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IMPROVEMENT OF SHELF LIFE OF ‘INJERA’ USING CHEMICAL PRESERVATIVES

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July, 2009
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DEDICATION

This work is dedicated to my family who I love most in this world more than anything.

To:  My father Zewdu Woldegiorgis (Fazuca)

My mother Yeshareg Bezabh (Mazuca)

My brother Estifanos Zewdu (Adinee)

My sisters Kidest Zewdu (Krro) and Hiwot Zewdu (Dudu)
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LIST OF ABBREVIATIONS AND ACRONYMS

ADI- Average Daily Intake
\(a_w\)- Water Activity
CSA- Central Statistical Agency (of Ethiopia)
CYP- Czapek’s Solution Agar
Eh or O/R- Oxidation reduction potential
EHNRI- Ethiopian Health and Nutrition Research Institute
FAO-Food and Agriculture Organization
FDA- Food and Drug Administration (of USA)
g- Gram
GRAS- Generally Regarded As Safe
ISA-Injera Sucrose Agar
JECFA- Joint Expert Committee on Food Additives
Kg- Kilogram
l- Litre
MAP- Modified Atmosphere Packaging
MEA- Malt Extract Agar
ml- Millilitre
NA-Nutrient Agar
PDA-Potato Dextrose Agar
ppm- Parts Per Million
SPSS- Statistical Product and Service Solutions
WHO- World Health Organization
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Injera is staple Ethiopian fermented bread which can be made from different types of cereals, particularly from tef (*Eragrostis tef*). About two-third of Ethiopian diet consists of injera and it accounts for about two-third of the daily protein intake of Ethiopian population. It has a very high nutritional value, as it is rich in calcium and iron. Unfortunately, injera has a short shelf life of 3-4 days essentially due to mould spoilage. The use of weak organic acid as preservatives is allowed in acidic foods, primarily as mould inhibitor. In this study, the influence of some commonly used chemical preservatives, benzoic acid, sodium benzoate, potassium sorbate and calcium propionate was investigated by isolation of moulds before and during its ambient storage for 12 days. The preservatives were added immediately before baking at the concentration of 0.1% of benzoic acid/ sodium benzoate, 0.2 % of potassium sorbate, 0.3 % of calcium propionate and 0. 2 % blend of the four (wt/wt) as recommended by Food and Drug Administration. Three fungal species: *Aspergillus niger*, *Penicillium sp* and *Rhizopus sp* found to be responsible in injera spoilage. *Penicillium* and *Rhizopus* are more dominant at the temperature of between 16-20°c, while *Aspergillus niger* is more dominant at higher temperature of between 25-32°c. Injera samples had a pH and moisture content of 3.38-3.45 and 62-65%, respectively. Antimicrobial activities of the preservatives investigated prolong the shelf life of injera up to12 days. The effectiveness of preservation was ranked as sodium benzoate>benzoic acid>potassium sorbate>blend >calcium propionate showing that benzoates and benzoic acid are the most effective. The incorporation of benzoic acid and its salt while preparing injera at home or in larger scale processing should be practiced so as to save a significant amount of injera that is lost due to mould spoilage.
CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Injera is a thin, fermented Ethiopian traditional bread made from flour, water and starter (ersho) which is a fluid saved from previously fermented dough. Tef (*Eragrostis tef* (Zucc) Trotter) is the most popular grain for making injera; although other grains such as sorghum, maize, barley, wheat and finger millet are sometimes used. The major quality attribute of a good injera is its slightly sour flavour (Adamu Zegeye, 1997). Fellow (1997) reported that normal and typical injera is round, soft, spongy and resilient, about 6 mm thick, 60 cm in diameter with uniformly spaced honeycomb-like ‘eyes’ on the top. Injera has a very high nutritional value, as it is rich in calcium and iron. ‘Wot’ in the Ethiopian national language (Amharic) means a stew which is made from plant and animal products is served with injera. As a result of this, injera is not only a kind of bread –it is also an eating utensil (Science of cooking, n.d). Injera is the undisputed national food of Ethiopians (Blandino *et al*., 2003). According to Ball *et al*., (1996) about two-third of Ethiopian diet consists of injera and it accounts for about 2/3 of the daily protein intake of Ethiopian population (Arogundade, 2006). Unfortunately, injera storage period does not usually exceed 3 days at ambient temperature under the traditional storage conditions essentially due to mould spoilage.

Food preservation has been a long lasting desire of human beings (Juneja *et al*., 2008). It was the prerequisite to man settling down in one place, instead of moving from place to place in the never ending hunt for fresh food. The earliest preservation technologies developed were drying, smoking, chilling and heating. Later on, the art of controlling these technologies was
developed. The use of various compounds such as salt and spices to preserve foods was also used in ancient times (Zeuthen and Bogh-Soresen, 2003).

Early history is evident of the chemical preservation of food and its various products. The addition of chemicals to food is not a recent innovation; it is being practiced throughout the world (Bohra and Pariah, 2006). Food preservatives are added to stop or delay nutritional losses due to microbiological, enzymatic or chemical changes of foods during its shelf life. They also prevent consumer hazards due to the presence of microbial toxins or pathogenic microorganisms and economic losses due to spoilage (Russell and Gould, 2003).

Benzoic acid, sorbic acids, and propionic acid and their respective sodium, potassium and calcium salts are the most commonly used preservatives in foodstuffs. They are generally used to inhibit yeast and mold growth, being also effective against a wide range of bacteria. These compounds are most active in foods of low pH value and essentially ineffective in foods at neutral pH values (Santini et al., 2009).

1.2 STATEMENT OF THE PROBLEM

Quite a substantial amount of food is lost at household level due to two major reasons. The first is the direct loss due to microbial spoilage. Though no statistical data is available, it is assumed that a significant amount of injera is lost due to moulding at household level per baking cycle. The second type of loss is rather indirect. In many instances, people tend to consume more than they require because most households cannot afford cooling devices to extend the keeping quality of the food. Food item, which could last much longer under proper processing or storage, are consumed, thus ‘lost’, in much shorter period of time without the
physiological need to do so. Microbiological studies to improve the keeping quality of indigenous foods through microbial processing, use of food preservatives or combination of both would significantly contribute to curb problems of food shortage at household level (Mogessie Ashenafi, 2002; 2006).

Several management tools such as the use of chemical preservatives are available to help control mold contamination (Higgins and Brinkhaus, 1999). Sorbic acid, propionic acid and benzoic acid and their potassium, calcium and sodium salts are permitted in breads, cakes, certain cheese, and other foods, primarily as a mould inhibitor (Jay, 2000). According to Carson, (2000) it took more than 27 days for bread with chemical preservatives to be 100% covered by moulds and other microorganisms. Mogessie Ashenafi (2006) suggested the inclusion of sorbates or benzoates in the right proportion after completion of fermentation and immediately before baking may improve the spoilage of injera due to moulding.

There are no reports on the preservative efficiency of the above chemicals in any of the traditional Ethiopian fermented foods. This study, therefore, was designed to evaluate the possibility of using these chemicals at their permissible level to control mould spoilage and improve shelf life of injera.
1.3 OBJECTIVES OF THE STUDY

1.3.1 General Objective

❖ To improve the shelf life of injera using benzoic acid, sodium benzoate, potassium sorbate and calcium propionate as preservative.

1.3.2 Specific Objectives

❖ To isolate, cultivate and identify the moulds which are responsible in spoiling injera.

❖ To examine the efficacy of sorbic acid, propionic acid and benzoic acid on the identified injera moulds.

❖ To evaluate injera based agar media for the cultivation of moulds.
CHAPTER 2

LITERATURE REVIEW

2.1 MOULDS

2.1.1 Food Spoilage by Moulds

Moulds, those dusty little spots found spreading over bread, cheese, books and other things in the home, cause the loss of millions of dollars to our economy every year and even worse, are menace to our health (Malloch, 1981). There is an increasing knowledge and understanding of the role played by moulds in food spoilage. Especially the discovery of mycotoxin production in foods has highlighted the importance of moulds in food quality. It is, however, only within the last 5–10 years that major progresses have been made towards the prevention of spoilage caused by moulds. This is due to recent international agreements on taxonomy and analytical methods for food borne moulds, which has led to the discovery, that a specific, very limited funga (= mycobiota) is responsible for the spoilage of each kind of food. This is called the associated or critical funga and has been shown to consist of less than ten species (Filtenborg et al., 1996).

The microbial spoilage of foods may be viewed simply as an attempt by the food biota to carry out what appears to be their primary role in nature. This should not be taken in the teleological sense. In spite of their simplicity when compared to higher forms, microorganisms are capable of carrying out many complex chemical reactions essential to their perpetuation. To do this, they must obtain nutrients from organic matter, some of which constitutes our food supply (Jay, 2000).
Moulds are ubiquitous which can be found in a wide variety of environments, such as in plants, animal products, soil, water and insects. This broad occurrence can be explained by the fact that moulds can utilize a variety of substrates such as pectines and other carbohydrates, organic acids, proteins and lipids. Moreover, moulds are relatively tolerant to low pH, low water activity, low temperature and the presence of preservatives (Huis in’t Veld, 1996).

In addition to visible spoilage, moulds can also accumulate toxins hazardous to health. It has now been established that more than 200 different types of moulds do form substances that are orally toxic to man, when growing in certain foods. Although most research has been carried out on the metabolites of *Aspergillus flavus*, it is quite obvious that in addition to the so-called aflatoxins, many other mycotoxins may be of great significance (Huis in’t Veld, 1996). Certain moulds are capable of producing toxic and carcinogenic metabolites. Proliferation of these organisms on foods must be regarded as a potential health hazard. A further consequence of such mould growth is economic loss due to poor appearance, off flavours and need for severe trimming (Skirdal and Eklund, 1993).

### 2.1.2 Characteristics of Moulds

Molds are filamentous fungi that grow in the form of a tangled mass that spreads rapidly and may cover several inches of area in 2 to 3 days. The total of the mass or any large portion of it is referred to as **mycelium**. Mycelium is composed of branches or filaments referred to as **hyphae** (Heritage *et al.*, 1996).

Those of greatest importance in foods multiply by ascospores, zygospores, or conidia. The **ascospores** of some genera are notable for their extreme degrees of heat resistance. One group
forms pycnidia or acervuli (small, flask-shaped, fruiting bodies lined with conidiophores). **Arthrospores** result from the fragmentation of hyphae in some groups.

There were no radical changes in the systematic of food borne fungi during the 1980s. The most notable changes involve the discovery of the sexual or perfect states of some well-known genera and species. In this regard, the **ascomycete** state is believed by mycologists to be the more important reproductive state of a fungus, and this state is referred to as the **teleomorph**. The species name given to a teleomorph takes precedence over that for the **anamorph**, the imperfect or conidial state. **Holomorph** indicates that both states are known, but the teleomorph name is used (Jay, 2000).

Some of the genera of moulds that commonly cause spoilage of foods and grains are:  
*Penicillium, Aspergillus, Eurotium, Fusarium, Endomyces, Rhizopus, Mucor, Monilia* and *Cladosporium*
2.2 FACTORS THAT AFFECT MICROBIAL GROWTH ON FOOD

Fungal growth is influenced by a variety of complex interactions between intrinsic and extrinsic factors (Lopez-Malo et al., 2005).

2.2.1 Intrinsic Parameters

The parameters of plant and animal tissues that are an inherent part of the tissues are referred to as intrinsic parameters (Jay, 2000, Huis in’t Veld, 1996). These parameters are Nutrient content, pH, Moisture content, Oxidation-reduction potential (Eh), Antimicrobial constituents and Biological structure.

A) Nutrient Content of the Food

Microorganisms can use foods as a source of nutrients and energy as well as to derive the chemical elements that constituent microbial biomass. The inability of an organism to utilize a major component of a food material will limit its growth. Thus, the ability to synthesize starch-degrading enzymes will favour the growth of an organism on cereals and other farinaceous products. The concentration of key nutrients can, to some extent, determine the rate of microbial growth (Bohra and Pariah, 2006). Microorganism require a minimum of a carbon source and energy source, a nitrogen(amino acids and vitamins) source, inorganic nutrients, water for growth, and these are readily provided by food (Omonigho and Ikenebomeh, 2000).
B) pH

The acidity or alkalinity of an environment has a profound effect on the activity and stability of macromolecules such as enzymes, so it is not surprising that the growth and metabolism of microorganisms are influenced by pH (Bohra and Pariah, 2006). It has been well established that most microorganisms grow best at pH values around 7.0 (6.6-7.5), whereas few grow below 4.0. Bacteria tend to be more fastidious in their relationships to pH than molds and yeasts, with the pathogenic bacteria being the most fastidious (Jay, 2000). According to (Lucke, 2003) pH is an important factor affecting growth of microorganisms in food because it affects:

1) Microbial energy metabolism involving the build up of gradients of hydrogen ion concentration across membrane and

2) Microbial enzyme activity and stability of cellular macromolecules.

C) Moisture Content

One of the oldest methods of preserving foods is drying or desiccation. The preservation of foods by drying is a direct consequence of removal or binding of moisture, without which microorganisms do not grow. It is now generally accepted that the water requirements of microorganisms should be described in terms of the water activity ($a_w$) in the environment (Jay, 2000). The concept of water activity ($a_w$) introduced by Scott 1957 is the most useful expression of the water availability for microbial growth and enzyme activity (Abdullah et al., 2000). This parameter is defined by the ratio of the water vapor pressure of food substrate to the vapour pressure of pure water at the same temperature $aw = p/p_o$, where $p$ is the vapor pressure of the solution and $p_o$ is the vapor pressure of the solvent (usually water).
In general, bacteria require higher values of aw for growth than fungi, with gram-negative bacteria having higher than gram positives. Most spoilage bacteria do not grow below $a_w = 0.91$, whereas spoilage molds can grow as low as 0.80 (Jay, 2000). Salt and sugar have long been used as effective means of extending shelf life of various products as these solutes bind water, leaving less water available for the growth of microorganisms. Essentially the water activity (aw) of the product is reduced, and since most microorganisms require a high water activity, they are unable to survive (Morris et al., 2004).

**D) Oxidation- Reduction Potential (Eh or O/R)**

Microorganisms display varying degrees of sensitivity to the oxidation-reduction potential (O/R, Eh) of their growth medium. The O/R potential of a substrate may be defined generally as the ease with which the substrate loses or gains electrons. Aerobic microorganisms require positive Eh values (oxidized) for growth, whereas anaerobes require negative Eh values (reduced). Among the substances in foods that help to maintain reducing conditions are -SH groups in meats and ascorbic acid and reducing sugars in fruits and vegetables (Jay, 2000). The O/R potential of a food is determined by the following:

- The characteristic O/R potential of the original food
- The *poising capacity;* that is, the resistance to change in potential of the food
- The oxygen tension of the atmosphere about the food
- The access that the atmosphere has to the food.
E) **Antimicrobial Constituents**

The stability of some foods against attack by microorganisms is due to the presence of certain naturally occurring substances that have been shown to have antimicrobial activity. Some species are known to contain essential oils that possess antimicrobial activity. Among these are eugenol in cloves, allicin in garlic, cinnamic aldehyde and eugenol in cinnamon, allyl isothiocyanate in mustard, eugenol and thymol in sage, and carvacrol (isothymol) and thymol in oregano. Cow's milk contains several antimicrobial substances, including lactoferrin, conglutinin, and the lactoperoxidase system (Jay, 2000).

F) **Biological Structures**

The natural covering of some foods provides excellent protection against the entry and subsequent damage by spoilage organisms. In this category are such structures as the testa of seeds, the outer covering of fruits, the shell of nuts, the hide of animals, and the shells of eggs. In the case of nuts such as pecans and walnuts, the shell or covering is sufficient to prevent the entry of all organisms. Once cracked, nutmeats are subject to spoilage by molds. The outer shell and membranes of eggs, if intact, prevent the entry of nearly all microorganisms when stored under the proper conditions of humidity and temperature (Jay, 2000).

2.2.2 **Extrinsic Parameters**

According to Huis in’t Veld (1996) and Jay (2000) extrinsic parameters of foods are those properties of the storage environment that affect both the foods and their microorganisms. Those of greatest importance to the welfare of food borne organisms are temperature of storage, relative humidity of environment, presence and concentration of gases and presence and activities of other microorganisms.
A) Temperature of Storage

Microorganisms, individually and as a group, grow over a very wide range of temperatures. The lowest temperature at which a microorganism has been reported to grow is -34°C; the highest is somewhere in excess of 100°C. It is customary to place microorganisms into three groups based on their temperature requirements for growth. Those organisms that grow well at or below 7°C and have their optimum between 20°C and 30°C are referred to as psychrotrophs. Those that grow well between 20°C and 45°C with optima between 30°C and 40°C are referred to as mesophiles, whereas those that grow well at and above 45°C with optima between 55°C and 65°C are referred to as thermophiles (Jay, 2000).

B) Relative Humidity of Environment

Relative humidity is essentially a measure of the water activity of the gas phase. When food commodities have a lower water activity are stored in an atmosphere of high relative humidity water will transfer from the gas phase to the food. It may take a very long time for the bulk of the commodity to increase in water activity, but condensation may occur on surface giving rise to localized regions of high water activity. It is in such regions that propagules, which have remained viable, but unable to grow, may now germinate and grow. Once microorganisms have started to grow and become physiologically active they usually produce water as an end product of respiration. Thus they increase the water activity of their own immediate environment so that microorganisms are able to grow and spoil a food, which was initially considered to be microbiologically stable (Jay, 2000).
C) Presence and Concentration of Gases

Oxygen is the most important gas in contact with food. Its presence and its influence on redox potential are important determinants of microbial associations that develop their rate of growth. The inhibitory effect of carbon dioxide on microbial growth is applied in modified atmosphere packing (MAP) of food and is an advantageous consequence of its use at elevated pressures in carbonated mineral waters and soft drinks (Bohra and Pariah, 2006). Since molds are strictly aerobic, for the attainment of long shelf lives the levels of residual O$_2$ must be kept below 1% (Guynot et al., 2003; 2004).

D) Presence and Activities of Other Microorganisms

Some food borne organisms produce substances that are either inhibitory or lethal to others; these include antibiotics, bacteriocins, hydrogen peroxide, and organic acids (Jay, 2000).

2.2.3 Hurdle Technology

Under intrinsic and extrinsic parameters, the effect of single factors on the welfare of microorganisms is presented. In the hurdle concept, multiple factors or techniques are employed to affect the control of microorganisms in foods. Barrier technology, combination preservation, and combined methods are among some of the other descriptions of this concept. Foods that employ the hurdle concept in their formulation would embody a series of the above parameters, thus making for a multi targeted approach to preventing germination and growth of microorganisms. In order to grow, the organism must "hurdle" a series of barriers (Jay, 2000; Lombard et al, 2000; Knochel and Gould, 1995).
2.3 METHODS OF PRESERVATION AND EXTENSION OF SHELF LIFE OF FOOD

2.3.1 Spoilage and Shelf life of food

With few exceptions all foods lose quality and potential shelf life at some rate following harvest, slaughter or manufacture in a manner that is very dependent on food type, composition, formulation (for manufactured foods), packaging and storage conditions. Spoilage, or other changes that lead to loss of shelf life, may occur at any of the many stages between the acquisition of raw materials and the eventual consumption of a finished product (Gould, 1996). The shelf-life of a product is defined as the expected time duration that a product will remain organoleptically acceptable. It is a function of holding temperature and the number of microorganisms remaining in it after processing (Doughari et al., 2007).

The principal reactions that lead to spoilage and that are consequently also the principal targets for effective preservation and controls are well known, and relatively few. They include some that are essentially physical, some that are chemical, some that are enzymic and some that are microbiological (Huis in’t Veld, 1996, Morris et al., 2004). The high increase in food deterioration is due to contamination with micro-organism since the entire environment in which we live is colonized by micro-organisms (Akpan and Kovo, 2005).

The schematic representation of the complex mechanisms of food is presented in Figure 1.
**External conditions**

Storage conditions

Humidity Gas atmosphere (O₂, N₂, CO₂)

Packaging (materials)

Light

![Diagram showing quality deterioration during storage of foods](image)

- **Product conditions**
  - Initial quality
  - Intrinsic factors
  - Raw materials
  - Ingredients
  - Hygienic processing

- **Biochemical damages**
  - Rancid flavour
  - Warmed-over flavour
  - Change of texture

- **Microbiological deterioration**
  - Discoloration
  - Production of off-flavours: Rancid flavour, Warmed over flavour, Putrid flavour, Sour flavour
  - Loss of Nutrients
  - Production of toxics
  - Changes of texture
  - Putrid flavour
  - Sour Flavors
  - Slime
  - Gas

**Figure 1. Quality deterioration during storage of foods**

(Adapted from Huis in’t Veld, 1996)
2.3.2 Preservation of Food

Food preservation is an action or a method of maintaining food at desirable level of properties or nature for their maximum benefits (Rahman, 2007). All foods begin to spoil as soon as they are harvested or slaughtered. Some spoilage is caused by such microorganisms as bacteria and moulds. Other spoilage resulted from chemical changes within the food itself due to natural processes such as enzyme action or oxidation (Jay, 2000). Preservation is based firstly on the delay or prevention of microbial growth. It must therefore operate through those factors that most effectively influence the growth and survival of microorganisms. Such factors are not numerous. They include a number of essentially physical factors, some predominantly chemical ones and some microbial ones which depend on the nature of the microorganisms that are present. These factors have been categorized in a number of ways but the most widely quoted categorizations (Huis in’t Veld, 1996) separate the major factors into intrinsic factors, and extrinsic factors (section 2.2).

2.3.3 Major Food Preservation Technologies

The major food preservation techniques that are employed are therefore all based on a relatively limited set of factors, so that their range is necessarily limited also (Gould, 1996; Morris et al., 2004; Jay, 2000). The major food preservation methods are summarized in Table 1 below.
### Table 1. Major methods of preservation (Adapted from Huis in’t Veld, 1996)

<table>
<thead>
<tr>
<th>Cold</th>
<th>Heat</th>
<th>Drying</th>
<th>Fermentation</th>
<th>Physical</th>
<th>Chemical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freezing</td>
<td>Cooking</td>
<td>Tunnel</td>
<td>Alcoholic</td>
<td>Filtration</td>
<td>Sugar, alt spices, acid preservatives e.g. benzoates, nitrites</td>
</tr>
<tr>
<td>Chilling</td>
<td>Pasteurization</td>
<td>Solar</td>
<td>Acetic</td>
<td>Separation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Canning</td>
<td>Spray</td>
<td>Lactic</td>
<td>Distillation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vacumm</td>
<td></td>
<td>Irradiation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Freeze Drying</td>
<td></td>
<td>Concentration</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Modified/controlled atmosphere packing (MAP)</td>
<td></td>
</tr>
</tbody>
</table>

#### 2.3.2.2 Food Preservation with Chemicals

According to Jay 2000, the use of chemicals to prevent or delay the spoilage of foods derives in part from the fact that such compounds are used with great success in the treatment of diseases of humans, animals, and plants. This is not to imply that any and all chemotherapeutic compounds can or should be used as food preservatives. Although a large number of chemicals have been described that show potential as food preservatives, only a relatively small number are allowed in food products, due in large part to the strict rules of safety adhered to by the Food and Drug Administration (FDA) and to a lesser extent to the fact that not all compounds that show antimicrobial activity in vitro do so when added to certain foods. Below are described those compounds most widely used, their modes of action where known, and the types of foods in which they are used. Those chemical preservatives Generally Recognized as Safe (GRAS) are summarized in Table 2.
Table 2. Summary of some GRAS chemical food preservatives (Adapted from Jay, 2000)

<table>
<thead>
<tr>
<th>Preservatives</th>
<th>Maximum Tolerance</th>
<th>Organisms Affected</th>
<th>Foods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propionic acid/propionates</td>
<td>0.32%</td>
<td>Molds</td>
<td>Bread, cakes, some cheeses, rope inhibitor in bread dough</td>
</tr>
<tr>
<td>Sorbic acid/Sorbates</td>
<td>0.2%</td>
<td>Molds</td>
<td>Hard cheeses, figs, syrups, salad dressings, jellies, cakes</td>
</tr>
<tr>
<td>Benzoic acid/benzoates</td>
<td>0.1%</td>
<td>Yeasts and molds</td>
<td>Margarine, pickle relishes, apple cider, soft drinks, tomato catsup, salad dressings</td>
</tr>
<tr>
<td>Parabens*</td>
<td>0.1%</td>
<td>Yeasts and molds</td>
<td>Bakery products, soft drinks, pickles, salad dressings</td>
</tr>
<tr>
<td>SO₂/sulfites</td>
<td>200-300 ppm</td>
<td>Insects, microorganisms</td>
<td>Molasses, dried fruits, wine making, lemon juice (not to be used in meats or other foods recognized as sources of thiamine)</td>
</tr>
<tr>
<td>Ethylene/propylene oxides</td>
<td>700 ppm</td>
<td>Yeasts, molds, vermin</td>
<td>Fumigant for spices, nuts</td>
</tr>
<tr>
<td>Sodium diacetate</td>
<td>0.32%</td>
<td>Molds</td>
<td>Bread</td>
</tr>
<tr>
<td>Nisin</td>
<td>1%</td>
<td>Lactics, clostridia</td>
<td>Certain pasteurized cheese spreads</td>
</tr>
<tr>
<td>Dehydroacetic acid</td>
<td>65 ppm</td>
<td>Insects</td>
<td>Pesticide on strawberries, squash</td>
</tr>
<tr>
<td>Sodium nitrite</td>
<td>120 ppm</td>
<td>Clostridia</td>
<td>Meat-curing preparations</td>
</tr>
<tr>
<td>Ethyl formate</td>
<td>15-220 ppm§</td>
<td>Yeasts and molds</td>
<td>Dried fruits, nuts</td>
</tr>
</tbody>
</table>

Note: GRAS (generally recognized as safe) per Section 201 (32) (s) of the U.S. Food, Drug, and Cosmetic Act as amended.
* Methyl-, propyl-, and heptyl-esters of p-hydroxybenzoic acid.
§ As formic acid
2.3.2.2.1 Benzoic acid / Benzoates

Benzoic acid and its salt have been widely used in the food industry for many years as important food preservatives in order to inhibit various bacteria, yeasts and fungi growth in acidic media. They are also used in other products, such as pharmaceuticals and cosmetics. As one kind of common chemical, 638,000 tons of benzoic acid is approximately produced globally per year (Qi et al., 2009). According to Jay 2000, sodium benzoate was the first chemical preservative permitted in foods by the FDA in 1908 as a preservative in certain foods and it continues in wide use today in a large number of foods.

Benzoic acid is an effective antimicrobial agent for the purpose of preservation. However, sodium benzoate is more effective and preferred because it is approximately 200 times more soluble than benzoic acid. The soft drink industry is the largest user of benzoate as a preservative due to the amount of high fructose corn syrup in many carbonated beverages (DINOX, 2004).

Benzoic acid has been widely employed as an antimicrobial agent in foods and it occurs naturally in cranberries, prunes, cinnamon and cloves. It is well suited for acid foods such as fruit juices, carbonated beverages, pickles and sauerkraut. Benzoic acid has been found to cause no deleterious effect when used in small amounts. It is, however, readily eliminated from the body after conjugation with glycine to form hippuric acid (Doughari et al., 2007).
Benzoic acid (C₆H₅COOH) and its sodium salt (C₇H₅NaO₂), along with the esters of p-hydroxybenzoic acid (parabens) are permissible in foods up to 0.1%. The approved derivatives of benzoic acid have structural formulas as noted below:

The parabens appear to be more effective against molds than against yeasts. As in the case of bacteria, the propyl derivative appears to be the most effective where 100 ppm or less is capable of inhibiting some yeasts and molds, whereas for heptyl- and methyl parabens, 50-200 and 500-1,000 ppm respectively, are required. Like benzoic acid and its sodium salt, the methyl- and propyl parabens are permissible in foods up to 0.1%, and heptyl parabens is
permitted in beers to a maximum of 12 ppm and up to 20 ppm in fruit drinks and beverages. The pK for these compounds is around 8.47, and their antimicrobial activity is not increased to the same degree as for benzoate with the lowering of pH as noted. They have been reported to be effective at pH values up to 8.0 (Jay, 2000; Ibekwe et al., 2007).

The antimicrobial activity of benzoate is related to pH, the greatest activity being at low pH values. The antimicrobial activity resides in the undissociated molecule. These compounds are most active at the lowest pH values of foods and essentially ineffective at neutral values. The pK of benzoate is 4.20 and at a pH of 4.00, 60% of the compound is undissociated, whereas at a pH of 6.0, only 1.5% is undissociated. This results in the restriction of benzoic acid and its sodium salts to high-acid products such as apple cider, soft drinks, tomato catsup, and salad dressings. High acidity alone is generally sufficient to prevent growth of bacteria in these foods but not that of certain molds and yeasts. As used in acidic foods, benzoate acts essentially as a mold and yeast inhibitor, although it is effective against some bacteria in the 50- to 500- ppm range. Against yeasts and molds at around pH 5.0-6.0, from 100 to 500 ppm are effective in inhibiting the former, whereas for the latter, from 30 to 300 ppm are inhibitory (Jay, 2000; Akpan and Kovo, 2005)

2.3.2.2 Sorbic acid/ Sorbates

Sorbic acid (CH-CH=CH-CH=CH-COOH) is employed as a food preservative, usually as the calcium, sodium, or potassium salt (Jay, 2000; Venturini et al., 2002 and Santini et al., 2009). Sorbic acid and its potassium salt are the most widely used forms of the compounds and are collectively known as sorbates. Sorbate was first patented by Gooding 1945 as an antifungal agent, and has been used to a growing extent to protect a variety of foods against spoilage by
microorganisms (Razavi-Rohani and Griffiths, 1999). Sorbic acid was approved by FDA for use as a food preservative in 1955 (Jay, 2000). Potassium salt is commonly used because it is more stable. Furthermore, its greater solubility extends the use of sorbate to solutions appropriate for dipping and spraying (Gonzalez-Fandos et al., 2005). These compounds are permissible in foods at levels not to exceed 0.2%. The widest use of sorbates is as fungistats in products such as cheeses, bakery products, fruit juices, beverages, salad dressings, and the like (Jay 2000). The acceptable daily intake (ADI) values, determined by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), is 25 mg/kg of body mass for sorbic acid and sorbates salts(Santini et al., 2009).

The sorbates are primarily effective against molds and yeasts, but research has shown them to be effective against a wide range of bacteria (Jay, 2000, Venturini et al., 2002 and Santini et al., 2009). In general, the catalase-positive cocci are more sensitive than the catalase negatives, and aerobes are more sensitive than anaerobes. The resistance of the lactic acid bacteria to sorbate, especially at pH 4.5 or above, permits its use as a fungistatic in products that undergo lactic fermentations.

In the case of molds, inhibition may be due to inhibition of the dehydrogenase enzyme system. Against germinating endospores, sorbate prevents the outgrowth of vegetative cells (Figure 5).
Inhibition of microorganisms by sorbates is dependent on microbial types, species, and strains and on environmental factors. There are reports that lower pH and higher NaCl concentrations increase the inhibitory effect of sorbates against fungi (Razavi-Rohani and Griffiths, 1999). Some moulds are sensitive to the action of sorbic acid, while others can grow in the presence of large amounts, metabolize or degrade it. For example some penicillia degraded sorbic acid and produced 1,3-pentadiene, a volatile compound with an extremely strong kerosene-like odour (Skirdal and Eklund, 1993).

As with other food preservatives, the use of sorbate has advantages as well as limitations, but when used with proper planning and under the correct conditions, the advantages outweigh the disadvantages (Razavi-Rohani and Griffiths, 1999). Sorbic acid and its salts have several advantages as food preservatives. Initially thought to have only antimycotic activity, they are now known to also inhibit a wide range of bacteria, particularly aerobic catalase-positive organisms. Effective concentrations do not normally alter product taste or odor. These
preservatives are also considered harmless (Gonzalez-Fandos et al., 2005). Sorbic acid and its derivatives are commonly used to inhibit mold growth and extend the shelf life of several foods because of physiological harmlessness and organoleptic neutrality (Venturini et al., 2002). Other studies showed that sorbic acid has low toxicity, explained by the fact that it is rapidly metabolised by pathways similar to those of other fatty acids. In humans a few cases of idiosyncratic intolerance to sorbic acid have been reported (non-immunological contact urticaria and pseudo-allergy). For the above mentioned reasons, sorbic acid and sorbates salts (especially potassium sorbate) have become the leading preservatives for a wide variety of food products (Santini et al., 2009).

Like sodium benzoate, they are more effective in acid foods than in neutral foods and tend to be on par with the benzoates as fungal inhibitors. Sorbic acid works best below a pH of 6.0 and is generally ineffective above pH 6.5. These compounds are more effective than sodium benzoate between pH 4.0 and 6.0. At pH values of 3.0 and below, the sorbates are slightly more effective than the propionates but about the same as sodium benzoate. The pK of sorbate is 4.80, and at a pH of 4.0, 86% of the compound is undissociated, whereas at a pH of 6.0, only 6% is undissociated (Jay, 2000; Skirdal and Eklund, 1993). Therefore, the antimicrobial activity of sorbates is known to be pH dependent and its antimicrobial effectiveness increases as the pH value approaches its dissociation constant (Razavi-Rohani and Griffiths, 1999). Sorbic acid can be employed in cakes at higher levels than propionates without imparting flavour to the product (Jay, 2000).
2.3.2.2.3 Propionic acid/Propionates

Propionic acid is a three-carbon organic acid with the structure $\text{CH}_3\text{CH}_2\text{COOH}$. This acid and its calcium and sodium salts are permitted in breads, cakes, certain cheese, and other foods (Jay 2000). The antimicrobial effect of propionic acid has been known since 1913. Propionic acids and their salts are primarily inhibitory to molds; however, some species of molds are resistant and can grow in media containing substantial amounts of propionic acid and its salts (Razavi-Rohani and Griffiths, 1999). They tend to be highly specific against moulds, with the inhibitory action being primarily fungistatic rather than fungicidal. Propionic acid is employed also as a "rope" inhibitor in bread dough (Jay, 2000). Propionic acid has previously been shown to inhibit moulds and Bacillus spores, but not yeasts to a large extent, and has therefore been the traditional chemical of choice for bread preservation (Ryan et al., 2005).

Similar to other lipophilic acid preservatives, the optimum pH level for the inhibitory effect of propionic acid is around 5.0, with a maximum effective pH of 6.0. Propionic acid was most effective at lower pH’s, but propionic acid was never as effective as sorbic acid at any of the pH’s tested. The antifungal activity of sodium and calcium propionate did not approach propionic acid in effectiveness (Razavi-Rohani and Griffiths, 1999). The tendency toward dissociation is low with this compound and its salts, and they are consequently active in low-acid foods. With respect to the antimicrobial mode of action of propionates, they act in a manner similar to that of benzoate and sorbate. The pK of propionate is 4.87 and at a pH of 4.00, 88% of the compound is undissociated, whereas at a pH of 6.0, only 6.7% remains undissociated. The undissociated molecule of this lipophilic acid is necessary for its antimicrobial activity (Jay, 2000). The mode of action of propionic acid is similar as noted below with benzoic acid and sorbic acid on section 2.3.2.2.4.
Concentrations of propionic acid and propionates ranging from 8 to 12% were effective in controlling mold growth on the surface of cheese and butter (Razavi-Rohani and Griffiths, 1999). Legislation implemented under the European Parliament and Council Directive No. 95/2/EC requires that Propionic acid may only be added to bread in a concentration not exceeding 3000 ppm. However, recent studies have shown that under these conditions Propionic acid is not effective against common bread spoilage organisms. Additionally, a reduction of preservatives to sub-inhibitory levels might stimulate the growth of spoilage fungi and/or mycotoxin production. Recent trends in the bakery industry have included the desire for high-quality foods, which are minimally processed and do not contain chemical preservatives, thus increasing the interest toward natural preservation systems (Ryan et al., 2008).

2.3.2.2.4 Mode of Action of Weak Organic Acids

As lipophilic acids sorbate, benzoate, and propionate appear to inhibit microbial cells by the same general mechanism. The mechanism involves the proton motive force (PMF). Briefly, hydrogen ions (protons) and hydroxyl ions are separated by the cytoplasmic membrane, with the former, outside the cell, giving rise to acidic pH and the latter, inside the cell, giving rise to pH near neutrality. The membrane gradient thus created represents electrochemical potential that the cell employs in the active transport of some compounds such as amino acids. Weak lipophilic acids act as protonophores. After diffusing across the membrane, the undissociated molecule ionizes inside the cell and lowers intracellular pH. This result in a weakening of the transmembrane gradient such that amino acid transport is affected adversely (Figure 6). Although alteration of the PMF by lipophilic acids has wide support, other factors may be involved in their mode of action (Jay, 2000; Gould, 1996; Brul and Coote, 1999).
Figure 5. A schematic representation of the stress response of a yeast cell challenged with weak organic acids (Adapted from Brul and Coote, 1999)

According to Gould (1996) the element important in their modes of action is the dissociation constant of the acid, because it is the undissociated form that is most lipophilic and therefore most readily permeates the membrane, and it is the pH value and the dissociation constant together that determine the proportion of the acid that is in this form. The pK values of the common weak organic acid preservatives range from 4.2 (benzoic) to 4.87 (propionic), so that at pH values much above these, activity is greatly reduced.
The inhibition by organic acids has been attributed to the protonated form of these acids, which are uncharged and may therefore cross biological membranes (Figure 7). The resulting inhibition of growth may be due to acidification of the cytoplasm and/or accumulation of anions inside the cell (Sahlin, 1999).

Figure 6. Diffusion of a weak organic acid into a microbial cell and its dissociation yielding protons (H⁺) and potentially toxic anions (A⁻)
(Adapted from Sahlin, 1999)

The ability of an acid to inhibit bacteria depends principally on the pKa of the acid: the higher the pKa of the acid, the greater the proportion of undissociated acid, and the more inhibitory the acid is likely to be. The degree of dissociation of weak acids in acidic environment can be calculated using the Hendersen-Hasselbalch equation:

\[
\text{pH} = \text{pKa} + \log \frac{[A^-]}{[HA_c]}
\]
2.4 INJERA

2.4.1 Product Description

Injera is Ethiopian fermented bread made from different types of cereals. It is large, flat, round and uniformly spaced honeycomb-like eyes, each measuring about 60 cm in diameter and the base has a smooth surface. Injera looks whitish cream, reddish–brown or brown depending on the type of cereal flour used (Fellow, 1997). Injera can be made from tef (Eragrostis tef), wheat, barley, sorghum, or maize or a combination of some of these cereals. Whenever the soil type and rainfall patterns are suitable for the cultivation of tef, injera from tef is more favoured than from the other cereals (Mogessie Ashenafi, 2006). According to EHNRI (1997) the various types of injera produced from the different varieties of cereals do not have significant variation in their calorie, moisture, protein, carbohydrate, or phosphorus nutrients (Table 3). Significant variations are, however, observed in the other nutrients.

According to CSA (2008) report, tef [Eragrostis tef (Zucc) Trotter] has the largest share of area (23.42 %, 2.6 million hectares) under cereal cultivation and third (i.e. after maize and wheat) in terms of grain production (18.57 %, 29.9 million quintals) in Ethiopia. Tef grain commands premium price among other cereals cultivated in Ethiopia. There is a growing interest on tef grain utilizations because of nutritional merits (whole grain); the protein is essentially free of gluten the type found in wheat and it accounts (Arogundade, 2006) for about 2/3 of the daily protein intake in the diet of Ethiopian population. The grain proteins are also presumed easily digestible because prolamins are very small. Tef grain micronutrient is also apparently high, particularly in iron, a result of agronomic practices used in Ethiopia and fermentation on injera making. Because of this, the prevalence of iron deficient anaemia among tef injera consumers in Ethiopia is low (Geremew Bultosa, 2007).
Table 3. Composition of various varieties of injera in terms of 100 grams of edible portion (EHNRI 1997)

<table>
<thead>
<tr>
<th>Injera ingredient</th>
<th>Energy (calories)</th>
<th>Moisture %</th>
<th>Nitrogen g</th>
<th>Protein g</th>
<th>Fat g</th>
<th>CHO g</th>
<th>Fibre g</th>
<th>Ash g</th>
<th>Ca mg</th>
<th>P mg</th>
<th>Fe mg</th>
<th>Thiamine (µg)</th>
<th>Riboflavin (µg)</th>
<th>Niacin (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barely (Black)</td>
<td>124.9</td>
<td>68.3</td>
<td>0.54</td>
<td>3.4</td>
<td>0.10</td>
<td>27.6</td>
<td>0.80</td>
<td>0.60</td>
<td>34.0</td>
<td>96.0</td>
<td>3.60</td>
<td>0.13</td>
<td>0.07</td>
<td>1.00</td>
</tr>
<tr>
<td>Barely (white)</td>
<td>125.8</td>
<td>68.5</td>
<td>0.42</td>
<td>2.6</td>
<td>0.20</td>
<td>28.4</td>
<td>0.80</td>
<td>0.30</td>
<td>5.00</td>
<td>72.0</td>
<td>2.10</td>
<td>0.08</td>
<td>0.05</td>
<td>1.00</td>
</tr>
<tr>
<td>Maize (yellow)</td>
<td>161.20</td>
<td>60.80</td>
<td>0.55</td>
<td>3.4</td>
<td>1.20</td>
<td>34.20</td>
<td>0.80</td>
<td>0.40</td>
<td>4.00</td>
<td>86.00</td>
<td>2.10</td>
<td>0.14</td>
<td>0.07</td>
<td>0.40</td>
</tr>
<tr>
<td>Maize (White)</td>
<td>153.00</td>
<td>62.60</td>
<td>0.57</td>
<td>3.6</td>
<td>1.00</td>
<td>32.40</td>
<td>0.80</td>
<td>0.40</td>
<td>22.00</td>
<td>82.00</td>
<td>1.80</td>
<td>0.09</td>
<td>0.04</td>
<td>0.50</td>
</tr>
<tr>
<td>Millet (black)</td>
<td>156.00</td>
<td>60.50</td>
<td>0.53</td>
<td>2.9</td>
<td>0.50</td>
<td>35.00</td>
<td>2.90</td>
<td>1.10</td>
<td>1.29</td>
<td>88.00</td>
<td>16.80</td>
<td>0.09</td>
<td>0.04</td>
<td>0.20</td>
</tr>
<tr>
<td>Millet (mixed)</td>
<td>174.20</td>
<td>55.80</td>
<td>0.71</td>
<td>3.9</td>
<td>0.60</td>
<td>38.30</td>
<td>3.20</td>
<td>1.40</td>
<td>122.00</td>
<td>86.00</td>
<td>18.90</td>
<td>0.13</td>
<td>0.06</td>
<td>0.30</td>
</tr>
<tr>
<td>Sorghum (red)</td>
<td>136.10</td>
<td>66.10</td>
<td>0.42</td>
<td>2.30</td>
<td>0.50</td>
<td>30.60</td>
<td>0.70</td>
<td>0.50</td>
<td>10.00</td>
<td>90.00</td>
<td>1.70</td>
<td>0.16</td>
<td>0.27</td>
<td>0.60</td>
</tr>
<tr>
<td>Sorghum (mix)</td>
<td>168.10</td>
<td>58.00</td>
<td>0.66</td>
<td>3.60</td>
<td>0.50</td>
<td>37.30</td>
<td>1.60</td>
<td>0.60</td>
<td>13.00</td>
<td>100.00</td>
<td>2.90</td>
<td>0.11</td>
<td>0.09</td>
<td>1.30</td>
</tr>
<tr>
<td>Tef (red)</td>
<td>155.90</td>
<td>60.20</td>
<td>0.58</td>
<td>3.40</td>
<td>0.70</td>
<td>34.00</td>
<td>1.80</td>
<td>1.70</td>
<td>50.00</td>
<td>115.00</td>
<td>14.70</td>
<td>0.09</td>
<td>1.16</td>
<td>0.60</td>
</tr>
<tr>
<td>Tef (white)</td>
<td>145.00</td>
<td>63.80</td>
<td>0.51</td>
<td>3.00</td>
<td>0.60</td>
<td>31.90</td>
<td>1.00</td>
<td>0.70</td>
<td>56.00</td>
<td>100.00</td>
<td>7.00</td>
<td>0.21</td>
<td>0.11</td>
<td>0.50</td>
</tr>
<tr>
<td>Tef (mixed)</td>
<td>150.20</td>
<td>62.20</td>
<td>0.64</td>
<td>3.80</td>
<td>0.60</td>
<td>32.40</td>
<td>1.40</td>
<td>1.00</td>
<td>68.00</td>
<td>105.00</td>
<td>13.80</td>
<td>0.21</td>
<td>0.17</td>
<td>0.50</td>
</tr>
<tr>
<td>Wheat (black)</td>
<td>147.70</td>
<td>63.20</td>
<td>0.87</td>
<td>4.90</td>
<td>0.50</td>
<td>30.90</td>
<td>1.10</td>
<td>0.50</td>
<td>17.00</td>
<td>91.00</td>
<td>2.20</td>
<td>0.16</td>
<td>0.19</td>
<td>1.00</td>
</tr>
<tr>
<td>White (white)</td>
<td>145.60</td>
<td>63.40</td>
<td>0.55</td>
<td>3.10</td>
<td>0.40</td>
<td>32.40</td>
<td>1.10</td>
<td>0.70</td>
<td>21.00</td>
<td>147.00</td>
<td>4.40</td>
<td>0.15</td>
<td>0.17</td>
<td>1.00</td>
</tr>
<tr>
<td>White (mixed)</td>
<td>157.40</td>
<td>60.80</td>
<td>0.65</td>
<td>3.40</td>
<td>0.60</td>
<td>34.60</td>
<td>1.20</td>
<td>0.60</td>
<td>25.00</td>
<td>104.00</td>
<td>1.80</td>
<td>0.16</td>
<td>0.17</td>
<td>1.30</td>
</tr>
<tr>
<td>Mean</td>
<td>148.66</td>
<td>62.44</td>
<td>0.59</td>
<td>3.38</td>
<td>0.57</td>
<td>32.86</td>
<td>1.37</td>
<td>0.75</td>
<td>32.02</td>
<td>97.29</td>
<td>6.7</td>
<td>0.14</td>
<td>0.19</td>
<td>0.73</td>
</tr>
<tr>
<td>SD</td>
<td>12.53</td>
<td>3.57</td>
<td>0.12</td>
<td>0.63</td>
<td>0.28</td>
<td>3.01</td>
<td>0.78</td>
<td>0.41</td>
<td>32.93</td>
<td>18.00</td>
<td>6.39</td>
<td>0.04</td>
<td>0.29</td>
<td>0.36</td>
</tr>
<tr>
<td>%CV</td>
<td>8.4</td>
<td>5.7</td>
<td>19.9</td>
<td>18.5</td>
<td>48.7</td>
<td>9.1</td>
<td>57.2</td>
<td>54.3</td>
<td>102.8</td>
<td>18.5</td>
<td>95.3</td>
<td>30.9</td>
<td>151.3</td>
<td>49.8</td>
</tr>
</tbody>
</table>
2.4.2 Processing of Injera

Cereal grains had been one of man’s earliest sources of food. One way of processing the grains into food is through fermentation (Taiwo, 2009 and Blandino et al., 2003). The preparation of tef injera consists of two stages of natural fermentation, which last for about 24 to 72 hours, depending on ambient temperatures. Temperature in the highlands of Ethiopia is generally between 17 and 25°C. The only required ingredients are the tef flour and water (Berhanu Abegaz Gashe, 1985). The method of processing of injera from its raw materials to the final product is summarized by Fellow, (1997) as described below on Table 4. It involves preparing and mixing the ingredients to dough, which is fermented and subsequently thinned to a batter. The batter is then poured onto a hot griddle in a thin layer to cook, developing its colour, flavour and texture.

**Table 4. Processing of injera from tef (Source: Fellow, 1997)**

<table>
<thead>
<tr>
<th>Process</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw materials</td>
<td>Tef (<em>Eragrostis tef</em>) is an indigenous cereal for making injera. Other cereals which may be used are sorghum, millet, barely, wheat or a combination of cereals.</td>
</tr>
<tr>
<td>Clean</td>
<td>All impurities are removed by hand and winnowed in the case of sorghum, millet, barely, and wheat. Tef is simply winnowed and sifted through a fine sieve.</td>
</tr>
<tr>
<td>Hull</td>
<td>Sorghum, barley and wheat are usually dampened and pounded traditionally in a wooden mortar and pestle to remove the bran. Mechanical hullers are also available.</td>
</tr>
<tr>
<td>Grind</td>
<td>The sifted tef is ground through a stone mill.</td>
</tr>
<tr>
<td>Step</td>
<td>Description</td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>Mixing and first fermentation</strong></td>
<td>Mix one part of flour, two parts of water and about 16 percent ersho (a starter saved from previously fermented dough) by weight of the flour. Mix very well and leave it to ferment for three days.</td>
</tr>
<tr>
<td><strong>Thin and heat</strong></td>
<td>Discard the surface water formed on the top of the dough. For every 1kg of original flour, take about 200ml of the fermented mixture and add twice as much water, mix and bring to a boil (traditionally known as ‘absit’ making). It should be cooled to about 46(^\circ)C/115(^\circ)F before it is mixed into the main part of the dough. Thin the main dough by adding water equal to the original weight of the flour.</td>
</tr>
<tr>
<td><strong>Batter making and second fermentation</strong></td>
<td>Add the absit to the thinned dough and mix very well (known as batter making). Leave the batter for about 30 minute to rise (the second fermentation), before baking commences. A small portion or the batter is saved to serve as a starter (ersho) for the next batch.</td>
</tr>
<tr>
<td><strong>Griddle</strong></td>
<td>Injera is griddled by pouring about two-thirds of a litre of the batter onto the hot greased electrical ‘metad’ (injera griddle made of clay) using circular motion from the outside towards the center. It is cooked in about 2-3 minutes at a temperature of the metad reaching 90-95(^\circ)C. Rapeseed oil is used to grease the metad between each one.</td>
</tr>
<tr>
<td><strong>Store</strong></td>
<td>Several layers of injera can be stored in a ‘messhob’ (traditional straw basket) with a tight cover for three days in a cool, dry, ventilated place.</td>
</tr>
</tbody>
</table>
CHAPTER 3
MATERIALS AND METHODS

3.1 STUDY SITE

Experiments were carried out from October 2008-April 2009 in the Mycological Research Laboratory of the Biology Department and the Food Microbiology Laboratory of Food Science and Nutrition Program, at the Science Faculty, Addis Ababa University. Addis Ababa is the capital city of Ethiopia with the altitude of 2444 meter above sea level with a mean annual rainfall of 1196mm and located 9°01 N and 038°45 E. The minimum and maximum temperatures were 9.9°C and 24.6°C, respectively (Fissha Itana & Olsson, 2004).

3.2 PREPARATION OF INJERA

The tef injera samples were prepared at home in almost the same way as reported by Fellow (1997), except that the art of my mother was also included in this traditional method. Accordingly, the tef flour mixed 1:2 (w/w) with clean water and 16% of ersho by the weight of the flour was kneaded by hand in a bowl in the traditional way. The resultant dough was allowed to ferment for 3 days at ambient temperature. After this primary fermentation, the dough was mixed 1:3 (v/v) with boiling water, and heated for 15min with continuous stirring. The hot cooked dough (absit) was then mixed back into the fermenting dough, and sufficient clean water was added to make a batter. The batter was left covered for 2 h of secondary fermentation. Some more clean water was added to thin down and form the right consistency batter. Finally, about half a litre of batter was poured onto the hot clay griddle in a circular motion from the outside, working towards the centre. After 2-3 min of cooking using electric
injera baking equipment (‘metad’), it was removed and stored in a basket called “messhob”. The injera was then transported from home to Addis Ababa University, Faculty of Science: Food Microbiological laboratory of Food science and Nutrition program for further study.

3.3 MICROBIOLOGICAL ANALYSIS

3.3.1 Isolation and Cultivation of Moulds that Spoil Injera

The injera samples were kept in the laboratory at ambient temperature for at least 4-5 days until moulds starts to appear visually on it. After 4 or 5 days of storage at ambient temperature, depending upon their difference in color and other morphology, the spoiling moulds were directly transferred into a growth media of potato dextrose agar (PDA) and Injera sucrose agar (ISA)[as described in section 3.5] amended with 60mg/l Chloramphenicol, in order to suppress the growth of bacteria. The cultures were incubated at room temperature to induce the growth of mycelium and fruiting structures (spores) for five to seven days. To get a pure culture, each of the emerging mycelium or spores was transferred to fresh growth media. The pure colony from the isolation were incubated again at ambient temperature and kept in slant at refrigeration at $4^\circ$C for further identification.

3.3.2 Characterization and Identification of Moulds that Spoil Injera

The identification of the fungal isolates was based on morphological characterization that emphasizes on colony characteristics, conidial features and vegetative compatibility of isolates within genus level. For this purpose slide cultures were prepared for each of the isolates. Identification to the genus level was made according to Barnett & Hunter, (1972). In addition
to these, the fungal isolates were sent to Biotechnology Researches Institute, Braunshweig, Germany, for confirmation of their identity.

3.4 INJERA AGAR MEDIA

3.4.1 Preparation of Injera Sucrose Agar (ISA) Media

Freshly baked injera was sundried and changed to ‘derkosh’ (dried injera). The ‘derkosh’ was powdered using mortar and pestle, 100g of the powder was mixed with 500ml of water and kept in a shaker for 15 minutes. The injera broth was filtered using cheese cotton cloth, proportional to the volume of the broth 2 % sucrose and 2 % agar was then added. It was mixed and heated until boiling. After sterilization (15 minutes at 121\(^0\)C), the media was poured on Petri dishes. The ISA (Injera sucrose agar) media was kept in a refrigerator for future practical studies on fungi and for cultivation of the injera moulds.

3.4.2 Comparison of ISA with other Mycological Media

For evaluating the effectiveness of injera sucrose agar media as compared to other mycological media, *Aspergillus niger*, *Aspergillus flavus*, *Rhizopus* and *Penicillium* were sub cultured on PDA at ambient temperature for 3-4 days. A sterile cork borer (6-mm diameter) was used to bore and transfer each young culture to the newly prepared media (Gupta et al., 2000) of potato dextrose agar, malt extract agar, nutrient agar, czapek’s solution agar and injera sucrose agar media. The plates were then incubated at ambient temperature. The evaluations were carried out by means of daily measurement of the colony diameter, starting at 24 h after the experiment began and finishing when the entire plate surface of the control treatment was covered by the fungus.
3.5 ANALYZING THE EFFECT OF TEMPERATURE

Temperature optima for the common injera moulds were investigated. Growth and sporulation of *Aspergillus niger*, *Aspergillus flavus*, *Penicillium* and *Rhizopus* were assessed by inoculating them on injera sucrose agar media. The cultures were incubated at temperatures of $4^\circ$C (refrigeration temp), $16^\circ$C (room temperature during the experiment days), $20^\circ$C, $25^\circ$C and $32^\circ$C. The growth (colony size) of each of the moulds was determined as – no growth, + slight mould growth, ++ moderate mould growth and +++ high mould growth after 5 days of incubation. A duplicate of each Petri dishes were used in each temperature treatments.

3.6 DETERMINATION OF pH AND MOISTURE CONTENT OF INJERA

3.6.1 Sampling Method

From each of the baking cycles, samples were taken to determine pH and moisture content of injera. The sampling was done by taking pieces of injera from every quarter of the injera roll and blending all together.

3.6.2 Measurement of pH

An electronic pH meter (*JENWAY MODEL 370 pH/mV METER, England*) was used to measure p. After calibration using standard solutions at pH 4 and 7, each injera suspension (10 g of ground injera was added to 100 g distilled water and the dispersion was homogenised using a shaker) was measured.
3.6.3 Moisture Content Determination

The moisture content was determined by placing a weighed quantity (5g) of part of the injera samples first at 65°C. Further drying was done at temperature of 100°C in an air oven until constant weight was recorded (AOAC, 2000). Moisture content was expressed as:

\[
\text{% Moisture content} = \frac{M_{\text{initial}} - M_{\text{final}}}{M_{\text{initial}}} \times 100\%
\]

3.7 EVALUATION OF EFFICACY OF CHEMICAL PRESERVATIVES

3.7.1 Addition of Chemical Preservatives

Injera was prepared in the same way as described in section 3.2. Each of the chemical preservatives were added on the injera batters at the concentration of 0.3 % calcium propionate, 0.2 % potassium sorbate, 0.1 % benzoic acid and sodium benzoate and 0.2 % blend of the four just immediately before baking. The concentration of preservatives in breads is 0.3% of final weight for calcium propionate, 0.1% for sodium benzoate and benzoic acid and 0.2% for potassium sorbate. These values correspond to 1.5 grams of propionate per pound of flour, 0.5 grams of benzoate and 1grams of sorbate per pound of flour (Jay, 2000). After the baking process, the experimental and control samples were stored in the laboratory at ambient temperature. (The chemicals were added immediately before baking to prevent the chemicals from retarding the 2º fermentation of the injera preparation process).
Figure 8. Injera samples that kept in the laboratory at ambient temperature

3.7.2 Comparing the Rate of Mould Invasion

Mould outgrowth on each of the injera treatments was monitored daily over a period of 12 days (injera rolls were left at room temperature) and photos of each was taken. Injera spoilage was evaluated based on the percentage of the total surface area of each roll where fungal outgrowth occurred. The measurement of the mould outgrowth from the photos was done using the Photoshop elements of Adobe Photoshop Version 10.0. An injera rolls was deemed positive if more than 1% of the total surface area was covered with fungi (Ryan et al., 2008).

3.7.3 Evaluation of Shelf Life of Injera

The injera samples were examined for visible signs of moulds growth on the crust every day. The microbial shelf life is defined as the period in days in which the spoilage caused by microorganisms was first observed. The shelf life was expressed in relation to the corresponding control (Katsinis et al., 2008).
3.8 STATISTICAL ANALYSIS

The statistical analysis of the colony diameter of moulds at different media by compare means one way ANOVA (P<0.05) and the line graph relating mould invasion (%) over time were conducted using SPSS version 15.0. Differences between media were determined by using LSD.
CHAPTER 4

RESULTS AND DISCUSSION

4.1 MOULDS ISOLATED FROM INJERA

Under favourable conditions moulds can grow on a large variety of substrates. In bakery processing, the most common type of microbial spoilage is mould growth and in many cases it is the major factor governing shelf-life (Marin, 2005). The most wide spread and probably most important moulds, in terms of biodeterioration of bakery products are species of *Aspergillus*, *Penicillium*, *Eurotium* and *Fusarium* (Guynot *et al.*, 2005; Gerez *et al.*, 2009). Other species, such as those of *Cladosporium*, *Mucor* and *Rhizopus*, have been found less frequently (Guynot *et al.*, 2003). According to Filtenborg *et al.*, (1996) each individual food type is normally infected by a limited number of fungi.

In this study, small white fungal colonies (visible to the naked eye) appeared on injera after staying 4-5 days of storage in a messhob. Accordingly, the small colonies gradually grew and started to show different kind of colony color on sporulation. The injera moulds were isolated at different times of storage at ambient temperature and detected by microscopic evaluation, after observing their morphological characteristics of the colony. It was then found that three types of fungal species that belonged to the genera of *Penicillium*, *Aspergillus (Aspergillus niger)* and *Rhizopus* species were responsible in spoiling injera, although their extent of growth varied depending up on the ambient temperature of the day (The effect of temperature on the growth rate of the injera moulds was discussed on section 4.3). The three fungi isolated from injera were discussed below:
4.1.1 *Penicillium* species

Penicillium is a deuteromycetes which is greenish or blue green colonies; conidia in long chains on repeatedly branched conidiophores resembling a brush like head (Fig.9d) (penicillus) as described by Aneja, (2003). *Penicillium* species greenish colonies were seen on injera (Fig.9a), on PDA (Fig.9b) and also on sterile injera plated on a Petri dish (Fig.9c). According to Gock *et al.*, (2003) *penicillium* species are xerophilic fungi that frequently bring about spoilage of intermediate and low water activity baked goods.

![Image of Penicillium growing on injera](image1)

![Image of Penicillium growing on PDA](image2)

![Image of Penicillium grown on sterile injera plate](image3)

![Image of microscopic brush like structure of Penicillium](image4)

**Figure 9. *Penicillium* isolated from injera**
4.1.2 Aspergillus niger

*Aspergillus niger* is very common imperfect fungi with typically black colony. Microscopically (Fig. 10d) conidiophores arising from a foot-cell, catenate (basipetal) conidia on phialides (1or 2 series) on vesicle as described by Aneja (2003). In this study also *Aspergillus niger* black colonies were seen on injera (Fig. 10a), on PDA (Fig. 10b) and on a sterile injera plated on a Petri dish (Fig. 10c). Further *Aspergillus niger* black colonies became more dominant and visible to the naked eye when the ambient temperature got warmer. Similarly Shur and Nielsen, (2004) also reported that *Aspergillus niger* is more favoured in warmer climates.

![Aspergillus niger grown on injera](imageA) ![Aspergillus niger grown on PDA](imageB)

![Aspergillus niger grown on sterile injera plate](imageC) ![Microscopic structure of A. niger](imageD)

*Figure 10. Aspergillus niger isolated from injera*
4.1.3 *Rhizopus* species

Rhizopus is member of the common bread moulds. Its colony on injera (Fig.11a), potato dextrose agar (Fig.11b) and on sterile injera plated on a Petri dish (Fig.11c) was white to dark grey. As described by Aneja (2003) rhizopus has a nonseptate mycelium with root like rhizoids; black columellate, sporangiophores, in clusters.

![Rhizopus grown on injera](image1)

![Rhizopus grown on PDA](image2)

![Rhizopus grown on sterile injera plate](image3)

![Microscopic structure of Rhizopus](image4)

Figure 11. *Rhizopus* isolated from injera
The overall analysis of the identification and isolation process implied various causes for the growth of the moulds on injera. As clearly described by Marin et al., (2002) and Gock et al., (2003) the baking temperature is generally sufficient to destroy these organisms, contamination might arise from mould spores derived from the atmosphere or from surfaces during the cooling, finishing and wrapping procedures Therefore, good hygienic practices should be carried out with respect to food handlers, raw materials, equipment and premises. In relation to this the fermenting vat should be well cleaned after each batch of injera making. Potable water should be used. The straw basket (messhob) used to store injera should have tight covers, and be kept in a ventilated, cool, dry places, raised of the floor. Packing also protects to some extent against insects, animals and soils. When empty, the baskets should regularly be cleaned and stored, raised off the floor in a dry place (Fellow, 1997).

Apart from the repelling sight of visible growth, fungi are also responsible for off-flavour formation and the production of mycotoxins and allergenic compounds (Gutierrez et al., 2009). In this study a yellowish pigment on the surface of a spoiled injera had been observed when injera was kept for 12 days (Figure 12). Since both Penicillium and Aspergillus sp. are toxin producing moulds, a spoiled injera could also be a source of toxic compounds that might be menace to human health, although further investigation is needed to know for sure whether this yellowish pigment released by this moulds was actually a toxin or not.
4.2 MEDIA FROM INJERA

4.2.1 Growth of Moulds on Injera Sucrose Agar (ISA) Medium

The medium prepared from injera with sugar supplement, Injera sucrose agar (ISA) was found to be effective in supporting the growth of moulds such as *Aspergillus niger*, *Penicillium*, *Aspergillus flavus* and *Rhizopus*. In this experiment injera sucrose agar media had been used as any other kind mycological media for the cultivation of injera moulds due to its effectiveness and cheaper price. The injera sucrose agar media were also used to evaluate the effect of temperature on the growth rate of injera moulds which was discussed in section 4.3.

Figure 12. Yellowish pigment released by injera moulds after 12 days of storage
 Whenever a particular mould is needed, it must be supplied with some form of organic carbon for energy, a source of nitrogen for protein and vitamin synthesis, and several minerals (Malloch, 1981) and these could be easily provided by injera. EHNRI, 1997 reported that injera in general can be considered as a good source of energy, fibre, iron and vitamins (Table 1). This might be also one of the reason that injera was spoiled easily after 3 days of storage at ambient temperature.

ISA medium was used in this study for getting a pure culture of each of the moulds for identification. Since, many moulds have quite specific nutrient requirements and are specialized to use materials that other fungi use with difficulty or not at all. We can take advantage of this for the isolation of fungi by presenting a particular substance to the environment for colonization and then later recovering it for isolation of the fungi that occupied it (Malloch, 1981). This way of isolating fungi is usually called baits (baiting). ISA had used as a bait in this study to favoured only moulds that could utilize injera as their source of energy.

The contribution of injera sucrose agar media in this study had been also for founding out the growth temperature range of injera moulds so as to use the appropriate incubation temperature.
while cultivating them. Accordingly using the result that was found in section 4.3, *Penicillium* and *Rhizopus* species were incubated at ambient temperature while *Aspergillus niger* was incubated at 25°C and more.

### 4.2.2 Effectiveness of Injera Sucrose Agar (ISA) Medium

The media prepared from injera, injera sucrose agar media was found to be as effective as other mycological media. Table 5 shows the colony diameter of *Rhizopus* on media of potato dextrose agar (PDA), Czapek’s solution agar (CZP), Nutrient agar (NA), Malt extract agar (MEA) and Injera sucrose agar (ISA) on day 1-5. Although the colony diameter of *Aspergillus niger*, *Aspergillus flavus* and *Penicillium* were difficult to measure due to their non uniform outgrowth on all the media.

<table>
<thead>
<tr>
<th>Media Type</th>
<th>Colony diameter of in mm on day 1-5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>PDA</td>
<td>0</td>
</tr>
<tr>
<td>MEA</td>
<td>0</td>
</tr>
<tr>
<td>CYP</td>
<td>0</td>
</tr>
<tr>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>ISA</td>
<td>0</td>
</tr>
</tbody>
</table>

The ANOVA (Appendix 1) showed that the colony diameter of *Rhizopus* grown on injera sucrose was not significantly (p<0.05) different from the colony diameter of the other mycological media evaluated in this study.
Injera sucrose agar media had been the cheapest and conducive way in cultivating those moulds isolated from a spoiled injera. However, the effectiveness of injera sucrose agar media to support the growth of other types of moulds should be evaluated further in order to use it as one kind of mycological media.

4.3 EFFECT OF TEMPERATURE ON THE GROWTH RATE OF INJERA MOULDS

Temperature is generally considered to be one of the most important environmental factors that seriously affect not only the growth and germination of spoilage moulds in vitro, but also in vivo (Tian and Bertolini, 1995). This study revealed that *Penicillium* and *Rhizopus* were more dominant in spoiling injera at lower temperature, while *Aspergillus niger* grew much faster as the temperature gets higher (25-32°C). None of the moulds grew when the temperature was kept at 4°C (Table 6). The reduced activity of a fungus at lower temperature may have corresponded with reduction of nutrient utilization, so that the fungus used the colonized substrate more slowly (Ibid, 1995).

**TABLE 6. Growth rates of injera moulds on ISA medium at different temperature**

<table>
<thead>
<tr>
<th>Moulds</th>
<th>4°C</th>
<th>16°C</th>
<th>20°C</th>
<th>25°C</th>
<th>32°C</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. niger</em></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td><em>Penicillium spp</em></td>
<td>-</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>Rhizopus spp</em></td>
<td>-</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

- No growth at all
- Slight mould growth

++ Moderate mould growth
+++ High mould growth
Aspergillus niger grew in a very fast and furious way as the storage temperature rose from 25°C to 32°C. Tyson & Fullerton (2004) mentioned that Aspergillus niger require a higher temperature with an optimum temperature range of 28-34°C for its growth. Similarly Shur and Nielsen (2004) also confirmed that Aspergillus niger is favoured more in warmer climates.

From literature most fungi are mesophilic and are inhibited or killed at unusually high or low temperatures. There are some, however; that actually require unusual temperatures: thermophiles (>40°C) and psychrophilic (15°C or less) (Malloch, 1981). Obviously from the analysis of the study the moulds spoiling injera were mesophilic since their growth temperature range was between 16 and 32°C. One of the possible explanations for the isolated moulds to be only mesophilic could be related to climatic condition of the study site, where a moderate climatic condition of maximum temperature of 24.6°C was recorded. According to fellow, (1997) contamination of moulds is less important in cool highland regions than warm lowland regions. With a significant altitude difference in Ethiopia, obviously the dominant mould spoiling injera might vary from the colder to the hotter parts of the country.

In the final analysis temperature could be one of the hurdles that could be applied to prolong the shelf life of injera in addition to the use of the preservatives.
4.4 pH AND MOISTURE CONTENT OF INJERA

4.4.1 pH of Injera

Results of this investigation showed that injera was generally acidic irrespective of the type of preservatives added (Table 7). The pH value of the samples stored at ambient temperature ranges from 3.45-3.38. The pHs were 3.38 for the sample with benzoic acid, 3.39 for the sample with sodium benzoate, 3.45 for the sample with potassium sorbate and 3.44 for the sample with calcium propionate. The pH of the control sample without preservatives and blend of all the four preservatives sample were 3.40 and 3.38 respectively. The results indicated no variation in temperature (24.7°C) for all the samples. The acidity decreased slightly for the samples containing benzoic acid, sodium benzoate and blend of the four, while it slightly increased for the samples containing potassium sorbate and calcium propionate as compared to the control.

TABLE 7. pH of injera with and without preservatives

<table>
<thead>
<tr>
<th>Injera samples</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.40</td>
</tr>
<tr>
<td>0.1 % benzoic acid</td>
<td>3.38</td>
</tr>
<tr>
<td>0.1 % sodium benzoate</td>
<td>3.39</td>
</tr>
<tr>
<td>0.2 % Potassium sorbate</td>
<td>3.45</td>
</tr>
<tr>
<td>0.3 % calcium propionate</td>
<td>3.44</td>
</tr>
<tr>
<td>0.2 % Blend of the four</td>
<td>3.38</td>
</tr>
</tbody>
</table>
The pH values obtained, when compared with the pH of bread which is mostly between 4.7 and 7.4 (FDA, 2007) was quite low. The high acidity of injera could account for the low numbers and few types of organisms isolated, although the isolates have been found to be associated with food spoilage as described by Ryan, et al., (2008). This might implied that the moulds that spoiled injera were only those which could overcome such acidic environment like *Penicillium* and *Aspergillus* species.

Gould (1996) reported that it is the pH value of the food and dissociation constant of the acid that determine the proportion of the acid that is in the undissociated form. The antimicrobial effect of the undissociated acid is much stronger than the dissociated acid because the undissociated form is lipophilic and therefore most readily permeates the microbial membrane. In the present study therefore, one of the reasons for the better effectiveness of benzoic acid (discussed in section 4.5) in preserving injera than any of the preservatives could be due to the lower pH of injera in addition to its dissociation constant (pKa). According to Shur and Nielsen (2004) the pKa value of propionic acid, sorbic acid and benzoic acid are 4.88, 4.76 and 4.2, respectively which demonstrate the more acidic nature of benzoic acid than sorbic and propionic acid.
4.4.2 Moisture Content of Injera

The moisture content of fresh injera samples (as soon as it was baked) were ranging from 62-65% (Table 8). There was no significant difference in the moisture content of injera with and without preservatives. However, there was a gradual decrease in the moisture content as the number of days of storage gone from the 1st day to the 5th day at ambient temperature.

**TABLE 8. Moisture Content of the Injera with and without preservatives**

<table>
<thead>
<tr>
<th>Injera samples</th>
<th>Moisture content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st day</td>
</tr>
<tr>
<td>Control</td>
<td>64.2 %</td>
</tr>
<tr>
<td>0.1 % benzoic acid</td>
<td>63.7 %</td>
</tr>
<tr>
<td>0.1 % sodium benzoate</td>
<td>63.4 %</td>
</tr>
<tr>
<td>0.2 % Potassium sorbate</td>
<td>64.3 %</td>
</tr>
<tr>
<td>0.3 % calcium propionate</td>
<td>64.4 %</td>
</tr>
<tr>
<td>0.2 % Blend of the four</td>
<td>63.5 %</td>
</tr>
</tbody>
</table>

According to Guynot et al., (2003) even if bakery products are important staple foods in most countries and cultures, mold growth and drying are two problems that limit the shelf life of both high and intermediate-moisture bakery products. In this study also the moisture content of injera had decreased significantly when kept for 5 days.

The moisture content of the control injera obtained in this study was comparable with the value reported in the Ethiopian Food composition table (Table 3) of Ethiopian Health and Research Institute of Ethiopia (EHNRI, 1997) although this study concentrated on injera baked at home. From the composition table it can be seen that injera made from any of the cereals has higher moisture content than any of the breads.
According to Ayub et al., (2003), the overall mean moisture content of different kinds of bread made from wheat range from 37-47 % during storage. In the contrary, injera had a higher moisture content of 62-65% that made injera more perishable than most bread. The other reason injera was more perishable than bread could be due the larger the surface area of injera which made it more accessible by fungal spores to land and grew. According to Fellow (1997), to prevent mould growth the product should be properly cooled and dried before packing. Hence, any surface moisture will encourage contamination and the injera must not be handled with wet hands as surface moisture will facilitate mould growth.
4.5 EFFICACY OF THE CHEMICAL PRESERVATIVES ON INJERA

4.5.1 Extent of Mould Invasion on Injera

Investigation of the antimicrobial activity of the preservatives tested in this study revealed that the chemical preservatives were effective against injera moulds. This was shown by the reduction in percentage of mould invasion of the samples containing preservatives as compared to the sample without preservative (Table 9 and Figure 14). Antifungal activity of preservatives investigated in this study also showed that injera samples containing benzoic acid and its sodium salt were the most effective of all the preservatives tested. Even after 12 days of preservation, samples containing benzoic acid and its sodium salt had more keeping quality than samples with other preservatives. Samples without preservatives (control), however, exhibited the highest mould invasion from day 1-12 of preservation and consequently, had the poorest keeping quality as compared to those with preservatives.

Chemical preservatives has become an increasingly important practice in modern food technology with the increase in production of processed and convenience foods. These preservatives are deliberately added to stop or delay nutritional losses due to microbiological, enzymatic or chemical changes and thus increasing its shelf-life. Benzoic acid, sorbic acid and propionic acid are generally effective to control mould and inhibit yeast growth, and against a wide range of bacterial attack (Tfouni and Toledo, 2002).
TABLE 9. Percentage of the invaded surface as compared to the total surface of injera

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Invasion of injera(%) over a number of days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>0.1 % benzoic acid</td>
<td>0</td>
</tr>
<tr>
<td>0.1 % sodium benzoate</td>
<td>0</td>
</tr>
<tr>
<td>0.2 % potassium sorbate</td>
<td>0</td>
</tr>
<tr>
<td>0.3 % calcium propionate</td>
<td>0</td>
</tr>
<tr>
<td>0.2 % blend of the four</td>
<td>0</td>
</tr>
</tbody>
</table>

Depending up on the type of mould that dominated the injera samples at the given condition there might be a variation in the degree of invasion that was reported on table 8. If the injera was first dominantly invaded by for *Rhizopus* it might invade the whole surface of injera in a very short period of time due to many zygomycetes are fast growing and coarse which can take over the culture they are growing in a few days (Malloch, 1981). As clearly depicted in the graph (Figure14) the sample containing 0.3 % calcium propionate (purple line) had shown a drastic change than the control (blue line) in its degree of mould invasion between the 6 and 8 day of storage.
Figure 14. Mould invasion of injera with and without preservatives in 12 days

The effectiveness of the organic acids used in this study generally increase in effectiveness in the order of propionic acid < sorbic acid < benzoic acid which is in agreement with the results of Gould, (1996) which reported the order reflects their increasing lipophilicity. The sample containing benzoic acid and its salt (sodium benzoate) best preserved injera of all the preservatives tested could be due to the antimicrobial activity of benzoic acid is principally in the undissociated form and benzoic acid is relatively acidic (pKa = 4.2) which it inhibits the growth of spoilage moulds. It has the ability of the dissociated molecule to interface with membrane energetic (Akpan & Kovo, 2005). Further it was speculated that effectiveness of sodium benzoate than benzoic acid could be due to the fact that the salt is 200 times more soluble than the acid (DIONEX, 2004).
Similarly, the smaller effect of propionic acid in contrast to benzoic acid and sorbic acid confirms earlier results of Razavi-Rohani and Griffiths, (1999) who reported that even if propionic acid and its salts are primarily are inhibitory to moulds; some species of moulds are resistant and grow in media containing substantial amounts of propionic acid and its salts. Moreover when compare with propionic acid and sodium propionate, calcium propionate (also used in this study) is least effective on moulds.

Ryan et al., (2008) stated that the optimum pH level for the inhibitory effect of propionic acid is 5.0 with a maximum effect of pH of 6.0. In contrary to this, the pH of injera containing calcium propionate was highly acidic (around 3.44) and made it very far from the optimum pH for it to be effective. In addition to this, the least effectiveness of calcium propionate on injera could be due to the concentration used. Propionic acid should only be added in bread in a concentration not exceeding 3000ppm (also the concentration used in this study); recent studies have shown that under these conditions propionic acid is not effective against common bread spoilage organisms.

Finally, the combined (blended) effect of the four preservatives used for this study had been also evaluated. According to Martinez-Flores et al., (2004) the combined action of two or more preservatives is more effective in inhibiting the growth of microorganism, which could be due to a synergistic effect among the preservatives added to the product. However in this study there seem to be no synergistic effect since the blended sample was only more effective than calcium propionate.
4.5.2 Shelf Life of Injera with and without Preservatives

The effect of chemical preservatives on the shelf life of injera was shown in Table 10 and it was determined as the day in which mould was visible to the naked eye. The shelf life of injera without preservatives (control) was 3-4 days while injera containing 0.3 % calcium propionate was 4-5 days depending upon the storage temperature. Shelf life of injera containing 0.1 % benzoic acid and sodium benzoate were the better preservatives of all tested which prolonged the shelf life of injera up to 10 and 12 days respectively. 0.2 % of potassium sorbate and 0.2 % of blend of the four preservatives had similarly prolonged the shelf life of injera 6-8 days at 20±2°c.

Table 10. Shelf life of injera containing preservatives at 20± 2°c

<table>
<thead>
<tr>
<th>Injera Treatments</th>
<th>Shelf life ( days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3-4 days</td>
</tr>
<tr>
<td>0.1 % benzoic acid</td>
<td>8-10 days</td>
</tr>
<tr>
<td>0.1 % sodium benzoate</td>
<td>10-12 days</td>
</tr>
<tr>
<td>0.2 % potassium sorbate</td>
<td>6-8 days</td>
</tr>
<tr>
<td>0.3 % calcium propionate</td>
<td>4-5 days</td>
</tr>
<tr>
<td>0.2 % blend of the four</td>
<td>6-8 days</td>
</tr>
</tbody>
</table>

Fungi are the most common spoilers in bakery products. Commonly a shelf life of 3-4 days may be expected when they are unpreserved (Ryan et al., 2008). Preservatives may be microbiidal and kill the target organisms or they may be microbiostatic in which case they simply prevent them from growing, thus prolonging the shelf life of the product (Jay, 2000). The preservatives used in this study had prolonged the shelf life of injera from just 3-4 days to
up to 10 days. In these 10 days injera could be consumed before it loses its acceptability, since
the shelf life of a product is the time a product will remain organoleptically acceptable
(Doughari, 2007).
CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Even though injera is one of the most popular Ethiopian indigenous fermented food, it did not receive the scientific attention it deserves for many years as far as preservation concerned. Its storage period does not usually exceed 3 days at room temperature. To the best of our knowledge, there are no previous reports on the use of chemical preservatives in any of the Ethiopian traditional foods. This study not only identified the moulds that were responsible in spoiling injera but also evaluated the common chemical preservatives used in the food industry for the control of injera moulds.

In the present study three fungal species from *Penicillium*, *Rhizopus* and *Aspergillus (Aspergillus niger)* species were found spoiling injera while keeping it at ambient temperature. The first two species were more dominant in spoiling injera as the storage temperature lowers between 16-20°C, while *Aspergillus niger* is more dominant in spoiling injera stored at higher temperatures.

The study also showed that the shelf life of injera can be extended by the use of preservatives and storage conditions that will not favour mould outgrowth. Injera sample without preservatives exhibited the highest percentage of moulds from day 1 to 12 days of preservation and consequently had the poorest keeping quality as compared to those with preservatives. Investigation of the antimicrobial activity of the preservatives also revealed that sodium benzoate was the most effective of all the preservatives tested in inhibiting mould growth while calcium propionate was least effective of all the preservatives tested.
5.2 Recommendations

- In developing country like Ethiopia which is still under a struggle to be food secured, losing a substantial amount food due to spoilage is a disaster. Applying the result of this study can contribute a lot in saving a significant amount of injera that is lost in every household per every baking cycle. Moreover, prolonging the shelf life of injera can curb the problem faced to export injera to other parts of the world and to the many Diasporas that miss their home country as well as injera.

- As the study was conducted in Addis Ababa with specific climatic condition, further investigation on the type and kind of moulds that spoil injera on the warmer parts of Ethiopia should be evaluated.

- Further studies on the sensory attributes of injera with preservatives must be conducted to see if any of the additives has effect on health and nutritional status.

- Even if, the use of preservatives is an attractive means to diminish spoilage and insure food safety, consumers today are not in favour of additives as preservatives and an urge to reduce the quantities used exists within the bakery industry. Reduction of preservatives to sub-inhibitory levels has nevertheless been shown to stimulate growth of spoilage fungi in some cases. As a result of this, the application of the hurdle technology (combined technology) should be evaluated to minimize the amount of the chemical preservatives used in injera. So the application of other alternative preservation methods such as:
  - Modified atmosphere packaging (MAP),
  - Irradiation,
❖ Pasteurization of the packaged injera

❖ Natural plant extracts (also called green chemicals) and

❖ Bio-preservation [the use of microorganisms and/or their metabolites] to prevent spoilage and extend the shelf life of injera (and other Ethiopian traditional foods) should also be evaluated.
REFERENCES


APPENDIX 1. ANOVA of Rhizopus colony diameter on different kind of media

### Descriptives

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<tr>
<th>Colony Diameter in mm</th>
<th>N</th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>Std. Error</th>
<th>95% Confidence Interval for Mean</th>
<th>Minimum</th>
<th>Maximum</th>
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<td>5</td>
<td>58.000</td>
<td>37.8562</td>
<td>15.9352</td>
<td>10.980 to 105.920</td>
<td>.0</td>
<td>100.0</td>
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<td>51.800</td>
<td>37.3738</td>
<td>18.7141</td>
<td>5.194 to 98.905</td>
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<td>Total</td>
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<td>7.0851</td>
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### ANOVA

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<th>F</th>
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<td>.989</td>
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<td>29,352,000</td>
<td>20</td>
<td>1,487,000</td>
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<tr>
<td>Total</td>
<td>30,128,000</td>
<td>24</td>
<td>1,487,000</td>
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### Post Hoc Tests

**Multiple Comparisons**

<table>
<thead>
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<th>(I) Media Type</th>
<th>(J) Media Type</th>
<th>Mean Difference (I-J)</th>
<th>Std. Error</th>
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DECLARATION

I, the undersigned, declare that this thesis is my original work and that all sources of materials used for the thesis have been correctly acknowledged.

**Name:** Ashagrie Zewdu

Signature: ________________

Date: ________________

The thesis has been submitted with my approval as a supervisor.

**Name:** Dawit Abate (PhD) Signature: ________________

Date: ________________
RESEARCHABLE TITLES

While conducting this research I came across many researchable areas that need to be addressed in the future. For anyone who is interested I suggested the following titles as research topics:

1. Improvement of shelf life of “injera” using parabens (Esters of p-hydroxy benzoic acid)

2. The use of antifungal LAB to reduce the amount of calcium propionate, benzoic acid or sorbic acid added on injera

3. An attempt to minimize chemical preservatives added in injera by modified atmosphere packing combination to prevent fungal spoilage

4. Influence of temperature, water activity and pH on the growth of injera moulds

5. Effect of some naturally occurring antimicrobial compounds on the shelf life of injera

6. Preservation of Ethiopian injera using hurdle technology

7. *In vitro* study of the efficacy of several organic acids against molds isolated from injera

8. Effectiveness of sorbic acid in Ethiopian dairy products