Addis Ababa University

School of Graduate Studies

Institute of Biotechnology

Isolation of Yeast and Lactic Acid Bacteria from Selected Ethiopian Fermented Foods and Evaluation of their Leavening Capacity

MSc Thesis:

Submitted to the school of Graduate Studies of Addis Ababa University in Partial Fulfillment of the Requirement for the Degree of Master of Science in Biotechnology (Industrial Biotechnology)

BY:

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Advisor: Diriba Muleta (PhD, Assoc. Prof.)

October, 2024
Addis Ababa, Ethiopia

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Examiner's Approval Sheet

We certify that Martha Yishak's M.Sc. thesis entitled **" Isolation of Yeast and Lactic Acid Bacteria from Selected Ethiopian Fermented Foods and Evaluation of their Leavening Capacity"** has been conducted under our direct supervision.

Therefore; we, as examining board, approved it as the final document to be accepted as fulfilling the requirement for the degree of Master of Science in Biotechnology, Industrial Biotechnology.

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LIST OF ABBREVIATIONS

BSA	Bismuth Sulfite Agar
YEPD	Yeast Extract Peptone Dextrose
KIA	Kliger Iron Agar
MRS	De Man, Rogosa and Sharpe medium
ANOVA	Analysis of Variance
CFU	Colony Forming Unit
OD	Optical Density
GRAS	Generally Regarded as Safe
LAB	Lactic Acid Bacteria
H_2S	Hydrogen Sulfide
SEM	Scanning Electron Microscopy
Rpm	Revolution per Minute
α	Alpha

Evaluation of the Leavening Capacity of Yeast and Lactic Acid Bacteria Isolated from Ethiopian Traditional Fermented Products

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Abstract

There is a high demand for baker's yeast for various Ethiopian foods and bioprocess industries. Currently, the baker's yeast for the country's demand is fully imported from abroad, and a huge amount of foreign currency has to be spent for this purpose, which necessitates the need for alternative import substitution products. Therefore, the main objective of the current study was to screen indigenous yeasts and lactic acid bacteria isolates from *Borde*, Tella, and *Teff* dough having superior bread leavening abilities and evaluating their impact on bread's sensory quality and shelf life investigation. A total of 230 yeasts and 42 lactic acid bacteria were isolated and purified. Out of which, 47 yeast and 20 lactic acid bacteria isolates were selected for further analysis based on their baking ability; carbon dioxide production, growth rate, hydrogen sulfide (H_2S) production, temperature tolerance, flocculation, and ethanol tolerance, within 24 hours of incubation. Four yeasts and five lactic acid bacteria isolates were chosen to develop starter cultures for bread making. The combined effect of two selected yeast isolates was also tested. The findings demonstrated that the five most potent yeast isolates (Y3, Y1, Y2,Y4 and Y5) tolerated different values of temperature that ranged up to 45°C and alcohol concentrations up to 16%. The isolate from *Tella* (Y3), had a maximum leavening capacity of (251.67 ±7.6 ml) at 4 hours of fermentation, but the commercial strain had a maximum leavening capacity of (245.67 ± 5.9 ml) at 3 and 4 hours. There was no significant difference ($p>0.05$) between *Tella* and the commercial strain. Effect of the three co-inoculated isolates (Y3 + Y1 + Y2) was found the highest at 275 ml at 120 min, compared co-inoculation of two combinations (Y3+Y1) 261 ml at 120 min. The five screened lactic acid bacteria isolates (L1, L2, L5, L4 and L3) revealed very poor leavening capacity; none of the isolates were selected for further testing. A panel of judges assessed the bread's organoleptic quality based on its appearance, color, texture, flavor, and overall acceptability. A nine-point "Hedonic scale" was used to gauge consumer acceptability of the items, and the commercial strain scored the highest (6.18 ± 0.17), isolates from *Tella* (Y3) scored (5.70 ± 0.57) although there was no statistically significant difference ($p>0.05$) between the two isolates. The bread made with *Tella* isolates (BY3) had the highest shelf life of 5 days, compared the bread made with commercial yeast (BCY) 4 days. The results of the present study indicated that mixed cultures showed superior leavening potential than that of single cultures and commercial yeast, and the wild yeasts isolated from Ethiopian traditional fermented products can be used for the development of bakery yeast.

Keywords: Fermentation, Leavening, mixed cultures, sensory quality, shelf life, starter culture.

1. INTRODUCTION

Background of the study

Traditional fermented food and beverage products are an integral part of diets in developing countries. Various micronutrients are undersupplied, and millions of people in many poor countries struggle to get enough food to eat each day (Ramos *et al.*, 2023). The large levels of CO_2, proteins, vitamins, minerals, and flavorings that are produced by baker's yeasts contribute to the bread's overall flavor, color, and texture. Fermentation that occurs naturally or spontaneously is thought to increase the nutritional value and safety of food. Additionally, it contributes to the products' improved palatability, acceptability, and sensory quality (Marshall and Mejia, 2011). Moreover, fermentation helps to extend the shelf-life of foods by preventing the outgrowth of spoilage microorganisms and foodborne pathogens (Motarjemi, 2002). The significance of yeasts in food technology in a world of low agricultural production and rapidly increasing population makes the production of food-grade yeasts extremely important (Bekatorou *et al.*, 2006). Since a large part of the earth's population is malnourished, due to poverty and food insecurity, scientists are concerned whether the food supply can keep up with the world population increase (Mbow *et al.*, 2020).

Bread is one of the most consumed foods worldwide, has a high energy value and is low in cost. It is one of the main foods consumed daily in all parts of the world and used as food for different social classes (Balarabe *et al*., 2017; Hager *et al*., 2012). In addition to its good flavor, bread has crucial nutritional value, being also a source of protein, fiber, and minerals (Malomo *et al*., 2012; Tulha *et al*., 2012). It may be described as a fermented confectionery product produced mainly from wheat flour, water, yeast, and salt by a series of processes involving mixing, kneading, proofing, shaping, and baking (Dewettinck *et al*., 2008). The dough is inoculated with baker's yeast and incubated at the required temperature and time. The CO2 produced during the incubation period of the baking process results in the raised dough with a specific taste and smell. The resulting bread structure depends on dough ingredients, yeast activity, fermentation temperature, and gas bubble formation (Ali *et al*., 2012). No other ingredient can successfully replace the function of yeast in bread making (which considered as a staple food for a large section of the world's population), and thus it is vital to the baking industry. As industrialization

increased, the manufacture of fermented products and the demand for yeast grew exponentially. Nowadays, modern industries require vast amounts of selected yeasts to obtain high-quality, reproducible products and to ensure fast and complete fermentation (Gómez-Pastor *et al*., 2011).

Baker's yeast is a high volume, low-value product, with more than 100,000 tons produced per year on a global scale (Ugalde and Castrillo, 2002). Additionally, with the increasing shift towards a bio-based economy, there is a rising demand for developing efficient cell factories that can produce fuels, chemicals, pharmaceuticals, and food ingredients using yeasts. Among the yeasts, *Saccharomyces cerevisiae* is exceptionally well suited for the above purposes. Yeasts can be propagated using cheap raw materials and easily harvested due to their larger cell sizes and flocculation abilities. The raw materials used as substrates for industrial yeast biomass production are usually agricultural, forestry, and food wastes. These are materials like starch, molasses, distiller's wash, whey, fruit and vegetable wastes, wood, and straw (Chilakamarry *et al.*, 2022; Drzymała *et al.*, 2020). So, there are numerous possibilities to isolate and screen wild *S. cerevisiae* strains which have the superior leavening capacity. However, the presence of yeasts from a local source is yet to be exploited, especially in bakery products as a leavening agent (Elsa Beyene *et al.*, 2020).

Baked foods are widely consumed in Ethiopia and play an essential role in the local economy (Mogessie Ashenafi, 2006). In Ethiopia, a wide range of traditional fermented foods and beverages are produced from different raw materials such as cereals, Enset (false banana), honey, and milk (Mogessie Ashenafi *et al.*, 2006; Kebede Abegaz *et al.*, 2002). Some of the most popular Ethiopian traditional fermented foods and beverages are *Injera, Dabo, Ambasha, Kocho, Bulla, Ergo, Siljo, Tella, Tej, Arkie, Borde, Shamita,* and *Kineto* (Mooha *et al.*, 2015; Mogessie Ashenafi, 2006). Most of the customs and rituals involving the Ethiopian traditional fermented foods and beverages are still prevalent in urban areas, village communities, and rural households. Bread is also a staple food in Ethiopia, consumed by a large portion of the population (Mihratu Amanue *et al.*, 2018). Traditionally, bread fermentation relies on the use of wild yeast and lactic acid bacteria present in the environment (Pérez-Alvarado *et al.*, 2022; Zerihun Tsegaye *et al.*, 2018a). These microorganisms play a crucial role in the leavening process, contributing to the flavor, texture, and overall quality of the bread (Mollakhalili-Meybodi *et al.*, 2023). Numerous food and beverage sectors, the manufacture of single-cell proteins, bread making, and more

recently, the production of bioethanol, have all benefited from the helpful physiological characteristics of yeasts (Wani *et al.*, 2023; Bilal *et al.*, 2022). For flour dough to rise, mature, and develop fermentation flavor, carbon dioxide production is essential (De Vuyst *et al.*, 2023; Amr and Alkhamaiseh, 2022). However, there is limited scientific knowledge on the specific strains of yeast and bacteria present in Ethiopian bread and their leavening capacity. Currently, baker's yeast is used in Ethiopian baking operations; this yeast is mainly imported (Elsa Beyene *et al.*, 2019a; Zerihun Tsegaye *et al.*, 2018a).

Therefore, this study was aimed to characterize and optimize the bread leavening capacity of wild yeast and lactic acid bacteria in Ethiopia. Moreover, the study was aimed to identify the predominant yeast and LAB present in traditional food, evaluate their leavening capacity, and optimize fermentation conditions to enhance their performance for bread baking.

1.1. Statement of the problem

Ethiopia is a developing country with a high demand for baker's yeast for various food and bioprocess industries. Thus, the use of commercial baker's yeast is increasing day by day, and several alcohol and beverage industries (beer and wine) are also locally prepared, and these industries need tons of yeast. However, there is no baker's yeast producing plant in the country. Ethiopia imports all of its baker's yeast needs, primarily from China and Europe, despite the fact that its annual baker's yeast consumption is expanding. According to the data of the Ethiopian Statistical Services (2022), Ethiopia imported baker's yeast in 2022 for a cost of around 896,696,819.00 ETB for active yeasts and 28,904,717.00 ETB for inactive yeasts; other single-cell micro-organisms, dead ones and which is equivalent to 16,528,598 US Dollars. So, a considerable amount of foreign currency has to be spent for this purpose, and this necessitates the need for alternative import substitution of baker's yeasts for the national development by screening native yeasts isolates from local substrates.

Previous studies (Elsa Beyene *et al.*, 2020; Zerihun Tsegaye *et al.*, 2018a) have confirmed that there is a high chance to isolate and characterize novel baker's yeast isolates from local substrates with high dough leavening ability. Therefore, the purpose of the present study was to screen out novel bakery yeast and LAB isolates from indigenous sources for bread production.

These isolates can give a unique flavor to bread and can form an identity for the bread industry of Ethiopia.

1.2. Objectives

1.2.1. General objective

- ❖ To isolate, characterize, and screen yeast and lactic acid bacteria from selected Ethiopian fermented foods and investigate their bread leavening, sensory, and shelf life analysis.

1.2.2. Specific objectives

- ❖ To identify, characterize, and screen the different isolates of wild yeast and LAB present in *Borde*, Tella, and *Teff* dough that can be potentially used for bread leavening.
- ❖ To compare the performance of wild yeast and LAB isolates with commercially available yeast in terms of bread leavening capacity and bread quality.
- ❖ To evaluate sensory evaluation of bread baked with local yeast isolates and commercial yeast.
- ❖ To examine the shelf life of bread baked with local isolates and commercial yeast.

2. LITERATURE REVIEW

2.1. Taxonomy of Yeast

Yeasts are single-celled eukaryotic microorganisms classified in the kingdom Fungi, with about 1,500 species currently described (Roychoudhury, 2020). This includes a wide range of organisms like molds, mushrooms, and lichens. Yeast cells are usually spherical, oval, or cylindrical in shape (Umeh *et al.*, 2022). Yeast cell size can vary greatly depending on the species, typically measuring from 2–3 µm to 20–50 µm in length and 1–10 µm in width. *Saccharomyces cerevisiae*, commonly referred to as brewer's or baker's yeast, is generally ellipsoid in shape with a large diameter of 5–10 µm and a small diameter of around 5 µm (Walker and White, 2017). There is great diversity in cell shapes and modes of cellular reproduction in the yeasts.

Fungi are distinct from plants and animals and are characterized by their cell walls made of chitin. Many types of yeast belong to the Phylum *Ascomycota*, which is one of the largest phyla of fungi. Ascomycota are characterized by the production of sexual spores in sac-like structures called asci. Within the phylum *Ascomycota*, yeasts are placed in the subphylum *Saccharomycotina* (Kurtzman and Sugiyama, 2015). This subphylum includes yeasts that ferment sugars and reproduce asexually by budding. *Saccharomycetes* are a class of fungi that includes yeasts with simple morphology and unique metabolic characteristics. Yeasts are further classified into different orders based on their genetic relationships and characteristics. Some common orders of yeasts include *Saccharomycetales* and *Brettanomyces*, among others. *Saccharomycetaceae* is a family of yeasts within the order *Saccharomycetales* (Dujon and Louis, 2017). Yeasts are classified into different genera and species based on their genetic and physiological characteristics. Some well-known genera of yeasts include *Saccharomyces*, *Candid*a, *Cryptococcus*, and *Pichia*. Yeasts are further classified into individual species based on specific characteristics. For example, *Saccharomyces cerevisiae*, *Candida albicans*, and *Cryptococcus neoformans* are examples of yeast species. Within a species, yeasts can further be divided into strains based on genetic differences, physiological characteristics, and geographic origin. Strains may exhibit variations in traits such as sugar metabolism, tolerance to environmental stress, and pathogenicity. Traditionally, yeasts were classified based on their

morphological characteristics, such as cell shape, size, and mode of reproduction. However, with the advent of molecular techniques, the taxonomy of yeasts has been revised to reflect their genetic relationships.

Scientific Classification

Domain: Eucaryota

Kingdom: Fungi

Phylum: *Ascomycota*

Subphylum: *Saccharomycotina*

Class: *Saccharomycetes*

Subclass: *Saccharomycetidae*

Order: *Saccharomycetales*

Family: *Saccharomycetaceae*

Genus: *Saccharomyces*

Species: *Saccharomyces cerevisiae*

It's important to note that there are many different species of yeasts, and new species are continually being discovered and classified. Yeasts have significant ecological, industrial, and medical importance, and understanding their taxonomy and diversity is essential for studying their ecology, genetics, and biotechnological applications.

2.2.Ecology of yeasts

Yeasts are ubiquitous in nature and can thrive in diverse environments. They are commonly found in soil, water bodies, plant surfaces, flowers, fruits, and the gastrointestinal tracts of animals. The distribution of yeast species is influenced by factors such as temperature, moisture, pH, nutrient availability, and competition with other microorganisms (Starmer and Lachance, 2011). Yeasts interact with a wide range of organisms in their environment, including bacteria, fungi, plants, insects, and animals. These interactions can be beneficial, such as in mutualistic relationships with plants or in symbiotic associations with insects, or detrimental, such as in competitive interactions with other microorganisms (Lachance and Walker, 2018). Yeasts exhibit high species diversity, with thousands of known species belonging to different genera. Yeast communities can vary significantly between different habitats and are shaped by factors such as environmental conditions, host specificity, and interspecies interactions. Yeasts play important

roles in nutrient cycling and decomposition processes in ecosystems (Mittelbach and Vannette, 2017). They are involved in the breakdown of organic matter, fermentation of sugars, and recycling of nutrients like carbon, nitrogen, and phosphorus. Yeasts contribute to the decomposition of plant materials, fruits, and other organic substrates, releasing nutrients that can be utilized by other organisms. They have evolved a variety of adaptations to survive and thrive in different environments. Some yeast species have developed tolerance to extreme conditions, such as high temperatures, low pH, or high salinity, allowing them to colonize specialized habitats. The biogeography of yeast species reflects their distribution patterns and the environmental factors that shape their geographic ranges.

2.3.Yeast reproduction

2.3.1. Vegetative reproduction

Budding is the form of vegetative reproduction that occurs in *S. cerevisiae* (Fischer *et al.*, 2021; Hittinger, 2013). The division of budding yeast is asymmetrical; the daughter cell is smaller than the mother. Buds may arise from any point on the cell wall; however, they do not arise from the same point more than once (Briggs, 2004). In the budding process, a new cell forms as a small outgrowth of the mother cell when mother cells attain a critical cell size. After enlargement of the bud, nuclear division, cell wall formation and finally, separation of the daughter cell from the parent cell take place (Briggs, 2004; Walker, 1998). Scanning electron micrograph of the budding yeast is shown in Figure 1. Haploid cells of the same sex also unite occasionally to form abnormal diploid cells (a/a or α/ α) that can reproduce only asexually, by budding in the usual way. The majority of industrial yeasts reproduce by budding (Kurtzman *et al.*, 2011a).

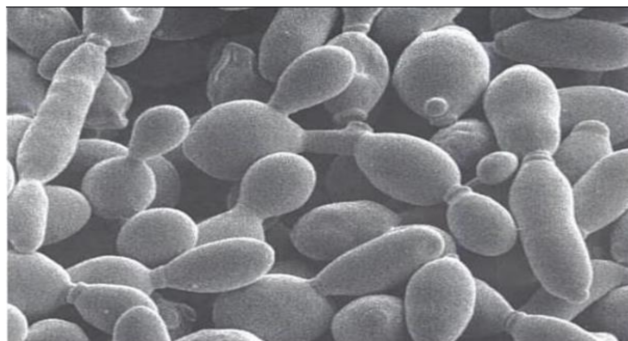

Figure 1. Scanning electron microscope observation of *S. cerevisiae* cells during budding (Chapman and Roberts, 1997; Pringle *et al.*, 1979).

2.3.2. Sexual reproduction

Some cells show sexual reproduction via mating process, in which two yeast cells come together and fuse (Heitman *et al.*, 2013; Knop, 2011). Figure 2 shows the sexual life cycle of *S. cerevisiae*. There are two different mating types of haploid *Saccharomyces cerevisiae* designated as a and α. Mating types can be considered analogous to male and female gametes. Haploids of mating type a produce “a factor”. Also, haploids of mating type α produce “α factor." Both of these factors are peptide hormones (pheromones) that attract cells of the opposite mating type. That is, cells of type a mate only with cells of type α, or vice versa; this stimulates the fusion of the two cells to produce an a/α diploid cell (de Sá, 2010; Greig and Leu, 2009). When the environment becomes stressful, *S. cerevisiae* switches to sexual reproduction, and each diploid cell produces four similar-sized haploid gametes (spores) of two mating types (MATa and MATalpha) within a capsule (ascus). Spores do not engage in further mitotic divisions (germinate) until the environmental conditions have improved. During germination, spores resume vegetative growth, and a haploid cell of one mating type, guided by mating pheromones, mates with a cell of the opposite mating type to regenerate a diploid cell (Butler, 2010; Chang, 2013; Dohlman, 2002).

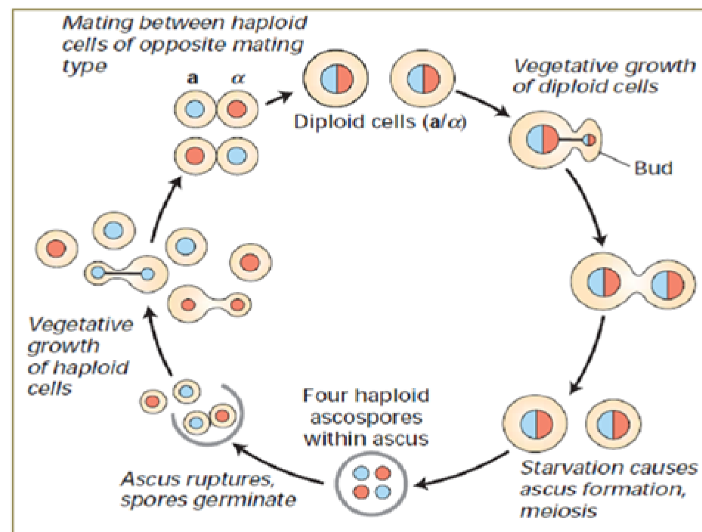

Figure 2. Sexual and vegetative reproduction of the yeast *S. cerevisiae* (Schaechter, 2011)

Saccharomyces cerevisiae is a unicellular fungus, possessing a nuclear genomic DNA of 12068 kilobases (kb) organized in 16 chromosomes (Goffeau *et al.*, 1996). Its genome has been completely sequenced and was found to contain approximately 6000 genes, of which, 5570 are predicted to be protein-encoding genes (Moore and Frazer, 2002; Wood *et al.*, 2001). It is one of the earliest domesticated organisms in human civilization history, and it has been exploited for thousands of years in traditional fermentation processes to produce beer, wine, and bread (Schaechter, 2011). It is a yeast that has been utilized by mankind for several purposes, commonly known by names like Brewer's yeast, Ale yeast, Top-fermenting yeast, Baker's yeast, Budding yeast, etc. (Caspeta and Navarrete, 2020; Kandhare *et al.*, 2023). It is one of the most intensively studied eukaryotic model organisms in molecular and cell biology, much like Escherichia coli as the model prokaryote. *Sacharomyces cerevisiae,* which was first introduced by Meyen in 1837 (Martini, 1993; Ohya and Kashima, 2019) is a small, single-celled member of the kingdom of fungi (Quain, 2006; Suh *et al.*, 2006) and belongs to the phylum *Ascomycetes* and genus *Saccharomyces*. The phylum Ascomycetes takes its name from the production of asci (singular, ascus) in which the mating of two haploid nuclei from different mating types occurs and results in a diploid nucleus that then undergoes meiosis to give haploid ascospores (Moore and Frazer, 2007; Watkinson *et al.*, 2015).

2.4.Characteristics of bakery yeasts:

The cells of *S. cerevisiae* are characteristically spherical, oval, or cylindrical in shape and range from 4-10 μm in diameter (Briggs, 2004; Greig and Leu, 2009). The sizes of haploid and diploid cells show variations depending on the phase of growth and type of the strain (Esslinger, 2009). Generally haploid cells are 4 μm diameter spheroids, and diploid cells are 5 x 6 μm ellipsoids. Also, for a haploid and diploid cell, mean cell volumes are 29 and 55 μm3, respectively (Walker, 1998). Furthermore, mean cell size of S. cerevisiae increases with cell age (Hu *et al.*, 2013; Longo and Fabrizio, 2012). Yeasts, distinguished from most fungi by their everyday existence as single ovoid cells about 8 μm long and 5 μm in diameter, doubling every 1-3 hours in favorable media (Wayman and Parekh, 1990). Physiological characteristics are also used to a great extent in determining yeast species (Walker and White, 2017). Yeasts may reproduce asexually or sexually. Yeasts are classified based on the microscopic appearance of the cells, the mode of sexual

reproduction, certain physiological features (especially metabolic capabilities and nutritional requirements), and biochemical features (Kurtzman *et al.*, 2011a; Walker and White, 2017).

The physiological features that distinguish different yeasts include the range of carbohydrates (mono-, di-, tri-, and polysaccharides) that a given organism can use as a source of carbon and energy under semi-anaerobic and aerobic conditions, the relative ability to grow in the presence of 50-60% (*w/v*) D-glucose or 10% (*w/v*) sodium chloride plus 5% (*w/v*) glucose (a measure of osmo-tolerance) and the relative ability to hydrolyze and utilize lipids. These properties help investigators determine which yeast strains merit investigation for a particular application (Griffin, 1996).

Saccharomyces cerevisiae has structural compartments such as nucleus, mitochondria, Golgi apparatus, secretory vesicles, endoplasmic reticulum, vacuoles, and microbodies in the cell structure, like in other higher eukaryotes (Frankl *et al.*, 2015; Walker and White, 2017). Besides these features, in common with other fungi, they have a cell wall that is thick and tough. The presence of a cell wall differentiates a yeast cell from an animal cell. Due to not having a chloroplast compartment in the cell, they are not plants, either. So, yeast cells are considered to be close to both animal and plant cells (Russell, 2003; Walker, 1998). Cell wall of *S. cerevisiae* contains structural components such as proteins, lipids, pigments, carboxylate, phosphate, sulfhydryl, amine groups, as distinct, potential metal-complexing sites. Mannan-β-glucan stands out as the main structural polymer of the cell wall (Klis *et al.*, 2006; Orlean, 2012). Vacuole is the other cellular component, which is the largest compartment of the cell. It is involved in numerous functional processes, including the homeostasis of cell pH and the concentration of ions, storage of polyphosphate and amino acids, osmoregulation, and degradation processes (Li and Kane, 2009; Veses *et al.*, 2008).

Saccharomyces cerevisiae is a facultative anaerobic organism which is able to grow on various fermentable and non-fermentable carbon sources. It is a desirable organism because it is relatively cheap to grow in large quantities on simple media (Amata, 2013; Cartwright, 2013; Raines-Casselman, 2005). If yeast is grown on fermentable carbon sources such as glucose, it produces its metabolic energy primarily from glycolysis. Due to its ability to ferment sugars to ethanol and carbon dioxide, it has long been utilized for the production of food and alcoholic beverages. It is

remarkably important for the baking industry for raising dough. Carbon dioxide is trapped within tiny bubbles and causes the dough to rise. Also, these organisms can be taken as a vitamin supplement due to their high content of B vitamins, niacin, and folic acid (Amata, 2013; Heitmann *et al.*, 2018).

Saccharomyces cerevisiae, a model organism and the first eukaryote to have its genome completely sequenced, is of great importance for eukaryotic cellular and molecular biology for more than 50 years (Duina *et al.*, 2014; Müller and Grossniklaus, 2010). The complete sequence of its genome functions as a reference towards the sequences of other higher eukaryotic genes, including those of humans. A reason for its popularity in basic and applied research is its classification as GRAS (generally regarded as safe) by the U.S. Food and Drug Administration (FDA) (Nevoigt, 2008; Porro *et al.*, 2005).

2.5. Factors influencing yeast growth

Several factors influence both yeast growth (and fermentation) and therefore should be considered when propagating and maintaining the yeast. The most important are carbon source, aeration, pH, temperature, and ethanol.

2.5.1. Carbon source

The primary carbon and energy source for most yeast is glucose, which is converted via the glycolytic pathway to pyruvate and by the Krebs cycle to analytes and energy in the form of ATP (Rodrigues *et al.*, 2006). Yeasts are further classified according to their modes of energy production from pyruvate: respiration and fermentation. These processes are regulated by environmental factors, mainly glucose and oxygen concentrations. In respiration, pyruvate is decarboxylated in the mitochondrion to acetyl-CoA, which is completely oxidized in the citric acid cycle to CO_2, energy, and intermediates to promote yeast growth (Flores *et al.*, 2000).

In anaerobic conditions, glucose is slowly utilized to produce the energy required just to keep the yeast cells alive. This process is called fermentation, in which the sugars are not entirely oxidized, yielding CO_2 and ethanol (Bekatorou *et al*., 2006) as final products. When the yeast cell is exposed to high glucose concentration, catabolite repression occurs, during which gene expression and synthesis of respiratory enzymes are repressed, and fermentation prevails over respiration (Dueñas-Sánchez *et al.*, 2012).

2.5.2. Nitrogen source

Nitrogen is an essential nutrient for the growth and metabolism of yeast. Yeast requires nitrogen in the form of amino acids, ammonia, and peptides to synthesize proteins, enzymes, and other vital molecules necessary for its growth and reproduction (Su *et al*., 2021). The availability and type of nitrogen source in the environment can significantly impact the growth of yeast. Some nitrogen sources, such as proteins and complex nitrogen compounds, may require additional enzymatic breakdown by yeast before they can be utilized. These complex sources may result in slower growth rates compared to assimilable nitrogen sources. Inadequate nitrogen availability can lead to nitrogen limitation, which can negatively impact yeast growth and fermentation capacity (Zhang *et al*., 2018). Nitrogen limitation can result in reduced cell growth, sluggish fermentation, lower alcohol production, and off-flavors in the final product. In industrial fermentation processes, the choice of nitrogen source and its concentration in the fermentation medium are critical factors that can influence the growth and performance of yeast. Optimal nitrogen supplementation is essential to ensure robust yeast growth, productivity, and the production of high-quality fermented products such as beer, wine, and bread.

2.5.3. Oxygen transfer/Aeration

Oxygen is essential for the growth and yield of metabolites in aerobic organisms (Kuriyama and Kobayashi, 1993). For the oxygen to be absorbed by microorganisms, it must be dissolved in aqueous solution along with the nutrients. The air used in fermentation, whether sterile or not, is forced under pressure into the bottom of the fermenter just below the lowest impeller. The air enters through a sparger, which is a pipe with fine holes (Tervasmäki *et al.*, 2018). The smaller the holes, the more adequate the bubbles and the more influential the supply of oxygen to the microorganisms (Carvalho *et al.*, 2006). Agitators with their attached impellers serve several ends. They help to distribute the incoming air as fine bubbles, mix organisms uniformly, create local turbulence, as well as ensure a uniform temperature. Sterile air is needed in some aerobic fermentation, and it is produced in several ways including irradiation, electrostatic precipitation of particles, the use of heat resulting from the compression of the gas. But the most commonly used method is the passage of the air through filters either made of materials such as cellulose nitrate, or more commonly of cotton and sometimes other materials (Berovic, 2005; Carvalho *et al.*, 2006).

Glucose and oxygen are significant factors in aerobic fermentation. When oxygen is limited, glucose is metabolized to ethanol, and less cell mass is produced (Alfenore *et al.*, 2004). When the objective is the production of cell mass such as baker's yeast or single-cell protein (SCP), oxygen is the most important determinant of cell mass yield. Oxygen must be supplied to the liquid by aeration and agitation so that cell growth remains aerobic. Highly aerobic culture conditions are used in the production of yeast, specifically baker's yeast, to maximize cell growth (Campelo and Belo, 2004).

2.5.4. Temperature

Many fermentation processes release heat, which must be removed to maintain the optimum temperature for the productivity of the organism. In small laboratory fermenters, temperature control may be achieved by immersing the tank in a water bath; in medium-sized ones, control may be achieved by a jacket of cold water circulating outside the tank or merely by bathing the unjacketed cylinder with water. In large fermenters, the temperature is maintained by circulating refrigerated water in pipes within the fermenter and sometimes outside it as well. A heating coil is also provided to raise the temperature when necessary (Chisti and Moo-Young, 2006; Okafor, 2007). The temperature of anaerobic fermentation affects both the solubility of oxygen and the mass transfer coefficient. The solubility of oxygen drops when the temperature increases; thus, the driving force for oxygen transfer is reduced. At the same time, the mass transfer coefficient (k_L) increases because the diffusivity of oxygen in the liquid film is increased. The net effect of temperature on oxygen transfer depends on the range of temperature considered. The oxygen transfer rate will increase when the temperature increases from 10°C to 40°C (Yang and Wang, 1992). Above 40°C, the solubility of oxygen drops significantly, adversely affecting the driving force and rate of oxygen transfer (Breznak and Costilow, 2007; Vogelaar *et al.*, 2000).

2.5.5. pH

The importance of the control of pH in microbial growth is well known. In some industrial fermentations, good yield depends on accurate control (and hence accurate measurement) of the pH of the fermentation broth. Sometimes the control of pH is achieved by natural buffers present in the medium; phosphates and calcium carbonate may also be used for this purpose. The buffering effect of these compounds is, however, usually temporary. The broth must, therefore, be

sampled and the pH adjusted as desired with either acid or base (Boulton and Quain, 2008; Reddy and Reddy, 2011). This method is laborious and may not accurately reflect the continuous change taking place in the pH of the broth. Sterilizable pH probes have become available, and these are inserted in the fermenter or in a suitable projection from there in which the broth bathes the electrode. With these electrodes, it is now possible to use an arrangement that will monitor pH changes and automatically induce the introduction into the medium of either acid or alkali (De Farias Silva *et al.*, 2017). In most fermentation, acidity rather than alkalinity is the situation to be combated. Such acidity usually arises from microbial activity. It is, therefore, usual to arrange for the introduction of anhydrous ammonia as acidity increases (Sarao and Arora, 2017). Hydrogen ion concentration has a significant influence on industrial fermentation due as much to its importance in controlling bacterial contamination as its effect on yeast growth, fermentation rates, and by-product formation (Beckner *et al.*, 2011; Walker and Stewart, 2016). Under fermentation conditions, the intracellular pH of *S. cerevisiae* is usually maintained between 5.5 and 5.75 when the external pH is 3.0 or between 5.9 and 6.75 when the external pH is varied between 6.0 and 10.0. The gap between the extracellular pH and the intracellular pH widens, tremendous stress is placed on the cells, and more energy is expended to maintain the intracellular pH within the range that permits growth and survival of the yeast (Quayum, 2011; Rahman, 2013). In the industrial production of baker's yeast, the optimum pH ranges are 4.5-5.5.

2.5.6. Carbon dioxide concentration

Water and carbon dioxide are two of the most common end-products of aerobic fermentations. The measurement of CO_2, therefore, helps determine the course of the fermentation as well as the carbon balance (Arslan *et al.*, 2012). At least three principles are employed in current equipment for CO_2 determination. The first method, which is the most widely used, depends on the ability of CO_2 to absorb infrared rays. A sensitive sensor translates this absorption to a gauge or record, from which it can be read. In another principle, the effluent gas emerging from the broth is bubbled through a dilute solution of NaOH containing phenol red. The change in color of the phenol red is reflected in a photocell, and the amount of CO_2 may be calculated from a standard curve. The third method depends on the thermal conductivities of the various gases in a mixture (Okafor, 2007).

2.5.7. Ethanol

The presence of ethanol has a significant effect on yeast growth and activity, especially during the latter stages of fermentation when the ethanol reaches its highest concentrations. The presence of ethanol prevents growth and decreases viability, resulting in an extension of the fermentation duration (Pratt *et al.*, 2003). Research showed that the addition of ethanol decreases the speed of growth initiation and nitrogen assimilation (Ribéreau-Gayon *et al.*, 2006). The inhibition of ethanol on yeast growth can also be enhanced by other factors such as temperature, exposure to oxygen, osmotic pressure, and nutrient deficiency (Laluce *et al.*, 2009). Ethanol not only inhibits cell growth but also represses glucose transport through the modification of the cell membrane (Rolland *et al.*, 2002; Santos *et al.*, 2008). Consequently, the metabolism and energy supply of yeast can also be affected (Dos Santos *et al.*, 2013; Zhao and Bai, 2009). Therefore, it can be inferred that the increase in ethanol leads to a decrease in the rate of both yeast growth and fermentation.

2.5.8. Foam

Foams in industrial fermentations are controlled either by chemical or mechanical means (Koller, 2004). Chemicals controlling foams have been classified into antifoams, which are added to the medium to prevent foam formation, and defoamers, which are added to knock down foams once these are formed (Denkov *et al.*, 2014; Junker, 2007). Ideally, the antifoam should be miscible with the foaming liquid (Etoc *et al.*, 2006). Antifoams used in industrial fermentation should ideally have the following properties. They should: (i) be non-toxic to microorganisms and higher animals, especially if the fermentation product is for internal use; (ii) not affect taste and odor, as a change in the usual organoleptic properties of the finished goods due to the antifoam or other components of the medium may result in consumer rejection of the goods; (iii) be autoclavable. (iv) not be metabolized by the microorganisms; sometimes, as when natural oils are used, the antifoam may be utilizable, in which case they must be replaced regularly (Wang *et al.*, 2021). (v) not impair oxygen transfer. (vi) be active in small concentrations, cheap, and persistent.

Antifoams may be added manually when the foam is observed. This entails close watch and may be expensive. Automatic antifoam additions are now very common and depend on a probe that is activated when foams rise and make contact with the probe. One of the earliest is the wick defoamer in which the foam drew some antifoam on making contact with a wick. Modern

methods are electrically activated systems (Junker, 2007). Other systems that have been used include antifoam introduction via the sparging air or continuous drip-feeding. Mechanical defoamers of various designs have been described. In general, they act by physically dispersing the foams by rapidly breaking them up.

2.6. Substrates for yeast biomass production

For their nutrition, yeasts require a source of carbon for growth and energy, a nitrogen source for the synthesis of protein and other nitrogenous materials, inorganic nutrients for the buildup of the normal functioning and structure of the cell, as well as vitamins (Amata, 2013). The raw materials used as substrates for industrial yeast biomass production are usually agricultural, forestry, and food waste by-products. There are two types of raw materials depending on the grown microorganism: conventional materials like starch, molasses, distiller's wash, whey, fruit and vegetable wastes, wood, straw, etc., and unconventional ones like petroleum by-products, natural gas, ethanol and methanol (Marova *et al.*, 2012).

2.6.1. Molasses

Molasses is used in the baker's yeast production, in the fermentation technology for ethanol, citric, lactic and gluconic acids production, as well as glycerol, butanol and acetone production, as an ingredient of mixed feeds or in the production of amino acids (Mamun-Or-Rashid *et al.*, 2022; Nasir *et al.*, 2017). Molasses is a byproduct of the sugar industry. It is the residue after the crystallization of the main fraction. When no more sugar can be crystallized out of solution, the resulting liquid (molasses), containing about 50% sucrose is eliminated. For every 100 Kg of plant, some 3.5 to 4.5 Kg of molasses may be obtained (Gabriel *et al.*, 2014; Tirsit Tibebu, 2020). Based on its origin, it can be called cane molasses or beet molasses (Valli *et al.*, 2012) and is the cheapest source of carbohydrate (Shahzad *et al.*, 2017; Tripathi *et al.*, 2012). It contains 45-55% fermentable sugars, including sucrose, glucose, fructose, raffinose, melibiose, and galactose (Gabriel *et al.*, 2014). The use of molasses for the production of yeast biomass has simplified the manufacturing process in many ways. Its cost is reduced as compared with the use of grain and other raw materials (Malik, 2016; Merrylin *et al.*, 2020). The molasses is used as a source of carbon, energy, and other essential nutrients.

Molasses could not supply all the essential nutrients for yeast growth (Lino *et al.*, 2018; Walker and Stewart, 2016). Therefore, the addition of supplements such as $(NH_4)_2SO_4$, urea, yeast extract, or peptone as the nitrogen source, KH_2PO_4, H_3PO_4 as the phosphorus source, other macro elements such as calcium in the form of calcium salts, magnesium in the form of magnesium salts, and microelements such as iron, zinc, copper, and manganese is necessary for maximizing biomass yield of *Saccharomyces cerevisiae* or any other types of yeasts. Vitamins are also required for yeast growth (biotin, inositol, pantothenic acid, and thiamine) (Julies, 2019; Perli *et al.*, 2020). The composition of molasses may vary quite widely depending on the location, soil type, the climatic conditions, and the production process of each sugar factory (Bhatnagar *et al.*, 2016; Palmonari *et al.*, 2020; Santos *et al.*, 2020). Table 1 shows the percentage composition of both beet and cane molasses. Molasses replaced malted grain for yeast production during the First World War (Gélinas, 2012; Meussdoerffer, 2009). It is then becoming common practice to develop yeast on beet and cane molasses and ammonium salts (such as sulfate, phosphate, or chloride), with aqua ammonia as an additional source of nitrogen and as an aid in controlling the pH of the medium (Zabed *et al.*, 2017).

Table 1. Percentage composition of cane and beet molasses

	Molasses Type			
	Cane (% Min-Max)	**Average (%)**	**Beet (% Min-Max)**	**Average (%)**
Dry matter (%)	75.7-79.6	76.8	67-80.9	77.6
Protein	2.2-9.3	6.07	10.7-15.6	13.5
Total sugars	57-71	62.3	50.6-68.4	62.1
Sucrose	39.2-67.3	48.8	46.5-66.1	60.9
Glucose	1.3-12	5.26	0.02-1.96	0.28
Fructose	2.3-14.2	8.07	0.01-0.87	0.29
Starch	0.06-1.07	0.33	0.02-0.17	0.08
Ash	10.2-16.3	13.1	6.5-18.5	11.7
Ca	0.82-3.13	1.39	0.02-1.24	0.3
Mg	0.19-0.63	0.43	0.00-0.09	0.02
Na	0.01-0.42	0.08	0.05-1.45	0.62
K	0.31-7.99	1.82	0.65-5.54	2.44
Sulphates	0.81-4.09	2.09	0.17-1.84	0.61
Phosphates	0.7-2.97	2.03	0.31-1.65	0.76
Nitrates, mg.kg	17-999	464	16-116	55
Chlorides, mg.kg	1-340	60	411-8056	3974

Source: (Palmonari *et al.*, 2020)

2.6.2.Whey

Whey is the leading waste of the dairy industry. It is produced worldwide in large amounts, and its disposal causes severe environmental problems due to its high organic load, which makes its full treatment impossible (Bekatorou *et al.*, 2006). On the other hand, whey has a significant nutritional value since it contains respectable amounts of proteins, lactose, organic acids, fat, vitamins, and minerals (Ahmad *et al.*, 2019). Therefore, its conversion to products of added value is a significant concern for science and industry. The composition (high salt concentrations) and temperature of whey at the moment of its production in the factory do not allow easy microbial utilization. Lactose, the primary sugar constituent in whey, can be metabolized only by a few species of the *Kluyveromyces* and *Candida* yeasts. The yeast *S. cerevisiae* cannot utilize lactose because it lacks the enzyme ßgalactosidase and lactose permease. *Kluyveromyces marxianus* is one of the strains with high lactose utilization used for biomass production from whey on a commercial scale (Karim *et al.*, 2020; Saini *et al.*, 2017a).

2.6.3. Starch

Saccharomyces cerevisiae can utilize starch, only after its conversion to fermentable sugars, glucose, and maltose (Akbas and Stark, 2016; Saini *et al.*, 2017b). Hydrolysis of starch to glucose can be done either by treatment with acid or non-yeast enzymes. Enzymatic treatment includes three different processes: gelatinization by heating, liquefaction by thermostable a-amylases, and saccharification by mixed enzyme activities (Patil *et al.*, 2021; Sundarram and Murthy, 2014). Nevertheless, processes like these imply considerable costs, which is the main limiting factor in industrial utilization of starch for yeast biomass production. Starch can be utilized by mixed cultures of yeasts and amylolytic fungi like *Aspergillus* species for SCP or ethanol production (Chi *et al.*, 2009; Tiwari *et al.*, 2015).

2.6.4. Residues of Forestry and agriculture

Wastes of agriculture and forestry are rich in cellulose, hemicellulose, and lignin (Millati *et al.*, 2019; Petre *et al.*, 2013). Their enzymatic conversion to fermentable sugars requires chemical pretreatment that leads to various polymer fragments. *Saccharomyces cerevisiae* does not have the variety of enzymes required to hydrolyze these polymers. As a result, yeast biomass

production on lignocellulosic wastes implies a high economic cost. A solution to this problem could be the use of mixed cultures of *Saccharomyces cerevisiae* and cellulolytic microorganisms, but this process is today applied for ethanol production in pilot plants only (Bekatorou *et al.*, 2006; Dashtban *et al.*, 2009; Favaro *et al.*, 2019).

2.7. Application of yeasts

The yeast is synonymous with *S. cerevisiae*, and other yeast species of biotechnological importance have been introduced to produce industrial products beyond traditional foods as shown in Table 2. They have much industrial importance in scientific, food, medical, and agricultural disciplines (Schaechter, 2011; Vilela, 2019). In addition to traditional industrial applications of yeasts in several food fermentations such as alcoholic beverages, bakery products, cheese, sausages, and other fermented foods, the modern applications of yeasts involve the production of fuel ethanol, single cell protein (SCP), feeds and fodder, industrial enzymes, and small molecular weight metabolites (Johnson, 2013a; Kieliszek *et al.*, 2017). More recently, *Komagataella (Pichia) pastoris, Saccharomyces cerevisiae, Ogataea (Hansenula) polymorpha,* and certain other yeast species have been developed as industrial organisms for the heterologous production of enzymes and proteins, including protein pharmaceuticals (Nandy and Srivastava, 2018; Vieira Gomes *et al.*, 2018). Yeasts, especially *S. cerevisiae*, are increasingly being used as hosts for expression of protein biocatalysts and multi-enzyme pathways for the synthesis of fine chemicals and small molecular weight compounds of medicinal and nutritional importance (Johnson and Echavarri-Erasun, 2011). Yeasts have important roles in agriculture as agents of biocontrol, bioremediation, and as indicators of environmental quality (Kowalska *et al.*, 2022). Several of these processes and products have reached commercial utility, while others are in development (Branduardi and Porro, 2012; Johnson and Echavarri-Erasun, 2011).

Table 2. Industrial application of yeasts

Type of product	**Examples**	**References**
Potable alcoholic beverage	Beer, Wine, and cider	(Gutiérrez *et al.*, 2018; Maicas, 2020)
Distilled beverages	Whisky, Vodka, Rum, brandy, sake	(Black and Walker, 2023; Walker and Stewart, 2016)
Food and animal feed	Baker's yeast, yeast extracts, fodder yeast, livestock growth, feed pigments	(Ferreira *et al.*, 2010; Shurson, 2018)

Fermentation products (chemicals)	Fuel ethanol (bioethanol), carbon dioxide, glycerol, citric acid, vitamins	(Parapouli *et al.*, 2020; Walker, 2009)
Enzymes for food industry	Invertase, galactosidase, pectinase, lactase, lipase, inulinase,	(Sharma *et al.*, 2021; Sunmonu and Sanusi, 2022)
Recombinant proteins	Hormones (e.g., insulin), viral vaccines, antibodies, growth factors, interferon, blood proteins and enzymes	(Demain and Vaishnav, 2009; Kulagina *et al.*, 2021)

2.7.1. Application of yeasts in food, beverage and feed industry

Yeasts play vital roles in the production of many traditional fermented foods and beverages across the world, signifying the food culture of the regions and the community (Aidoo and Nout, 2010; Nduko *et al.*, 2016). The common fermented foods and beverages, mostly prepared by yeasts, or in combination with bacteria and molds, are: beers and ales, breads and bakery products, cachaça, cheeses, kefir, kimçi, dairy products (e.g., kefir; yoghurt, fermented milk), cocoa, coffee, fermented meat and sausages, fermented olives and cucumbers, soy paste, tea fungus, silage and probiotics (Anal, 2019; Snauwaert, 2014; Stewart and Russell, 2014).

Saccharomyces cerevisiae, also known as baker's yeast,is the most common yeast species in bread and sourdoughs (Heitmann *et al.*, 2018). Bread production requires the mixing of flour, water and sourdough (Catzeddu, 2019). Depending on the culture and geographic location, different kinds of flours were used, including wheat, barley, emmer, einkorn, khorasan, rye, spelt, teff, maize, or sorghum, while the sourdough was a mix of flour and water, containing fermenting yeast and LAB (Carbonetto *et al.*, 2018; Petrova and Petrov, 2020). *Saccharomyces cerevisiae* is generally inoculated into bread dough at a concentration of 2% of the total ingredients. The oxygen from the air entrapped in the dough during mixing is consumed in a couple of minutes by the respiration of yeast cells, and under the anaerobic conditions that are formed, yeast cell reproduction is slowing down and the fermentation reaction takes place (Parapouli *et al.*, 2020). The optimal conditions for fermentation in the dough are around 34–38°C, at pH 4.0–5.2, using fresh cells because older cells require longer fermentation time. A possible factor that delays yeast multiplication is the addition of fat, salt, or spices (Hidalgo and Brandolini, 2014b).

Yeasts have long been cultivated as rich sources of protein, minerals, vitamins (particularly B vitamins), and other nutrients for humans and animals (Bratosin *et al.*, 2021). Production of yeast SCP has certain advantages compared to plant, animal, and other microbial sources of SCP,

including rapid growth and accumulation of biomass, high protein content (up to 50%), high contents of vitamins and minerals, and ability to grow on a wide variety of substrates, including various industrial waste streams (Jach and Malm, 2022; Jach *et al.*, 2022). The production of value-added SCP from inexpensive substrates could help to alleviate world shortages in the food supply, particularly in developing countries. Substrates utilized have included molasses, starch, cassava, Jerusalem artichoke, whey products, sulfite waste liquor, potato wastes, brewery wastes, and other waste streams from agricultural processes, food processing, and industrial processes (Hashempour-Baltork *et al.*, 2022; Kaur *et al.*, 2023). Due to the relatively large cell size and flocculation abilities of yeasts, they can be more easily harvested than bacteria from the fermentation liquor. Compared to bacteria, many types of yeast contain low quantities of nucleic acids, which can have detrimental nutritional effects. Certain yeast species have been used as prebiotic and probiotic agents for preventing or treating various intestinal, nutritional, and toxicological disorders (Anadón *et al.*, 2021; Markowiak and Śliżewska, 2017). Some yeasts allowed and commonly used in animal feeds as probiotic additives are *Candida peltata*, *C. saitoana* and *S. cerevisiae (*Bekatorou *et al.,* 2006).

2.7.2. Application of yeasts in biopharmaceutical, enzyme and chemical industry

Biopharmaceuticals are used to compensate for deficiency or lack of body proteins important for normal functioning of the organism. They can be divided mainly into the following categories: blood factors, thrombolytics and anticoagulants, hormones, enzymes, growth factors, interferons and interleukins, vaccines and monoclonal antibodies. Since the early 1980s, yeasts have been utilized for heterologous production of a variety of proteins (Kulagina *et al.*, 2021; Roohvand *et al.*, 2017). The production of heterologous proteins in yeasts holds enormous potential for biotechnological processes. A major breakthrough in heterologous protein expression in yeast was the cloning, expression, processing, and secretion of human proinsulin in *S. cerevisiae* in the 1980s (Branduardi and Porro, 2012; Mattanovich *et al.*, 2012). Yeasts are intensively being developed as protein expression systems and in comparison to mammalian cell lines have higher productivity, higher cell yields, shorter fermentation cycles, can be cultured in defined media under relatively inexpensive conditions, can efficiently secrete proteins, possess posttranslational modification pathways, nonpathogenic and non-pyrogenic (Çelik and Çalık, 2012; Demain and

Vaishnav, 2009). Table 3 shows examples of commercial recombinant protein pharmaceuticals and vaccines produced by yeasts.

Table 3. Commercial vaccines and recombinant protein pharmaceuticals produced in yeasts

Product	Use	Yeast Host
Short-acting recombinant insulin	Diabetes Mellitus	*Saccharomyces cerevisiae*
Granulocyte macrophage colony-stimulating factor (GM-CSF)	Bone marrow transplantation, Regulation of hematopoiesis	*S. cerevisiae*
Hirudin/lepuridin	Anticoagulant	*S. cerevisiae*
Urate oxidase	Hyperuricemia	*S. cerevisiae*
Platelate-derived growth factor	Diabetic ulcers	*S. cerevisiae*
Human serum albumin	Excipient, shock, cirrhosis	*S. cerevisiae*
Erythropoietin	Renal disease	*S. cerevisiae*
Glucagon	Hypoglycemia	*S. cerevisiae*
Human growth hormone	Dwarfism, tissue repair	*S. cerevisiae*
Insulin	Diabetes	*S. cerevisiae*
Hepatitis A vaccine	Hepatitis A	*S. cerevisiae*
Hepatitis B vaccine	Hepatitis B	*S. cerevisiae, O. polymorpha,*
Diphtheria, tetanus, pertussis	Hepatitis B	*S. cerevisiae*
Haemophilus influenzae type B	Combnination vaccines and polio	*S. cerevisiae*
Human papillomavirus (HPV) antigen	Human papillomavirus	*K. pastoris*

The enzyme market is generally categorized into four utility classes: (a) technical enzymes, which comprise about 65% of the market, including enzymes used in the detergent, starch, textile, leather, pulp and paper, and personal care industries; (b) food enzymes, about 25% of the market, including enzymes used in brewing, dairy, wine and juice, fats and oils, and baking industries; (c) feed enzymes, about 10% of the market, used in animal feeds; and (d) diagnostic enzymes, comprising a small portion of the overall enzyme market (Raveendran *et al.*, 2018; Robinson, 2015; Sanchez and Demain, 2017). In recent years, growth of the bulk enzyme industry has been particularly robust in the baking and animal feed sectors, in industrial organic syntheses for fine chemicals and pharmaceuticals, and to a lesser degree for paper and pulp processing, production of biofuels, and for personal care (Sanchez and Demain, 2017; Singhania *et al.*, 2015). However, several yeast enzymes have found application in the production of high-value specialized fine chemicals and for biotransformation of pharmaceutical intermediates. Certain yeasts, especially *Komagataella pastoris* and *S. cerevisiae*, are increasingly being

utilized for heterologous production of enzymes and a variety of other proteins (Barone *et al.*, 2023; Fischer and Glieder, 2019; Gündüz Ergün *et al.*, 2019). The use of yeasts in "White Biotechnology" applications, or production of low-cost, high-volume products, is accelerating. *Saccharomyces cerevisiae*, *K. lactis* and *Schef. stipitis* have been developed for the production of L-lactic acid (Branduardi and Porro, 2012; Singh, 2014). *Saccharomyces cerevisiae* and *R. glutinis* have been used for medium-scale production of amino acids including lysine, methionine, phenylalanine, and proline. Yeasts have also been used for the production of alcohols used in bulk processes (Johnson and Echavarri-Erasun, 2011). Mannitol, widely used in the food, pharmaceutical, and chemical industries, is produced by various yeast species from glycerol. Glycerol is a commodity chemical that is used in a number of industrial products. It has been traditionally produced by chemical synthesis from propylene and is also recovered following hydrolysis of fats.

2.7.3. Application of yeasts in agricultural and environmental protection

Yeasts have important roles in agriculture as bio-control agents (Godana *et al.*, 2020; Kakraliya *et al.*, 2020). Postharvest losses of stored fruits, vegetables, and grains due to decay by fungal pathogens can be very significant, and in fact, addressing this problem is receiving increasing importance as worldwide demand for food increases (Chowdhury *et al.*, 2022; Villalba *et al.*, 2016). There is an increasing concern about the environmental effects and safety of chemical pesticides and fungicides all over the world. Traditionally, the postharvest diseases are controlled by fungicides. The main problems involved in fungicide utilization are related to environmental pollution and public health concerns (Alengebawy *et al.*, 2021; Asogwa and Dongo, 2009). Research on the use of yeasts as BCAs has mainly focused on their use for managing postharvest diseases, mainly of fruit; however, this application represents only a small portion of the complete spectrum of plant disease management (Buzzini and Margesin, 2014; Usall *et al.*, 2016). Yeasts also have important roles in the biotransformation and degradation of pollutants and xenobiotics (Schlüter and Schauer, 2017; Shrie and Vijayalakshmi, 2022). This activity is thought mostly to be due to the production of solubilizing acids within microbial consortia such as biofilms. The colored recalcitrant compounds can be removed from molasses-based wastewaters by the yeast *Issatchenkia orientalis,* which is a major problem in the baker's yeast industry (Mahgoub *et al.*, 2016; Tondee *et al.*, 2008). Basidiomycetous yeasts were shown to

degrade starch, pullulan, dextran, xylan, polygalacturonate, galactomannan, and tannic acid as sole carbon sources (Johnson, 2013b; Middelhoven, 2009). They were unable to grow on cellulose, chitin, arabinogalactan, and xanthan gum. Several basidiomycetous yeasts grow on phenolic compounds. These results indicate that basidiomycetous yeasts are involved in the natural biodegradation of biomass and may have potential for remediation of various industrial waste streams. It has been reported that *Pseud. jejuensis* is capable of degrading certain plastic wastes (Seo *et al.*, 2007). The ability of yeasts to degrade mycotoxins has important health implications, since these toxicants occur in commodities, such as feeds, foods, and beverages, and are considered as serious health hazards (Schisler *et al.*, 2011).

2.8. Taxonomy of Lactic Acid Bacteria (LAB)

Lactic acid bacteria (LAB) are a heterogeneous group of phylogenetically closely related microorganisms that produce lactic acid as the major or sole product of carbohydrate fermentation (Bintsis, 2018). LAB are Gram-positive, non-sporulating, catalase-negative, acid-tolerant, non-respiring but aerotolerant, usually non-motile cocci or rods with low Guanidine + Citosine (G + C) content (Ameen and Caruso, 2017; Comi and Iacumin, 2017; Negm, 2018). Except for a few species belonging to the *Streptococcus*, *Enterococcus*, *Lactococcus*, and *Carnobacterium* genera, LAB are non-pathogenic with a generally recognized as safe (GRAS) or food-grade status (Sin *et al.*, 2013). LABs were classified based on their morphology, mode of glucose fermentation, ability to grow at different temperatures, and use of sugars as carbon substrates. Other features, such as configuration of lactic acid produced and tolerance to salt, acid, and alkali, were also considered (Liu *et al.*, 2014. According to the current taxonomic classification, LAB belong to the phylum Firmicutes, class Bacilli, order *Lactobacillales*. The families include *Aerococcaceae*, *Carnobacteriaceae*, *Enterococcaceae*, *Lactobacillaceae*, *Leuconostocaceae*, and *Streptococcaceae*. Four main genera of LAB, namely *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*, were originally described; however, recent taxonomic revisions have proposed the following new genera: *Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Dolosigranulum*, *Enterococcus*, *Globicatella*, *Lactococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus*, and *Weisella* (Stefanovic *et al.,* 2017). Of all the genera mentioned, the lactobacilli and carnobacteria are rods; the remaining genera are cocci, except for *Weisella* species, which may be either rods or cocci. Lactobacillus is one of the most well-known and extensively studied genera of LAB. It

includes a wide range of species such as *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus casei*, and *Lactobacillus rhamnosus*. These bacteria are commonly used in food fermentation, probiotics, and animal feed applications (Goldstein *et al*., 2015). *Lactobacillus plantarum* is a versatile and industrially important lactic acid bacterium that can be found in fermented pickles (Ming *et al.*, 2018). Streptococcus is another important genus of LAB that includes species like *Streptococcus thermophilus* and *Streptococcus salivarius*. *Streptococcus thermophilus* is used in the fermentation of dairy products like yogurt and cheese (Widyastuti and Febrisiantosa, 2014). Pediococcus is a genus of LAB that includes species such as *Pediococcus acidilactici* and *Pediococcus pentosaceus*. These bacteria are commonly found in fermented foods and are used as probiotics and in animal feed formulations (Liu *et al.*, 2014). Leuconostoc species, like *Leuconostoc mesenteroides*, are also classified under LAB. They are important in food fermentation, particularly in the fermentation of vegetables and dairy products. Enterococcus is another genus of LAB that includes species such as *Enterococcus faecalis* and *Enterococcus faecium*. While some species can be pathogenic, certain strains are used as probiotics and in food fermentation. Weissella species, such as *Weissella cibaria* and *Weissella confusa*, are also classified under LAB (Fusco *et al*., 2015). They are commonly found in fermented foods and can be used as probiotics. *Oenococcus oeni* is a genus within LAB that is primarily associated with the fermentation of wine. The classification of LAB continues to evolve as new species are discovered and characterized. LABS are widely used in various industries, including food and beverage fermentation, probiotics, and animal feed formulations, due to their beneficial properties and applications. These microorganisms have beneficial effects on human health, such as treating allergies, increasing the immune system, reducing urogenital infections, treating ulcers, preventing the initiation of colon cancer, decreasing cholesterol levels in the blood, treating the causes of diarrhea and constipation, and treating lactose-intolerance cases (Ayivi *et al.*, 2020).

2.8.1. Ecology of LAB

LABs are widely distributed in various ecological niches, including the gastrointestinal tract of animals, plants, fermented foods, and natural environments. The ecology of LAB is diverse, and they play essential roles in various ecosystems. Their ability to inhabit different niches is a trait of their metabolic diversity and adaptability. LABs are prevalent in the gastrointestinal tract of

animals, including humans, where they contribute to gut health and microbial balance (Arqués *et al.*, 2015). They help in food digestion, nutrient absorption, and protection against pathogenic bacteria by producing antimicrobial compounds. LABs are vital in the fermentation of various foods such as yogurt, cheese, sauerkraut, kimchi, and sourdough bread. They convert sugars into lactic acid, imparting characteristic flavors, textures, and preservation properties to the fermented products. LABs can outcompete harmful bacteria and fungi during fermentation, enhancing food safety (Ayivi *et al.*, 2020).

LABs are present in various natural environments including soil, water, plants, and air. They may contribute to plant growth promotion, nutrient uptake, disease resistance, and stress tolerance. Some LAB strains have been used as plant probiotics or biocontrol agents in agriculture (Raman *et al.,* 2022). They have adapted to diverse ecological niches, playing roles in nutrient cycling, decomposition, and interactions with other microorganisms. LABs interact with other microorganisms in complex ecosystems (Canon *et al.*, 2020). They may exhibit antagonistic activity against pathogenic bacteria, fungi, and spoilage organisms, contributing to food preservation. LAB can also form symbiotic relationships with yeast or other bacteria in mixed-culture fermentations. LAB have evolved mechanisms to tolerate and thrive in various environmental conditions, including pH, temperature, salt concentration, and oxygen levels. They can utilize a wide range of carbohydrates for energy metabolism and adapt to stress conditions such as low pH or high osmotic pressure during fermentation. Understanding the ecology of lactic acid bacteria is crucial for their applications in food fermentation, probiotics, animal nutrition, agriculture, and environmental biotechnology.

2.8.2. Reproduction of LAB

2.8.2.1. Asexual reproduction

Binary fission is a common mode of asexual reproduction in lactic acid bacteria (Bradley, 2019). In the process of binary fission, a single parent cell divides into two daughter cells, each containing a complete copy of the genetic material (DNA) of the parent cell. This division allows for rapid multiplication of LAB populations under favorable growth conditions. Binary fission begins with the replication of the bacterial DNA. The DNA molecule undergoes replication, resulting in the formation of two identical DNA molecules attached to the cell membrane at the

origin of replication. As the DNA replicates, the cell elongates to accommodate the increased genetic material. The bacterial cell grows in size as it prepares for division. A septum (cell wall membrane) forms at the center of the elongated cell, dividing it into two compartments. The septum is formed by the synthesis of new cell wall and cell membrane components. The septum continues to grow inward, eventually dividing the cell into two daughter cells. Each daughter cell contains a copy of the genetic material and other cellular components necessary for independent viability (Zhu *et al*., 2018). Once the septum is fully formed, the two daughter cells separate from each other, becoming individual, independent cells capable of growth and further division. It is a highly efficient method of reproduction for LAB, allowing for rapid population growth in favorable conditions (Bhuyan and Das, 2018). This process is essential for maintaining LAB populations in various environments, including food fermentations, probiotic products, and the human microbiota.

2.8.2.2. Sexual reproduction

Conjugation is a process of genetic exchange between two bacterial cells involving the transfer of DNA from a donor cell to a recipient cell (Morelli and Wright, 2019). It is one of the common modes of sexual reproduction in LAB. Conjugation allows for the exchange of genetic material, such as plasmids containing beneficial traits, antibiotic resistance genes, or other genetic elements. The donor cell, which possesses the genetic material to be transferred, contains a plasmid or conjugative transposon that carries the genes required for conjugation. The donor cell forms a pilus, a hair-like appendage that makes contact with the recipient cell. The plasmid or genetic material is transferred from the donor cell to the recipient cell through the pilus. This process may involve the physical contact and fusion of the cell membranes of the donor and recipient cells (Plavec and Berlec, 2020). The transferred genetic material integrates into the recipient cell's genome or remains as an extrachromosomal element, providing the recipient cell with new genetic information. Conjugation allows LAB to exchange genetic material, leading to genetic diversity within populations. This genetic diversity can contribute to adaptation, survival in changing environments, and the acquisition of beneficial traits.

2.9. Characteristics of lactic acid bacteria (LAB)

Lactic acid bacteria (LAB) are a diverse group of microorganisms that belong to the phylum Firmicutes. They are Gram-positive, non-spore-forming, rod-shaped or cocci-shaped bacteria that predominantly ferment carbohydrates into lactic acid (Negm, 2018). LABs are defined as chemotrophic microbes as they generate energy (ATP) by substrate-level phosphorylation. Although these bacteria ferment sugars when they grow under anaerobic conditions, they can also grow in the presence of oxygen. Sugars are the primary carbon and energy sources for LAB grown on substrates used for fermented foods and feed production, as well as in laboratory media. According to the way LAB ferment carbohydrates, they are classified as homofermentative or heterofermentative (Fuhrmann, 2021). Homofermentative LAB (*Lactococcus*, *Streptococcus*, *Pediococcus*, *Enterococcus*, and some *Lactobacillus* species) ferment sugars by the Embden–Meyerhoff–Parnas pathway to pyruvate, which is converted into lactic acid by lactate dehydrogenase (Souza *et al*., 2023). Heterofermentative LAB such as *Leuconostoc*, *Oenococcus*, and certain *Lactobacillus* species ferment sugars generally by the phosphoketolase pathway (PKP, also known as the pentose phosphoketolase shunt or the 6-phosphogluconate pathway). Fermentation of pentoses such as xylose and ribose leads to the formation of pyruvate and acetyl-P and their subsequent conversion to lactate and acetate, respectively. Hexoses (i.e., glucose, fructose, and mannose) can be converted to lactate, CO_2, and ethanol. CO_2 is a product of 6-P-gluconate degradation, which occurs during conversion of hexoses to pentoses. The specific enzyme of the heterofermentative pathway is d-xylulose-5P phosphoketolase, which catalyzes the conversion of xylulose-5P into glyceraldehyde-3-phosphate (GAP) and acetyl-P (Souza *et al*., 2023).

The fermentation type (homolactic or heterolactic) is an important taxonomic criterion. The genera *Leuconostoc*, *Oenococcus*, and *Weisella* as well as certain *Lactobacillus* species such as *Lactobacillus buchneri*, *Lactobacillus brevis*, *Lactobacillus fermentum*, and *Lactobacillus reuteri,* are obligate heterofermentative. Other lactobacilli, including *Lactobacillus acidophilus*, *Lactobacillus delbrueckii*, *Lactobacillus helveticus*, and *Lactobacillus salivarius*, are obligate homofermentative and not able to metabolize pentoses. Finally, *Lactobacillus casei*, *Lactobacillus curvatus*, *Lactobacillus plantarum*, *Lactobacillus sakei*, and most LABs can

homofermentatively ferment hexoses and pentoses and are known as facultatively heterofermentative (Khubber *et al*., 2022).

2.10. Major factors that influence the growth of lactic acid bacteria (LAB)

2.10.1. Carbon Source

The carbon source plays a crucial role in the growth of lactic acid bacteria (LAB) as it serves as the primary energy and carbon substrate for their metabolism. Glucose, lactose, sucrose, and maltose are some of the common carbon sources that LAB can utilize (Tian *et al*., 2021). The type and availability of carbon sources in the growth medium can impact the growth rate and metabolism of LAB.

2.10.2. Nitrogen, Vitamins and Minerals Source

LAB needs nitrogen for the synthesis of proteins and nucleic acids. Peptones, amino acids, and ammonium salts are some of the nitrogen sources that can support the growth of LAB. The quality and quantity of nitrogen sources in the growth medium can influence the growth and productivity of LAB (Tian *et al*., 2021). LAB also requires various vitamins and minerals as cofactors for their metabolic pathways. B-vitamins, folic acid, and minerals like iron, magnesium, and zinc are important for the growth and metabolism of LAB. The presence of essential vitamins and minerals in the growth medium is essential for optimal growth of LAB.

2.10.3. Temperature

Temperature plays a crucial role in the growth and metabolic activity of lactic acid bacteria (Wang *et al*., 2021). Each species of LAB has an optimal temperature range at which they grow and metabolize most efficiently. For most LAB commonly used in food fermentation, the optimal growth range is typically between 30-45°C, with some species thriving at lower temperatures (such as Lactobacillus plantarum) and others at higher temperatures (such as Streptococcus thermophilus). LABs exhibit varying degrees of tolerance to temperature fluctuations (Mbye *et al*., 2020). Some LAB are mesophilic, preferring moderate temperatures around 20-45°C, while others are thermophilic, thriving at higher temperatures above 45°C. Temperature tolerance is important for determining the range of environmental conditions in which LAB can grow and compete with other microorganisms. Certain LAB species, such as

Lactobacillus sakei and Lactobacillus plantarum, are psychrotrophic, meaning they can grow at refrigeration temperatures (4-7°C). This cold tolerance is important for the fermentation and preservation of refrigerated foods, such as sausages, dairy products, and pickles (Taormina, 2021).

2.10.4. pH and Acidity

LABs are acid-tolerant bacteria that can grow in acidic environments within a range of pH 4.0-7.0. The pH of the growth medium can impact the growth of LAB, as some strains prefer slightly acidic conditions, while others can tolerate a wider pH range (Wang *et al*., 2021). The acidic conditions help LAB outcompete harmful bacteria. However, extreme pH levels, either extremely acidic or alkaline, can hinder the growth of LAB.

2.10.5. Oxygen

Different LAB strains may have varying oxygen requirements for optimal growth. Some LAB species are strictly anaerobic and cannot survive in the presence of oxygen, while others are aerotolerant or facultative anaerobes that can grow under both aerobic and anaerobic conditions. The oxygen requirements of LAB can vary based on their metabolic pathways and adaptation to different environmental conditions. LABs are well-known for their ability to ferment sugars into lactic acid, a process that occurs under anaerobic conditions. The fermentation pathway enables LAB to produce energy in the absence of oxygen, leading to the accumulation of lactic acid as the primary fermentation product. This acidification of the environment plays a crucial role in various applications of LAB, such as food fermentation and probiotic production (Deshwal *et al*., 2021).

2.11. Major applications of Lactic Acid Bacteria (LAB)

2.11.1. Applications of LAB in food fermentation

Lactic acid bacteria (LAB) play a crucial role in the fermentation of various foods and beverages (Bintsis, 2018). They are responsible for the production of lactic acid, which is crucial for flavor development, preservation, and shelf-life extension. LABs are commonly used in the fermentation of dairy products (yogurt, cheese), sauerkraut, pickles, fermented meats, sourdough bread, and many other fermented foods. Since early times, humans have exploited food

fermentation without knowing the microorganisms involved. Traditionally, fermented foods are made through native microorganisms such as LAB from the available resources that precede the fermentation process. During the fermentation, the food components are converted into products with increased organoleptic properties and/or increased shelf life. Today, the majority of fermented foods are manufactured with the addition of selected, well defined, starter cultures with well characterized traits, specific for each individual product (Bintsis and Athanasoulas, 2015).

Lactic acid bacteria (LAB), particularly strains of *Lactococcus* and *Streptococcus*, are used as starter cultures in the production of yogurt, cheese, buttermilk, sour cream, and other fermented dairy products. They convert lactose into lactic acid, which lowers the pH, improves texture, enhances flavor, and provides preservation benefits. LAB, such as *Lactobacillus plantarum* and *Lacticaseibacillus brevis*, are used as starter cultures in the fermentation of vegetables, including sauerkraut, pickles, kimchi, and other fermented vegetable products. They initiate the fermentation process, acidify the vegetables, inhibit the growth of spoilage bacteria, and contribute to the development of characteristic flavors (Zhang *et al*., 2018). LAB, particularly strains of *Lactobacillus* and *Pediococcus*, are essential in sourdough bread fermentation. They colonize the dough, producing lactic acid and various flavor compounds, which contribute to the sour taste, aroma, and improved texture of the bread. LABs in sourdough bread fermentation are also involved in gluten breakdown, making the bread easier to digest (Holzapfel and Wood 2014).

Lactic acid bacteria (LAB), such as *Lactobacillus sakei* and *Lacticaseibacillus curvatus*, are used as starter cultures in the fermentation of meats, including dry-cured sausages, salami, and fermented sausages. They contribute to the development of desired flavor, texture, and enhanced food safety by acidification, production of antimicrobial compounds, and control of spoilage bacteria. LAB, including strains of *Lactobacillus*, *Leuconostoc*, and *Pediococcus*, are used as starter cultures in the production of various fermented beverages. This includes kefir, kombucha, kvass, and other probiotic drinks. They convert sugars into lactic acid and other metabolites, improving the taste, aroma, and nutritional qualities of the beverages. By using LAB as starter cultures in food fermentation, food producers can ensure consistency, improve food safety,

enhance product quality, and create unique flavors and textures in a wide variety of fermented foods and beverages (Hansen 2002; Kaviteke *et al*. 2018).

2.11.2. Applications of LAB as probiotics

Lactic acid bacteria (LAB) strains with proven health benefits are widely used as probiotics. Probiotics are live microorganisms that, when consumed in adequate amounts, confer health benefits to the host (Corcionivoschi *et al.*, 2010). The main probiotic microorganisms used belong to the *Bifidobacterium* and *Lactobacillus* genera (Goyal *et al.*, 2012). They occupy different ecological positions in the human gastrointestinal tract. Lactobacilli are normal inhabitants of the intestine, whereas *Bifidobacteria* reside in the colon (Pedretti, 2013; Wang *et al.*, 2015). These bacteria are generally regarded as safe (GRAS) because they can reside in the human body without causing harm. Probiotic bacteria are helpful in maintaining good health and in fighting intestinal and other disease disorders when consumed as a food component (Hussain, 2013). There is a significant number of studies indicating the benefits of probiotics, which include reducing antibiotic-associated diarrhea, allergy, lactose intolerance, prevention of colon cancer, prevention of eczema in children, reduction of cholesterol, as well as development of the immune system and protection against gut pathogens (Jankovic *et al.*, 2010). LAB strains such as *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium lactis* can improve digestion by promoting the breakdown of carbohydrates and supporting the absorption of nutrients. They also help maintain a balanced gut microbiota and alleviate symptoms of digestive disorders like irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD). LAB strains, including *Lactobacillus rhamnosus*, *Lactobacillus fermentum*, and *Bifidobacterium bifidum*, have been shown to enhance the immune response. They stimulate the production of immune cells, modulate the activity of the immune system, and help protect against pathogens by strengthening the gut barrier (Rijkers *et al.*, 2010).

2.11.3. Applications of LAB as bio-preservation

Lactic acid bacteria (LAB) strains produce antimicrobial substances, such as organic acids, hydrogen peroxide, and bacteriocins, which inhibit the growth of harmful bacteria and extend the shelf-life of perishable foods (Ross *et al.*, 2002). LAB-based bio-preservation is commonly employed in the meat, seafood, and dairy industries. LABs play a crucial role in the fermentation

and preservation of dairy products. They are used as starter cultures for the production of yogurt, cheese, and other fermented dairy products. LAB strains, such as *Lactobacillus acidophilus* and *Bifidobacterium bifidum*, produce lactic acid and other antimicrobial compounds during fermentation, which help control the growth of spoilage bacteria and increase the shelf-life of dairy products (Mesele Admassie, 2018). LABs are also used in the preservation of meat and poultry products. They compete with and inhibit the growth of harmful bacteria, such as *Listeria monocytogenes* and *Salmonella*, thereby reducing the risk of contamination and spoilage. LAB strains, such as *Lactobacillus sakei* and *Lactobacillus curvatus*, have been widely studied for their effectiveness in inhibiting the growth of spoilage organisms in cured and fermented meat products. The use of LAB as bio-preservatives offers a natural and safe approach to extend the shelf-life of various food products while maintaining their sensory and nutritional quality. These applications provide an alternative to chemical preservatives and contribute to the development of clean label and minimally processed foods (Schnürer and Magnusson, 2005). Overall, LABs have a wide range of practical applications in the food, healthcare, environmental, and industrial sectors due to their beneficial properties and versatility.

3. MATERIALS AND METHODS

3.1. Sampling site and sample collection

In order to isolate wild yeasts and LAB, samples of *Teff* dough, *Borde*, and *Tella* were obtained from different households' grocery stores in the Yeka, Gulele, and Arada sub-cities of Addis Ababa, Ethiopia (Figure 3). Hundred ml of each sample were collected in sterile screw-capped bottles, transported to the microbiology laboratory at the Addis Ababa University's Department of Microbial, Cellular, and Molecular Biology using an ice box in order to minimize the risk of contamination and maintain the original microbial composition of the sample during transportation. Finally, the samples were kept in a refrigerator at 4°C for further study. A total of 54 samples (18 from each *Borde*, Tella, and *Teff*) were collected from the study sites.

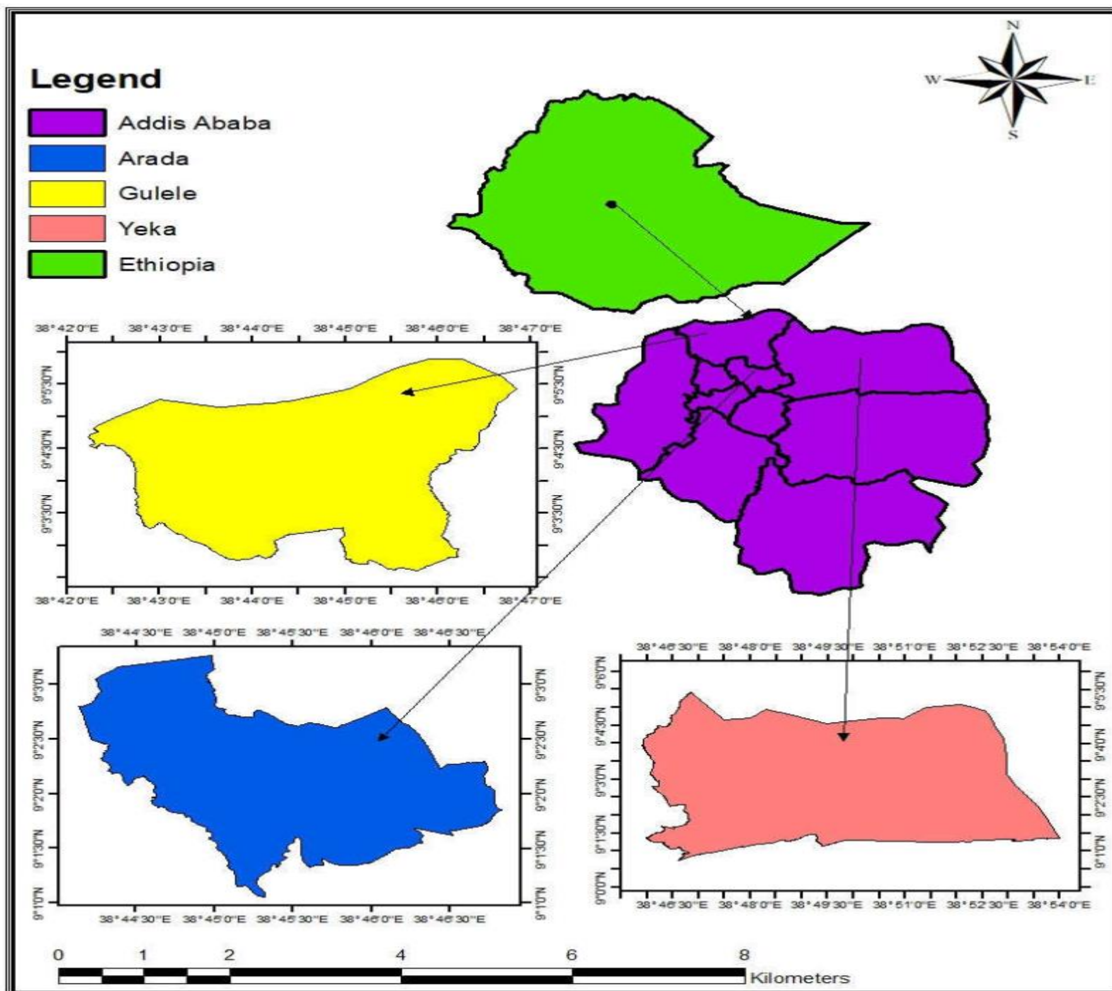

Figure 3. The location map of Addis Ababa showing the three sub-cities where *Teff*, *Borde*, and *Tella* samples were collected (source: Google satellite map).

3.2. Isolation and purification of wild yeast and active dry yeast

The isolation procedures for each sample and active dry baker's yeast were done by serial dilution agar plate technique (Qureshi *et al*., 2007). Yeast extract peptone dextrose (YEPD) agar (Conda, Spain) with the composition of (yeast extract, 10g; peptone, 20g; glucose, 20g; agar, 15g; distilled water 1000 ml) was used to isolate and screen the yeasts. Before sterilization, the pH of the medium was adjusted to 5 and after sterilization; chloramphenicol (0.1 g/L) was added to inhibit the growth of other bacteria. One ml of each sample (*Tella, Borde*, and *Teff* dough) was mixed with 9 ml of sterile distilled water in a sterile flask for serial dilutions. Then the mixture was homogenized for 30 seconds using a vortex mixer, and one ml of the mix was taken for serial dilution and added into a test tube containing 9ml sterile distilled water. From appropriate serial dilutions,0.1 ml was plated onto potato dextrose agar (PDA) and incubated for 48-72 hrs at 30°C to isolate morphologically well-formed discrete colonies (Barnett, 2000). The yeast isolates were further sub-cultured (streak plated) on the respective medium to establish pure cultures. The pure colonies were transferred to PDA slant cultures and kept at 4°C for further study. In order to designate the yeast isolates from *Tella, Borde*, and *Teff*, the first letter of each sample was combined with numbers. The coding of the yeast isolates was restructured as follows: B144 to Y1, B255 to Y2, Tl241 to Y3, B2414 to Y4, B141 to Y5 and Commercial yeast to CY.

The active dry baker's yeast Saccharomyces cerevisiae, by the name (Just Instant, Netherlands) was used for the investigation as a standard culture. A 0.5 g of this yeast was suspended in 50 ml of sterile distilled water aseptically. Serial dilutions (10^{-1}-10^{-6}) were made to reduce the number of yeast cells, as described above. Aliquots of 0.1 ml of the suspensions were spread plated on PDA. The cultures on the agar plate were incubated at 30°C for 48 hrs. The colonies were transferred to slant PDA cultures and preserved at 4°C for further study.

3.3.Isolation and purification of LAB isolates

For isolation of LAB, 1 ml of each sample from traditional fermented foods (*Teff* dough*, Tella* and *Borde)* was mixed with 9 ml of sterile distilled water. Then, a sequential decimal dilution of the homogenate was obtained. From the appropriate dilutions, 0.1 ml aliquots were spread plated in duplicates on pre-dried surfaces of de Man, Rogosa, and Sharp (MRS) agar (Himedia, India) plates (peptone 10 g, beef extract 10 g, yeast extract 5 g, glucose 20 g, Tween 80 (1 mL),

K_2HPO_4 2 g, $MgSO_4.7H_2O$ 0.2 g, $MnSO_4.4H_2O$ 0.05 g, sodium acetate 5 g, triammonium citrate 2 g, agar 20 g, bromocresol purple 0.04 g, and distilled water 1000 mL). The inoculated plates were incubated under anaerobic conditions using a candle jar at 37°C for 48 hrs. Then, some distinct colonies were randomly picked from countable MRS plates for purification and further identification. The isolated colonies suspected as LAB were transferred into about 5 ml MRS broth (Himedia, India) and purified by repeated streaking on MRS agar. Pure cultures of LAB were then streaked on slants of MRS agar and stored at 4°C for further screening and characterization (Kebede Abegaz, 2007). In order to designate LAB isolates from *Tella*, *Borde*, and *Teff*, the first letter of each sample was combined with different numbers. The coding of LAB isolates was restructured as follows: B343 to L1, Tl336 to L2, Tf55 to L3, Tf56 to L4 and Tl133 to L5.

3.4.Identification and characterization of yeast isolates

3.4.1. Cultural characterization

Cultural characteristics of yeast isolates were performed by streak culturing of the isolates on MEAB or YEPD plates and incubated at 30°C for 48 hrs. Then the colonies were examined based on their cultural characteristics (colony shapes, size, pigment, elevation, edge, and surface appearance) by comparing with the commercial strain.

3.4.2. Morphological characterization

A single colony of yeast was mixed in a droplet of sterile normal saline on a glass slide and smeared until the smear dried off. The smear was then stained using methylene blue dye, air-dried, and a drop of oil immersion and observed under a light microscope at 1000× magnification (Guimarães *et al.*, 2006).

3.4.3. Carbohydrate utilization test

The carbohydrate utilization test was performed using 10 mL of peptone broth (peptone: 10g; NaCl: 5g; phenol red: 0.018g; distilled water: 1000 mL; carbohydrate: 10 g) along with the inverted Durham tubes in the broth (Appendix 6). The carbohydrates used were dextrose, fructose, lactose, galactose, maltose, and sucrose. The culture media were inoculated with purified yeast isolates and incubated for 24 hours. The color change from red to yellow indicated the fermentation using carbon sources (Karki *et al.*, 2017).

3.5. Identification and characterization of LAB isolates

3.5.1. Cell shape and cell arrangement

Light microscope was used to examine overnight pure broth cultures to determine the shape and arrangement of the cells on clean microscope slides (Kebede Abegaz, 2007).

3.5.2. Motility test

Motility was examined by taking a fresh culture that had been growing for 24 hrs and incubating it in a test tube with Sulfide indole motility medium (SIM) agar (Himedia, India) using a sterile needle. For 24-48 hrs, the tubes were incubated at 37°C. While non-motile bacteria had growth that was restricted to the stab line with distinct edges without spreading to the surrounding area, motile bacteria showed diffused growth that extended from the surface and line of inoculation (Okoro *et al.*, 2011).

3.5.3. Gram reaction

Each isolate was thinly smeared on a grease-free slide, air-dried, and heat-fixed by passing each slide over the blue flame of a burning Bunsen burner repeatedly. Each slide was flooded with a crystal violet solution, dried for a minute, washed with distilled water, and treated with iodine for a minute. The slides were decolorized by ethanol, rinsed with distilled water, and counterstained using safranin for one minute. They were rewashed, air-dried, and observed under an oil immersion objective lens (X100) of the light microscope. Gram negative bacteria appeared pink or red, while gram positive bacteria appeared purple (Onyeagba, 2004).

3.5.4. Spore production test

Each isolate of LAB was grown in MRS agar slant for 24-36 hrs. The clean isolates were prepared on a pure slide, heat-fixed, and flooded with 5% malachite green solution and steamed for one minute. The stain was washed off with distilled water and counter-stained with 2 drops of safranin solution for 20 seconds. The slides were allowed to air dry and examined under an oil immersion objective (100×) lens. Endospores stained green (Patil *et al.*, 2010).

3.5.5. Catalase test

By putting a 24-hour-old bacterial culture onto a slide with a drop of 3% hydrogen peroxide (H_2O_2) solution, the catalase test was carried out. Catalase enzyme was shown by gas bubbles that

appeared as white foam (Patil *et al.*, 2010). Isolates with gram positive and catalase negative reactions were finally used for further identification.

3.5.6. Sugar fermentation

A sugar fermentation test including the fermentation of glucose, lactose, sucrose, fructose, mannose, xylose, and mannitol was conducted to ascertain the homofermentative and heterofermentative properties of isolates. A peptone water medium containing 1% fermentable sugar and 0.01% phenol red was used to make the sugar indicator broth. Each test tube received ten milliliters of sugar broth, and the inverted Durham tubes were carefully inserted within the tube. The test tubes and inverted Durham tubes were both autoclaved, inoculated with a loopful of fresh overnight culture, and then incubated at 37°C for 24-48 hours. According to (Ali and Mustafa, 2009), the Durham tubes' bubble formation indicated the production of gas, while the yellow coloration indicated the production of acid.

3.6.Screening parameters of wild yeast and LAB isolates

3.6.1.Carbon dioxide production tests for yeast isolates

Purified yeast isolates were screened for fermentative ability using the method of (Barnett *et al*. 2000). Carbon dioxide production was detected by using YEPD broth (Conda, Spain) medium (yeast extract, 10g; peptone, 20g; glucose, 20g; distilled water 1000ml) for the formation of gas (CO_2) in Durham tubes. Test tubes were filled with 10ml of YEPD broth medium containing inverted Durham tubes. Each tube was inoculated with 1ml (~1.2×10^8 cfu/ml) of yeast cells, taken from an actively growing culture. To adjust the number of yeast cells using a spectrophotometer at 550 nm involves diluting a known concentration of the microbial sample with a suitable diluent to achieve the desired concentration. The tubes were incubated at 30˚C within 24-48 hours and examined for the formation of gas bubbles in the inserted tubes. Isolates were selected based on the volume of gas in Durham tube during the incubation period. Potent gas producers were selected for further tests (Appendix 1).

3.6.2. Carbon dioxide production tests for LAB isolates

Purified LAB isolates were screened for fermentative ability using the method of (Fawole *et al.* 2004). Carbon dioxide production (CO_2) from glucose was determined in modified MRS broth

containing inverted Durham tubes with 1% glucose. MRS broth (8 ml) in separate tubes containing 1% glucose with inverted Durham tubes was prepared and inoculated separately with 50 µl of 1% overnight fresh LAB cultures. Then the test tubes were incubated at 37°C for 3 days. The presence of gas in Durham tubes during 5 days of observation indicates CO_2 production from glucose (Bulut, 2003). Potent gas producers were selected for further tests.

3.6.3. Growth rate of yeast and LAB isolates

Yeast and LAB isolates from various traditional sources were grown in YEPD and de Man Rogosa and Sharpe broth, respectively, for quantifying the cell density. Microbial cells were detected by measuring optical density at 600 nm using a spectrophotometer (Scott, 2011 and Anna *et al*., 2020). Isolates with maximum/ heavy biomass were selected for subsequent studies.

3.6.4. H_2S production test of yeast isolates

Potent gas producers were streaked on Bismuth Sulfite Agar (BSA) (Himedia, India) and incubated at 30°C for 3 days to determine H_2S production, which is linked to an off-flavor and unpleasant taste. Hydrogen sulfide production is indicated by colonies that showed black color on BSA plates along the line of inoculation (Jiranek *et al.*, 1995). The positive isolates were discarded. Isolates with potent carbon dioxide production and isolates with none or slight H_2S production were selected for further analysis.

3.6.5. H_2S production test of LAB isolates

Selected LAB isolates were streaked on Kligler Iron Agar (KIA) (Himedia, India) containing plates and incubated anaerobically at 37°C for 48 hrs. Colonies that exhibited black color along the line of inoculation or throughout the butt indicate hydrogen sulfide production were discarded (Jiranek *et al.,* 1995). The positive isolates were discarded. Isolates with potent carbon dioxide production and isolates with none or slight H2S production were selected for further analysis.

3.6.6. Flocculation test of yeast isolates

The screened yeast isolates were inoculated in 10 ml of YPG broth and incubated at 30°C for 3 days. They were agitated to observe the flocculation forming. The best flocculent isolates were prioritized for further testing.

3.7.Stress tolerance of yeast and LAB isolates

3.7.1. Temperature tolerance test of yeast isolates

The ability of screened yeast cells to grow at different temperature values was characterized by inoculating yeast isolates in 5 ml YEPD broth medium. The pH of the medium was adjusted to 5 before autoclaving. Tubes containing this medium were inoculated with the same number of actively growing yeast cells (approximately 1.2 x 10^8 cell/ml) and incubated at 5 different temperatures: 25, 30, 37, 45 and 50°C (Guimarães *et al.*, 2006). Optical density (OD) at 550 nm was determined using a spectrophotometer (Jenway, United Kingdom) at 48 hours. The increase in optical density of the yeast cultures in a tube was recorded as evidence of growth. The growth of yeast cells was further confirmed by plating them on YEPD agar medium (Appendix 2). Potent gas producers, none H_2S producers, and thermotolerant isolates were selected for further analysis.

3.7.2. Temperature tolerance test of LAB isolates

The tolerance test of LAB isolates to different temperatures was examined by inoculating a pure culture (approximately 1.2 x 10^8 cell/ml) in 5 ml MRS broth and incubating for 48 hours at 10, 15, 37, and 45°C to assess their growth (Kimaryo *et al.*, 2000). Their turbidity on the broth medium was used to estimate growth (Askal Desiye and Kebede Abegaz, 2013). The growth of LAB isolates was further confirmed by plating them on MRS agar medium. LAB isolates with potent carbon dioxide production and thermotolerant ability were selected for further testing.

3.7.3. pH tolerance test of yeast isolates

The ability of screened yeast cells to grow at different pH values was also conducted by growing them in YEPD, and the pH was adjusted to 4, 5 and 6 using 1N HCl and 1N NaOH. The yeast isolates were inoculated (approximately 1.2 × 10^8 cells/ml) in 5 ml YEPD broth and incubated at 30°C. The growth was determined at 48 hours by measuring the OD at 550nm using a spectrophotometer (Jenway, United Kingdom). Further investigation was done by plating them on YEPD agar medium.

3.7.4. pH tolerance test of LAB isolates

The growth of screened LAB isolates at various pH levels was examined through the inoculation of a loopful of overnight culture into MRS broth that had been pH-adjusted to 4, 6, 8 and 10. They were kept in an anaerobic environment at 37°C for 48 hours. Their turbidity on the broth

medium was used to gauge growth (Askal Desiye and Kebede Abegaz, 2013). The growth of LAB isolates was further confirmed by plating them on MRS agar medium.

3.7.5. Ethanol tolerance test

The ability of screened yeast cells to grow in higher ethanol concentrations medium was characterized by growing them in YEPD broth medium with a pH value of 5 containing five different concentrations of ethanol: 2, 6, 10, 14 and 16% (*v/v*), respectively, and incubated at 30°C (Guimarães *et al.*, 2006). The growth was determined at 48 hours by measuring the OD at 550nm using a spectrophotometer (Jenway, United Kingdom). The growth of yeast was further confirmed by plating them on to YEPD agar medium.

3.7.6. Hyperosmotic tolerance test

Screened yeast isolates were cultured on the YEPD broth medium containing 30, 40, and 50% dextrose with a pH value of 5 and incubated at 30°C for 48 hours. The cell density of different yeast isolates in response to high dextrose concentration was measured using a spectrophotometer at 550 nm (Jenway, United Kingdom) (Karki *et al.*, 2017). The growth of yeast was further confirmed by plating them on to YEPD agar medium.

3.7.7. Salt tolerance test of yeast isolates

The growth of screened yeast cells at different NaCl, yeast extract, 5g/L; peptone, 5g/L; 5, 10, and 15% (*w/v*) NaCl broth was prepared with a pH value of 5. The isolates with the same number of actively growing (approximately 1.2 x 10^8 cells/ml) were inoculated in 50 ml flasks and incubated at 30°C. The growth was determined at 48 hours by measuring the optical densities at 550nm using a spectrophotometer (Jenway, United Kingdom). The growth of yeast was further confirmed by plating them on to YEPD agar medium.

3.7.8. Salt tolerance test of LAB isolates

The ability of LAB isolates to grow at different NaCl concentrations were examined by inoculating a loopful of overnight culture in a test tube containing MRS broth adjusted to 2, 4, 6.5, 8, and 10% NaCl. MRS medium without salt was used as a control. In order to evaluate growth based on the turbidity of the broth, they were then incubated at 37°C for 48 hours (Askal Desiye and Kebede Abegaz, 2013). The growth of LAB isolates was further confirmed by plating

them on MRS agar medium. LAB isolates with good salt tolerance ability were selected for further testing.

3.8. Evaluation of selected yeast and LAB isolates for making bread

Selected yeast cells and LAB isolates were grown in YEPD and MRS broth respectively at 30°C for yeast cells and 37°C for LAB in a shaker incubator at 150 rpm for 72 hrs. After 48 hrs, yeast cells were harvested through membrane filtration with a pore size of 0.45µm in order to obtain the yeast cake (Ríos *et al.*, 2012). The yeast cake (Appendix 3) was then dried at 50°C for 24 hrs before the estimation of dry weight (Almeida and Pais, 1996). The dried yeast was weighed and used as yeast starter culture for making bread. LAB isolates were collected through centrifugation for 25 min at 5,000 rpm, washed twice with distilled water, and the supernatants were discarded. The pellets were dried and weighed in order to use as LAB starter culture for making bread.

The screened yeast and LAB isolates were evaluated for their dough raising capacity. The four single yeast isolates (Y3, Y1, Y2 and Y4), and the combined effect of two yeast isolates (Y3 + Y1) and (Y3 + Y1 + Y2) were tested. Single LAB isolates (L1, L2, L3, L4 and L5) were also tested. Using selected yeast isolates, five types of bread were prepared in duplicate. BY3: bread made with yeast isolate Y3; BY1: bread made with yeast isolate Y1; BY2: bread made with yeast isolate Y2; BB141: bread made with yeast isolates Y5; BY4: bread made with yeast isolate Y4; and control: bread made with commercial bakery yeast, BCY. Commercially available wheat flour (Kojj all-purpose flour) was used for dough preparation. Bread was prepared by using wheat flour (50 g), yeast pellet (0.6 g), table sugar (3 g), salt (0.5 g), and distilled water (40 ml). The sugar and yeast pellets were first dissolved in lukewarm water in order to allow yeast activation (Karki *et al.*, 2017). The flour was added to the activated yeast mixture and thoroughly combined. Proofing was done by incubating the dough at 30°C for 3 hours. The commercial yeast strain was used as a positive control, whereas the dough without any yeast was used as a negative control. Then, the dough samples were baked in a microwave oven at 180°C for 20 minutes. Additionally, 10g of the dough mixture were kept in the measuring cylinder to monitor the rise of the dough level at 30°C and room temperature conditions. Every hour, the level was recorded while it was incubated for consecutive 6 hours (Aboaba and Obakpolor,

2010). The test was carried out in duplicates, and the promising yeast isolates were preserved for further analysis.

3.8.1. Bread Sensory Evaluation

According to Iwe *et al.* (2014), ten panelists were used to evaluate the sensory properties of five types of bread samples baked with local yeast starter for various aspects, including taste, color, texture, appearance, and overall acceptance. For sensory evaluation, a nine-point hedonic scale was employed: Dislike extremely: 1, dislike very much: 2, dislike moderately: 3, dislike slightly: 4, neither like nor dislike: 5, like slightly: 6, like moderately: 7, like very much: 8, and like extremely: 9 (Appendix 4). Before and after testing each piece of bread, the judges were asked to rinse their mouths with water.

3.8.2. Shelf Life Analysis of Bread

The bread samples were placed in sterile polyethylene bags and stored at room temperature until mold growth was observed visually on the surface of the bread samples. Visual observation of bread was carried out every day (Gámbaro et al., 2006).

3.9. Statistical Data Analysis

The data were subjected to statistical analysis using one-way analysis of variance (ANOVA). Means and standard deviations were calculated for leavening activities at room temperature and 30°C using SPSS version 25 software. The comparisons for each mean were performed by using Fisher's least significant difference (LSD) at $P<0.05$ level of significance (Chim *et al.*, 2015).

4. RESULTS

4.1 Isolation and purification of yeast and LAB isolates

In this study, a total of 54 samples (18 from each *Bord*e, *Tella*, and *Teff* dough) were collected from the study sites. A total of 230 yeast and 42 LAB isolates were purified and subsequently characterized (Table 4 and 6). They were compared to the commercial yeast in terms of cultural traits.

4.2 Identification and characterization of yeast isolates

4.2.1 Cultural characteristics of yeast isolates

The majority of colonies showed characteristics including an entire edge, a circular form with raised elevation, and a creamy to white color. The cultural characteristics of the commercial *Saccharomyces cerevisiae* strain were compared. According to the results of the cultural characteristics study, a greater percentage (69%) of *Tella* isolates, 68% of *Borde* isolates, and 47% of *Tella* isolates had an entire edge, a circular form with raised elevation, and a creamy to white color (Table 4). Fewer percentages of the isolates, 15% from *Tella*, 10% from *Borde*, and 13% from *Teff*, had rhizoid shape, smooth margin, and flat elevation with white colony color. After being incubated for 48 hours at 30°C, selected yeast isolates developed butyrous and smooth white elevated colonies on YEPD agar medium. Heavy, dry climbing pellicles formed on the surface of the YEPD broth medium after three days of incubation at 30°C (Figure 4).

Table 4. Cultural characteristics of yeast isolate (representative isolates)

Substrates	Shape	Color	Elevation	Margin	No. of isolate
Tella	Circular	Creamy white	Raised	Entire	9
	Irregular	Creamy	Convex	Serrate	2
	Rhizoid	White	Flat	Smooth	2
Borde	Circular	Creamy white	Raised	Entire	13
	Irregular	Creamy	Convex	Serrate	4
	Rhizoid	White	Flat	Smooth	2
Teff	Circular	Creamy white	Raised	Entire	7
	Irregular	Creamy	Convex	Serrate	6
	Rhizoid	White	Flat	Smooth	2
Total number of yeast isolates					47

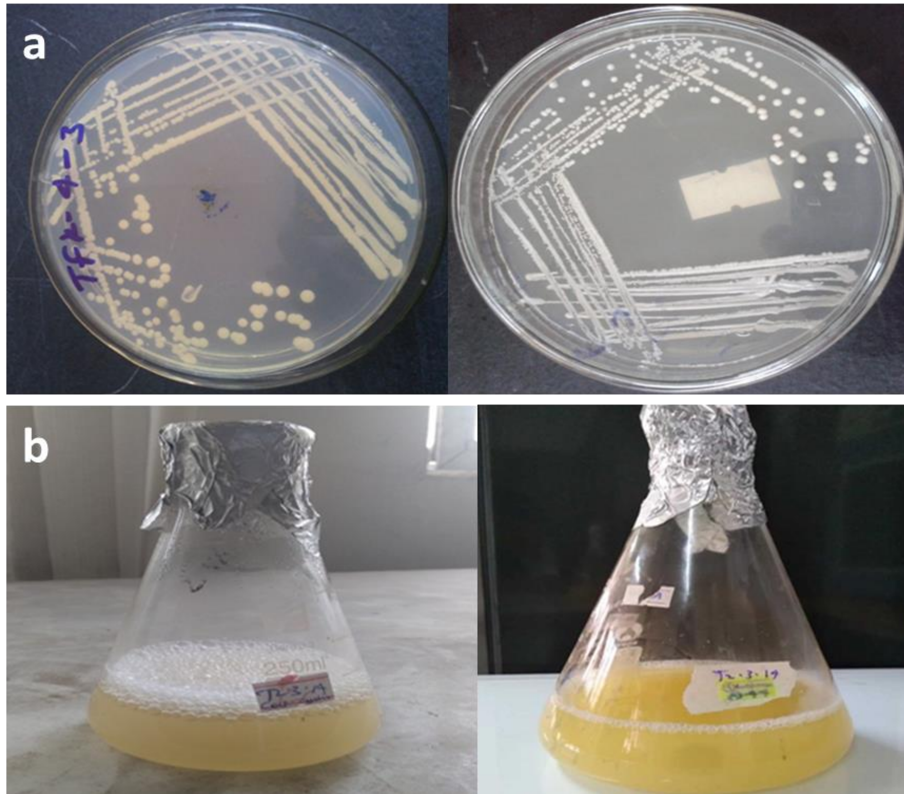

Figure 4. (a) Colonies on YEPD agar medium and (b) growth in YEPD broth medium

4.2.2 Morphological characterization of selected yeast isolates

The cell morphology of the yeast isolates was compared with the commercial yeast under a compound microscope (Olympus, Japan). Microscopic observation of the selected yeast isolates such as Y3, Y1, and Y4 showed a circular cellular shape, while Y2, Y5, and the commercial yeast showed an oval shape with clustered budding.

4.2.3 Biochemical characterization of selected yeast isolates

In the present study, all yeast isolates utilized glucose, sucrose, fructose, and maltose but failed to grow on lactose; that is, they were unable to utilize lactose and thus were possible Saccharomyces genera. Fermentation was observed by the presence of a gas bubble trapped inside the Durham tubes (Table 5). The color change from red to yellow indicated the fermentation using carbon sources (Appendix 5).

Table 5. Sugar utilization test of yeast isolates

Isolates	Dextrose Colour	Gas	Fructose Colour	Gas	Lactose Colour	Gas	Sucrose Colour	Gas	Maltose Colour	Gas
Y4	Yellow	2	Yellow	3	Red	0	Yellow	5	Yellow	2
Y5	Yellow	5	Yellow	2	Red	0	Yellow	2	Yellow	4
Y2	Yellow	4	Yellow	5	Red	0	Yellow	4	Yellow	4
Y3	Yellow	5	Yellow	5	Red	0	Yellow	4	Yellow	5
Y1	Yellow	5	Yellow	5	Red	0	Yellow	4	Yellow	5
Commercial yeast	Yellow	5	Yellow	5	Red	0	Yellow	5	Yellow	5

No bubble present, 0; the presence of a small bubble, 1; bubble filled ¼ of Durham tube, 2; bubble filled ½ of the Durham tube, 3; bubble filled ¾ of the Durham tube, 4; bubble filled the Durham tube, 5.

4.3 Identification and characterization of LAB isolates

A total of forty-two LAB isolates were identified from *Teff, Tella,* and *Borde* samples. Most LAB isolates had a white colony appearance and a rod shape, while some spherical isolates were also discovered. The bulk of the LAB isolates failed to produce gas from glucose, but five of the twenty LAB isolates that were chosen did. As a result, the majority of the isolates showed characteristics of being Gram-positive, catalase-negative, non-motile, and non-spore forming (Table 6). Fifteen isolates were homo-fermentative, meaning they did not produce CO_2 when fermenting carbohydrates and 5 isolates were heterofermentative, meaning they did. While some LAB isolates can ferment sucrose and lactose, all LAB isolates can ferment glucose and mannitol (Table 7). Finally, for further investigation, isolates having Gram-positive and endospore negative reactions were selected for further analysis.

Table 6. Morphological and physiological characteristics of LAB isolates

Characteristics	Isolates				
	L1	L2	L3	L4	L5
Colony color	White	White	White	White	White
Colony shape	Circular	Circular	Circular	Circular	Circular
Margin	Entire	Entire	Entire	Entire	Entire
Cell shape	Rod	Rod	Rod	Rod	Spherical
Gram stain	+	+	+	+	+
Spore staining	-	-	-	-	-
Motility	-	-	-	-	-
Catalase test	-	-	-	-	-

+ = Gram positive and - = Gram negative

Table 7. Biochemical identification of LAB isolates

Carbohydrate fermentation	Isolates				
	L1	L2	L3	L4	L5
Glucose	+	+	+	+	+
Lactose	+	+	-	+	+
Sucrose	+	+	+	+	+
Fructose	-	+	+	+	+
Mannose	+	+	+	-	-
Xylose	-	+	-	+	+
Mannitol	+	+	+	+	+
Gas	+	+	+	+	+

Positive (+) = showed yeast cell growth, and Negative (-) = showed absence of yeast cell growth.

4.4 Screening parameters of yeast and LAB isolates

4.4.1 CO_2 production test of yeast isolates

Yeast isolates had different degrees of carbon dioxide production capacity, and they were grouped as follows: strong gas producers, moderate gas producers, weak gas producers, and none gas producers. Within 24 hours of incubation at 30°C, forty-seven strong gas-producing yeast isolates were selected for further testing (Table 8). Compared to the other substrates, isolates from *Borde* were potent gas producers (Appendix 1). The selected yeast isolates have been preserved for further investigation.

Table 8. Isolates producing more CO_2 from each substrate at 24 hours of incubation

Samples	Total number of isolates	Best gas producers	Designation of best gas producers
Teff	45	11	Y47. Y41, Y44, Y13
Tella	133	13	Y3, Y46, Y31, Y38, Y24, Y32
Borde	52	23	Y5, Y1, Y14, Y35, Y2, Y16, Y4, Y15

4.4.2 CO_2 production test of LAB isolates

Based on their carbon dioxide production, LAB isolates were categorized as weak and none gas producers. LAB isolates showed very poor carbon dioxide production as compared to yeast isolates. From those forty-two purified LAB isolates, twenty weak gas producers were chosen for further study.

4.4.3 Growth rate of yeast and LAB isolates

Yeast and LAB isolates exhibited different growth rates as measured by their optical density (OD). From those forty-seven yeast isolates, high growth rates (OD value ≥ 1.7), were subjected to further testing. From those selected twenty LAB isolates, high growth rates (OD value ≥ 0.6) were selected for further testing.

4.4.4 H_2S production test of yeast and LAB isolates

On the basis of hydrogen sulfide (H_2S) production, yeast isolates were grouped into four categories (non-producers, slight level, moderate level, and intense level of H_2S producers). Accordingly, 13 yeast isolates were none H_2S producers (green), 6 yeast isolates were slight H_2S producers (light brown), 5 yeast isolates were moderate H_2S producers (dark brown), and 23 yeast isolates were intense H_2S producers (black color) (Figure 5). A total of 19 yeast isolates; none of the H_2S producers (13 isolates) and the low H_2S producers (6 isolates) were selected for further testing (Table 9). Regarding LAB isolates, 4 none H_2S producers and 3 low H_2S producers were selected for further testing.

Table 9. Test for H_2S Production of yeast isolates

Samples	Intense H_2S response (+++)	Moderate H_2S response (++)	Slight H_2S response (+)	No H_2S response (--)
Teff	7	1	1	2
Tella	4	1	1	6
Borde	12	3	4	5
Commercial yeast	0	0	0	2

+++: intense response; ++: moderate response; +: Slight response; --: no response

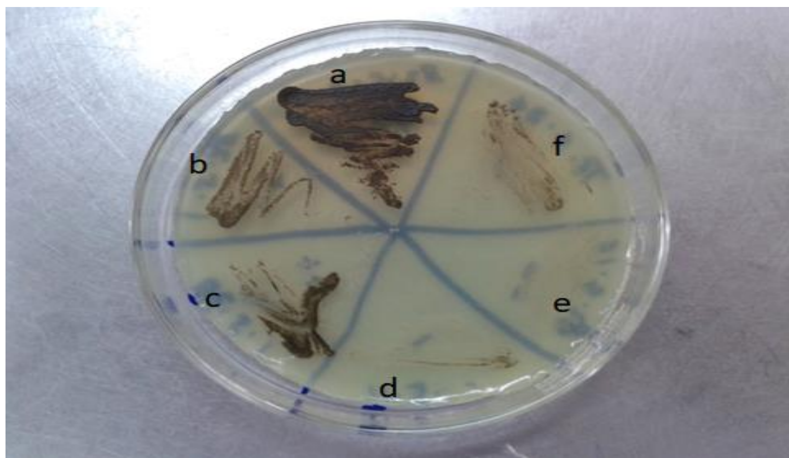

Figure 5. H_2S production test on BSA agar medium (a) isolates that produced intense amounts of H_2S (b) and (c) isolates that produced moderate amounts of H_2S (d) and (f) isolates that produced slight amounts of H_2S (e) isolates that did not produce H_2S

4.4.5 Flocculation Test

Three days at 30°C was used for incubating the yeast isolates after they had been injected in 10 ml of YPG broth. They were agitated to observe the flocculation forming. The majority of isolates had effective flocculation characteristics (Table 10).

Table 10. Flocculation Test of yeast isolates

Samples	Flocculation		Best flocculant isolates
	Positive	**Negative**	
Teff	7	3	Y42, Y45, Y41
Tella	12	2	Y3, Y34, Y31, Y35, Y26
Borde	20	3	Y1, Y5, Y4, Y21, Y2
Commercial yeast	2	0	Commercial yeast

4.5 Stress tolerance of yeast and LAB isolates

4.5.1 Thermo-tolerance test of yeast isolates

Yeast isolates grew at different temperature values and showed different responses (Table 11). The maximum biomass yield for all the isolates and the commercial yeast was observed at 30°C. OD reading value 2.39±0.04 for Y3-*Tella* isolate was noted maximum growth at 30°C. The commercial yeast, isolates from *Borde* (Y1, Y5, Y2, and Y4) and *Tella* (Y3) can withstand temperatures of up to 45°C. The optimum temperature for their maximum growth was 30°C. All yeast isolates were not grown at 50°C. The minimum biomass yield was observed at 45°C for all yeasts.

Table 11. Temperature tolerance test for yeast isolates

Temperature tolerance (°C)	Isolates at different temperatures					
	Y1	**Y2**	**Y3**	**Y4**	**Y5**	**CY**
25	0.41±0.03^{ac}	0.54±0.05^{ac}	0.69±0.04^{c}	0.57±0.04^{ac}	0.60±0.15^{c}	1.44±0.04^{b}
30	2.01±0.03^{d}	1.74±0.05^{b}	2.39±0.04^{d}	1.87±0.04^{b}	1.92±0.19^{b}	2.24±0.04^{d}
37	0.56±0.23^{c}	0.51±0.02^{c}	0.78±0.25^{c}	0.68±0.02^{c}	0.59±0.03^{c}	1.66±0.15^{b}
45	0.46±0.17^{ac}	0.40±0.16^{ac}	0.57±0.01^{c}	0.41±0.20^{ac}	0.53±0.19^{c}	0.96±0.17^{c}

Note: Means with the same letter are not significantly different at $p<0.05$.

4.5.2 Thermo-tolerance test of LAB isolates

Based on the influence of temperature on the growth of LAB, it was determined that the *Tella* (L2 and L5), *Borde* (L1) and *Teff* isolates (L4 and L3) all grew best at 37°C. In both 15 and 45°C, *Tella* and *Borde* isolates grew successfully (Table 12). At 10°C, *Borde* isolates grew well, *Tella* isolates grew slowly at this temperature, and *Teff* isolates (L4 and L3) were unable to grow there. The growth rate of LAB generally decreases as the temperature decreases.

Table 12. Temperature tolerance test for LAB isolates

LAB Isolates	Different temperatures			
	10°C	**15°C**	**37°C**	**45°C**
L1	+	+	+	+
L2	+/-	+	+	+
L3	-	+/-	+	+
L4	-	+/-	+	+
L5	+/-	+	+	+

+ = showed presence of LAB growth and - = showed no LAB growth

4.5.3 pH tolerance test of yeast isolates

Yeast isolates grew at different pH values and showed different responses. All the isolates were able to grow at all pH levels. For both isolate, pH 5 was the optimum value (Table 13). The findings indicated that the maximum biomass yield was achieved by the isolates Y1, Y2, Y3, Y4, Y5 and commercial yeast, were 1.87±0.05, 1.63±0.02, 1.91±0.07, 1.68±0.07, 1.79±0.01 and 2.29±0.03 respectively. Y3 isolates from *Tella* were more pH-tolerant than the *Borde* and *Teff* isolates.

Table 13. PH tolerance test of yeast isolates

pH tolerance	Isolates at different pH values					
	Y1	Y2	Y3	Y4	Y5	CY
4	1.21±0.03^{a}	1.40±0.05^{a}	1.50±0.04^{b}	1.07±0.04^{a}	0.98±0.01^{a}	1.68±0.01^{bc}
5	1.87±0.05^{b}	1.63±0.02^{bc}	1.91±0.07^{b}	1.68±0.07^{bc}	1.79±0.01^{bc}	2.29±0.03^{d}
6	1.56±0.02^{bc}	1.51±0.02^{bc}	1.38±0.03^{a}	1.38±0.02^{a}	1.58±0.01^{bc}	1.79±0.01^{bc}

Note: Means with the same letter are not significantly different at $p<0.05$.

4.5.4 pH tolerance test of LAB isolates

In general, it was found that isolates from Tella and Borde performed effectively at all pH levels, however isolates from *Teff* were less resistant to changes in pH. *Borde* isolates grew at pH 4, *Tella* isolates grew at pH 6, and *Teff* isolates were unable to grow at pH 2 and pH 4 (Table 14). Except for isolates from *Borde*, all isolates from LAB had optimal pH values of 6.

Table 14. PH tolerance test of LAB isolates

LAB IsolatesIsolates	Effect of pH on LAB isolates				
	2	4	6	8	10
L1	-	+	+	+	+
L2	-	+	+	+	+
L3	-	-	+	+	+
L4	-	-	+	+	+
L5	-	+	+	+	+

+ = showed presence of LAB growth and - = showed no LAB growth.

4.5.5 Ethanol tolerance

Both the commercial isolate and (Y3) isolate from *Tella* were highly tolerant to ethanol and grew up to 16% *(v/v)* alcohol concentration. The yeast cell species Y2, Y1, Y5, and Y4 isolated from *Borde* were moderately tolerant to ethanol and survived in environments with alcohol concentrations of between 10 and 14% *(v/v)* (Table 15).

Table 15. Ethanol tolerance test of yeast isolates

Ethanol tolerance (%)	Isolates at different ethanol concentrations					
	Y1	Y2	Y3	Y4	Y5	Commercial yeast
2	1.81±0.03^{a}	1.74±0.05^{a}	1.70±0.04^{a}	1.70±0.04^{a}	1.78±0.01^{a}	1.93±0.12^{a}
6	1.57±0.05^{ab}	1.63±0.02^{ab}	1.69±0.07^{ab}	1.59±0.07^{ab}	1.62±0.01^{ab}	1.78±0.02^{a}
10	0.67±0.00^{b}	1.51±0.02^{ab}	0.81±0.00^{b}	0.59±0.00^{bc}	1.58±0.00^{b}	1.32±0.00^{ab}
14	0.59±0.03^{bc}	0.43±0.21^{c}	0.79±0.12^{b}	0.45±0.03^{c}	0.64±0.13^{bc}	0.91±0.01^{b}

16	0.26±0.02[c]	0.24±0.13[c]	0.36±0.02[c]	0.27±0.01[c]	0.23±0.01[c]	0.49±0.03[c]

Note: Means with the same letter are not significantly different at $p<0.05$.

4.5.6 Salt-tolerance test of yeast isolates

The results of the study revealed the resistance of both yeast isolates to increasing osmotic pressure. All yeast isolates grew at their fastest rates on media containing 5% (*w/v)* NaCl, then gradually slowed down to 10% NaCl (*w/v*) and no growth at 15% NaCl (*w/v)* (Table 16). For both commercial and all yeast isolates, 5% NaCl (*w/v*) concentration produced the best growth.

Table 16. Salt tolerance test of yeast isolates

Salt tolerance (%)	**Isolates at different salt concentrations**					
	Y1	**Y2**	**Y3**	**Y4**	**Y5**	**Commercial yeast**
5	1.05±0.08[a]	0.97±0.02[a]	1.48±0.06[a]	1.28±0.04[a]	1.30±0.02[a]	1.73±0.01[a]
10	0.90±0.01[b]	0.87±0.03[b]	1.10±0.01[b]	0.59±0.07[c]	0.72±0.01[bc]	1.32±0.02[b]
15	0.70±0.02[bc]	0.65±0.01[bc]	0.82±0.01[b]	0.68±0.01[bc]	0.58±0.10[c]	0.92±0.01[b]

Note: Means with the same letter are not significantly different at $p<0.05$.

4.5.7 Salt tolerance test of LAB isolates

The results demonstrated the resistance of LAB isolates to medium osmotic pressure. LAB isolates grew at their fastest rates in media containing 2, 4, and 6% (*w/v*) NaCl, then at their slowest rates in media containing 8% (*w/v*) NaCl and 10% (*w/v*) NaCl. The two isolates from *Tella* were resistant to increased osmotic pressure at 8% and 10% salt, respectively (Table 17). For all LAB isolates, the 4% (*w/v*) NaCl concentration exhibited the best growth.

Table 17. Salt tolerance test for LAB isolates

Isolates	**Salt tolerance**				
	2%	**4%**	**6%**	**8%**	**10%**
L1	+	+	+	-	-
L2	+	+	+	+	+
L3	+	+	+	-	-
L4	+	+	+	-	-
L5	+	+	+	+	+

+ = showed presence of LAB growth and = showed no LAB growth.

4.5.8 Hyperosmotic tolerance test of yeast isolates

When exposed to varied dextrose concentrations, the cell density of several yeast isolates was compared to that of the commercial strain. The majority of isolates showed a comparable level of tolerance at both glucose concentrations. Isolate Y2 from *Borde showed* less tolerance to glucose in both concentrations. With the exception of Y2, all yeast isolates grew at high concentrations in media that contained up to 30% D-glucose, then decreased gradually in media that contained 40% D-glucose and even more in those that contained 50% D-glucose.

4.6 Dough raising capacity of local yeast and LAB starters

The rise of dough level versus the speed of the fermentation process was assessed by noting the rise of dough mixture kept in a measuring cylinder (Appendix 6). The dough in each container was similarly incubated for six hours at room temperature and 30°C. Before and after fermentation, the dough's height was measured in milliliters from the graduated surface of the cylinder.

The screened yeast and LAB isolates were evaluated for their dough raising capacity. The four yeast cell isolates (Y3, Y1, Y2 and Y4) and the combined effects of three yeast cell isolates (Y3+Y1, Y3+Y2 and Y3 + Y1 + Y2) were tested. Isolate Y3 from *Tella* revealed excellent leavening capacity, having the highest capacity (251.67 ± 7.6 ml) at four hours of fermentation, and dough fermented with the commercial strain having the highest capacity (245.67 ± 5.9 ml) at three and four hours. Between *Tella* (Y3) and *Borde* isolates (Y1, Y4, Y5 and Y2), there was a significant difference ($p<0.05$). Negative control dough made without yeast did not produce a volume increment (75 ml) within six hours of fermentation. In terms of leavening activity, there was no significant difference between Y3 and the commercial strain ($p>0.05$) (Table 18). After two hours of fermentation, the leavening activity of all the yeast isolates reached its peak. The leavening ability of yeast isolates at room temperature also showed a significant difference ($p<0.05$). The results showed that the isolate from *Tella* had a slightly larger rise in dough level (251.6 ± 77.6 ml), although it took 4 hrs for the fermentation time to reach its maximum. Therefore, the dough rose even higher than the commercial yeast tested, but the commercial strain's fermentation took three hrs to reach its peak leavening (Table 18).

The combined effect of the selected yeast isolates (Y3+Y1) and (Y3 + Y1 + Y2) on dough rising were evaluated. Results of the three co-inoculated isolates (Y3 + Y1 + Y2) showed the highest (275 ml) at 120 min; while the rising volume of dough of the two combinations (Y3+Y1) was 261 ml at 120 min. The combined effect of the isolates displayed better leavening capacity with short fermentation time as compared to the single isolates.

The five screened LAB isolates (L1, L2, L5, L4 and L3) were evaluated for their dough raising capability test and revealed very poor leavening capacity compared with co-inoculated isolates; therefore, none of the isolates were selected for further testing.

Table 18. Leavening action of yeast isolates at 30°C

Fermentation time (hrs)	Isolates				
	Y1	**Y2**	**Y3**	**Y4**	**CY**
0 hr	78.3±2.89^{a}	78.3±2.89^{a}	75±5^{a}	78.3±2.89^{a}	73.3±2.89^{a}
1 hr	78.3±2.89^{a}	78.3±2.89^{a}	128.7±6.51^{b}	78.3±2.89^{a}	167±4^{b}
2 hr	81±1.73^{a}	78.3±2.89^{a}	197±8^{c}	78.3±2.89^{a}	231.7±5.03^{c}
3 hr	123.3±8.62^{b}	84.3±4.51^{a}	226±7^{d}	83.3±4.04^{a}	245.7±5.86^{d}
4 hr	136.3±20.10^{b}	96.7±6.03^{b}	251.7±7.57^{e}	95.7±6.11^{b}	245.7±5.86^{d}
5 hr	169.7±15.94^{c}	159.7±12.22^{c}	252±7.81^{e}	129±11.79^{c}	220.3±6.11^{de}
6 hr	195.7±6.50^{d}	194.7±6.03^{d}	237.3±12.34^{de}	164±11.14^{d}	193.7±8.08^{de}

Note: Means with the same letter are not significantly different at $p<0.05$.

4.7 Evaluation of bread quality made with yeast starters

4.7.1 Sensory analysis of bread

Using the screened single yeast isolates, five types of bread were prepared in duplicate. BY3: bread made with yeast isolate Y3; BY1: bread made with yeast isolate Y1; BY2: bread made with yeast isolate Y2; BY4: bread made with yeast isolate Y4; and control: bread made with commercial bakery yeast, BCY. A nine-point hedonic scale was used to analyze the data and perform sensory perception of appearance, color, texture, flavor, and overall acceptability (Appendix 4). The sensory evaluation of the bread made with commercial yeast compared with local yeast isolated from *Tella* and *Borde*. The bread made with commercial yeast (BCY) scored higher than the bread made with *Tella* isolates (BY3) in every sensory category (Table 19). Between the two breads (BY3), bread made with Y3 and (BCY) bread made with commercial

yeast, there were no significant variations in the sensory scores for any of the qualities. The average acceptability rating for bread made with commercial yeast (BCY) was 6.18 ± 0.17, compared to 5.70 ± 0.57 for bread (BY3) made with Y3.

Table 19. Sensory evaluation of bread made with local yeast starter and commercial strain

	Characteristics and desirability of bread				
Breads	**Taste**	**Color**	**Texture**	**Appearance**	**Overall Acceptability**
BY1	3.43±0.59[a]	4.90± 0.35[a]	3.94± 1[a]	3.09± 0.72[a]	3.84± 0.27[ab]
BY3	5.45±0.68[a]	5.82±0.38[a]	5.74± 0.61[b]	5.55± 0.55[bc]	5.64± 0.13[ac]
BY2	2.94±0.43[a]	5.49±0.85[a]	3.94± 1[a]	4.95± 0.3[bc]	4.33± 0.33[ab]
BY4	3.09±0.72[a]	5.15±0.56[a]	5.37± 0.62[b]	4.67± 0.89[b]	4.57± 0.14[b]
BCY	6.06±0.2[a]	5.86±0.61[a]	6.20± 0.08[b]	6.00± 0.52[c]	6.03± 0.25[c]

Mean values ± Standard deviation of three independent experiments and means with the same letter are not significantly different at $p<0.05$.

4.7.2 Shelf Life Analysis of Bread

Visual observation of mold growth on the surface of the bread was used to determine the shelf life of the bread. When mold growth begins visual observation on the surface of bread, it was spoiled. Based on this principle, the shelf lives of the bread made with commercial yeast (BCY), Y3 (TlB24), Y1 (BY1), Y2 (BY2), and Y4 (BY4) breads were 4, 5, 3, 4 and 3 days respectively. The bread (TlB24) made with *Tella* isolates had the highest shelf life than the bread made with commercial yeast (BCY).

5. DISCUSSION

In this study, a total of 230 yeast and 40 LAB isolates were purified from local fermented food and drinks and allowed to be characterized. The morphological characteristics of the screened yeast isolates observed using a microscope under (1000X magnification) showed that the screened yeast isolates were unicellular with spherical or oval cell shapes, compared to commercial yeast cells. This result is in agreement with (Greame, 2005) who confirmed the characteristics of *Saccharomyces.* Similar conclusion was reached on the basis of growth pattern studies in liquid and solid YPD media; in all cases, white and creamy colonies with smooth colony textures were obtained after subculture (Thapa *et al*., 2015). The result of the cultural characteristics showed that the colonies of the yeast isolates were creamy to creamy white, circular, with entire/ smooth margins, and raised elevation after 48 hrs of incubation at 30ºC (Table 7). The colonies exhibiting characteristics such as creamy to white color, fluffy, and smooth margins were designated as the genus Saccharomyces. As reported by (Walker and White, (2017); Graeme and Nia, (2005), cream colonies are the characteristic of yeast, especially Saccharomyces. Furthermore, based on the ovoid microscopic shape, the presence of ascospores in asci, and budding pattern (multipolar), Rahman (2013) determined that all isolates were found to belong to the Saccharomyces type of unicellular ascomycete. The results of this study verified earlier research indicating that yeast from *Teff* dough and *Tella* are of the *Saccharomyces* type. The yeast isolates were proven to be yeast when they were examined under a 40x microscope during the budding stage (Tamene Milkessa and Dawit Abate, 2009).

All 20 of the isolates that were chosen for this investigation were determined to be LAB through an examination of their morphological, biochemical, and physiological traits. As a result, while the majority of the isolates were determined to be homofermentative types, some were found to be heterofermentative types. This result is consistent with the findings of Asnake Dessalegn and Mogessie Ashenafi (2010), who found that small percentages (6%) of the isolates were heterofermentative, the bulk (94%) of the LAB isolated from teff dough, awaze, and kotchqotcha were homofermentative. Gram-positive lactic acid bacteria are grouped together based on common morphological, metabolic, and physiological traits. They are catalase-negative, anaerobic or microaerophilic, non-sporulating rods or cocci, and acid-tolerant. Furthermore, lactose and other carbohydrates can be converted to lactic acid by the majority of LAB members

(Chowdhury and Ferdouse, 2012). Despite being mesophilic, some species can flourish at temperatures as low as 5°C and as high as 45°C. While most LABs thrive in the pH range of 4.0-4.5, some can grow in an acidic pH (3.2), while others may grow in an alkaline pH (9.6) (Ratau, 2018). Although there can be differences in their reactivity, salt tolerance (6.5% NaCl) can also be utilized to identify LAB.

In our study, yeast cells and LAB isolates exhibited an intense, moderate, slight, and none response to hydrogen sulfide production. H_2S is an undesirable compound associated with an off-flavor and unpleasant taste that must be absent in processed foods (Winter, 2012). Yeasts and LAB isolates that showed high production of H_2S are undesirable for bread making because it confers flavor and taste that compromise the quality of the bread (Castillo Lozano *et al*., 2007). Therefore, the yeast and LAB species that did not produce H_2S or low level of this compound could be recommended as the best candidate isolates in bread making. Nineteen yeast isolates (13 none-H_2S producers and 6 slight H_2S producers) and seven LAB isolates (4 none H_2S producers and 3 low H_2S producers) were selected from the screened 47 yeast and 20 LAB isolates. This result was comparable to the work of (Tika *et al*., 2017), who evaluated both none-H_2S producers and low-H_2S producers for bread production. The non-sulfide generating yeast isolates, which exhibited green colonies included Y1, Y2, Y3, Y4, Y5, etc., did not produce H_2S. Isolates exhibited much black color on Bismith Sulphate Agar and Kliger Iron Agar medium are designated as heavy H_2S producers (Jiranek *et al*., 1995) as noted in this study. Therefore, some of the wild yeast and LAB isolates in the present study could be potential candidates for wheat dough leavening for bread making since they showed no or light production of H_2S and also had better fermentation ability than commercial yeast.

Yeast cells and LAB isolates showed different carbon dioxide production capacities (strong, medium, low, and none). Based on carbon dioxide production, 47 strong gas-producingyeast and 20 low gas-producingLAB were selected from 230 yeast and 42 LAB isolates. Carbon dioxide production is the basic criterion for selecting high-performing bakery yeasts. Yeasts produce carbon dioxide, ethanol, and other secondary metabolic products during the fermentation of carbohydrates. These products help to formulate flavor and aroma, and the carbon dioxide produced acts as a leavening agent in the dough. Strains can also be chosen according to gas

generation, as this is the most crucial criterion for leavening and can also be an indicator of strong invertase activity (Zerihun Tsegaye *et al.*, 2018b). A gas bubble that was caught inside the Durham tubes revealed fermentation. Because *S. cerevisiae* cannot ferment lactose, it may be identified. This study's results were similar to earlier reports that the strain lacked the β-galactosidase system or lactase (Xavier *et al.*, 2018).

Bai *et al.* (2022) claim that yeast cells with the ability to flocculate due to a process called cell adhesion are a fascinating characteristic in the brewing and bread-making industries. Because it can eliminate the need for extra filtration and centrifugation stages during bulk production at the industrial scale, this phenomenon has an impact on the cost of producing yeast biomass. Additionally, yeast species' flocculation qualities guarantee a high cell density and a sizable volume of harvested cells, and they can also increase the fermentation process's ethanol productivity.

The effect of temperature on growth and maximal biomass yield revealed that all yeast isolates in this study produced their maximum biomass at 30°C, which is in good agreement with the finding of Nitayavardhana *et al*. (2010) that the optimal temperature is approximately 30°C. Certain yeast isolates from *Tella* and *Borde* (Y3, Y1, Y2, Y5 and Y4) in this investigation were able to withstand temperatures as high as 45°C. These results were consistent with what was reported by (Umeh *et al*., 2019), who stated that some yeast isolates could grow at 45°C. The findings of this study also agreed with previous studies that reported yeasts could withstand temperatures as high as 40°C (Elsa Beyene *et al.*, 2020; Choi *et al.*, 2010). Most of the *Teff* isolates in this study were tolerated at temperatures of up to 37°C only, and the outcome of this study agreed with the conclusions of (Maaruf *et al.*, 2011), who reported that yeast isolates could only withstand temperatures of up to 37°C. Given that yeast can handle high temperatures, it is possible to utilize the isolates to carry out fermentation at a variety of temperature ranges since they are able to tolerate the excess heat generated during the fermentation process. They can also be added to bread dough to quicken the proofing process, produce more carbon dioxide, and improve taste and aroma development. Stress related to baking can cause significant harm to yeast isolates with low thermotolerance (Munna *et al.*, 2015).

It has been discovered that yeast cells can survive in a pH range of 3.6 to 6.0, most especially *S. cerevisiae*, or baker's yeast (Pitt and Hocking, 2022). Depending on the temperature, oxygen content, and yeast strain, the ideal pH range for yeast growth can range from pH 4.0 to 6.0. It has been suggested that utilizing yeast in less aseptic equipment can minimize loss from bacterial contamination because of the yeasts' capacity to function well below pH 4.0 (Appiah, 2013). Isolates (Y3) from *Tella* and commercial isolate grew effectively at alcohol concentrations ranging from 14 to 16% and showed a strong tolerance to ethanol. *Borde's* isolated yeast species (Y2, Y1, and Y4) grew well at alcohol concentrations of 10 to 14 and had a moderate ethanol tolerance. The results of this study are consistent with the research of (Maaruf *et al.*, 2011), which found that certain yeast species could grow in media with ethanol concentrations of 10 and 13% (*v/v*), but only a small number of yeast species could grow in media with ethanol concentrations of 15% (*v/v*). According to (Stanley *et al.*, 2010), a high alcohol concentration is damaging to yeast cells because it damages the cell membrane, which prevents the cell from growing. The current study explained that the majority of the isolates' grow slow at 15% concentration. For bread to have the desired flavor, the alcohol content must be at an appropriate level (Stanley *et al.*, 2010).

The growth of both isolates reached a peak in media containing 5% NaCl and progressively decreased in media containing 10 and 15% NaCl. This result is consistent with the findings of Meier-Dörnberg *et al.* (2017), which stated that the majority of *Saccharomyces* isolated from traditional fermentation techniques had extreme conditions integrated into their physiological makeup. The ideal growth was achieved with 5% NaCl and 30% D-glucose. This implies that along with commercial yeasts, they are able to withstand a greater osmotic pressure. A yeast strain must withstand the low osmotic pressure during baking. Cell survival is largely dependent on the medium's osmotic pressure (Maaruf *et al.*, 2011).

The maximum dough-raising volumes and times required to reach such levels varied among different yeast isolates. The screened wild yeast isolates displayed relatively higher baking potential than commercial yeast. In this study, the maximum leavening capacity was achieved by *Tella* isolate Y3 followed by the commercial strain. After four hours of fermentation at 30°C, the isolate Y3 (251.67 ± 7.6 ml) achieved the highest leavening volume. In a related investigation, commercial yeast (245.67 ± 5.9 g) fermented for the lowest number of time-three hrs. The results

of the study showed that the yeast isolates' fermentative power is superior to that of the tested commercial yeast. According to studies conducted by Appiah (2013), yeast species isolated from native sources showed superior characteristics in fastening the fermentation process. While the growth in Y3 was the largest and reached its peak in four hrs of fermentation, the rise in commercial yeast was the fastest and reached its peak in just three hrs. After two hrs of fermentation, all of the yeast isolates in this investigation reached their peak leavening activity. This finding is consistent with a study by Nasr and Zaky (2011) that examined the dough rising power of various brands of baker's yeasts sold in Egypt, including those from Turkey, China, the UK, and Egypt. In addition, the results of this study contradict the conclusions of Elsa Beyene *et al.* (2019a), who reported that the powerful yeast cell isolates displayed their maximum leavening activity during the first two hrs of fermentation. The experiments revealed that the native yeast isolate's fermentative power is superior to that of the tested commercial yeast. Therefore, when it comes to fermenting bread dough, yeast isolate can be a great substitute for commercial baker's yeast.

In comparison to dough fermented with a separate yeast isolate, almost all dough fermented with a mixture of yeasts rose in volume more with a shorter fermentation time. The combined effect of the three co-inoculated isolates (Y3 + Y1 + Y2) was found the highest 275 ml at 120 min; while the raising volume of dough as a result of co-inoculation of two isolates (Y3+Y1) was found 261 ml at 120 min. This result was consistent with the conclusion reached by (Liliane *et al*., 2019). The ability of yeast isolates to raise dough varied. The diversity of yeast species, which affects how much gas is produced, may be the cause of this variation (Jelana *et al*., 2011).

The sensory evaluation of the bread made with commercial yeast compared with local yeast isolated from *Tella* and *Borde*. Five types of bread were prepared from the screened separate yeast isolates. Sensory perception in relation to appearance, color, texture, flavor, and overall acceptability was conducted, and a nine-point hedonic scale was used to analyze the results. The bread made with commercial yeast (BCY) scored a bit higher than the bread made with *Tella* isolates (BY3) in every sensory category (Table 12). Between the two breads (BY3); bread made with Y3 and (BCY) bread made with commercial yeast, there were no significant variations in the sensory scores for any of the qualities. The average acceptability rating for bread made with

commercial yeast (BCY) was 6.18 ± 0.17, compared to 5.70 ± 0.57 for bread (BY3) made with *Tella* isolates Y3. The results of the present study were greater than the findings of (Mehmet, 2023) who reported that the color, taste, odor, texture, and overall acceptability scores of breads made with yeast isolates were 5.4, 4.0, 4.0, 3.8, and 3.8, respectively. The outcome of the study was fairly consistent with Malik *et al.* (2021).

The shelf lives of the bread made with commercial yeast (BCY), Y3 (BY3), Y1 (BY1), Y2 (BY2), and Y4 (BY4) breads were 4, 5, 3, 4 and 3 days respectively. The bread (BY3) made with *Tella* isolates had the highest shelf life than the bread made with commercial yeast (BCY), which is in good agreement with the report made by Melkam Dessalegn and Berhanu Andualem (2023).

In general, the study focused on understanding and enhancing the leavening capacity of wild yeast and lactic acid bacteria in bread had revealed best performance compared to commercial yeast. Characterizing the properties of these microorganisms and optimizing conditions to improve their performance in leavening dough is very essential to achieve better bread quality through a deeper understanding of the fermentation process and the interactions between wild yeast and lactic acid bacteria in the context of bread making. Overall, the study provided a comprehensive analysis of the optimization process, compared the performance of wild yeast and lactic acid bacteria to commercial yeast, discussed sensory and nutritional aspects, addressed practical implications for the baking industry, and outlined potential areas for future research.

6. CONCLUSION AND RECOMMENDATION

6.1. Conclusion

In conclusion, the study confirms the accessibility of yeast isolates from local fermented foods and drinks such as *Tella*, *Borde* and *Teff* dough and demonstrating excellent dough leavening capabilities suitable for industrial baking. Among the tested attributes, the yeast species isolated, specifically Y3, outperformed commercial yeast, exhibiting tolerance to temperatures up to 45°C and alcohol concentrations up to 16%. These isolates, along with commercial yeast, demonstrated fermentation abilities for various sugars but not lactose. The outcome of the present study also demonstrates that the isolate from *Tella* (Y3), had a maximum leavening capacity (251.67 ±7.6 ml) than the commercial strain (245.67 ± 5.9 ml). The combined effect of the isolates displayed improved leavening capacity with short fermentation time as compared to the single isolates. The average acceptability rating for bread made with commercial yeast was 6.18 ± 0.17, compared to 5.70 ± 0.57 for bread made with *Tella* isolates (Y3). The bread made with *Tella* isolates (BY3) had the highest shelf life (5 days) than the bread made with commercial yeast (4 days). The screened LAB isolates revealed very low leavening capacity compared to yeast isolates. These findings concluded that local fermented foods and drinks can serve as a rich source of baker's yeast, offering a potential alternative for dough leavening.

6.2.Recommendations

1. Molecular characterization of potential yeast and LAB isolates.
2. Further study on modeling and optimizing of yeast and LAB isolates using RSM.
3. Further investigation of sourdough starter culture

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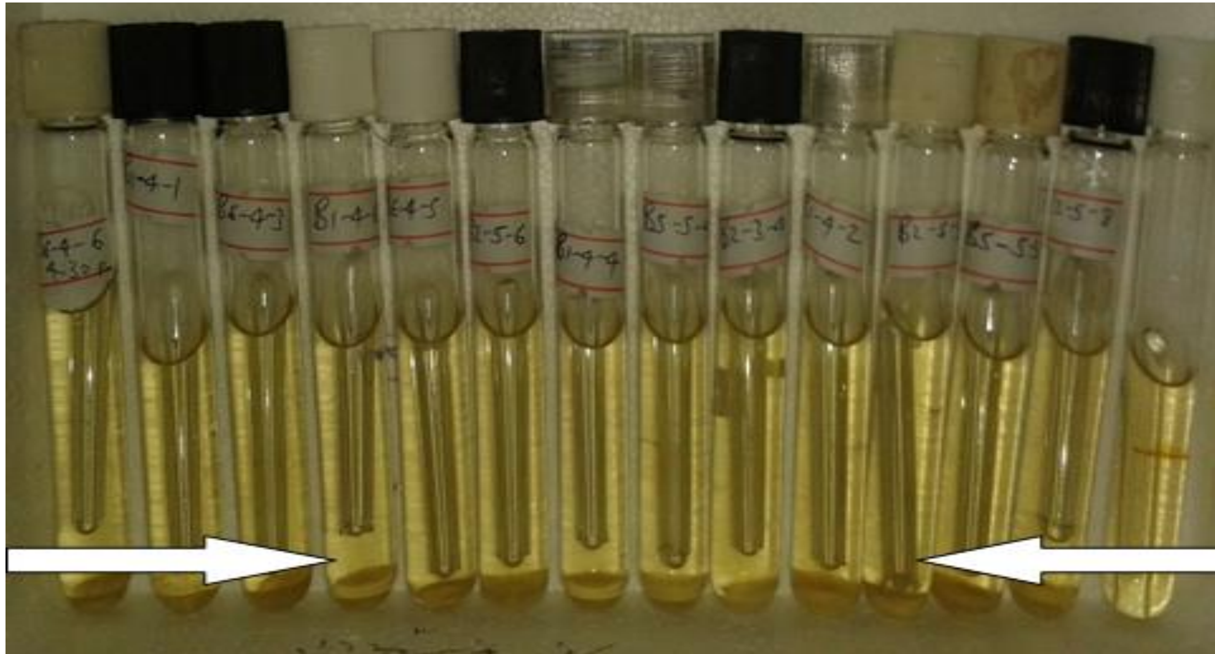
Appendices

Appendix 1: Best CO_2 producers of *Borde* isolates



Appendix 2: Effect of different temperatures on yeast growth

Yeast isolates	Effect of different temperatures on yeast growth				
	25°C	**30°C**	**37°C**	**45°C**	**50°C**
Y2312	+	+	+	-	-
Yl649	+	+	+	-	-
Y48	+	+	+	-	-
Y412	+	+	-	-	-
Y4	+	+	+	+	-
Y249	+	+	+	-	-
Y5	+	+	+	+	-
Y2315	+	+	+	-	-
Y45	+	+	+	-	-
Y52	+	+	-	-	-
Y451	+	+	-	-	-
Y411	+	+	-	-	-
Y2	+	+	+	+	-

Y44	+	+	-	-	-
Y43	+	+	+	-	-
L4	-	+	-	-	-
Y46	+	+	+	-	-
Y41	+	+	+	-	-
Y554	+	+	+	-	-
Y1333	+	+	+	-	-
Y6422	+	+	+	-	-
Y645	+	+	-	-	-
Y142	+	+	+	-	-
Y234	+	+	+	-	-
Y253	+	+	+	-	-
Y6424	+	+	+	-	-
Y51	-	+	-	-	-
Y657	+	+	+	-	-
Y3	+	+	+	+	-
Y1	+	+	+	+	-
Y415	+	+	-	-	-
Y414	+	+	-	-	-
Y553	+	+	+	-	-
Y239	+	+	+	-	-
Y134	+	+	+	-	-
Y1315	+	+	+	-	-
Y57	+	+	-	-	-
Y643	+	+	+	-	-
Y34	+	+	+	-	-
Y53	+	+	-	-	-
Y49	+	+	-	-	-
Y6421	+	+	+	-	-
Y451	+	+	-	-	-
Y42	+	+	-	-	-
Y644	+	+	+	-	-
Y38	+	+	+	-	-
Y6418	+	+	+	-	-
Commercial yeast	+	+	+	+	-

Appendix 3: Collected yeast cake

Appendix 4: Ballot for the sensory evaluation of bread

Sensory scorecard using nine-point hedonic scale for bread leavened with wild yeast isolates.

Panelist code/Name____________________Date__________________Age________

Instruction: Kindly TASTE and EVALUATE each bread sample using the scale provided below and place the corresponding score on the space provided that best reflects your feelings about the sample. Choose the descriptor which, in your opinion, is the most applicable to the characteristics being evaluated.

You are presented with a food sample. Please taste and tick that which describes how you feel about the sample.

Characteristics anddesirability	Sample code	S1	S2	S3	S4	S5	S6
Taste							
Colour							
Texture							
Appearance							
Overall acceptability							
Comment							

Characteristics

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

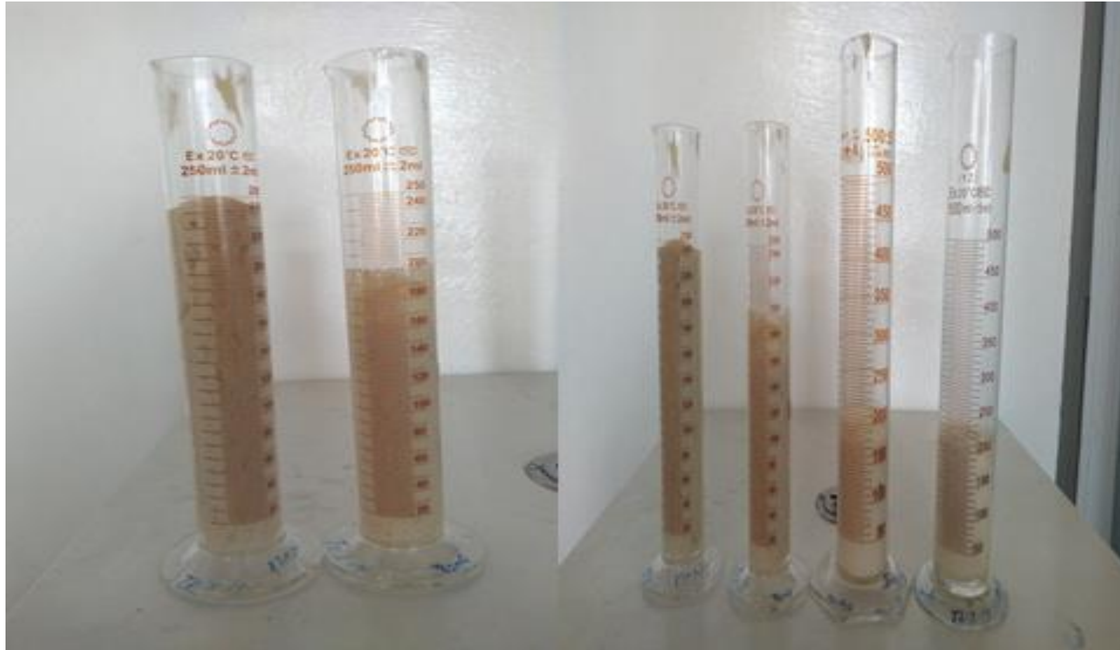
Overall acceptability

1. Acceptable slightly 2.Acceptable moderately
3. Acceptable, very much 4.Acceptable extremely
5. Extremely unacceptable 6.Very much unacceptable
7. Moderately unacceptable 8.Slightly unacceptable
9. Neither acceptable nor unacceptable

Please rinse your mouth with water before testing each sample.

Appendix 5: Carbohydrate utilization test before and after fermentation

Appendix 6: Wheat dough fermentation in a measuring cylinder



Appendix 7: Wheat dough preparation for baking