EFFECT OF FERMENTATION TEMPERATURE ON CUCUMBER PICKLE (KHALPI) FERMENTATION KINETICS

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2024

Effect of Fermentation Temperature on Cucumber Pickle (Khalpi) Fermentation Kinetics

A dissertation submitted to the Department of Food Technology, Central Campus of Technology, Tribhuvan University, in partial fulfillment of the requirements for the degree of B. Tech. in Food Technology

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December, 2024

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Approval Letter

This *dissertation* entitled *Effect of Fermentation Temperature on Cucumber Pickle (Khalpi) Fermentation Kinetics* presented by Gaurab Luitel has been accepted as the partial fulfillment of the requirement for the B. Tech. degree in Food Technology

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December 10, 2024

Acknowledgements

I am sincerely grateful to my supervisor, Mr. Om Prakash Panta (Asst. prof. , Central Campus of Technology, Dharan), for his valuable guidance, encouragement, and motivation throughout my research work.

My heartfelt thanks go to Ast. prof. Dr. Dil Kumar Limbu (Campus Chief), Professor Basanta Kumar Rai and Assistant Professor Navin Gautam (Head of Department, Department of Food Technology) for providing essential resources, insights, and support, as well as answering my questions and helping me complete this work.

I would also like to thank my teachers and the library and laboratory staff, including Mr. Mahesh Shrestha, Mr. Suman Pulami, Mrs. Meera Shrestha, and Ms. Dikshya Rai, for their guidance and direct and indirect assistance.

I want to thank my friends, especially Pritam Khatri, Prekshya Timsina, Suman Bisunke, Abhinaya Limbu, my senior Dinesh Subedi and my junior Darpan Pandey, for their support during this project. I appreciate everyone who offered insights or assistance, directly or indirectly.

Finally, I am deeply thankful to my parents, brothers, and family for their unwavering love, encouragement, and moral support.

Date of submission: December 10, 2024

(Gaurab Luitel)

Abstract

The aim of this work was to investigate the kinetics of lactic acid production and the growth of lactic acid bacteria during the fermentation of radish pickles. The pickles were prepared following a traditional pickle-making process. Over a fermentation period of sixteen days, changes in lactic acid bacteria counts, titratable acidity (expressed as lactic acid), pH, TSS, and sensory attributes were systematically analyzed. The fermentation was conducted under anaerobic conditions using airtight jars, with samples incubated at both a controlled temperature of 37°C and room temperature 28 ± 6 °C.

Over the 16-day fermentation period, acidity, pH, total soluble solids (TSS), lactic acid bacteria (LAB) count, and sensory scores of Khalpi were evaluated under two different temperature conditions, revealing distinct variations. The acidity, expressed as % lactic acid, increased from 0.236% on Day 1 to 1.5733 ± 0.0116% at 37°C and from 0.2333% to 1.6467 ± 0.0058% at room temperature by Day 16. Time and temperature significantly ($p > 0.05$) affected acidity in both fermentation conditions. TSS rose from 8.766°Bx on Day 1 to 12.23 ± 0.057°Bx at 37°C and from 8.76°Bx to 12.03 ± 0.057°Bx at room temperature by Day 16, with a significant ($p > 0.05$) impact of time and temperature. The pH decreased from 5.216 on Day 1 to 3.81 ± 0.015 at 37°C and to 3.74 ± 0.017 at room temperature by Day 16, with significant ($p > 0.05$) effects of both factors.LAB counts increased from 4.505 Log CFU/mL on Day 1 to 10.4623 Log CFU/mL on Day 10, followed by a decline to 5.06 Log CFU/mL on Day 16 at 37°C. Similarly, LAB counts rose from 4.4913 Log CFU/g on Day 1 to 9.176 Log CFU/g on Day 10, then dropped to 5.535 Log CFU/g by Day 16 at room temperature, with significant ($p < 0.05$) differences observed among all samples during fermentation. Sensory scores for overall acceptance improved from 3.80 ± 0.45 on Day 1 to 8.00 ± 0.71 at room temperature and 7.80 ± 0.83 at 37°C on Day 16. While time significantly ($p > 0.05$) influenced overall acceptance, temperature had no significant effect.

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List of abbreviations

Abbreviation	Full form
RT	Room temperature
ANOVA	Analysis of variance
db	Dry-basis
wb	Wet-basis
AOAC	Association of Official Analytical Chemists
LAB	Lactic Acid Bacteria
TSS	Total Soluble Solid
pH	Potential of hydrogen
MRS Agar	De Man, Rogosa and Sharpe agar
Tukey's HSD	Tukey's Honestly Significant Difference
TOM	Total organic matter

Part I

Introduction

1.1 General Introduction

Cucumis sativus L., often known as cucumber, belongs to the Cucurbitaceae family of plants, which has 750 species and 90 genera. It is one of the earliest vegetable crops to be grown, it is grown in almost every country in every temperature range (Tatlioglu, 1993). As the fourth most important vegetable crop in the world, cucumbers are a model crop for the Cucurbitaceae family, which also includes other important crops including melon, watermelon, pumpkin, and squash. Cucumber was first domesticated about 3000 years ago in India, and it quickly spread to Western Asia and then to Southern Europe. Cucumber was brought to North China via the Silk Road, and it reached South China via Burma and the border between India and China. This initial introduction paved the way for its subsequent spread throughout East Asia (Lv *et al.*, 2012). Cucumber reached a significant global production of 70 million tons in 2013, as reported by FAOSTAT. Approximately 70% of the world's cucumber production comes from the Asian continent, which is the dominant producer. China is by far the largest producer, followed by Russia, Turkey, and Iran (Naegele and Wehner, 2016).

Pickling is a traditional culinary practice that dates back to approximately 2400 BC and includes preserving food in vinegar or brine. Throughout history, pickling has been a fundamental part of food preservation in a wide range of societies and civilizations (Chakraborty *et al.*, 2018). Pickling has been a centuries-old method of preserving fruits, vegetables (including roots and tubers), fish, and meat. It is thought that this method of preserving fruits and vegetables was learned by the ancient Mesopotamians (Kawahara *et al.*, 2010). Pickling was an ancient food preservation technique used by the Chinese, Indians, and Egyptians, among others (Chakraborty *et al.*, 2018).

The most common vegetable used to make pickles worldwide is cucumber. When pickling cucumbers, acid fermentation begins soon after the cucumbers are submerged in brine and continues for two to six weeks in order to preserve the cucumbers. Cucumber fermentation is best started with facultative homo-fermentative lactic acid bacteria (*Pediococcus sp.*) and hetero-fermentative lactic acid bacteria (*Lactobacillus plantarum* and

Lactobacillus pentosus). These bacteria are more likely to dominate cucumber fermentation and produce lactic acid and carbon dioxide (Zhai *et al.*, 2018).

Khalpi, a pickled cucumber delicacy originating from Nepal, is typically consumed as a pickle, often combined with mustard oil, salt, and powdered chillies. The fermentation process of khalpi commonly involves following steps - ripe cucumbers are cut into the proper size and sun-dried for a few days, they are put inside a bamboo container and sealed tightly with dried leaves. The cucumbers naturally ferment at ambient temperature for three to five days, giving them a sour flavor. After adding mustard oil, salt, and powdered chili, the pickled product—known as khalpi—is usually consumed. The months of September and October are used to prepare for khalpi. *Leuconostoc fallax, L. plantarum*, and *L. brevis* are among the microorganisms found in khalpi (J. P. Tamang *et al.*, 2009)

Lactate fermentation, a widely adopted preservation technique, continues to function as an alternative in situations where refrigeration and other preservation methods are unavailable for ensuring the safety of food (Holzapfel, 2002). Some lactic acid bacteria (LAB) strains that have been found in fermented vegetable products before show both functional and protective traits. In North East India, these strains can be used as starting cultures to enable the regulated and efficient manufacture of fermented vegetable products (B. Tamang, 2006). Bacteria including Lactobacillus plantarum, Lactobacillus brevis, and *Leuconostoc Fallax* are commonly involved in the fermentation of khalpi. These bacteria appear as a rise in the number of bacteria, an increase in acidity, a drop in pH, and the production of a distinct flavor (B. Tamang and Tamang, 2010b).

1.2 Statement of the problem

The number of traditional foods in diets has significantly decreased in the modern period, especially in a number of developing Asian countries. There is a growing trend in the consumption of foreign foods. The production of jobs and revenue is significantly influenced by traditional meals. The changes in dietary habits are mostly linked to changes in wealth and income, easier access to a wider range of alternative foods, changing attitudes and beliefs about food, population movements, and increasing trade worldwide (Dahal *et al.*, 2007).

The most promising indigenous products might be made commercially viable, opening up new revenue streams for a lot of people. Most traditional products are now made at the household level, either for limited sales or direct consumption, with little to no

commercialization. A focus on maintaining quality and hygiene together with little technical assistance could help many of these items find success in the marketplace (Roshan Shrestha *et al.*, 2012).

The renowned traditional Nepalese pickle known as khalpi has long been a beloved staple, yet there remains much to uncover about its economic potential and the science behind its production. This research takes a fresh approach to refining traditional methods, with the aim of transforming khalpi into a high-quality, marketable product. By delving into the specific aspects of fermentation—such as the patterns of cell growth, the types of lactic acid bacteria involved, and the effects of temperature on pH, lactic acid production, and TSS—we seek to optimize the production process and ensure consistent quality. This understanding will enhance the commercial viability of khalpi, offering valuable insights that contribute to the preservation and modernization of this treasured Nepali culinary tradition.

1.3 Objectives

1.3.1 General objectives

The primary goal of this dissertation is to study the changes that occur in khalpi pickle during its fermentation and effects of temperature difference in khalpi pickle fermentation.

1.3.2 Specific objectives

1. To analyze the effect of different times and temperatures on Lactic acid production, TSS, and pH changes during khalpi fermentation.
2. To explore the pattern of cell growth of lactic acid bacteria during the fermentation of pickles kept at different temperature conditions.
3. To analyze the effects of different times and temperatures on sensory score during khalpi fermentation.
4. To identify the optimum time for the completion of Khalpi fermentation under different temperature conditions.

1.4 Significance of the work

This research is crucial in advancing the understanding and application of fermentation processes in traditional foods like khalpi pickle. By exploring the patterns of cell growth and identifying the types of lactic acid bacteria that thrive under different temperatures during fermentation, this study aims to provide foundational knowledge that can enhance the quality and consistency of fermented products. Understanding how temperature affects pH levels, lactic acid production, and TSS changes during khalpi pickle fermentation offers valuable insights into optimizing fermentation conditions. Furthermore, the identification and potential use of specific lactic acid bacteria as starter cultures can lead to the development of more standardized and reliable fermentation processes, paving the way for product innovation and commercialization. This research holds significant potential to benefit small-scale industries and technologists by providing them with the tools and knowledge needed to produce high-quality, standardized khalpi pickles, thereby contributing to the preservation and modernization of traditional food practices.

1.5 Limitation of the work

i. The temperature at different days of fermentation at room temperature could not be made constant.

ii. Due to time constraints, the shelf life of the product was not determined.

iii. Due to resource limitations, individual species of lactic acid bacteria present during the fermentation were not separately quantified.

Part II

Literature review

2.1 Fermentation

Fermentation is among the most ancient techniques in food technology. It is a natural and environmentally friendly process that results in products with enhanced flavors and improved nutritional value. This method has been validated over thousands of years as an effective means of food preservation. It aligns with consumer preferences and expectations, and its popularity continues to grow steadily (Gaggia *et al.*, 2011).

Food and beverage fermentation knowledge dates back at least 6,000 years and likely originated from natural, spontaneous microbial interactions. Today, there are two primary methods of achieving food fermentation. The first method relies on the native microflora present in the raw food materials or processing environment, as seen in the fermentation of sauerkraut, pickle, kimchi, and certain soy products. These are often referred to as “wild ferments” or “spontaneous ferments” by researchers and food technologists. The second method involves adding starter cultures to initiate fermentation, known as “culture-dependent ferments,” which includes products like kefir, yogurt, kombucha, and natto (Voidarou *et al.*, 2020).

Fermentation has long been one of the most effective traditional methods for preserving food, enabling people across the globe to efficiently manage valuable food resources for thousands of years. This process transforms perishable foods and beverages into stable, safe products that can be stored and transported with ease. Additionally, fermented foods develop enhanced organoleptic qualities and become easier to digest. By preventing food spoilage, fermentation increases food availability and reduces waste. Humans have harnessed microorganisms to preserve food from ancient civilizations like Assyria, Babylonia, Egypt, and Persia to the Slavs. Over time, they gained experience in identifying which products could be fermented, how to accelerate the process, and methods to extend the shelf life of food products while preventing spoilage (Niakousari *et al.*, 2021).

2.2 Pickling

The technique of preserving food through pickling has long served as a method to extend the shelf life of food and vegetables. The term "pickle" finds its roots in the Dutch word 'pekel', denoting a brine solution often infused with spices, employed for both preserving and enhancing the flavor of food (Dt and N, 2003a). Pickling is a method of preserving or prolonging the shelf life of food through either anaerobic fermentation in brine or submersion in vinegar. This preservation process often influences the texture and flavor of the food. The outcome of this method is referred to as a pickle, and when named, the term is typically preceded by word 'pickled'. A wide range of foods, including vegetables, fruits, mushrooms, meats, fish, dairy, and eggs, can undergo pickling(Contributors). The primary goal of pickling is to inhibit the spoilage and contamination of food items caused by natural microflora. Additionally, incorporating certain ingredients, such as spices, during pickling process enhances the flavor and nutritional value of the final product. Pickling entails preserving food items under low pH conditions, achieved through the use of brine, vinegar, or other acidic substances(Seervi *et al.*, 2014). With the presence of low pH and elevated acid levels, food items can undergo successful bio-preservation, remaining viable for over two years without the need for refrigeration (J. P. Tamang, 1998).

While the precise origin of pickling remains uncertain, insights from archaeologists and anthropologists suggest it's root can be traced back to around 2400 B.C. It is believed that the ancient Mesopotamians possessed knowledge in the preservation of fruits and vegetables through pickling techniques. Various ancient societies, including the ancient Egyptians, Chinese, and Indians, employed pickling techniques as a method of preserving food(Chakraborty *et al.*, 2018). The significance of pickling become evident around 2030 B.C. when cucumbers, transported from distant regions of India subcontinent, were preserved in the Tigris valley, marking the initiation of a new tradition (Dt and N, 2003b). In the Indian subcontinent, particularly in the himalayan and nearby regions, pickles are commonly referred to as 'achar' and a meal is often considered incomplete without a touch of pickle. The condiments are frequently enjoyed alongside main course meals, serving as both appetizers and digestive aids(Kumari *et al.*, 2016). Furthermore, the inclusion of pickles promptly contributes additional taste, flavor and texture to conventional fruits and vegetables. From an economic standpoint, pickling effectively mitigates the price fluctuations observed between peak harvesting periods and off seasons. Additionally, it aids

in diminishing losses attributed to post harvest spoilage of fruits and vegetables(Sultana *et al.*, 2014).

2.3 Khalpi

In Nepal, people enjoy a variety of chutneys and pickles made from different vegetables, pulses, and spices, which they eat with rice and breakfast dishes. Many studies have looked at how these pickles and chutneys are made, including how they're optimized and standardized, and how they change over time. However, there's not much information available about khalpi pickle, a traditional Nepali pickle served with dal, bhat, and tarkari. khalpi pickle is made from ripe cucumbers, often large ones grown locally. It has a nice flavor with a bit of sourness that makes it a tasty appetizer. Adding salt and mustard seed powder during preparation helps good bacteria grow and keeps bad bacteria away from the pickle. This study aims to learn more about khalpi pickle and how it's made, which will help us understand traditional food preservation better.

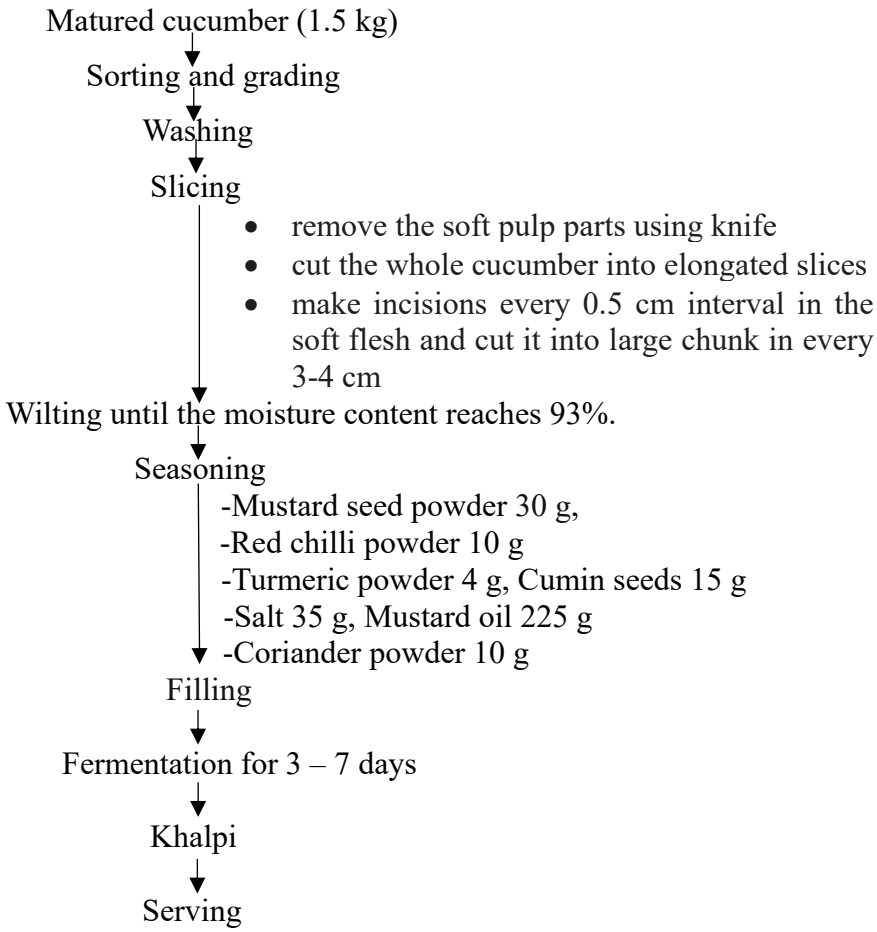

Fig 2.1 Preparation of Khalpi pickle

2.4 Lactic acid bacteria

Lactic acid bacteria (LAB), characterized as gram-positive microorganisms, serve as the primary safe industrial-scale generators of lactic acid (LA). The production of LA occurs through the glycolysis pathway under anaerobic conditions, utilizing hexoses and pentoses from LAB metabolism pathways. The yield and productivity of LA are contingent on factors such as pH (ranging from 3.5 to 9.6), temperature (5-45 °C), the presence of nutrients (including amino acids, peptides, nucleotides, and vitamins), and the specific LAB strain used. Various LAB strains, encompassing genera like *Leuconostoc, Lactococcus, Lactobacillus, Pediococcus, Enterococcus, Streptococcus, Vagococcus, Aerococcus, Carnobacterium, Tetragenococcus, Oenococcus,* and *Weissella*, have been employed for LA production(Abedi and Hashemi, 2020). Nevertheless, LAB types like *Lactobacillus, Lactococcus, Leuconostoc, Streptococcus, and Pediococcus* are employed as starter cultures in food fermentations in industries. Within LAB varieties, Lactobacillus is particularly valuable commercially because of its strong acid tolerance, high production yield, and productivity. Furthermore, these strains can be modified for targeted production of L/D-lactic acid (Kylä-Nikkilä *et al.*, 2000). There are two fermentative LAB pathways:

2.4.1 The homofermentative LAB

LAB possesses the aldolase enzyme, enabling the conversion of glucose predominantly into LA. Homofermentative LAB typically utilizes hexose and pentose sugars through the Embden-Meyerhof pathway (employing glycolysis and the pentose phosphate pathway). As a major end-product, homofermentative LAB generates two LA molecules per mole of consumed glucose, exhibiting a theoretical yield of 1g per 1 g .Experimental yields vary based on the type of carbon source used(Martinez *et al.*, 2013). For the commercial production of lactic acid (exceeding 100 g/L), only homofermentative LAB is utilized due to its high yield, nearing the maximal theoretical value, along with enhanced productivity and optical purity of lactic acid (>99%). Homofermentative LAB comprises Streptococcus, Lactococcus, Enterococcus, Pediococcus, and some Lactobacillus. Notable homofermentative *Lactobacillus spp*. includes *L. delbruckii* subsp. *bulgaricus*, *L. acidophilus, Streptococcus salivarius subsp. thermophilus, and L. helveticus*. Additionally, studies by Abdel-Rahman et al. have demonstrated that Enterococcus mundtii QU 25 and genetically modified Lactobacillus plantarum exhibit the ability to metabolize

homofermentative pentoses into lactic acid(Abdel-Rahman *et al.*, 2013; Abdel-Rahman *et al.*, 2011).

2.4.2 The heterofermentative LAB

LAB has the capability to transform glucose into lactic acid (LA), acetic acid (AA), formic acid, ethanol, diacetyl, acetoin, and carbon dioxide (CO_2 gas detection serves as a diagnostic test to distinguish between heterofermentative and homofermentative fermentation)(Abdel-Rahman *et al.*, 2011). Heterofermentative LAB has the capability to utilize both the phospho-gluconate pathway (with a theoretical yield of 0.5 g/g) and the phospho-ketolase pathway (with a theoretical yield of 0.6 g/g) when metabolizing hexose and pentose sugars, respectively (Abdel-Rahman *et al.*, 2013; Abdel-Rahman *et al.*, 2011). The application of heterofermentative LAB as dairy starter cultures is infrequent due to the release of CO_2 and concurrent production of lactic acid (LA) and other organic acids, which are perceived as defects leading to various issues. These problems include bloated packaging and cracks in dairy products and hard cheeses. Heterofermentative LAB primarily consists of *Oenococcus, Leuconostoc*, and some *Lactobacillus spp*. Main *Bacillus spp*. have obtained accreditation from the European Food Safety Authority (Hazards) and the Food and Drug Administration (FDA), being listed under the Qualified Presumption of Safety (QPS) and Generally Recognized as Safe (GRAS) categories for applications in livestock production [18]. Certain Bacillus strains, such as *B. coagulans, B. sterothermophilus, B. licheniformis* (both thermophilic and non-thermophilic), B. subtilis, Bacillus sp., and alkaliphilic bacilli like *B. circulans var. alkalophilus ATCC 21783, B. alkalophilus sp. halodurans* ATCC 27557, *B. alcalophilus* ATCC 27647, *alkaliphilic B. sp.* WL-S20, and B. sp. 17-1 ATCC 31007, have the potential to produce LA(Abedi and Hashemi, 2020).

2.5 Lactic acid bacteria in vegetable fermentation

The ancient practice of fermenting plant materials, with its roots traced back to Asia, emerges as a captivating preservation technique. Buckenhuskes and colleagues posit fermented plant products as the "food of the future," supported by compelling factors:

- Exceptional hygienic safety through the suppression of pathogenic bacteria growth.
- Products earn the coveted labels of "natural" or "biological."

- Enrichment of desired metabolites like L-lactic acid and amino acids.
- Formation of flavorful compounds and the elimination of undesirable flavor compounds like glucosinolates.
- Lower energy input compared to alternative preservation methods.
- User-friendly handling and storage without the need for cooling.
- Simplicity in pre-handling raw materials before subsequent processing (Salminen *et al.*, 2004).

The journey of vegetable fermentation unfolds in four stages: initiation of fermentation, primary fermentation, secondary fermentation and post-fermentation. Considering the initial microbial population in fresh vegetables, dominated by aerobic organisms, facultatively anaerobic enterobacteria, LAB, and yeast, the growth dynamics depend on various factors. As fermentation progresses, the pH of the material drops rapidly due to acid production and the limited buffering capacity of most vegetables. Simultaneously, the redox potential decreases, creating a favorable environment for the selection of LAB. Secondary fermentation and post-fermentation stages are instigated by spoilage bacteria, yeasts, or molds, utilizing residual sugars or fermentation acids as substrates. This intricate dance of microbial interactions adds a layer of fascination to the art and science of plant material fermentation(Salminen *et al.*, 2004).

In the enchanting world of khalpi fermentation, heterofermentative lactic acid bacteria (LAB) like *Leuconostoc fallax, L. brevis,* and *P. pentosaceus* initiate the fermentation journey, weaving their transformative spells, and fermentation is finally completed by *L. planatarum* (B. Tamang and Tamang, 2010a).

In vegetable fermentations, key lactic acid bacteria (LAB) from *Lactobacillus, Leuconostoc,* and *Pediococcus* genera play a crucial role. While Enterococcus and Lactococcus show up at fermentation's start, their role in spontaneous fermentation is unclear. The species normally found in fermented vegetables don't usually reduce nitrate and require fermentable carbohydrates for growth. Homofermentative lactobacilli yield 85% lactic acid from glucose, while heterofermentative strains produce lactic acid, CO_2, ethanol, and/or acetic acid in equal amounts. Fructose acts as a hydrogen acceptor, converted to

mannitol by heterofermentative LAB. *L. plantarum* displays phenotypic variability, affirmed by genomic heterogeneity through DNA/DNA homology studies. Acidulant effectiveness relies on dissociation constant, pKa, which is most organic acids lies between pH 3 and 5 with antimicrobial impact of fermentation acids based on undissociated acid concentrations and low pH synergy. *L. plantarum's* acid tolerance stems from pH homeostasis even at low external pH. The antimicrobial effect of fermentation acids depends on undissociated form concentrations and low pH.

2.6 Antimicrobial Fermentation end products

The microbiota in fresh vegetables is initially dominated by Gram-negative aerobic bacteria and yeasts, with lactic acid bacteria forming a minor portion. Yet, when conditions shift to anaerobic with adjusted moisture levels, salt concentration, and temperature, spontaneous lactic acid fermentation occurs. This process gives lactic acid bacteria a competitive advantage, leading to a specific and reproducible succession of bacteria during fermentation. Salt and rapid organic acid production suppress Gram-negative bacteria early in fermentation. Bacteriocins, antimicrobial peptides or proteins produced by bacteria, play a crucial role in this process, with lactic acid bacteria, renowned for their bacteriocin production, influencing the ecology of traditionally fermented foods(Wood, 2012). Various strategies have been employed to explore how organic acids impact microbial activity. The inhibitory impact of acids is compared based on factors like pH, concentration, chain length, type, and branching degree to target a broad range of microorganisms. Challenges arise from experiments expressing concentration in different units, making it somewhat challenging to compare acids or draw broad conclusions about the most suitable acid for a specific effect in a category of foods. Historically, lactic acid has been recognized more for its sensory attributes than its antimicrobial qualities, but recent applications include using it as a rinse for beef, pork, and chicken carcasses. Its inhibitory power lies in lowering pH to levels where bacteria cannot initiate growth. In fermented foods, lactic acid, combined with other growth-inhibiting factors from lactic acid-producing microorganisms, hinders the growth of competing microorganisms. Notably, lactic acid excels at inhibiting spore-forming bacteria at pH 5.0, surpassing malic, citric, propionic, and acetic acid by fourfold in limiting the growth of Bacillus coagulans, the organism responsible for flat-sour spoilage in tomato juice(Doores, 2005). The utilization of lactic acid alone to lower the pH to 3.74 is inadequate

to hinder the proliferation of spoilage yeast in fermented vegetables. The presence of additional organic acids is deemed beneficial for this purpose(Savard *et al.*, 2002) .

CO_2 accumulation in fermented products extends microbial lag phases and decreases growth rates. CO_2's inhibitory effects vary based on factors like partial pressure, concentration, storage temperature, and microorganism type. While CO_2's bacteriostatic effect is known, its precise mechanism remains unclear. Gram-negative bacteria are generally more sensitive to CO_2 than gram-positive bacteria, with lactobacilli often among the most resistant (Salminen *et al.*, 2004) .

2.7 Lactic Acid Fermentation

The term 'fermentation' originates from the Latin verb fervere, meaning to boil, describing the bubbling action of yeast on fruit or malted grain extracts. This bubbling results from the anaerobic breakdown of sugars, producing carbon dioxide. However, the term holds distinct interpretations for biochemists and industrial microbiologists. Biochemically, it refers to energy generation through the catabolism of organic compounds, while in industrial microbiology, its scope tends to be more comprehensive(Stanbury *et al.*, 2013).

Lactic acid fermentation, practiced for millennia, serves the dual purpose of preserving surplus and perishable food while enhancing their organoleptic qualities. A diverse array of fruits and vegetables has traditionally been employed as substrates for lactic acid fermentation, yielding a rich spectrum of final products, some uniquely tied to specific geographical regions. The pivotal role of lactic acid bacteria in shaping the microecosystem is crucial for the success of the process. The swift acidification resulting from the metabolic activities of lactic acid bacteria effectively suppresses the growth of competing micro-organisms. Incorporating salting during the initial stages of fermentation aids in the proliferation of lactic acid bacteria. Additionally, maintaining the proper temperature and ensuring the quality of the raw material are pivotal factors influencing the outcome of the fermentation process (Paramithiots, 2021).

In lactic acid fermentation, the achieved high acidity, low pH, and reduced oxidation-reduction potential act as guardians, inhibiting unwanted organisms and averting detrimental chemical shifts. In fact, lactic acid bacteria carry on essential metabolic biological processes without oxygen by means of a complex series of intermolecular oxidation and reduction. These organisms are sometime referred as micro-aerophilic. these organisms bring about

transformations without decomposing foods into basic components like carbon dioxide and water. Instead, their metabolic masterpiece often yields lactic acid from sugars, marking a key output. While gently modifying other components, some species produce additional products. This bacterial brigade, with its acidic alchemy converting carbohydrates into lactic acid, acetic acid, alcohol, and carbon dioxide, stands as a vital guardian of preserving delectable and nutritious food for humankind (Rekha Shrestha, 2002).

2.8 Lactic acid

Discovered by Scheele in 1780, lactic acid, also known as 2-hydroxypropionic acid, is a naturally occurring organic acid found in sour milk(Ferguson *et al.*, 2018). Industrial production of lactic acid (LA) was initiated by Fremi in 1881 through fermentation, marking a significant milestone in its manufacturing history(Choudhary *et al.*, 2021). Lactic acid (LA) can be generated either through chemical synthesis or microbial fermentation utilizing sugars sourced from renewable resources, including agricultural waste materials(Martinez *et al.*, 2013). Lactic acid is considered safe and falls under the classification of generally Recognized As Safe (GRAS)(Datta *et al.*, 1995). Lactic acid holds significant biotechnological value due to it's extensive applications in the cosmetics, pharmaceutical, medical, and chemical industries(P Pawar *et al.*, 2014). Lactic acid ($CH_3CHOHOOH$) is a chiral molecule with two enantiomeric forms: L-lactic acid and D-lactic acid (Figure 1). Its optically active forms, L (+) or D (-), or a racemic mixture (L (+) and D (-), can be determined based on the chosen production processing routes.

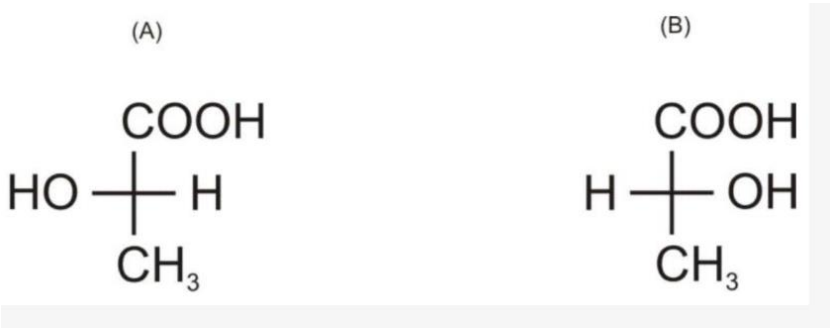

Fig 2.2 Three-dimensional structure of optical L-lactic acid (**A**) and D-lactic acid (**B**) (Adapted from Pohanka (2020) (Pohanka, 2020).

Lactic acid, existing as a yellow to colorless liquid at 15 °C and 1013 bars, exhibits solubility solely in water, ethanol, and other water-soluble miscible organic solvents. Its

hygroscopic nature typically results in a colorless concentrated solution (up to 90%). Odorless and less volatile, lactic acid stands as the simplest hydroxycarboxylic acid with diverse physico-chemical properties, including melting points of 53.0 °C (L-lactic acid), 52.8 °C (D- lactic acid), and 16.8 °C (racemic LD-lactic acid). Boiling points vary under different pressures; for instance, at 1.87 ka, lactic acid boils at 103 °C, rising to 122 °C at 1.99 kPa. At 20 °C, its solid density is 1.249 g/L, while in the aqueous solution at 25 °C, the density stands at 1.057 g/mL (for 20% wt.) and 1.201 g/mL (for 88.6% wt.).The lactic acid dissociation constants (pa) at 25 °C for L and D isomers are 3.79 and 3.83, respectively (Dusselier *et al.*, 2013).

2.9 Mechanism of Lactic Acid Fermentation

The primary pathways of hexose fermentation within lactic acid bacteria are well-known and have been extensively described. These pathways, illustrated in Fig. 1, share a common feature of targeting only hexose phosphates with a specific configuration. However, they differ in how they split the carbon skeleton, resulting in distinct sets of end-products(Kandler, 1983).

Glycolysis, found in *Streptococci, Pediococci*, and homofermentative lactobacilli. involves the cleavage of fructose 1,6-bisphosphate with aldolase into two triose phosphate moieties, ultimately converted to lactate. Thus, glycolysis leads to homolactic fermentation (Kandler, 1983).

Hetero-fermentation in *leuconostoc* and beta-bacteria begins with the oxidation of glucose 6-phosphate to gluconate 6-phosphate, followed by decarboxylation and splitting of the resulting pentose 5-phosphate into C-2 and C-3 moieties. This process generates equimolar amounts of CO_2, lactate, and acetate or ethanol from hexose. The acetate/ethanol ratio depends on the system's oxidation-reduction potential. In the presence of an additional hydrogen acceptor like O_2 or fructose, ethanol formation is bypassed, and O_2 is reduced to H_2O_2 or H_2O, while fructose is reduced to mannitol (Kandler, 1983).

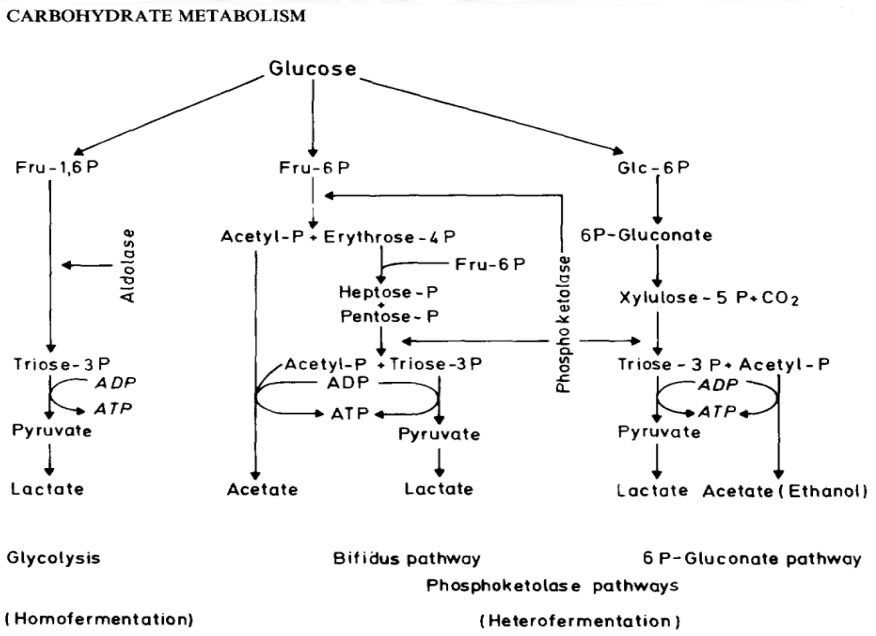

Fig 2.3 Schematic presentation of the main pathways of hexose fermentation in lactic acid bacteria (Kandler, 1983).

Hetero-fermentation in bifidobacteria starts by splitting fructose 6-phosphate with phospho-ketolase into C-2 and C-4 moieties. The C-2 moiety is converted to acetate, while the C-4 moiety, along with a triose moiety from an additional fructose 6-phosphate molecule, forms heptose 7-phosphate through transketolase action. Consecutive splitting of heptose 7-phosphate and resulting pentose 5-phosphate by phospho-ketolase ultimately yields acetate and lactate at a molar ratio of 3:2(Kandler, 1983).

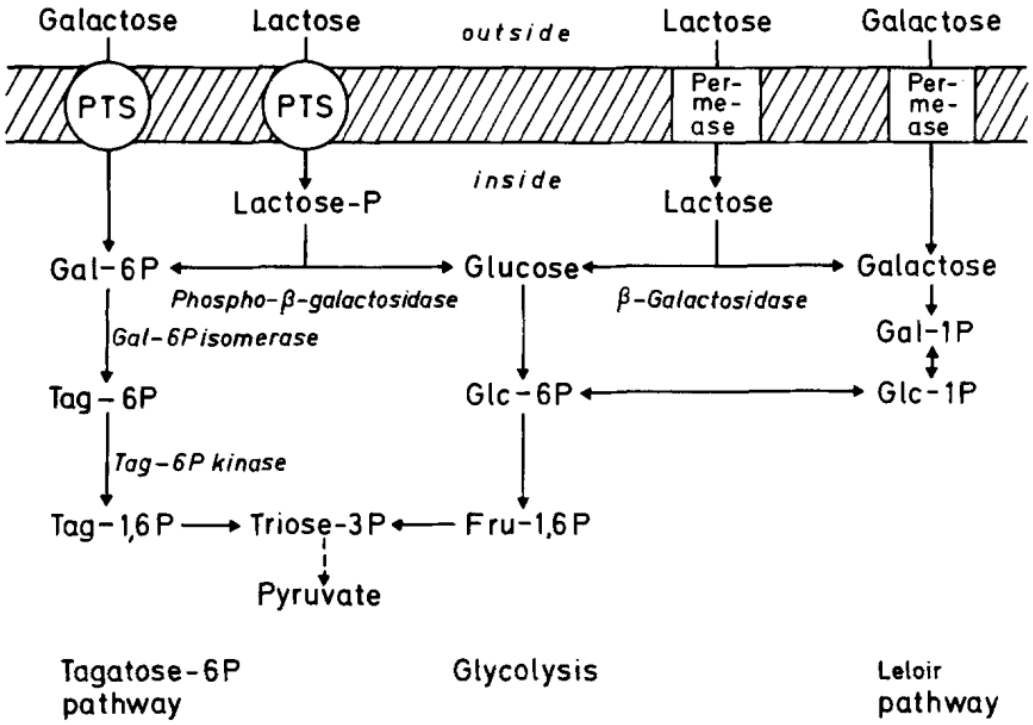

Fig 2.4 Scheme of lactose and galactose uptake and dissimilation in some lactic acid bacteria (Kandler, 1983).

These mechanisms allow clear differentiation between homolactic fermentation and the two types of hetero-fermentation by analyzing fermentation end-products and conducting specific enzyme tests. However, complexities arise when fermenting complex substrates with compounds beyond fermentable hexoses, such as pentoses or organic acids. This is often encountered in natural materials like fruit juices and vegetables. Moreover, pyruvate may not only be reduced to lactate but also converted to various other products depending on the growth conditions and properties of the specific organism (Kandler, 1983).

Part III

Materials and methods

3.1 Materials

3.1.1 Raw Materials

Matured cucumber (45 days after flowering), Mustard seed powder, Red chilli powder, Turmeric powder, Cumin seeds, Salt, Mustard oil, Fresh chilli, Coriander seed was purchased from the local market of Itahari.

3.1.2 Chemical, equipment, and other material

All the chemicals, laboratory glassware, and equipment used for the study were obtained from the Central Campus of Technology laboratory. The apparatus and chemicals required are listed in Appendix F.

3.2 Methods

3.2.1 Preparation of raw materials for fermentation

Matured cucumbers (*Cucumis sativus*) was purchased from the local market. The initial step was meticulously cleaning the whole cucumbers and chilies. The cucumbers and chilies was washed thoroughly to ensure cleanliness. Subsequently, each cucumber was delicately sliced into elongated pieces, with the removal of its soft pulp (seed part). To enhance the removal of moisture during drying and to facilitate the absorption of spices, incisions was made at 1 cm intervals, and the cucumbers was then be cut into larger, enticing chunks measuring 4×3×2 cm. Both the cucumber chunks were exposed to the sun's invigorating rays for 1 days until the moisture content reaches 93%.

3.2.2 Seasoning

For 1.5 kg cucumber 30 gm roasted mustard seed powder,15 gm roasted cumin seed powder, 10 gm roasted coriander powder, 4 gm turmeric powder, 35 gm salt, 50 gm solar dried chilli, 225 gm mustard oil will be used for seasoning, they will be mixed properly with cucumber.

3.2.3 Fermentation

Approximately 200 grams of perfectly seasoned cucumbers were taken and they were placed in a 250 ml, 23gm PET jar then the jar was tightly capped and it was allowed to undergo fermentation.

3.2.4 Sampling

Samples were taken in every 48 hours till 16 days of fermentation for analyses.

3.2.5 Microbial analysis

In the future, samples (10 g) of each product was mixed with 90 ml of 0.85% (w/v) sterile physiological saline and homogenized in a lab blender for 1 min. A serial dilution in the same diluents was made. LAB was isolated on plates of MRS agar (M641, HiMedia) supplemented with 1% $CaCO_3$ and incubated at 30°C in an anaerobic gas-jar (LE002, HiMedia) for 48-72 h. Colonies of molds and yeasts was examined on potato dextrose agar (MO96, HiMedia) and yeast-malt (YM) agar (M424, HiMedia), supplemented with 10 IU/ml benzylpenicillin and 12 µg/ml streptomycin sulfate, respectively, which was incubated aerobically at 28°C for 72 h. Isolated colonies based on colony morphology was selected randomly from the highest diluted plates. Purity of the isolates was checked by streaking again and sub-culturing on fresh agar plates of the isolation media, followed by microscopic examinations. Purified isolates of LAB was preserved at -20°C in MRS broth (M369, HiMedia) with 15% (v/v) glycerol added (B. Tamang and Tamang, 2010b).

3.2.6 Characterization and identification

The cell morphology and motility of all bacterial isolates was examined using a phase contrast microscope. The LAB isolates was undergo Gram staining and be assessed for catalase production by introducing a drop of 10% hydrogen peroxide solution to the isolates. Preliminary identification was based on carbon dioxide production from glucose. These procedures was follow the established methods of (Schillinger and Lücke, 1987).

3.2.7 Analytical procedure

The sample's pH was directly determined using a digital pH meter that was calibrated with standard buffer solutions from Merck. Titratable acidity was represented as a percentage of

lactic acid in the sample. Following that, the percentage of lactic acid was transformed into grams per liter.

3.2.8.1 Determination of moisture content

The moisture content of the sample was determined by measuring the weight loss during heating in a thermostatically controlled oven at 100°C or 105°C, following the hot air oven method as described by AOAC (2005).

$$\text{Moisture (\%)} = \frac{\text{Inital weight(g)} - \text{Final weight(g)}}{\text{Inital weight(g)}} \times 100(\%)$$

3.2.8.2 Determination of crude protein content

The Kjeldahl method was used to determine the nitrogen content, and a factor of 6.25 was multiplied to the nitrogen concentration to get total protein (AOAC, 2005).

The nitrogen content was calculated using the following equation:

$$\text{Nitrogen}(\%) = \frac{(\text{titer} - \text{blank})\text{ml} \times \text{Molarity of HCL} \times 14 \times 100 \times 100}{\text{Aliquoit(ml)} \times \text{wt of sample} \times 1000}$$

The protein content was then determined using the following equation:

$$\%\ \text{Protein} = \%\ \text{Nitrogen} \times \text{Conversion Factor}$$

Conversion factor 6.25 was used to convert the nitrogen content to crude protein.

3.2.8.3 Determination of crude fat content

The crude fat content of the sample was determined by solvent extraction method as described by AOAC (2005).

The percentage of fat content was calculated using the following formula:

$$\%\ \text{fat content} = \frac{\text{Weight of cup with fat} - \text{Weight of empty cup}}{\text{Weight of sample}} \times 100\%$$

3.2.8.4 Determination of ash content

The ash content of the samples was determined as per the AOAC (2005) method. A 5 g sample was placed in a pre-weighed crucible, dried, and then heated in a muffle furnace at

550°C for 3 h. The total ash content of the food sample was calculated using the following formula:

$$\text{Ash Content}(\%) = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100\%$$

3.2.8.5 Determination of crude fiber content

A chemical procedure was used to determine crude fiber; the sample was first treated with boiling dilute sulphuric acid, then with boiling sodium hydroxide, and finally with alcohol as the standard method of AOAC (2005).

$$\text{Crude fiber}(\%) = \frac{(\text{Residue} - \text{Ash})\text{g} \times (100 - \text{Fat})}{\text{Sample(g)}}$$

3.2.8.6 Determination of carbohydrate content

Carbohydrate content was determined by the difference method (AOAC, 2005). For many years, the total carbohydrate content of foods has been calculated indirectly instead of being measured directly. In this method, the amounts of other components in the food (protein, fat, water, crude fiber, ash) are measured individually. These values are then added together and subtracted from the total weight of the food. This method is called "total carbohydrate by difference" and is calculated using the following formula:

$$\text{Carbohydrate}(\%) = 100 - (\text{Moisture} + \text{Protein} + \text{Fat} + \text{Ash} + \text{Crude fiber})$$

3.2.8.9 pH measurement

The pH was measured according to the method described by AOAC (2005). First, 10 g of crushed pickle was weighed and placed in an Erlenmeyer flask, then 100 ml of distilled water at 25ºC was added. The mixture was stirred with an electronic agitator for 30 min. After stirring, the contents were transferred to a beaker and allowed to rest for 10 min before measuring the pH.

3.2.9 Sensory Analysis

The Khalpi pickle were evaluated based on appearance, flavor, texture, juiciness, taste, and overall palatability using a 9-point hedonic scale. Panelists rated the samples, with 9 points

for "extremely liked" and 1 point for "extremely disliked." The samples were presented randomly.

For this test, semi-trained panelists from the B.Tech 4th year and teachers from the Central Campus of Technology participated. For all the sensory attributes, they were encouraged to rate according to their preferences.

The sensory evaluation focused on several parameters: appearance, texture and tenderness, taste, flavor, and overall palatability. The panelists were untrained, and differences in quality were analyzed statistically, following (Ranganna, 1986) method. For more details, refer to the specimen card in Appendix A.

Part IV

Result and discussion

The analysis of cucumber was studied as described in material and method section and this cucumber was used in khalpi pickle preparation. Khalpi pickle was prepared and fermentation was carried out at room temperature shown in Appendix E, fig E and in constant temperature of 37°C. The change in percentage acidity, TSS, PH and microbial load was studied as described in material and method section. Results and discussion of the overall study are described in the following headings.

4.1 Chemical Properties of raw cucumber

The moisture content was found to be 96.12 % in this analysis. According to Suma *et al.* (2016), the moisture content of different variety of cucumber was found to be in between ranged from 93% to 97%.

The protein content of dried cucumber was determined to be 7.179% and raw cucumber protein content was found to be 0.28%. According to Bw *et al.* (2017), the protein content was in range of 0.29-0.84% in different variety of cucumber, which is slightly higher than this result.. These discrepancies might be due to the loss of nitrogenous material during digestion, species variations, and environmental conditions.

The fat content of raw cucumber was measured at 0.24% in this analysis. According to Agatemor *et al.* (2018) fat content was found to be 0.55% which is slightly higher than this result.This indicates that this moisture fat% is higher than those reported by other researchers, likely due to factors such as genetic diversity and climate conditions.

The crude fiber content of raw cucumber was found to be 0.47 % in this analysis. According to Agatemor *et al.* (2018), the fiber content was 1.02. These differences can likely be attributed to environmental conditions.

The ash content of raw cucumber was determined to be 0.42 % in this analysis. Which is slightly lower compared to the ash content determined by Agatemor *et al.* (2018), which is 0.94%. The difference may be due to factors such as genetic diversity and climate conditions.

The carbohydrate content was found to be 2.57 % in this analysis which is slightly lower than the carbohydrate determined by Xu *et al.* (2010), which is 2.63%.These differences can

be attributed to factors such as environmental conditions, cucumber variety, and experimental error.

The chemical properties of raw cucumber is presented in Table 4.1.

Table 4.1 Chemical properties of cucumber

Attributes	Cucumber
Moisture (wb)	96.12 ± 0.06
Protein (wb)	0.28 ± 0.02
Fat (wb)	0.24 ± 0.03
Crude Fiber (wb)	0.47 ± 0.1
Ash (wb)	0.42 ± 0.01
Carbohydrate (wb)	2.57 ± 0.17

* Values are represented as mean ± standard deviation of triplicate determinations.

4.2 Effects of fermentation period and temperature conditions on Acidity

4.2.1 Effects of fermentation period at 37°C on Acidity

The effect of fermentation time on acidity during pickle fermentation at 37°C was studied. The results from the one-way analysis of variance (ANOVA) demonstrated significant changes in acidity over time, highlighting the progression of lactic acid fermentation. The graph indicate a consistent increase in acidity as fermentation progressed from Day 1 to Day 16 (Fig 4.1). The mean acidity values ranged from 0.2367 ± 0.0116 on Day 1 to 1.5733 ± 0.0116 on Day 16. The increase was particularly notable in the initial stages of fermentation (Days 1 to 7), followed by a more gradual rise in acidity from Days 10 to 16.

The results obtained from ANOVA indicated a significant effect of time on acidity ($p < 0.05$). The steep increase in acidity observed between Days 1 and 7 corresponds to the exponential growth phase of lactic acid bacteria (LAB). These results are consistent with the findings of Xu *et al.* (2010), who reported rapid acid production during the early stages of

fermentation due to the high availability of fermentable sugars like glucose and fructose. As fermentation progresses, organic acid accumulation lowers the pH, which slows microbial activity and results in a more gradual increase in acidity. Also The results are in accordance with Ghimire *et al.* (2020a) who concluded that as the fermentation days increases, acidity increases while pH decreases till maximum value.

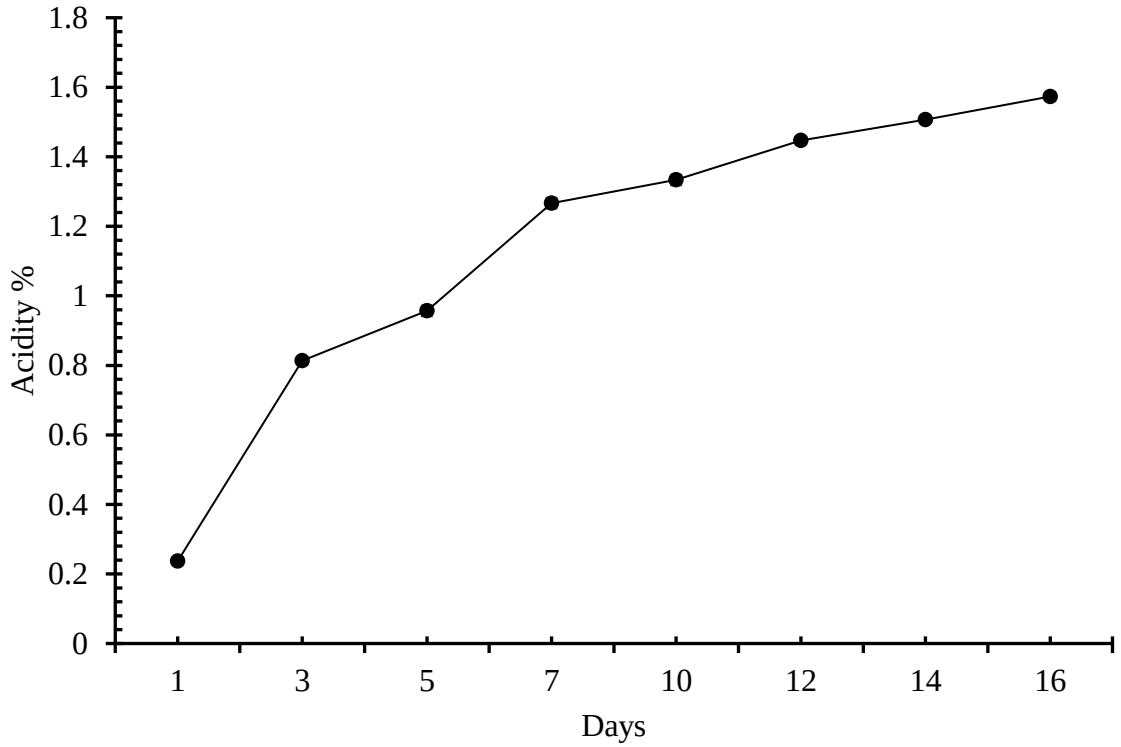

Fig. 4.1 Mean Acidity of the sample at different times during pickle fermentation at 37°C

4.2.2 Effect of fermentation period at room temperature on Acidity

The changes in acidity over time during pickle fermentation at RT was evaluated. The results from the analysis of variance (ANOVA) revealed significant increases in acidity as fermentation progressed. The mean acidity values increased significantly from 0.23 ± 0.0058 on Day 1 to 1.65 ± 0.0058 on Day 16 (Fig 4.2). The increase in acidity was more pronounced during the earlier days of fermentation (Days 1 to 7) and continued steadily through the later stages, reflecting ongoing microbial activity and acid production. The ANOVA results showed that time had a significant effect on acidity ($p < 0.05$). The results are in accordance with (Ghimire *et al.*, 2020b) who concluded that as the fermentation days increases, acidity increases while pH decreases till maximum value.

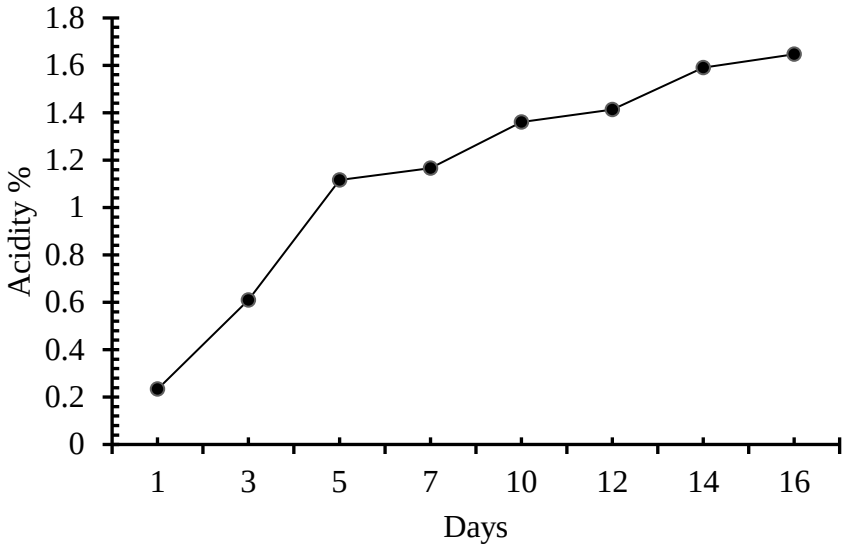

Fig. 4.2 Mean acidity of the sample at different times during pickle fermentation at room temperature

4.3 Effects of fermentation period and temperature conditions on TSS

4.3.1 Effect of fermentation period at 37°C on TSS

The changes in Total Soluble Solids (TSS) over time during pickle fermentation at 37°C was studied. The results from the analysis of variance (ANOVA) revealed significant increases in TSS as fermentation progressed. The mean TSS values increased significantly from 8.77 ± 0.058 on Day 1 to 12.23 ± 0.058 on Day 16 (Table 1). The rise in TSS was more pronounced in the early days (Days 1 to 7) and slowed as fermentation approached later stages, indicating a progressive solubilization of compounds during fermentation. The ANOVA results show that time had a significant effect on TSS ($p < 0.05$). The increase in TSS during fermentation of khalpi pickle is shown in figure 4.3

The result complied well with Haokip *et al.* (2022), who also found a significant increase in TSS during 90 days of storage period of mango pickle. A similar trend was reported by Singh *et al.* (2021) in cauliflower pickle. The increase in tss may be due to well mixing and dissolving of salt and other ingredients during the storage period and also it may be due to reduction in moisture content of cucumber since the addition of salt which causes osmosis which reduces the moisture content of cucumber ultimately resulting in the increase of TSS of khalpi pickle (Haokip *et al.*, 2022) .

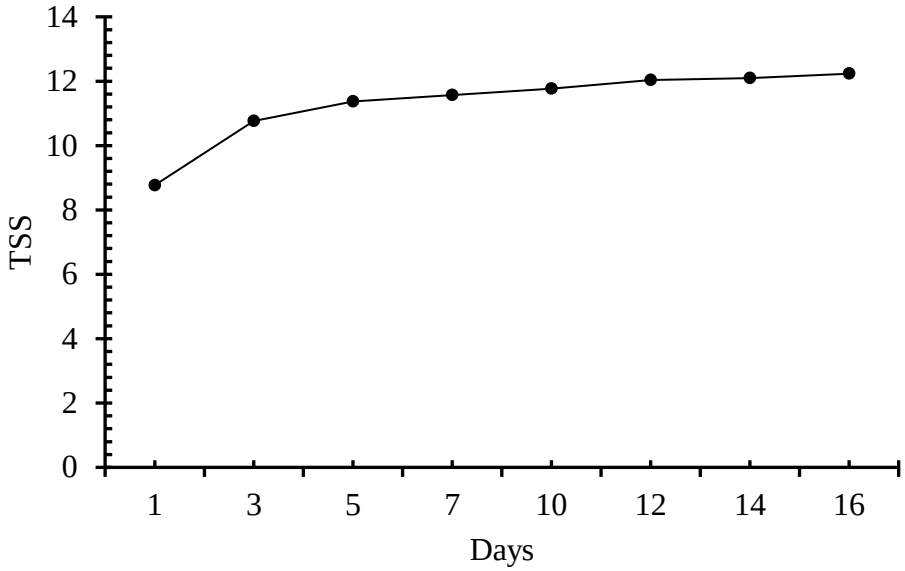

Fig. 4.3 Mean TSS of the sample at different times during pickle fermentation at 37°C

4.3.2 Effect of fermentation period at room temperature on TSS

This study evaluated the changes in TSS over time during pickle fermentation at room temperature. The results from the analysis of variance (ANOVA) revealed significant increases in TSS as fermentation progressed.

The mean TSS values increased significantly from 8.77 ± 0.058 on Day 1 to 12.03 ± 0.058 on Day 16 (Fig 4.11). The increase in TSS was more pronounced during the early fermentation days (Days 1 to 7) and gradually slowed in the later stages, indicating continuous solubilization of compounds during fermentation. The ANOVA results showed that time had a highly significant effect on TSS ($p < 0.05$). The increase in TSS during fermentation of khalpi pickle is shown in Fig 4.4 .

The result complied well with the findings of Haokip *et al.* (2022), he also found a significant increase in TSS during 90 days of storage period of mango pickle. A similar trend was reported by Singh *et al.* (2021) in cauliflower pickle. The increase in TSS may be due to well mixing and dissolving of salt and other ingredients during the storage period and also it may be due to reduction in moisture content of cucumber since the addition of salt which causes osmosis which reduce the moisture content of cucumber ultimately resulting in the increase of TSS of khalpi pickle (Haokip *et al.*, 2022) .

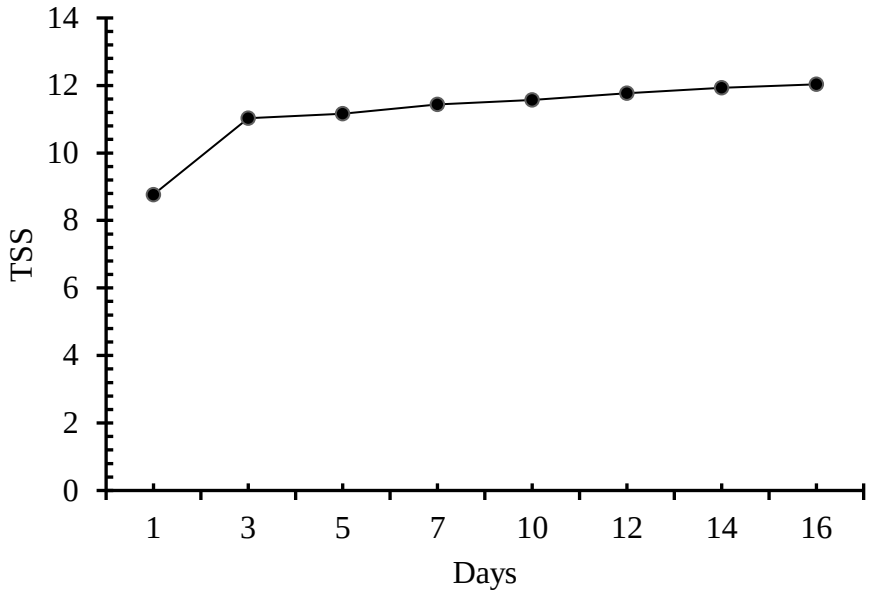

Fig. 4.4 Mean TSS of the sample at different times during pickle fermentation at room temperature

4.4 Effects of fermentation period and temperature conditions on pH

4.4.1 Effect of fermentation period at 37°C on pH

The changes in pH over time during pickle fermentation at 37°C was studied. The findings reveal a significant decline in pH throughout the fermentation period, reflecting the progression of lactic acid fermentation. The decline was more pronounced during the early stages (Days 1 to 7) and became gradual as fermentation progressed toward the later stages. The ANOVA results showed that time had a significant effect on pH ($p < 0.05$). The rapid reduction in pH observed during the initial days (Days 1–7) is consistent with the active growth phase of lactic acid bacteria (LAB), during which fermentable sugars are converted into organic acids such as lactic acid, resulting in a drop in pH.

This result was similar to the findings of Fleming *et al.* (1989) who observed the pH of fermented cucumber at the end of fermentation to be 3.7. This value is a little lower than the value that we found in our research. The difference may be because of the pH of fermented cucumber being observed for 30 days till no fermentable sugar was remained in product, in the study conducted by Fleming *et al.* (1989), but here in our research we have observed the pH for only 16 days so there still may be little amount of fermentable sugar in khalpi pickle

which would be converted to Lactic acid by lactic acid bacteria thus resulting in the decrease of pH.

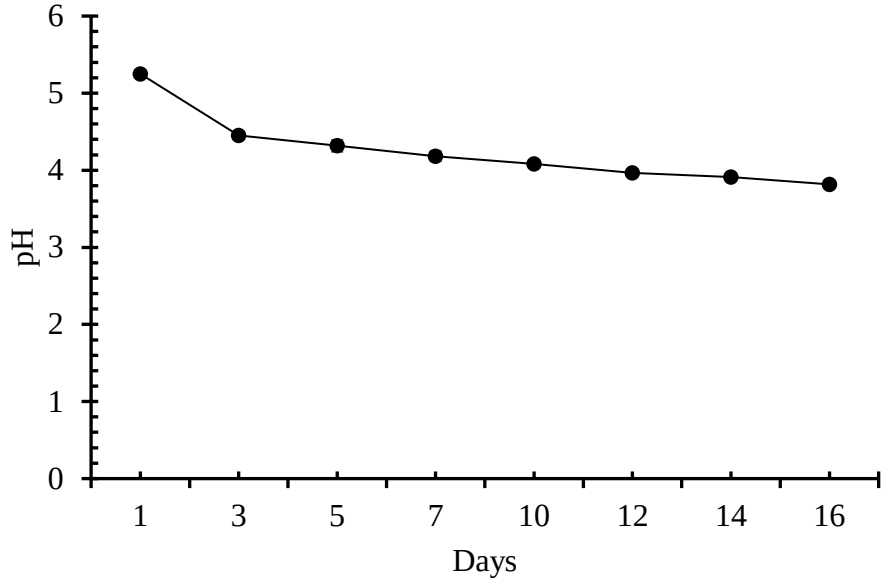

Fig. 4.5 Mean pH of the sample at different times during pickle fermentation at 37°C

4.4.2 Effect of fermentation period at room temperature on pH

This study evaluated the changes in pH over time during pickle fermentation at room temperature. Results from ANOVA demonstrated a significant decrease in pH as fermentation progressed, indicative of increased acid production. The descriptive statistics indicate a significant reduction in pH, from 5.167 ± 0.059 on Day 1 to 3.757 ± 0.059 on Day 16 (Fig 4.6). The decline was most pronounced in the early stages (Days 1 to 7), after which the rate of decrease slowed, indicating a stabilization phase in fermentation. The ANOVA results revealed a significant effect of fermentation time on pH ($p < 0.05$). The sharp decline in pH during the initial stages (Days 1–7) corresponds to the exponential growth phase of lactic acid bacteria (LAB).

This result was similar to the findings of Fleming *et al.* (1989), who observed the pH of fermented cucumber at the end of fermentation to be 3.7. This value is little lower than the value that we found in our research. The difference may be because of the pH of fermented cucumber being observed for 30 days till no fermentable sugar was remained in product, in the study conducted by Fleming *et al.* (1989), but here in our research we have observed the pH for only 16 days so there still may be little amount of fermentable sugar in

khalpi pickle which would be converted to Lactic acid by lactic acid bacteria thus resulting in the decrease of pH.

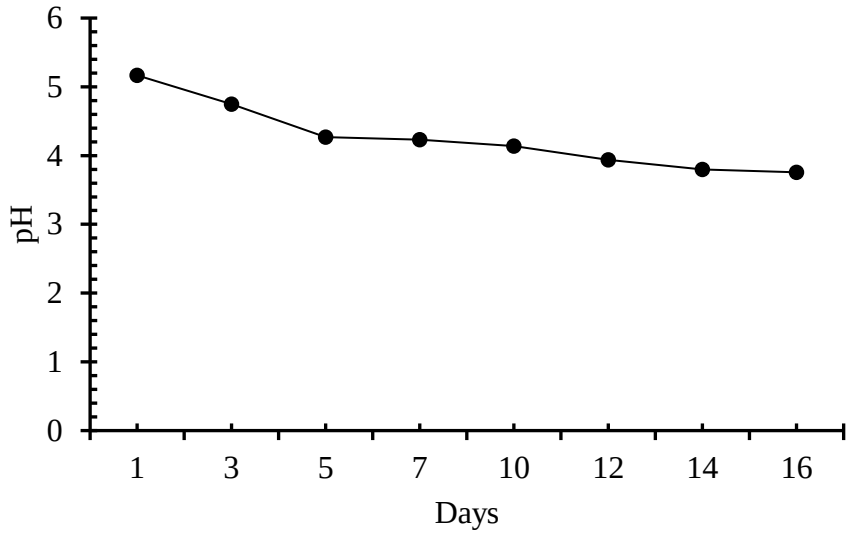

Fig. 4.6 Mean pH of the sample at different times during pickle fermentation at room temperature

4.5 Effects of fermentation periods and temperature conditions on LAB counts

4.5.1 Effect of fermentation period at 37°C on LAB counts

The relationship between fermentation time and the microbial load of lactic acid bacteria (LAB) at 37°C was studied. The findings reveal significant changes in microbial load throughout the fermentation process. The ANOVA results show that microbial load varies significantly across fermentation days ($p < 0.05$). The microbial load increased rapidly in the early stages, peaking on Day 10 at 10.4623 Log CFU/mL, before sharply declining by Day 16 to 5.06 Log CFU/mL (Fig 4.7). The ANOVA test indicates that time had a significant effect on microbial load ($p < 0.05$). The microbial load increased exponentially from Day 1 to Day 10, corresponding to the logarithmic growth phase of LAB. The subsequent decline reflects the depletion of nutrients and the accumulation of inhibitory byproducts, such as organic acids.

The result complied well with Lu *et al.* (2012) who also found the similar result of initial lactic acid bacteria 10*4 CFU/ml and final lactic acid bacteria level of 2* 10^5 CFU/ml and 6* 10^3 on day 30 and day 90 of fermentation.

The early exponential rise of LAB population and acid percentage at may have been caused by the quick development of homofermentative bacteria as such effect the acidification rate of vegetable and promote the growth of single microbial species giving it a competitive edge over other species (Sharma, 2007) . The decrease in lactic acid bacteria during fermentation can be attributed to end-product inhibition, where undissociated lactic acid penetrates the cytoplasmic membrane, causing cytoplasmic acidification. This disrupts the proton motive force and transmembrane pH gradient, reducing the energy available for cell growth (Othman *et al.*, 2017)Fig. 4.4 Mean Lactic Acid Bacteria (LAB) Counts of the sample at different times during pickle fermentation at 37°C

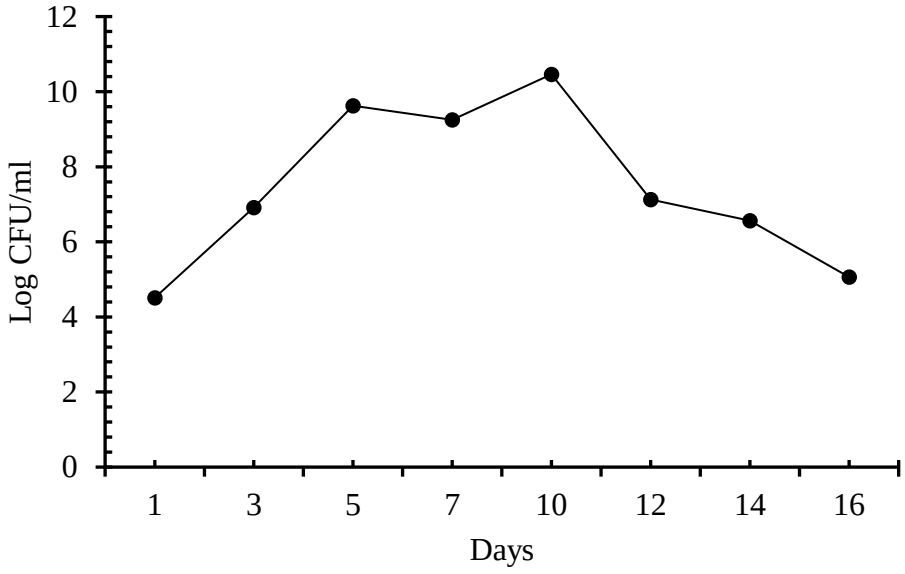

Fig. 4.7 Mean Lactic Acid Bacteria (LAB) Counts of the sample at different times during pickle fermentation at 37°C

4.5.2 Effect of fermentation period at room temperature on LAB count

This study evaluated changes in the microbial load of lactic acid bacteria (LAB) over time during pickle fermentation at RT. The results from ANOVA revealed significant changes in microbial load throughout the fermentation process. The mean microbial load increased substantially from 4.4913 Log CFU/g on Day 1 to 9.176 Log CFU/g on Day 10, before decreasing significantly to 5.535 Log CFU/g on Day 16 (Fig 4.8). The microbial load peaked during the mid-fermentation stage (Days 7–10), likely due to optimal conditions for LAB growth, followed by a decline, possibly as a result of nutrient depletion or the accumulation

of inhibitory byproducts. The ANOVA results showed that time had a significant effect on microbial load ($p < 0.05$).

The result complied well with Lu *et al.* (2012), who also found the similar result of initial lactic acid bacteria 10*4 CFU/ml and final lactic acid bacteria level of 2* 10^5 cfu/ml and 6* 10^3 on day 30 and day 90 of fermentation. The early exponential rise of LAB population and acid percentage may have been caused by the quick development of homofermentative bacteria as such effect the acidification rate of vegetable and promote the growth of single microbial species giving it a competitive edge over other species (Sharma, 2007) . The decrease in lactic acid bacteria during fermentation can be attributed to end-product inhibition, where undissociated lactic acid penetrates the cytoplasmic membrane, causing cytoplasmic acidification. This disrupts the proton motive force and transmembrane pH gradient, reducing the energy available for cell growth (Othman *et al.*, 2017).

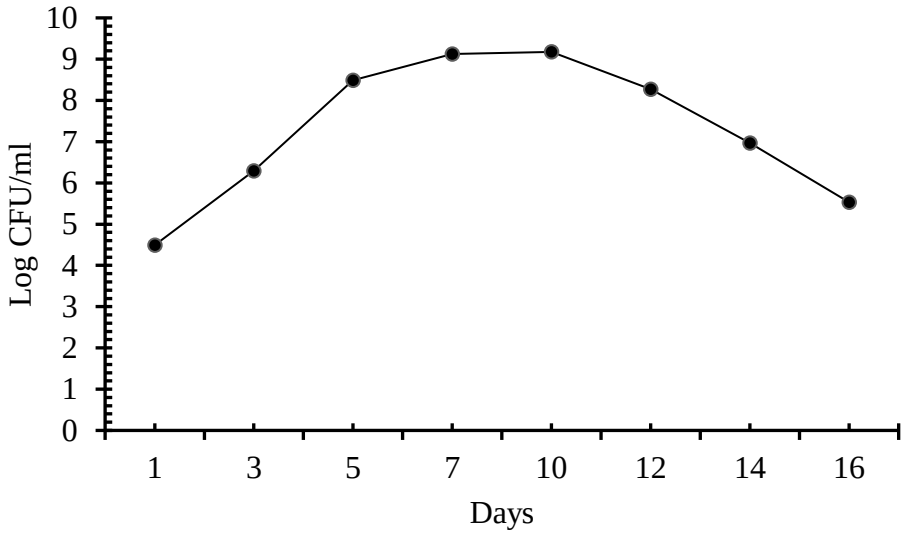

Fig. 4.8 Mean lactic acid bacteria (LAB) count of the sample at different times during pickle fermentation at room temperature

4.6 Effects of fermentation period and temperature conditions on appearance

4.6.1 Effect of fermentation period at 37°C on appearance

This study assessed the effect of fermentation time at 37°C on the appearance score of pickles. The sensory evaluation used a 9-point hedonic scale, where higher scores indicate

greater preference for appearance. The results revealed significant improvements in appearance scores over time.

The ANOVA results indicate a significant effect of time on appearance scores ($p < 0.05$). Post hoc Tukey's test revealed significant differences between the earlier days (Days 1 to 5) and later days (Days 10 to 16). The improvement in appearance is attributed to the effects of microbial activity and biochemical changes that enhance color uniformity, texture, and clarity.

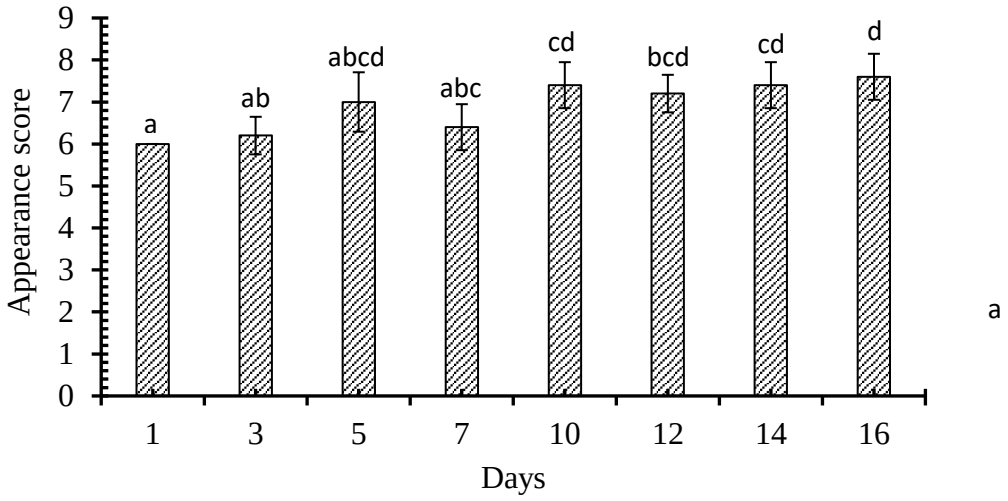

Fig. 4.9 Mean appearance score of the sample at different times during pickle fermentation at 37°C.

4.6.2 Effect of fermentation period at room temperature on appearance

This study analyzed the impact of fermentation time at room temperature on the appearance scores of pickles. A 9-point hedonic scale was used, where higher scores indicate greater preference. The results revealed significant improvements in appearance scores over time, with higher ratings in later fermentation stages. The mean appearance score increased from 6.00 ± 0.00 on Day 1 to 7.60 ± 0.55 on Day 16 (Fig 4.10). Early fermentation stages were rated between "Like slightly" and "Like moderately," while later stages achieved ratings close to "Like very much." The ANOVA results revealed a significant effect of fermentation time on appearance scores ($p < 0.05$).

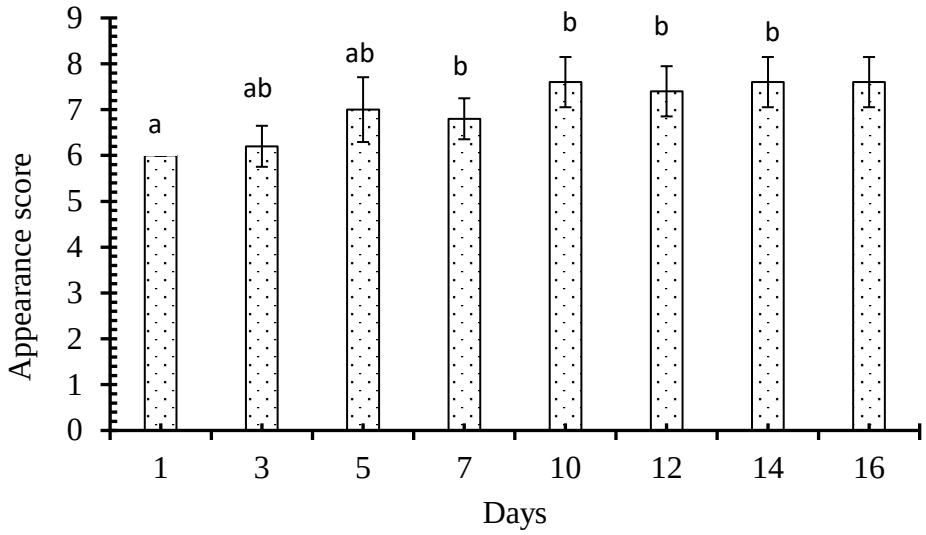

Fig. 4.10 Mean appearance score of the sample at different times during pickle fermentation at room temperature

4.7 Effects of fermentation periods and temperature conditions on taste

4.7.1 Effect of fermentation period on taste at 37°C

This study evaluated changes in the taste score of pickles during fermentation at 37°C. A 9-point hedonic scale was used, where higher scores indicate greater taste preference. The results demonstrated significant improvements in taste scores over time.

The ANOVA results reveal a significant effect of fermentation time on taste scores ($p < 0.05$).

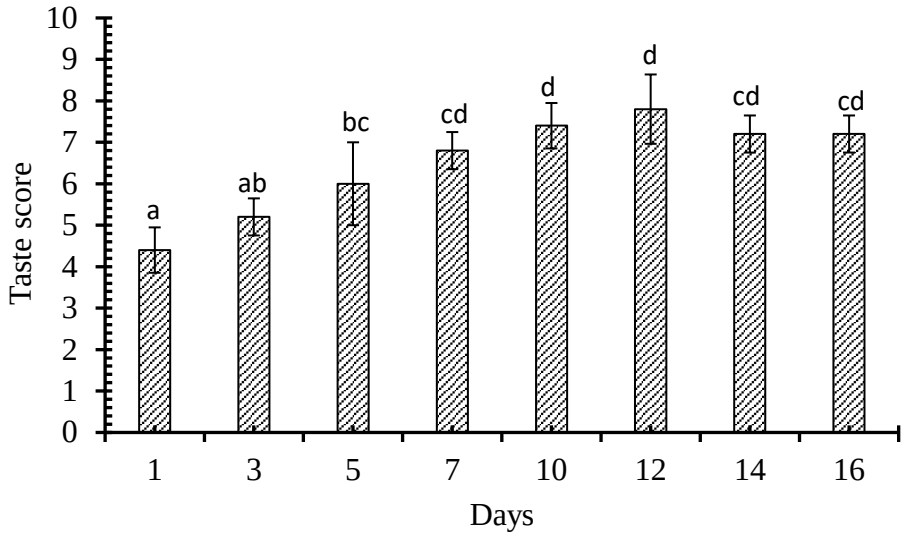

Fig. 4.11 Mean taste score of the sample at different times during pickle fermentation at 37°C.

4.7.2 Effect of fermentation period at room temperature on taste

This study evaluated the effect of fermentation time at room temperature on the taste scores of pickles. A 9-point hedonic scale was used, where higher scores indicate greater preference. The results from the one-way analysis of variance (ANOVA) demonstrated significant changes in taste scores over time, highlighting the progression of flavor development during fermentation.

The ANOVA results revealed a significant effect of time on taste scores ($p < 0.05$). Tukey's post hoc test identified significant differences in taste scores between earlier days (e.g., Day 1) and later days (e.g., Day 10 and beyond). These findings correspond to the increasing acid production and breakdown of complex sugars into flavor-enhancing compounds by lactic acid bacteria during fermentation.

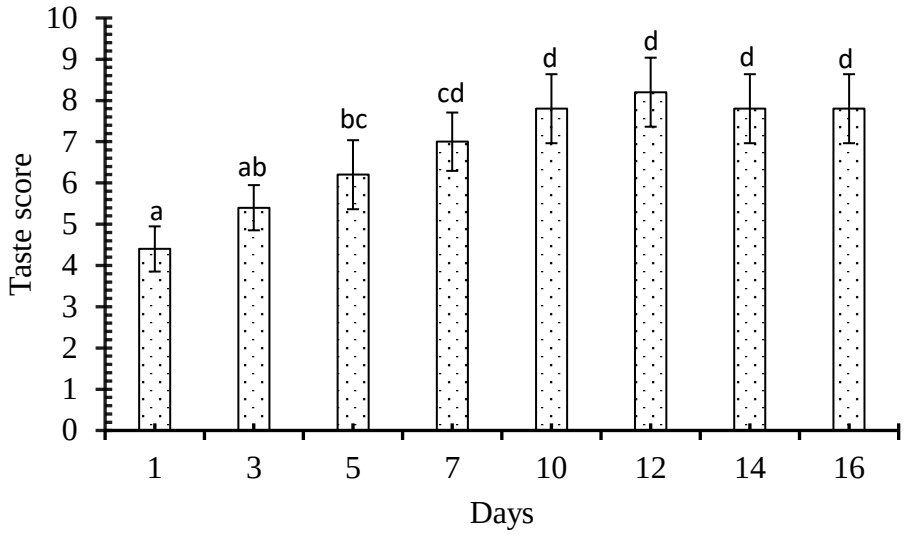

Fig. 4.12 Mean taste score of the sample at different times during pickle fermentation at room temperature

4.8 Effects of fermentation periods and temperature conditions on smell

4.8.1 Effect of fermentation periods at 37°C on smell

This study evaluated the changes in the smell score of pickles during fermentation at 37°C. A 9-point hedonic scale was used, where higher scores indicate greater preference for the smell. The results demonstrated significant improvements in smell scores over time.

A gradual improvement was observed throughout the fermentation process, with significant increases after Day 10 (Fig 4.13). The ANOVA results reveal a significant effect of fermentation time on smell scores ($p < 0.05$).

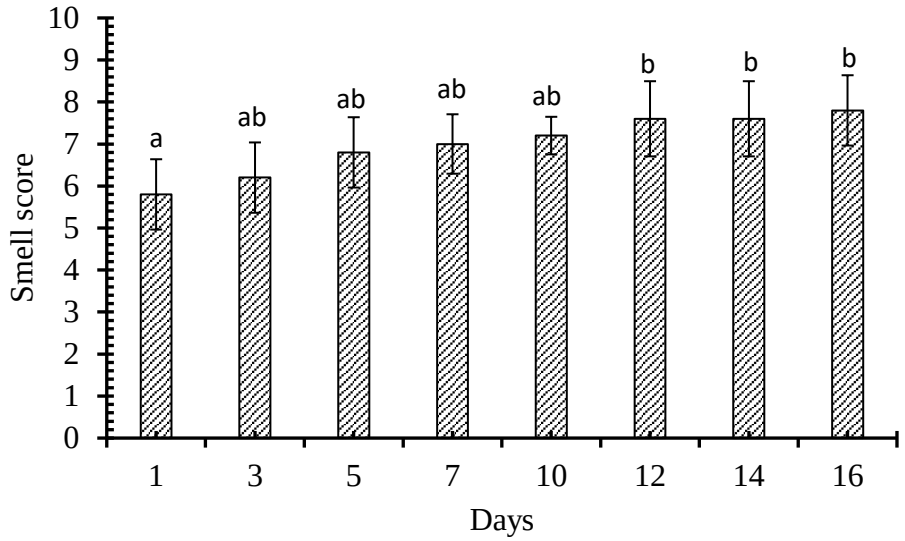

Fig. 4.13 Mean smell score of the sample at different times during pickle fermentation at 37°C

4.8.2 Effect of fermentation periods at room temperature on smell

This study evaluated the effect of fermentation time at room temperature on the smell of pickles. A 9-point hedonic scale was used, where higher scores indicate greater preference. The results revealed significant improvements in smell scores over time, with the highest ratings observed in later fermentation stages.

The mean smell score increased from 5.80 ± 0.84 on Day 1 to 8.00 ± 0.71 on Day 16 (Fig 4.14). Early fermentation stages were rated between “Dislike slightly” and “Like slightly,” while later stages achieved ratings close to “Like very much.” The ANOVA results revealed a significant effect of fermentation time on smell scores ($p < 0.05$).

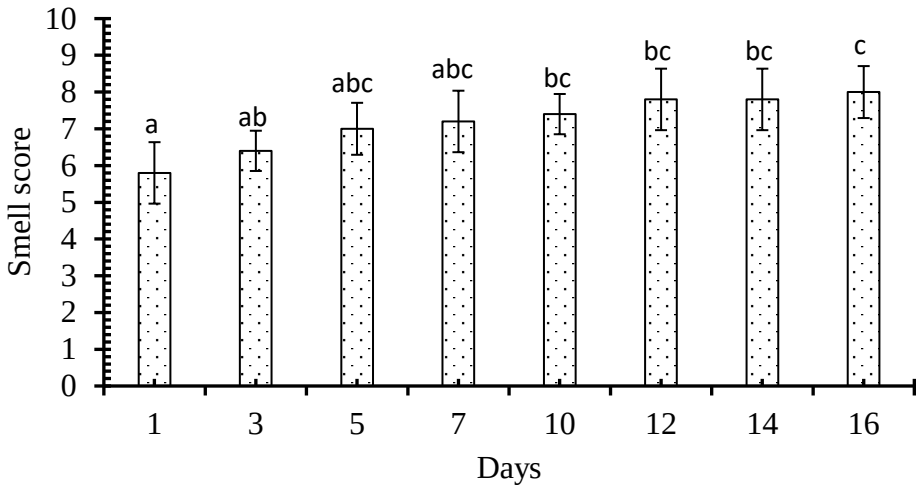

Fig. 4.14 Mean smell score of the sample at different times during pickle fermentation at room temperature

4.9 Effects of fermentation periods and temperature conditions on mouthfeel

4.9.1 Effect of fermentation periods on mouthfeel at 37°C

This study analyzed the effect of fermentation time on the mouthfeel of pickles at 37°C. A 9-point hedonic scale was used, where higher scores indicate greater preference..The ANOVA results indicate a significant effect of fermentation time on mouth feel scores ($p < 0.05$). Notably, Day 12 scores are significantly higher than most earlier days, reflecting the culmination of fermentation's impact on texture.

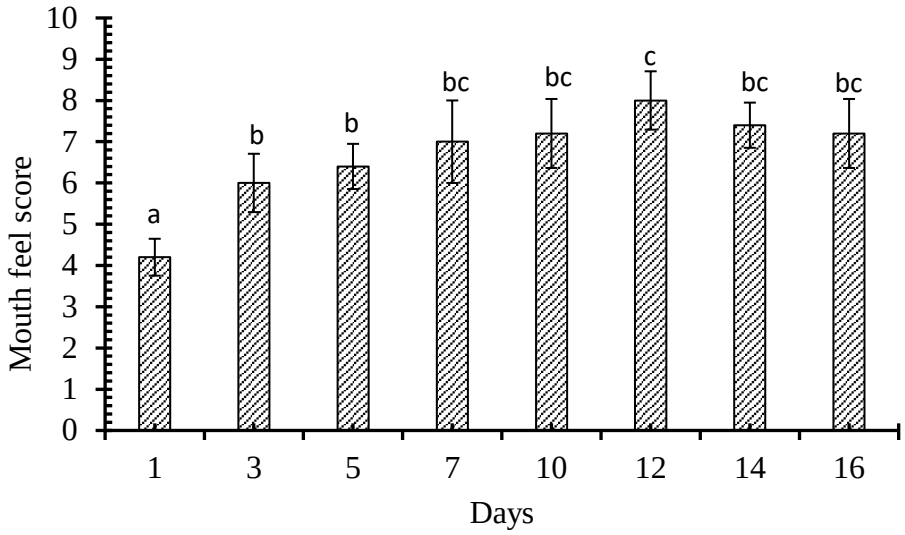

Fig. 4.15 Mean mouth feel score of the sample at different times during pickle fermentation at 37°C

4.9.2 Effect of fermentation periods at room temperature on mouthfeel

This study examined the impact of fermentation time at room temperature on mouth feel scores, assessed using a 9-point hedonic scale. ANOVA results showed significant changes in mouth feel scores over time, indicating the influence of fermentation conditions on textural development.

Scores improved steadily until Day 12, after which slight decrease in texture score was observed on subsequent days (Fig 4.16). The ANOVA results reveal a significant effect of fermentation time on mouth feel scores ($p < 0.05$). The peak score on Day 12 suggests optimal textural qualities during this stage.

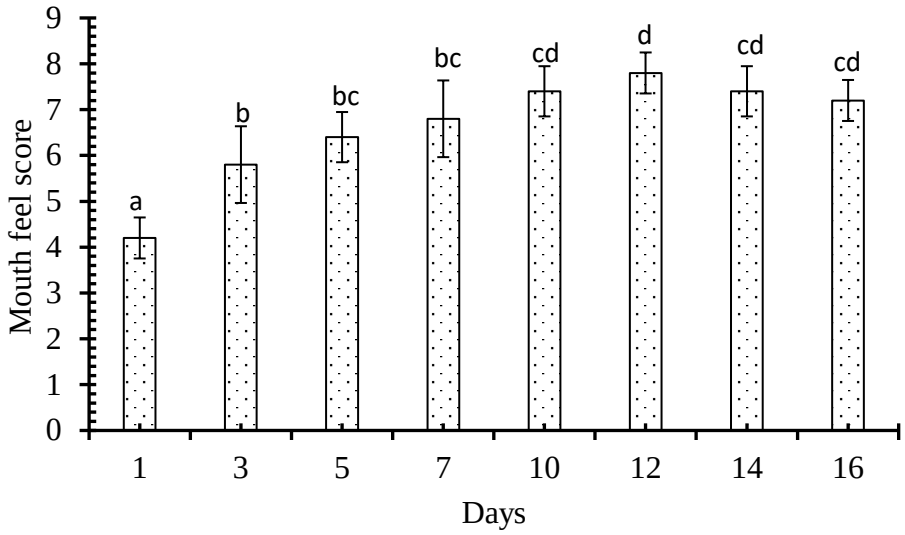

Fig. 4.16 Mean mouth feel score of the sample at different times during pickle fermentation at room temperature

4.10 Effect of fermentation periods and temperature conditions on overall acceptance

4.10.1 Effect of fermentation periods at 37°C on overall acceptance

This study analyzed the effect of fermentation time on the overall acceptance of pickles at 37°C. A 9-point hedonic scale was used, where higher scores indicate greater overall preference. The results demonstrated significant improvements in overall acceptance scores as fermentation progressed.

The ANOVA results indicate a significant effect of fermentation time on overall acceptance scores ($p < 0.05$).Scores improved progressively with fermentation time, with notable increases observed from Day 7 onward, stabilizing at Day 12. The stabilization observed after Day 12 suggests that the optimal sensory balance is achieved by this stage.

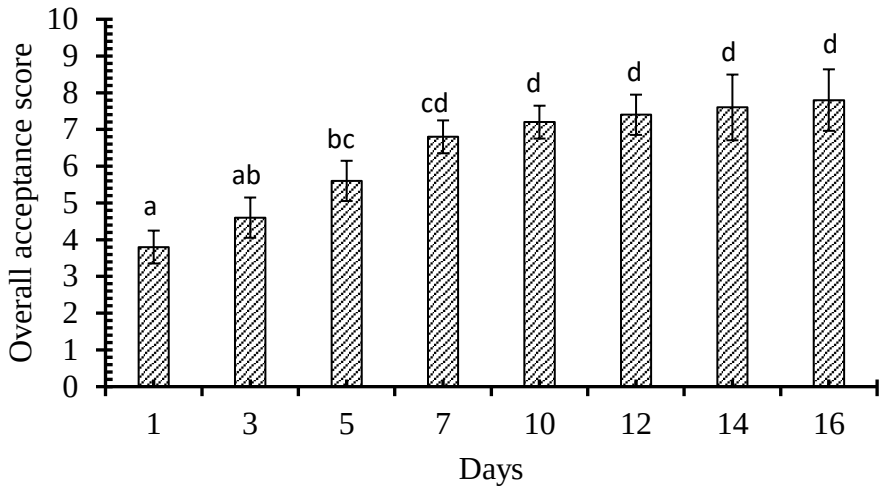

Fig. 4.17 Mean overall acceptance score of the sample at different times during pickle fermentation at 37°C

4.10.2 Effect of fermentation periods at room temperature on overall acceptance

This study evaluated the effect of fermentation time at room temperature on the overall acceptance score of pickles. A 9-point hedonic scale was used, where higher scores indicate greater preference. The results showed significant improvements in overall acceptance scores as fermentation progressed.

The ANOVA results revealed a significant effect of fermentation time on overall acceptance scores ($p < 0.05$). The scores increased significantly between Days 1 and 7, followed by further improvements until Day 16. The largest improvements occurred between Days 1 and 7, followed by smaller yet significant increases between Days 7 and 16.

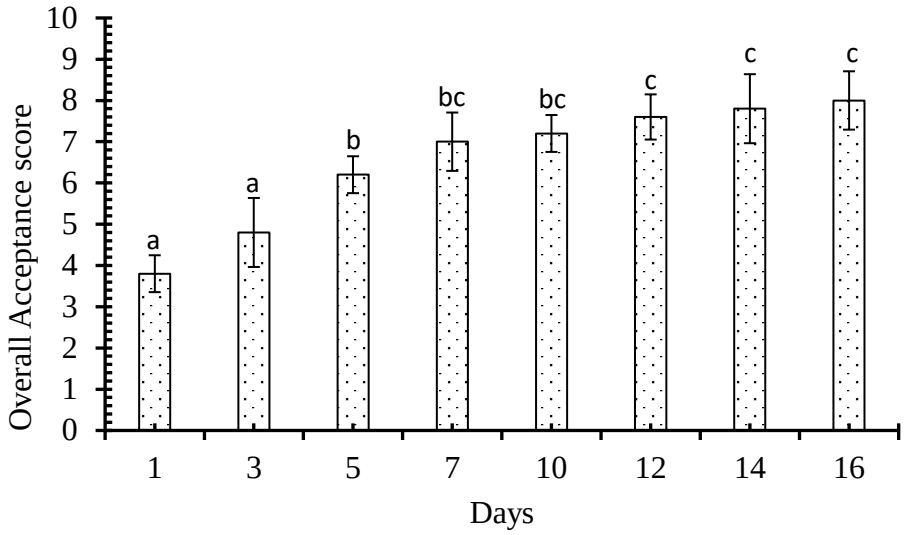

Fig. 4.18 Mean overall acceptance score of the sample at different times during pickle fermentation at room temperature

PART V

Conclusions and recommendations

5.1 Conclusions

Based on the result and discussion following conclusions can be drawn:

1. The acidity of the pickle (% lactic acid) increased from 0.2333% to 1.646% at room temperature and 1.573% at 37°C. While no significant differences were observed on Days 1 and 10, statistically significant variations occurred on Days 5, 7, and 16.

2. TSS increased from 8.767 to 12.03 at room temperature and 12.233 at 37°C, with significantly higher values in pickles fermented at 37°C.

3. The pH dropped from 5.1667 to 3.7567 at room temperature and 5.25 to 3.8167 at 37°C. While early and mid-stage pH changes were similar, a significant difference emerged by Day 16, with higher pH in pickles fermented at 37°C.

4. LAB counts peaked at Day 10, rising from 4.491 to 9.176 Log CFU/mL at room temperature and 10.46 Log CFU/mL at 37°C, then declined to 5.53 Log CFU/mL at room temperature and 5.06 Log CFU/mL at 37°C by Day 16 of fermentation.

5.2 Recommendations

The following suggestions for future research might be made based on the current study's findings.

1. The identification of specific species of lactic acid bacteria involved in the fermentation process could be studied to provide valuable insights for process optimization and quality control.

2. Further research could focus on conducting comprehensive shelf-life studies to determine the optimal storage conditions and shelf life of the product under various packaging and storage methods.

3. Investigating the biochemical changes occurring during fermentation, including the formation of flavor compounds and the degradation of nutrients, can be explored in future research.

PART VI

Summary

Pickling is a traditional culinary practice that dates back to approximately 2400 BC and includes preserving food in vinegar or brine. Throughout history, pickling has been a fundamental part of food preservation in a wide range of societies and civilizations. Khalpi is a fermented cucumber pickle that is originated from Nepal. In this study, Cucumber and other ingredients used in khalpi pickle was purchased from local Itahari market of Sunsari district, Nepal. The study was done to examine the effect of Fermentation Time and Temperature on khalpi pickle.

The khalpi pickle sample were placed at two storage condition one at room temperature and other at 37°C in an incubator. The changes that occur during fermentation time period were observed. The observation results showed significant increases in acidity to 1.5733 ± 0.0116% at 37°C and to 1.6467 ± 0.0058% at room temperature, TSS to 12.23 ± 0.058 °Bx at 37°C and to 12.0333 ± 0.05774 °Bx at room temperature, pH to 3.8167 ± 0.0153 at 37°C and to 3.74 ± 0.02 at room temperature and LAB counts were changing throughout fermentation at both 37°C and room temperature. Acidity increased rapidly in the initial stages, followed by a more gradual rise. TSS also increased, indicating the release of soluble compounds. LAB counts exhibited a characteristic growth pattern with an initial increase followed by a decline. pH decreased steadily throughout the fermentation process in both temperature conditions. Sensory evaluation revealed significant improvements in appearance, taste, smell, mouthfeel, and overall acceptance as fermentation progressed. While minor differences were observed between the two temperature conditions, pickles fermented at room temperature generally exhibited higher acidity levels at later stages and overall sensory score was slightly higher compared to pickle fermented at 37°C.

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Appendices

Appendix A

Specimen card for sensory evaluation

Hedonic rating test

Name of the panelist: ……

Date: …….

Product: Khalpi pickle

Dear panelist, you are given 5 coded samples of beetroot ready to serve (RTS) on each proportion with variation TSS, please give points for your degree of preference on the following parameter using the table given;

Sample code	Appearance	Taste	Smell	Mouthfeel	Overall acceptance
A					
B					
C					
D					
E					
F					
G					
H					
I					
J					
K					
L					
M					
N					
O					
P					

Judge the characteristics on the 1-9 scale as below:

Like extremely – 9	Like slightly – 6	Dislike moderately – 3
Like very much – 8	Neither like nor dislike – 5	Dislike very much – 2
Like moderately – 7	Dislike slightly – 4	Dislike extremely – 1

Any comments:

……

…….

Signature:

Appendix B

A. Analysis of Variance different time period during fermentation at 37°C

Table A.1 One-way ANOVA for acidity at different time period during fermentation at 37°C

	Sum of Squares	df	Mean Square	F	Sig.

Between Groups	4.415	7	.631	3983.684	.000
Within Groups	.003	16	.000		
Total	4.418	23			

Table A.2 One-way ANOVA for Lactic Acid Bacteria(LAB) Count at different time period during fermentation at 37°C

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	212582462004 4594400000.00 0	7	30368923143494 2050000.000	7611.996	.000
Within Groups	638338173800 666500.000	16	39896135862541 656.000		
Total	212646295821 8395000000.00 0	23			

Table A.3 One-way ANOVA for Ph at different time period during fermentation at 37°C

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.377	7	.625	220.707	.000
Within Groups	.045	16	.003		

Total	4.423	23			

Table A.4 One-way ANOVA for TSS at different time period during fermentation at 37°C

	Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**
Between Groups	27.118	7	3.874	929.771	.000
Within Groups	.067	16	.004		
Total	27.185	23			

Table A.5 One-way ANOVA for Mouth feel score at different time period during fermentation at 37°C

	Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**
Between Groups	47.975	7	6.854	13.054	.000

Within Groups	16.800	32	.525
Total	64.775	39	

Table A.6 One-way ANOVA for Overall acceptance score at different time period during fermentation at 37°C

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	79.100	7	11.300	30.133	.000
Within Groups	12.000	32	.375		
Total	91.100	39			

Table A.7 One-way ANOVA for Smell score at different time period during fermentation at 37°C

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	17.600	7	2.514	3.944	.003
Within Groups	20.400	32	.638		
Total	38.000	39			

Table A.8 One-way ANOVA for taste score at different time period during fermentation at 37°C

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	49.600	7	7.086	18.286	.000

Within Groups	12.400	32	.387		
Total	62.000	39			

Table A.9 One-way ANOVA for appearance score at different time period during fermentation at 37°C

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	13.200	7	1.886	7.184	.000
Within Groups	8.400	32	.262		
Total	21.600	39			

B. Analysis of Variance different time period during fermentation at room temperature

Table B.1 One-way ANOVA for lactic acid % at different time period during fermentation at room temperature

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5.059	7	.723	5981.631	.000
Within Groups	.002	16	.000		
Total	5.061	23			

Table B.2 One-way ANOVA for Lactic acid bacteria (LAB) at different time period during fermentation at room temperature

	Sum of Squares	df	Mean Square	F	Sig.

Between Groups	828685338347428 9700.000	7	11838361976391 84130.000	282.777	.000
Within Groups	669835468686666 56.000	16	41864716792916 66.000		
Total	835383693034295 6000.000	23			

Table B.3 One-way ANOVA for pH at different time period during fermentation at room temperature

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.934	7	.705	132.878	.000
Within Groups	.085	16	.005		
Total	5.019	23			

Table B.4 One-way ANOVA for TSS at different time period during fermentation at room temperature

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	23.073	7	3.296	988.839	.000
Within Groups	.053	16	.003		
Total	23.126	23			

Table B.5 One-way ANOVA for appearance score at different time period during fermentation at room temperature

	Sum of Squares	df	Mean Square	F	Sig.

Between Groups	14.575	7	2.082	7.932	.000
Within Groups	8.400	32	.263		
Total	22.975	39			

Table B.6 One-way ANOVA for mouthfeel score at different time period during fermentation at room temperature

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	47.775	7	6.825	18.828	.000
Within Groups	11.600	32	.363		
Total	59.375	39			

Table B.7 One-way ANOVA for overall acceptance score at different time period during fermentation at room temperature

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	80.700	7	11.529	27.948	.000
Within Groups	13.200	32	.413		
Total	93.900	39			

Table B.8 One-way ANOVA for smell score at different time period during fermentation at room temperature

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	20.175	7	2.882	5.240	.000
Within Groups	17.600	32	.550		
Total	37.775	39			

Table B.9 One-way ANOVA for taste score at different time period during fermentation at room temperature

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	65.375	7	9.339	16.242	.000
Within Groups	18.400	32	.575		
Total	83.775	39			

fermentation at room temperature

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	65.375	7	9.339	16.242	.000
Within Groups	18.400	32	.575		
Total	83.775	39			

Appendix C

Table C: Cost evaluation of the khalpi pickle

Ingredients	Cost per 1000g (NPR)	Quantity used (g)	Cost (NRP)
Cucumber	13	690	8.97
Salt	25	40	1
Oil	330	205	82.5
Mustard	425	28	11.9
Red Chilli Powder	600	9.5	5.7
Turmeric Powder	450	3.8	1.71
Cumin Seed	800	14.2	11.36
Coriander	450	9.5	4.37
Total		1000	127.51

Note: Actual price of cucumber was Rs 10 per kg. considering 30% loss from moisture and seed i.e by product loss its price reached to Rs 13 per kg.

Appendix D

Table D: Different days Quality parameter analysis results.

Days	Temp	Acidity %	TSS	pH	LAB Count CFU/ml
1	Room	0.233	8.7667	5.1667	4.491
1	37°C	0.236	8.7667	5.25	4.5
3	Room	0.61	11.0333	4.75	6.292
3	37°C	0.813	10.7667	4.45	6.91
5	Room	1.116	11.1667	4.2667	8.4913
5	37°C	0.956	11.3667	4.3167	9.62
7	Room	1.166	11.4333	4.2333	9.1249
7	37°C	1.266	11.5667	4.1833	9.25
10	Room	1.36	11.5667	4.14	9.176
10	37°C	1.333	11.7667	4.08	10.46
12	Room	1.413	11.7667	3.94	8.274
12	37°C	1.446	12.0333	3.9667	7.12
14	Room	1.59	11.9333	3.8	6.9653
14	37°C	1.506	12.1	3.9133	6.56
16	Room	1.646	12.2333	3.7567	5.535
16	37°C	1.573	12.0333	3.8167	5.06

Appendix E

Table E: Different days Sensory score result.

Days	Temp	Appearance Score	Taste Score	Mouthfeel score	Smell Score	Overall Acceptance Score
1	Room	6	4.8	4.2	6.2	3.8
1	37**°C**	6.2	4.8	4.2	6.2	4
3	Room	6.2	5.2	5.4	6.6	5.2
3	37**°C**	6.8	5.8	5.2	6.4	5
5	Room	6.8	6.6	6.4	7	6.6
5	37**°C**	7.2	6.2	6.2	6.8	5.6
7	Room	7.2	7.6	7.6	7.6	7.6
7	37**°C**	7.2	6.8	7.2	7.4	6.6
10	Room	8.2	8.4	7.8	8.2	8
10	37**°C**	7.8	7.8	7.6	8	7.6
12	Room	8.2	8.6	8.2	8.2	8.2
12	37**°C**	8.2	7.8	8	7.8	7.8
14	Room	8.2	8.6	8.2	8.4	8.4
14	37**°C**	8.2	7.8	8.2	8	7.8
16	Room	8.4	8.8	8.2	8.6	8.2
16	37**°C**	8.6	8.2	8.2	8.2	8.2

Appendix F

Table F.1 Apparatus used

Equipments	**Manufacturer**
Autoclave	Shiva, India
Bacteriological incubator	Vitco, India
pH meter	Labtronics Panchkula, India
Petri-dish	
Phase contrast microscope	Olympus BX41
Colony counter	Stuwart scientific
Pipette, burette, conical flask, beakers, test tubes	
Mercury thermometer	
Electronic balance	MRRS Digi Model MTT-T
Mortar and pestle	
Water bath	

Table F.2 Chemicals used

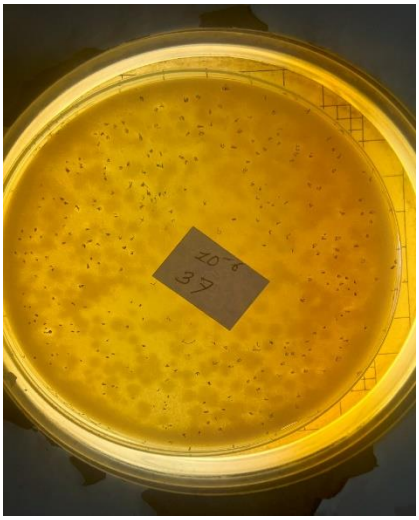
Chemicals	Manufacturer
Sodium hydroxide	Qualigens fine chemicals
Oxalic acid	Thermofisher scientific India Pvt. Ltd
Calcium carbonate	Qualigens
Lactobacillus MRS -M641 HiMedia	Tmmedia

Color plates

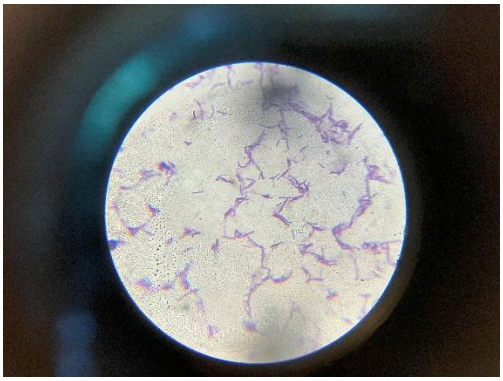
P1 Cucumber

P2 Cucumber after cutting

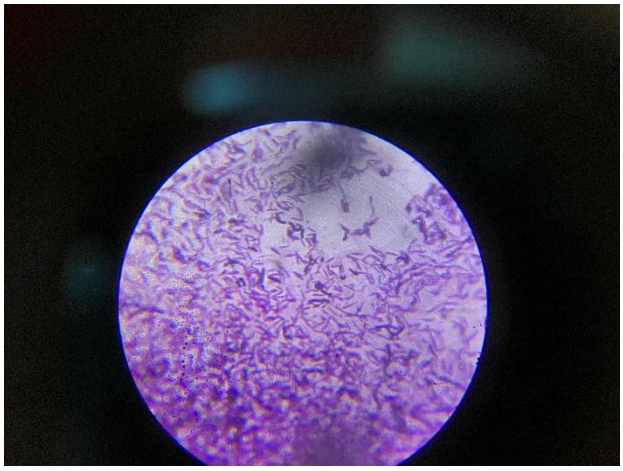
P3 Khalpi Placed in incubator



P4 Mrs Agar Plate of LAB



P5 Microscopic view of LAB from MRS Agar Plate



P6 Microscopic view of LAB after propagating in MRS Agar Plate