B1-1	3.96 ± 0.05
B1-2	3.90 ± 0.01
B1-3	3.80 ± 0.10
B1-4	3.66 ± 0.06
B2-0	4.03 ± 0.05
B2-1	4.00 ± 0.01
B2-2	3.90 ± 0.01
B2-3	3.76 ± 0.05
B2-4	3.80 ± 0.01

Values given in the table are mean± standard deviation



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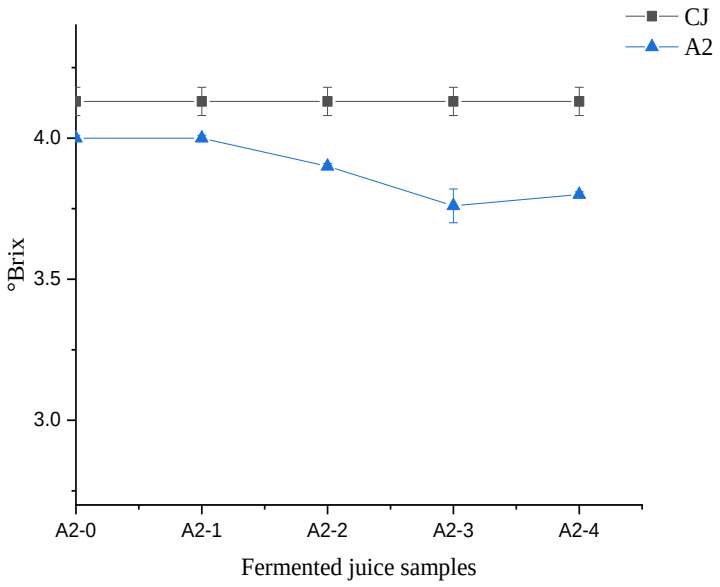

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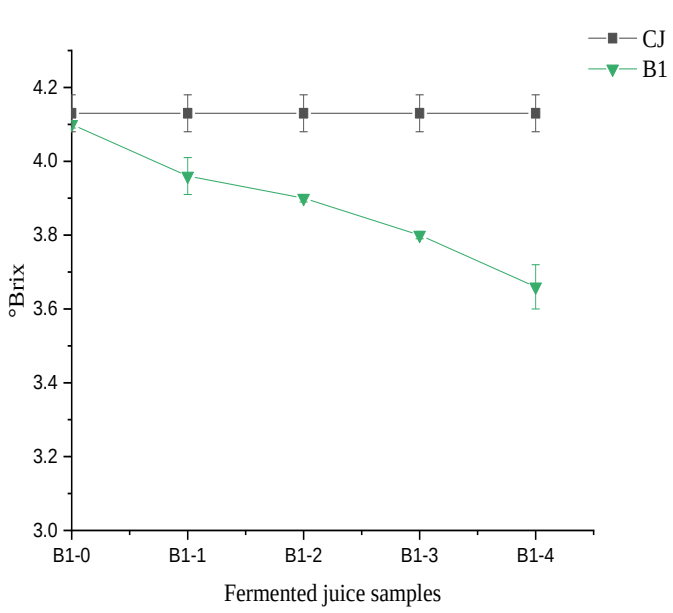

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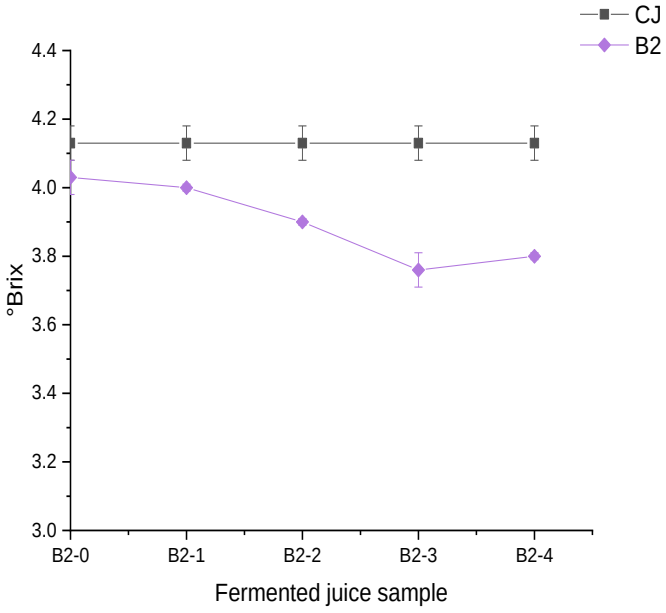

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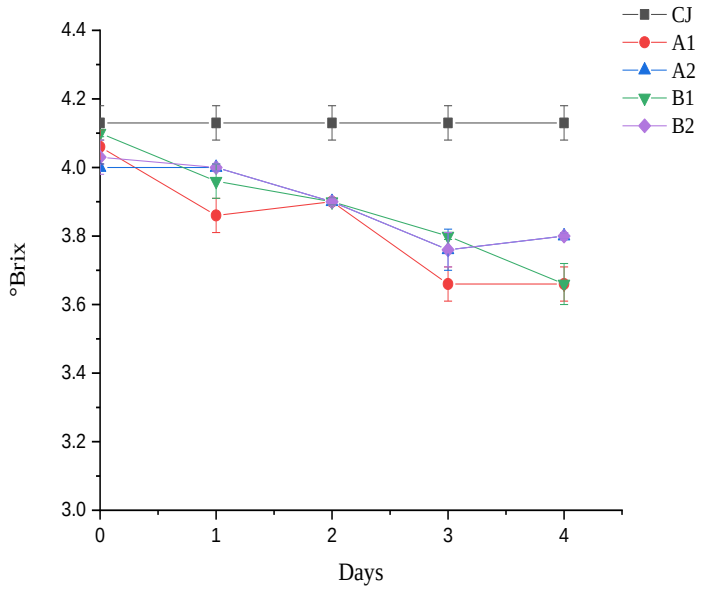


(e)

Figure 19: °Brix of fermented juice samples from 0th to 4th day

The results of pH and TSS (°B) of fermented samples are given in **Table 19 and Table 20** respectively. Data indicated that the pH of the control sample (non-fermented guava juice) was

4.24± 0.02. During fermentation hours pH of the juice significantly got reduced. The pH of the juice was decreased due to the organic acid especially the lactic acid produced during the fermentation days. In A1 samples, pH reduced from 4.11 ± 0.01 on 0^{th} day to 2.75 ± 0.01 on 4^{th} day **(Figure 18 (a))**. In A2 samples, pH reduced from 4.21 ± 0.03 on 0^{th} day to 2.86 ± 0.02 on 4^{th} day **(Figure 18 (b))**. Sample A1 and A2 at 4^{th} day shown the pH 2.75 ± 0.01and 2.86 ± 0.02 which was decreased from initial pH 4.11 ± 0.01 and 4.21 ± 0.031 respectively.

Similarly, at 37 °C, pH of the B1 fermented samples reduced from 4.12± 0.01 on 0^{th} day to 2.83 ± 0.06 on 4^{th} day **(Figure 18 (c))**. Similarly, pH of the B2 fermented samples was reduced from 4.22 ± 0.01 on 0^{th} day to 3.18 ± 0.05 on 4^{th} day **(Figure 18 (d))**. The results depicted that the lowest pH came out at 24°C due to successive growth of bacteria at this particular temperature. Also, juice inoculated with cell culture have shown the maximum pH reduction as compared to the juice samples inoculated with cell pellet of starter culture. pH of all the samples was compared with the pH of control juice **(Figure 18 (e))**.

For determining the total soluble solids i.e., solids such as sugars, acids, proteins and other salts etc. present in the dissolved form in the juice, data was represented in °B. Initial degree Brix of the juice was found to be 4.13 ± 0.057 **(Table 20)**. During fermentation hours, the fermented juice samples A1, °B were reduced from 4.0 ± 0.057 on 0^{th} day to 3.6 ± 0.05 on 4^{th} day **(Figure 19 (a))**. In A2 samples, °B were reduced from 4.00 ± 0.01 on 0^{th} day to 3.8 ± 0.01 on 4^{th} day **(Figure 19 (b))**. In B1 samples, °B were reduced from 4.1 ± 0.01 on 0^{th} day to 3.6 ± 0.06 on 4^{th} day **(Figure 19 (c))**. In B2 samples, °B were reduced from 4 ± 0.05 on 0^{th} day to 3.8 ± 0.01 on 4^{th} day **(Figure 19 (d))**. The comparison among all the samples was done with control juice **(Figure 19 (e))**.

Thus, TSS in samples significantly got reduced from 4.1 to 3.6 in the samples having culture as inoculum and the samples those were inoculated with cell pellet of LAB, have shown slight decrease in the °B from 4.1 to 3.8. As per the results, pH reduced in the fermented juice sample at maximum rate as compared to samples inoculated with cell pellet. It could be the reason that samples having bacterial culture that were able to grow actively as MRS media components flourished the bacterial growth whereas the harvested cells grow slow due to slow adaptation in the medium. It can be revealed that decrease in the pH of the medium due to organic acid such as

lactic acid that may increase the shelf life of probiotic beverage and prevent it from getting contaminated with other types of pathogens. Similar results have shown in the recent study where three strains of probiotic LAB have significantly developed a functional beverage of star fruit juice where pH have significantly reduced from 5.91 ± 0.01 to 4.76 ± 0.11 (*Lactobacillus helveticus* L10), 4.71 ± 0.03 (*Lactobacillus paracasei* L26) and 4.41 ± 0.02 (*Lactobacillus rhamnosus* HN001) and °B reduced from 7.09 ± 0.01 to 6.97 ± 0.03 (*Lactobacillus helveticus* L10), 6.98 ± 0.03 (*Lactobacillus paracasei* L26) and 6.93 ± 0.01 (*Lactobacillus rhamnosus* HN001) during the 8 days of fermentation due to consumption of sugars and production of lactic acid and other organic acids such as acetic acid (Lu et al., 2018).

4.6.3 Determination of Total Phenolic content

Calibration curve for gallic acid standard was prepared from 0 to 30 µg/ml concentration **(Table 21** & **Figure 20)** and A1, A2, B1 and B2 samples were analyzed for the total phenolic content. The concentration of phenolic content presented in **Table 22** and **Figure 21** for control and fermented juice samples.

Table 21: Standard curve of gallic acid

Gallic acid (µg/ml)	O. D. at 750 nm
0	0
2.5	0.0488
5	0.1138
7.5	0.1742
10	0.2388
12.5	0.3265
15	0.3825
17.5	0.4466
20	0.5011
22.5	0.5503
25	0.6083
27.5	0.6559
30	0.7147

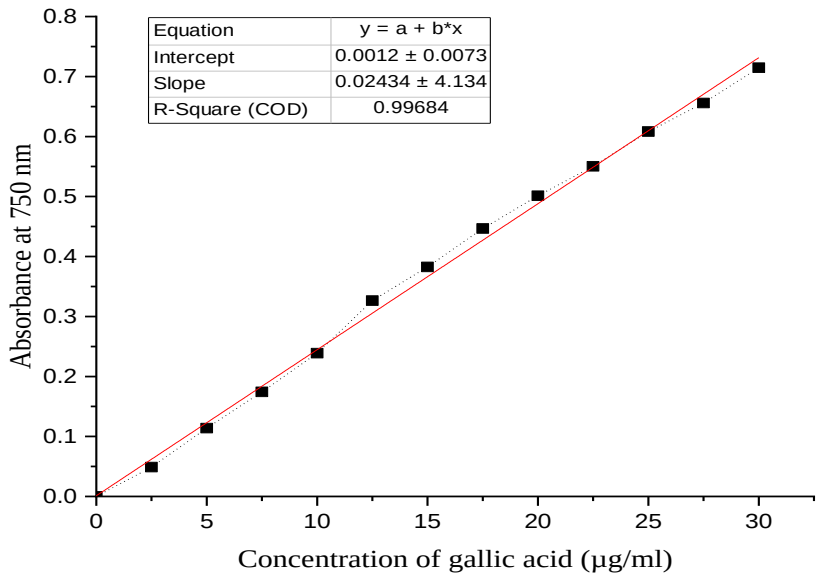


Figure 20: Calibration curve of gallic acid (µg/ml)

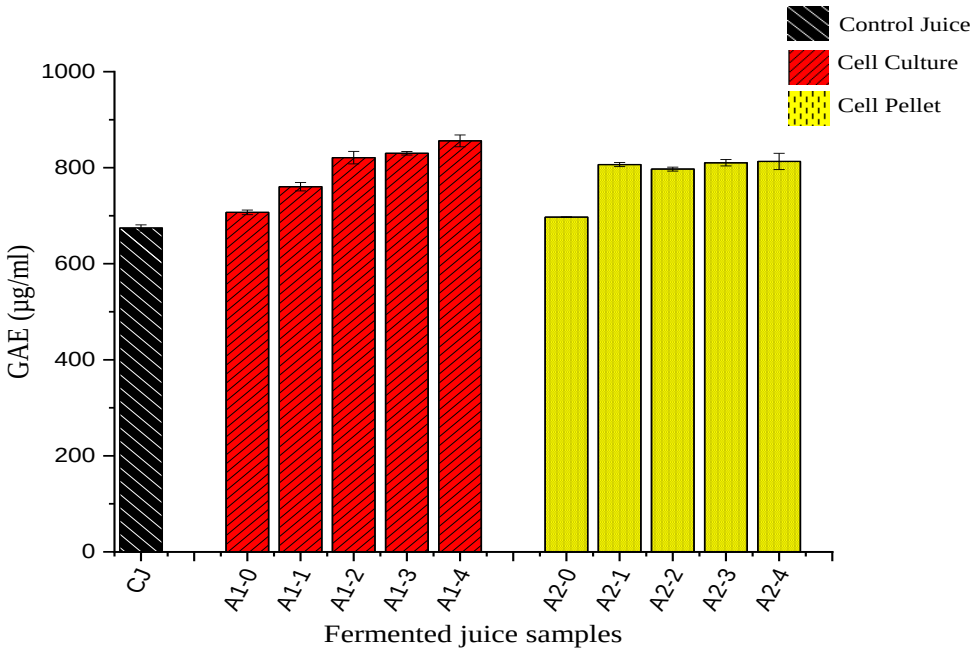
Table 22: Total phenolic content in control and fermented juice samples

Sample	µg GAE /ml
CJ	674.48 ± 6.53
A1-0	706.99 ± 4.76
A1-1	760.49 ± 8.82
A1-2	820.98 ± 12.97
A1-3	830.00 ± 3.60
A1-4	855.96 ± 12.37
A2-0	697.11± 0.62
A2-1	806.58 ± 4.14
A2-2	797.11 ± 4.17
A2-3	810.28 ± 6.61
A2-4	812.95 ± 16.98

Values given in the table are mean± standard deviation

Sample	µg GAE /ml
CJ	674.48 ± 6.53
B1-0	702.46 ± 1.85
B1-1	788.06 ± 8.17
B1-2	813.99 ± 12.57
B1-3	830.86 ± 22.43
B1-4	831.36 ± 2.07
B2-0	725.51 ± 11.05
B2-1	737.03 ± 19.00
B2-2	760.08 ± 12.64
B2-3	783.53 ± 16.10
B2-4	785.25 ± 14.63

Values given in the table are mean± standard deviation



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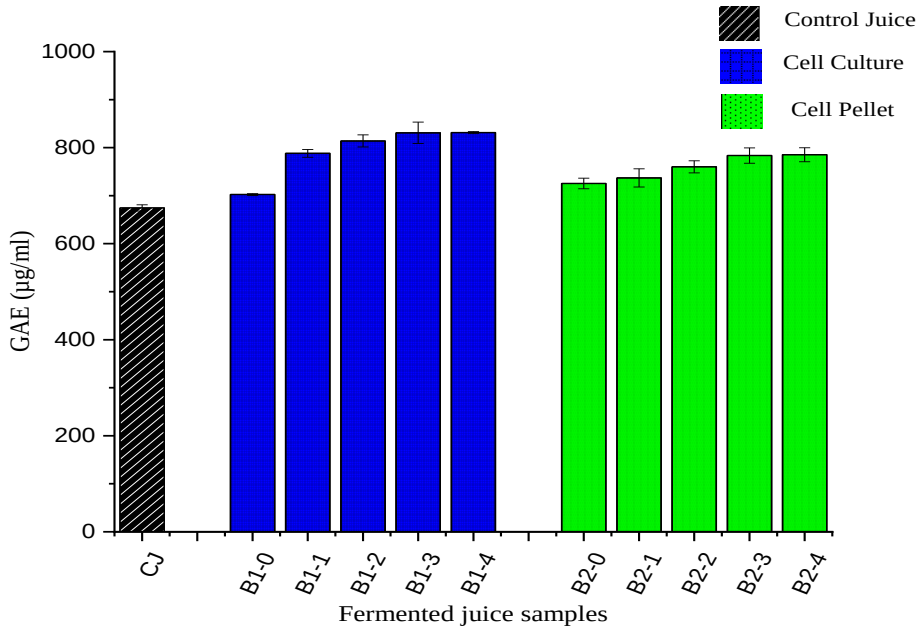


(b)

Figure 21: Total phenolic content (µg GAE/ml) in control juice (CJ) and fermented juice samples from 0^{th} to 4^{th} day

The initial phenolics of the control juice was 674.48 ± 6.53 µg GAE/ml **(Table 22)** During the fermentation days, phenolics have increased in fermented juice samples. In A1 samples, phenolic content increased from 706.99 ± 4.76 µg GAE/ml on 0^{th} day to 855.96 ± 12.37 µg GAE/ml on 4^{th} day and in A2 samples, phenolic contents increased from 697.11± 0.62 µg GAE/ml on 0^{th} day to 812.95 ± 16.98 µg GAE/ml on 4^{th} day **(Figure 21)**.

With increase in the temperature of fermentation less phenolics have been produced. In B1 samples, phenolic content increased from 702.46 ± 1.85 µg GAE/ml on 0^{th} day to 831.36 ± 2.07 µg GAE/ml on 4^{th} day and in B2 samples, phenolic contents increased from 725.51 ± 11.05 µg GAE/ml on 0^{th} day to 785.25 ± 14.630 µg GAE/ml on 4^{th} day **(Figure 21).**

Lactic acid fermentation of guava juice showed the increased phenolics as compared to control juice. At 24°C maximum phenolics were produced **(Figure 21)**. In A2, B1 and B2 samples phenolic content increased upto 3^{rd} day but on 4^{th} day these samples showed insignificant increase in total phenolic content.

Increase in the phenolic content may occur due to the enzymatic degradation of various polyphenols during the fermentation days by LAB. Another study reported the 66.48 % increase (11800 µg GAE/ml) in phenolics during fermentation as compared to control juice (2830 µg GAE/ml) when *Lactobacillus plantarum* was used for the inoculation of guava fruit extract (Bhat et al., 2015).

It has been reported in a study that on fermentation of medicinal plants with lactic acid bacteria (*Lactobacillus acidophilus*, *Bifidobacterium breve* and the mixture of both of the bacteria) to develop a functional beverage, the highest polyphenols were found in the range from 182.50 to 315.62 µg GAE/ml during the 30 days of fermentation period (Gadhoumi et al., 2021).

4.6.4 Estimation of Total Flavonoid Content

Calibration curve for Quercetin (µg QE/ml) was prepared from 100-900 µg/ml **(Table 23** & **Figure 22)** and A1, A2, B1 and B2 samples were analyzed for the total flavonoid content estimation. The concentration of flavonoid content presented in **Table 24** and **Figure 23** for control juice and fermented juice samples.

Table 23: Standard curve of quercetin

Quercetin (µg/ml)	O. D. 510 nm
100	0.066
200	0.168
300	0.249
400	0.321
500	0.424
600	0.514
700	0.604
800	0.711
900	0.817

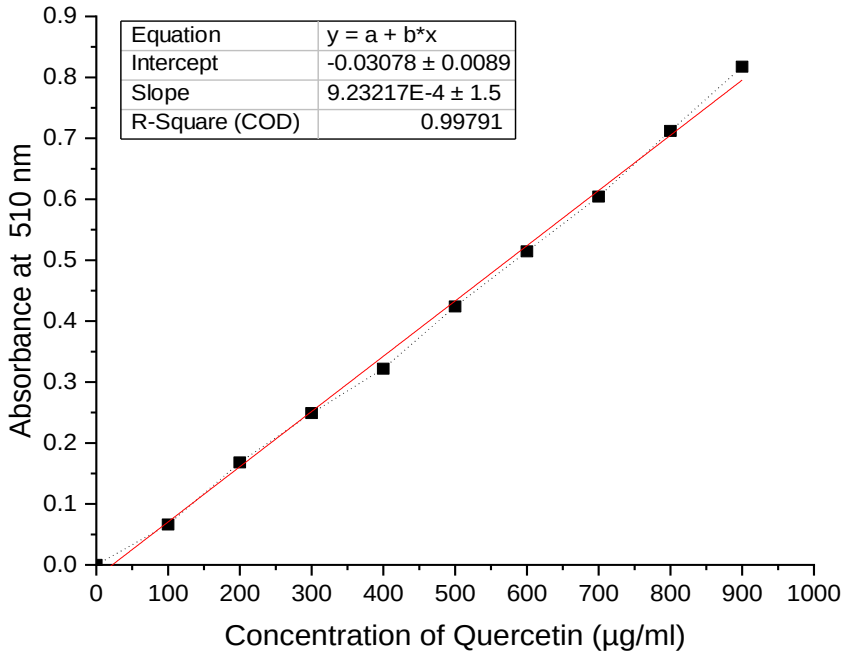


Figure 22: Calibration curve of total flavonoids (µg QE/ml)

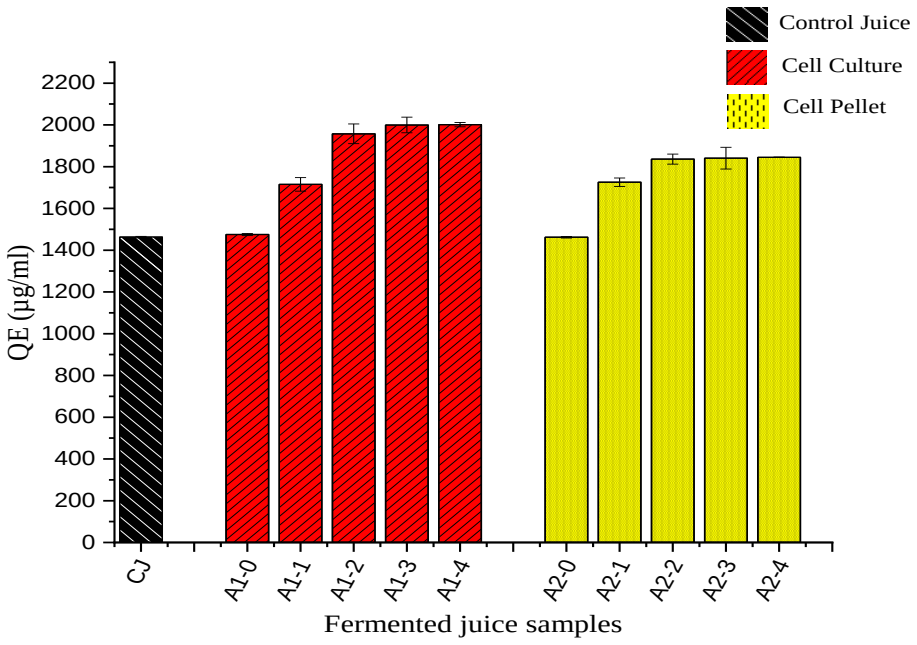
Table 24: Flavonoid content in control and fermented juice samples

Sample	Quercetin Equivalent (µg/ml)
CJ	1462.59 ± 3.57
A1-0	1475.00± 5.00
A1-1	1715.18 ± 32.77
A1-2	1957.40 ± 47.27
A1-3	1999.63 ± 37.45
A1-4	2001.44 ± 10.59
A2-0	1462.00±3.6
A2-1	1725.55 ± 20.00
A2-2	1836.29 ± 24.19
A2-3	1840.74 ± 51.88
A2-4	1845.00 ± 1.00

Values given in the table are mean± standard deviation

Sample	Quercetin Equivalent (µg/ml)
CJ	1462.59 ± 3.57
B1-0	1476.33 ± 3.78
B1-1	1578.37 ± 10.1
B1-2	1748.88 ± 20.57
B1-3	1750.14 ± 0.25
B1-4	1877.00 ± 7.21
B2-0	1467.66 ± 2.51
B2-1	1632.59 ± 27.26
B2-2	1653.33 ± 24.36
B2-3	1659.40 ± 28.61
B2-4	1767.63 ± 45.01

Values given in the table are mean± standard deviation



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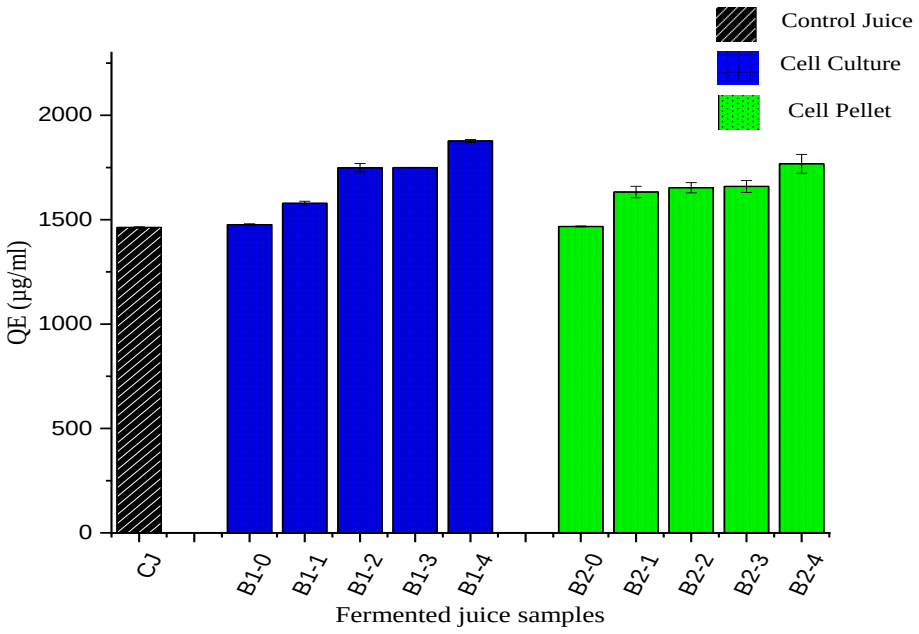


(b)

Figure 23: Total flavonoids (µg QE/ml) in control juice (CJ) and fermented juice samples from 0^{th} to 4^{th} day

Flavonoid content of the fermented juice samples significantly increased from the flavonoids present in control juice sample (**Table 24**). During the hours of fermentation, A1 and A2 have 1475 ± 5 µg QE/ml and 1462 ± 3.6 µg QE/ml respectively and B1 and B2 have 1476 ± 3.78 µg QE/ml and 1467 ± 2.5 µg QE/ml respectively. These values were close to flavonoids present in control juice 1462.59 ± 3.57 µg QE/ml. During the fermentation days, flavonoid content increased to 2001.44 ± 10.59 µg QE/ml and 1845.00 ± 1.00 µg QE/ml of A1 and A2 samples on 4^{th} day respectively **(Figure 23 (a))**. B1 and B2 on 4^{th} day, showed increased flavonoid content to 1877 ± 7.211 µg QE/ml and 1767.63 ± 45.01 µg QE/ml respectively **(Figure 23 (b))**.

It has been reported in a study that on fermentation of medicinal plants with lactic acid bacteria (*Lactobacillus acidophilus*, *Bifidobacterium breve* and the mixture of both of the bacteria) to develop a functional beverage, the highest flavonoid content was found as 152.13 µg QE/mL during the 30 days of fermentation period (Gadhoumi et al., 2021).

4.6.5 Estimation of Reducing sugars

Calibration curve for glucose (µg/ml) was prepared from 100-1000 µg/ml **(Table 25** & **Figure 24)** and samples were analyzed for the reducing sugar estimation. The concentration of reducing sugars presented in **Table 26** & **Figure 24** for control and fermented juice samples.

Table 25: Calibration curve of glucose standard

Glucose (µg/ml)	OD at 540 nm
100	0.1364
200	0.3384
300	0.5356
400	0.75
500	0.9441
600	1.1488
700	1.3579
800	1.5697
900	1.73845
1000	1.95105

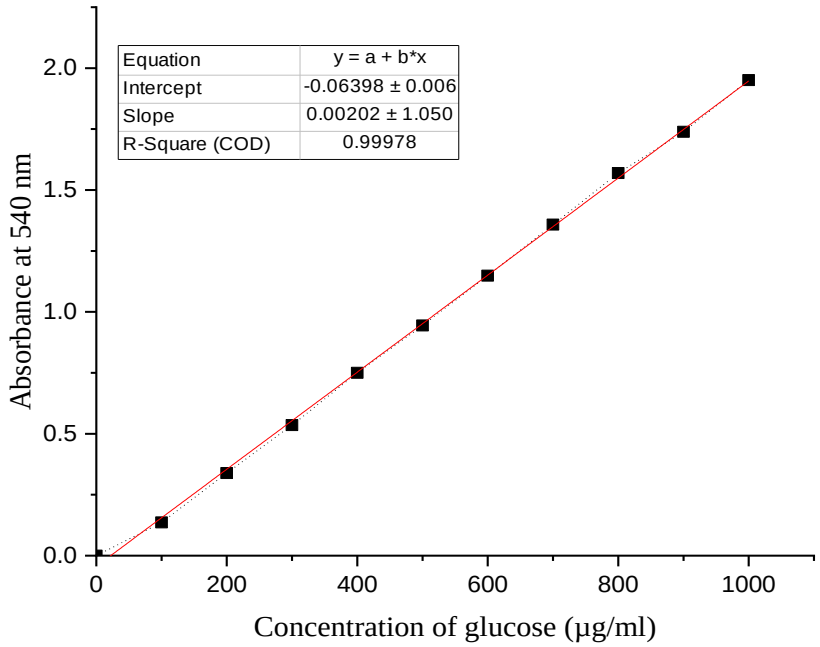


Figure 24: Calibration curve of glucose (µg/ml)

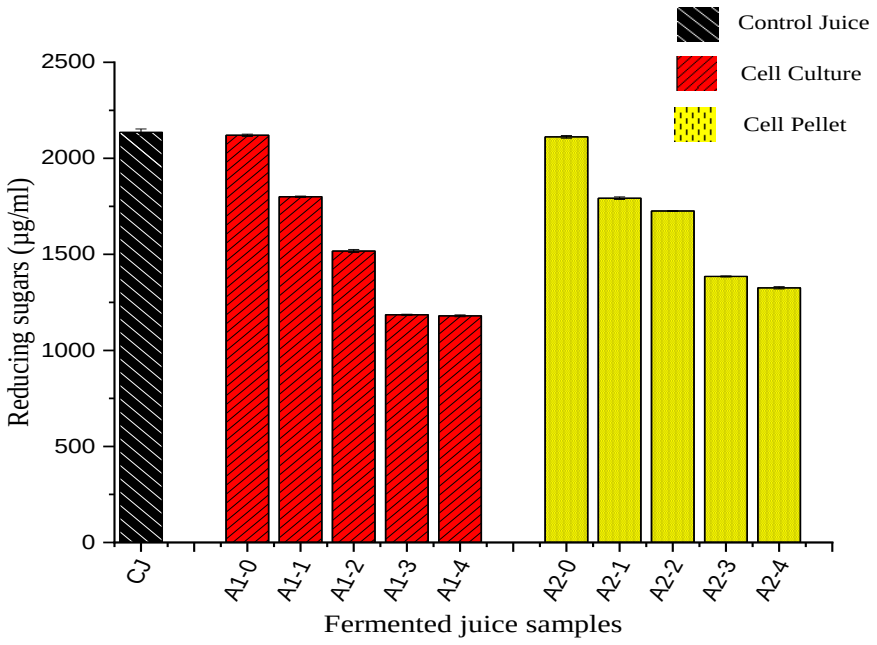
Table 26: Reducing sugars content in control and fermented juice samples

Sample	Reducing sugars (µg/ml)
CJ	2135.00 ± 17.78
A1-0	2120.00 ± 6.24
A1-1	1800.00 ± 3.78
A1-2	1517.30 ± 8.15
A1-3	1185.50 ± 3.12
A1-4	1180.00 ± 5.00
A2-0	2112.00 ± 7.04
A2-1	1792.50 ± 6.61
A2-2	1725.50 ± 2.25
A2-3	1385.00 ± 2.56
A2-4	1326.00 ± 6.03

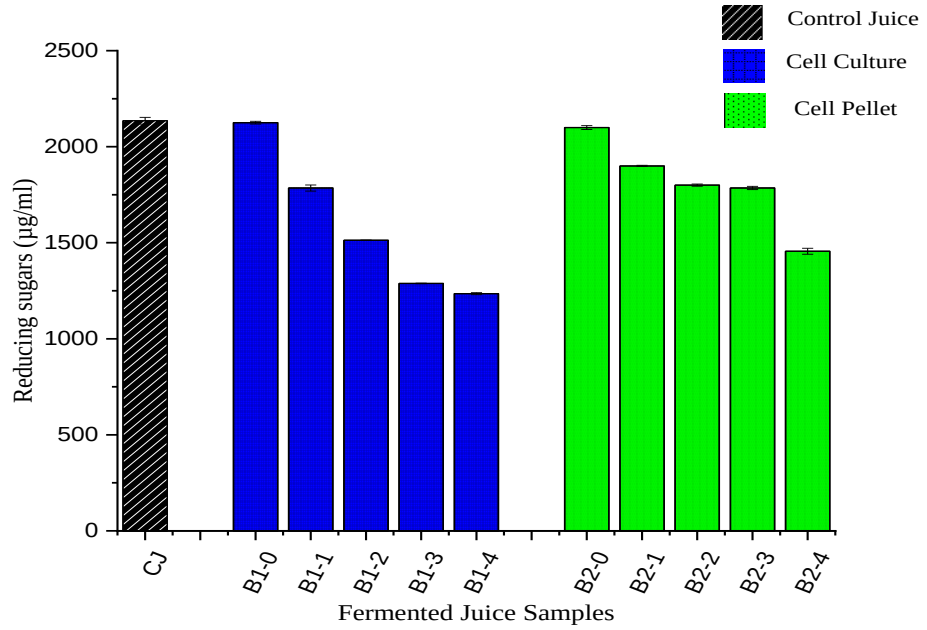
Values given in the table are mean± standard deviation

Sample	Reducing sugars (µg/ml)
CJ	2135.00 ± 17.78
B1-0	2125.00 ± 7.00
B1-1	1785.00 ± 16.07
B1-2	1513.00 ± 1.00
B1-3	1288.00 ± 1.55
B1-4	1235.00 ± 5.39
B2-0	2100 ± 10.00
B2-1	1900 ± 3.40
B2-2	1800 ± 5.68
B2-3	1785 ± 8.57
B2-4	1456 ± 15.55

Values given in the table are mean± standard deviation



(a)



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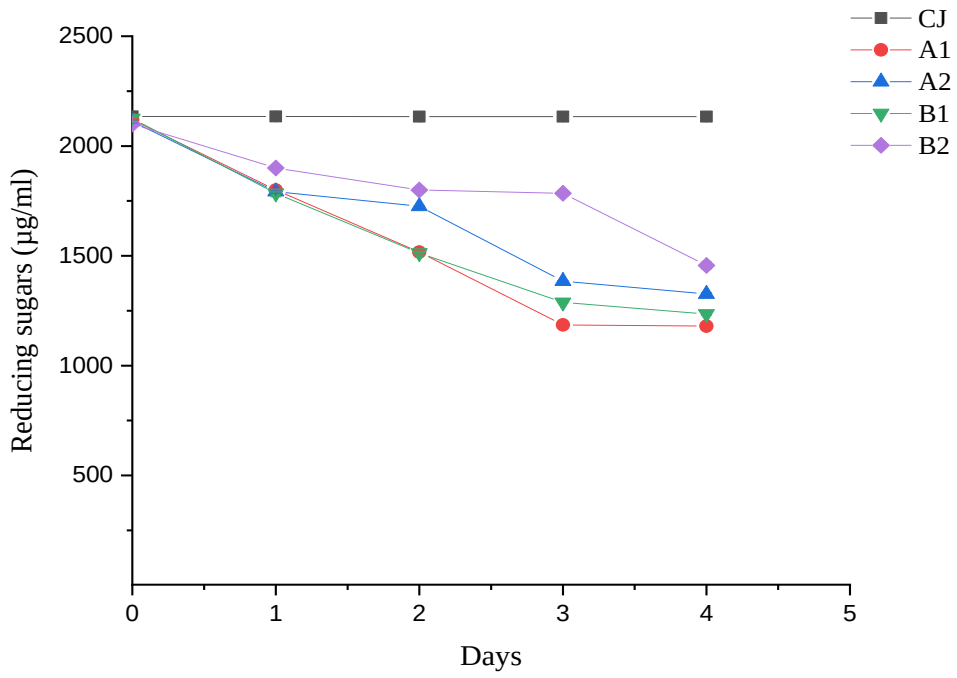


(c)

Figure 25: Reducing sugars content (µg/ml) in control juice (CJ) and fermented juice samples from 0^{th} to 4^{th} day

Initial juice contained 2135 ± 17.78 µg/ml of reducing sugars. In fermented samples A1 and A2, initial reducing sugars were reduced to 1180 ± 5.00 µg/ml and 1326 ± 6.03 µg/ml on 4th day of fermentation **(Figure 25 (a)**). Similarly, in B1 and B2 samples, 1235 ± 5.395 µg/ml and 1525.00 ± 15.55 µg/ml were the final reducing sugars at 4th day **(Figure 25 (b)**). The comparison of control juice with fermented samples showed that reducing sugar content in control juice remain constant whereas, in fermented samples reducing sugar content reduced during the fermentation period **(Figure 25 (c)**). LAB in the medium utilized available sugars for growth and production of metabolites in the juice during fermentation. Thus, reducing sugars have been shown to decrease as the days of fermentation increased. The concentration of reducing sugar was reduced from 19500 µg/ml to 6500 µg/ml during fermentation of guava juice by using *Lactobacillus plantarum* (Bhat et al., 2015).

4.6.6 Determination of microbial cell count

The viability of the probiotic starter culture *Pediococcus pentosaceus* VNK-1 in the probiotic guava beverage was assessed by CFU/ml on MRS agar plates. A1, A2, B1 and B2 samples were withdrawn from fermentation bottles on 0th day to 4th day **(Table 27 and Figure 26)**.

Table 27: Viable microbial cell count in fermented juice samples

Sample	Viable cells (CFU/ml)	Sample	Viable cells (CFU/ml)
A1-0	$2.13 \times 10^9 \pm 1.53 \times 10^8$	B1-0	$2.27 \times 10^9 \pm 1.53 \times 10^8$
A1-1	$1.97 \times 10^9 \pm 1.53 \times 10^8$	B1-1	$1.9 \times 10^9 \pm 1.53 \times 10^8$
A1-2	$3.23 \times 10^9 \pm 2.52 \times 10^8$	B1-2	$3.00 \times 10^9 \pm 2.00 \times 10^8$
A1-3	$3.80 \times 10^9 \pm 2.00 \times 10^8$	B1-3	$3.70 \times 10^9 \pm 4.58 \times 10^8$
A1-4	$3.77 \times 10^9 \pm 2.52 \times 10^8$	B1-4	$3.27 \times 10^9 \pm 3.06 \times 10^8$
A2-0	$2.00 \times 10^9 \pm 2.00 \times 10^8$	B2-0	$1.97 \times 10^9 \pm 1.53 \times 10^8$
A2-1	$1.90 \times 10^9 \pm 1.00 \times 10^8$	B2-1	$1.83 \times 10^9 \pm 1.5 \times 10^8$
A2-2	$2.30 \times 10^9 \pm 2.65 \times 10^8$	B2-2	$2.73 \times 10^9 \pm 2.08 \times 10^8$
A2-3	$1.60 \times 10^9 \pm 2.00 \times 10^8$	B2-3	$1.73 \times 10^9 \pm 2.8 \times 10^8$
A2-4	$9.00 \times 10^8 \pm 1.00 \times 10^8$	B2-4	$8.67 \times 10^8 \pm 1.53 \times 10^8$
Values given in the table are mean± standard deviation		**Values given in the table are mean± standard deviation**	

Sample	Viable cells (log CFU/ml)
A1-0	9.33 ± 0.03
A1-1	9.29 ± 0.03
A1-2	9.51 ± 0.03
A1-3	9.58 ± 0.02
A1-4	9.58 ± 0.03
A2-0	9.30 ± 0.04
A2-1	9.28 ± 0.02
A2-2	9.36 ± 0.05
A2-3	9.20 ± 0.05
A2-4	8.95 ± 0.05

Values given in the table are mean± standard deviation

Sample	Viable cells (log CFU/ml)
B1-0	9.35 ± 0.03
B1-1	9.29 ± 0.03
B1-2	9.48 ± 0.03
B1-3	9.57 ± 0.06
B1-4	9.51 ± 0.04
B2-0	9.29 ± 0.03
B2-1	9.26 ± 0.04
B2-2	9.44 ± 0.03
B2-3	9.24 ± 0.05
B2-4	8.93 ± 0.08

Values given in the table are mean± standard deviation

Guava juice was inoculated with 12% inoculum size. In A1, A2, B1 and B2 at the time of inoculation cell count was 2.13 × 10^9 CFU/ml (9.33 log CFU/ml), 2 × 10^9 CFU/ml (9.3 log CFU/ml), 2.27 × 10^9 CFU/ml (9.35 log CFU/ml) and 1.97 × 10^9 CFU/ml (9.29 log CFU/ml) respectively. The growth of *Pediococcus pentosaceus* VNK-1 in guava fruit juice during 4 days of fermentation is shown in **Figure 26**.

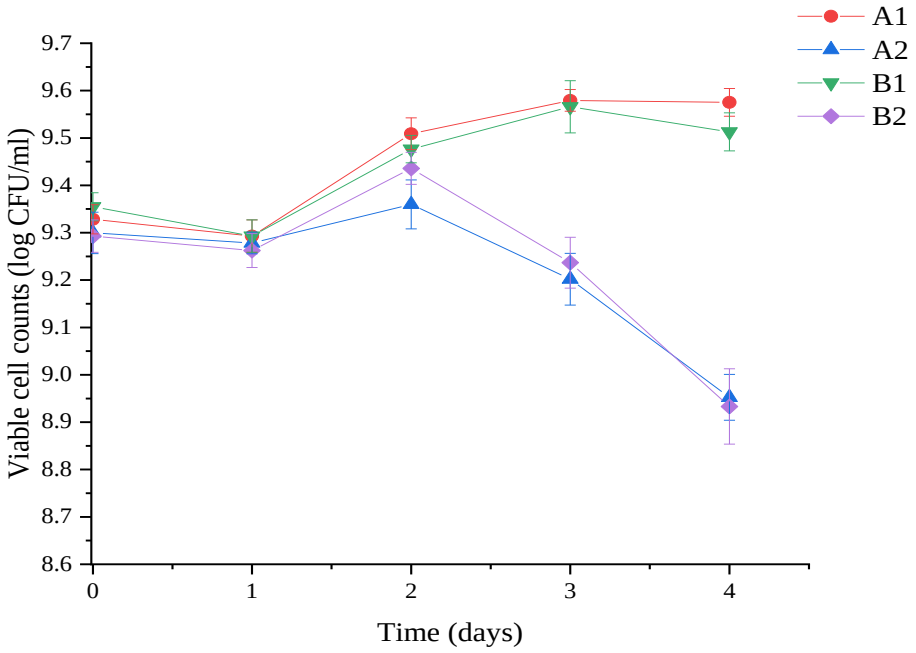


Figure 26: Viable cell counts (log CFU/ml) in fermented juice samples from 0^{th} day to 4^{th} day

On 1st day, CFU/ml in fermented samples was found to be reduced approximately 1.9 × 10^9 CFU/ml (approximately 9.29 log CFU/ml) for A1, A2 and B1 samples and 1.83 × 10^9 CFU/ml (9.26 log CFU/ml) for B2 samples. Lag phase was found to be elongated because pH of MRS broth was approximately 5 and pH of the guava juice was approximately close to 4. Due to differences in the pH as well as the availability of the nutrients in both MRS media and guava juice exerted the changes in growth kinetics of starter culture. Therefore, lag phase was extended due to decreased growth rate. On 2nd day and 3rd day, A1 and B1 fermented samples shown the exponential rise in CFU/ml. In A1 sample, 3.2× 10^9 CFU/ml (9.51 log CFU/ml) on 2nd day and 3.8 × 10^9 CFU/ml (9.58 log CFU/ml) on 3rd day was detected. Similarly, B1 samples were found with 3× 10^9 CFU/ml (9.48 log CFU/ml) on 2nd day and 3.7 × 10^9 CFU/ml (9.57 log CFU/ml) on 3rd day which shown the exponential increase from the first day. A2 and B2 samples also shown rise in CFU/ml. On 2nd day, A2 samples were found with 2.3 × 10^9 CFU/ml (9.36 log CFU/ml) and B2 samples were found with 2.73 × 10^9 CFU/ml (9.44 log CFU/ml). These cell counts reduced to 1.6 × 10^9 CFU/ml (9.2 log CFU/ml) in A2 samples and 1.7 × 10^9 CFU/ml (9.24 log CFU/ml) in B2 samples on 3rd day. In A1 samples, the stationary phase was observed on 4th day where, A1 samples were found with approximately equal number of cell counts as found on 3rd day i.e., 3.77 × 10^9 CFU/ml (9.58 log CFU/ml). Due to depletion of the nutrients, B1, A2 and B2 samples were found with reduced CFU/ml at the end of fermentation day. For B1 samples, cell count was 3.27 × 10^9 (9.51 log CFU/ml) CFU/ml, for A2 samples cell count was 9 × 10^8 CFU/ml (8.95 log CFU/ml) and B2 samples were found with 8.67 × 10^8 CFU/ml (8.93 log CFU/ml) that showed the decreased growth of bacterial culture on 4th day.

In this study, extended lag phase in the growth kinetics of starter culture was observed. These results were consistent with the results reported by (Lu et al., 2018) that explained the longer lag phase when juice of star fruit was fermented for period of 8 days at 30°C with three different lactobacillus species. Also, this study reported the continuous increase of the CFU/ml till 3rd day and *L. rhamnosus* reached stationary phase on 4th day and *L. paracsei* entered the stationary phase on 6th day of fermentation of juice of star fruit.

In another study, elongated lag phase was observed in pomegranate juice when four different species of *Lactobacillus* were used for fermentation of pomegranate juice at 30°C (Mousavi et al., 2011). It was reported that adaption of starter culture in a fresh fruit juice medium may also cause

reduction in the growth of the cells resulting in elongated lag phase. Thus, availability of the nutrients and pH of the guava juice affects the growth kinetics of *Pediococcus pentosaceus* VNK-1 at 24°C and 37°C. During fermentation of apple juice, *Lactobacillus* counts were reduced from 8.37 log CFU/ml to 7.85 log CFU/ml. The reduction in the cell counts may occur due to the depletion of carbohydrates that were metabolized by probiotic bacteria and resulting in the production of lactic acid in the medium. This caused reduction in the pH of the medium at the end of the fermentation and thus, affecting the growth of bacteria (Li et al., 2018).

4.6.7 Organoleptic studies

Sensory analysis of probiotic beverage was done by team of 10 panelist and scores were given according to the hedonic scale **(Table 28)**. From the results, it was analyzed that probiotic guava juice (A1) fermented at 24°C was found as the best due to enhanced phenolics, flavonoids and antioxidant capacity. Thus, A1 sample was selected for sensory analysis.

Table 28: Sensory analysis score of probiotic guava beverage

	Hedonic scale	
Parameter	**Control guava Juice**	**Guava probiotic beverage**
Appearance/color	8.1 ± 0.56	8 ± 0.63
Mouth feel	7 ± 0.47	7 ± 0.51
Aroma/odor	8.3 ± 0.67	8 ± 0.56
Taste	7.2 ± 0.42	7 ± 0.31
Overall acceptance	7.6 ± 0.51	7 ± 0.42

Data given in the table are mean values $\pm$ standard deviation (n=10).

Scores given by panelist were recorded and analyzed for overall acceptance of the product. Equal scores for control guava juice and beverage in terms of appearance showed that product's color was liked very much by the panelist as there were no notable changes in the appearance before and after fermentation.

In context with mouth feel and taste, product was moderately liked by the panelist. It may be occurred due to acidic mouth feel and less sugar content as production of organic acids in the juice after fermentation made it acidic and there was no supplementation of the sugar or citric acid to enhance the flavor/taste of the product. Aroma of the probiotic beverage was liked very much by the panelist due to the natural fragrance of guava fruit. Overall acceptance score of the beverage was 7 ± 0.42 which was approximately equal to the control juice that was 7.6 ± 0.51. Probiotic beverage was equally preferred by the panelist for the consumption.

4.7 Bioactive potential of the probiotic guava beverage

The bioactive potential of the developed probiotic beverage was assessed by determination of antioxidant activity and antimicrobial property. The control and fermented samples (A1, A2, B1 and B2) were analyzed for assessment of bioactive potential.

4.7.1 Antioxidant potential

Antioxidant activity of control and fermented juice samples was estimated by DPPH free radical scavenging assay **(Table 29** and **Figure 27).**

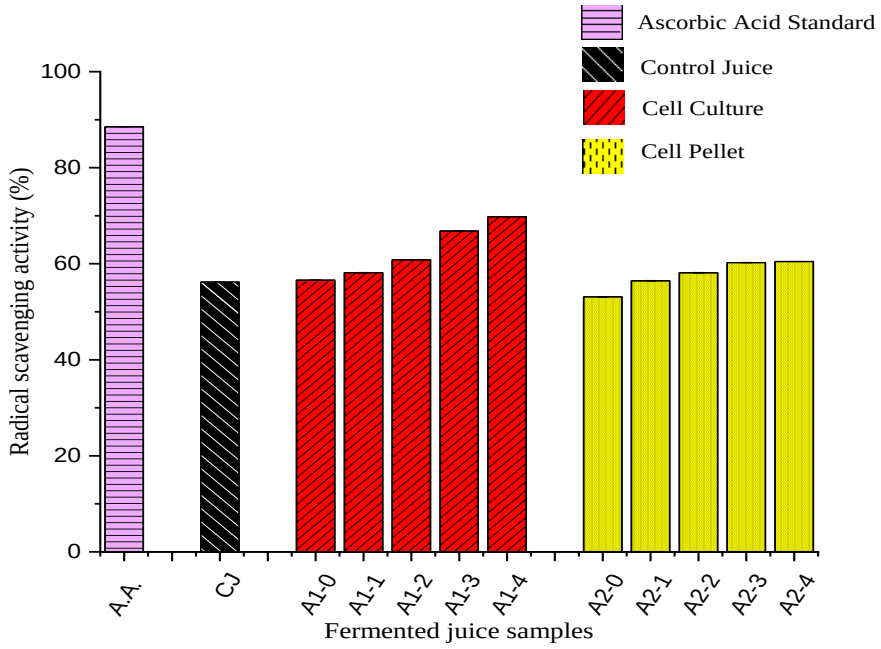
Table 29: Radical scavenging activity in control juice and fermented juice samples

Sample	% Antioxidant activity
A.A. standard	88.53 ± 0.01
CJ	56.20 ± 0.05
A1-0	56.60 ± 0.01
A1-1	58.14 ± 0.07
A1-2	60.84 ± 0.04
A1-3	66.83 ± 0.03
A1-4	69.79 ± 0.04
A2-0	53.10 ± 0.08
A2-1	56.46 ± 0.07
A2-2	58.12 ± 0.07
A2-3	60.23 ± 0.06
A2-4	60.46 ± 0.02

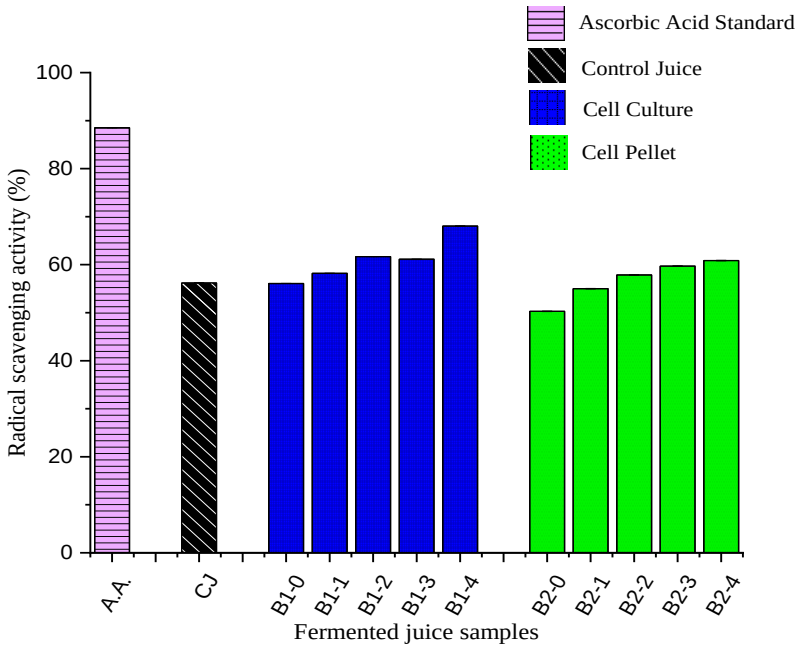
Values given in the table are mean± standard deviation

Sample	% Antioxidant activity
A.A. standard	88.53 ± 0.01
CJ	56.20 ± 0.05
B1-0	56.09 ± 0.01
B1-1	58.20 ± 0.02
B1-2	61.69 ± 0.02
B1-3	61.13 ± 0.05
B1-4	68.04 ± 0.02
B2-0	50.29 ± 0.06
B2-1	55.00 ± 0.06
B2-2	57.86 ± 0.05
B2-3	59.71 ± 0.05
B2-4	60.86 ± 0.051

Values given in the table are mean± standard deviation



(a)



(b)

Figure 27: Antioxidant activity (%) of ascorbic acid (A. A.) standard, control juice (CJ) and fermented juice samples from 0^{th} to 4^{th} day

The fermented juice samples showed the increased antioxidant potential/ free radical scavenging activity **(Table 29, Figure 27)**. Ascorbic acid standard showed the 88.53 $\pm$ 0.01 % antioxidant capacity whereas control juice (non-fermented juice) showed the 56.2 $\pm$ 0.05 % antioxidant activity. At 24 °C, A1 samples shown the gradual shift from 56.6 $\pm$ 0.01 % on 0^{th} day to 69.79 $\pm$ 0.04 % on 4^{th} day. In A2 samples, free radical scavenging activity increased from 53.1 $\pm$ 0.08 % on 0^{th} day to 60.46 $\pm$ 0.02 % on 4^{th} day **(Figure 27 (a))**.

In B1 samples, similar results were observed as found in A1 samples. B1 samples showed gradual increment of antioxidant activity which increased from 56.09 $\pm$ 0.01 % to 68.04707 $\pm$ 0.02 % at the 4^{th} day. Whereas, B2 samples showed increased antioxidant activity from 50.29 $\pm$ 0.06 % on 0^{th} day to 60.86 $\pm$ 0.051 % on 4^{th} day respectively **(Figure 27 (b))**.

Thus, juice samples at 4^{th} day, exhibited highest DPPH activity of 69.79 $\pm$ 0.04% and 68.04 $\pm$ 0.02 % of A1 and B1 samples respectively **(Figure 27)**. In relevant study, 82.51% was the highest percent of antioxidant activity found in fermented product at 8 hours of fermentation when guava juice was inoculated with *Lactobacillus plantarum.* This activity was reduced to 77 % at 16 hours of fermentation (Bhat et al., 2015). The another study reported increased antioxidant activity by 30% when juice of Myrtle berries were fermented with *Lactobacillus plantarum* via lactic acid fermentation (Curiel et al., 2015). The enhanced phenolic and flavonoid compound may occur due to the hydrolysis of polyphenolic compounds by enzymes produced by LAB into its derivatives which are simpler and possess biological activity. These compounds enhanced antioxidant activity of the fermented product.

4.7.2 Antimicrobial activity of probiotic beverage

Control juice and fermented juice samples (A1, A2, B1 and B2) were tested against three bacteria on MHA plates for analyzing antibacterial activity which was confirmed by observing and measuring diameter of zone of inhibition **(Table 30** and **Table 31)**. The MHA plates were observed after 24 hours and zone of inhibition was measured in millimeters (mm).

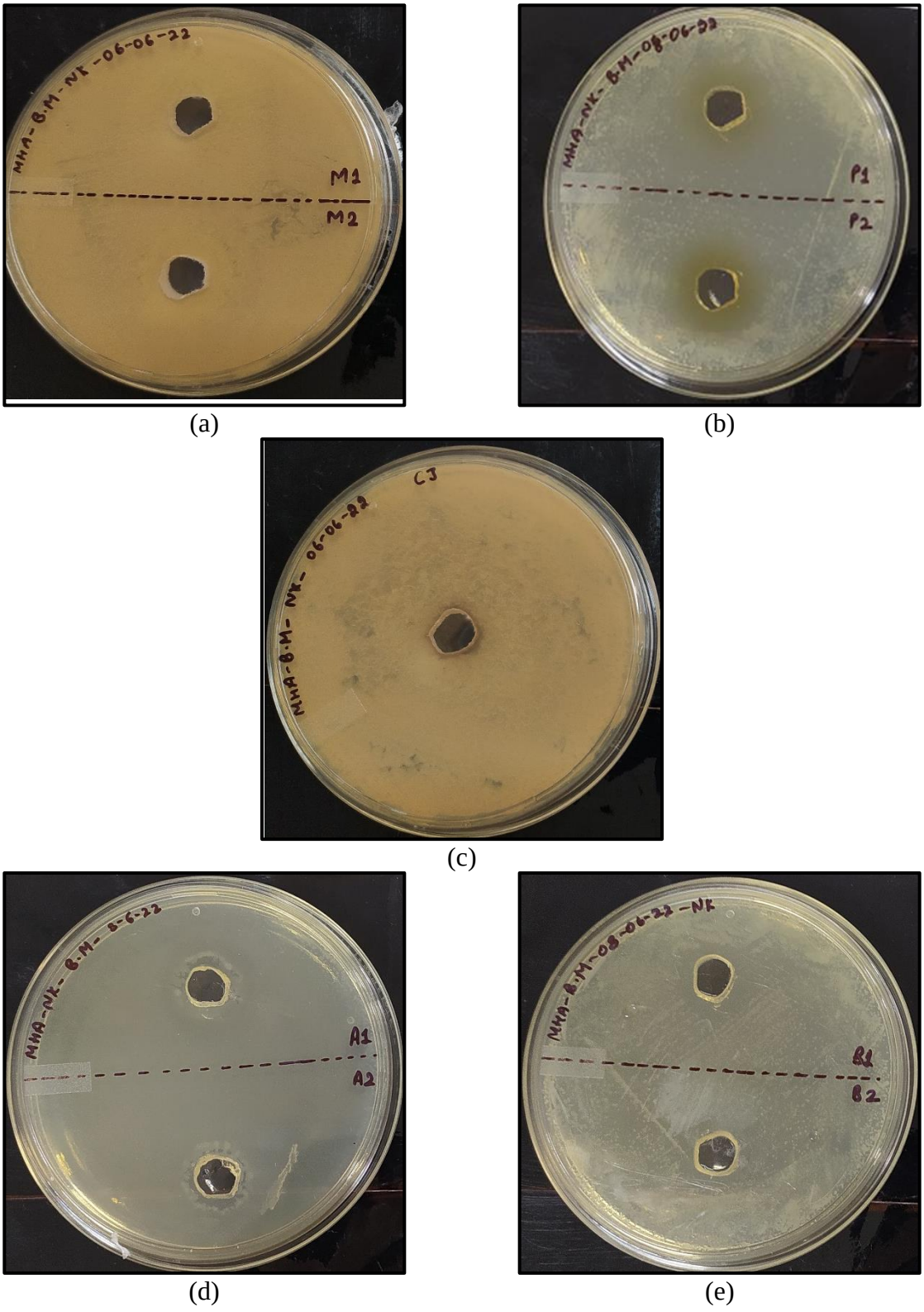
Table 30: Antibacterial activity of fermented product against *Bacillus megaterium* and *Staphylococcus aureus*

Bacteria tested	Sample Name	Sample Volume (µl)	Diameter of zone of inhibition (mm)	Figure No.
Bacillus megaterium	MRS media - control (M1)	50	--	28 (a)
	MRS media - control (M2)	100	--	
	Pediococcus pentosaceus- cell culture supernatant (P1)	50	19	28 (b)
	Pediococcus pentosaceus - cell culture supernatant (P2)	100	24	
	Control juice (C)	100	--	28 (c)
	A1 (juice + cell culture)	100	15	28 (d)
	A2 (juice+ cell pellet)	100	14	
	B1 (juice + cell culture)	100	18	28 (e)
	B2 (juice+ cell pellet)	100	16	
Staphylococcus aureus	MRS media - control (M1)	50	--	29 (a)
	MRS media - control (M2)	100	--	
	Pediococcus pentosaceus- cell culture supernatant (P1)	50	18	29 (b)
	Pediococcus pentosaceus - cell culture supernatant (P2)	100	24	
	Control juice (C)	100	--	29 (c)
	A1 (juice + cell culture)	100	20	29 (d)
	A2 (juice+ cell pellet)	100	18	
	B1 (juice + cell culture)	100	20	29 (e)
	B2 (juice+ cell pellet)	100	15	

Table 31: Antibacterial activity of fermented product against *Bacillus subtilis*

Bacteria tested	Sample Name	Sample Volume (µl)	Diameter of zone of inhibition (mm)	Figure No.
Bacillus subtilis	MRS media - control (M1)	50	--	30 (a)
	MRS media - control (M2)	100	--	
	Pediococcus pentosaceus- cell culture supernatant (P1)	50	15	30 (b)
	Pediococcus pentosaceus - cell culture supernatant (P2)	100	20	
	Control juice (C)	100	--	30 (c)
	A1 (juice + cell culture)	100	17	30 (d)
	A2 (juice+ cell pellet)	100	14	
	B1 (juice + cell culture)	100	18	30 (e)
	B2 (juice+ cell pellet)	100	15	

Results for antibacterial activity of non-fermented, fermented juice and MRS- cell free supernatant was assessed by agar-well diffusion method (**Table 30 and Table 31; Figure 28, 29 and 30**). The wells having control MRS media components (50 µl and 100 µl) and 100 µl of control juice didn't show any antimicrobial activity against tested bacterial strains (**Figure 28 (a and c)**). P1 and P2 showed potent antimicrobial property as the sample volume increased from 50 µl to 100 µl, the zone of inhibition also increased which was given as 19 mm to 24 against *Bacillus megaterium* (**Figure 28 (b)**). A1, A2, B1, B2 constantly exerted the antibacterial effects against gram positive bacteria. Zone of inhibition appeared around the wells confirmed the antibacterial activity. Wells having A1 and A2 samples showed maximum zone 15 mm and 14 mm respectively **(Figure 28 (d))**. B1 and B2 showed 18 mm and 16 mm zone of diameter respectively against *Bacillus megaterium* **(Figure 28 (e))**.

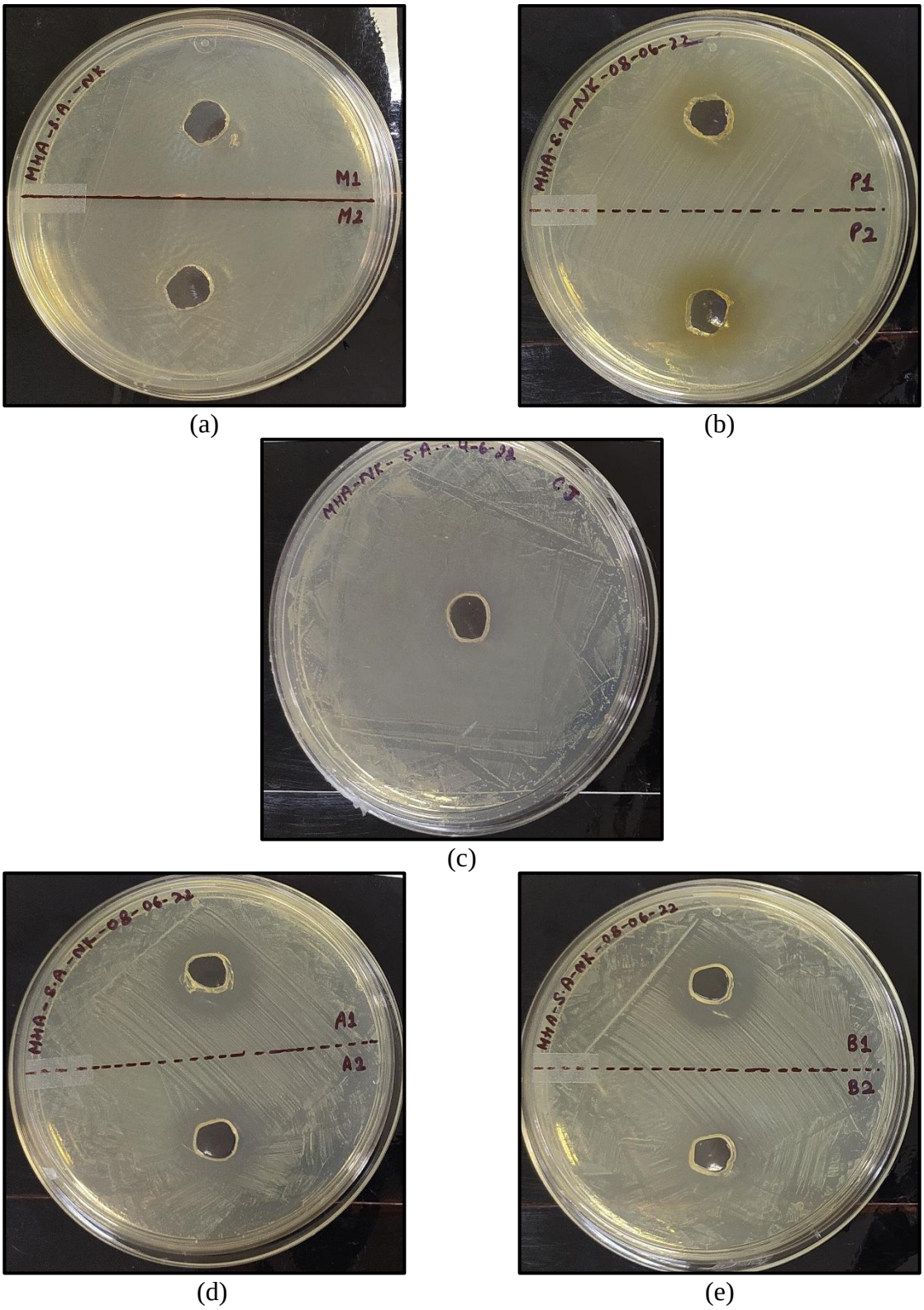


(a) (b)

(c)

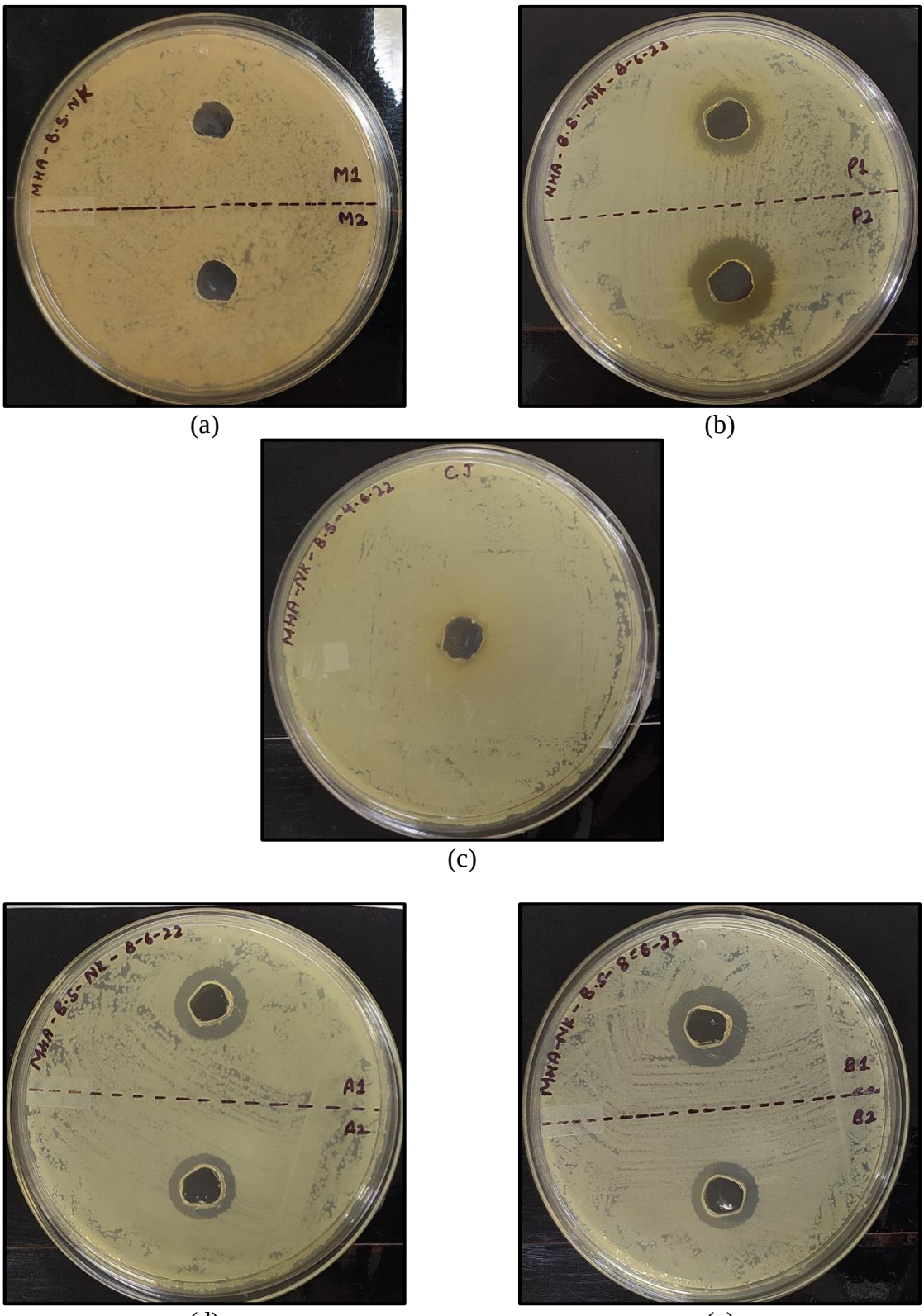
(d) (e)

Figure 28: Antibacterial activity of MRS cell free culture supernatant and fermented guava juice samples (A1, A2, B1, B2) against *Bacillus megaterium*



(a) (b) (c) (d) (e)

Figure 29: Antibacterial activity of MRS cell free culture supernatant and fermented guava juice samples (A1, A2, B1, B2) against *Staphylococcus aureus* on MHA plates



(a) (b) (c) (d) (e)

Figure 30: Antibacterial activity of MRS cell free culture supernatant and fermented guava juice samples (A1, A2, B1, B2) against *Bacillus subtilis* on MHA plates

The wells having control MRS media components (50 µl and 100 µl) and 100 µl of control juice didn't show any antimicrobial activity against pathogenic bacterial strain *Staphylococcus aureus* **(Figure 29 (a and c))**. P1 and P2 showed potent antimicrobial property as the sample volume increased from 50 µl to 100 µl, the zone of inhibition also increased which was given as 18 mm to 24 against *Staphylococcus aureus* **(Figure 29 (b))**. A1, A2, B1, B2 showed the antibacterial effects against gram positive bacteria. Zone of inhibition appeared around the wells confirmed the antibacterial activity. Wells having A1 and A2 samples showed maximum zone 20 mm and 18 mm respectively against *Staphylococcus aureus* **(Figure 29 (d))**. B1 and B2 showed 20 mm and 15 mm zone of diameter respectively against *Staphylococcus aureus* **(Figure 29 (e))**.

The wells containing control MRS media components (50 µl and 100 µl) and 100 µl of control juice didn't show any antimicrobial activity against pathogenic bacterial strain *Bacillus subtilis* (**Table 31; Figure 30 (a and c)**). P1 and P2 showed potent antimicrobial property as the sample volume increased from 50 µl to 100 µl, the zone of inhibition also increased which was given as 15 mm to 20 against *Bacillus subtilis* **(Figure 30 (b))**. A1, A2, B1, B2 showed the antibacterial effects against gram positive bacteria. Zone of inhibition appeared around the wells confirmed the antibacterial activity. Wells having A1 and A2 samples showed maximum zone 17 mm and 14 mm respectively against *Bacillus subtilis* **(Figure 30 (d))**. B1 and B2 showed 20 mm and 15 mm zone of diameter respectively against *Bacillus subtilis* **(Figure 30 (e))**.

A1 and A2 samples exerted the equal antibacterial effect on *Staphylococcus aureus* showing zone of diameter 20 mm whereas A2 and B2 gave the diameter of 18 and 15 mm.

Bacillus subtilis showed the maximum susceptibility in the presence of A1, A2, B1 and B2 samples. A1 and B1 have shown the maximum zone of inhibition i.e., 17 mm and 18 mm, whereas small zone found around the wells containing A2 and B2 samples i.e., 14 and 15 mm. It may be particularly due to a smaller number of cells counts of LAB present in these samples.

Similar, studies have shown the antimicrobial activity of cell free supernatant of *Pediococcus pentosaceus* against test organism which were *Staphylococcus aureus KCTC-1621* and *E. coli O157:H7* confirmed by zone of inhibition using agar-well diffusion assay (Bajpai et al., 2016). Thus, cell free supernatant of various strains of *Pediococcus pentosaceus* have been proven to be potent alternative solution for preserving the food grade products from contamination.

Above results for antimicrobial activity were found to be in line with the results reported in recent study where *Lactobacillus plantarum* mediated guava juice fermentation produced probiotic guava beverage significantly inhibiting the growth of *Escherichia coli, Staphylococcus aureus* and *Pseudomonas* species (Sourangshu C, 2015) .

Another study reported that *Pediococcus pentosaceus* has the capability to produce bacteriocin like substance (like Pediocin or Nisin) which is a peptide that is released into the extracellular environment or remains intact with the outer membrane of bacterial cell., that specifically inhibit the growth the gram positive and negative bacteria as well as against different fungal species even under wide range of pH and possess higher thermal stability (de Souza de Azevedo et al., 2020).

Thus, *Pediococcus pentosaceus* mediated lactic acid fermentation of guava juice to develop a probiotic beverage exhibited the antibacterial activity and has the potential to diminish the growth of foodborne pathogens. Consequently, this antibacterial property improves the shelf life of the product. Also, this probiotic guava beverage would be safe to consume because *Pediococcus pentosaceus* falls under the category of generally regarded as safe (GRAS) for human consumption.

CHAPTER 5

CONCLUSIONS & SCOPE

Conclusions & Scope

Fermentation of fresh fruits and fruit juices by probiotic bacteria provide value-added products that enhance the nutritional value of the fruit juices. These nutritionally enriched products are widely accepted and preferred worldwide as these products provide health benefits to the host system.

Aiming for a healthy life-style, the demand for fermented and fortified food-products have been increased by the consumers. Guava (*Psidium guajava*), a cost-effective fruit, possess polyphenols, antioxidants, high fiber content and easily available in the tropical regions. This nutritionally rich fruit can be used as a potential substrate for the growth of LAB.

In the present study, fermentation technology has been exploited to develop a novel probiotic *Psidium guajava* beverage using a potential lactic acid bacterium, *Pediococcus pentosaceus* VNK-1. This bacterium was isolated from the surface of fresh guava. Being the part of normal gut microbiota, various strains of *Pediococcus pentosaceus* have been proven to be effective in maintaining gut health, prevention of different types of cancers and inflammatory disorders. In addition to this, recent studies have shown the antioxidant potential, immune system regulation and lowering the blood cholesterol levels as the major benefits provided to the body.

In this study, optimization of parameters for lactic acid production was carried out using three independent factors: (a) temperature in the range of 15 to 32°C, agitation in the range of 50-100 rpm and inoculum concentration 5-12%, using response surface methodology (RSM) by following the central composite design (CCD) in design expert software. The optimum conditions obtained after the analysis for maximum lactic acid production were: 24°C temperature, 12% of the inoculum concentration of the starter culture and agitation rate was 60 rpm. Fermentation was carried out for 4 days and samples were collected for the evaluation of the probiotic beverage.

The developed probiotic beverage using culture broth (12 % inoculum concertation) at 24°C has shown the maximum increase in the lactic acid concentration, antioxidants, phenolics, flavonoids. The *Pediococcus pentosaceus* VNK-1 was able to grow and survive in the guava juice medium with final cell viability of 3.77×10^9 CFU/ml (9.58 log CFU/ml) on 4^{th} day of fermentation. Total phenolic content was significantly increased from 674.48$\pm$ 6.53 µg GAE/ml in control juice to 855.967 $\pm$ 12.37 µg GAE/ml in A1 samples. Fermentation led to increase in the total flavonoids

in guava juice. Initial in control juice flavonoids were 1462.59 ± 3.57 µg QE/ml, that significantly increased to 2001 ± 10.5 µg QE/ml A1 samples. The maximum antioxidant activity to scavenge the free radicals was observed as 69.79 ± 0.04 % in A1 fermented samples. At 4th day, maximum LA production was found as 561.75 ± 1.44 µg/ml for A1 samples.

The increased lactic acid concentration in the guava juice led to reduction of pH of the control juice from 4.24 ± 0.0264 to pH 2.75± 0.01 for A1 fermented samples. TSS of the guava juice also reduced from 4.13 ± 0.057 °B to 3.66 ± 0.05 for A1 samples. Control juice was found with 2135 ± 17.78 µg/ml of reducing sugars that further reduced to 1180 63 ± 5 µg/ml in A1 samples. The overall acceptance of the score of the beverage was 7 ± 0.42 and accepted by the panelists for consumption.

A1 and B1 fermented samples showed maximum zone of inhibition against *Bacillus megaterium, Staphylococcus aureus* and *Bacillus subtilis* respectively. The antimicrobial activity of peptides produced by LAB bacteria mainly pediocin (bacteriocin) and lactic acid concentration would satisfactorily provide properties together in the fermented beverage that becomes a substantial benefit to the product in order to resist the growth of various types of pathogens and thus increasing the shelf life of the product.

Thus, in this study, 24°C and 37°C both the temperatures were found to be suitable for the lactic acid fermentation. The inoculation of pasteurized guava juice with culture broth was found to be more efficient in this fermentation as compared to usage of harvested cells for the inoculation. For maximum lactic acid production, total phenolic content, total flavonoid content and highest potential to scavenge the free radical/oxidizing agents, 24°C temperature was found to be optimum for the growth of *Pediococcus pentosaceus* VNK-1 in guava juice and to develop a probiotic guava juice.

As explained in this study, the probiotic beverage has shown significant antimicrobial activity against *Bacillus megaterium*, *Bacillus subtilis* and *Staphylococcus aureus*.

For further investigations, the cell cytotoxicity of the developed guava beverage can be performed on murine cell lines (fibroblasts Balb3T3) to ensure the safety for consumption. The viability of fibroblasts can be assessed through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium

bromide (MTT) assay. In order to study the shelf life of the probiotic beverage, beverage needs to be stored at 4 °C for 1-4 weeks.

Thus, it would be a promising ideology for formulation and development of probiotic-ready-to-drink beverage from nutritionally rich fruit guava using optimized fermentation parameters. This probiotic guava beverage can manifest benefits to the health of consumers who prefer fruit juices, fruitful for diabetic patients, also one who face intolerance to dairy products and milk-protein allergies. In addition to these benefits, probiotic guava beverage would be able to prevent diseases occurring due to oxidative stress.

CHAPTER 6

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