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School of Chemical and Bio-engineering

Stream of Biochemical Engineering

Identification, Isolation and Characterization of Suitable Lactic Acid Bacterial Strain for Minimizing Fermentation Time of Kocho

A thesis submitted to School of Chemical and Bio-engineering in partial fulfillment of the requirements for Master's Degree in Biochemical Engineering

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This is to certify that the thesis prepared by **Mr. Redae Nuguse** entitled "***Identification, Isolation and Characterization of Suitable Lactic Acid Bacterial Strain for Minimizing Fermentation Time of Kocho***" and presented in fulfillment of the requirements for the degree of Master of Science in Biochemical Engineering complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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DEDICATION

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ABSTRACT

Kocho is a traditional food prepared through fermentation of decorticated Enset in Southern Ethiopia. It has been produced for many years and is being consumed in the region. Various research findings were reported on Enset with major concerns such as food safety and security issues, microbial dynamics of enset fermentation, microbial spoilage and accompanying changes, biochemical changes during fermentation and the effect of altitude on microbial successions. However, gaps were observed during Kocho fermentation, involving lactic acid bacterial (LAB) strain for traditional Kocho fermentation, standardization and optimization of the process. Kocho fermentation has been done in underground pit for a minimum of four months. This long time of fermentation has become a problem of food insecurity, and its minimization is the main concern of this thesis research. Three Kocho samples (Month-1, Months-3 and Months-6) and three Enset trial varieties (Agade, Disho and Gimbo) were taken from Morsito Wereda of SNNP, Ethiopia. The temperature and pH of fermented mass samples (Month-1, Months-3 and Months-6) were measured and recorded as 32, 27 and 29^0C and 4.78, 4.47 and 4.39, respectively. From each of the three samples, 1mg was taken and serial dilution was prepared, thereafter 0.1ml from each serial dilution factor was inoculated to 54 petri plates and incubated at 25, 30 and 35^0C temperatures. Growth of mesophilic microbes was recorded from each plate. The results showed that colony forming units of microbes were the highest in Month-1 sample at the temperature of 30^0C. Based on morphological structure, color and size, LAB were identified, isolated, re-cultured and purified to single strain. White, small and round structure of the strain were stored at 4^0C. By using Enset trial variety samples as a source of carbon, broth media were prepared according to MRS composition. 30-35 CFU of the pure strain was inoculated to each broth medium, and lactic acid concentration was determined by pH meter for 36 days with 4 days interval at 35 and 30^0C incubation temperatures. Generally, pH dropped down linearly for both temperatures and for the three Enset trial variety samples up to 23^{rd} day of fermentation and became constant (~pH=4.50) at 24-28^{th} day and reached ~pH=5.30 at 36^{th} day for all varieties. However, pH decreased faster at 35^0C for all Enset trial varieties, and Disho fermentation dropped faster for eight days than others, and long-delayed from 8-16^{th} day. The pH and temperature of the experiment corresponded with pH and temperature that were recorded during sample collection of sample Month-6. The result showed that only 24-28 days are needed to get Kocho with good texture, flavor, and smell and color which is equivalent to the naturally fermented Kocho in six Months.

Key words: Decortication, Enset, Fermentation, Kocho Lactic Acid Bacteria

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List of Acronyms

AFB	Aflatoxin B
AIDS	Acquired Immunodeficiency Syndrome
FDA	Food and Drug Administration,
GI	Gastro Intestinal
GMO	Genetically Modified Organisms
GRAS	Generally Regarded As Safe
IgA	Immunoglobulin A
LAB	Lactic Acid Bacteria
LD50	Lethal Dose 50
Spp	Species
Van A, Van B Van C	Vancomycin A, B and C

CHAPTER ONE

1. INTRODUCTION

As the world's population has been alarmingly increasing (Lamichhane, 2014), food security, energy and environmental protection are becoming current issues of the century (Pender and Gebremedhin, 2007). To solve these problems, scientists have been looking for different problem solving methodologies (Babu, 2000). Some of the means for problem solving include application of modern technologies and research outputs. Traditionally, some societies have their own social, cultural, religious and moral backgrounds solving such problems and adjusting themselves with new technologies and findings. As example from above, to solve food security, there are ample opportunities around the world, as the world is full of diversified high yielding potential plants. One of the high yielding potential plants in Ethiopia is enset (Ensete ventricosum Cheesman). Due to its drought tolerance and high productivity, Enset is regarded as a priority food security crop in Ethiopia (Habte, 2013), where it makes a major contribution to food security of the country. Enset is also known by the name of "Tree against hunger" (Stone *et al*., 2011).

Enset [false banana, Ensete ventricosum (Welw.) Cheesman, Musaceae] is a monocarpic short-lived perennial plant which is cultivated in Ethiopia since ancient times. It is drought tolerant; withstanding droughts that seriously damage cereals (Habte, 2013). About 20% of the human population in Ethiopia depends on enset as a food source (Brandt *et al*., 1997). Enset contributes to the local environment by improving the nutrient balance in soil (Elias *et al*., 1998), providing shadow, thus moderating temperature, and being part of farming systems with high biodiversity (Tesfaye, 2008).

Ethiopia, being a food insecure country and in protracted crisis (FAO, 2010), would be benefited from increased and improved use of enset. Enset cultivation is a straight-forward method to facilitate for people to achieve independent livelihood security. Enset can improve food security in drought-prone areas where the climate is warm but not too hot, thus in much larger areas than where currently used (Negash and Niehof, 2004).

There are four major farming systems in Ethiopia: pastoralism, shifting cultivation, the seed-farming complex, and the enset (Ensete ventricosum)-planting complex (Westphal, 1975). Of these, the enset-planting complex is the most sustainable indigenous farming system that can support the densely populated highlands of the south and south-western parts of Ethiopia. Enset is one of the oldest cultivated plants in Ethiopia. Anthropologists, archaeologists, historians, and other scholars argue that domestication of enset in Ethiopia occurred as early as 10,000 years ago (Brandt *et al*., 1997). The highlands of southern Ethiopia form the geographical centre of enset cultivation (Vavilov and Rodin, 1997), and the various ethnic groups in this region recognize and exploit many enset landraces.

Within the enset production systems, seven to ten million people cultivate the crop as a staple food, or as a co-staple with cereals and root- and tuber crops. Enset produces a starchy food from its vigorous pseudostem, its corm and from the stalk. A mixture of scraped pseudostem pulp, the pulverized corm, and the stalk of the in or escence is fermented in a pit. The resultant product is called '**kocho**' locally. Although many different dishes are prepared from kocho, most common are a pancake-like bread and porridge. Furthermore, the corm can be cooked fresh and consumed in a way similar to Irish potato, sweet potato, or cassava. Local people also believe that particular enset landraces have various medicinal properties (Tsegaye and Struik, 2000).

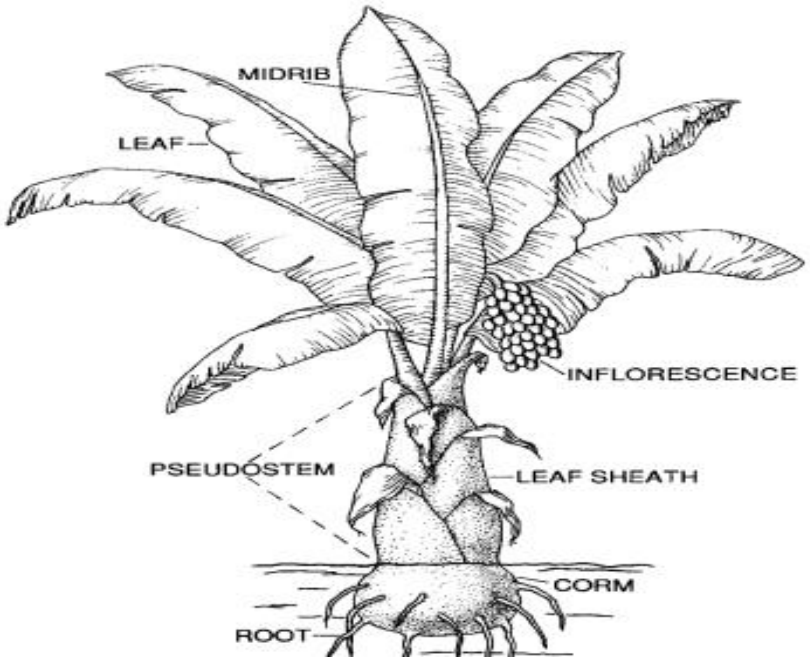


Figure 1: Enset plant (Enset ventricosum)

Enset (false banana), is usually larger than banana, reaching up to 10 meters and with a pseudostem up to one meter in diameter. The leaves are more erect than those of a banana plant, have the shape of a lance head and may be five meters long and nearly one meter wide. Its pseudostem dilates at the base to a circumference of 1.5 to 3.0 m. Depending on the variety and ecological condition of its cultivation, the pseudostem length ranges from 2 to 5 m. The pseudostem and leaf midribs color vary considerably; some are purple to dark red but most are light green with variegated brown patches (Taye, 1984).

Enset is usually harvested at onset of flowering, approximately, 5 to 8 years after planting, and is grown with generations of plants mixed, thus being a reliable food source overtime (Dalbato, 2000). The vegetative growth habit of enset is similar to banana plants, but enset is not grown for the fruits; enset fruits contain mostly large and very hard seeds (Karlsson *et al.*, 2011).

According to the farmers enset is the enemy of hunger, and human and livestock life is impossible without it. Despite its importance for food security and environmental sustainability, however,

little research and development work has been done on enset production systems. The Ethiopian government has focused its agricultural research and development efforts mainly on high-yielding annual crops that can be marketed. The attention of the government towards encouraging the adoption of these new technologies has resulted in a shift from enset to cereal-based agriculture. As a result, some enset growing regions have experienced famine in recent years, something unknown to past generations (Tsegaye and Struik, 2002).

The pseudostem, the main food source, was rich in soluble carbohydrates (80%) and starch (65%), but had low protein content (4%). An enset based diet should be supplemented with protein and complementary amino acids; for example from beans, which are suitable to intercrop with enset (Mohamed *et al.*, 2013). For humans, edible parts of enset are the pseudostem (squeezing and fermenting gives the main food source from enset). The main food items obtained from Enset are Kocho, Bulla and Amicho. Kocho is more fibrous, major product of Enset fermentation and starchy mineral rich food. Bulla is less fibrous, unfermented, partially fermented or sometimes fermented byproduct during Enset fermentation (Ashenafi, 2006), whereas Amicho is unfermented and underground part of Enset (the corm) (Karssa *et al*., 2014). All the three parts of the food items are rich in starch and mineral contents (Tadele, 2009), even though less in protein, fat and vitamins (Hunduma and Ashenafi, 20011).

Kocho, which can be baked to a thin bread, provides a good source for Ca and Fe (Abebe *et al*., 2007) but is low in protein; this can be substantially improved by adding for example kidney beans to the diet; beans are frequently intercropped with enset in home garden agriculture (Abebe *et al.*, 2010). The chemical composition and mineral content of enset make it is a relatively suitable fodder for ruminants (Nurfeta *et al*., 2008a), which in turn provide proteins to humans.

Sensory attributes of Kocho are not acceptable by most of none Enset producing areas since they are not familiar to the odor, taste and flavor of the food. Most of the uncommon sensory attributes are the results from microbial spoilage due to high moisture content of Kocho. High moisture content supports the growth of spoilage microorganisms which in turn produce unpleasant organic compounds. The nutritional and organoleptic qualities of Kocho could therefore, be process related. Nutrient loss and time taking fermentation processes are common and vary from place to place (Ashenafi, 2006).

Recently, awareness of the importance of enset for food security and environmental sustainability has increased. For instance, some communities that had shifted from enset to cereal production have started to grow enset again as their recent experience with famine has caused them to appreciate Enset's capacity to prevent hunger. In addition, enset production systems are being promoted outside of the traditional enset region, not only in adjacent areas but also in areas far to the north and east of Ethiopia where, historically, the population has depended mainly on cereal crops (Tsegaye and Struik, 2002).

1.1. Problem Statements

Various research findings were reported by different investigators on Enset with the major concerns such as Enset cultivation and productivity (Tsegaye and Struik, 2001), food safety and security issues (Kanshie, 2002). Moreover, Microbial dynamics of Enset fermentation (Gashe, 1987a), microbial spoilage and accompanying changes (Gashe, 1987b) and biochemical changes during fermentation and the effect of altitude on microbial successions (Hunduma and Ashenafi, 2011), chemical composition and degradability in different morphological fractions (Nurfeta *et al.,* 2008), mineral content (Atilabachew and Chandravanshi, 2008) and mineral absorption inhibitory factors, improving the indigenous processing of Kocho using different cultivars of Enset (Yirmaga, 2013), differences between the pits and jars of Kocho fermentation (Karssa *et al*., 2013) were evaluated, assessed and investigated.

Besides, time of Kocho fermentation was differ from place to place and from condition to condition, it takes up to seven months. This long time of traditional Kocho fermentation is a problem for food security. Moreover, WHO food safety unit has given high priority to the research area of fermentation as a technique for preparation/storage of food. The main reason for this is that in developing countries, one tenth of the children under five years of age dies due to dehydration. The dehydration is mainly caused by incidences of diarrhoea. The main cause for getting diarrhoea is the ingestion of food not having the appropriate standard regarding the hygienic condition. The hygienic standard of a food is based on the processing and handling of the food, as well as on the conditions of the raw materials.

The gap of long time of fermentation of Kocho processing was filed using lactic acid bacteria in this thesis. To make Kocho security stable in the country, the time that spent on the fermentation will be minimized using lactic acid bacteria or other responsible. Therefore, this study mainly carried out to minimize the fermentation process of kocho including identification and isolation of suitable lactic acid bacteria and biochemical changes that occurred during fermentation process of Kocho under different treatment conditions. Lactic acid fermentation of food has been found to reduce the risk of having growth of pathogenic microorganisms in the food. The current study was, therefore, initiated to bridge the aforementioned knowledge gaps.

1.2. Objectives

1.2.1. General Objective

The main objective of this thesis was minimization of fermentation time of Kocho production using lactic acid bacterial strain.

1.2.2. Specific Objective

- ✓ To know the concentration of microbial growth and their growth rate kinetics
- ✓ To identify, isolate and characterize specific strain of lactic acid bacteria
- ✓ To purify lactic acid bacteria in separate and sterile MRS media

- ✓ To measure the concentration and production of lactic acid, from Enset powder samples (Agade, Disho and Gimbo)

1.3. Significance of the Study

- o To distribute lactic acid bacterial strain for local farmers for the fermentation of decorticated Enset during traditional Kocho fermentation as a starter culture
- o To produce Kocho in large scale, and standardize the traditional Kocho processing and to open a door for commercialization of Kocho world-wide.
- o To increase sustainable food security in the country through quick Kocho fermentation process to reduce the possible risks of harmful microbial growth

1.4. Scope of the Study

The study was conducted in Hadiya zone, Morsito Wereda, and Addis Ababa University-Addis Ababa Institute of Technology Biochemical Engineering Department Laboratory. The study was mainly concerned in identification, isolation and purification of lactic acid bacteria from samples of (Month-1, Months-3 and Months-6), biochemical changes of identified and isolated lactic acid bacteria and concentration of lactic acid production from Enset powder samples (Agade, Disho or Gimbo) as a carbon source during lactic acid bacterial growth in the laboratory.

CHAPTER TWO

2. LITERATURE REVIEW

2.1. Relative Chemical Composition of Enset Plant

The enset is a plant of which every single part is used. There are a variety of not -alimentary purposes which the plant is designed to: (a) the building function (enset is used to cover the roofs or the house's walls, to baste materials, to make containers, ropes, mats, bags, ropes and sieves); (b) the economic function (the leaves of the plant, and the strong fibers which are extracted from it, are important articles of barter, an integral part of the inter-tribal trade, and goods through which to get money); (c) the domestic function (the stems and central nervatures, once dried, feed the fire; the pulp extracted from these parts can be used as a duster or a brush, as a pillow or a nappy for children, as a support for pots; the fresh leaves are used as serving dishes or as a protective covering inside the pits where the enset is fermented or to pave the ground where the processing of the plant takes place); (d) the protective function (the fresh leaves wrap kocho, honey, tobacco, butter, bread, crops, but also, in the past, the newborn babies; they cast a shadow over the other crops or men, screening them from the light of the s un, the wind, the rain; they allow the packaging and therefore the transport of objects and goods to the local markets; the enset plants surround the house providing security, primarily in the form of a supply of close and always available food); (e) the dietary function, but for animals as forage (since the plant contains a lot of water and is quite resistant to drought, some specimens of enset are cut down to feed the animals especially in the dry season when the grass is spare); (f) the healing function, specific clones and parts of the enset are used for therapeutic purposes, both for humans and animals, to treat fractures and broken bones, problems related to childbirth, for example, as an aid to release the placenta, for diarrhea and as a means of birth control for abortion (Tesfaye, 2008). Since the existence of a close intertwining of food and eating with so many other subjects, the enset might become a privileged pass key to the study of ecology, small-scale agriculture, and nutrition and gender relations. The specificity of the Enset's case suggests the existence of more flexible and varied working patterns (Pender and Gebremedhin, 2007).

Dry matter (DM) content of enset was 11 to 15% of fresh weight and the organic matter fraction of DM was around 90% (Table 1). As fractions of DM, crude protein content was 3 to 13%, crude fat 0.4 to 5%, crude fibre 6 to 24% and soluble carbohydrates approximately 50 to 80% for different parts of enset (Table 1). Leaves had the highest concentration of protein, fat, sugar, fibre, cellulose, hemicellulose and lignin, and least of soluble carbohydrates and starch (Table 1). The corm had the highest concentration of most soluble carbohydrates and starch, and least of protein, fibre, cellulose and sugar, while the pseudostem had least of lignin (Table 1).The mineral content was 3 to 4% of potassium (K) and below 2%, in most cases below or much below 1%, of all other (Table 2). Leaves had the highest concentration of P, Ca, Mg, Se and Mn, the pseudostem had the highest concentration of Zn and corm had the least of K and Mn (Table 2). The two tables are given below (Mohammed *et al*., 2013).

Variable	Corm	Pseudostem	Whole enset
Dray matter	14.08	14.59	14.34
Organic matter	91.83	92.54	90.87
Ash	8.17	7.47	9.13
Crude protein	3.33	3.65	5.98
Crude fat	0.41	0.36	0.84
Crude fibre	5.65	7.51	9.48
Soluble carbohydrate	82.44	81.02	74.57
Cellulose	8.75	10.81	14.95
Hemicellulose	5.93	8.61	9.39
Lignin	2.11	0.79	1.97
Starch	71.19	64.93	60.62
Sugar		2.28	0.85

Table 1: Relative composition of enset

Enset (Ensete ventricosum) has many usages: food, fodder, fibres and traditional medicine. Being perennial, enset improves local climate and soil conditions. It could contribute to improved food security in several drought-prone parts of the world. The aims of this study were to reveal the amino acids of enset corm, which can be cooked as a root crop, and to increase the general knowledge regarding chemical composition and energy values of different enset fractions. Water content was high, 85 to 90%, which is beneficial when used as fodder during dry periods. Enset corm contained 17 of 20 amino acids and had similar or higher concentration than potato of 12 of these. Leaves had 13% protein, among the highest available in Ethiopia, 20% crude fibre and 10% sugar; a good fodder and suitable for ensilage. The pseudostem, the main food source, was rich in soluble carbohydrates (80%) and starch (65%), but had low protein content (4%) (Mohammed *et al*, 2013).

Mineral	Corm	Pseudostem	Whole enset
Na	0.03	0.02	0.01
P	0.11	0.12	0.15
K	3.06	3.07	3.40
Ca	0.37	0.41	0.33
Mg	0.15	0.15	0.13
Se	0.07	0.06	0.05
Cu	2.9	2.7	1.3
Mn	43.3	61.0	64.6
Co	-	0.01	0.01
Zn	90.0	116.0	48.0

Table 2: Mineral composition of enset

Little is known about enset-based farming systems, however, particularly with respect to production systems, cultivation methods and genetic diversity. Average farm sizes have decreased with increasing population, and traditional technologies and practices have failed to produce enough enset to feed the population. The crop has a long growing cycle and gives low yields in traditional production systems. Because enset cultivation spans several different ethnic groups and agro-ecological zones, production methods and processing procedures vary greatly. In order to improve traditional enset production systems, indigenous knowledge from the different enset growing regions needs to be analyzed and understood.

Such an improved understanding of indigenous knowledge related to enset production can help to identify guidelines for selecting potentially interesting topics for scientific research (De Walt, 1994). Diseases, insect pests and drought have also threatened the production of enset. Though some characterization of the crop's genetic diversity has been done, identification of plant material with resistance to diseases and pests, or with the capacity to excel in specific environments, has not begun yet.

The Hadiya zone falls within the area of the combined enset, which is used as a staple crop, being however combined with cereals and tubers. The groups that use this model are Gamo, Hadiya, Wolayta and Ari. Within the Hadiya group there are considerable differences among the households: richer families with more resources use more cereals at the expense of the enset, families with fewer resources depend entirely on it. Livestock is important to get the manure; the oxen are used to plow. The density of the population is high, sometimes more than 200 people per km^2. The main products derived from the enset are kocho, bulla and amicho; they presuppose different methods of production and are consumed at different times throughout the year and even in special occasions (Tsegaye and Struik, 2002).

Hadiya people are nowadays mainly settled along the Ethiopian Rift Valley, between the Gibe river basin and the northern part of the Omo River. The southern administrative region which they belong to is one of the most densely populated areas of Ethiopia. Usually a community of a certain size is inhabited by clans (sulla) that are divided into lineages (moollo) and sub-lineages (mine). Each of these is chaired by its respective judge or head (daannuwwa), which are also responsible for the agricultural associations of mutual assistance. The farmers generally live in village communities, as opposed to the isolated farms in the north; they give great weight to the kinship relations because of the more immediate ties they keep with household. They have a structured legal system; the domestic group and the political institutions are able to keep a close watch on the behavior of the individual (Tsegaye and Struik, 2002).

The Hadiya society is characterized by a predominant commitment to agricultural activities, especially the enset-growing, which is often combined with that of grain, barley and maize, as well as the breeding of domestic animals. The methods of cultivation and harvesting of the plant follow a specific and detailed cycle. Enset seems to play a key role in both the economic and the family life. As witnessed by some surveys (AAAS, 1997), the peasants are accustomed to say in a strong

evidence of self-awareness that "enset is their food, our clothes, their beds, our homes, the food for their animals, their dishes".

One of the most impressive feature of the area is the flourishing, luxuriant vegetation. The single plot resembles a botanical garden, filled with countless species of plants. The first striking thing for the observer is the size: the largest exemplars can reach 10 meters in height, with a false trunk up to one meter in diameter. It is a tough plant, with thick and hard leaves: it grows at best at an altitude between 2000 and 2750 meters; it does not tolerate the frost, but it survives the drought; it is not affected by heavy rains; in case of inclement weather or limited rain it may stop to grow but never dies completely. This characteristic, the persistence in extreme situations, that Dessalegn Rahmato (1995) called “resilience” plays an important role in the environmental conservation: the enset protects the soil from the erosion due to rainwater; it positively alters the land because of a continuous application of manure. Many fields keep productive for decades, if not for centuries. Around the house, where commonly it is grown, it provides protection from the wind and the sun. It is considered aesthetically desirable, since it beautifies the landscape and gives shape to the human settlements which result to be close-knit and encircled by the plantations (Negash and Niehof, 2004).

2.2. Definition of Fermented Food

Emmanuel (2011) has defined fermented foods as those foods which have been subjected to the action of micro-organisms or enzymes so that desirable biochemical changes cause significant modification to the food. However, to the microbiologist, the term “fermentation” describes a form of energy-yielding microbial metabolism in which an organic substrate, usually a carbohydrate is incompletely oxidized, and an organic carbohydrate acts as the electron acceptor (Adams, 1990). This definition describes that processes involving ethanol production by yeasts or organic acids by lactic acid bacteria are considered as fermentations. Whichever definition used, foods submitted to the influence of lactic acid producing microorganisms is considered a fermented food (Emmanuel, 2011).

2.3. Classification of Fermented Foods

Fermented foods can be classified in many different ways, Dirar (1993) says that in Southeast Asia the classification often is according to the kind of microorganism involved. Other classifications are based on commodity (Campbell-Platt, 1987). Dirar presents the traditional Sudanese classification that is based on the function of the food. The different classifications show the different viewpoints of the authors, and often a classification that works very well in one part of the world is not suitable in other parts. Once the classification scheme is made up, it can be difficult to distribute the foods.

2.4. Benefits of Fermenting Food

Many fermented milk products, which are eaten as they are, contain living microorganisms. Acidofilus milk, filmjolk, yoghurt, junket and kefir are fermented milks containing either Lactic Acid Bacteria (LAB) alone or both LAB and yeast or mixed cultures producing mainly lactic acid

or a combination of lactic acid and small amounts of alcohol. Kumiss is fermented milk made of mare's milk using a mixed culture. Lassi in India, a fermented milk consumed as a beverage after dilution with water, and Yakultin Japan and China are typical fermented milk products made of mixed culture by spontaneous fermentation. Other milk based products which are fermented with some cereals are flummery which is fermented yoghurt like product containing boiled whole grains and prokllada which is mainly fermented whey with addition of taste enhancing substances. Lao-chao, a fermented, glutinous, slightly alcoholic, steam cooked rice, maheua non-alcoholic beverage from maize, sorghum or millet, pozol which is either a thick porridge like food or a thin beverage made of maize flour, a thick alcoholic beverage similar to beer made of sorghum, and tapea thick pasty fermented food containing alcohol made from millet or maize but also some times from cassava are typical examples of fermented foods made of cereals (Adesulu and Awojobi, 2014).

Foods like injera from tef, and kisra from sorghum are commonly made after fermenting dough for two or three days with or without starter. The common fermented legume products include hama-natto which is a soybean paste, used for flavoring, oncom made of groundnut presscake, or soybean presscake used as a relish, fermented soy milk and sufu made of soybean curd, mold, salt and alcohol. Kimchiis a popular fermented food made mainly of vegetables in Korea. Pickled fruits and vegetable are common in many countries and sauerkrautis a well-known product made by fermenting cabbage. German salami (smoked), Italian salami, Lebanon bologna (sausage), Longaniza (sausage), and Teewurstare typical fermented meat products of Europe. While paak made of fish and cereal by lactic acid fermentation and pin dang and tarama made of fermented roe are typical fermented fish products of the Far Eastern countries (Sahlin, 1999).

Lactic acid bacteria (LAB) were first isolated from milk (Carr *et al*., 2002). LAB occur naturally in fermented food and have been detected in soil, water, manure and sewage. LAB exist inhuman and in animal Lactic acid bacteria are among the important groups of bacteria providing health benefits to human, animal, and plant (Rodriguez *et al*, 2012). Using LAB in food fermentation is one of the ancient known food preserving techniques. Lactic acid is used in the food industry as flavor agent, preservative and acidifier. It is also used in other industries that manufacture leather, textile, pharmaceuticals, pesticides, herbicides and cosmetics.

The estimated world demand for lactic acid is about 130,000-150,000 metric tons per year and demand continues to rise because of the increasing demand for green or Earth-friendly materials. Lactic acid is the monomer for the production of polylactic acid (PLA), which is a bioplastic material that can be used to manufacture disposable kitchen wares and even high-end biomedical products. PLA is used in making biodegradable implants to repair fractures and injuries such as broken bones (Toleco *et al*, 2016). Because of its biocompatibility, PLA has been approved by the US FDA in 2004 for the treatment of facial fat loss, to thicken skin and improve the appearance of folds and sunken areas. Lactic acid may be produced chemically or through microbial fermentation (Wee *et al*., 2008). While chemical synthesis produces racemic mixtures, some microorganisms can selectively produce pure optical isomers. This gives fermentation an advantage over chemical

synthesis, as it eliminates the costly process of enantiomeric separation, when pure isomers are needed, especially for the synthesis of high quality PLA. However, most lactic acid bacteria are not able to assimilate more complex carbon sources like starches and dextrins and rely on simple sugars, like glucose, for their metabolism. In addition most lactic acid bacteria optimally grow on a rather costly medium called de Man, Rogosa and Sharpe (MRS) medium which contain proteose peptone, yeast extract and beef extract that play important roles as nitrogen and vitamin sources (Altaf *et al*., 2005).

In order to increase fermentation to a larger-scale, a medium with components derived from industrial by-products would be more viable. Studies made on alternative large scale fermentation media for lactic acid fermentation were made primarily to reduce costly nitrogen sources. These include the use of corn steep liquor or CSL, whey, trub or lees, soybean flour, tofu liquid waste and others (Wee *et al*., 2008).

Investigations of growth and physiology of microorganisms are most often performed with batch grown cultures. The main characteristics of a batch system are: excess of all nutrients, drastic change of population density during few generation times, drastic change of the ratio substrates/products during few generation times and (ideally) a completely homogeneous medium with no fluxes of components into or out of the system. The phenotype of an organism is highly affected by its growth medium, therefore the outcome of a batch culture is also a very specific phenotype which is not necessarily the most abundant form. It is important to emphasize that batch-culture-like systems are not abundant in nature, microorganisms do not often grow under unlimited nutrient conditions at maximal rate. Characteristics of natural ecosystems are instead: limited nutrient supply, long generation times, mixed populations as well as continuous fluxes of components into and out of the system (Altaf *et al.*, 2005)..

In 1950 Jacques Monod formulated the concept of steady-state cultures and the cultivation of continuous cultures in laboratory chemostats. This technique offered a variety of new possibilities for the investigation of microbial physiology. Microorganisms can be cultivated under all kind of desired limitations. Long-term competition, symbiosis-or mutant selection experiments can be carried out using pure cultures or mixtures of microorganisms, and above all, the growth rate of the culture can be fixed by the operator of the chemostat at all desired levels between almost zero and the maximal growth rate of the organisms.

A small scale chemostat was run in this study. As a model organism a lactic bacteria was chosen that had been enriched from an intestinal swab in rich yeast-glucose medium at 37°C. The organism was gram-positive, non-spore-forming, cocci-shaped, catalase-negative and fermented glucose to lactate in a 1:2 molar ratio in batch culture.

Lactic acid bacteria are subdivided into obligatory homo-fermentative lactobacilli (group A), facultative hetero-fermentative lactobacilli (group B) and obligatory hetero-fermentative lactobacilli (group C) Whereas group A lactobacilli degrade hexoses almost exclusively by the

Embden-Meyerhof pathway to lactate and cannot use pentose or gluconate, group B lactobacilli can additionally ferment pentoses via inducible pentose phosphoketolase and produce acetic acid, formic acid and ethanol beside lactic acid under glucose limitation. Group C lactobacilli ferment hexoses to lactic acid, acetic acid, ethanol and CO_2 via the phospho-gluconate pathway.

Properties such as nutritional, environmental, and adhesion adaptations have provided LAB with the ability to adapt and present in different environments ranging from food matrices such as dairy products, meats, vegetables, sourdough bread, and wine to human mucosal surfaces such as oral cavity and gastrointestinal tract (Morelli *et al.*, 2011). LAB is known for their fastidious nutritional requirements which may vary among species and even among strains (Vera Pingitore *et al.*, 2009). Strains of LAB are also known as fast growing microorganisms that can explore different metabolic activities. Metabolic activities are associated with production of many beneficial compounds such as organic acids and antimicrobial compounds, unique enzymes that can breakdown complex organic compounds into simple functional compounds. Thus the fast growing characteristics and the metabolic activity are the keys of LAB benefits and applications.

Metabolic activities of LAB which are necessary for survival and growth are also important for any application. The primary metabolic activity in LAB is degradation of carbohydrates and related compounds to obtain mainly energy and carbon molecules (Sanchez and Demain, 2008). However, proteinases and peptidases activities of LAB have gained much attention due to their importance in the accelerated maturation and enzyme modification of different food products especially cheese (Kirilov *et al*., 2009). Other metabolic activities including lipolysis and degradation of complex compounds such as polyphenols and being of lavones have their important role in food industry and human health. However, the metabolic activities of LAB are not naturally optimized for maximal production rates of biotechnologically important compounds (Sanchez and Demain, 2008). The growth and metabolic activities of LAB could be affected by both biochemical and biophysical environments. Biochemical environment is made available through culture media that are utilized for bacterial growth and referred to as nutrients or nutritional requirements. Depending upon the special nutritional requirements of particular LAB species a large variety of culture media have been developed with different purposes and uses. Coordination by growth factors and optimization of nutritional requirements could ensure that necessary enzymes and correct amount of each beneficial compound are made at any given time (Hoefnagel *et al.*, 2002). Knowledge of factors affecting metabolic activities of LAB is important to optimize the activities and to achieve better controlled processes. Thus a large amount of research was established with regard to the relationship between LAB's nutritional requirements and metabolic activities

By tradition, lactic acid bacteria (LAB) are the most commonly used microorganisms for preservation of foods. Their importance is associated mainly with their safe metabolic activity while growing in foods utilizing available sugar for the production of organic acids and other metabolites. Their common occurrence in foods and feeds coupled with their long-lived use contributes to their natural acceptance as GRAS (Generally Recognized As Safe) for human consumption (Aguirre & Collins, 1993).

However, there are many kinds of fermented foods in which the dominating processes and end products are contributed by a mixture of endogenous enzymes and other microorganisms like yeast and mold. Very often, a mixed culture originating from the native micro-flora of the raw materials is in action in most of the food fermentation processes. However, in an industrial scale a particular defined starter culture, which has been developed under controlled conditions, is of first preference so that the qualities of the finished product could be consistently maintained day after day. Moreover, modern methods of gene-technology make it possible for the microbiologists to design and develop starter cultures with specific qualities.

Single as well as mixed culture fermentation of pearl millet flour with yeast and lactobacilli significantly increased the total amount of soluble sugars, reducing and non-reducing sugar content, with a simultaneous decrease in its starch content (Khetarpaul and Chauhan, 1990). The digestibility of starch in bengal gram, cowpea and green gram was increased by fermentation (Urooj and Puttaraj, 1994).

Many microbiological studies deal with identification of organisms isolated from various fermented foods. Lactic acid bacteria isolated from tomatoes that were naturally fermented under partial anaerobic conditions were found to be Leuconostoc mesenteroides, Lactobacillus brevis and Streptococcus spp. (Beltran-Edeza and Sánchez, 1989).

Kocho production is done in a ground pit, at a temperature (about $30^{o}C$), anaerobic fermentation (as air is limited); the starter culture is taken from amicho (formerly fermented decorticated enset pseudosteam), which facilitates fermentation of Kocho (used as inoculation of microorganism) and from some literatures the dominant microbes that facilitates fermentation of Kocho are lactic acid bacterial species.

In spite of the fact that Kocho is very important source of food, energy and industrial raw material (Olango, 2009), in Ethiopia, its usage is known by insignificant regions of the country as only 67,000 sq. km of the total land (about 1.2 millions of sq. km) was cultivated by Enset plant (Ashenafi, 2006). The main reasons to limited cultivation and consumption of Kocho could be related to uncommon inherent sensory attributes for non-consumers, nutrition loss, long fermentation period, lack of awareness and its short shelf-life. Sensory attributes of Kocho are not acceptable by most of none Enset producing areas since they are not familiar to the odor, taste and flavor of the food. Most of the uncommon sensory attributes are the results from microbial spoilage due to high moisture content of Kocho. High moisture content supports the growth of spoilage microorganisms which in turn produce unpleasant organic compounds. The nutritional and organoleptic qualities of Kocho could therefore, be process related. Nutrient loss and time taking fermentation processes are common and vary from place to place (Ashenafi, 2006). Food taboo, lack of knowledge, experience, skills and technology in Enset cultivation, fermentation and consumption are the other drawbacks (Stone *et al.*, 2011).

To improve Kocho related problems and increase its productivity, shelf-life, food safety and quality of the products, among numerous techniques, fermentation methods and addition of traditional preservatives of plant sources are very important activities. Such traditional preservatives contain chemical constitutes with characteristic of flavors, antioxidant as well as antimicrobial activities (Marshall and Mejia, 2012).

In Ethiopia, enset is fermented to produce kocho, the main food product obtained by fermenting the mixture of the scraped pulp of the pseudostem, pulverized corm and stalks of inflorescence. Other food types obtained from enset are: bulla (extracted juice from edible part of enset) and amicho (non-fermented corm consumed after boiling). Scientific information is available on the microbiology of enset fermentation (Ayele and Gashe, 1994). These studies indicated that lactic acid bacteria are the dominant microorganisms involved during the traditional fermentation of enset for kocho production. Kocho also contains a diverse group of microorganisms at the beginning of fermentation as aerobic and anaerobic spore-formers, enterobacteriaceae, lactic acid bacteria and yeasts (Gashe, 1987).

The traditional processing of enset has two Phases namely: Phase I (surface fermentation), beginning of fermentation and continued for about 15 days and Phase II (pit fermentation), completion of fermentation for about 15 additional days and stay for a maximum one year (the present survey data). The corms of selected mature enset plants were used as major raw material for the preparation of starter culture (Amcho). After removing the soil and other unwanted parts of the corm with knife (Shole), it was slightly pulverized with a serrated and sharp-edged tool made from animal bone (scapula) to remove the remaining unwanted part and to make it ready for fermentation. All the prepared corms were wrapped with fresh enset leaves near the farm site and left at ambient temperature for about 8 days. At the 5^{th} day, it was exposed to the sun for 5 to12 hours and again wrapped with fresh enset leaves and allowed to further ferment for 3 to 5 days. Traditionally, kocho was fermented with following procedures (Shank and Ertiro, 2008).

2.5. Traditional Kocho Preparation

Enset is usually harvested just before flowering, the preferred harvesting time is just when the plant flowers. The time duration required to flower depends upon climatic conditions, clone type, and management. Hence, the flowering time varies from 3 to 15 years but is optimally around 6 or 7 years (Shank and Ertiro, 2008).

2.5.1. Enset Washing

The enset is washed to remove the soils, insects, dusts and any unwanted impurities which may decrease the quality of the product. After harvesting leaves and older leaf sheaths are first removed from the designated plants. The internal leaf sheaths (commonly up to two meters in length) are separated from the pseudostem down to the true stem, which is about a 20 centimeter section between corm and pseudostem. Then the true stem is separated or stumped from the underground corm. The concave side of the leaf sheath is peeled and cut into pieces of about one meter length

and split lengthwise in order to shorten the leaf sheath to a workable size (Asres and Omprakash, 2014).

2.5.2. Decorticating, Grating and Squeezing

Then the leaf sheath is decorticated using a decorticating machine which helps to reduce the size. There is variation in the way that the corm is grated in different places. One practice is to uproot the corm and remove any soil from its surface at this stage the pseudostem and corm reduced to small pieces and ready for squeezing. Squeezer is used to separate kocho and bulla from decorticated and chopped enset by adding water, which added to aid the extraction of bulla, with the application of some force using presser. Then the Kocho is send to the fermenter and Bulla is collected in received tank (Asres and Omprakash, 2014).

2.5.3. Fermentation

According to (Asres and Omprakash, 2014), after the completion of decorticating and grating, the leaf sheath pulp is spread on fresh enset leaves covering the tank in the ground, after which the grated corm is spread on the processed pulp. A starter is added to aid in fermentation. This starter consists either of already fermented kocho to which various spices and herbs are added or fermenting agents are prepared from the inner portion of the corm and then mixed with the decorticated pulp and grated corm after some weeks. Turning, mixing, rinsing, and chopping continue over a period of time until the mixture partially ferments, when it is then referred to as kocho. The total time period for this fermentation to occur ranges from two to five Months. Then the fermented kocho is stored in fermenter tank that placed in the ground. The kocho must be left in a storage tank for a minimum of a month, but it can be stored for many months and even for several years. The fermenter tank is opened at intervals to allow aeration. This is repeated until the desired fermentation quality is reached or the food is needed and increasingly exported to urban markets.

2.5.3.1. Microflora in Fermented Foods

Traditionally, lactic acid bacteria (LAB) are the most commonly used microorganisms for preservation of foods. Their importance is associated mainly with their safe metabolic activity while growing in foods utilizing available sugar for the production of organic acids and other metabolites (Zhou, 2012).

However, there are many kinds of fermented foods in which the dominating processes and end products are contributed by a mixture of endogenous enzymes and other microorganisms like yeast and mold. Very often, a mixed culture originating from the native microflora of the raw materials is in action in most of the food fermentation processes. However, in an industrial scale a particular defined starter culture, which has been developed under controlled conditions, is of first preference so that the qualities of the finished product could be consistently maintained day after day. Moreover, modern methods of gene-technology makes it possible for the microbiologists to design and develop starter cultures with specific qualities (Sahlin, 1999).

Many microbiological studies deal with identification of organisms isolated from various fermented foods. Lactic acid bacteria isolated from tomatoes that were naturally fermented under partial anaerobic conditions were found to be Leuconostoc mesenteroides, Lactobacillus brevis and Streptococcus spp. (Zhou, 2012). In Asia mainly molds of the genera Aspergillus, Rhizopus, Mucor, Actinomucor, Amylomyces, Neurospora and Monascus are used in the manufacture of fermented foods. In Europe, mold-ripened foods are primarily cheeses and meats, usually using a Penicillium-species (Leistner, 1990). Gari made by fermenting cassava slurry was found to contain Bacillus, Aspergillus and Penicillium spp. as the predominant organisms (Wadamori, 2014). The micro-organisms present in a fermented food made in Ghana called dawadawa after 24h of fermentation, predominantly were Bacillus sp. with small numbers of (0.3%) Staphylococcus sp., after 36h 60% Bacillus sp., 34% Staphylococcus sp. and after 48 h 56% Bacillus sp. and 42% Staphylococcus spp. (Zhou, 2012). Indonesian tape ketan, a sweet, sour and alcoholic rice product, is produced using a starter culture containing molds, yeasts and bacteria. After 72h of fermentation, the pH was 3.5 while the biomass of the hyphae of the molds was 15.3mg/g and of the yeast 3.3mg/g. (Cook *et al.,* 1991). In Okpiye, which is a food condiment prepared by the fermentation of Prosopis Africana seeds, several species of bacteria especially Bacillus subtilis, B. licheniformis, B. megaterium, Staphylococcus epidermis and Micrococcus spp. were found to be the most active organisms. In trahanas, a fermented food prepared in Greece from a mixture of milk and wheat flour, Streptococcus lactis, Streptococcus diacety lactis, Leuconostoc cremoris, Lactobacillus lactis, Lactobacillus casei, Lactobacillus bulgaricus and Lactobacillus acidophilus were found to play the major role in producing acid and aroma (Wadamori, 2014)

2.5.3.2. Nutritional Value of Fermented Foods

According to (Wadamori, 2014), a significant increase in the soluble fraction of a food is observed during fermentation. The quantity as well as quality of the food proteins as expressed by biological value, and often the content of water soluble vitamins is generally increased, while the antinutritional factors show a decline during fermentation. Fermentation results in a lower proportion of dry matter in the food and the concentrations of vitamins, minerals and protein appear to increase when measured on a dry weight basis (Adams, 1990). Single as well as mixed culture fermentation of pearl millet flour with yeast and lactobacilli significantly increased the total amount of soluble sugars, reducing and non-reducing sugar content, with a simultaneous decrease in its starch content (Onda *et al.,* 2003). Combination of cooking and fermentation improved the nutrient quality of all tested sorghum seeds and reduced the content of antinutritional factors to a safe level in comparison with other methods of processing. Mixed culture fermentation of pearl millet flour with Saccharomyces diastaticus, Saccharomyces cerevisiae, Lactobacillus brevis and Lactobacillus fermentum was found to improve its biological utilization in rats (Onda *et al.*, 2003). Fermentation induced a significant decrease in lipid and lignin contents of okara, which is an insoluble residue obtained as a by-product in the manufacture of soybean milk. The fermented okara on the other hand neither increased PER nor the weight gain in rats (Ashenafi, 2006) compared to non-fermented samples. The digestibility of starch in bengal gram, cowpea and green

gram was increased by fermentation. Cooking of these fermented legumes further increased the starch digestibility (Ashenafi, 2006).

2.5.3.3. Microbial Activities in Enset Fermentation Processes

According to Gashe (1987a), on the day zero, Kocho has high moisture content, low titrateble acidity, near neutral pH and high soluble reducing sugar concentration when compared to the final fermentation days of Kocho. During the initial period, Kocho contained a diverse group of microorganisms such as aerobic and anaerobic spore formers, Gram negative bacteria including members belonging to the Enterobacteriaceae, lactic acid bacteria and yeasts. In indigenous fermented foods, the microorganisms responsible for the fermentation are usually the microbial flora naturally present on the raw substrate (Ashenafi, 2006).

It has also been indicated that Leuconostoc mesenteroides is responsible for initiating the fermentation of Enset during initiation period. As it was described in the previous study, because of the activities of this species and to some extent, of Streptococcus faecalis, the pH of the fermenting Kocho was reduced from 6.5 to 5.6. These organisms may be then succeeded by some of the homo-fermentative Lactobacillus species. Through the activities of the Lactobacillus species, the pH can be further reduced to 4.2. The microorganisms are also temperature dependent. For instance, if Pediococcus cerevisiae present in Kocho, it can't achieve prominence in relatively low fermentation temperature between 14°-18°C. Spore-formers may be present in fairly high numbers during the first 15 days of fermentation. The butyrous odor usually detected during the first two weeks in fermenting Kocho is due to the activities of certain clostridial species, and yeasts can be also present in fairly high numbers (Gashe, 1987a).

2.5.3.4. Metabolic Activity of Lactic Acid Bacteria

Metabolic activity of LAB has gained much focus in research and industry. The main metabolic activity of LAB is breaking down different carbohydrates and related compounds to obtain energy and carbon molecules. Other metabolic activities such as breaking down proteins, lipids, and other compounds are also important for normal growth. Thus, the metabolic activities of LAB can include: carbohydrates metabolism, protein metabolism, lipids metabolism, and other metabolic activity (Rodríguez, 2012).

2.5.3.5. Carbohydrates Metabolism

Carbohydrates are the main source of energy for bacterial growth. LAB metabolizes carbohydrates into different useful compounds (mainly lactic acid) through a common process known as fermentation. Fermentation is a well-documented process and thus only short summary of fermentation was included in this review. Fermentation is the metabolism of sugar in which energy is derived from partial oxidation of an organic compound using organic intermediates as electron donors and electron acceptors (Axelsson, 2011). No outside electron acceptors are involved; no membrane or electron transport system is required; and all ATPs are produced by the substrate level of phosphorylation. According to the mode of splitting carbon skeleton thus leading to different sets of end-products, three major pathways of hexoses fermentation were described to

occur within LAB (Axelsson, 2004). Based on fermentation pathways, LAB can be divided into two physiological groups: homo-fermentative (e.g., Lactococcus lactis, L. delbrueckii, and L. casei) and hetero-fermentative (e.g., L. amylovorus, L. reuteri, and L. manihotivorans) (John *et al*., 2007). Homo-fermentative LAB metabolize one molecule of hexose sugars such as glucose to two molecules of lactic acid and two molecules of ATP resulting in more than 85% lactic acid from one molecule of glucose (Von Wright A. and Axelsson, 2011). Hetero fermentative LAB produce only 50% lactic acid fermenting one molecule of glucose to one molecule of lactic acid, one molecule of ethanol/acetate, one molecule of CO_2, and only one molecule of ATP (Axelsson, 2004). The ratio of acetate/ethanol depends on the oxidation reduction potential of the system. The difference in acid production and change in pH could be used as a basis for differentiation of these two groups of LAB. With regard to disaccharides and oligosaccharides, they are taken up with the help of specific permeases then will be split inside the cell into mono-saccharides to be phosphorylated. For example, lactose is taken up by a specific permease and is split, in most lactobacilli, by β-galactosidase to form monosaccaride (John *et al.,* 2007).

2.5.3.6. Protein Metabolism

LABs have gained much attention due to their proteolytic activities, which are of especial importance in the accelerated maturation and enzyme modification of different food products such as cheese. Proteolysis is the process in which proteins are broken down by proteinases and peptidases into polypeptides, amino acids, and peptides (Savijoki *et al*., 2006). Proteinases and peptidases can be found as extracellular and secreted as free enzymes outside the cell or intracellular inside the cell. The proteolytic systems of LAB are important as a means of making protein, peptide, and amino acids available for bacterial growth, but these systems can also form the rheological and organoleptic properties of fermented foods (Bintsis *et al.*, 2003). Proteolysis has been particularly well documented in relation to the growth of lactobacilli and lactococci in milk, where they are largely responsible for flavor development during cheese production. Proteinase also helps to reduce the allergic properties of milk and milk products for infants who can lead to a severe nutritional problem of protein-energy deficiency (Yuan and Furuta, 2003). Of cheese making LAB strains, L. helveticus and Lactococcus lactis have been studied in greatest detail (Savijoki *et al.*, 2006).

2.5.3.7. Lipid Metabolism

Lipid metabolism is the break-down of lipid by lipases into fatty acids and glycerol. LAB strains have either intracellular or extracellular lipases (Meyers, 1996.). In addition, LAB strains perform unique fatty acid transformation reactions including is omerization, hydration, dehydration, and saturation. These functions can be used in food industry and probiotics. For example, lipolyses of milk fat by LAB constitute the main biochemical changes in cheese flavor development (Katz *et al.*, 2002). However, not all LAB strains can metabolize lipids. Meyers and others (1996) screened over 100 different LAB strains for lipase production and identify only 29 lipase-producing strains.

Lipase activity of LAB has shown to provide different health benefits to the host. Lipases are useful in the preparation of dietetic formulations for infants, geriatrics, and convalescents (Ogawa

et al., 2005). Evidence from mice, preclinical and clinical trials has revealed that lactobacilli can also break down cholesterol into serum lipids. The hypolipemic effect of Lactobacillus could is due to a lower intestinal absorption of lipids or a higher lipid catabolism.

2.5.3.8. Other Metabolic Activities

LAB strains express several other metabolic activities that are major contributors to sensory changes in fermented foods such as flavor, astringency, and color by breaking down different organic compounds in the food matrix (Rodriguez, 2012). These enzymes also play an important role in the probiotic characteristics of LAB contributing to a variety of health benefits in humans, animals, and plants. LAB metabolize different simple and complex functional compounds such as terpenoids, carotenoids, sterols, polyphenols, and isoflavones (Chen *et al*., 2012). In general these complex functional compounds are known for their health benefits but they are unavailable for gut absorption. The metabolic process in food fermentation or in the gut will degrade these compounds to smaller metabolites that can be absorbed and benefit the host organism (Chen *et al.*, 2012).

Diacetyl is produced during conversion of citric acid in milk to pyruvate and pyruvate is converted to α-actolactate and then to the precursor for diacetyl. Most LAB strains can decarboxylate α-acetolactate by α-acetolactate decarboxylase to the metabolic end-product acetoin and aromatic whereas some LAB strains do not contain the responsible enzyme, resulting in accumulation of α-acetolactate and high production of diacetyl in dairy products (Hoefnagel *et al.*, 2002). Acetaldehyde is a major contributor to flavor in dairy products, and it is produced mainly by LAB. In addition, LAB can be used to inhibit the growth of harmful microorganisms by producing different antimicrobial compounds including bacteriocin, hydrogen proxide, carbon dioxide, and diactyle in addition to the rapid production of lactic acid. Wine LAB play a preeminent role in the production of grape wines where their growth and metabolism may positively or negatively affect wine quality (Hoefnagel *et al.*, 2002). LAB may also metabolize diacetyl and acetaldehyde during malolactic fermentation in wine and the remove Ochratoxin A from wines (Jussier *et al*., 2006).

2.5.3.9. Biochemical Environment of Lactic Acid Bacteria

Bacteria, in general, require an appropriate biochemical and biophysical environment to grow and express normal metabolic activities. Biophysical environmental factors including temperature, pH, water activity, redox potential, and the presence of inhibitory compounds produce a wide range of variations among LAB strains (Lechiancole *et al.*, 2002). The biochemical environment conditions are made available through nutrients in the culture media. LABs are known as fastidious microorganisms that cannot grow on simple mineral media supplemented only with a carbon source. In addition to carbohydrates (carbon source), culture media of LAB are usually supplemented with various free amino acids, peptides, nucleic acid derivatives, fatty acids esters, minerals, vitamins, and buffering agents (John *et al.*, 2007).

The fastidious characteristics of LAB, the ability of LAB strains to produce acid and antimicrobial compounds, and the variations in nutritional requirements among LAB strains have added additional limitations and challenges with regard to developing general growth media. In addition,

metabolites that are produced by some LAB strains may inhibit the growth of other strains or even the same strain such that the case of bacteriocin production. On the other hand, low nutrient concentrations may cause fast depletion in the essential nutrient which may negatively affect growth whereas high nutrient concentration such as salts could also negatively affect growth or could be insoluble in water (Lechiancole *et al*., 2002).

2.5.3.10. Health effects of fermented foods

One of the reasons for the increasing interest in fermented foods is its ability to promote the functions of the human digestive system in a number of positive ways. This particular contribution is called probiotic effect. Already early in 1900, Metchnikoff pointed out the use of fermented milks in the diet for prevention of certain diseases of the gastrointestinal tract and promotion of healthy day to day life. Since then a number of studies have now shown that the fermented food products do have a positive effect on health status in many ways. The human intestinal microbial flora is estimated to weigh about 1000 grams and may contain 10^{16}–10^{17} colony forming units representing more than 500 strains (Lechiancole *et al*., 2002). For physiological purposes, it can be considered to be a specialized organ of the body with a wide variety of functions in nutrition, immunology and metabolism. Studies on mice have shown that the indigenous microorganisms in the stomach are Lactobacillus, Streptococcus and Torulopsis, while in the small intestine, ceacum and colon several different species (Bacteroides, Fusobacterium, Eubacterium, Clostridium, etc.) coexist. The gastrointestinal microflora in humans are also known to contain hundreds of species (Sahlin, 1999). Even though there is a wide variation among individuals, the number of species and size of the population are usually kept stable in normal healthy subjects. There is a constant struggle in maintaining the desirable balance and a dynamic equilibrium between microbial populations within the intestinal flora. The anaerobic organisms, which outnumber the gram negative enteric bacteria by about 10000:1, are associated with the intestinal epithelium limiting adherence of potential pathogens by effective colonization. The stability of the intestinal microflora is affected by many factors including dietary habits (Zhang *et al*., 2009).

Decrease in the number of anaerobic bacteria is associated with increase in the number of gram negative pathogens in the intestinal tract and their translocation to extra-intestinal tissues. Under normal conditions the intestinal wall prevents translocation of organisms both dead and living as well as microbial products like toxins from the gut to the blood. However, in patients with systemic insult like starvation, shock, injury and infection or specific insult of the gastrointestinal canal through inflammation, chemotherapy or radiation, the gut mucosal permeability will be increased leading to translocation of microbes (Sahlin, 1999). A fermented food product or live microbial food supplement which has beneficial effects on the host by improving intestinal microbial balance is generally understood to have probiotic effect.

2.5.3.11. Anticholesterolemic Effect

Hepner *et al.* (1979) reported hyper-cholesterolemic effect of yoghurt in human subjects receiving a one-week dietary supplement. Studies on supplementation of infant formula with lactobaccillus acidophilus showed that the serum cholesterol in infants was reduced from 147 mg/ml to

119mg/100 ml (Rupasinghe and Yu, 2012). In an in vitro study the ability of 23 strains of lactic acid bacteria isolated from various fermented milk products the bacterial cells to bind cholesterol was investigated. No cholesterol was found inside the cells.

2.5.3.12. Anticancerogenic Effect

Apart from this, there are interesting data on anticarcinogenic effect of fermented foods showing potential role of lactobacilli in reducing or eliminating procarcinogens and carcinogens in the alimentary canal (Sahlin, 1999). The enzymes b-glucuronidase, azoreductase and nitroreductase, which are present in the intestinal canal, are known to convert procarcinogens to carcinogens. Oral administration of lactobacilli rhamnosus GG was shown to lower the faecal concentration of b-glucuronidase in humans implying a decrease in the conversion of procarcinogens to cancinogens. Fermented milk containing Lactobacillus acidophilus given together with fried meat patties significantly lowered the excretion of mutagenic substances compared to ordinary fermented milk with Lactococcus fed together with fried meat patties. The process of fermentation of foods are also reported to reduce the mutagenicity of foods by degrading the mutagenic substances during the process.

2.5.3.13. Immunoactive Effects

Some lactic acid bacteria which are present in fermented milk products, are found to play an important role in the immune system of the host after colonization in the gut. Oral administration of Lactbacillus casei caused an improvement of the function of the peritoneal macrophages and increased the production of IgA. The mechanism of this effect is not clearly known, but it is speculated that the lactobacilli, their enzymes or the metabolic products present in the fermented food product may act as antigens, activating production of antibodies.

Marin *et al*. (1997) have studied the influence of lactobacilli used in fermented dairy products on the production of cytokines by macrophages. The results indicated that for most strains, direct interaction with macrophages caused a concentration dependent increase in tumor necrosis factor and interleukin. A study by Perdigon *et al*. (1995) showed that the Lactobacillus casei could prevent enteric infections and stimulate secretory IgA in malnourished animals but also translocate bacteria, while yoghurt could inhibit growth of intestinal carcinoma through increased activity of IgA, T cells and macrophages. In a review by Marteau and Rambaud (1993) the authors concluded that there is a potential of using lactic acid bacteria for therapy and immunomodulation in mucosal diseases, especially in the gastrointestinal tract.

Isolauri (1996) have presented a study suggesting that Lactobacillus sp. strain GG could be used in the prevention of food allergy. It is suggested that dietary antigens induce immunoinflamatory response that impairs the intestine's barrier function and that probiotic organisms could be a means of introducing a tool to reinforce the barrier effect of the gut.

2.5.4. Toxins and Toxin Producing Organisms in Fermented Foods

Lactic starter culture were found to be effective in preventing the formation of botulin toxin, even in the absence of nitrate. No aflatoxin production was reported in tempe and miso prepared using Rhizopus oligosporus and Aspergillus oryzae on soya bean, chickpea and horse bean. Aspergillus flavus grown in broth had a lower aflatoxin production when 10% cell free supernatant culture fluid from lactobacilli was added. This effect could not be explained on the basis of pH or competition. Studies, mainly with Aspergillus oryzae, have shown no traces of aflatoxin production in traditional mold-fermented products. However when an aflatoxin producing strain was inoculated at the same time, large amounts of aflatoxin was found. The aflatoxin production of Aspergillus parasiticus was studied and found to increase in the presence of Lactococus lactis. In contaminated peanut press-cake, Rhizopus oligosporus and Neurospora sitophila were found to reduce the aflatoxin content by 50 and 60% (Takala *et al*., 2003).

The length of fermentation of cassava roots, the type of gari flour and the source of the flour affected the levels of HCN detected (Ishibashi and Yamazaki, 2001). During the fermentation of melon seeds for the preparation ogiri, the aflatoxin content was reduced, and after 4 days of the 7 days of fermentation no aflatoxin was found (Sanders, 2003). These results were sustained by the analysis of 26 market samples that were found negative in aflatoxin. Still the microorganism Aspergillus flavus was found in the fermenting ogiri at all stages. Apricot seeds were tested for the production of tempe. Amygdalin, a toxic, cyanogenic substance in particularly the bitter seeds, was reduced by 70% during the tempe process (Zendo *et al.*, 2003)

More than 90% of 488 cassava products from villages in Africa were fermented. Some microorganisms can hydrolyse linamarin, 52% of isolates from gari had the ability, but studies have shown that in grated products, like gari, it is primarily edogenous linamarase that hydrolyses the linamarin. The cyanide levels in blood of humans in Nigeria was found to be 0,294µmol/l, which is higher than the cyanide level of a non-tropic population, 0.13µmol/l. This could be attributed to a higher degree of exposure to cyanide since the staple diet in this part of Nigeria is cassava based meals. Grating prior to fermentation, fermentation, and the garrification process itself are all three components that together decides the residual cyanide content of garri produced from cassava (Reid *et al*., 2003)

The results indicate that consumption of fermented dairy products may provide protection against the development of tumors. The underlying mechanism is unknown, but may involve stimulation of the immune system. In a Salmonella typhimurium mutagenicity assay using nitrosated beef extract, eight of ten isolated Lactobacillus strains reduced the number of revertants back to the levels of untreated controls. Rats given Lactobacillus casei before a genotoxic carcinogen, had higher levels of undamaged colon cells than rats who did not recieve Lactobacillus casei before the genotoxic carcinogen (McAuliffe *et al*., 2001).

Aflatoxins, secondary metabolites, have been demonstrated to be carcinogenic, teratogenic, and mutagenic. Lactic acid bacteria such as Lactobacillus spp. were found to inhibit aflatoxin

biosynthesis. The studies showed that the aflatoxin inhibition was probably due to an inhibitory metabolite other than hydrogen peroxide and low pH. However, other lactic bacteria such as Lactococcus lactis were found to stimulate growth and aflatoxin production of Aspergillus parasiticus and at the same time promote the transformation of aflatoxin B1 which is highly mutagenic to nontoxic aflatoxinB2a and less toxic aflatoxicol. Gourama who studied ability of Lactobacillus spp to inhibit the growth of Penicillium and its production of mycotoxin, found that two of lactic acid bacteria isolates which was identified as Lactobacillus casei spp showing high inhibitory activity against Penicillium citrinum and P. expansum. Further, the cell-free supernatants of the two Lactobacilli was shown to have inhibitory activity, independent of their production of lactic acid or hydrogen peroxide, which was sensitive to proteolytic enzymes like trypsin and pepsin as well as to higher temperatures (100^{o}C). This could be an indication that the antimycotic and antimycotoxigenic activity could be due to LAB metabolites that are proteinaceous in nature (Danielsen and Wind, 2003).

Milk fermented with Lactobacillus acidophilus LA-2 was demonstrated to suppress faecal mutagenicity in the human intestine. Faecal mutagenicity and bacterial composition of 6 healthy subjects consuming their regular diet were investigated before and during the administration of milk fermented with Lactobacillus acidophilus LA-2. The result has shown that administration of fermented milk caused a remarkable decrease (71.9% on average; with a range of 19.4-90.6%) in faecal mutagenicity compared to that before the administration as well as an increase in population of Lactobacillus spp. and Bifidobacterium spp. in the faeces of all subjects (Takala *et al.*, 2003).

Studies on the antimutagenic activity of the milk fermented with mixed-culture with various lactic acid bacteria and yeast, showed that the fermented milks produced with mixed cultures of lactic acid bacteria had a wider range of activity against mutagens than those produced with a single strain of lactic acid bacteria (Thomas *et al.*, 2000).

Antimutagenic properties of the methanol extract of kimchi, a fermented food from Korea were evaluated by using Ames test for its antimutagenic activities towards aflatoxin B (AFB). Mutagenicity of AFB was reduced by 35-75% upon addition of kimchi methanol extracts and the inhibition of mutagenicity was highest in those samples of kimchi which were fermented for 3 weeks. Studies effects of fermentation of cassava by Aspergillus niger B-1 on the cyanide and protein contents of cassava. It was shown that the fermentation process reduced the cyanide content of cassava by 95% to 2 mg/kg, and increased its total protein content by 50%. A significant decrease in cyanogenic glycosides was detected after 3 days of fermentation (Wiedemann *et al.*, 2004).

2.5.5. Bacteriocins Produced by Lactic Acid Bacteria

According to Wiedemann *et al*. (2004) a group of children fed with lactic acid fermented gruel had a mean number of 2.1 diarrhoea episodes compared to 3.5 for the group fed with unfermented gruel. Although Salmonella, Campylobacter, Shigella, Vibrio, Yersinia and Escherichia are the most common organisms associated with bacterial diarrhoea diseases, other enterotoxigenic

genera, including Pseudomonas, Enterobacter, Klebsiella, Serratia, Proteus, Providencia, Aeromonas, Achromobacter and Flavobacterium, have also been reported. In addition, it was found that there was no significant difference between the behavior of the pathogens in fermented porridge or acid-supplemented nonfermented porridge, which implies that the anti-microbial effect is due to presence of lactic and acetic acids at reduced pH, and that other anti-microbial substances do not play a detectable role. Similarly, Adams (1990) suggested that lactic acid bacteria are inhibitory to many other microorganisms when they are cultured together, and this is the basis of the extended shelf life and improved microbiological safety of lactic-fermented foods. Lactobacillus species can produce a variety of metabolites, including lactic and acetic acids which lower pH, that are inhibitory to competing bacteria, including psychrotrophic pathogen. This effect could be due to a combination of many factors. The inhibition by organic acids has been attributed to the protonated form of these acids, which are uncharged and may therefore cross biological membranes. The resulting inhibition of growth may be due to acidification of the cytoplasm and/or accumulation of anions inside the cell (Adams, 1990).

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 un-dissociated acid, and the inhibitory the acid is likely to be. On this basis, one would expect acetic acid (pKa = 4.75) to be a more effective antimicrobial agent than lactic acid (pKa =3.86) (Adams, 1990). Lactobacillus acidophilus and L. bulgaricus inhibit activities of a wide variety of Gram-positive and Gram-negative organisms. Mold growth was prevented in high-moisture maize samples (27% moisture) that were inoculated with Lactobacillus plantarumor Propioni bacterium shermanii and stored for 60 days at 26°C and the initial yeast population was drastically reduced in samples inoculated with Propioni bacterium shermanii while samples inoculated with Lactobacillus plantarum had an accelerated acid production in the early stage of. Studies with porridges inoculated with pathogenic bacteria showed that acidification, either by adding acids or by fermentation, prevented the bacterial growth. The most resistant Salmonella died at a rate of 1.2 log cycle/h, the most resistant Shigella at 0.9 log cycle/h, the most resistant Escherichia coli at 0.6 log cycle/h. Drum dried and reconstituted porridge also showed the same characteristics (Reid *et al*., 2003).

Bacteriocins are bactericidal proteins that can have either a narrow spectrum (inhibit closely related bacteria) or a wide spectrum (inhibit a diverse group of Gram-positive bacteria). Bacteriocins mentioned are: nisin, pediocin A, diplococcin, lactacin B, lactacin F, lactocin 27, helveticin J, lactostrepcins. These compounds are important in the preservation of fermented foods, and helps the producing microorganism to dominate the flora of the substrate. There are numerous reports on natural antimicrobials produced by lactic acid bacteria: nisin, acidoline, acidophiline, lactacine, lactocidine, lactocine, helveticine, bulgarican, plantaricin, reuterin, diplococcin, lactostrepcin etc (Ishibashi and Yamazaki, 2001).

Inhibitory effects of lactic acid bacteria against enteropathogenic microorganisms were measured, using the agar spot test and disc assay method. Cell-free supernatants of the lactic acid bacteria inhibited growth of Staphylococcus aureus, Salmonella mumum, Escherichia coli, Bacillus cereus

and B. subtilis. It is suggested that inhibitory substances other than lactic acid were present in supernatant preparations, and that production of these antibacterial substances may be plasmid directed. Antimicrobial activity of 241 lactic acid bacteria belonging to Lactobacillus plantarum, Pediococcus pentosaceus, L. fermentum/reuteri and L. brevis isolated from various processing stages of maize (corn) dough fermentation (for kenkey) were shown to inhibit other Gram-positive and Gram-negative bacteria. The antimicrobial effects were explained by the combined influence of acids, compounds sensitive to proteolytic enzymes and other compounds with antimicrobial activity, with acid production being the most important factor (Sanders, 2003).

Some LAB strains ribosomally synthesize antimicrobial peptides, or bacteriocins, targeted to inhibit other Gram-positive bacteria. Even though antimicrobial peptides occupy an inhibition spectrum narrower than that of antibiotics, bacteriocins produced by LAB have been reported to permeate the outer membrane of Gram-negative bacteria and to induce the inactivation of Gram-negative bacteria in conjunction with other enhancing antimicrobial environmental factors, such as low temperature, organic acid and detergents.

The pattern of antimicrobial factors was not species-specific as well as the safety and storage stability of fermented maize is suggested to depend on a mixed population of lactic acid bacteria with different types of antimicrobial characteristics. It is concluded that the introduction of pure cultures as starters may, therefore, impose a risk to the final product. Fermented meats have caused food-borne illness due to enterohemorrhagic Escherichia coli. Consumption of Lebanon bologna, a moist fermented sausage manufactured from lean beef, was epidemiologically associated with outbreak of salmonellosis. A study was conducted to determine the effects of pH (after the fermentation step), final heating temperature, and time on destruction of E.coli O157:H7 and Salmonella tryhimurium in Lebanon bologna. The results showed that fermentation alone reduced populations of both pathogens by <2 log units and heating alone reduced populations of E.coli O157:H7 by <3 log units. A combination of fermenting to either pH 5.2 or 4.7, followed by heating at 110^{o}F (43.3^{o} C) for 20 h, 115^{o}F (46.1^{o}C) for 10 h, or 120^{o}F (48.9^{o}C) for 3h reduced populations of both pathogens by >7 log units (Sanders, 2003).

Bacteriocins produced by LAB are classified into three main groups, lantibiotics being the most documented and industrially exploited. The groups are lantibiotics (Class I), nonlantibiotics, small heat-stable peptides (Class II) and large heat-labile protein (Class III). The lantibiotic nisin naturally produced by Lactococcus lactis spp. Lactis is commercially available as food additive. The nisin variants A and Z, differing by one amino acid, are approved for use in foodstuffs by food additive legislating bodies (Food and Drug Administration, FDA). In addition, a new nisin variant, nisin Q, has been isolated from a L. lactis strain found in river water in Japan. Nisin Q differs in four amino acids as a mature peptide and in two amino acids of the leader sequence (Zendo *et al*., 2003).

All forms of nisin are antimicrobials active against Gram-positive bacteria, such as LAB, Listeria spp., Micrococcus spp. And spore forming bacteria like Bacillus spp. and Clostridium spp. The

inhibiting mode of nisin towards vegetative cells consists of several phases. Nisin most probably inactivates sulfhydryl groups in the cytoplasmic membrane, thereby acting as an inhibitor of both spore outgrowth (at the stage of swelling) and vegetative growth. From a number of toxicological studies the nontoxic and nonimmunogenic character of nisin has become clear. Streptococcus lactis produces the polypeptide nisin, active against gram-positive organisms including Streptococcus cremoris which, in turn, produces "diplococcin" active against gram-positive organisms including Streptococcus lactis. Thus these microorganisms compete in the fermentation of milk products while inhibiting growth of other gram-positive bacteria (Reid *et al*., 2003).

Nisin accumulates on the cell membrane and inserts into it, then aggregates within the membrane to form a water-filled pore (McAuliffe *et al.,* 2001). Another model suggests that nisin molecules bind by electrostatic interactions to the anionic membrane surface, leading to a high local concentration that disturbs the lipid dynamics and causes localized strains, forcing the nisin into the membrane. At this stage, a voltage-dependent pore is formed leading to the dissipation of the bacterial proton motive force. Loss of the proton motive force, required for ATP synthesis and the transport of ions, causes cell death through depletion of energy dependent reactions. Nisin is also known to inhibit peptidoglycan biosynthesis by interacting with cell wall precursors, lipid I and lipid II. (Zendo *et al*., 2003) concluded that nisin lipid II interaction stabilized the pore complex. The electric transmembrane potential is strongly reduced in the presence of nisin and lipid II (Wiedemann *et al*., 2004).

The bacteriocin production is highest at the end of the exponential and early stationary phase and reduction is caused by proteolytic degradation of the bacteriocin (Thomas *et al.,* 2000). Some bacterial strains, such as Clostridium botulinum 169B and Streptococcus bovis JB1 are resistant to nisin. Resistance is assumed to be based on the enzymatic decomposition of nisin. Nisin resistance in spore forming strains has been associated with an enzyme produced during germination acting on the C-terminal lanthionine ring of nisin (Zendo *et al.,* 2003).

2.5.6. Safety of Lactic Acid Bacteria

The use of LAB as a probiotic requires a safety assessment. The functional properties of the strains should be well studied and documented (Holzapfel *et al.,* 2001). Generally recognized health-promoting properties are non-pathogenic behavior, the ability to persist within the GI tract and adhesion, and the ability to modulate immune responses. Reid *et al*, (2003) pointed out the importance of considering the possible side effects of probiotics on the consumer, e.g. bloating or blocking the normal functional gut transit. Ishibashi and Yamazaki (2001) pursued the research of bacteria converting food components or biological secretions into secondary substances harmful to the host. Lactobacilli and lactococci commonly hold a GRAS status. Japan legally recognizes functional foods (Foods for Specified Health Use). Lethal dose (LD50) of LAB was measured for mice by oral administration and found to be > 10 cfu/kg, depending on the strain (Ishibashi and Yamazaki, 2001). The safety of two Bifidobacterium longum strains of human origin was evaluated on healthy adult volunteers: no side effects were reported and the immune parameters measured remained without undesirable changes. However, some enterococci such as E. faecalis

and E. faecium are classified in risk group II as pathogens. Special concern has been expressed on the potential risk arising from the existence of antibiotic transferable genes among lactobacilli. Some species of LAB (L. acidophilus, L. reuteri, L. rhamnosus, Leuconostoc spp.) commonly used in the food industry or naturally occurring in raw food materials are resistant to glycopeptide antibiotics such as teicoplanin and vancomycin (Goldstein *et al.,* 2000). Antibiotic resistance encoding genes may transfer into a susceptible strain via a mobile genetic such as plasmids and transposons to produce new resistant bacterial strains. Conjugative transposons are commonly found in enterococci and streptococci as well as in some Lactococcus lactis strains reported to contain a chromosomally located transposon. Plasmids of LAB do not commonly carry transmissible antibiotic resistance genes but can take in conjugative transposons and plasmids. Some plasmids, such as those with bacteriocin immunity genes, can integrate into the chromosome. Plasmid-linked antibiotic resistance therefore poses a hazard (Danielsen and Wind, 2003).

Resistance to glycopeptides in clinical isolates are classified as high-level resistance as well by inducible and constitutively low-level resistance. Vancomycin resistance in enterococci is associated with the presence of nucleotide sequences related to vanA, vanB and vanC. Use of feeds containing antibiotics and antibiotics for promoting growth in animals, such as fluoroquinolones for poultry, were shown to correlate with antibiotic-resistant bacteria in the animals. Several Enterococcus strains and some of Lactobacillus spp. (L. casei, L. plantarum, and L. rhamnosus) with transferable vancomycin resistance have been isolated from clinical samples, indicating that antibiotic medication may be involved in such cases. Lactobacilli appear to be sensitive to penicillins but less so to oxacillin, cefoxitin, ceftriaxone, metronidazole, cephalothin and imipenem (Danielsen and Wind, 2003). Low sensitivity to ampicillin and piperacillin has been fully observed as well. L. acidophilus and L. reuteri as well as the genus Enterococcus are examples of probiotic bacteria resistant to some degree to vancomycin (Danielsen and Wind, 2003)

The antibiotic resistance genes serving as selective markers in LAB have been replaced by food-grade cloning systems based on nisin immunity, complementation of deficiency in lactose utilization (Takala *et al.,* 2003), and suppression of nonsense mutation for positive selection of transformants. The term food-grade can be used when the modified microorganism contains such elements not harming the consumer when present in foods. Food-grade cloning systems need to be based on DNA from LAB or other microbes with a long history of safe use in the food industry. Genetically modified LAB can in future be utilized as improved starters in food fermentation and for the safe production of metabolites used as food additives (Takala *et al*., 2003).

The isolation of LAB from clinical samples has raised debate over the safety of probiotic bacteria and whether or not the bacteria are actually infectious (Ishibashi and Yamazaki, 2001). Some LAB have been implicated in local systemic infections including septicemia and endocarditis as well as liver abscesses. In most cases of infection, the organisms were shown to be of host origin. Some cases have been linked to the consumption of probiotics (Salminen *et al*., 2004). Except for enterococci and streptococci, the clinical significance of LAB is low, L. rhamnosus being the most

frequently isolated LAB from clinical samples. The isolation of LAB from infections is likely to be the result of opportunist pathogens on an immunosuppressed host (Salminen *et al.,* 2002). Many factors may promote translocation of intestinal bacteria, such as intestinal mucosal injury, immunodeficiency of the host, an abnormal intestinal bacterial microbiota, and previous antibiotic treatment, complications from Acquired Immunodeficiency Syndrome (AIDS) and prior hospitalization and surgery (Salminen *et al.,* 2004).

The development of novel approaches in food and in pharmaceutical clinical therapies allow broadening the potential for using lactic acid bacteria in food and pharmacology (Renault, 2002). The nature of genetic modifications can be divided into three groups: 1) one-step genetic events like deletions, gene amplifications, plasmid insertions and losses, 2) multi-step genetic rearrangements with DNA of the same species, and 3) trans-species genetic modifications.

Steidler *et al.,* (2003) has emphasized the effective use of gene manipulated LAB in the battle against food spoilage and pathogenic bacteria. As examples, genetically modified LAB have been utilized to improve cheese ripening, produce phage resistant starter strains, and protect against tetanus toxin and bovine rotavirus. It can be used to treat Shiga toxigenic Escherichia coli infections and dysentery in humans, prevent dental caries and treat inflammatory bowel disease. Grangette *et al.,* (2001) studied spontaneous gene transfer in the GI tract and observed that in vivo transfer rate in the gut was 0.03 transconjugants per recipient cell. All new ingredients and genetically modified organisms (GMO) in foods fall under the Novel Foods Regulation of the Europe legislation. No GMO has yet been authorized as a feed additive in Europe. Renault (2002) discussed the use of genetically engineered LAB in foods, emphasizing the value of risk assessment in correlation with the expected benefits of modified strains. The objective of risk assessment is to identify and evaluate the potential adverse effects of GMOs. The cumulative and long-term effects on human health and the environment have also to be taken into account. Assessment focuses on GM development and the possible gene transfer to host microbiota. (Renault, 2002).

2.5.7. Exploitation of Probiotic Lactic Acid Bacteria

The methods for selection of probiotic bacterial strains are discussed in the literature. Host specificity, the generally regarded as safe (GRAS) status, colonization, antimicrobial activity, and desirable metabolic activity are generally agreed upon (Reid *et al.,* 2003), but issues such as the effect of living versus nonliving probiotics or even their survival in the intestinal tract (Reid *et al.,* 2003) remain open. Criteria for quality, including the sensory characteristics of probiotic strains, is well established as are those for technological. In addition to in vitro experiments (Gibson and Fuller, 2000), animal models and GI tract simulation studies have been employed for probiotic detection. The ultimate test for probiotic functionality is a double blind, placebo-controlled and randomized human study. Prebiotic and probiotic based biotherapy has shown potential as an alternative for medical treatment. The demonstration of probiotic activity of a given strain requires a well-designed, double blind, placebo-controlled host-specific study also showing resistance to technological processes, meaning viability and activity throughout processing phases. Each

potential probiotics train must be documented independently, without extrapolating any data from closely related strains and employing only well-defined strains (Dunne and Shanahan, 2003).

2.5.8. Effect of Prebiotics on Probiotic Bacteria

The ability of a probiotic LAB strain to survive in the GI tract may be promoted by oligosaccharides facilitating the metabolism and growth of LAB in the lumen. Dietary fibre, mainly oligosaccharides and polysaccharides fermented in the colon may act as prebiotics. The importance of prebiotics as enhancers of the growth and performance of probiotic bacteria has been documented in humans (Crittenden *et al*., 2002). Bifido-bacterium species. And Lactobacillus sp. especially produce a positive effect on human health. The significance of prebiotics in animal diet has also been studied and represents a growing field of research (Gibson and Fuller, 2000).

2.5.9. Sedimentation

This helps to separate bulla from impurities before it left for fermentation and drying by density difference the water becomes at the top and bulla settles down. The bulla easily collected at the cone shape of the cylinder and the water left either for further process or drainage system. Filter Bag is used to remove fine fibers and other impurities by filtering Kocho to get purified product and also increasing the efficiency of the dryer by removing some amounts of water.

2.5.10. Drying

The moisture contained in bulla and kocho is removed by using drying process. The drying performed by either in open air (sun drying) or using industrial drying equipment like that of rotary drum dryer in order to conduct the process in short period of time. Pulverization used for cutting to reduce the size of dried kocho and bulla prepared for sieving of flavored product in the standard size. Sieve separates big size products from specified sizes. The oversize left at the top and the size that we need to use only left at end of the sieve passing through series of sieves of different sizes. Finally the products stored in storage tanks and distributed to the market by packing and making it easy for handling.

Foods prepared under unhygienic conditions and frequently heavily contaminated with pathogenic organisms play a major role in child mortality through a combination of diarrhoea diseases, nutrient malabsorption, and malnutrition. All food items contain microorganisms of different types and in different amounts. Which microorganisms that will dominate depends on several factors, and sometimes microorganisms initially present in very low numbers in the food, for example lactic acid bacteria (LAB), will outnumber the other organisms inhibiting their growth. In contrast to fermented meat, fish, dairy and cereal products, fermented vegetables have not been recorded as a significant source of microbial food poisoning (Fleming and McFeeters, 1981).

It is not known, which variety of Enset could ferment faster and which one slower and whether environmental factors in respective samples have any influence on lactic acid bacteria activities. Understanding the varieties of Enset and effect of temperature could help to scientifically define and improve the product of Kocho. Improving, standardizing and modernizing

of traditional enset fermentation process could help to minimize time and energy needed, enhance quality and quantity of the food product and also minimize wastage and related public health problems.

The aim of this thesis was, therefore, to isolate, purify and inoculate lactic acid bacteria into decorticated and chopped Enset varieties and to know the optimum temperature of lactic acid bacterial growth over fermentation period of experimental Enset trial varieties.

CHAPTER THREE

3. MATERIALS AND METHODS

3.1. Materials

Fermented Kocho samples (Month-1, Months-3 and Months-6), decorticated pseudostem of Enset varieties (Agade, Disho and Gimbo), light microscope, pH meter, oven, distillated water, biosafety-Hood, autoclave, inoculating loop, petri plates, incubators, busser burner, flasks (250 ml, 500 ml and 1000ml), measuring cylinder, test tubes, sample collecting bottles, mechanical reduction, analytical balance, glove, 75% alcohol, colony counter (CFUs), incubators, refrigerator, digital stirrer.

3.2. Chemicals

De Man Rogosa Sharpe (MRS) agar media, peptone, beef extract, yeast extract, sodium acetate, tween 80, disodium phosphate, potassium phosphate, ammonium citrate, magnesium sulfate, manganese sulfate and distilled water.

3.3. Methods

3.3.1. Preliminary Survey and Study Site

A preliminary survey was done in Enset growing area of Hadiya Zone (Morsito town) of SNNP Regional State, Ethiopia in September 2016. Actual experimental pits were prepared on enset farms of a selected household at the selected site in October 2016 (figure 3a and f). As processing of Enset fermentation was traditionally the responsibility of the women, knowledgeable woman was employed for this experimental purpose. The study was undertaken on the mentioned area. The study area was selected mainly in consideration of the availability of Enset varieties and mild temperature. Enset processing mainly takes place from October to early December and occasionally from May to mid-June (Belay *et al*., 2008). According to all local respondents, Enset can be processed throughout the year as long as fresh Enset leaves are available for use at some critical steps during the processing. Enset growers could process enset almost throughout the year in high altitude area since enset plants retain sufficient number of leaves for the whole year. However, rainy season is not preferred for processing as the area becomes muddy and occasionally flooded (Hunduma and Ashenafi, 2011).

At the study site, mature Enset plants (mostly Agade, Disho and Gimbo) were decorticated and further processed traditionally. The fermentation pit was prepared and the floor and walls of the pit were longitudinally lined with layers of fresh Enset leaves. Layers of more fresh leaves were put on the surface as shown in figure 3a and f blow. Heavy logs and stones were put as weight over the tightly wrapped and sealed mass possibly to ensure the creation of airtight conditions in the pit according to Ashenafi (2006). The proliferation of yeasts requires an abundant continuous supply of oxygen. The low number of yeasts in fermenting enset could be due to unavailability of sufficient oxygen in the tightly packed and sealed fermenting mass. Surviving mold spores may

later result in post-processing food loss and health risk by mycotoxin producing species. Development of dark discoloration on loosely wrapped market Kocho and bulla, products of Enset fermentation, was caused by molds (Ashenafi and Abebe, 1996). According to Gashe (1987b), Kocho became easily contaminated with microorganisms when removed from fermenting pits and the major spoilage fungi belonged to Penicillium and Trichoderma species.

3.3.2. Sample Collection and Transportation

On October 01/2016, different types of Enset varieties (mostly Agade, Disho and Gimbo) were chopped together and allowed to ferment in a prepared ground pit traditionally for six Months. Repeating the same process, on January 01/2017 and February 01/2017 and allowed to ferment for three Months and one Month respectively (figure 2-b and f). The pit sizes, geographical area, amount of the chopped enset varieties and quantity of samples (~50kg each) were the same for each fermentation process. The only difference was time of fermentation (Months-6, 3 and 1). The process was supported with Miss. Abebayo, who was well known for traditional Kocho making process in the preferred area.

Samples, approximately 500g of each fermented (Month-1, Months-3 and Months-6) were collected randomly from different parts of the fermenting mass aseptically using sampling tongs, wrapped with enset leave, "Koba" (figure 2-c) and pooled into a sterile polyethylene bag (figure 2-e) separately. They were assigned 1M, 3M and 6M (figure 2-d), representing Month-1, Months-3 and Months-6 fermented Kocho respectively. Finally they were transported using ice cream sample handling mechanism to Addis Ababa. While collecting these samples, the temperatures and pH of the fermented mass were recorded using temperature sensor and pH meter respectively. The samples were taken to the laboratory and stored at a temperature of 4^0C in Addis Ababa Institute of Technology, Biochemical Engineering laboratory. Experimental analysis was done within two days of sample collection.

A) Pit for Kocho fermentation

B) Fermented Kocho

c) *Sample collection procedure: the fermented mass was wrapped with enset leaves (koba)*

Figure 2: Sample collection

Besides, three mature Enset plant varieties (Agade, Gimbo and Disho) were collected from the same area and chopped into small pieces separately using traditional Kocho making processing. These chopped varieties were allowed to dry at open air on separate plates without mixing with each other for three days (figure 3-a). Due to high content of moisture they had, it was very hard to crush them to powder form. As a result, they were over-dried for overnight. The oven was set to temperature of 105^0C (figure 3-b). The dried Enset varieties were added to crusher and converted to powder particles (figure 4) and allowed to pass through 150 micro size sieve.

a) *b)*

Figure 3: Enset trial varieties at room temperature and oven

Figure 4: Powder of Enset passing through 150 µ meter sieve size

3.3.3. Chemical Availability

De Man Rogosa Sharpe (MRS) media agar and all chemicals that were described above were purchased from chemical laboratory suppliers and the pH of the media were adjusted to 6.2-6.5 during media preparation.

3.3.4. Serial Dilution Preparation

1mg from each Kocho samples (Month-1, Months-3 and Months-6) was mixed thoroughly with the help of a shaker (IKA MS 3 digital) with 9 ml of distilled water and a serial dilution was prepared 1 to 5 (10^{-1} to 10^{-5}) dilution factor. Five test tubes for each sample and a total of 15 test tubes were used for the three samples. From the appropriate dilutions, 0.1 ml volumes of aliquots were spread-plated in triplicates on pre-dried surfaces of de Man Rogosa Sharpe (MRS) Agar media plates to count aerobic mesophilic bacteria. Colonies were counted after incubation at 25, 30 to 35^{o}C of temperatures for 24, 48 and 72 hours of incubation period following in figure 5 blow.

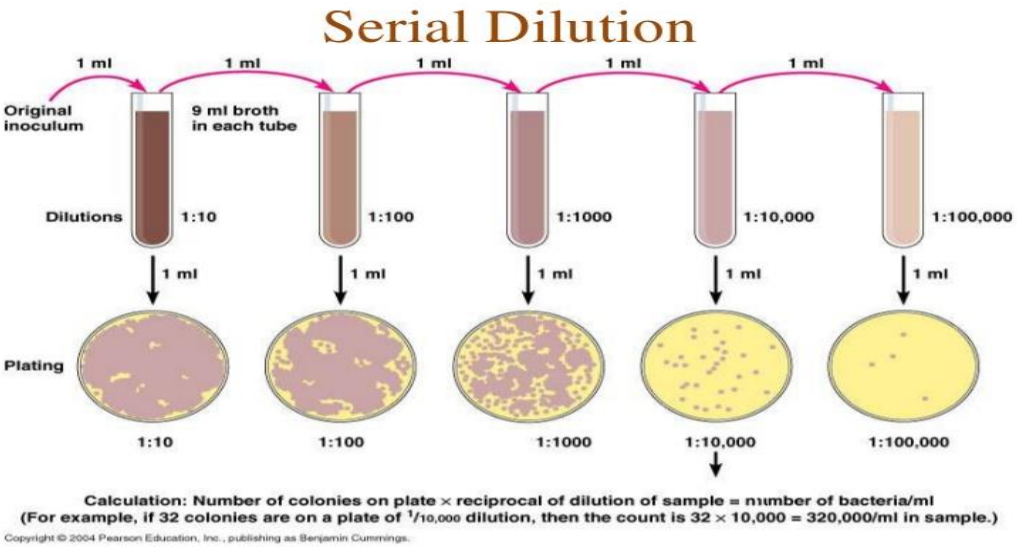


Figure 5: Procedures of serial dilution preparation

3.3.5. Media preparation

a) Heating before autoclaving *b) 54 plates in biosafety hood for MRS Media*

Figure 6: Media preparation

3.3.5.1. Calculation

1 petri plate requires 20 - 25 (an average of 22.5 ml) media. For this experiment, 54 petri plates were used. So, for 54 petri plates 1,215 ml media were required. 500g MRS agar media needed 8 liter and for 1,215 ml, approximately 152 gram of RMS agar medium was used.

1,215 ml MRS agar medium was prepared through 5 (500 ml see figure 6b) flasks with 243 ml each. 54 petri plates were prepared with an average composition of 22.5 ml each (see figure 6a). Then from each sample prepared in the above serial dilution preparation, 4-5 (10^{-4} to 10^{-5}) dilution factor was spread to 54 petri plates and 10^{-1} to 10^{-3} factorial dilution was rejected due to the assumption of high growth of microbes.

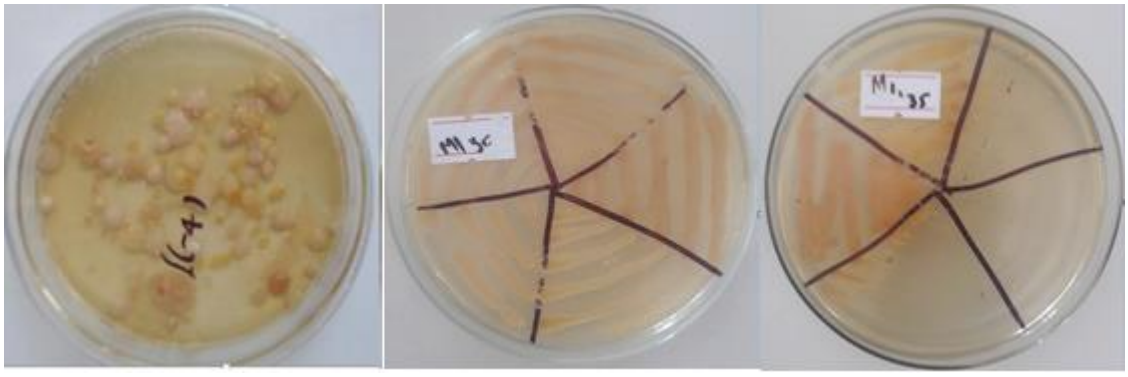
Inoculation of the medium was done, with three replications to get accurate results. Six plates (3 times from 10^{-4} and 3 times from 10^{-5}) were incubated at temperature of 25, 30 and 35^0C for 24, 48 and 72 hours with a total 54 plates for three samples (18 for each samples) as shown in figure 6 above.

3.3.6. Microbiological Analysis (Microbial Counts)

The viable cells of microbes were counted with colony counter and recorded as colony forming units (CFU) in 24 hours interval (24, 48 and 72 hours). Isolates from MRS agar plates, which were Gram-positive, non-spore forming, catalase negative rods or cocci were considered as LAB and were grouped as homofermentative or heterofermentative by their ability to produce gas in 5% glucose in MRS broth after incubation at 30-32^0C for 4-5 days (Hunduma and Ashenafi, 2011).

3.3.7. Identification, Optimization and Isolation of Suitable LAB Strain

Knowing morphological appearance (structure, size, color etc.) of the suitable LAB, it was identified, isolated, and re-cultured in a new media for 48 hours at a temperatures of 30 and 35^{o}C from sample Month-1 (figure 7).



1(-4) defined sample Month-1 at dilution factor 4, $M1_{30}$ and $M1_{35}$ describes LAB from sample Month-1 at temperature of 30 and 35^oC

Figure 7: Identification of LAB based on structure, size, and color

3.3.8. Serial Dilution Preparation with Identified LAB

4-5 colony of LAB from sample Month-1 were thoroughly mixed with the help of shaker with 9 ml of distilled water. A serial dilution was prepared with 1 to 5 (10^{-1} to 10^{-5}) dilution factor. Five test tubes were used for this experiment.

3.3.9. Media Preparation for Re-culturing of LAB

3.3.9.1. Calculation

1 petri plate requires 20-25 (an average of 22.5 ml) media, for this experiment 15 petri plates were used. So, for 15 petri plates 338 ml media were required. 500g MRS Agar media needed 8 liter and for 338 ml, approximately 21gram of RMS agar medium was used.

3.3.10. Plate Spreading and Incubation

0.1 ml from each serially prepared (10^{-1} to 10^{-5}) LAB sample was plate spread into petri plates in a replication of three with pour plating method and incubated at a temperature of 30^{o}C for 48 hours.

3.3.11. Microbiological Analysis

Viable cells of microbes were counted with colony counter and recorded as colony forming units (CFU) in 48 hours. Pure colony were obtained from this experiment and stored in refrigerator at temperature of 4^{o}C until another media containing carbon source Enset trial varieties (Agade, Disho and Gimbo) powder would be prepared.

3.3.12. Biochemical Analysis

3.4.12.1. Gram's staining

A 24 h old LAB was used for Gram's staining. The colonies were spread over a thin layer across a microscope slide containing a drop of water and allowed to air dry. The smear was fixed and covered with crystal violet for 1 min. After rinsing the slide under gently running tap water, the smear was then flooded with Gram's iodine solution for 1 min and washed gently with organic solvent (70% ethanol). After rinsing the slide, it counter stained with Safranin for 1 min. The stain was gently rinsed off by tilting the slide under a lightly and regular running tap water until the water runs clear. The stain was blot drying with clean cloth. Gram-positive cells were 36appeared purple and Gram-negatives red after proper staining under oil immersion objective (magnification 1000X) microscope.

3.4.12.2. KOH String Test

KOH string test was carried out using a drop of 3% potassium hydroxide (w/v) on a glass slide. A visible loopful of cells from a single, well-isolated colony was mixed into the drop. The colony which didn't turn viscous was considered as gram-positive.

3.4.12.3. Catalase Test

To check the production of enzyme catalase, a clean microscopic slide was used. A drop of 3% H_2O_2 (v/v) was put on the microscopic slide aseptically. A loop of bacterial culture was taken

and mixed with the H2O2 solution on the slide and the absence of bubble production was observed.

3.3.13. Broth Preparation

3.3.13.1. Calculation of Media for Agade, Disho and Gimbo

Beef extract (10 g), Yeast extract (5.0 g), Sodium acetate (5.0 g) Tween 80 (1.0 g), Disodium phosphate (2.0 g), Potassium phosphate (2.0 g), Ammonium citrate (2.0 g), Magnesium sulfate (0.2 g), Manganese sulfate (0.05 g), Enset Powder (Agade 31 g). The media were prepared in 3 (500 ml) flasks separately with approximately 343 ml after thoroughly mixed with shaker for each experiment below. From literatures the 50 to 80% (average ~65%) composition of enset was soluble carbohydrates, to get 20 gram of glucose 31 gram of Enset powder (Agade, Disho and Gimbo) was required. The media were prepared with the composition of the above nutrients (figure 8a).

3.3.14. Inoculation with LAB and Incubation of Broth Media

An average of 30 colonies were selected and picked with ring rod which was sterilized with alcohol and flame and inoculated for each broth medium prepared. There were 12 flasks with 500ml (four flasks with the same sample). After inoculation with purified LAB, the media were incubated at temperatures of 30 and 35ºC for about 36 days in shaker incubator (figure 9b).

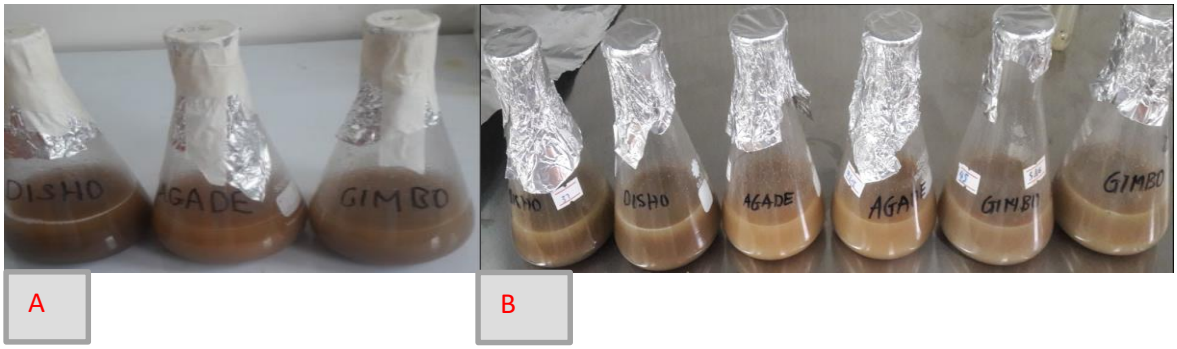


Figure 8: Broth media of enset trial varieties (Agade, Disho and Gimbo)

3.3.15. Physico-chemical Determination

pH of the fermentation process of the Enset trial varieties were measured for over one Month (36 days) in ten days at interval of 4 (0, 4, 8, 12, 16, 20, 24, 28, 32 and 36) using a digital pH meter as shown in the table 4 below.

3.3.16. Statistical Analysis and Study Variables

Data analysis, charts and trend lines were generated using Design Expert 6.0.8 Potable version. The averages were compared at 5% significance level. The variables that were studied in this thesis were mainly ***fermentation temperature, Colony forming units, pH (concentration of lactic acid) and fermentation time***. During the isolation and identification of lactic acid bacteria, three different fermentation time ranges (24, 48 and 72 hours) and three temperature ranges (25, 30 and

35°C) for three samples (Month-1, Months-3 and Months-6) were analyzed and the experiment was repeated three times. For experimental Enset trial, three samples (Agade, Disho and Gimbo), two incubation temperature ranges (30 and 35°C) and ten fermentation times (36 days) were used, the experiment was repeated two times and concentration of lactic acid was analyzed with digital pH meter. The experimental design for identification and isolation of suitable lactic acid bacterial strain for minimizing fermentation time of Kocho was a full factorial design. That means four factors (fermentation temperature, sample, dilution factor and incubation time), three levels for isolation and identification and three factors (fermentation temperature, sample, and incubation time) two levels for experimental trial. Response for isolation and identification and experimental trial was colony forming units and pH of fermentation respectively.

3.4. General Experimental Procedures for Study

The block flow diagram below indicated the overall process starting from sample collection to experimental trial in a clear and precise way (figure 9).

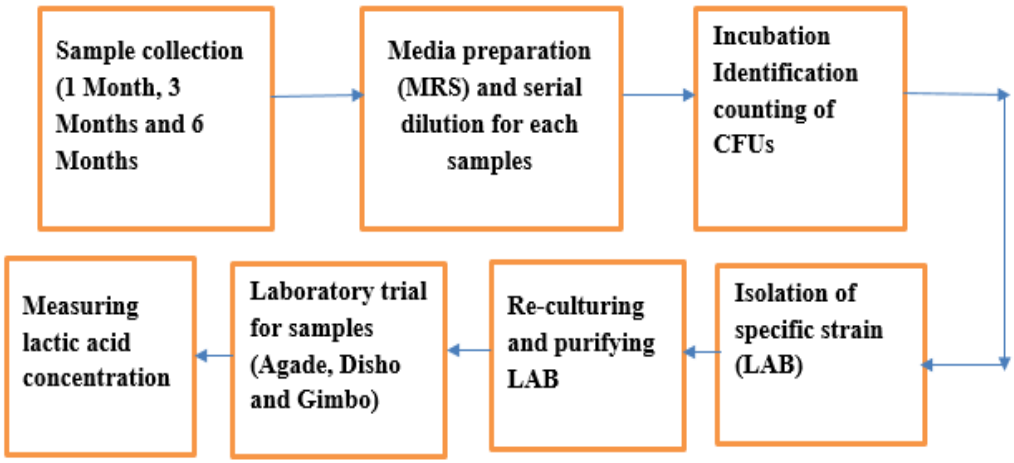


Figure 9: Overall design and experimental procedure for this thesis

Microbial growth was analyzed for each sample (Month-1, Months-3 and Months-6) using serial dilution factor 10^{-4} and 10^{-5} in tri-replicate experiment as shown in the table 3 below for sample Month-1 as example.

Samples 1	Dilution factor	Temperature (0C)	Replication	Results (CFU)		
				24 Hours	48 Hours	72 Hours
	4	25				
		30				
		35				
	5	25				
		30				
		35				

Table 3: Method of microbial growth analysis

Concentration of lactic acid was analyzed for each enset trial sample (Agade, Disho and Gimbo) in duplicate experimental procedure for 36 days at temperature of 30 and 35^0C as shown in the table 4 below. At each time interval fermentation, pH was recorded according to the schedule.

Samples	Temperature (^{0}C)	Replication	pH Records									
			Fermentation time (days)									
			0	4	8	12	16	20	24	28	32	36
Agade	30	1										
		2										
	35	1										
		2										
Disho	30	1										
		2										
	35	1										
		2										
Gimbo	30	1										
		2										
	35	1										
		2										

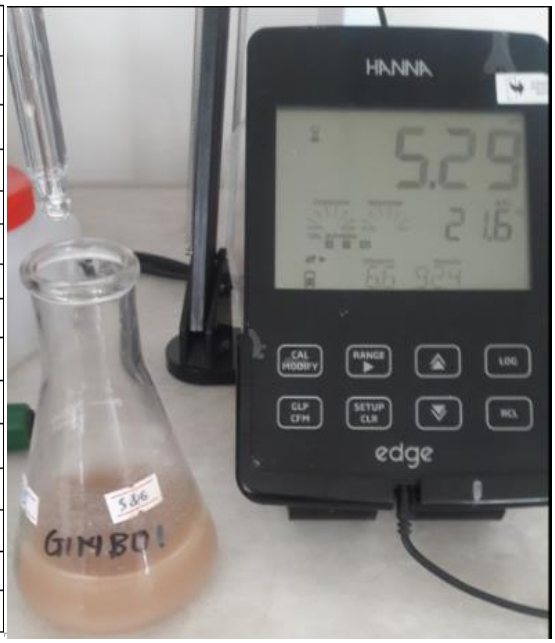


Table 4: Analysis of concentration of lactic acid bacteria in laboratory enset trial varieties

CHAPTER FOUR

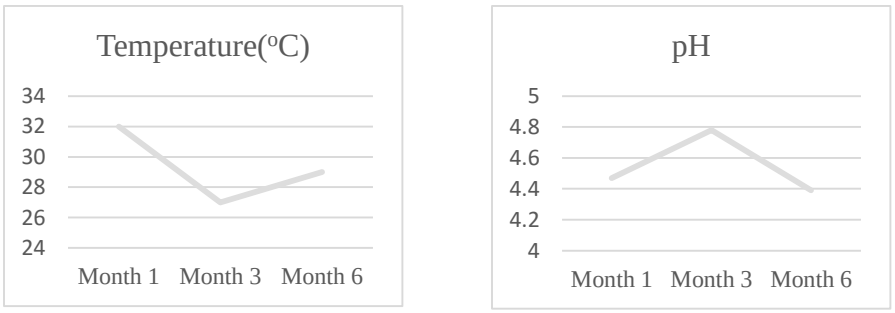
4. RESULTS AND DISCUSSION

4.1. Sample Collection and Preparation

General steps like decortication and pulverization of the pseudostem, wrapping of fermentable mass with Enset leaves, digging of the pit for fermentation around the homestead and allowing fermentation of mass in the pit were basically similar in all Enset samples (Months-1, 3 and 6).

The increase in LAB counts and the corresponding fall in pH, resulted in low levels of these groups of bacteria. According to Jay *et al.* (2005), products that contain polysaccharides but no significant levels of simple sugars are normally stable to the activities of yeasts and lactic acid bacteria due to the lack of amylase in most of these organisms.

During sample collection, the temperature of the fermented masses of samples (Month-1, Months-3 and Months-6) were measured and recorded as an average of 32, 27 and 29^0C respectively. Due to the warmer temperature and fermentable sugars produced by the amylolytic microorganisms, coliforms and other members of enterobacteriaceae grew faster and reached significantly higher than other microbes in sample Month 1. Coliforms were called "pioneer species" (Scott and Sullivan, 2008). However, these groups of microorganisms towards the proliferation of LAB were not detected due to the production of antimicrobial substances during fermentation of different foods and the temperature decreased in sample Month-3. At the end of Enset fermentation, biochemical changes of LAB were assumed to increase in higher rate at the same time the temperature increased (figure 10 a).



a) Temperature *b) pH*

Figure 10: Graphs of temperature and pH in samples (Months-1, 3 and 6)

Moreover, pH of the fermented masses of samples (Month-1, Months-3 and Months-6) were also recorded as average of 4.47, 4.78 and 4.39 respectively. The pH of fermenting mass was significantly related with the number of availability of biochemical and kinetic behavior of microorganisms in fermenting mass. As number of counts of fermenting microbes increased, the

biochemical and kinetic behavior of a group of microbes and temperature increased but the pH value of the fermenting mass fell down as shown in figure-10 b above.

4.2. Microbiological Analysis (Microbial Counts)

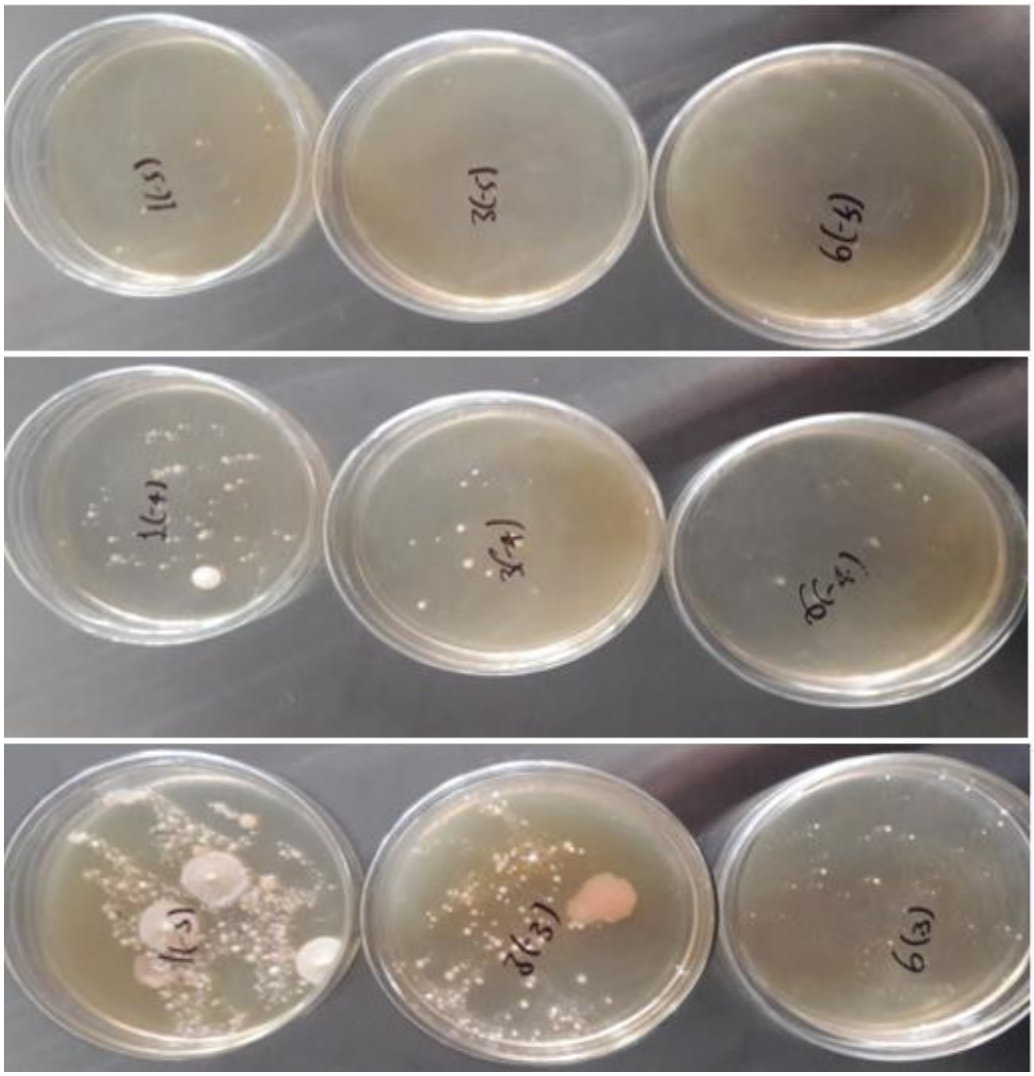


Figure 11: Growth of microbes in samples (1, 3 and 6 Months) for 48 hours

1(-5), 1(-4) and 1(-3) are representing samples with Month-1 in serial dilution factor 5, 4 and 3 (10^{-5}, 10^{-4} and 10^{-3}) respectively. It is the same for Month-3 and Month-6 accordingly.

The result of microbial growth of all 54 petri plates were recorded in the table below with 162 treatments within 72 hours.

Samples	Dilution factor	Temperature (0C)	Replication	Results (CFU/ml)		
				24 Hours	48 Hours	72 Hours
Month-1	4	25	0	$1.28x10^6$	$1.33x10^6$	$1.37x10^6$
			1	$1.21x10^6$	$1.22x10^6$	$1.3x10^6$
			2	$1.1x10^6$	$1.11x10^6$	$1.13x10^6$
		30	0	$2.31x10^6$	$2.35x10^6$	$2.42x10^6$
			1	$2.43x10^6$	$2.40x10^6$	$2.45x10^6$
			2	$2.11x10^6$	$2.11x10^6$	$2.12x10^6$
		35	0	$2x10^6$	$2.01x10^6$	$2.1x10^6$
			1	$1.99x10^6$	$2x10^6$	$2.03x10^6$
			2	$1.89x10^6$	$1.9x10^6$	$1.93x10^6$
	5	25	0	$0.4x10^6$	$0.9x10^6$	$1.1x10^6$
			1	$0.5x10^6$	$0.5x10^6$	$0.6x10^6$
			2	$0.3x10^6$	$0.6x10^6$	$0.7x10^6$
		30	0	$1.4x10^6$	$1.7x10^6$	$1.8x10^6$
			1	$1.8x10^6$	$1.9x10^6$	$2x10^6$
			2	$1.3x10^6$	$1.5x10^6$	$1.6x10^6$
		35	0	$0.4x10^6$	$0.5x10^6$	$0.6x10^6$
			1	$0.4x10^6$	$0.9x10^6$	$1.1x10^6$
			2	$1x10^6$	$1.2x10^6$	$1.3x10^6$
	4	25	0	$0.6x10^6$	$0.9x10^6$	$1x10^6$
			1	$0.3x10^6$	$0.5x10^6$	$0.5x10^6$

			2	$1x10^6$	$1.1x10^6$	$1.2x10^6$
		30	0	$1x10^6$	$1.1x10^6$	$1.11x10^6$
			1	$1.1x10^6$	$1.12x10^6$	$1.13x10^6$
			2	$1x10^6$	$1.11x10^6$	$1.12x10^6$
		35	0	$1x10^6$	$1x10^6$	$1x10^6$
			1	$0.9x10^6$	$1.1x10^6$	$1.1x10^6$
			2	$0.5x10^6$	$0.7x10^6$	$0.7x10^6$
Month-3	5	25	0	$0.0x10^6$	$0.1x10^6$	$0.3x10^6$
			1	$0.2x10^6$	$0.5x10^6$	$0.6x10^6$
			2	$0.2x10^6$	$0.1x10^6$	$0.5x10^6$
		30	0	$0.0x10^6$	$0.1x10^6$	$0.3x10^6$
			1	$0.7x10^6$	$0.5x10^6$	$0.9x10^6$
			2	$0.3x10^6$	$0.5x10^6$	$0.6x10^6$
		35	0	$0.5x10^6$	$0.7x10^6$	$0.7x10^6$
			1	$0.2x10^6$	$0.3x10^6$	$0.9x10^6$
			2	$0.6x10^6$	$0.7x10^6$	$0.5x10^6$
	4	25	0	$0.019x10^6$	$0.02x10^6$	$0.021x10^6$
			1	$0.015x10^6$	$0.016x10^6$	$0.018x10^6$
			2	$0.017x10^6$	$0.016x10^6$	$0.018x10^6$
		30	0	$0.024x10^6$	$0.025x10^6$	$0.027x10^6$
			1	$0.026x10^6$	$0.027x10^6$	$0.027x10^6$
			2	$0.025x10^6$	$0.029x10^6$	$0.03x10^6$
		35	0	$0.019x10^6$	$0.022x10^6$	$0.024x10^6$
			1	$0.01x10^6$	$0.012x10^6$	$0.013x10^6$

			2	$0.018x10^6$	$0.016x10^6$	$0.018x10^6$
Month-6	5	25	0	$0.0x10^6$	$0.2x10^6$	$0.2x10^6$
			1	$0.0x10^6$	$0.0x10^6$	$0.2x10^6$
			2	$0.0x10^6$	$0.0x10^6$	$0.0x10^6$
		30	0	$0.1x10^6$	$0.1x10^6$	$0.1x10^6$
			1	$0.2x10^6$	$0.2x10^6$	$0.3x10^6$
			2	$0.0x10^6$	$0.0x10^6$	$0.1x10^6$
		35	0	$0.1x10^6$	$0.2x10^6$	$0.2x10^6$
			1	$0.1x10^6$	$0.0x10^6$	$0.2x10^6$
			2	$0.1x10^6$	$0.1x10^6$	$0.3x10^6$

Table 5: Microbial counts within 72 hours and for three temperature ranges (25, 30 35^{0}C)

As shown in the table and figure 11 above, the growth of mesophilic microbes decreased as fermentation time of Kocho increased, from sample Month-1 to Month-6. This decrement of mesophilic microbial count did not correspond to the lactic acid bacterial growth but other amylolytic microorganisms, even yeast and molds were incorporated. When the pH of the fermenting media fell down, the microbe that could survive at lower pH (LAB) could grow. So, as fermentation time (Months) increased, only specific strain could dominate the process. As the count of different groups of microorganisms decreased towards the completion of fermentation, Bacillus species, in the form of their spores, appeared at a higher frequency. Otherwise the active vegetative forms are usually inhibited at the pH values observed towards the completion of fermentation. Gram positive cocci and Gram negative rods, consisting of Acinetobacter, Pseudomonas and members of enterobacteriaceae, might have contributed to amylolytic actions at lower levels. However, amylolysis may not be desirable in the fermented product. Amylolytic Gram-negative rods such as Acinetobacter and non-lactic Gram-positive cocci such as micrococci and staphylococci were isolated from spoiled Kocho and bulla (Ashenafi and Abebe, 1996).

Bacillus species are reported to break down starch and make fermentable sugars available during cassava fermentation (Oyewole, 1992). On sample Month-6 for example, lactobacillus species only dominated. This was because the microbes secreted secondary metabolites and antimicrobial products that could kill other microbes, and only the microbes that could resist for that secrete and acidic environment could grow. Amylolytic lactobacilli were known to develop during the fermentation of plant materials (Fitzsimons *et al.*, 1994). Morphologically, from sample Months-6 specific strains were grown, identified and isolated specifically. On the other hand, there were different microbes (size, color, structure etc.) grown in the sample Month-1.

Higher temperature of incubation contributed to rapid proliferation of microorganisms and rapid fermentation process and hence shorter fermentation time. Lactic acid bacteria dominated the fermentative microflora at later stages of fermentation. The coliforms and other members of enterobacteriaceae contributed to initial lowering of the pH of the fermenting mass. At some later days the homofermentative lactobacilli took over the process and dominated the flora until the end of fermentation. Following the proliferation of the lactobacilli the pH decreased and titratable acidity increased. Count of yeasts remained low throughout the fermentation process. The coliforms and enterobacteriaceae were undetectable following domination of the microflora by LAB. The optimum temperature for the growth of mesophilic microbes was approximately 30^0C and the highest number of growth of microbes was obtained from sample Month-1 within 72 incubation period and the active exponential growth rate of microbes was found around sample Month-1. The growth of microbes linearly decreased from sample Month-1 to Month-3 and to Month-6 within 72 hours (in 24 hours range) at 25, 30 and 35^0C temperatures. This was because the nutritional content responsible for growth of amylolytic microbes was diminished and afterwards the accumulation of secondary metabolites and the computation of microbes for nutrition affected the concentration of microbes in the sample. Almost the same result was obtained from different dilution factors (10^{-4} and 10^{-5}) incubated at temperature 25, 30 and 35^0C and 24, 48 and 72 hours. As the optimum temperature is 30^0C, single Petri plate was selected from each sample and the growth of microbes were plotted for 72 hours in eight hours interval as shown in the figure 12.

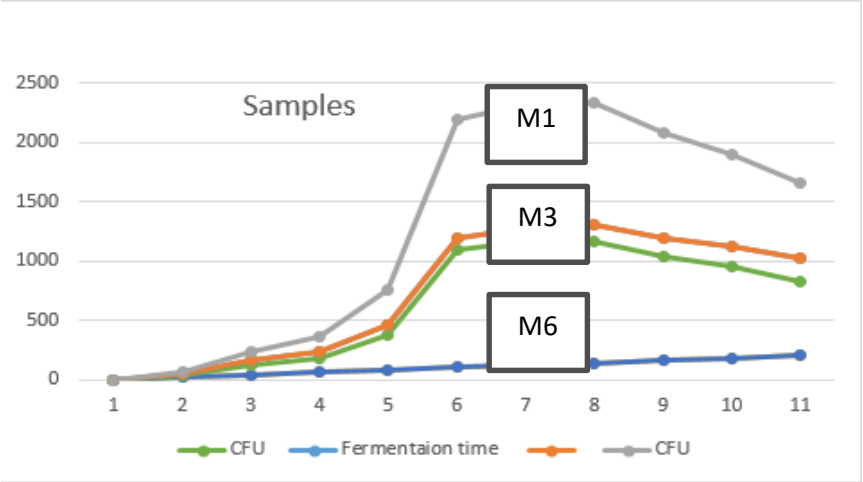


Figure 12: Microbial growth kinetics of samples at temperature of 30^0C

The graph approximately corresponded with well know Monod rate growth kinetics of microorganisms. This graph did not corresponded to growth of lactic acid bacteria but described for the growth of all mesophilic microorganisms. The growth of lactic acid bacteria was increased towards the end of fermentation.

1. **Lag phase (nutritional content of the medium)**

When microorganism is introduced into the fresh medium, it takes some time to adjust with the new environment. This phase is termed as Lag phase, in which cellular metabolism is accelerated, cells are increasing in size, but the bacteria are not able to replicate and therefore no increase in cell mass. The length of the lag phase depends directly on the previous growth condition of the organism. When the microorganism growing in a rich medium is inoculated into nutritionally poor medium, the organism will take more time to adapt with the new environment. The organism will start synthesizing the necessary proteins, co-enzymes and vitamins needed for their growth and hence there will be a subsequent increase in the lag phase. Similarly when an organism from a nutritionally poor medium is added to a nutritionally rich medium, the organism can easily adapt to the environment, it can start the cell division without any delay, and therefore will have less lag phase as shown in the figure 12 above.

2. **Exponential or Logarithmic (log) phase**

During this phase, the microorganisms are in a rapid growing and dividing state. Their metabolic activity increases and the organism begin the DNA replication by binary fission at a constant rate. The growth medium is exploited at the maximal rate, the culture reaches the maximum growth rate and the number of bacteria increases logarithmically (exponentially) and finally the single cell divide into two, which replicate into four, eight, sixteen, thirty two and so on (That is 2^0, 2^1, 2^2, 2^3.....2^n, n is the number of generations) This will result in a balanced growth. The time taken by the bacteria to double in number during a specified time period is known as the generation time as shown in the figure 12 above.

3. **Stationary phase**

As the bacterial population continues to grow, all the nutrients in the growth medium are used up by the microorganism for their rapid multiplication. This result in the accumulation of waste materials, toxic metabolites and inhibitory compounds such as antibiotics in the medium. This shifts the conditions of the medium such as pH and temperature, thereby creating an unfavorable environment for the bacterial growth. The reproduction rate will slow down, the cells undergoing division is equal to the number of cell death, and finally bacterium stops its division completely. The cell number is not increased and thus the growth rate is stabilized. If a cell taken from the stationary phase is introduced into a fresh medium, the cell can easily move on the exponential phase and is able to perform its metabolic activities as usual as shown in the figure 12 above.

4. **Decline or Death phase**

The depletion of nutrients and the subsequent accumulation of metabolic waste products and other toxic materials in the media will facilitates the bacterium to move on to the death phase. During this, the bacterium completely loses its ability to reproduce. Individual bacteria begin to die due to the unfavorable conditions and the death is rapid and at uniform rate. The number of dead cells exceeds the number of live cells. Some organisms, which can resist this condition can survive in the environment by producing endospores as shown in the figure 12 above.

For all three samples, microbial counts were highr in dilution factor four than dilution facor five and higher counts were recorded at 30^0C of temperature and 72 hours incubation period. The microbial counts that were recorded here not directly related to viable cell counts but dead cells and viable cells as they were recorded with colony counter.

The result of above Table of mesophilic microbial growth was fed and analyzed through design expert software with full factorial design and the following results were generated.

Every count of microbial growth record of the above table was fed into design expert software to predict the model terms and analysis of variance of the experiment as follows.

Response: CFU (10^6)
Hierarchical Terms Added after Manual Regression BD
ANOVA for Selected Factorial Model
Analysis of variance table [Partial sum of squares]

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	75.83	21	3.61	124.92	< 0.0001	significant
A	*50.99*	*2*	*25.49*	*881.85*	*< 0.0001*	
B	*5.22*	*2*	*2.61*	*90.29*	*< 0.0001*	
C	*0.74*	*2*	*0.37*	*12.72*	*< 0.0001*	
D	*6.54*	*1*	*6.54*	*226.12*	*< 0.0001*	
AB	*5.45*	*4*	*1.36*	*47.13*	*< 0.0001*	
AD	*5.53*	*2*	*2.77*	*95.72*	*< 0.0001*	
BD	*0.063*	*2*	*0.031*	*1.08*	*0.3415*	
CD	*0.23*	*2*	*0.11*	*3.90*	*0.0225*	
ABD	*1.08*	*4*	*0.27*	*9.37*	*< 0.0001*	
Residual	4.05	140	0.029			
Lack of Fit	*0.34*	*32*	*0.011*	*0.31*	*0.9998*	*not significant*
Pure Error	*3.71*	*108*	*0.034*			
Cor Total	79.88	161				

The Model F-value of 124.92 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant.
In this case A, B, C, D, AB, AD, CD, ABD are significant model terms.
Values greater than 0.1000 indicate the model terms are not significant.
If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The "Lack of Fit F-value" of 0.31 implies the Lack of Fit is not significant relative to the pure error. There is a 99.98% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Std. Dev.	0.17	R-Squared	0.9493
Mean	0.73	Adj R-Squared	0.9417
C.V.	23.43	Pred R-Squared	0.9322
PRESS	5.42	Adeq Precision	38.161

The "Pred R-Squared" of 0.9322 is in reasonable agreement with the "Adj R-Squared" of 0.9417.

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 38.161 indicates an adequate signal. This model can be used to navigate the design space.

Final Equation in Terms of Coded Factors:

CFU (10^6)	=
+0.73	
+0.71	* A[1]
-0.052	* A[2]
-0.23	* B[1]
+0.21	* B[2]
-0.083	* C[1]
+1.154E-003	* C[2]
-0.20	* D
-0.29	* A[1]B[1]
+0.085	* A[2]B[1]
+0.33	* A[1]B[2]
-0.13	* A[2]B[2]
-0.20	* A[1]D
-0.047	* A[2]D
+0.023	* B[1]D
+2.173E-003	* B[2]D
-0.042	* C[1]D
-7.327E-003	* C[2]D
+0.074	* A[1]B[1]D
-0.031	* A[2]B[1]D
+0.081	* A[1]B[2]D
-0.081	* A[2]B[2]D

4.3. Identification and Isolation of Suitable LAB Strain

Even if the media were selective for lactobacilli, the samples were composed of many growth of microbes such as molds, lactic acid bacteria, yeast and other nondeductible microbes (figure 13 left). Figure 13 of left image showed different types of growth of microorganisms. Different structure, size, color and appearance corresponded to multiple microorganisms. From the above microbiological analysis, microbes from samples (Months-3 and Months-6) were rejected; due too few number of microbial growth on the medium and they were in stationary phase and death phase respectively. As well as microbes that were grown from sample (Month-1) at a temperature of 25 and 35^{o}C were rejected. This was also because of small number of counts of microbes as the optimum temperature for mesophilic microbes was 30^{0}C. Microbes from sample Month-1 that were grown at 30^{0}C were in active exponential growth phase kinetics. Knowing the morphological appearance (structure, size, color) of lactobacilli, lactic acid bacteria had semi-round structure, small size and white color (figure 13 left), they were identified, isolated, and re-cultured in a new media for 48 hours at a temperature of 30^{0}C from sample Month-1 only (figure 13 right).

Figure 13: Growth of different microbes

4.4. Microbiological Analysis (Microbial Counts of Purified LAB)

After a specific strain was identified, isolated and optimized, serial dilution was made with the purified LAB. And then the serially diluted (serial dilution factor 10^{-4} and 10^{-5}) of purified LAB were inoculated in new MRS media at temperature 30^{0}C for 48 hours. Colonies of acid-producing bacteria, identified by a clear zone around each colony, were randomly selected from MRS-agar plates and purified by re-plating on MRS-agar plates. Semi-round structure, small size and white color lactobacilli were observed. Growth distribution, like structure and color of lactobacilli (serial dilution factor10^{-5}) was compared and gotten similar appearance with the standard lactobacilli growth distribution as shown below in the figure 14. The lactobacilli colony counts were approximately 6.2X10^{6}. This pure LAB were stored at a temperature of 4^{0}C refrigerator for further analysis.

Figure 14: Comparison standard and isolated lactobacilli

4.5. Analysis of Lactic Acid Concentration

Lactic acid concentration was measured through pH meter for 36 days with 4 days interval at two temperature ranges (30 and 35^{0}C) as shown in table below.

Samples	Temperature (^{0}C)	Replication	pH									
			Fermentation time (days)									
			0	4	8	12	16	20	24	28	32	36
Agade	30	1	6.92	6.65	6.17	5.43	4.91	4.75	4.51	4.50	4.90	5.33
		2	6.90	5.95	5.21	5.16	5.27	4.98	4.47	4.47	5.37	5.36
	35	1	6.98	5.70	5.61	5.31	4.59	4.57	4.41	4.39	4.60	5.59
		2	6.95	5.64	5.55	5.40	5.00	5.03	4.76	4.75	5.09	5.40
Disho	30	1	6.96	5.81	5.16	5.62	5.47	5.13	4.64	4.65	5.45	5.52
		2	6.93	6.29	5.37	5.16	5.15	4.86	4.43	4.44	5.05	5.12
	35	1	6.89	5.69	5.09	5.21	4.93	4.87	4.49	4.49	4.83	5.80
		2	6.98	5.77	5.26	5.17	4.89	4.85	4.52	4.51	4.79	5.77
Gimbo	30	1	6.99	6.19	5.79	5.58	5.47	5.09	4.71	4.70	5.57	5.58
		2	6.89	6.25	5.42	5.27	5.07	4.83	4.38	4.37	5.21	5.24
	35	1	6.92	6.49	5.34	5.29	4.91	4.90	4.63	4.62	4.71	5.91
		2	6.97	5.66	5.18	5.19	4.79	4.80	4.39	4.39	4.87	5.99

Table 6: pH records in enset trial varieties for 36 days at temperature 30 and 35^{0}C

Initial pH of the pulverized and decorticated mass of trial Enset (Agade, Disho and Gimbo) varieties was approximately 6.50 during sample collection. Although the final pH values of the experimental trial were comperably similar, the longer duration of fermentation at 30^{0}C

temperature could result in the production of more acid thereby retarding the growth of undesirable microorganisms. The dominance of LAB during the active stages of Enset fermentation is in agreement with the report of Gashe (1987a). However, according to this author, heterofermentative cocci were important in initiating Enset fermentation. As all LAB were homofermentative, more acid would be produced per mole of fermentable sugar and the rate of pH fall would be faster. The lowest pH achieved during the fermentation was approximately 4.50 for all varieties. Similar pH value was reached only towards the end of fermentation at day of 28^{th}.

In general, as fermentation time was going for all experimental Enset trial varieties, the pH value decreased and finally reached approximately 4.50 for all varieties within 28 days. The lower pH value showed high growth of LAB in the media beyond that LAB could not grow. So the maximum growth was attained at pH value about 4.50 (table 6 above). After a minimum pH value was attained for all, the pH value started to increase as fermentation time was running. This pH value increment showed the fermentatble nutrient decreased and there was high competition of microbes in the fermenting media.

Comparing each experimental Enset trial varieties, pH value for Disho fermentation deropped faster within eight days than the other two varieties and from 8-16 days the Disho resisted for fermentation. This faster fermentation showed that carbohydrates that found in Disho variety were more suitable for the growth of LAB. For the other two varieties pH value dropped almost in a linear form. However, experimental Enset trial varieties attained almost the same pH value at 24-28^{th} and then it started to increase for all varieties.

Comparing temperatures of fermentation, the pH value dropped faster at 35^0C than at 30^0C temperature. An initial sharp decline in pH corresponded to a similar increase in count of all groups of microorganisms. This showed that at temperature of 35^0C the break down of disaccharides that available in experimental Enset trial varieties to glucose molecule was higher than at 30^0C. When temperature increased to some level, the disentegration of molecules also increased. As result, the LAB adapted easily and early to the fermenting media and fermenting mass.

Generally, either at temperature of 30 or 35^0C the pH value was approximately 4.50 for all experimental Enset trial varieties at 24^{th} -28^{th} days of fermentation. And the maximum growth for LAB was at temperature of 30^0C from the previous experiments. Consequently, inoculation of LAB with decorticated and chopped Enset varieties at temperature of 30^0C took to ferment a maximum of 26 days. The minimum pH value obtained from this experiment was 4.50 and corresponded to that obtained from sample Month-6 during sample collection. This pH value indicated that, to get kocho as if the experimental enset trial varieties were fermented for approximately 6 Months.

Every pH record of the above table was fed into design expert software to predict the model terms and analysis of variance of the experiment as follows.

Response: pH

ANOVA for Selected Factorial Model

Analysis of variance table [Partial sum of squares]

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	59.25	19	3.12	78.44	< 0.0001	significant
B	*0.40*	*1*	*0.40*	*10.12*	*0.0019*	
C	*57.24*	*9*	*6.36*	*159.97*	*< 0.0001*	
BC	*1.61*	*9*	*0.18*	*4.51*	*< 0.0001*	
Residual	3.98	100	0.040			
Lack of Fit	*1.09*	*40*	*0.027*	*0.57*	*0.9703*	*not significant*
Pure Error	*2.88*	*60*	*0.048*			
Cor Total	63.23	119				

The Model F-value of 78.44 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant.
In this case B, C, BC are significant model terms.
Values greater than 0.1000 indicate the model terms are not significant.
If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The "Lack of Fit F-value" of 0.57 implies the Lack of Fit is not significant relative to the pure error. There is a 97.03% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Std. Dev.	0.20	R-Squared	0.9371
Mean	5.33	Adj R-Squared	0.9252
C.V.	3.74	Pred R-Squared	0.9095
PRESS	5.72	Adeq Precision	29.812

The "Pred R-Squared" of 0.9095 is in reasonable agreement with the "Adj R-Squared" of 0.9252.
"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 29.812 indicates an adequate signal. This model can be used to navigate the design space.

Term	Coefficient Estimate	DF	Standard Error	95% CI Low	95% CI High	VIF
Intercept	5.33	1	0.018	5.29	5.36	
B-Temperature	-0.058	1	0.018	-0.094	-0.022	1.00
C[1]	1.61	1	0.055	1.51	1.72	
C[2]	0.68	1	0.055	0.57	0.79	
C[3]	0.10	1	0.055	-4.918E-003	0.21	

C[4]	-9.917E-003	1	0.055	-0.12	0.098
C[5]	-0.29	1	0.055	-0.40	-0.18
C[6]	-0.44	1	0.055	-0.55	-0.33
C[7]	-0.80	1	0.055	-0.91	-0.69
C[8]	-0.80	1	0.055	-0.91	-0.69
C[9]	-0.29	1	0.055	-0.40	-0.18
BC[1]	0.066	1	0.055	-0.042	0.17
BC[2]	-0.12	1	0.055	-0.23	-0.016
BC[3]	-0.033	1	0.055	-0.14	0.075
BC[4]	3.750E-003	1	0.055	-0.10	0.11
BC[5]	-0.13	1	0.055	-0.24	-0.020
BC[6]	6.250E-003	1	0.055	-0.10	0.11
BC[7]	0.063	1	0.055	-0.045	0.17
BC[8]	0.060	1	0.055	-0.049	0.17
BC[9]	-0.16	1	0.055	-0.27	-0.055

Final Equation in Terms of Coded Factors:

pH	=
+5.33	
-0.058	* B
+1.61	* C[1]
+0.68	* C[2]
+0.10	* C[3]
-9.917E-003	* C[4]
-0.29	* C[5]
-0.44	* C[6]
-0.80	* C[7]
-0.80	* C[8]
-0.29	* C[9]
+0.066	* BC[1]
-0.12	* BC[2]
-0.033	* BC[3]
+3.750E-003	* BC[4]
-0.13	* BC[5]
+6.250E-003	* BC[6]
+0.063	* BC[7]
+0.060	* BC[8]
-0.16	* BC[9]

CHAPTER FIVE

5. CONCLUSION AND RECOMMENDATION

5.1. Conclusion

This thesis showed notable differences from previous researches that were done by different researchers based on different fermentation conditions. It indicated that fermentation time of decorticated and chopped Enset varieties was minimized using lactic acid bacterial strain, that was obtained from Months-1 fermented kocho through microbiological analysis. The time of fermentation of kocho in laboratory scale was minimized from about six Months to about one Month through laboratory trial analysis. The minimum pH value that had obtained from experimental trial and the pH value obtained from sample Month-6 during sample collection were the same, approximately 4.50. As a result of this thesis, using LAB as starter culture, kocho was produced within one Month. The kocho that had been produced through this experiment assumed to have the same texture, flavor, smell and color as if it was fermented for six Months through natural process. Even if the aseptic technique in laboratory differed to aseptic technique in field, the result indicated that fermentation time of Enset could minimized though LAB. The result of this thesis helps to optimize processing techniques, standardize, open a door for industrialization of kocho and produce microbial starter culture for large scale kocho fermentation.

5.2. Recommendation

Enset has untapped potential in ensuring food security through fermentation producing Kocho using lactic acid bacterial strain. The thesis was basically in identification, isolation and characterization of lactic acid bacteria through morphological, physiological and biochemical analysis. Apart these measurements genotypic and genetic sequence measurements of a specific lactic acid bacterial stain were not conducted.

Taking the above mentioned measurements in to account, it was recommended that morphologically, physiologically, and genetically serious and detailed investigation of specific lactic acid bacterial species that could be ferment decorticated Enset should be carried in detail. Like polymerase chain reaction (PCR) amplification, 16 rRNA sequencing and gene sequencing.

The thesis was limited only in the laboratory as a result it was suggested that intensive research has to be conducted while using lactic acid bacterial species as a starter culture during decorticated enset fermentation.

At the end of this paper appendix was attached whenever further information was needed regarding the analysis of microbial counts and lactic acid concentration that was generated through Design Expert 6.0.8 Portable software.

6. REFERENCES

Abebe, T., Wiersum, K. F., and Bongers, E. (2010). Spatial and temporal variation in crop diversity in agroforestry home gardens of southern Ethiopia. Agroforest. Syst.78:309-322.

Abebe, Y., Bogale, A., Hambidge, K. M., Stoecker, B. J., Bailey, K., and Gibson, R. S. (2007). Phytate, zinc, iron, and calcium content of selected raw and prepared foods consumed in rural Sidama, Southern Ethiopia, and implications for bioavailability. J. Food Compos. Anal. 20:161-168.

Adam, M. R. (1990). Topical aspects of fermented foods. Trends in Food Science & Technology 1, 141-144.

Adesulu, A. T., and Awojobi, K. O. (2014). Enhancing sustainable development through indigenous fermented food products in Nigeria. African Journal of Microbiology Research, 8(12): 1338-1343.

Altaf, M., Naveena, B. J. and Reddy, G. (2005). Screening of inexpensive nitrogen sources for production of lactic acid from starch by amylolytic Lactobacillus amylophilus GV6 in single step fermentation. Food Technology and Biotechnology 43: 235-239.

Ashenafi, M. (2006). A review on the microbiology of indigenous fermented foods and beverages of Ethiopia', Ethiopopian Journal of Biological Sciences, Vol. 5, pp189-245.

Asres, A. and Omprakash, S. (2014). Extension of Enset Plant Product for Rural Development in Ethiopia, Wollo University, Kombolcha, Ethiopia.

Atlabachew, M., and Chandravanshi, B. S. (2008). Levels of major, minor and trace elements in commercially available enset (Ensete ventricosum (Welw.), Cheesman) food products (Kochoand Bulla) in Ethiopia. Journal of Food Composition and Analysis, 21(7): 545-552.

Axelsson, L. (2004). "Lactic Acid Bacteria: Classification and Physiology," In: S. Salminen, A. von Wright and A. C. Ouwehand, Eds., Lactic Acid Bacteria: Microbiology and Functional Aspects, Marcel Dekker, New York.

Axelsson, L. and Wright, A. (2011). "Lactic Acid Bacteria: An Introduction," In: S. Lahtinne, S. Salminen, A. Von Wright and A. Ouwehand, Eds., Lactic Acid Bacteria: Microbiological and Functional Aspects, CRC Press, London

Ayele, N. and Berhanu, A. G. (1994). Survival and growth of selected pathogens in fermented kocho (Enseteventricosum). East Africa Medical Journal.

Babu, S. C. (2000). Rural nutrition interventions with indigenous plant foods-a case study of vitamin A deficiency in Malawi. Agron. Soc. Environ. 4(3): 169-179.

Belay, A., Hunduma, T., Fekadu, E., Negisho, K., and Ali, A. (2008). Gender based analysis of production system in Ambo. In: Chiche, Y. and Kelemu, K. (eds.), Proceedings of the workshop on gender analysis in agricultural research (pp. 27-29) Addis Ababa, Ethiopia.

Berhanu, A. G. (1987). Kocho fermentation. Journal of Applied Bacteriology.

Bintsis, T. A., Vafopoulou-Mastrojiannaki, E., Litopoulou T., and Robinson, R. K. (2003): "Protease, Peptidase and Esterase Activities by Lactobacilli and Yeast Isolates from Feta Cheese Brine," Journal of Applied Microbiology.

Brandt, S. A, Spring, A., Hiebsch, C., McCabe, J.T., Tabogie, E., Diro, M., Wolde-Michael, G., Yntiso, G., Shiegeta, M., and Tesfaye, S. (1997). The "Tree Against Hunger": Enset-Based Agricultural Sy stems in Ethiopia. American Association for the Advancement of Science, Washington.

Bringel, F. (1998). "Carbamoyl phosphate and Natural Auxotrophies in Lactic Acid Bacteria,"

Campbell-Platt, G. (1987). Fermented foods of the world-a dictionary and guide. London, Butter worths.

Carr, F. J., Hill, D., and Maida, N. (2002). The lactic acid bacteria: A literature survey. Crit. Rev. Microbiol. 28, 281-370.

Chen, H., Hayek, S., Guzman, J. R., Gillitt, N. D., Ibrahim, S. A., Jobin C., and Sang, S. (2012). "The Microbiota Is Essential for the Generation of Black Tea Theaflavins-Derived Metabolites," PloS One, Vol. 7, No. 12, Article ID: e51001.

Cook, P. E., Owens, J. D, and Campbell P. G. (1991). Fungal growth during rice tape fermentation. Letters in Applied Microbiology 13, 123-125.

Crittenden, R., Karppinen, S., Ojanen, S., Tenkanen, M., Fagerström, R., Mättö, J., Saarela, M., Mattila-Sandholm, T., and Poutanen, K. (2002). In vitro fermentation of cereal dietary fibre carbohydrates by probiotic and intestinal bacteria. J. Sci. Food Agric. 82, 781-789.

Dalbato, A. L. (2000). An overview of population and food production situation in SNNPR. Population Newsletter, Awassa. Eth. 4:10-13.

Danielsen, M. and Wind, A. (2003). Susceptibility of Lactobacillus spp. to antimicrobial agents. Int. J. Food Microbiol. 82, 1-11.

De Walt, R. B. (1994). Using indigenous knowledge to improve agriculture and natural resource management. Human Organization 53(2):123-130.

Dirar, H. A. (1993). The indigenous foods of the Sudan, a study in African food and nutrition. ISBN: 0-85198-858.

Dunne, C., and Shanahan, F. (2003). Biotherapeutic agents in the treatment of gastrointestinal disorders. Curr. Treatm. Opin. Inf. Dis. 5, 183-188.

Elias, E., Morse, S., and Belshaw, D. G. (1998). Nitrogen and phosphorus balance of Kindo Koisha farms in southern Ethiopia. Agr. Ecosyst. Environ. 71:93-113.

FAO (2010). The state of food insecurity in the world. FAO, Rome.

Gashe, B. A. (1987a). Kocho fermentation. Journal of Applied Bacteriology, 62(6): 473-477.

Gashe, B. A. (1987b). Spoilage organisms of Kocho. MIRCEN Journal of Applied Microbiology and Biotechnology, 3(1): 67-73.

Gibson, G. R., and Fuller, R. (2000). Aspects of in vitro and in vivo research approaches directed toward identifying probiotics and probiotics for human use. J. Nutr.

Goldstein, E. J. C., Citron, D. M., Merriam, C. V., Warren, Y. and Tyrrell, K. L. (2000). Comparative in vitro activities of ertapenem (MK-0826) against 1,001 anaerobes isolated from human intra-abdominal infections. Antimicrob. Agents Chemoth. 44, 2389-2394.

Grangette, C., Müller-Alouf, H., Goudercourt, D., Geoffroy, M. C., Turneer, M., and Mercenier A. (2001). Mucosal immune responses and protection against tetanus toxin after intranasal immunization with recombinant Lactobacillus plantarum. Infect. Immun. 69, 1547-1553.

Habte, T. K., Abegaz K. A., and Negera E. G. (2013). The microbiology of Kocho: An Ethiopian Traditionally fermented food from Enset (Ensete ventricosum). International Journal of Life Sciences, 8 (1): 7-13.

Hartemink, R., Domenech, V. R. and Rombouts F. M. (1997). "LAMVAB-A New Selective Medium for the Isolation of Lactobacilli from Faeces," Journal of Microbiological Methods

Hoefnagel, M. H., Starrenburg, M. J., Martens, D. E., Hugenholtz, J., Kleerebezem, M., Swam, I. I., Bongers, R., Westerhoff, H. V. and Snoep. J. L. (2002). "Metabolic Engineering of Lactic Acid Bacteria, the Combined Approach: Kinetic Modelling, Metabolic Control and Experimental Analysis," Microbiology.

Holzapfel, W. H., Haberer, P., Geisen, R., Björkroth, J., and Schillinger, U. (2001). Taxonomy and important features of probiotic microorganisms in food nutrition. Am. J. Clin. Nutr. 73, 365S-373.

Hunduma, T., and Ashenafi, M. (2011). Effect of altitude on microbial succession during traditional Enset (Ensete ventricosum) fermentation. International Journal of Food, Nutrition and Public Health, 4(1): 39-53.

Ikpeme-Emmanuel, C. A., Eneji, C. A., and Osuchukwu, N. C. (2011). Functional and sensory properties of iron fortified West African cassava fermented meals;"gari" and "fufu". African Journal of Food Science, 5(8): 484-489.

Ishibashi, N., and Yamazaki, S. (2001). Probiotics and safety. Am. J. Clin. Nutr. 73, 465S-470

Jay, M., Loessner, M. and Golden, D. (2005). Modern Food Microbiology. New York: Springer.

John, R. P., Nampoothiri, K. M. and Pandey, A. (2007). "Fermentative Production of Lactic Acid from Biomass: An Overview on Process Developmentsand Future Perspectives," Applied Microbiology and Biotechnology.

Jussier, D., Morneau, A. D., and de Orduña, R. M. (2006). "Effect of Simultaneous Inoculation with Yeast and Bacteria on Fermentation Kinetics and Key Wine Parameters of Cool-Climate Chardonnay," Applied and Environmental Microbiology, Vol. 72, No. 1, pp. 221-227.

Kanshie, K. T. (2002). Five thousand years of sustainability? A case study on Gedeo land use (southern Ethiopia). Treemail Publishers, Heelsum, the Netherlands: 16-100.

Karlsson, L. M., Dalbato, A. L., and Tamado, T. (2011). Seed morphology and fresh seed germination of "false banana", Enset ventricosum, (Musaceae). Informativo Abrates 21:132.

Karssa, T. H., Ali, K. A., and Gobena, E. N. (2014). The microbiology of Kocho: An Ethiopian traditionally fermented food from Enset (Ensete ventricosum). International Journal of Life Sciences, 8(1): 7-13.

Katz, M., Medina, R., Gonzalez, S. and Oliver, G. (2002). "Esterolytic and Lipolytic Activities ofLactic Acid Bacteria Isolated from Ewes Milk and Cheese," Journal of Food Protection.

Khetarpaul, N. and Chauhan, B. M. (1990). Fermentation of pearl millet flour with yeasts and lactobacilli: in vitro digestibility and utilisation of fermented flour for weaning mixtures. Plant Foods for Human Nutrition.

Kirilov, N., Petkova, T., Atanasova, J., Danova, S., Iliev, I., Popov, Y., Haertle, T. and Ivanova, I. (2009). "Proteolytic Activity in Lactic Acid Bacteria from Iraq, Armenia and Bulgaria," Biotechnology & Biotechnological Equipment.

König, H. and Fröhlich, J. (2009). "Lactic Acid Bacteria." Eds., Biology of Microorganisms on Grapes, in Must and in Wine, Springer, Verlag, Berlin Heidelberg.

Lamichhane, S. A. (2014). Genetically Modified Foods-Solution for Food Security. International Journal of Genetic Engineering and Biotechnology, 5(1): 43-48.

Lechiancole, T., Ricciardi, A. and Parente, E. (2002). "Optimization of Media and Fermentation Conditions for the Growth of Lactobacillus sakei," Annals of Microbiology.

Leistner, L. (1990). Mould-fermented foods: recent developments. (In `Proceedings of the International Conference on Biotechnology & Food, Food Biotechnology 4, 433-441.

Marshall, E., and Mejia, D. (2012). Traditional fermented food and beverages for improved livelihoods, Rural Infrastructure and Agro-Industries Division Food and Agriculture Organization of the United Nations Rome 2011, Diversification booklet number 21: 15-16.

McAuliffe, O., Ross, R.P. and Hill, C. (2001). Lantibiotics: structure, biosynthesis and mode of action. FEMS Microbiol. Rev. 25, 285-308.

Meyers, S. A., Cuppett, S. L. and Hutkins, R. W. (1996). "Lipase Production by Lactic Acid Bacteria and Activity on Butter Oil," Food Microbiology.

Mohammed, B., Martin, G. and Laila, M. K. (2013). Nutritive values of the drought tolerant food and fodder crop enset, School of Animal and Range Sciences, Hawassa University, Ethiopia.

Morelli, L., Calleagri, M. L., Vogensen, F. K. and Wright, A. V. (2011). "Genetics of Lactic Acid Bacteria," In: S. Lahtinne, S. Salminen, A. Von Wright and A. Ouwehand, Eds., Lactic Acid Bacteria: Microbiological and Functional Aspects, CRC Press, London.

Negash, A, Niehof, A. (2004). The significance of enset culture and biodiversity for rural household food and livelihood security in southwestern Ethiopia. Agric. Human Values. 21:61-71.

Nurfeta, A., Eik, L. O., Tolera, A., and Sundstøl, F. (2008). Chemical composition and in sacco dry matter degradability of different morphological fractions of 10 Enset (Ensete ventricosum) varieties. Animal Feed Science and Technology, 146(1): 55-73.

Ogawa J., Kishino, S., Ando, A., Sugimoto, S., Mihara, K. and Shimizu, S. (2005). "Production of Conjugated Fatty Acids by Lactic Acid Bacteria," Journal of Bioscience and Bioengineering

Olango, T., and Bizuayehu, T. (2009). Management of Enset (Ensete ventricosum (Welw.) Cheesman) diversity in Wolaita, southern Ethiopia: farmers' knowledge and implications for on-farm conservation: 1-9.

Onda, T., Yanagida, F., Tsuji, M., Shinohara, T., and Yokotsuka, K. (2003). Time series analysis of aerobic bacterial flora during miso fermentation. Letters in applied microbiology, 37(2): 162-168.

Onyenwoke, C. A. and Simonyan, K. J. (2014). Cassava post-harvest processing and storage in Nigeria. African Journal of Agricultural Research, 9(53): 3853-3863.

Pender, J., and Gebremedhin, B. (2007). Determinants of agricultural and land management practices and impacts on crop production and household income in the highlands of Tigray, Ethiopia. Journal of African Economies, 17(3): 395-450.

Pingitore, E. V., Hebert, E. M., Sesma, F. and Nader-Macias, M. E. (2009). "Influence of Vitamins and Osmolites on Growth and Bacteriocin Production by Lactobacillus salivarius CRL 1328 in a Chemically Defined Medium," Canadian Journal of Microbiology.

Reid, G., Sanders, M. E., Gaskins, H. R., Gibson, G. R., Mercenier, A., Rastall, R., Roberfroid, M., Rowland, I., Cherbut, C., and Klaenhammer, T.R. (2003). New scientific paradigms for probiotics and prebiotics. J. Clin. Gastroenterol. 37, 105-118.

Renault, P. (2002). Genetically modified lactic acid bacteria: applications to food or health and risk assessment. Biochimie 84, 1073-1087.

Rodríguez, H., Curiel, J. A., Landete, J.M., de las Rivas, B., de Felipe, F. L., Gómez-Cordovés, C., Mancheño, J. M. and Muñoz, R. (2012). "Food Phenolics and Lactic Acid Bacteria," International Journal of Food Microbiology.

Rupasinghe, H. V., and Yu, L. J. (2012). Emerging preservation methods for fruit juices and beverages. INTECH Open Access Publisher. ISBN: 978-953-51-0067-6.

Sahlin, P. (1999). Fermentation as a method of food processing production of organic acids, pH development and microbial growth in fermenting cereals. Lund Institute of Technology, Lund University: 1-63.

Salminen, M. K., Rautelin, H., Tynkkynen, S., Poussa, T., Saxelin, M., Valtonen, V., and Järvinen, A. (2004). Lactobacillus bacteremia, clinical significance and patient outcome, with special focus on probiotic L. rhamnosus GG. Clin. Infect. Dis. 38, 62-69.

Sanchez, S. and Demain. A. L. (2008). "Metabolic Regulation and Overproduction of Primary Metabolites," Microbiology and Biotechnology.

Sanders, M. E. (2003). Probiotics: Considerations for human health. Nutr. Rev. 61, 91-99.

Savijoki K., Ingmer, H. and Varmanen, P. (2006). "Proteolytic Systems of Lactic Acid Bacteria," Applied Microbiology and Biotechnology.

Scott, R. and Sullivan, W. C. (2008). Ecology of fermented foods. Human Ecology Review Vol.15, pp.25-31.

Shank, R. and Ertiro, C. (2008). A Linear Model for Predicting Enset Plant Yield and Assessment of Kocho Production in Ethiopia, Addis Ababa.

Steidler, L., Neirynck, S., Huyghebeart, N., Snoeck, V., Vermeire, A., Goddeeris, B., Cox, E., Remon, J. P. and Remaut, E. (2003). Biological containment of genetically modified Lactococcus lactisfor intestinal delivery of human interleukin 10. Nature Biotechn. 21, 785-789.

Stone, A., Massey, A., Theobald, M., Styslinger, M., Kane, D., Kandy, D., Tung, A., Adekoya, A., Madan, J., and Davert, E. (2011). Enset (Tree Against Hunger). Africa's indigenous crops.

Tadele, Z. (2009). Role of orphan crops in enhancing and diversifying food production in Africa. In African Technological Developmental Forum Journal: 9-15.

Tadesse, G., Ephraim, E. and Ashenafi, M. (2005). Assessment of the antimicrobial activity of lactic acid bacteria isolated from Borde and Shamita, traditional Ethiopian fermented beverages, on some food-borne pathogens and effect of growth medium on the inhibitory activity. Internet Journal of food safety. Vol. 5. pp. 13-20.

Takala, T. M., Saris, P. E. J. and Tynkkynen, S.H. (2003). Food-grade host/vector expression system for Lactobacillus caseibased on complementation of plasmid-associated phospho-galactosidase gene lacG. Appl. Microbiol. Biotechnol. 60, 564-570.

Tesfaye, B. (2008). The enset (Ensete ventricosum) gardens of Sidama: composition, structure and dynamics of a traditional poly-variety system. Genet. Resour. Crop Evol. 55:1347-1358.

Thomas, L. V., Clarkson, M. R., and Delves-Broughton, J. (2000). Nisin, p. 463-524. In Natural Food Antimicrobial Systems, ed. A. S. Naidu. CRC Press, Boca Raton.

Toleco, M. R. M., Duque, S. M. M., Tan, A. G., Kobayashi, V. B. and Flores, D. M. (2016). An optimized sustainable medium for the direct lactic acid fermentation of sago (Metroxylon sagu Rottb.) starch by Enterococcus faecium DMF78, International Food Research Journal 23(3): 1274-1279.

Tsegaye, A. and Struik, P. C. (2000). Research supporting the genetic diversity of enset in southern Ethiopia. In Encouraging diversity. The conservation and development of plant genetic resources, 245-249.

Tsegaye, A. and Struik, P. C. (2002). Analysis of enset (ensete ventricosum) indigenous production methods and farmbased biodiversity in major enset-growing regions of southern Ethiopia.

Tsegaye, A., and Struik, P. C. (2001). Enset (Ensete ventricosum (Welw.) Cheesman) kocho yield under different crop establishment methods as compared to yields of other carbohydrate-rich food crops. NJAS-Wageningen Journal of Life Sciences, 49(1): 81-94.

Tsehaye, Y., and Kebebew, F. (2006). Diversity and Cultural use of Enset (Ensete ventricosum (Welw.) Cheesman) in Bonga in Situ Conservation Site, Ethiopia. Ethnobotany Research and application, 4: 147-157.

Urooj, A. and Puttaraj, S. (1994). Effect of processing on starch digestibility in some legumes, an in vitro study.

Vavilov, N. I. and Rodin, L. E. (1997). Five Continents. Rome: International Plant Genetic Resource Institute.

Wadamori, Y., Vanhanen, L., and Savage, G. P. (2014). Effect of kimchi fermentation on oxalate levels in silver beet (Beta vulgaris var. cicla). Foods, 3(2): 269-278.

Wee, Y.J., Reddy, L. V. A., and Ryu, H. W. (2008). Fermentative production of L (+)-lactic acid from starch hydrolyzate and corn steep liquor as inexpensive nutrients by batch culture of Enterococcus faecalisRKY1. Journal of Chemical Technology and Biotechnology 83: 1387-1393.

Westphal, E. (1975). Agricultural Systems in Ethiopia. Wageningen: Centre for Agricultural Publication and Documentation.

Wiedemann, I., Benz, R., and Sahl, H. G. (2004). Lipid II-mediated pore formation by the peptide antibiotic nisin: a black lipid membrane study. J. Bacteriol. 286, 3259-3261.

Yirmaga, M. T. (2013). Improving the indigenous processing of kocho, an Ethiopian traditional fermented food. Journal of Nutrition and Food Science, 3(182): 1-6.

Yuan, Q. and Furuta, G. T. (2003). "Insights into Milk Protein Allergy: Microenvironment Matters," Gastroenterol.

Zendo, T., Fukao, M., Ueda, T. Higuchi, J., Nakayama, K., Sonomoto, K. (2003). Identification of the lantibiotic nisin Q, a new natural nisin variant produced by Lactococcus lactis61-14 isolated from a river in Japan. Biosci. Biotechnol. Biochem. 67, 1616-1619.

Zhang, G., Mills, D. A., and Block, D. E. (2009). "Development of Chemically Defined Media Supporting High-Cell-Density Growth of Lactococci, Enterococci, and Streptococci," Applied and Environmental Microbiology.

Zhou, W., and Therdthai, N. (2012). "Fermented bread". Handbook of plant-based fermented food and beverage technology (2nd edition.) CRC Press, 479.

7. APPENDICES

Response: CFU (10^6)

Hierarchical Terms Added after Manual Regression BD
ANOVA for Selected Factorial Model
Analysis of variance table [Partial sum of squares]

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	75.83	21	3.61	124.92	< 0.0001	significant
A	*50.99*	*2*	*25.49*	*881.85*	*< 0.0001*	
B	*5.22*	*2*	*2.61*	*90.29*	*< 0.0001*	
C	*0.74*	*2*	*0.37*	*12.72*	*< 0.0001*	
D	*6.54*	*1*	*6.54*	*226.12*	*< 0.0001*	
AB	*5.45*	*4*	*1.36*	*47.13*	*< 0.0001*	
AD	*5.53*	*2*	*2.77*	*95.72*	*< 0.0001*	
BD	*0.063*	*2*	*0.031*	*1.08*	*0.3415*	
CD	*0.23*	*2*	*0.11*	*3.90*	*0.0225*	
ABD	*1.08*	*4*	*0.27*	*9.37*	*< 0.0001*	
Residual	4.05	140	0.029			
Lack of Fit	*0.34*	*32*	*0.011*	*0.31*	*0.9998*	*not significant*
Pure Error	*3.71*	*108*	*0.034*			
Cor Total	79.88	161				

The Model F-value of 124.92 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant.
In this case A, B, C, D, AB, AD, CD, ABD are significant model terms.
Values greater than 0.1000 indicate the model terms are not significant.
If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The "Lack of Fit F-value" of 0.31 implies the Lack of Fit is not significant relative to the pure error. There is a 99.98% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Std. Dev.	0.17	R-Squared	0.9493
Mean	0.73	Adj R-Squared	0.9417
C.V.	23.43	Pred R-Squared	0.9322
PRESS	5.42	Adeq Precision	38.161

The "Pred R-Squared" of 0.9322 is in reasonable agreement with the "Adj R-Squared" of 0.9417. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 38.161 indicates an adequate signal. This model can be used to navigate the design space.

	Coefficient	Standard	95% CI	95% CI

Term	Estimate	DF	Error	Low	High	VIF
Intercept	0.73	1	0.013	0.70	0.75	
A[1]	0.71	1	0.019	0.67	0.75	
A[2]	-0.052	1	0.019	-0.089	-0.014	
B[1]	-0.23	1	0.019	-0.26	-0.19	
B[2]	0.21	1	0.019	0.18	0.25	
C[1]	-0.083	1	0.019	-0.12	-0.046	
C[2]	1.154E-003	1	0.019	-0.036	0.039	
D-Dilution factor	-0.20	1	0.013	-0.23	-0.17	1.00
A[1]B[1]	-0.29	1	0.027	-0.34	-0.23	
A[2]B[1]	0.085	1	0.027	0.032	0.14	
A[1]B[2]	0.33	1	0.027	0.28	0.39	
A[2]B[2]	-0.13	1	0.027	-0.18	-0.074	
A[1]D	-0.20	1	0.019	-0.24	-0.16	
A[2]D	-0.047	1	0.019	-0.084	-9.737E-003	
B[1]D	0.023	1	0.019	-0.014	0.060	
B[2]D	2.173E-003	1	0.019	-0.035	0.040	
C[1]D	-0.042	1	0.019	-0.079	-4.237E-003	
C[2]D	-7.327E-003	1	0.019	-0.045	0.030	
A[1]B[1]D	0.074	1	0.027	0.021	0.13	
A[2]B[1]D	-0.031	1	0.027	-0.083	0.022	
A[1]B[2]D	0.081	1	0.027	0.028	0.13	
A[2]B[2]D	-0.081	1	0.027	-0.13	-0.029	

Final Equation in Terms of Coded Factors:

CFU (10^6) =
+0.73
+0.71 * A[1]
-0.052 * A[2]
-0.23 * B[1]
+0.21 * B[2]
-0.083 * C[1]
+1.154E-003 * C[2]
-0.20 * D
-0.29 * A[1]B[1]
+0.085 * A[2]B[1]
+0.33 * A[1]B[2]
-0.13 * A[2]B[2]
-0.20 * A[1]D
-0.047 * A[2]D
+0.023 * B[1]D
+2.173E-003 * B[2]D
-0.042 * C[1]D
-7.327E-003 * C[2]D
+0.074 * A[1]B[1]D

-0.031 * A[2]B[1]D
+0.081 * A[1]B[2]D
-0.081 * A[2]B[2]D

Diagnostics Case Statistics

Standard Order	Actual Value	Predicted Value	Residual	Leverage	Student Residual	Cook's Distance	Outlier t	Run Order
1	1.28	1.19	0.094	0.136	0.593	0.003	0.592	129
2	1.21	1.19	0.024	0.136	0.150	0.000	0.150	108
3	1.10	1.19	-0.086	0.136	-0.546	0.002	-0.544	109
4	0.60	0.75	-0.15	0.136	-0.932	0.006	-0.932	161
5	0.30	0.75	-0.45	0.136	-2.830	0.057	-2.905	155
6	1.00	0.75	0.25	0.136	1.598	0.018	1.607	42
7	0.019	-0.024	0.043	0.136	0.270	0.001	0.270	29
8	0.015	-0.024	0.039	0.136	0.245	0.000	0.244	94
9	0.017	-0.024	0.041	0.136	0.258	0.000	0.257	139
10	2.31	2.26	0.052	0.136	0.326	0.001	0.325	127
11	2.43	2.26	0.17	0.136	1.085	0.008	1.086	144
12	2.11	2.26	-0.15	0.136	-0.939	0.006	-0.939	86
13	1.00	1.05	-0.046	0.136	-0.293	0.001	-0.292	103
14	1.10	1.05	0.054	0.136	0.340	0.001	0.339	110
15	1.00	1.05	-0.046	0.136	-0.293	0.001	-0.292	98
16	0.024	-0.015	0.039	0.136	0.246	0.000	0.245	38
17	0.026	-0.015	0.041	0.136	0.258	0.000	0.258	95
18	0.025	-0.015	0.040	0.136	0.252	0.000	0.251	50
19	2.00	1.94	0.058	0.136	0.368	0.001	0.367	105
20	1.99	1.94	0.048	0.136	0.305	0.001	0.304	78
21	1.89	1.94	-0.052	0.136	-0.328	0.001	-0.327	67
22	1.00	0.85	0.15	0.136	0.966	0.007	0.965	73
23	0.90	0.85	0.053	0.136	0.333	0.001	0.332	113
24	0.50	0.85	-0.35	0.136	-2.198	0.034	-2.229	75
25	0.019	-0.025	0.044	0.136	0.276	0.001	0.275	150
26	1.000E-002	-0.025	0.035	0.136	0.219	0.000	0.218	100
27	0.018	-0.025	0.043	0.136	0.270	0.001	0.269	51
28	1.33	1.24	0.094	0.136	0.593	0.003	0.592	125
29	1.22	1.24	-0.016	0.136	-0.103	0.000	-0.103	111
30	1.11	1.24	-0.13	0.136	-0.799	0.005	-0.798	116
31	0.90	0.80	0.10	0.136	0.649	0.003	0.648	130
32	0.50	0.80	-0.30	0.136	-1.881	0.025	-1.899	19
33	1.10	0.80	0.30	0.136	1.915	0.026	1.933	2
34	0.020	0.026	-6.259E-003	0.136	-0.040	0.000	-0.039	63
35	0.016	0.026	-0.010	0.136	-0.065	0.000	-0.065	16
36	0.016	0.026	-0.010	0.136	-0.065	0.000	-0.065	3
37	2.35	2.31	0.042	0.136	0.263	0.000	0.262	37
38	2.40	2.31	0.092	0.136	0.579	0.002	0.578	72
39	2.11	2.31	-0.20	0.136	-1.256	0.011	-1.258	84

40	1.10	1.10	3.741E-003	0.136	0.024	0.000	0.024	35
41	1.12	1.10	0.024	0.136	0.150	0.000	0.150	158
42	1.11	1.10	0.014	0.136	0.087	0.000	0.087	10
43	0.025	0.035	-0.010	0.136	-0.064	0.000	-0.064	97
44	0.027	0.035	-8.148E-003	0.136	-0.052	0.000	-0.051	4
45	0.029	0.035	-6.148E-003	0.136	-0.039	0.000	-0.039	26
46	2.01	1.99	0.018	0.136	0.115	0.000	0.115	145
47	2.00	1.99	8.185E-003	0.136	0.052	0.000	0.052	49
48	1.90	1.99	-0.092	0.136	-0.581	0.002	-0.580	136
49	1.00	0.90	0.10	0.136	0.649	0.003	0.648	120
50	1.10	0.90	0.20	0.136	1.282	0.012	1.285	104
51	0.70	0.90	-0.20	0.136	-1.249	0.011	-1.251	6
52	0.022	0.025	-3.370E-003	0.136	-0.021	0.000	-0.021	44
53	0.012	0.025	-0.013	0.136	-0.085	0.000	-0.084	121
54	0.016	0.025	-9.370E-003	0.136	-0.059	0.000	-0.059	34
55	1.37	1.26	0.11	0.136	0.691	0.003	0.689	58
56	1.30	1.26	0.039	0.136	0.248	0.000	0.247	39
57	1.13	1.26	-0.13	0.136	-0.828	0.005	-0.827	92
58	1.00	0.82	0.18	0.136	1.127	0.009	1.128	156
59	0.50	0.82	-0.32	0.136	-2.037	0.030	-2.060	81
60	1.20	0.82	0.38	0.136	2.392	0.041	2.434	85
61	0.021	0.051	-0.030	0.136	-0.189	0.000	-0.188	64
62	0.018	0.051	-0.033	0.136	-0.208	0.000	-0.207	24
63	0.018	0.051	-0.033	0.136	-0.208	0.000	-0.207	118
64	2.42	2.33	0.087	0.136	0.550	0.002	0.549	65
65	2.45	2.33	0.12	0.136	0.740	0.004	0.739	123
66	2.12	2.33	-0.21	0.136	-1.348	0.013	-1.352	157
67	1.11	1.12	-0.011	0.136	-0.068	0.000	-0.068	30
68	1.13	1.12	9.185E-003	0.136	0.058	0.000	0.058	11
69	1.12	1.12	-8.148E-004	0.136	-0.005	0.000	-0.005	80
70	0.027	0.060	-0.033	0.136	-0.207	0.000	-0.206	126
71	0.027	0.060	-0.033	0.136	-0.207	0.000	-0.206	14
72	0.030	0.060	-0.030	0.136	-0.188	0.000	-0.187	82
73	2.10	2.02	0.084	0.136	0.529	0.002	0.528	77
74	2.03	2.02	0.014	0.136	0.086	0.000	0.086	128
75	1.93	2.02	-0.086	0.136	-0.546	0.002	-0.545	62
76	1.00	0.92	0.078	0.136	0.494	0.002	0.493	15
77	1.10	0.92	0.18	0.136	1.127	0.009	1.128	88
78	0.70	0.92	-0.22	0.136	-1.404	0.014	-1.409	162
79	0.024	0.050	-0.026	0.136	-0.164	0.000	-0.163	148
80	0.013	0.050	-0.037	0.136	-0.234	0.000	-0.233	55
81	0.018	0.050	-0.032	0.136	-0.202	0.000	-0.201	151
82	0.40	0.50	-0.098	0.136	-0.617	0.003	-0.616	57
83	0.50	0.50	2.469E-003	0.136	0.016	0.000	0.016	47
84	0.30	0.50	-0.20	0.136	-1.250	0.011	-1.252	5
85	0.000	0.15	-0.15	0.136	-0.969	0.007	-0.968	52

86	0.20	0.15	0.047	0.136	0.297	0.001	0.296	117
87	0.20	0.15	0.047	0.136	0.297	0.001	0.296	23
88	0.000	-0.058	0.058	0.136	0.367	0.001	0.366	149
89	0.000	-0.058	0.058	0.136	0.367	0.001	0.366	25
90	0.000	-0.058	0.058	0.136	0.367	0.001	0.366	1
91	1.40	1.54	-0.14	0.136	-0.898	0.006	-0.898	8
92	1.80	1.54	0.26	0.136	1.632	0.019	1.642	138
93	1.30	1.54	-0.24	0.136	-1.531	0.017	-1.538	56
94	0.000	0.31	-0.31	0.136	-1.953	0.027	-1.973	20
95	0.70	0.31	0.39	0.136	2.476	0.044	2.523	115
96	0.30	0.31	-8.642E-003	0.136	-0.055	0.000	-0.054	9
97	0.100	-2.469E-003	0.10	0.136	0.648	0.003	0.647	96
98	0.20	-2.469E-003	0.20	0.136	1.281	0.012	1.284	33
99	0.000	-2.469E-003	2.469E-003	0.136	0.016	0.000	0.016	147
100	0.40	0.70	-0.30	0.136	-1.882	0.025	-1.900	32
101	0.40	0.70	-0.30	0.136	-1.882	0.025	-1.900	31
102	1.00	0.70	0.30	0.136	1.914	0.026	1.932	140
103	0.50	0.44	0.058	0.136	0.367	0.001	0.366	59
104	0.20	0.44	-0.24	0.136	-1.531	0.017	-1.538	17
105	0.60	0.44	0.16	0.136	1.000	0.007	1.000	53
106	0.100	0.020	0.080	0.136	0.508	0.002	0.506	153
107	0.100	0.020	0.080	0.136	0.508	0.002	0.506	89
108	0.100	0.020	0.080	0.136	0.508	0.002	0.506	124
109	0.90	0.62	0.28	0.136	1.796	0.023	1.811	13
110	0.50	0.62	-0.12	0.136	-0.734	0.004	-0.733	143
111	0.60	0.62	-0.016	0.136	-0.102	0.000	-0.101	36
112	0.100	0.27	-0.17	0.136	-1.086	0.008	-1.086	28
113	0.50	0.27	0.23	0.136	1.445	0.015	1.451	61
114	0.100	0.27	-0.17	0.136	-1.086	0.008	-1.086	87
115	0.20	0.060	0.14	0.136	0.883	0.006	0.882	46
116	0.000	0.060	-0.060	0.136	-0.383	0.001	-0.382	48
117	0.000	0.060	-0.060	0.136	-0.383	0.001	-0.382	12
118	1.70	1.66	0.040	0.136	0.250	0.000	0.249	106
119	1.90	1.66	0.24	0.136	1.515	0.016	1.522	142
120	1.50	1.66	-0.16	0.136	-1.015	0.007	-1.016	112
121	0.100	0.43	-0.33	0.136	-2.070	0.031	-2.095	99
122	0.50	0.43	0.073	0.136	0.461	0.002	0.460	160
123	0.50	0.43	0.073	0.136	0.461	0.002	0.460	102
124	0.100	0.12	-0.016	0.136	-0.102	0.000	-0.101	91
125	0.20	0.12	0.084	0.136	0.531	0.002	0.530	21
126	0.000	0.12	-0.12	0.136	-0.734	0.004	-0.733	7
127	0.50	0.82	-0.32	0.136	-2.000	0.029	-2.021	141
128	0.90	0.82	0.084	0.136	0.531	0.002	0.530	159
129	1.20	0.82	0.38	0.136	2.429	0.042	2.473	83
130	0.70	0.56	0.14	0.136	0.883	0.006	0.882	27
131	0.30	0.56	-0.26	0.136	-1.648	0.019	-1.658	54

132	0.70	0.56	0.14	0.136	0.883	0.006	0.882	154
133	0.20	0.14	0.062	0.136	0.391	0.001	0.389	68
134	0.000	0.14	-0.14	0.136	-0.875	0.005	-0.874	40
135	0.100	0.14	-0.038	0.136	-0.242	0.000	-0.241	18
136	1.10	0.75	0.35	0.136	2.195	0.034	2.226	93
137	0.60	0.75	-0.15	0.136	-0.969	0.007	-0.968	90
138	0.70	0.75	-0.053	0.136	-0.336	0.001	-0.335	133
139	0.30	0.41	-0.11	0.136	-0.687	0.003	-0.686	71
140	0.60	0.41	0.19	0.136	1.211	0.010	1.213	134
141	0.50	0.41	0.091	0.136	0.578	0.002	0.577	131
142	0.20	0.20	2.469E-003	0.136	0.016	0.000	0.016	69
143	0.20	0.20	2.469E-003	0.136	0.016	0.000	0.016	132
144	0.000	0.20	-0.20	0.136	-1.250	0.011	-1.252	45
145	1.80	1.80	2.469E-003	0.136	0.016	0.000	0.016	135
146	2.00	1.80	0.20	0.136	1.281	0.012	1.284	60
147	1.60	1.80	-0.20	0.136	-1.250	0.011	-1.252	122
148	0.30	0.56	-0.26	0.136	-1.672	0.020	-1.682	137
149	0.90	0.56	0.34	0.136	2.125	0.032	2.152	79
150	0.60	0.56	0.036	0.136	0.227	0.000	0.226	66
151	0.100	0.25	-0.15	0.136	-0.969	0.007	-0.968	107
152	0.30	0.25	0.047	0.136	0.297	0.001	0.296	119
153	0.100	0.25	-0.15	0.136	-0.969	0.007	-0.968	101
154	0.60	0.95	-0.35	0.136	-2.234	0.036	-2.267	74
155	1.10	0.95	0.15	0.136	0.929	0.006	0.929	146
156	1.30	0.95	0.35	0.136	2.195	0.034	2.226	43
157	0.70	0.70	2.469E-003	0.136	0.016	0.000	0.016	76
158	0.90	0.70	0.20	0.136	1.281	0.012	1.284	41
159	0.50	0.70	-0.20	0.136	-1.250	0.011	-1.252	114
160	0.20	0.28	-0.075	0.136	-0.476	0.002	-0.475	152
161	0.20	0.28	-0.075	0.136	-0.476	0.002	-0.475	22
162	0.30	0.28	0.025	0.136	0.156	0.000	0.156	70

Proceed to Diagnostic Plots (the next icon in progression). Be sure to look at the:

1) Normal probability plot of the studentized residuals to check for normality of residuals.
2) Studentized residuals versus predicted values to check for constant error.
3) Outlier t versus run order to look for outliers, i.e., influential values.
4) Box-Cox plot for power transformations.

Response: pH
ANOVA for Selected Factorial Model
Analysis of variance table [Partial sum of squares]

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	59.25	19	3.12	78.44	< 0.0001	significant
B	*0.40*	*1*	*0.40*	*10.12*	*0.0019*	
C	*57.24*	*9*	*6.36*	*159.97*	*< 0.0001*	
BC	*1.61*	*9*	*0.18*	*4.51*	*< 0.0001*	
Residual	3.98	100	0.040			
Lack of Fit	*1.09*	*40*	*0.027*	*0.57*	*0.9703*	*not significant*
Pure Error	*2.88*	*60*	*0.048*			
Cor Total	63.23	119				

The Model F-value of 78.44 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant.
In this case B, C, BC are significant model terms.
Values greater than 0.1000 indicate the model terms are not significant.
If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The "Lack of Fit F-value" of 0.57 implies the Lack of Fit is not significant relative to the pure error. There is a 97.03% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit.

Std. Dev.	0.20	R-Squared	0.9371
Mean	5.33	Adj R-Squared	0.9252
C.V.	3.74	Pred R-Squared	0.9095
PRESS	5.72	Adeq Precision	29.812

The "Pred R-Squared" of 0.9095 is in reasonable agreement with the "Adj R-Squared" of 0.9252.
"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 29.812 indicates an adequate signal. This model can be used to navigate the design space.

Term	Coefficient Estimate	DF	Standard Error	95% CI Low	95% CI High	VIF
Intercept	5.33	1	0.018	5.29	5.36	
B-Temperature	-0.058	1	0.018	-0.094	-0.022	1.00
C[1]	1.61	1	0.055	1.51	1.72	
C[2]	0.68	1	0.055	0.57	0.79	
C[3]	0.10	1	0.055	-4.918E-003	0.21	
C[4]	-9.917E-003	1	0.055	-0.12	0.098	
C[5]	-0.29	1	0.055	-0.40	-0.18	

C[6]	-0.44	1	0.055	-0.55	-0.33
C[7]	-0.80	1	0.055	-0.91	-0.69
C[8]	-0.80	1	0.055	-0.91	-0.69
C[9]	-0.29	1	0.055	-0.40	-0.18
BC[1]	0.066	1	0.055	-0.042	0.17
BC[2]	-0.12	1	0.055	-0.23	-0.016
BC[3]	-0.033	1	0.055	-0.14	0.075
BC[4]	3.750E-003	1	0.055	-0.10	0.11
BC[5]	-0.13	1	0.055	-0.24	-0.020
BC[6]	6.250E-003	1	0.055	-0.10	0.11
BC[7]	0.063	1	0.055	-0.045	0.17
BC[8]	0.060	1	0.055	-0.049	0.17
BC[9]	-0.16	1	0.055	-0.27	-0.055

Final Equation in Terms of Coded Factors:

pH	=
+5.33	
-0.058	* B
+1.61	* C[1]
+0.68	* C[2]
+0.10	* C[3]
-9.917E-003	* C[4]
-0.29	* C[5]
-0.44	* C[6]
-0.80	* C[7]
-0.80	* C[8]
-0.29	* C[9]
+0.066	* BC[1]
-0.12	* BC[2]
-0.033	* BC[3]
+3.750E-003	* BC[4]
-0.13	* BC[5]
+6.250E-003	* BC[6]
+0.063	* BC[7]
+0.060	* BC[8]
-0.16	* BC[9]

Diagnostics Case Statistics

Standard Order	Actual Value	Predicted Value	Residual	Leverage	Student Residual	Cook's Distance	Outlier t	Run Order
1	6.92	6.93	-0.012	0.167	-0.064	0.000	-0.064	79
2	6.90	6.93	-0.032	0.167	-0.174	0.000	-0.173	48
3	6.96	6.93	0.028	0.167	0.156	0.000	0.155	46
4	6.93	6.93	-1.667E-003	0.167	-0.009	0.000	-0.009	76
5	6.99	6.93	0.058	0.167	0.320	0.001	0.319	8

6	6.89	6.93	-0.042	0.167	-0.229	0.001	-0.228	100
7	6.98	6.95	0.032	0.167	0.174	0.000	0.173	14
8	6.95	6.95	1.667E-003	0.167	0.009	0.000	0.009	27
9	6.89	6.95	-0.058	0.167	-0.320	0.001	-0.319	26
10	6.98	6.95	0.032	0.167	0.174	0.000	0.173	17
11	6.92	6.95	-0.028	0.167	-0.156	0.000	-0.155	22
12	6.97	6.95	0.022	0.167	0.119	0.000	0.118	78
13	6.65	6.19	0.46	0.167	2.527	0.064	2.599	4
14	5.95	6.19	-0.24	0.167	-1.319	0.017	-1.324	69
15	5.81	6.19	-0.38	0.167	-2.088	0.044	-2.124	6
16	6.29	6.19	0.100	0.167	0.549	0.003	0.547	114
17	6.19	6.19	0.000	0.167	0.000	0.000	0.000	65
18	6.25	6.19	0.060	0.167	0.330	0.001	0.328	95
19	5.70	5.83	-0.13	0.167	-0.687	0.005	-0.685	105
20	5.64	5.83	-0.19	0.167	-1.016	0.010	-1.017	45
21	5.69	5.83	-0.14	0.167	-0.742	0.006	-0.740	59
22	5.77	5.83	-0.055	0.167	-0.302	0.001	-0.301	37
23	6.49	5.83	0.66	0.167	3.654	0.133	3.905 *	87
24	5.66	5.83	-0.17	0.167	-0.907	0.008	-0.906	50
25	6.17	5.52	0.65	0.167	3.571	0.128	3.804 *	43
26	5.21	5.52	-0.31	0.167	-1.703	0.029	-1.720	2
27	5.16	5.52	-0.36	0.167	-1.978	0.039	-2.008	57
28	5.37	5.52	-0.15	0.167	-0.824	0.007	-0.823	35
29	5.79	5.52	0.27	0.167	1.483	0.022	1.492	106
30	5.42	5.52	-0.10	0.167	-0.549	0.003	-0.547	81
31	5.61	5.34	0.27	0.167	1.493	0.022	1.502	25
32	5.55	5.34	0.21	0.167	1.163	0.014	1.165	20
33	5.09	5.34	-0.25	0.167	-1.364	0.019	-1.370	83
34	5.26	5.34	-0.078	0.167	-0.430	0.002	-0.429	16
35	5.34	5.34	1.667E-003	0.167	0.009	0.000	0.009	103
36	5.18	5.34	-0.16	0.167	-0.870	0.008	-0.869	40
37	5.43	5.37	0.060	0.167	0.330	0.001	0.328	62
38	5.16	5.37	-0.21	0.167	-1.154	0.013	-1.156	108
39	5.62	5.37	0.25	0.167	1.374	0.019	1.380	77
40	5.16	5.37	-0.21	0.167	-1.154	0.013	-1.156	33
41	5.58	5.37	0.21	0.167	1.154	0.013	1.156	112
42	5.27	5.37	-0.10	0.167	-0.549	0.003	-0.547	34
43	5.31	5.26	0.048	0.167	0.266	0.001	0.264	21
44	5.40	5.26	0.14	0.167	0.760	0.006	0.758	24
45	5.21	5.26	-0.052	0.167	-0.284	0.001	-0.283	60
46	5.17	5.26	-0.092	0.167	-0.504	0.003	-0.502	96
47	5.29	5.26	0.028	0.167	0.156	0.000	0.155	38
48	5.19	5.26	-0.072	0.167	-0.394	0.002	-0.392	18
49	4.91	5.22	-0.31	0.167	-1.721	0.030	-1.739	109
50	5.27	5.22	0.047	0.167	0.256	0.001	0.255	3
51	5.47	5.22	0.25	0.167	1.355	0.018	1.361	85

52	5.15	5.22	-0.073	0.167	-0.403	0.002	-0.401	71
53	5.47	5.22	0.25	0.167	1.355	0.018	1.361	91
54	5.07	5.22	-0.15	0.167	-0.842	0.007	-0.841	93
55	4.59	4.85	-0.26	0.167	-1.438	0.021	-1.445	107
56	5.00	4.85	0.15	0.167	0.815	0.007	0.814	23
57	4.93	4.85	0.078	0.167	0.430	0.002	0.429	82
58	4.89	4.85	0.038	0.167	0.211	0.000	0.210	94
59	4.91	4.85	0.058	0.167	0.320	0.001	0.319	73
60	4.79	4.85	-0.062	0.167	-0.339	0.001	-0.337	19
61	4.75	4.94	-0.19	0.167	-1.044	0.011	-1.044	36
62	4.98	4.94	0.040	0.167	0.220	0.000	0.219	53
63	5.13	4.94	0.19	0.167	1.044	0.011	1.044	49
64	4.86	4.94	-0.080	0.167	-0.440	0.002	-0.438	54
65	5.09	4.94	0.15	0.167	0.824	0.007	0.823	15
66	4.83	4.94	-0.11	0.167	-0.604	0.004	-0.602	51
67	4.57	4.84	-0.27	0.167	-1.465	0.021	-1.474	74
68	5.03	4.84	0.19	0.167	1.062	0.011	1.063	67
69	4.87	4.84	0.033	0.167	0.183	0.000	0.182	29
70	4.85	4.84	0.013	0.167	0.073	0.000	0.073	98
71	4.90	4.84	0.063	0.167	0.348	0.001	0.346	7
72	4.80	4.84	-0.037	0.167	-0.201	0.000	-0.200	5
73	4.51	4.52	-0.013	0.167	-0.073	0.000	-0.073	80
74	4.47	4.52	-0.053	0.167	-0.293	0.001	-0.292	119
75	4.64	4.52	0.12	0.167	0.641	0.004	0.639	116
76	4.43	4.52	-0.093	0.167	-0.513	0.003	-0.511	102
77	4.71	4.52	0.19	0.167	1.026	0.011	1.026	42
78	4.38	4.52	-0.14	0.167	-0.787	0.006	-0.786	11
79	4.41	4.53	-0.12	0.167	-0.678	0.005	-0.676	39
80	4.76	4.53	0.23	0.167	1.245	0.016	1.249	72
81	4.49	4.53	-0.043	0.167	-0.238	0.001	-0.237	68
82	4.52	4.53	-0.013	0.167	-0.073	0.000	-0.073	64
83	4.63	4.53	0.097	0.167	0.531	0.003	0.529	101
84	4.39	4.53	-0.14	0.167	-0.787	0.006	-0.786	110
85	4.50	4.52	-0.022	0.167	-0.119	0.000	-0.118	88
86	4.47	4.52	-0.052	0.167	-0.284	0.001	-0.283	1
87	4.65	4.52	0.13	0.167	0.705	0.005	0.703	41
88	4.44	4.52	-0.082	0.167	-0.449	0.002	-0.447	12
89	4.70	4.52	0.18	0.167	0.980	0.010	0.980	90
90	4.37	4.52	-0.15	0.167	-0.833	0.007	-0.832	47
91	4.39	4.53	-0.14	0.167	-0.742	0.006	-0.740	30
92	4.75	4.53	0.22	0.167	1.236	0.015	1.239	99
93	4.49	4.53	-0.035	0.167	-0.192	0.000	-0.191	32
94	4.51	4.53	-0.015	0.167	-0.082	0.000	-0.082	28
95	4.62	4.53	0.095	0.167	0.522	0.003	0.520	84
96	4.39	4.53	-0.14	0.167	-0.742	0.006	-0.740	92
97	4.90	5.26	-0.36	0.167	-1.969	0.039	-1.998	9

98	5.37	5.26	0.11	0.167	0.613	0.004	0.612	104
99	5.45	5.26	0.19	0.167	1.053	0.011	1.054	63
100	5.05	5.26	-0.21	0.167	-1.145	0.013	-1.146	97
101	5.57	5.26	0.31	0.167	1.712	0.029	1.729	13
102	5.21	5.26	-0.048	0.167	-0.266	0.001	-0.264	56
103	4.60	4.82	-0.22	0.167	-1.181	0.014	-1.184	66
104	5.09	4.82	0.27	0.167	1.511	0.023	1.521	113
105	4.83	4.82	0.015	0.167	0.082	0.000	0.082	58
106	4.79	4.82	-0.025	0.167	-0.137	0.000	-0.137	120
107	4.71	4.82	-0.11	0.167	-0.577	0.003	-0.575	10
108	4.87	4.82	0.055	0.167	0.302	0.001	0.301	75
109	5.33	5.36	-0.028	0.167	-0.156	0.000	-0.155	31
110	5.36	5.36	1.667E-003	0.167	0.009	0.000	0.009	70
111	5.52	5.36	0.16	0.167	0.888	0.008	0.887	61
112	5.12	5.36	-0.24	0.167	-1.309	0.017	-1.314	44
113	5.58	5.36	0.22	0.167	1.218	0.015	1.221	111
114	5.24	5.36	-0.12	0.167	-0.650	0.004	-0.648	55
115	5.59	5.74	-0.15	0.167	-0.842	0.007	-0.841	52
116	5.40	5.74	-0.34	0.167	-1.886	0.036	-1.911	117
117	5.80	5.74	0.057	0.167	0.311	0.001	0.310	115
118	5.77	5.74	0.027	0.167	0.147	0.000	0.146	86
119	5.91	5.74	0.17	0.167	0.916	0.008	0.915	89
120	5.99	5.74	0.25	0.167	1.355	0.018	1.361	118

* Case(s) with |Outlier T| > 3.50

Proceed to Diagnostic Plots (the next icon in progression). Be sure to look at the:

1) Normal probability plot of the studentized residuals to check for normality of residuals.
2) Studentized residuals versus predicted values to check for constant error.
3) Outlier t versus run order to look for outliers, i.e., influential values.
4) Box-Cox plot for power transformations.

If all the model statistics and diagnostic plots are OK, finish up with the Model Graphs icon.

THE END

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