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COLLEGE OF SCIENCE

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THE APPLICATION OF STARTER CULTURES IN THE FERMENTATION OF *PITO* TOWARDS INDUSTRIAL PRODUCTION

by

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A Thesis submitted to the Department of Food Science and Technology,

College of Science

in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

FEBRUARY 2018

DECLARATION

I hereby declare that this submission is my own work towards the PhD and that, to the best of my knowledge, it contains no material previously published by another person, nor material which has been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the text.

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ABSTRACT

Pito is a popular traditional sour sorghum beer widely consumed in Ghana and Nigeria. It is brewed mostly by women at an artisanal level but has huge economic potential for industrial production and commercialization. The brewing methods are not standardized and vary according to the ethnic group of the processor or the tribal area where it is brewed. The brewing processes are tedious and uncontrolled resulting in inconsistent product quality. There are two fermentation steps involved in the *pito* brewing process; an initial spontaneous lactic acid fermentation (souring) by lactic acid bacteria which come with the sorghum grains from the field and from the brewing environment followed by an inoculated alcoholic fermentation. The product is drunk while still fermenting and has a limited shelf life of 2 to 3 days. In this study, the fermentation performance of two commercial lactic acid bacteria, *L. delbrueckii* and *L. amylolyticus* and two commercial brewers' yeast strains of *S. cerevisiae*, Anchor Brewers' yeast and Munich Wheat Beer yeast as single strain starter cultures in *pito* wort were investigated using the Response Surface Methodology. The optimum fermentation conditions for their application in industrial production of *pito* were determined to be 12 h at 45°C for *L. delbrueckii*, 19 h at 45°C for *L. amylolyticus*, 71.6 h at 22.6°C for Munich Wheat Beer Yeast and 71.5 h for Anchor Brewer's Yeast at 24°C. Both lactic acid bacteria and and yeasts were found capable of achieving the desired end product characteristics of *pito*. *L. delbrueckii* and Anchor Sorghum Beer yeast were however selected preferentially on account of the economic

advantages of their use over the other two for industrial production. The fermentation profiles of the experimental *pito* brew fermented with pure single strain starter cultures of *L. delbrueckii* and Anchor Brewers' yeast using the derived optimal fermentation conditions was evaluated alongside those of a *pito* brew fermented using the traditional process. Lactic acid formation, pH change and extract utilization with time were monitored. Both brews followed the general lactic acid and alcoholic fermentation profiles but differences were observed which were on account of the intrinsic specific characteristics and capabilities of the microorganism to utilize the wort substrates and convert them into the fermentation products. Similar levels of sourness as indicated by pH and lactic acid content were achieved in both brews. The starter culture brew had a lower apparent degree of fermentation and lower alcohol level than the traditionally fermented brew. The product quality from the two optimized fermentation processes was also evaluated based on physicochemical analysis, shelf life, volatile fermentation by-products and consumer acceptance sensory evaluation. The *pito* brewed with the starter cultures compared favourably with *pito* brewed with the traditional process. Both had physicochemical analytical values within the range established for traditional *pito*. There was an improvement of shelf life of 2 days in *pito* brewed with pure single strain starter cultures of *L. delbrueckii* and Anchor Brewer's Yeast over traditionally brewed *pito*. The total level of volatile aroma compounds formed in the *pito* brewed with the starter cultures was higher (353.13 mg/l) than in the *pito* brewed with the traditional process (229.04 mg/l). The traditionally brewed

pito was characterized by higher levels of ethyl acetate and iso-amylalcohol while the pure single strain culture brew was characterized by higher levels of n-propanol, i-butanol and acetaldehyde. In the consumer acceptance sensory evaluation, there was no significant difference between the two *pito* products for overall liking and taste liking. There was however a statistical significant difference between them for aroma liking. The fermentation process became predictable and controlled through the application of starter cultures and provided a basis for standardization of the fermentation process towards consistency in product quality and industrial production.

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ACKNOWLEDGEMENT

There are many who provided invaluable assistance in the course of carrying out this work and to whom I owe a debt of gratitude. Foremost among them are my supervisors, Prof. W. O. Ellis, Prof. I. Oduro, and Prof. F. K. Saalia. I thank them for their guidance, advice and encouragement.

Many thanks go to the management of Food Research Institute in whose Sorghum Laboratory I carried out most of my laboratory work, to my brewing colleague and friend, Fash Sawyerr who was always available to share ideas, to Premium Foods Ltd for providing financial assistance and to Gregory Komlaga for his support since we both embarked on our studies.

Finally, I wish to express my profound appreciation to my family who have always been behind me and to my father, the source of inspiration and stimulation for the doctorial studies.

CHAPTER ONE

INTRODUCTION

1.1 Background

Pito is a popular traditional sour sorghum beer. It is widely consumed in Ghana and Nigeria and generally contains 3% alcohol (Egwim *et al.*, 2013). It is brewed from malted sorghum and is one of the wide varieties of indigenous African beers collectively called opaque or sorghum beer (Daiber and Taylor, 1995). It was originally a traditional drink of the people from the sorghum growing areas of West Africa but has become a popular drink all over the region, where it comes under various ethnic names, e.g. *pito* (Ghana and Nigeria), *dolo* (Burkina Faso, Niger, Mali and Ivory Coast) and *chapalo* (Togo and Benin). *Pito* is brewed mostly by women at an artisanal level (Sefa-Dedeh *et al*. 1999). It is a major income generating activity with a large local raw material base, i.e. local sorghum cultivated by subsistence farmers has a huge economic potential for industrial production and commercialization (Glover *et al.*, 2005). The brewing methods are varied according to the ethnic group or socio-cultural area. In Ghana, some of the popular *pito* types are the *Dagarti pito, Frafra pito, Kusasi pito, Kasena pito* and *Grushie pito* (UNIDO, 2007). *Pito* is sold at the premises where it is brewed and then served from traditional earthenware pots or by hawking where it is packaged in plastic bottles. It is consumed while still fermenting and has a limited shelf life of 2 days (Ellis *et al.*, 2005).

The biochemical processes involved in the production of *pito* and the production units of operation and sequences are basically the same as for the production of industrial commercial lager beers brewed from barley malt (Daiber and Taylor, 1995; Onyenekwe *et al.,* 2016). Both processes involve malting of the grain to develop amylolytic and proteolytic enzymes. The milled malt is mashed with water to make an infusion which is heated and held at specific temperatures at which the enzymes are active. The malt starch is converted into sugars for fermentation into alcohol (Djameh, 2010). There are two fermentation step involved in the *pito* brewing process; lactic acid fermentation (souring) followed by an alcoholic fermentation (Haggblade and Holzapfel, 1989a; Maoura and Pourquie, 2009; Ellis *et al.,* 2005). The lactic acid fermentation is carried out spontaneously by lactic acid bacteria which come with the sorghum grains from the field and from the brewing environment. The alcoholic fermentation is carried out by numerous types of yeasts and other microflora by back-slopping inoculation. A considerable amount of work has been done to identify and characterize the processes of *pito* production (Bansah, 1990; Sefa-Dedeh and Asante, 1998; Onaghise and Izuagbe, 2004). Work has also been done to identify the yeasts and other micro flora involved in the fermentation of *pito* (Demuyakor and Ohta, 1991; Sefa-Dedeh *et al.,* 1999) and on the microbiological safety of *pito* (Kolawole *et al.,* 2007). Very little however, has been done on improving the production processes towards industrial production despite the fact that a high proportion of the sorghum cultivated in Ghana goes into *pito* production (Awuni *et al.,*

2013; FRI, 2003). Out of the total of 239580 tons of sorghum consumed in 2005, 159,720 tons (66%) were used for the production of *pito* (Chopra, 2006). Towards improving the production processes towards scaling up to industrial level, a study was carried out by Djameh (2010) to improve the efficiency of the malting and mashing processes of *pito* brewing. The use of starter cultures has been proposed as a suitable approach to improving the African traditional fermented foods and beverages (Kirmayo *et al.,* 2002; Holzapfel, 2002). The use of suitable starter cultures improves the fermentation process, facilitates control over the initial phase of fermentation and the predictability of derivative products (Holzapfel, 2002). Jespersen (2003) and Nummer (1996) opined that microorganism for use as starter cultures must be isolated from the food in which it is intended to be used having been adapted to that environment. Studies have been carried out to investigate the quality of traditional sorghum beers fermented with starter cultures isolated from them (Coulibaly *et al.,* 2016; Lyumugabe *et al.,* 2014, Glover *et al.,* 2009; Ogunbanwo *et al.,* 2013; Oriji *et al.,* 2003) but not with commercial starter cultures that are used in industrial fermentation of beers. Sorghum, the cereal used in brewing *pito* however, depending on the variety, may have high polyphenol content which renders some of the processing methods incompatible with those of the standard methods of brewing with barley malt. Polymeric condensed tannins have been found to inhibit enzymes in the malt of bird-resistant sorghum and also impair lactic acid fermentation (Daiber, 1978). The commercial lactic acid bacteria and yeasts used in industrial

brewing of barley malt beers may not yield the same technological performance in sorghum wort on account of the inhibitory characteristics of the polyphenols. Towards industrial production of *pito*, this peculiarity of sorghum necessitates investigations into the application of commercial starter cultures in the fermentation process. In Southern Africa, in particular in the Republic of South Africa, Zimbabwe and Botswana, the production of indigenous sorghum malt beer *chibuku,* has become highly industrialized through the work of the Sorghum Beer Unit of South African Council for Scientific and Industrial Research (Haggblade and Holzapfel, 1989a).

1.2 Problem Statement

Pito brewing has remained at the artisanal level over the years with the processes remaining tedious, inefficient, not standardized, uncontrolled and unpredictable resulting in inconsistent product quality with very wide variations and a short shelf life. Inconsistency of product quality undermines the trust consumers have in a food product gives a negative perception on its safety. The lack of a controlled process for the fermentation step that will ensure consistent product quality and improve shelf life is a gap that must be given serious attention to enable scaling up of production to an industrial level.

1.3 Justification

Upscaling *pito* brewing from artisanal to industrial level will generate increased demand and market for sorghum and contribute towards the transformation of sorghum from a subsistence crop to an industrial raw material. Sorghum is still largely a subsistence crop, but is increasingly

becoming the foundation for successful food and beverage industries (Taylor, 2003). Industrially produced and packaged *pito* with extended shelf-life will increase its patronage. Product safety concerns which often deter potential consumers from drinking *pito* will be eliminated. The product will become available in shops using the usual distribution channels for existing commercial beers. *Pito* brewing is a key off-farm economic activity that provides income for the numerous households in Northern Ghana (PSI-Sorghum, 2006). The consequent increased production of sorghum will support food security and improve the incomes and livelihood of sorghum farmers. The industrial production of the *pito* beer would create employment opportunities and incomes for brewers and auxillary units in the beer value chain. In 2010, through a public-private –partnership project that developed a sorghum supply chain to two breweries, the average annual net income for sorghum farmers in Ghana increased from US$60 to US$110 per farming household in Ghana (EUCORD, 2010).

1.4 Aim

The aim of the research study was to investigate the effects of commercial starter cultures of lactic acid bacteria and brewers' yeasts on the fermentation characteristics and quality of *pito.*

1.5 Specific Objectives

1. To investigate the souring capacity and optimum fermentation conditions of single strain starter cultures of commercial lactic acid bacteria in *pito* wort.

2. To investigate the alcoholic fermentation capacity and optimum fermentation conditions of single strain commercial starter cultures of brewers' yeast, *Saccharomyces cerevisiae* in *pito* wort.

3. To evaluate the lactic acid fermentation (souring) and alcoholic fermentation profiles of *pito* wort fermented with single strain commercial starter cultures of lactic acid bacteria and brewers' yeast, *Saccharomyces cerevisiae* relative to those of *pito* wort fermented with the traditional spontaneous process as control.

4. To evaluate of the quality of pito brewed under conditions experimentally derived from the study with commercial starter cultures relative to *pito* brewed with the traditional process.

1.6 Outcomes

A controlled and predictable fermentation process for *pito* brewing that will achieve consistency in product quality and also enhance the product quality and shelf life over products from the traditional brewing process would be the outcome of the study. The study was also to contribute technological knowledge towards the scaling up of *pito* brewing from an artisanal to an industrial level.

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview of Traditional Sorghum Beers

Traditional sorghum beers or opaque beers are brewed from sorghum malt and sometimes together with starchy adjuncts such as maize and millets. Beers are the products of alcoholic fermentation of malted cereals in which sugars are converted into alcohol and carbon dioxide by yeasts following the EMP pathway. (Buglass, 2011). Fermented beverages have been produced by different human cultures for centuries Alba-Lois and Segal-Kischinevzky, 2010). Fermentation can be described as a desirable process of biochemical modification of primary food products brought about by microorganisms and their enzymes. These microorganisms are associated with raw material and/or utensils, or they may be added as starter cultures (Tamminen et al., 2004). The flavour and aroma compounds of the beer are metabolic by-products from yeast which are formed during fermentation and are passed into the beer. Yeast strain can affect the rate of fermentation, the efficiency and success of conversion of sugar to ethanol, and the nature and quantity of by-products.

Sorghum grain is the major cereal crop used to produce the traditional "opaque" beers (Asiedu, 1991). These beers are generally known by their vernacular names such as *ikigage* in Rwanda, *choukoutou* in Benin and Togo, *dolo* in Burkina-Faso, *pito* and *brukutu* in Nigeria and Ghana, *tchapalo* in Ivory Coast *amgba* in Cameroon, *doro* or *chibuku* in Zimbabwe, *merissa* in Sudan, *mtama* in Tanzania, *bili bili* in Chad and *kaffir* beer in South Africa

(Solange *et al.*, 2014). The traditional African beers are consumed while still fermenting, and the drink contains large amounts of fragments of insoluble materials (Rooney and Serna-Saldivar, 1991).

The processing methods of African traditional sorghum beer essentially involves malting, drying, milling, souring, boiling, mashing and fermentation, but variations may occur depending on the geographic localization (Haggblade and Holzapfel, 2004). There are two types of fermentation involved; lactic acid fermentation and alcoholic fermentation. The lactic acid fermentation or souring produces lactic acid which imparts the beer with its characteristic sour taste. It is carried out by a complex population of environmental microorganisms. The alcoholic fermentation is usually initiated by inoculating the soured wort with a portion of previous brew or dried yeast harvested from a previous fermented beverage. The microorganisms usually involved in the fermentations are mainly lactic acid bacteria and yeasts (Solange *et al.*, 2014). Lactic acid bacteria lower the pH of the beer which slows down the rate of microbial spoilage and inhibits the growth of pathogenic microorganism (Dendy, 1995). Two types of souring are distinguished, spontaneous and inoculated. The presence of lactic acid bacteria which form part of the natural microflora of the sorghum malt is responsible for the spontaneous souring. In inoculated souring a portion of the previous sour which contains a high concentration of viable lactic acid bacteria is used to inoculate the new sour. Spontaneous fermentation typically results from the competitive activities of different microorganisms; strains best adapted and with the highest growth rate

will dominate during particular stages of the process (Solange *et al.*, 2014). *Lactobacillus delbrueckii* is the dominant thermophilic bacterium active in sorghum beer production (Haggblade and Holzapfel, 1989a). At the end of the souring the pH lies between 3.0 and 3.5. A general physicochemical analytical profile of *pito* is shown in Table 1.

Table 1: Physical and Chemical Analytical Profile of *Pito* Courtesy UNIDO (2007)

PARAMETER	RANGE	AVERAGE
Original Extract, % w/w	8.0 – 14	11.0
Apparent Extract, %w/w	2.0 – 7.0	5.0
Real Extract, % w/w	3.0 – 8.0	6.0
Alcohol, vol%	2.0 -5.0	3.0
Ph	3.1 – 3.8	3.6
Total Titrable Acid as Lactic Acid, %	0.4 – 0.9	0.54
Colour, EBC	15 – 80	40
Turbidity, FTU	220 – 950	360

Several studies into the microbiological and biochemical characteristics of traditional sorghum beers as well as their technologies have been carried out and documented in different African countries (Chamunorwa *et al.*, 2002; Maoura *et al.*, 2005). A very varied yeast and lactic acid bacteria flora has been found in African sorghum beers, although *Saccharomyces cerevisiae* and heterofermentative *lactobacillus* usually predominate (Novellie, 1976; Sefa-Dedeh *et al*., 1999; Chamunorwa *et al*., 2002; Maoura *et al.*, 2005; Kayode *et al*., 2007; Lyumugabe *et al*., 2010).

2.2 Overview of *Pito* Brewing Process in Ghana

The brewing process for *pito* in Ghana involves malting of the grain, milling the malt, mashing, filtration and fermentation (Figure 1). There are variations to the process depending on the recipe handed down from the ancestors, type of grain, utensils available and the final nature of the end-product, e.g. taste, alcoholic content, viscosity, clarity. The brewing methods are varied according to the ethnic group or socio-cultural area (Djameh, 2010). In Ghana, some of the popular *pito* types are the *Dagarti pito*, *Frafra pito*, *Kusasi pito*, *Kasena pito* and *Grushie pito*. Unfermented *pito* and slightly fermented *pito* are consumed as food and are sources of nutrition for the inhabitants of the traditional *pito* brewing areas.

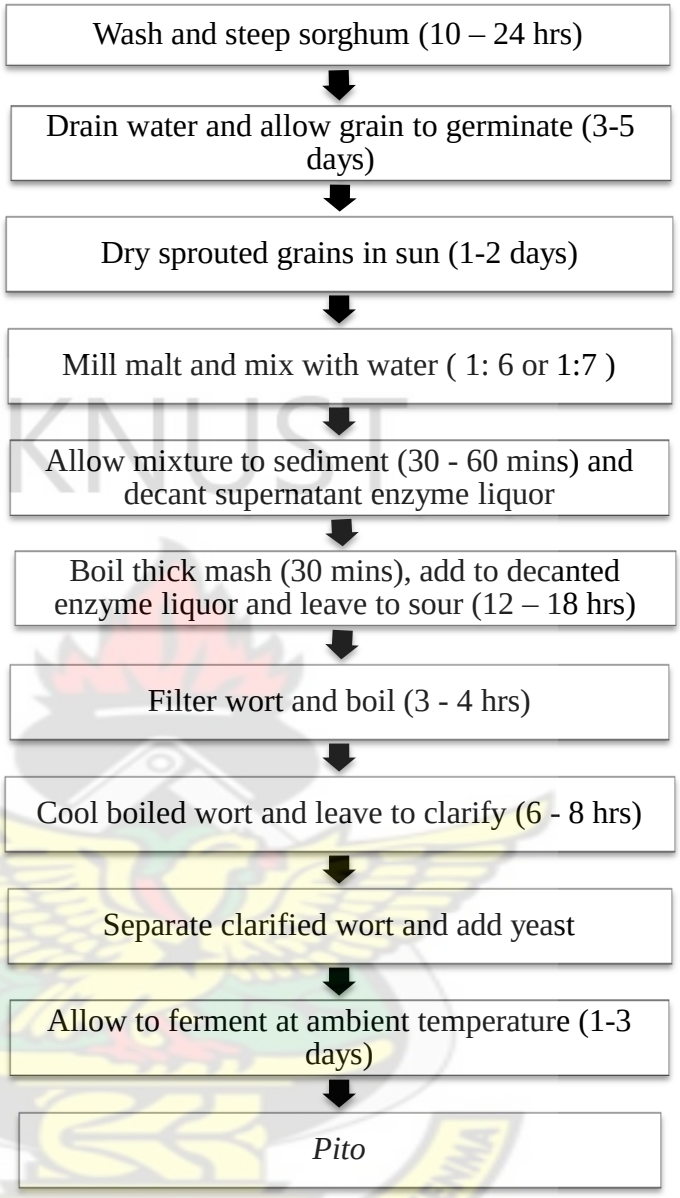

Figure 1: Flow diagram of Dargarti Pito brewing process, (Djameh, 2010)

2.2.1 Malting

The grains are washed in water to remove sand, dirt and other foreign materials. The washed grains are then steeped in water to take up water. After steeping between 12 to 16 hours, the grains are spread out in a thin layer on the

floor and left to germinate. The grains may be covered with jute sack and sprinkled with water daily.

After germinating adequately, i.e. between 4 and 6 days, the sprouted grains, now malt, are dried in the sun to terminate the germination and preserve it (UNIDO, 2007).

2.2.2 Mashing and Boiling

The malt is pounded into a coarse powder with a mortar and pestle or milled in a mill. The powder is mixed with water and a sedimentation agent, usually made from the bark of the okra plant or *Grewia venusta* and then left to sediment. The supernatant liquor, into which the malt enzymes are extracted, is decanted and kept aside and the thick sedimented mash is boiled to gelatinize the starch in the mash (Plate 1). After boiling for about an hour, the boiled mash is remixed with the decanted liquor and left to convert to a sugary wort and to become sour. When sufficient level of sourness is attained, the mash is filtered over grass placed in a basket or through a fine mesh nylon net. The filtered wort is boiled for two to three hours left to cool and clarify (UNIDO, 2007).

Plate 1: Heating of pito mash for starch gelatinization

2.2.3 Fermentation

The clarified cooled wort is decanted into fermentation pots and pitched with residues of a previous brew or inoculated with yeasts (Plate 2) trapped in the interstices of a belt woven from thin strips of straw or other plant material. The yeasts are trapped by dipping the belt into a previous fermenting *pito*. After fermenting for a day or two the finished *pito* is ready for consumption.

Plate 2: Cooled wort pitched with yeast under fermentation

Pito is sold at the premises where it is brewed and where it is served from traditional earthenware pots into calabashes or by hawking where it is filled in plastic bottles (Plate 3). *Pito* brewing in Ghana has remained at an artisanal level over the years. Even though the unit production processes are the same as for the production of European style beers, the technologies applied in their production are very different. Through scientific studies and applied research, lager beer brewing in the commercial breweries has evolved into a highly efficient industrialized process with consistent quality of the finished products.

Plate 3: Hawking of pito filled in recycled plastic water bottles (Djameh, 2010)

2.3 Industrial Brewing with Lactic Acid Bacteria

Sour sorghum beers, similar to *pito* known as *chibuku* are brewed commercially on industrial scale with lactic acid bacteria in eastern and southern Africa, mostly in South Africa, Zimbabwe, Zambia and Botswana (Daiber and Taylor, 1995). There are several sour beers also from Belgium and Germany in which lactic acid bacteria are used to produce a tasty and characteristic sourness. The Belgium sour beers are lambics, Witbier and the Flanders style beers. Examples of the German sour beers are Berliner Schultheiss and Berliner Kindl Weiss (Nummer, 1996). The European sour

beers are brewed from malted wheat and soured by cultures of lactic acid bacteria.

2.4 Characteristics of Lactic Acid Bacteria in Brewing

Lactic acid bacteria are Gram-positive, non–spore-forming rods (or *cocci),* and are obligate fermenters as they must ferment a carbohydrate source. They do not grow aerobically. Lactic acid bacteria include *Lactobacilli* and *Pediococci. Lactobacilli* are facultative anaerobes — they can ferment in the presence or absence of air, but prefer reduced oxygen levels (Kockova *et al.,* 2011). One important species to brewers is *Lactobacillus delbrueckii* named after Max Delbrück, a founding father of microbiology, who characterized bacteria in the sour beers of Berlin, the Berliner Weisse (Nummer, 1996). Besides *L. delbrueckii*, several othe *Lacobacilli* find their way into beer, including *L. brevis*, *L. acidophilus*, and *L. lactis.*

Lactic acid bacteria are divided into two categories based on their by-products of fermentation. The homofermentative group produces primarily lactic acid, whereas the heterofermentative group produces lactic acid, acetic acid, ethanol, and carbon dioxide (Kockova *et al.,* 2011; Rattanachaikunsopon and Phumkhachorn, 2010). Either group will cause spoilage in beers in which the bacterial by-products are not desired. Some lactic acid bacteria produce dextrans which can cover the surface of wort or beer, or form pellicles which are visually unappealing, but otherwise harmless (Nummer, 1996). The

homofermentative *Lactobacilli* and *Pediococci* and heterofermentative *Lactobacilli* each have different flavour profiles. Lactic acid bacteria provide a versatile tool for producing sour beers. For biological acidification of sour beers, a suitable strain of lactic acid bacteria is a prerequisite (Nummer, 1996). Pure cultures of *L. amylovorus* or *L. amylolyticus* are recommended for sour beers (Back, 1988). They have high acidification capacity of up to 2% lactic acid and are effective up to pH below 3. They are homofermentative and also ferment dextrins and starch. They grow at high temperatures of up to 52°C (Kunze, 2004a).

2.5 Quality defects of Traditional *Pito*

Inconsistent quality and limited shelf-life are the major quality issues associated with *pito*, just as reported for the other African traditional beers brewed from sorghum malt (Solange, *et al.*, 2014). The inconsistent product quality arises from non-standardized production methods and the use of rudimentary equipment. The wide variety of microorganisms present during the spontaneous fermentation also contributes to the widely varying quality (Lyumugabe *et al.*, 2014). The processes are not controlled and vary from batch to batch. The resident microflora which come with the sorghum grain from the field are active in the beer and their biochemical products are responsible for the early spoilage. The ambient temperatures at which alcoholic fermentation takes place are favourable for the growth of mesophilic lactic acid bacteria. The metabolic activities of mesophilic lactic acid bacteria are primarily responsible for the spoilage. These bacteria, along with other

undesirable bacteria (*Acetobacter*), produce acetic acid, volatile off-flavours, fruity odours, and pellicles which render the taste, odour and texture of the beer unacceptable to consumers (Dendy, 1995).

A study of isolates of lactic acid bacteria associated with sorghum beer spoilage indicated that *L. plantarum,* a mesophilic lactobacillus is the dominant species (Thord-Gray and Holzapfel, 2004). This together with a group of mesophilic homofermentative lactobacilli accounted for 70% of the spoilage organisms studied. According to Lyumagabe *et al.,* (2012), sorghum beers spoil rapidly because they are actively fermenting when sold, with organisms in addition to yeasts flourishing in the rich medium. They explained that during fermentation, yeasts initially increase in number. Then in the later stage of logarithmic growth the production of ethanol starts and proceeds during the stationary phase during which very little or no increase in the number of contaminating organisms seems to occur. However, at the end of fermentation, the yeasts die, or else they undergo autolysis and their cell constituents are released into the beer. With little or no competition from yeasts for the readily available nutrients, contaminating microorganisms increase rapidly in number and their metabolites change the flavour of the beer. Due to the favorable temperature of fermentation, these sequential events occur within a short time period. This period does not usually exceed more than 3 days in hot weather or 5 days in cold weather before spoilage occurs (Lyumugabe, 2013).

2.6 Addressing quality issues of *Pito*

Several methods have been proposed to address the quality issues of traditional African sorghum beers (Haggblade and Holzapfel, 1989b; Daiber and Taylor, 1995). The aim of these initiatives is to make the quality consistent and to improve the shelf life of the product.

2.6.1 Standardization of the production process

Consistency of product quality can be achieved by standardization of the process which entails specification for raw materials and process conditions. For any process that has more than one method of carrying it out, there is a potential issue with varying consistency of the output. Standardization of the process ensures that each time the process is carried out, it is completed in the same way. Standardization provides the baseline for quality and continuous improvement. It ensures that all work and procedures are done in the same way. Standardization helps stabilize a process to make it measurable, predictable and controllable (ABB, 2010)

2.6.2. Destruction of spoilage causing microorganism

Destruction of spoilage causing microorganisms will extend shelf life. According to Baba-Moussa *et al.,* (2012) in the manufacturing process of *tchakpalo,* the environment and the way of selling are responsible for contamination of the drink. The product can be stabilized by pasteurization or the use of chemical preservatives to prevent microbial activity of spoilage causing microorganism.

Microbial infection of Nigerian sorghum grain has been shown to be caused by the presence of *Aspergillus sp., Penicillium sp., Neuropora sp., Fusarium sp., Curvularia sp.,* and *Dreschelera sp*, (Boboye and Adetuyi, 1994). Radurization at 10 kGy of sorghum grains has proved effective in reducing the total microbial population of sorghum malt by about 99.5%. Lactic acid bacteria, fungi, and anaerobic endospores were reported to be eliminated to below the level of detection, extending the shelf life of the brewed beer by 2-3 days (Haggblade and Holzapfel, 1989b). Formaldehyde (0.1%) can be added to the steep water to retard fungal activity (Palmer, 1989). Lefyedi and Taylor (2006) investigated the effect of dilute alkaline steeping on microbial contamination, toxicity and diastatic power of sorghum malt and proposed the addition of 0.2% NaOH in steeping water for the control of bacterial and fungal contamination during sorghum malting.

2.6.3 Exclusion of spoilage causing microorganisms by the application of pure strain starter cultures

Exclusion of spoilage causing microorganisms can be achieved by the application of pure strains of starter cultures of desirable microorganisms which will be responsible for the fermentation process and keep away any microorganism whose activity or metabolite will adversely affect the quality of the product (Lyumugabe, 2013). Undesirable microorganisms can be prevented from getting access to the product by sterilizing the extract from the malt, wort, before introducing the starter culture. A starter culture may be defined as a preparation or material containing large numbers of viable microorganisms

selected for their properties and their harmlessness, which may be added to accelerate a fermentation process (Holzapfel, 2002).

It has been reported that the thermophilic lactic acid bacteria desired by brewers are not present on all malts (Novellie and De Schaepdrijver, 1986). The use of lactic acid bacteria in food products dates back to ancient times, and they are used mainly because of their contributions to flavour, aroma, and increased shelf life of fermented products (Nes *et al.,* 1996). Various strains of lactic acid bacteria are used commercially as starter cultures in the manufacture of food products, including dairy products, fermented vegetables, fermented doughs and alcoholic beverages (Patarata *et al.,* 1994 and Pattison *et al,* 1998). When the starter is adapted to the substrate, its use improves control of the fermentation process and the predictability of its products (Holzapfel, 1997). In addition, it facilitates control over the initial phase of fermentation (Holzapfel, 2002). The use of starter cultures also reduces the organoleptic variations and the microbiological instability of African fermented foods (Kirmaryo *et al.,* 2002). Research on improving the quality of traditional sorghum beer has focused on the adaptation of starter cultures. Sefa-Dedeh *et al.,* (1999) used a pure culture of *S. cerevisiae* and a mixed culture comprised of *S. cerevisiae* with *Kloeckera apiculata* or *Candida tropicalis,* to produce in the laboratory *pito* beer containing a high ethanol content compared to traditional *pito*. By contrast, they also found that a mixture of three cultures (*S. cerevisiae, K. apiculata* and *C. tropicalis*) as the starter culture produced a *pito* beer with a low ethanol content compared with the traditional *pito* beer.

N'Guessan *et al.,* (2010) successfully used *S. cerevisiae* in combination with *C. tropicalis* as starter cultures for the alcoholic fermentation of the *tchapalo* beer. Glover *et al.,* (2009) showed that *dolo* beer produced from starter culture combinations of a strain of *L. fermentum* and two strains of S. *cerevisiae* had a taste and aroma that did not differ significantly from the local *dolo* beer. N'Guessan *et al.,* (2010) tested starter cultures of *Candida tropicalis* and *Saccharomyces cerevisiae* isolated from *tchapalo* in pure culture and co-culture for their ability to ferment sorghum wort to produce *tchapalo.* Demuyakor and Ohta (1993) fermented *pito* using selected single yeast strains and mixed yeast culture collected from traditional breweries and compared the fermentation characteristics and product quality of the pure and mixed fermentations. Oriji *et al*. (2003) brewed *pito* in the laboratory with pure cultures of *Lactobacillus plantarum* in combination with *Saccharomyces cerevisiae* and *Pediococcus halophilus* in combination with *Candida tropicalis* isolated from a local brew. The pH, colour, titrable acidity, alcohol content, specific gravity, taste and flavour of *pito* produced by this method were found to compare favourably with that produced by the traditional method. Okoro *et al*. (2011) also used *Lactobacillus sake* as starter culture in producing *pito* and found significant variations in the quality of the product.

The use of starter cultures would be an appropriate approach for the control and optimization of the fermentation process in order to alleviate the problems of variations in organoleptic quality and microbiological stability observed in African traditional fermented beverages (Holzapfel, 1997; Achi, 2005; Viera-

Dalodé *et al.,* 2007; M u g u l a *et al.,* 2003). According to Sawadogo-Lingani *et al*. (2008), suitable microorganisms must be selected for use as starter culture based on technological properties such as rapid lactic acid formation. *L. delbrueckii* and *L. amylovorus* both resident in malt and used for industrial souring of sour beers being homofermentative and thermophilic which are desirable properties for production of sour beers with extended shelf-life could be suitable microorganisms to be used as starter cultures for the production of *pito*. Conditions of their use that will result in the most rapid and higher yield of lactic acid need to be investigated for optimization of the souring process of *pito*.

Most of the starter cultures used in studies were introduced during the fermentation in the form of fresh microbial suspensions which are difficult to maintain over extended periods. Coulibaly *et al.,* (2016) suggested the use of dried and active starter cultures which will be stable during storage. For industrial brewing of European types of beers, such dried active and stable commercial starter cultures of lactic acid bacteria and brewers' yeasts are available for the fermentation processes.

2.7 Tools for shelf life studies

According to Fu and Labuza (1993), shelf life of a food can be defined as the time period within which the food is safe to consume and/or has an acceptable quality to consumers. Shelf life testing consists basically of selecting the quality characteristics which deteriorate most rapidly with time and mathematical modeling of the change. Hedonic scoring, which indicates

the overall acceptance of a product or of a specific characteristic of it such as flavour, texture, appearance, aftertaste, etc. on a numerical scale, e.g. a 1-9 point scale labeled from "dislike extremely" to "like extremely", is also typically used to evaluate its shelf-life. Polhemus (2005) described a method by which a reasonable shelf life of a product can be established. One or more critical variables of the product associated with its effectiveness are measured at different lengths of time after production. A statistical model is then constructed with the data to predict that point in time after which the probability that the product will still be effective falls below a specified threshold. Ofosu *et al,* (2011) used modelling to study the shelf life of a formulated avocado product and determined its shelf life by determining the time it will take the peroxide value of the avocado product, which is a measure of fat rancidity and hence a spoilage indicator, to reach an unacceptable level. In a similar manner, the increasing sourness of *pito* with time can be used as a spoilage indicator to determine the time it will take the sourness of *pito* to reach a level at which it becomes unpleasant to drink. This time is the shelf life of *pito*.

CHAPTER THREE

Souring capacity of single strain commercial starter cultures of Lactic Acid bacteria *L. delbrueckii* and *L. amylolyticus* in Pito mash

3.1 Introduction

The lactic acid bacteria are a group of Gram positive bacteria, non-respiring, non-spore forming, cocci or rods, which produce lactic acid as the major end product of the fermentation of carbohydrates. They are some of the most important bacteria in desirable food fermentations, being responsible for the fermentation of sour dough bread, sorghum beer, all fermented milk, cassava (for gari and fufu production) and most "pickled" (fermented) vegetables (FAO, 1998). Lactic acid bacteria, including *L. delbrueckii*, are non-pathogenic. They are part of the normal microbiota in the human body where they help restore and maintain a healthy digestive system. Some of the strains of lactic acid bacteria used for biological acidification in breweries belong to the *L. delbrueckii* subsp. *delbrueckii, L. delbrueckii* subsp. *lactis* or *L. fermentum* and *L. amylolyticus* (Bohak *et al.,* 1998). These strains have adapted to the environment of beer wort as they belong to the microflora naturally resident on grains and malt. According to Nummer (1996), lactic acid bacteria provide a versatile tool for producing sour beers.

In traditional African sorghum beers, mash acidification, which is an important processing step, is carried out by lactic acid bacteria to convey the characteristic sourness to the beers. They form the second most prominent category of microorganisms in most of these beers. The most commonly

observed LAB are *Lactobacillus fermentum*, *Lactobacillus buchneri* (Lyumugabe *et al.,* 2010), *L. delbrueckii*, *Pediococcus acidilacti*, *Leuconostoc lactis*, and *Lactococcus lactis*

(Sawadogo- Lingani *et al.,* 2007). In the microbiological study of lactic acid bacteria in burukutu, a sorghum beer produced in Nigeria, Ghana and Benin, Faparusi *et al.* (1973) isolated *Leuconostoc mesenteroides, L. brevis, L. fermentum, L. delbrueckii* and *Streptococcus lactis*. Togo *et al.* (2002) isolated two strains of *L. plantarum* and two strains *L. delbrueckii* from *chibuku* the most common traditional sorghum beer sold in Zimbabwe for assessment of their potential as starter cultures to address the inconsistent quality of the product. In a study of the biodiversity of predominant lactic acid bacteria in *dolo* and *pito* wort for the production of sorghum beer, Sawadogo-Lingani *et al.,* (2007) found *L. fermentum* to be the dominant LAB species with *L. delbrueckii ssp. delbrueckii, L. delbrueckii ssp. bulgaricus and P. acidilactici* in lower numbers.

Lactic acid bacteria are divided into two categories based on their by-products of fermentation. The homofermentative group produces primarily lactic acid, whereas the heterofermentative group produces lactic acid, acetic acid, ethanol, and carbon dioxide (Kockova *et al.,* 2011; Blandino *et al.,* 2003). They are also classified as thermophilic or mesophilic depending on the temperature range at which they grow best. The desirable characteristics of lactic acid bacteria for use as a starter culture in brewing sour beers are rapid acid formation and non-development of off flavours in the beer.

Homofermentative and thermophilic strains are best suited for rapid acid formation and production of clean flavours. Heterofermentative and mesophilic strains are less desirable as they form less lactic acid and also tend to develop off flavours and early spoilage (Nummer, 1996).

The use of *L. delbrueckii* and *L. amylolyticus* is common with German brewers to provide sharpness of flavour in German wheat beers. Pure cultures of *Lactobacillus amylovorus* or *L. amylolyticus* are recommended for sour beers (Back, 1988). They have high acidification capacity of up to 2% lactic acid and are effective up to pH of below 3. They are homofermentative and also ferment dextrins and starch. They also grow at high temperatures up to 52°C (Kunze, 2004a) and their cultures are easy to handle.

L. amylolyticus belongs to the class of lactic acid bacteria known as amylolytic lactic acid bacteria (ALAB). These possess α-amylases that have the ability to partially hydrolyze raw starch and ferment different types of raw material that contain amylose, such as corn, potato, or cassava and different starchy substrates (Vishnu *et al.*, 2002; Naveena *et al.*, 2005) into lactic acid in a single step fermentation (Fossi and Tavea, 2013). ALAB could help improve starch conversion and fermentability of *pito* wort which is limited because of the inherent low level of beta amylase in sorghum malt.

L. delbrueckii appears to be the dominant thermophilic bacterium active in sorghum beer production (Haggblade and Holzapfel, 1989a) and yields a pH level between 3.0 and 3.5. However, since a sour inoculum obtained from malt may contain several other undesirable bacteria together with a mixed

population of thermophilic lactic acid bacteria with variable acid producing capabilities, the application of pure strains of starter cultures of *L. delbrueckii* became of interest for industrial brewing of sour sorghum beers.

In this study, the lactic acid production capacity of pure commercial strains of *L. delbrueckii* and *L. amylolyticus* used by breweries in the production of sour beers were investigated for their performance in *pito* wort as potential starter cultures for the industrial production of *pito*.

3.2 Materials and methods

3.2.1 Materials

Starter cultures of *L. delbrueckii* obtained from the Biological Laboratory of Versuchs- und Lehranstalt für Brauerei in Berlin (VLB) e.V., Berlin, Germany and *L. amylolyticus* obtained from Hefe Bank Weihenstephan GmbH, 84072 Au i. d. Hallertau, Germany were used in the study. The red sorghum variety, *kadaga* mostly used by *pito* brewers in Ghana was used as the base malting material. This was obtained from a Savannah Agricultural Research Institute, SARI, in Nyankpala.

3.2.2 Experimental Design

A response surface design comprising a 3 x 8 factorial design was used for the study. The factors considered for each of the lactic acid bacteria (*L. delbrueckii* and *L. amylolyticus)* were Temperature (35^0 C, 40^0 C, 45^0 C) and Time (0h, 8h, 16h, 24h, 32h, 40h, 48h, 56h). The measurable indicators were

pH, percent titratable acidity as lactic acid and percent extract. Each run was repeated and the mean values used in the analyses.

3.2.3 Preparation of starter cultures

The starter cultures were prepared according to the supplier's instruction (Appendix 1 and 2). The target values of pH and lactic acid at the sour point of *pito* were established as pH, 4.2 to 4.0 and lactic acid level of 0.4 to 0.6% from studies on traditional *pito* carried out by UNIDO (2007).

The starter culture of *L. amylolyticus* was prepared according to the supplier's instruction by propagating in 10-times multiplication-rate, 24h at 45°C. 1ml of the liquid culture was inoculated in 9ml of 11.0% in sterile *pito* wort and incubated at 45°C for 24h. The inoculum was further propagated in a similar fashion by inoculating 10ml culture in 90ml sterile wort to obtain 4 bottles of 100ml inoculum. To prepare *L. delbrueckii* "H1", 5 ml from the base culture was inoculated in 150ml of 12.4% sterile *pito* wort as specified by the supplier and incubated 24 hours at 48°C. The propagated cultures were kept refrigerated until use. The extract of the worts used to culture the lactic acid bacteria were chosen to obtain a starting extract of 12.3 ± 1% for all treatments. The cell population density of the cultures was determined as number of colony forming units, (cfu), by pour plate method using the serial dilution technique according to the procedure of microbiological control analysis specified by European Brewery Convention, Analytica EBC (1987). Incubation was carried out at 35 ± 2°C for 48h in anaerobic jars on de Mann Rogosa and Sharpe (MRS) agar as growth medium.

3.2.4 Preparation of Sorghum Malt

Sorghum malt for the entire study was prepared according to the procedure described by Djameh *et al.*, (2015). Twenty kilograms (20kg) of the red sorghum variety, *kadaga* was washed with tap water to remove dirt and other foreign materials. The cleaned grains were then sanitized by steeping in 0.2% caustic soda for 4 hours after which the caustic soda solution was drained and residual caustic soda on the surface of the grains was removed by rinsing with tap water. The grains were then given an air rest without steeping for 2 hours and then steeped in tap water for 12 hours followed by germination at 30°C for 5 days. The germinated grains were dried in a forced draught electric oven for 16 hours at 50˚C. The malt was then bagged and stored at ambient temperature for further use.

3.2.5 Preparation of sterile *Pito* wort.

Five kilograms (5kg) of sorghum malt was milled on a grain mill assesory of a Sonashi blender model No. SB-114 manufactured by Sonashi Electronics and Hardware Appliances, UAE. The milled malt was mixed with 25L of water at ambient temperature and left to sediment for 60 minutes. The clear supernatant enzyme extract was decanted and kept aside. The thick residual mash was boiled for 30 minutes and recombined with the decanted supernatant enzyme liquor. The temperature of the recombined mash was heated to 62˚C and maintained for 60 minutes and then raised to 72˚C and maintained until the mash saccharified adequately as indicated by negative starch test with iodine. The mash was then filtered through a fine mesh sieve to obtain a clear wort and

boiled to sterilize and concentrated to an extract level of 13.12%. The sterilized wort was filled into one litre (1L) and five litres (5L) glass flasks, re-sterilized and stored frozen until use.

3.2.6 Sample Preparation.

Prepared and frozen sterilized *pito* wort was thawed and 90ml measured 120ml High Density Polyethylene (HDPE) bottles. The filled worts were re-sterilized with steam at 104^0 C for 30 minutes and cooled. A total of 50 bottles were used. One set of 25 bottles of the cooled sterile wort was inoculated with 3ml of prepared *L. delbrueckii* culture and another set of 25 bottles inoculated with 10ml of prepared *L. amylolyticus* culture according to the suppliers' specifications (Appendix 1 and 2). The inoculated bottles were placed according to the experimental design in thermostatic water baths at 35^0 C, 40^0 C, and 45^0 C to undergo lactic acid fermentation. At 0h, 8h, 16h, 24h, 32h, 40h, 48h, 56h a bottle each of the fermenting *L. delbrueckii* and *L. amylolyticus* wort was taken out and rapidly cooled to 5^0 C (Table 3.1) to arrest any further fermentation and subsequently warmed up to 20^0 C for the determination of pH, extract and Lactic acid content.

A non-inoculated sterile sample kept at each of the experimental temperatures was analysed at the begining 0h and end of the experiment 56h as reference samples to ascertain that lactic acid in the treatments was produced by the inoculated lactic acid bacteria by testing to confirm no change in pH level.

3.2.7 Sample Analysis

3.2.7.1 pH

The pH of the system was measured using a Hanna digital pH meter, HI98190, Hanna Instruments, USA.

3.2.7.2 Extract Content

The extract content was measured using the Anton Paar DM 35N Density meter, manufactured by Anton Paar, Graz, Austria which was configured to convert density measurements automatically into Extract measurements (expressed as %) the quantity of soluble material extracted from the malt, based on a built-in conversion table.

3.2.7.3 Lactic Acid Content

The amount of lactic acid produced by the lactic acid bacteria during the fermentation was determined according to the procedure described by Kunze (2004a). Samples, (25ml) were take every 8h and titrated against 0.1N NaOH to a colour change from yellow to green at the endpoint at pH 7. Bromothymol blue (0.1 bromothymol blue in 100ml 20% ethanol) was used as indicator.

3.2.8 Statistical Analysis

The Minitab (Release 14) software was used to carry out regression analysis of the experimental data and plot the Main Effects Graphs, Surface Plots and Overlaid Contour Plots to depict how the responses relate to the levels of the variable factors. The Overlaid Contour Plots were used to determine optimal

conditions of the variables to achieve the desired values of Lactic acid and pH at the sour point. The lactic acid bacterium yielding the most efficient conditions for industrial production was selected and used in a confirmatory brew to validate the predicted conditions. The experimental data were statistically evaluated using Analysis of Variance (ANOVA). The adequacy of fit of the model was expressed by the coefficient of determination R^2 and R^2 adjusted.

3.3 Results and Discussion

The experimental data *for L. delbrueckii* and *L. amylolyticus* are reported in Tables 2 and 3 respectively. Regression analysis and analysis of variance of the data brought out the dependency of the responses on the investigated factors and explained the variation within the data. The p values indicate the significance of the dependency of the responses, lactic acid %, pH and Extract % on the process factors, time, h and temperature, T where $p < 0.05$ indicates that the dependence of the factor or term is significant.

Table 2: Fermenting capacity of *L. delbrueckii*

L. delbrueckii RUN	FACTORS		RESPONSES					
	Time, h	Temp, °c	Lactic acid %, Mean	Lactic acid %, Stdev	pH, Mean	pH, Stdev	Extract %, Mean	Extract %, Stdev
1	0	35	0.24	0.06	4.4	0.06	12.3	0
2	8	35	0.34	0.08	4.37	0.10	12.2	0.1
3	16	35	0.64	0.06	3.91	0.08	12	0.3
4	24	35	0.87	0.07	3.7	0.11	11.1	0.6
5	32	35	0.93	0.06	3.48	0.10	11	0.3
6	40	35	1.08	0.03	3.41	0.07	11	0.1
7	48	35	1.17	0.06	3.39	0.10	10.4	0.3
8	56	35	1.19	0.04	3.4	0.27	10.2	0.3
9	0	40	0.25	0.03	4.39	0.04	12.3	0
10	8	40	0.37	0.06	4.25	0.08	11.8	0.3
11	16	40	0.85	0.10	3.76	0.08	11	0.3
12	24	40	0.99	0.08	3.64	0.10	10.8	0.3
13	32	40	1.09	0.13	3.56	0.08	10.6	0.4
14	40	40	1.19	0.10	3.4	0.21	10.8	0
15	48	40	1.25	0.08	3.3	0.17	10.8	0.3
16	56	40	1.29	0.06	3.3	0.08	10.8	0.3
17	0	45	0.23	0.03	4.42	0.03	12.3	0
18	8	45	0.3	0.11	4.34	0.06	12	0.3
19	16	45	0.56	0.08	4.05	0.08	11.9	0.4
20	24	45	0.62	0.04	3.83	0.13	11.8	0.6
21	32	45	0.66	0.08	3.59	0.23	11.8	0.4
22	40	45	0.71	0.04	3.53	0.14	11.6	0.3
23	48	45	0.82	0.06	3.47	0.06	11.6	0.3
24	56	45	0.83	0.03	3.48	0.03	11.6	0.4

Table 3: Fermenting capacity of *L. amylolyticus*

L. amyloticus	FACTORS		RESPONSES					
RUN	Time, h	Temp, °c	Lactic acid %, Mean	Lactic acid %, Stdev	pH, mean	pH, stdev	Extract %, Mean	Extract %, Stdev
25	0	35	0.21	0.03	4.54	0.04	12.3	0
26	8	35	0.24	0.04	4.49	0.06	13	0.3
27	16	35	0.41	0.03	4.12	0.11	12.5	0.3
28	24	35	0.58	0.01	3.8	0.17	12.4	0.1
29	32	35	0.7	0.04	3.52	0.11	12.2	0.1
30	40	35	0.85	0.01	3.41	0.01	12.2	0.1
31	48	35	0.87	0.03	3.4	0.03	12.2	0.3
32	56	35	0.88	0.03	3.4	0.01	12.2	0.1
33	0	40	0.21	0.01	4.54	0.03	12.3	0
34	8	40	0.24	0.03	4.5	0.08	15	0.4
35	16	40	0.58	0.06	3.97	0.21	15	0.8
36	24	40	0.78	0.04	3.77	0.04	16	0.3
37	32	40	0.93	0.06	3.52	0.08	16	0.3
38	40	40	1.08	0.24	3.41	0.13	16	0.6
39	48	40	1.09	0.06	3.37	0.10	16	0.6
40	56	40	1.22	0.06	3.36	0.03	16	0.4
41	0	45	0.21	0.01	4.54	0.03	12.3	0
42	8	45	0.29	0.03	4.52	0.03	17.5	1.0
43	16	45	0.32	0.01	4.49	0.10	17.7	1.0
44	24	45	0.37	0.03	4.35	0.07	18.2	0.4
45	32	45	0.52	0.03	3.77	0.07	15.2	1.7
46	40	45	0.76	0.03	3.53	0.11	16	0.3
47	48	45	0.86	0.04	3.45	0.03	16	0
48	56	45	0.92	0.04	3.43	0.01	16.5	0.6

3.3.1 The dependence of Lactic Acid Formation on time and temperature

Response Surface Regression analysis (Tables 4 and 5) indicated that Lactic acid formation was significantly influenced by both factors time ($p < 0.001$) and temperature ($p < .001$) and their interactions ($p < 0.001$) for *L. delbrueckii*. For *L. amylolyticus*, only the factor time and the square interaction of

temperature significantly influenced lactic acid formation. The high values of R –squared (R-Sq, 95.9 % for *L. delbrueckii* and 93.3 % for *L. amylolyticus*) and R-squared adjusted (R-sq (adj), 94.8 % for *L. delbrueckii* and 91.5 % for *L. amylolyticus*) indicated that the models adequately explained the variation in the data. The accompanying ANOVA tables are presented in Tables 6 and 7.

Table 4: Response Surface Regression analysis of Lactic Acid, (%) versus Time, (h), Temp, (deg C), *L. delbrueckii*

Estimated Regression Coefficients for LACTIC ACID % for *l. delbrueckii*

Term	Coef	SE Coef	T	P
Constant	0.9978	0.03381	29.515	<0.001
TIME, (h)	0.4482	0.02487	18.023	<0.001
TEMP, (deg C)	-0.1081	0.01994	-5.423	<0.001
TIME, (h)*TIME),(h)	-0.2049	0.04352	-4.708	<0.001
TEMP, (deg C)*TEMP, (deg C)	-0.2106	0.03453	-6.099	<0.001
TIME, (h)*TEMP,(deg C)	-0.1019	0.03046	-3.345	<0.004

S = 0.07975 R-Sq = 95.9% R-Sq(adj) = 94.8%

Table 5: Response Surface Regression analysis of Lactic Acid, (%) versus Time, (h), Temp, (deg C), *L. amylolyticus*

Estimated Regression Coefficients for LACTIC ACID % for *l. amylolyticus*

Term	Coef	Coef SE	T	P
Constant	0.789792	0.03978	19.853	<0.001
TIME, (h)	0.438056	0.02926	14.970	<0.001
TEMP, (deg C)	-0.030625	0.02346	-1.305	0.208
TIME, (h)*TIME,(h)	-0.054931	0.05121	-1.073	0.298
TEMP,(degC)*TEMP(degC)	-0.204375	0.04064	-5.029	<0.001
TIME,(h)*TEMP(degC)	0.000208	0.03584	0.006	0.995

S = 0.09385 R-Sq = 93.3% R-Sq(adj) = 91.5%

Table 6: Analysis of Variance for dependence of lactic acid (%), on Time and Temperature for *L. delbrueckii.*

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	5	2.70200	2.70200	0.54040	84.96	<0.001
Linear	2	2.25323	2.25323	1.12662	177.12	<0.001
Square	2	0.37760	0.37760	0.18880	29.68	<0.001
Interaction	1	0.07117	0.07117	0.07117	11.19	0.004
Residual Error	18	0.11449	0.11449	0.00636		
Total	23	2.81650				

Table 7: Analysis of Variance for dependence of lactic acid, % on Time and Temperature for *L. amylolyticus*

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	5	2.22166	2.22166	0.444333	50.45	<0.001
Linear	2	1.98876	1.98876	0.994380	112.90	<0.001
Square	2	0.23290	0.23290	0.116451	13.22	<0.001
Interaction	1	0.00000	0.00000	0.00000	0.00	0.995
Residual Error	18	0.15854	0.15854	0.008808		
Total	23	2.38020				

The formation of lactic acid in *pito* wort with time by the two lactic acid bacteria was slow in the first 8 hours and increased rapidly thereafter. The first 8 hours of slow formation of lactic acid may be accounted for by the lag phase in which the bacteria get adapted to the fermentation environment. A comparison of the development of lactic acid with time by the bacteria at different temperatures is illustrated in Figure 2. The rate of lactic acid production slowed and levelled out for *L delbrueckii* after 48 hours at all the three temperatures but continued to increase for *L. amylolyticus* at 40°C and 45°C. The observed trends are in line with the reported characteristic of lactic acid bacteria to die off at a point when the level of lactic acid produced becomes toxic for them (Kunze, 2004a) and also confirmed that *L.*

amylolyticus has the ability to produce lactic acid at the high temperature of 45˚C.

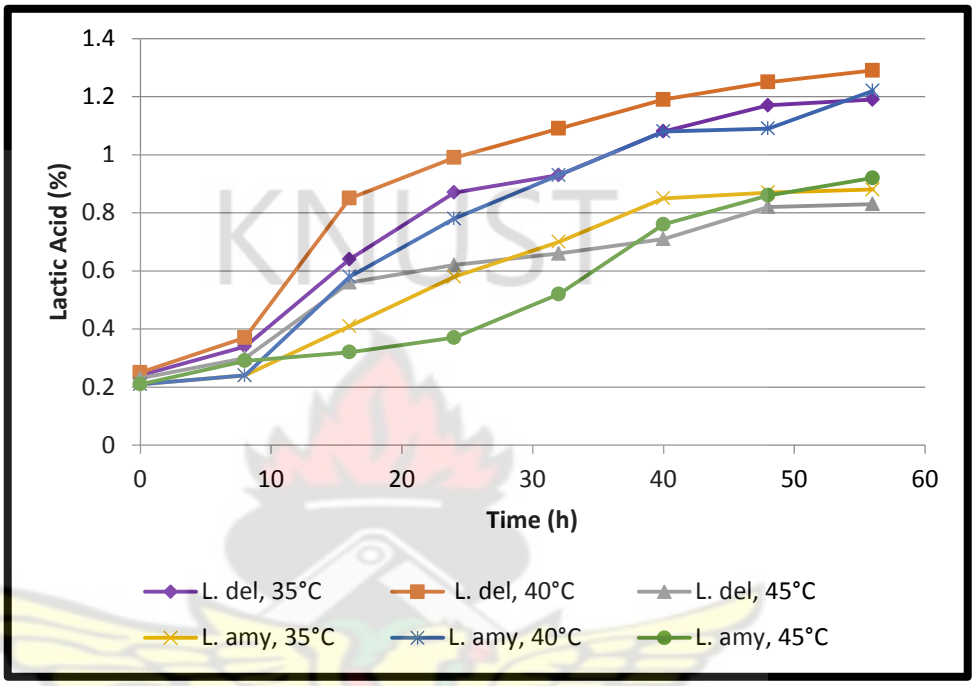

Figure 2: Comparison of lactic acid production in *pito* wort of *L. delbrueckii* and *L. amylolyticus* with time at different temperatures

Even though the strains of both *L. delbrueckii* and *L. amylolyticus* investigated are thermophilic, the experimental data indicated that lactic acid production by these bacteria peaks at 40˚C from 35˚C and decreases between 40˚C and 45˚C. Among the two lactic acid bacteria, *L. delbrueckii* has the highest capacity of lactic acid production at 40˚C and produced lactic acid at an average rate of 0.019% each hour. L. *amylolyticus* at 40˚C had the second highest average rate of formation lactic acid with 0.018% each hour.

Surface plots of the dependence of lactic acid formation on temperature and time for the two lactic acid bacteria are given in Figure 3 and Figure 4 to visualize the interaction of the factors. A curvilinear relationship between lactic acid formation and the factors time and temperature was indicated for both bacteria. The plots also depicted the increase in lactic acid production with time and the peaking of the production at 40˚C from 35˚C to 45˚C.

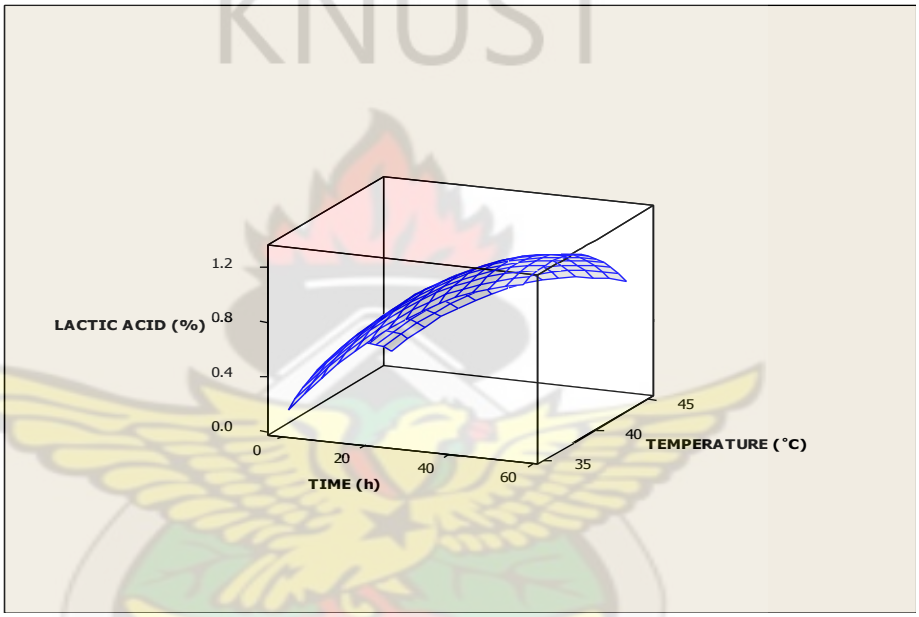

Figure 3: Surface plot of production of lactic acid by *L. delbrueckii* with time and temperature.

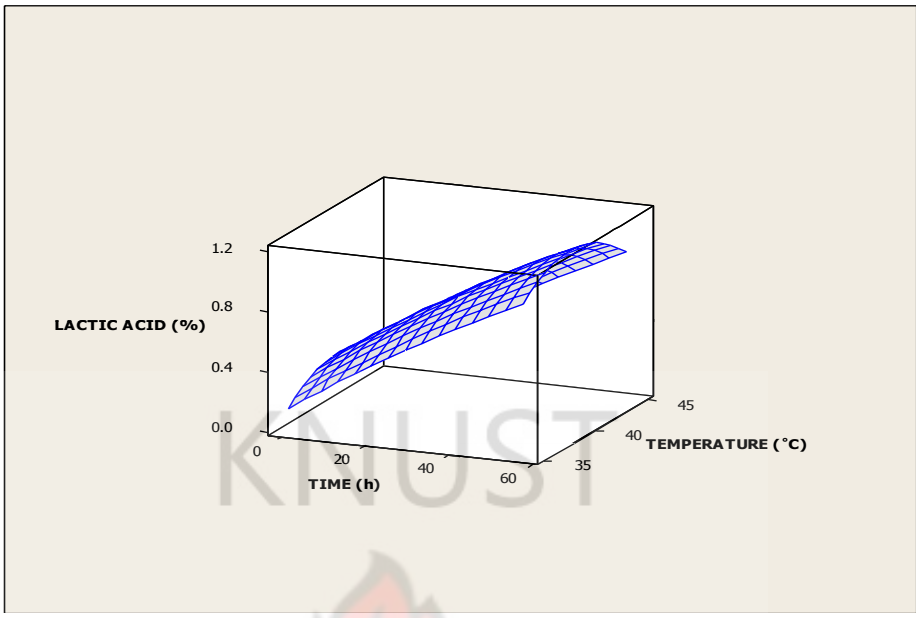

Figure 4: Surface plot of production of lactic acid by *L. amylolyticus* with time and temperature

3.3.2 Effect of time and temperature on pH

Response surface regression analysis and analysis of variance of the data on the dependence of pH on time and temperature for *L. delbrueckii* and *L. amyloliticus* are given in Tables 8, 9, 10 and 11.

Table 8: Response Surface Regression analysis of variation of pH with Time, h, and Temperature, deg C , for *L. delbrueckii*

Estimated Regression Coefficients for pH

Term	Coef	SE Coef	T	P
Constant	3.57844	0.03410	104.933	<0.001
Time, (h)	-0.55403	0.02508	-22.087	<0.001
TEMP, (deg C)	0.04062	0.02011	2.020	0.059
TIME, (h)*TIME,(h)	0.28365	0.04390	6.462	<0.001
TEMP, (deg C)*TEMP(deg C)	0.09812	0.03484	2.817	0.011
TIME, (h)*TEMP, (deg C)	0.01854	0.03072	0.604	0.554

S = 0.08045 R-Sq = 96.8% R-Sq(adj) = 95.9%

Table 9: Analysis of Variance for the variation of pH with Time, h, and Temperature, deg C , for *L. delbrueckii*

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	5	3.50750	3.50750	0.70150	108.39	<0.001
Linear	2	3.18357	3.18357	1.59179	245.95	<0.001
Square	2	0.32157	0.32157	0.16078	24.84	<0.001
Interaction	1	0.00236	0.00236	0.00236	0.36	0.554
Residual Error	18	0.11650	0.11650	0.00647		
Total	23	3.62400				

Table 10: Response Surface Regression analysis of variation of pH with Time, h, and Temperature, deg C, for *L. amylolyticus*

Estimated Regression Coefficients for pH *L. amylolyticus*

Term	Coef	SE Coef	T	P
Constant	3.72208	00.06472	57.513	<0.001
Time, (h)	-0.67028	0.04760	-14.080	<0.001
TEMP, (deg C)	0.08750	0.03817	2.292	0.034
TIME, (h)*TIME,(h)	0.19347	0.08331	2.322	0.032
TEMP, (deg C)*TEMP(degC)	0.11750	0.06611	1.777	0.794
TIME, (h)*TEMP, (deg C)	-0.01542	0.05830	-0.264	0.794

S = 0.1527 R-Sq = 92.2% R-Sq(adj) = 90.0%

Table 11: Analysis of Variance for the variation of pH with Time, h, and Temperature, deg C, for *L. amylolyticus*

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	5	4.94457	4.94457	0.98891	42.43	<0.001
Linear	2	4.74359	4.74359	2.37179	101.75	<0.001
Square	2	0.19935	0.19935	0.09968	4.28	0.030
Interaction	1	0.00163	0.00163	0.00163	0.07	0.794
Residual Error	18	0.41957	0.41957	0.02331		
Total	23	5.36413				

The variation in pH was significantly affected by time ($p < 0.001$), the square of time ($p < 0.001$) and the square of temperature ($p = 0.011$) for *L. delbrueckii*. For *L. amylolyticus*, the variation of pH is significantly influenced by time ($p < 0.001$), temperature ($p = 0.034$) and the square of time ($p = 0.032$). The high values of R –squared (R-Sq) and R-squared adjusted (R-sq (adj)) shown in Tables 8 and 10 indicated that the models adequately explained the variation in the data.

Parallel to the increase in lactic acid formation with time, there was a rapid decrease in pH for both lactic acid bacteria until it levelled off after a pH of 3.50 was attained. The changes in pH with time and temperature are depicted in Figures 5 and 6 respectively. The decease in pH was faster for *L. amylolyticus* relative to L *delbrueckii* with respect to time.

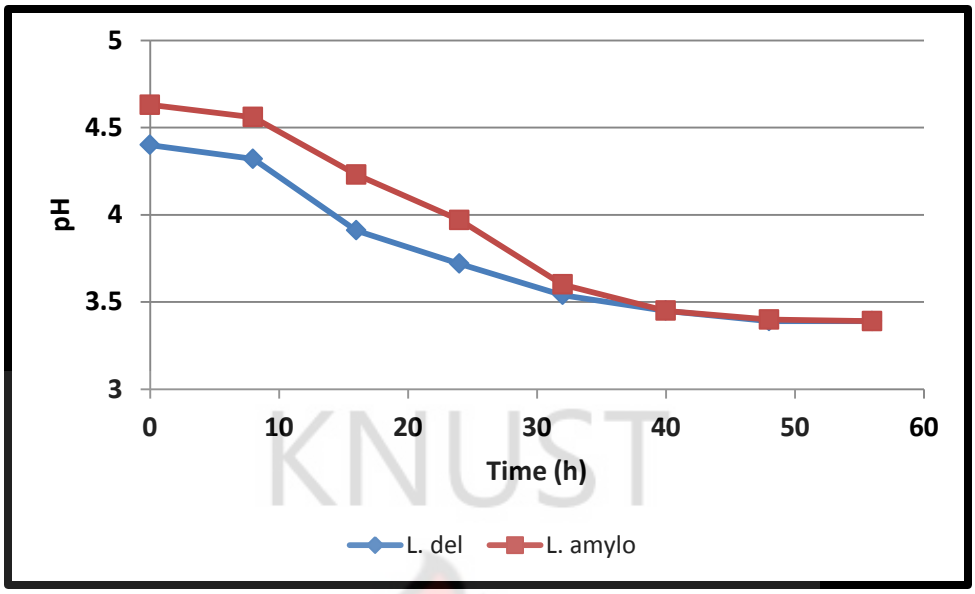

Figure 5: Variation of pH with time for *L. delbrueckii* and *L. amylolyticus*.

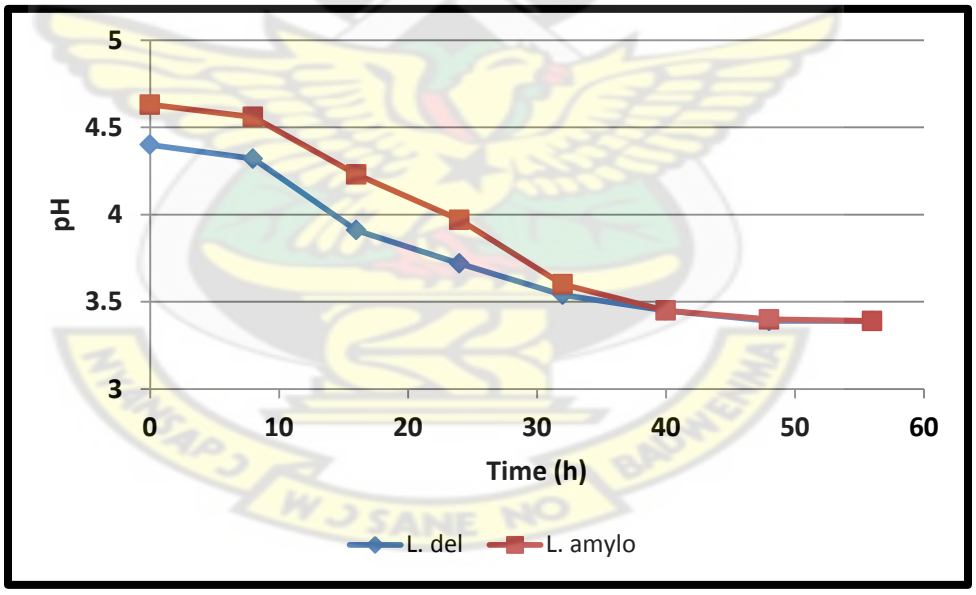

Figure 6: Variation of pH with temperature for *L. delbrueckii* and *L. amylolyticus*.

The variation of pH for both lactic acid bacteria with time and different temperatures are compared in Figure 7. There was not much change in pH in

the first 8 hours for both bacteria, indicative of a lag phase. This was followed by the rapid decrease up to the 32nd hour after which the pH levelled off. The decrease in pH was fastest for *L. delbrueckii* at 40°C with an average rate of 0.020 units per hour and slowest for *L. amylolyticus* at 45°C with an averagr rate of 0.017 units per hour.

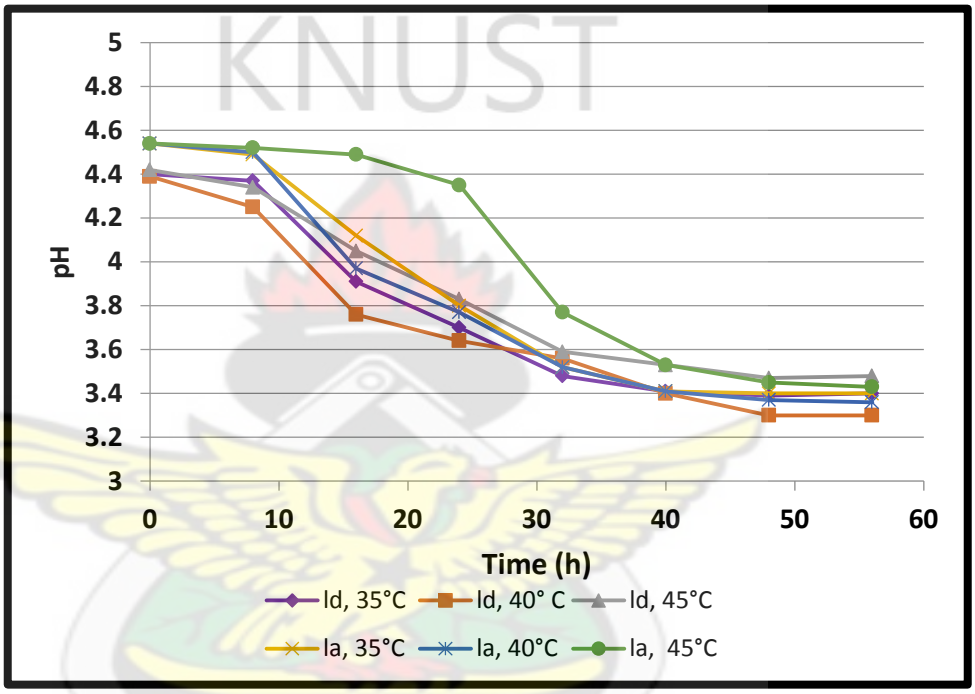

Figure 7: Comparing the variation of pH with time for *L. delbrueckii* (ld) and *L. amylolyticus* (la) at 35°, 40˚C and 45˚C.

Figure 8 and Figure 9 below give the response surface plots for the variation of pH with time and temperature for *L. delbrueckii* and *L. amylolyticus* respectively. A curvilinear dependence of pH on time and temperature was established for both bacteria.

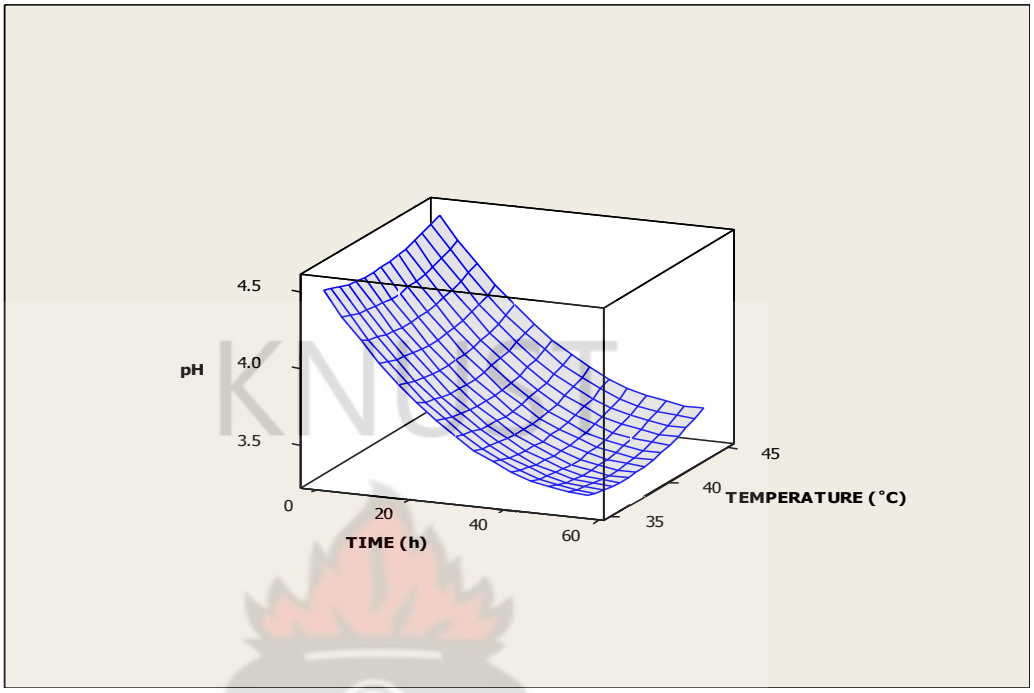

Figure 8: Variation of pH with time and temperature during lactic acid fermentation by *L. delbrueckii*

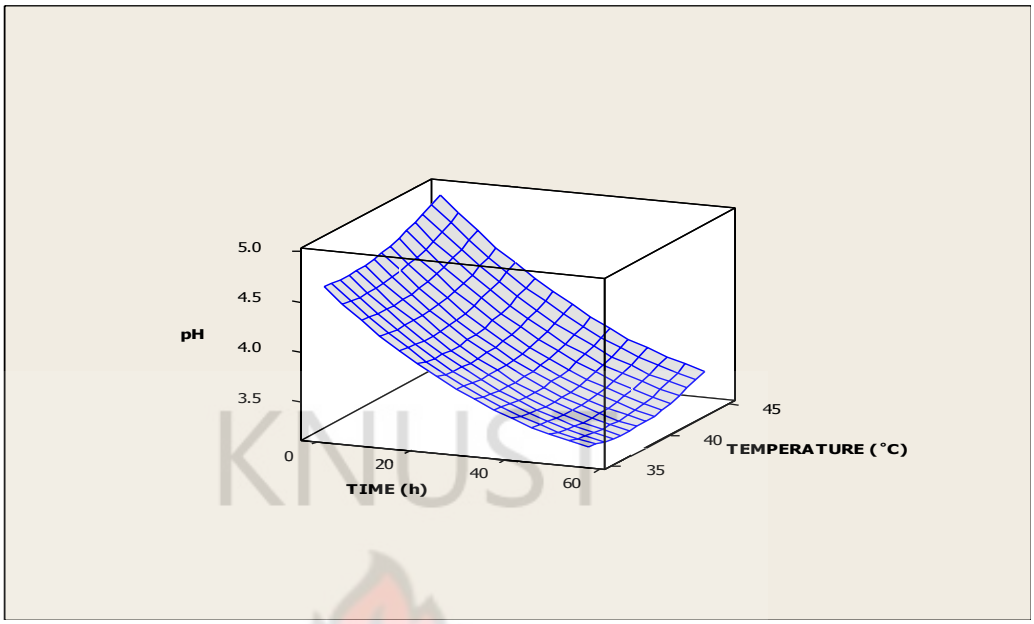

Figure 9: Variation of pH with time and temperature during lactic acid fermentation by *L. amylolyticus*.

3.3.3 Effect of time and temperature on extract utilization

Response surface regression analysis and analysis of variance of the data on the dependence of extract utilization with time and temperature are given in Tables 12 and 13 respectively for *L. delbrueckii* and for *L. amylolyticus* in Tables 14 and 15.

Table 12: Response Surface Regression analysis of Extract, % versus Time, h, Temp, deg C, for *L. delbrueckii*

Estimated Regression Coefficients for EXTRACT, % *L. delbrueckii*

Term	Coef	SE Coef	T	P
Constant	10.9573	0.09175	119.420	<0.001
Time, (h)	-0.7069	0.06749	-10.475	<0.001
TEMP, (deg C)	0.2750	0.05411	5.082	<0.001
TIME, (h)*TIME,(h)	0.3622	0.11811	3.066	0.007
TEMP, (deg C)*TEMP(deg C)	0.4375	0.09373	4.668	<0.001
TIME, (h)*TEMP, (deg C)	0.3958	0.08266	4.789	<0.001

S = 0.2165 R-Sq = 91.3% R-Sq(adj) = 88.9%

Table 13: Analysis of Variance for variation of Extract with Time and Temperature for L. *delbrueckii*

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	5	8.88623	8.88623	1.77725	37.93	<0.001
Linear	2	6.35050	6.35050	3.17525	67.77	<0.001
Square	2	1.46133	1.46133	0.73066	15.59	<0.001
Interaction	1	1.07440	1.07440	1.07440	22.93	<0.001
Residual Error	18	0.84335	0.84335	0.04685		
Total	23	9.72958				

Table 14: Response Surface Regression analysis of Extract, % versus Time, h, Temp, deg C, for *L. amylolyticus*

Estimated Regression Coefficients for EXTRACT, *L. amylolyticus*

Term	Coef	SE Coef	T	P
Constant	15.8844	0.5029	31.584	<0.001
Time, (h)	0.5819	0.3699	1.573	0.133
TEMP, (deg C)	1.9000	0.2966	6.406	<0.001
TIME, (h)*TIME,(h)	-1.3927	0.6474	-2.151	0.045
TEMP, (deg C)*TEMP(deg C)	-1.0125	0.5137	-1.971	0.064
TIME, (h)*TEMP, (deg C)	0.4083	0.4531	0.901	0.379

S = 1.186 R-Sq = 74.6% R-Sq(adj) = 67.5%

Table 15: Analysis of Variance for variation of Extract % with Time and Temperature for *L. amylolyticus*

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	5	74.3687	74.3687	14.8737	10.57	<0.001
Linear	2	61.2434	61.2434	30.6217	21.75	<0.001
Square	2	11.9820	11.9820	5.9910	4.26	0.031
Interaction	1	1.1433	1.1433	1.1433	0.81	0.379
Residual Error	18	25.3376	25.3376	1.4076		
Total	23	99.7063				

Extract utilization by *L. delbrueckii* is significantly influenced by time ($p < 0.001$), temperature ($p < 0.001$), the square of time ($p = 0.007$), the square of temperature ($p < 0.001$) and the interaction between time and temperature ($p < 0.001$). The variation within the data was adequately explained by the model (R-Sq (adj) = 88.9%). This indicates that time and temperature, have major impact on extract utilization and should be given attention. For *L. amylolyticus*, only temperature ($p < 0.001$) and the square of time ($p = 0.045$) affected the utilization of extract. For this bacterium, time did not significantly affect the utilization of extract ($p = 0.133$).

There was a distinct difference in the trend of extract utilization by the two bacteria as indicated in the main effects plot in Figure 10. Lactic acid bacteria utilize sugars, the main constituent of extract material in wort to produce lactic acid (Fossi and Tavea, 2013). In line with this extract level is expected to decrease with the formation of lactic acid as was observed for *L. delbrueckii*. However, for *L. amylolyticus*, there was an initial increase in extract, followed by a decrease and then an increase. This observation can be explained by the ability of *L. amylolyticus*, an amylolytic lactic acid bacterium (ALAB), to

convert starch and dextrines into sugars (Kunze, 2004a). *L. amylolyticus* initially must have converted residual starch and dextrines in the wort into sugars at a faster rate than the sugar was being converted into lactic acid resulting in a net increase in extract.

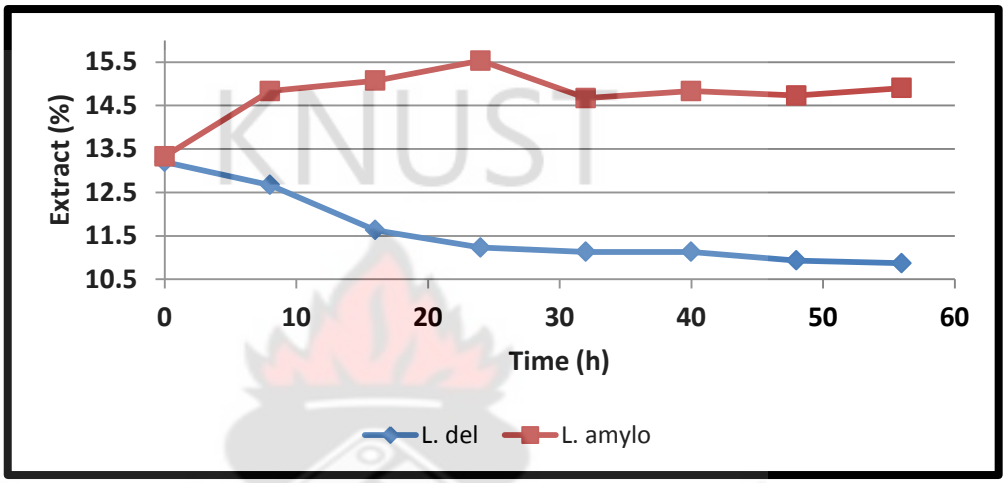

Figure 10: Utilization of extract with time for *L. delbrueckii* and *L. amylolyticus*.

Extract utilization at 35°C, 40°C and 45°C for the two bacteria during lactic acid fermentation is illustrated in Figure 11. At the higher temperatures of 40°C and 45°C a lot more residual extract was left for the subsequent alcoholic fermentation by yeast by *L. amylolyticus* than by *L. delbrueckii*. *L.*

amylolyticus at 35°C followed a similar profile of extract utilization with time as for *L. delbrueckii* at 35°C, 40°C and 45°C.

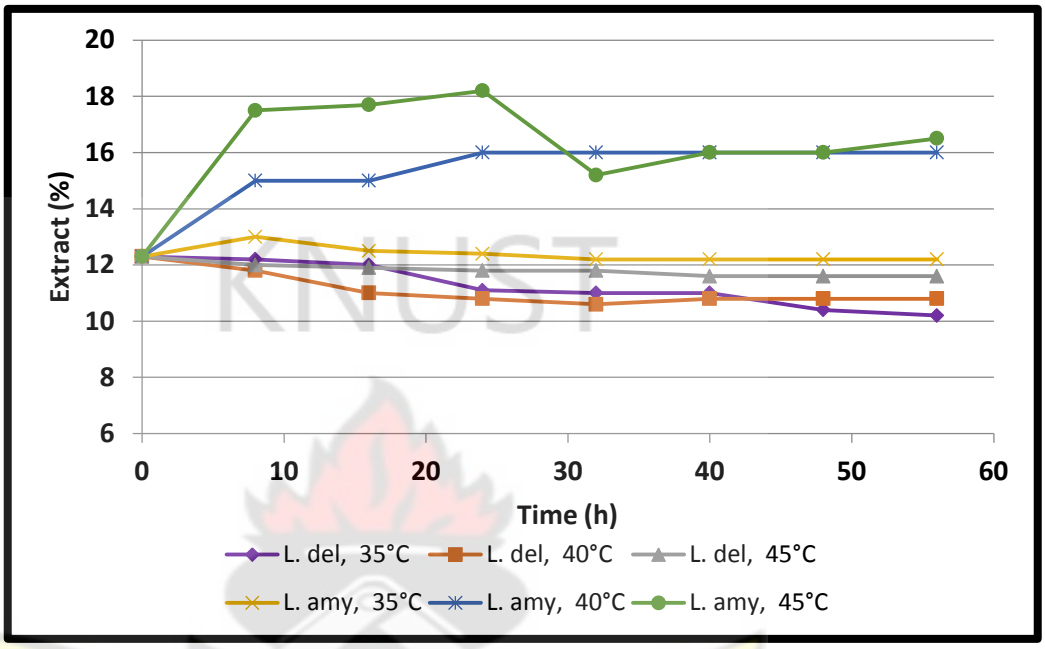

Figure 11: Comparison of the utilization of extract with time for *L. delbrueckii* and *L. amylolyticus* at 35°C, 40˚C and 45˚C

The surface plots for the variation of extract in Figure 12 and Figure 13 showed a curvilinear relationship with time and temperature depicted as inversions of each other reflecting the characteristic of *L. delbrueckii* as a solely extract consumer and *L. amylolyticus* as a net extract producer.

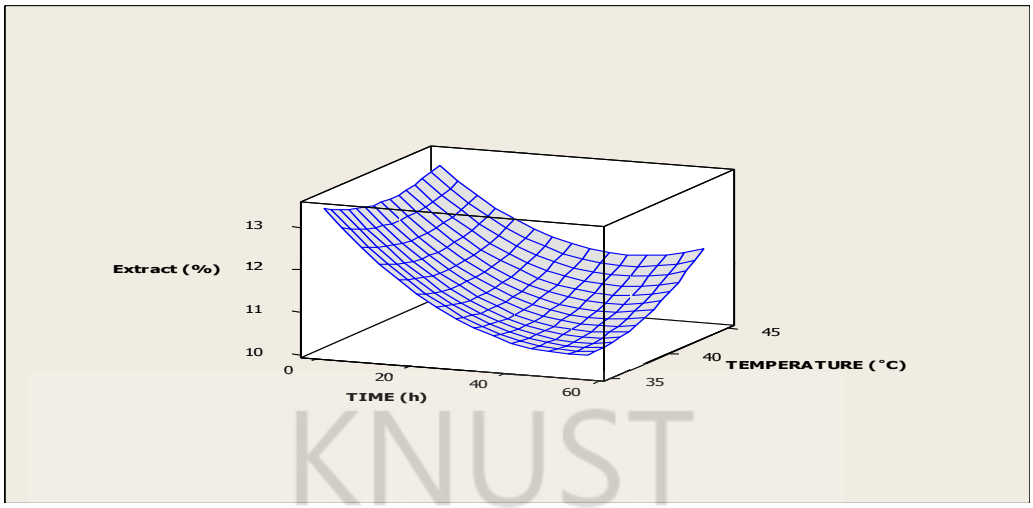

Figure 12: Utilization of extract with time and temperature during lactic acid fermentation by *L. delbrueckii*.

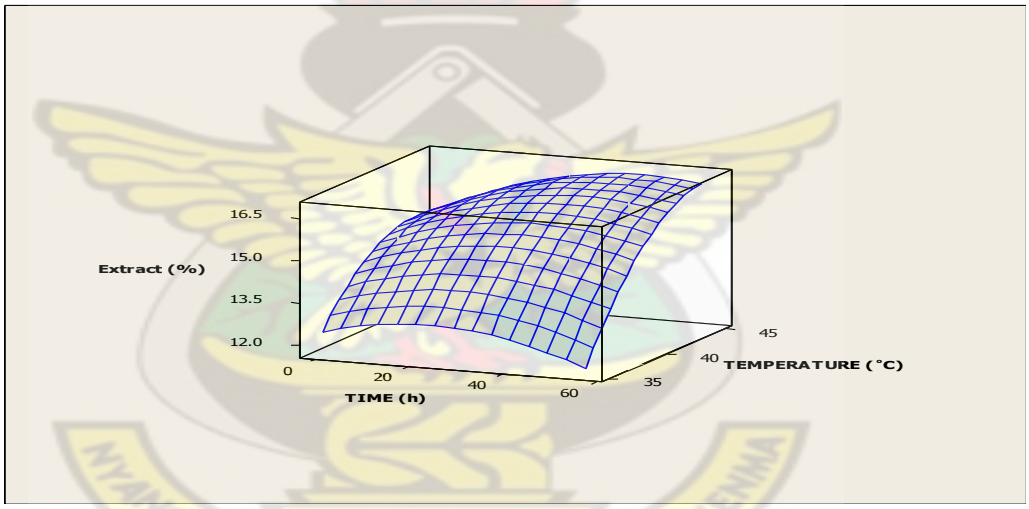

Figure 13: Utilization of extract with time and temperature during lactic acid fermentation by *L. amylolyticus*.

3.3.4 Optimization of conditions for souring

From overlaid contour plots, the optimal conditions of time and temperature for each of the lactic acid bacteria under study to attain the established target sour point of *pito* at pH level of 4,0 to 4.2 and lactic acid level of between 0.4

and 0.6% (UNIDO, 2007) were derived. Table 16 indicates the derived conditions from Figure 14 and Figure 15 which are the overlaid contour plots for *L. delbrueckii* and *L. amyloliticus* respectively. In both cases, the highest temperature in the feasible region of the plot was chosen to keep out the possible growth of any mesophilic contaminating microorganisms which are known to be responsible for early spoilage of the beer and which do not thrive at temperatures above 40°C (Haggblade and Holzapfel, 1989c).

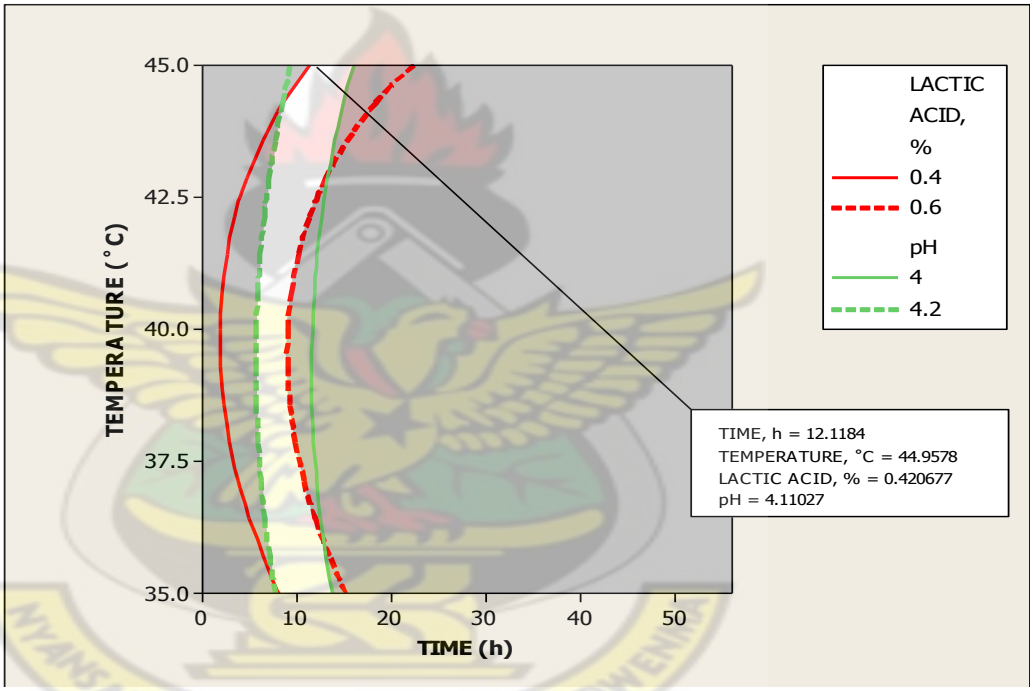

Figure 14: Overlaid contour plot showing the area (White) where conditions for attaining the targeted sour point of *pito* are achievable for *L. delbrueckii*.

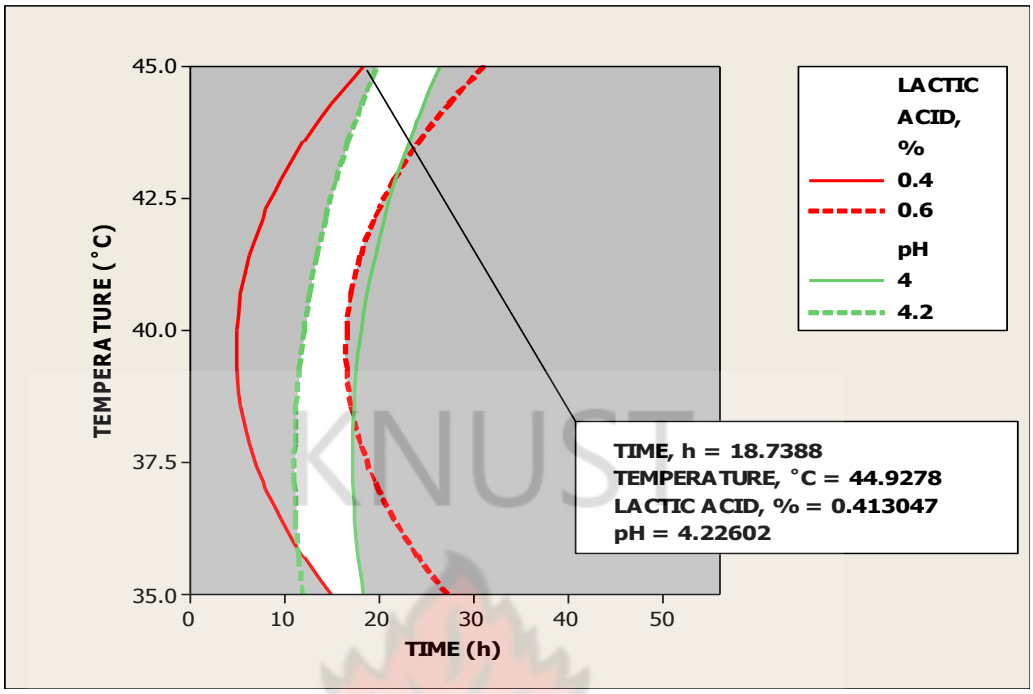

Figure 15: Overlaid contour plot showing the area (White) where conditions for attaining the targeted sour point of *pito* are achievable for *L. amylolyticus*.

Both investigated lactic acid bacteria were capable of achieving the desired sour point pH and lactic acid level of traditional *pito* (4.2 to 4.0 and 0.4 to 0.6 % respectively) at a temperature of 44.9 ˚C at which spoilage causing mesophiles do not thrive (Haggblade and Holzapfel, 1989c). However, using processing basis for choice of the one that will bring about the souring more efficiently for industrial application, *L. delbrueckii* is preferable over *L. amylolyticus* as it brings about the souring almost 3 hours shorter than *L. amylolyticus*.

Table 16: Optimal conditions for attaining Sour Point of *pito* wort by *L. delbrueckii* and *L. amylolyticus*

	Target Sour Point Conditions for Traditional *Pito* Wort (UNIDO, 2007)	**Optimal Sour Point Conditions Derived experimentally for *L. delbrueckii***	**Optimal Sour Point conditions derived experimentally for *L. amylolyticus***
Souring Culture	**Mixed microflora from sorghum malt and brewing environment**	**Single strain culture of *L. delbrueckii***	**Single strain culture of *L. amylolyticus***
Lactic acid %	**0.43**	**0.42**	**0.41**
pH	**4.0**	**4.1**	**4.2**
Souring duration, (h)	**16**	**12.08**	**18.9**
Souring Temp, (˚C)	**28**	**44.9**	**44.9**

According to Pratt *et al*. (2003), breweries all over the world, are continually seeking ways to reduce capital expenditure, labour, utilities, effluents and other operational costs while ensuring that the quality of their beers remains consistently high. A longer processing time at 44.9˚C comes with additional

the cost of heating energy, which must be kept at a minimum in an industrial production outfit. A shorter processing time, i.e. residence time in the fermenting vessel also results in a higher output of product over time, i.e. higher productivity. And according to Kunze (2004a), beer must be fermented and matured in the shortest possible time to make the processing plant economically viable. These economic benefits justify the choice of *L. delbrueckii* over *L. amylolyticus.*

Table 17 gives the souring duration and temperature for the industrial production of opaque sorghum beer in South Africa for comparison. In the use of mixed microflora from malt as the souring inoculum, the duration of souring is overnight (18h) just as for traditional *pito*. The shorter souring times for the iJuba process factory brewed beer is on account of the very high concentration of lactic acid bacteria starter culture employed (Haggblade and Holzapfel, 1989c); lactic acid production increases with increasing cell population density of the inoculating culture. In this study, supplier instructions for propagating and inoculation of the lactic acid bacteria were used for each of the lactic acid bacteria studied (Appendix 1 and 2).

Table 17: Souring duration and temperatures for South African opaque sorghum beers

	Home brewed with home malt	Factory brewed with industry malt	Home brewed with Commercial Malt	iJuba Type
Souring Temperature ˚C	30-60	49	Heated mash, temperature not specified	50
Duration of Souring, h	Overnight (12h)	8 -18	Overnight (12h)	4
Souring microorganism	Mixed microflora from malt	Mixed microflora from malt	Mixed microflora from malt	Concentrated culture of lactic acid bacteria

Source: Haggblade and Holzapfel, (1989c)

Souring temperatures between 48 and 50˚C are used for industrial souring in South Africa (Haggblade and Holzapfel, 1989c). The optimum lactic acid production temperature of lactic acid bacteria is strain specific. The two strains investigated in this study did not have their optimal lactic acid production above 40˚C. However, they produce lactic acid adequately at the 44.9˚C derived for their application to meet the souring requirement of *pito* and this temperature is also above the range (ambient to 37˚C) at which spoilage causing mesophilic lactic acid bacteria thrive.

3.3.5 Validation of the optimum conditions for souring

18 litres of sterile *pito* wort of 12.30 % extract and pH 5.0 was inoculated with 500 ml of *L. delbrueckii* starter culture resulting in 2.2 x 10^7 cells/ml population density as measured for the experimental brew and incubated at 45˚C for 12 hours as a confirmatory test to confirm the validity of the derived conditions in achieving the target sour point values of pH and lactic acid level for *pito* wort. The test was done in triplicate and yielded soured worts with average values of 0.43% lactic acid and pH of 4.0 as reported in Table 18. These values were close to the predicted values for the process and validated the predictability of the process conditions.

Table 18: Souring conditions used in confirmatory tests to validate prediction of *pito* sour point and results obtained.

Optimal Souring Conditions Derived For lactobacillus delbrueckii		**Souring Conditions Applied In Confirmatory Test Souring With lactobacillus delbrueckii**	
Temp, ˚C	45	Temp, ˚C	45
Time, h	12	Time, h	12
Initial Conditions of pito Wort Before Inoculation		**Initial Conditions of pito Wort Before Inoculation**	
Extract, %	12.3	Extract, %	12.3
pH	5.25	pH	5.25
Predicted Values For Sour Point		**Values Obtained At Sour Point**	
pH	4.1	pH	4.0
Lactic Acid, %	0.42	Lactic Acid, %	0.43

3.3.6 Conclusion and Recommendations

The two investigated lactic acid bacteria were capable of growing in *pito* wort and souring it adequately to achieve the desired sour point pH and lactic acid level of traditional *pito* (4.2 to 4.0 and 0.4 to 0.6% respectively) at a temperature of 44.9˚C for the subsequent alcoholic fermentation process. However, on the basis of the economic advantages offered by shorter process times for industrial productions, *L. delbrueckii* was selected the lactic acid bacterium of choice among the two commercial lactic acid bacteria for up-scaling the production of *pito* to industrial level. Inoculating 12.3% wort at a temperature of 45˚C with *L. delbrueckii* of cell population density of 2.2 X 10^7 cells/ml will attain the sour point in 12 hours.

L. amylolyticus with its displayed ability to convert starch and dextrines in *pito* wort into extract (sugars) would be suitable for souring *Frafra* type of *pito* which is characterized by its high alcohol content for which the brewing requires much higher malt input and prolonged boiling of the wort to concentrate it in order to obtain the high amount of extract necessary for the high alcohol level (UNIDO, 2007; Djameh, 2010). Since *pito* wort contains unconverted starch and dextrines, *L. amylolyticus* can be used to convert the residual starch and dextrins during the souring step to provide the needed additional extract for fermentation at a lower cost by dispensing with the extra malt input and extra wort boiling.

CHAPTER FOUR

Alcoholic fermentation capacity of single strain starter cultures of commercial brewers' Yeast, *Saccharomyces Cerevisiae* in *Pito* wort.

4.1 Introduction

Yeast is a unicellular microorganism which is used for brewing beers. According to Sicard and Legras (2011) yeasts of the saccharomyces sensu stricto species complex, which range from the industrial ubiquitous yeast *Saccharomyces cerevisiae* to those that are confined to geographically limited environmental niches, have been used for thousands of years by mankind for fermenting food and beverages. This complex which is reported to contain some of the most relevant species and strains for the fermentation industry represents the main group from which yeasts are selected for beer production. Yeasts in this group have the ability to convert sugar into ethanol and carbon dioxide (Vaughan-Martini and Martini, 2011).

The alcoholic fermentation of *pito* is carried out with yeasts recovered from a previous brew, i.e. the back slopping process. The froth of fresh foaming *pito* is also collected and dried for use as yeast containing inoculum. Some brewers also inoculate the wort with yeasts trapped into the interstices of a woven belt made from straw or sisal which is similar to the application of immobilized yeasts in modern commercial breweries for continuous fermentation in high cell density bioreactors. The fermentation is carried out at ambient temperature which varies with season, i.e. time of the year and

with location. Several studies have been carried out to identify the yeasts in *pito* brewing. Earlier studies by Demuyakor and Ohta (1991) reported *S. cerevisiae* as the predominant species (33%) in Ghanaian *pito*. Sefa-Dedeh *et al.,* (1999) reported a population of 38% as *S. cerevisiae*. Glover (2005) found a higher percentage using molecular methods. Most of the species of the other genera with which *S. cerevisiae* co-exists in the *pito,* e.g. *Pichia spp* and *Candida spp* tend to cause spoilage even though their metabolites together with those of *S. cerevisiae* collectively contribute to the flavour, i.e. aroma and taste of *pito* (Sefa-Dedeh *et al.*,1999).

Brewers' yeast are selected strains that are systematically isolated and grown as pure cultures. According to Lodolo *et al.* (2008), *S. cerevisiae* and *S. carlsbergensis* are the two species that are most often used as starter cultures in breweries. There is a distinction between yeasts belonging to *S. cerevisiae* and those belonging to *S. carlsbergensis.* They are respectively known as top fermenting yeasts and bottom fermenting yeasts. The distinction is based on their morphological, physiological and fermentation technological characteristics (Kunze, 2004b). Top fermenting yeasts are used in producing ale beers and bottom fermenting yeasts are used in producing lager beers (Iserentant, 1994). There is a difference in their temperature tolerances and hence the temperature applied during fermentations. Selection of a brewing strain for use in pitching wort is based on criteria such as fermentation behaviour, aroma profile of product, flocculation behaviour and fermentation performance. It is reported that in industrial brewing of

traditional opaque sorghum beer in South Africa, commercially produced active dried yeast is used to inoculate the wort (Novellie and De Schaepdrijver, 1986). These are strains of top fermenting *S. cerevisiae* which rapidly ferment the wort at ambient temperature (Daiber and Taylor, 1995). Beer must be fermented and matured in the shortest possible time to make the plant economically viable (Kunze, 2004a). Extract, which is the soluble material, mostly sugars and to a lesser extent protein, obtained from brewing malt is continually utilized during fermentation. The extent of conversion of extract to alcohol is the degree of attenuation (Kunze, 2004a). Some yeast strains flocculate early in the fermentation and do not achieve adequate attenuation.

In this study, fermentation performance, measured by fermentation rate and degree of fermentation, i.e. attenuation was used to select the yeast strain that can be used as a starter culture in the industrial production of *pito*. Pure strain cultures of two commercial brewers' yeast used in the industrial production of sour beers similar to *pito*, i.e. Anchor Brewers' Yeast, used in brewing sour traditional sorghum beers in South Africa and Munich Wheat Beer Yeast used in brewing sour wheat beers in Germany and Belgium were investigated for their performance in *pito* wort. Sachets of the two yeast types are depicted in Plate 4. The conditions that would optimize their performance with respect to the quality standards of *pito* were derived through Response Surface Methodology. The best performing of the two yeast types obtained

from the derived optimum performance conditions was selected for industrial application as starter culture.

Plate 4: Sachets of commercial brewers' yeast, Anchor Brewers' Yeast and Munich Wheat Beer Yeast investigated as starter cultures for their fermentation performance in pito wort.

Considering that the optimal pH range for the growth of *S. cerevisae* vary from 4 to 6 (Narendranath and Power, 2005) and that the pH of wort for alcoholic fermentation with brewers' yeast in beer production ranges from 5.0 to 5.7 (Narziss, 1985) three levels of initial pH of wort were investigated to determine if higher pH levels 4.5 and 5.0 would yield a better fermentation profile than the normal sour point fermentation pH of 4.0 for traditional *pito*. Thus, the pH ranges selected were 4.0, 4.5 and 5.0. The investigated yeasts have fermentation temperature tolerance ranging from 20˚C to 30˚C.

Therefore, the specific temperature within the range which would be optimal for fermenting *pito* wort in industrial production of *pito* needed to be identified. For this reason, three temperature levels, 20˚C, 25˚C and 30˚C were investigated. Fermentation at a temperature close to ambient temperature, 30 ˚C would reduce the energy costs of cooling the wort soured at 45˚C to the alcoholic fermentation temperature. Similarly, productivity will be higher with a shorter fermentation time than with a longer one. The objective was to find the fermentation conditions of temperature and time that would best yield end and product defining characteristics typical of a full bodied *pito,* i.e. pH of 3.6 to 3.8 and alcohol content of 3.0 to 3.5 % starting with an extract, also known as Original Gravity (OG) of 12 – 13 %.

4.2 Materials and methods

4.2.1 Materials

Two strains of brewers' yeast, *S. cerevisae*, Munich Wheat Beer Yeast (MWBY) and Anchor Brewers' Yeast (ABY) were investigated for their capacity to ferment soured *pito* wort using Response Surface Methodology. Munich Wheat Beer Yeast is an active dry top fermenting ale yeast, used by commercial breweries to produce sour German and Belgian wheat beers which are similar to *pito*. It is produced by Lallemand Inc, Canada and was obtained from MoreBeer Inc, Pittsburg, California. Anchor Brewers Yeast, a dry yeast developed for sour sorghum beers which are also similar to *pito,* was obtained from Rymco (PTY) Ltd. Bunsen Street. Industria, South Africa. The product information sheets of the yeasts are given in Appendices 3, 4 and 5. Both

yeasts are commercially available on the market and whichever is found suitable for the industrial production of *pito* can be readily utilized with the derived optimal fermentation conditions of wort temperature and pH at the pitching cell population density used for the investigations as according to supplier's instruction.

4.2.2 Experimental Design

A response surface design comprising a 3 x 3 x 7 factorial design was used in the study. The factors considered for each of the two S*accharomyces cerevisiae* strains (Munich Wheat Beer Yeast and Anchor Brewers' Yeast*)* were Initial pH of Wort (4.0, 4.5, 5.0), Fermentation Temperature (20°C, 25°C, 30°C) and Time (0h, 12h, 24h, 36h, 48h, 60h, 72h). The measurable indicators were pH and percent extract. The total runs comprising 63 for the entire design had each run replicated twice and the mean values used in the analyses. The same design was run for both Munich Wheat Beer Yeast and Anchor Brewers' Yeast.

4.2.3 Preparation of samples

Pito wort, six litres with 12.4 % extract and pH of 5.1 was boiled to sterilize and divided into three lots of 1800ml in glass flasks. Each lot of the 1800 ml was cooled to 40˚C and inoculated with 200 ml starter culture of *L. delbrueckii* and incubated at 40˚C. The pH of each lot was determined at 30 minute intervals and the incubation terminated when a pH level 4.0 was attained. The pH of the wort in one flask was adjusted to 4.5 and in a second

flask to 5.0 with 50% NaOH solution. The pH in the third flask was maintained at 4.0. The wort in each flask was then re-boiled to sterilize and concentrate to an extract of 13.2% and subsequently divided equally (330 ml) into six 500 ml Erlenmeyer flasks fitted with fermentation locks. One set of 9 flasks was pitched with Munich Wheat Beer Yeast (MWBY) and the other set of nine with Anchor Brewers' Yeast (ABY) after which the flasks were placed in controlled temperature rooms to ferment at 20˚C, 25˚C and 30˚C according to the Experimental Design indicated in Table 19. The pitching rate for both yeasts was 0.1% m/v, i.e. 0.1g in 100 ml as recommended by the suppliers. The dry ABY was sprinkled directly on the surface of the wort but MWBY was rehydrated according to the supplier's instruction and the slurry added to the wort. The product data and usage instruction for the yeasts are shown in Appendices 3, 4 and 5.

4.2.4 Determinations

Samples were taken from each flask at regular intervals (0h, 12h, 24h, 36h, 48h, 60h, 72h) to measure pH and Extract.

4.2.5 Analysis of Data

The experimental data was statistically evaluated using regression analysis and ANOVA. The adequacy of fit was evaluated with the coefficients of determination R^2 and the adjusted R^2. The fermentation capacity of each yeast was investigated using fermentation temperature, initial pH of the wort, (F pH) and fermentation time as factors with Extract and pH of the fermenting wort,

(R pH) as the responses. The numerical values for the factors, i.e. fermentation time and temperature that would yield the desired pH and Extract level typical of fresh *pito* were derived by prediction from overlaid contour plots. The derived values for the two yeasts, i.e. fermentation conditions were used to calculate their fermentation capacity, i.e. rate of fermentation and apparent degree of fermentation (attenuation). The calculated fermentation capacities of the yeasts were compared to enable the selection of the suitable one for use in the industrial production of *pito*. To check the adequacy of the model for the derivation of the fermentation conditions, test brews were made using the derived optimized values for fermentation time and temperature and comparing the experimental results with the predicted target values.

4.3 Results and Discussion

The data from the factorial experiments with Anchor Brewers' Yeast and Munich Wheat Beer Yeast to determine the important factors in their fermentation process are given in Tables 19 and 20 respectively.

Table 19: Response data for the factorial experiment for Anchor Brewers' Yeast (ABY).

ABY	FACTORS			RESPONSES			
RUN	TEMP °C	pH	TIME, h	pH, MEAN	pH, STDEV	EXTRACT %, MEAN	EXTRACT %, STDEV
1	20	5.0	0	5	0.14	13.2	0
2	20	5.0	12	4.65	0.04	11.6	0.1
3	20	5.0	24	4.36	0.03	11	0.3
4	20	5.0	36	4.24	0.07	9.6	0.3
5	20	5.0	48	4.12	0.04	7.8	0.3
6	20	5.0	60	4.06	0.03	7.2	0.1
7	20	5.0	72	4.02	0.11	7	0
8	25	5.0	0	5	0.06	13.2	0
9	25	5.0	12	4.22	0.04	10.4	0.3
10	25	5.0	24	4.01	0.04	8.2	0.4
11	25	5.0	36	3.89	0.13	7.4	0.3
12	25	5.0	48	3.88	0.03	7.2	0.1
13	25	5.0	60	3.8	0.07	7	0.3
14	25	5.0	72	3.78	0.06	7	0
15	30	5.0	0	5	0.06	13.2	0
16	30	5.0	12	3.98	0.08	9	0.6
17	30	5.0	24	3.73	0.03	7.4	0.3
18	30	5.0	36	3.7	0.08	7.1	0.3
19	30	5.0	48	3.58	0.04	7	0.3
20	30	5.0	60	3.57	0.08	7	0
21	30	5.0	72	3.47	0.04	7	0
22	20	4.5	0	4.5	0.11	13.2	0
23	20	4.5	12	4.25	0.04	11.6	0.3
24	20	4.5	24	4.06	0.08	11	0.3
25	20	4.5	36	3.96	0.08	9.6	0.3
26	20	4.5	48	3.9	0.07	7.4	0.3
27	20	4.5	60	3.86	0.08	7	0.4
28	20	4.5	72	3.84	0.11	7	0
29	25	4.5	0	4.5	0.06	13.2	0
30	25	4.5	12	4.02	0.03	10.8	0.3
31	25	4.5	24	3.77	0.07	8	0.3
32	25	4.5	36	3.67	0.10	7.2	0.1
33	25	4.5	48	3.71	0.08	7.2	0.3

ABY	FACTORS			RESPONSES			
RUN	TEMP °C	pH	TIME, h	pH, MEAN	pH, STDEV	EXTRACT %, MEAN	EXTRACT %, STDEV
34	25	4.5	60	3.63	0.08	7	0
35	25	4.5	72	3.63	0.06	7	0
36	30	4.5	0	4.5	0.03	7	0
37	30	4.5	12	4.25	0.13	13.2	0
38	30	4.5	24	3.65	0.10	10	0.3
39	30	4.5	36	3.58	0.04	7.4	0.1
40	30	4.5	48	3.47	0.08	7.2	0.1
41	30	4.5	60	3.46	0.08	7	0
42	30	4.5	72	3.38	0.14	7	0
43	20	4.0	0	4	0.06	13.2	0
44	20	4.0	12	3.98	0.04	12.2	0.3
45	20	4.0	24	3.79	0.06	10.4	0.3
46	20	4.0	36	3.74	0.11	8.4	0.3
47	20	4.0	48	3.7	0.07	7.2	0.3
48	20	4.0	60	3.66	0.13	7	0
49	20	4.0	72	3.62	0.14	7	0
50	25	4.0	0	4	0.03	13.2	0
51	25	4.0	12	3.77	0.13	11	0.4
52	25	4.0	24	3.65	0.10	8.2	0.4
53	25	4.0	36	3.61	0.10	7.5	0.1
54	25	4.0	48	3.62	0.08	7.4	0.1
55	25	4.0	60	3.55	0.08	7	0
56	25	4.0	72	3.55	0.18	7	0
57	30	4.0	0	4	0.04	13.2	0
58	30	4.0	12	3.76	0.11	10	0.4
59	30	4.0	24	3.55	0.10	8	0.4
60	30	4.0	36	3.51	0.13	7.8	0.3
61	30	4.0	48	3.43	0.11	7.2	0.3
62	30	4.0	60	3.38	0.06	7	0
63	30	4.0	72	3.35	0.08	7	0

Table 20: Response data for the factorial experiment for Munich Wheat Beer Yeast (MWBY)

MWBY	FACTORS			RESPONSES			
RUN	TEMP °C	pH	TIME, h	pH, AVG	pH, STDEV	EXTRACT %, AVG	EXTRACT %, STDEV
1	20	5.0	0	5	0	13.2	0
2	20	5.0	12	4.78	0.06	11	0.6
3	20	5.0	24	4.51	0.06	10.4	0.6
4	20	5.0	36	4.42	0.06	9	0.3
5	20	5.0	48	4.27	0.07	7	0.4
6	20	5.0	60	4.22	0.06	6.5	0.4
7	20	5.0	72	4.18	0.03	6	0.3
8	25	5.0	0	5	0.00	13.2	0
9	25	5.0	12	4.22	0.17	10.4	0.6
10	25	5.0	24	4.18	0.07	8.4	0.6
11	25	5.0	36	4.16	0.04	6.8	0.1
12	25	5.0	48	4.09	0.04	6	0.3
13	25	5.0	60	4	0.00	6	0
14	25	5.0	72	4	0.06	6	0
15	30	5.0	0	5	0.00	13.2	0
16	30	5.0	12	4.27	0.07	10	0.6
17	30	5.0	24	3.97	0.04	7.8	0.3
18	30	5.0	36	3.9	0.07	6.4	0.6
19	30	5.0	48	3.85	0.08	6	0
20	30	5.0	60	3.84	0.03	6	0
21	30	5.0	72	3.8	0.03	6	0
22	20	4.5	0	4.5	0.00	13.2	0
23	20	4.5	12	4.4	0.14	11.4	0.3
24	20	4.5	24	4.25	0.04	10.8	0.3
25	20	4.5	36	4.16	0.04	9	0.3
26	20	4.5	48	3.99	0.16	7.4	0.1
27	20	4.5	60	3.98	0.01	6.5	0.1
28	20	4.5	72	3.96	0.03	6	0
29	25	4.5	0	4.5	0.00	13.2	0
30	25	4.5	12	4.21	0.10	10.4	0.3
31	25	4.5	24	4.06	0.06	8.4	0.4
32	25	4.5	36	3.9	0.06	6.8	0.3
33	25	4.5	48	3.89	0.06	6	0.1

MWBY	FACTORS			RESPONSES			
RUN	TEMP °C	pH	TIME, h	pH, MEAN	pH, STDEV	EXTRACT %, MEAN	EXTRACT %, STDEV
34	25	4.5	60	3.84	0.03	6	0.0
35	25	4.5	72	3.84	0.01	6	0.0
36	30	4.5	0	4.5	0.00	13.2	0.0
37	30	4.5	12	4.1	0.17	10.4	0.3
38	30	4.5	24	3.94	0.03	7.4	0.4
39	30	4.5	36	3.9	0.03	6.4	0.1
40	30	4.5	48	3.8	0.03	6	0.0
41	30	4.5	60	3.8	0.03	6	0.0
42	30	4.5	72	3.79	0.01	6	0.0
43	20	4.0	0	4	0.00	13.2	0.0
44	20	4.0	12	4	0.04	11.4	0.6
45	20	4.0	24	3.98	0.03	10.4	0.4
46	20	4.0	36	3.93	0.04	9	0.3
47	20	4.0	48	3.83	0.04	6.6	0.4
48	20	4.0	60	3.8	0.00	6	0.0
49	20	4.0	72	3.75	0.04	6	0.0
50	25	4.0	0	4	0.00	13.2	0.0
51	25	4.0	12	3.9	0.14	10.8	0.4
52	25	4.0	24	3.81	0.10	8.8	0.6
53	25	4.0	36	3.74	0.11	7	0.3
54	25	4.0	48	3.73	0.04	6	0.0
55	25	4.0	60	3.69	0.04	6	0.0
56	25	4.0	72	3.69	0.06	6	0.0
57	30	4.0	0	4	0.00	13.2	0.0
58	30	4.0	12	3.83	0.06	10	0.4
59	30	4.0	24	3.71	0.01	7.2	0.6
60	30	4.0	36	3.69	0.06	6.4	0.3
61	30	4.0	48	3.65	0.01	6.2	0.1
62	30	4.0	60	3.64	0.01	6	0.0
63	30	4.0	72	3.62	0.01	6	0.0

4.3.1 Effect of fermentation temperature, time and initial pH on extract of fermenting wort

The results of the regression analysis are reported in Tables 21, 22, 23, and 24 indicated high values of coefficients of determination (R^2 = 93.8%, R^2 adjusted = 92. 7%) for Anchor Sorghum Beer Yeast and (R^2 = 96.3%, R^2 adjusted = 95.7%) for Munich Wheat Beer Yeast. This indicated that the model was adequately fitted to explain variances in the experimental data and capable of use for predictive purposes. Analysis of variance (ANOVA) also confirmed the significance of the model.

Table 21: Estimated Regression Coefficients for R Extract (%) for Anchor Brewers Yeast

Term	Coef	SE Coef	T	P
Constant	7.55714	0.19806	38.156	<0.001
F Temp, (˚C)	-0.54524	0.09639	-5.657	<0.001
F Ph	-0.03333	0.09639	-0.346	0.731
F Time, (h)	-2.95952	0.11805	-25.070	<0.001
F Temp,(˚C)*F Temp,(˚C)	0.34524	0.16695	2.068	0.044
F pH*F pH	0.06667	0/16695	0.399	0.691
F Timw,(h)*F Time,(h)	2.27857	0.20447	11.144	0.001
F Time,(˚C)*F pH	-0.16071	0.11805	-1.361	0.179
F Temp, (˚C)*F Time,(h)	0.37143	0.14458	2.569	0.013
F pH*F Time,(h)	0.08929	0.14458	0.618	0.540
S = 0.6247 R-Sq = 93.8% R-Sq(adj) = 92.7%				

Table 22: Analysis of Variance for Response Extract% verus Factor Temp, pH and Time for Anchor Brewers' Yeast

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	311.414	311.414	34.6016	88.67	<0.001
Linear	3	257.778	257.778	85.9262	220.20	<0.001
Square	3	50.188	50.188	16.7295	42.87	<0.001
Interaction	3	3.447	3.447	1.1491	2.94	0.041
Residual Error	53	20.681	20.681	0.3902		
Total	62	332.096				

Table 23: Estimated Regression Coefficients for R Extract (%) Munich Wheat Beer Yeast

Term	Coef	SE Coef	T	P
Constant	7.22804	0.17170	42.098	<0.001
F Temp, (˚C)	-0.57619	0.08356	-6.896	<0.001
F pH	-0.01667	0.08356	-0.199	0.843
F Time, (h)	-3.53095	0.10234	-34.503	<0.001
F Temp, (˚C) *F Trmp,(˚C)	0.30952	0.14473	2.139	0.037
F pH*F pH	-0.06905	0.14473	-0.477	0.635
F Timw,(h)*F Time,(h)	2.18333	0.17725	12.318	<0.001
F Time,(˚C)*F pH	-0.00357	0.10234	-0.035	0.972
F Temp, (˚C)*F Time,(h)	0.20000	0.12534	1.596	0.117
F pH*F Time,(h)	0.07857	0.12534	0.627	0.533

S = 0.5415 R-Sq = 96.3% R-Sq(adj) = 95.7%

Table 24: Analysis of Variance for Response Extract% versus Factor Temp, pH and Temp for Munich Wheat Beer Yeast.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	409.811	409.811	45.535	155.28	<0.001
Linear	3	363.049	363.049	121.016	412.68	<0.001
Square	3	45.899	45.899	15.300	52.17	<0.001
Interaction	3	0.862	0.862	0.287	0.98	0.409
Residual Error	53	15.542	15.542	0.293		
Total	62	425.353				

Analysis of the regression data showed that time ($p < 0.001$) and temperature ($p < 0.001$) had a significant effect on extract utilization in the fermenting *pito* wort for both Anchor Brewers' Yeast and for Munich Wheat Beer Yeast. For both yeasts, extract utilization increased with time and temperature. This observation was in agreement with the general profile of fermentation where yeasts utilize sugars continually until the sugars are depleted or until the level of the alcohol produced becomes toxic to them. Studies on fermentation of sugar by *S. cerevisiae* have shown that rate of fermentation, i.e. utilization of extract increases with temperature and correlates with the yeast growth. Yalcin and Ozbas, (2008) reported a positive linear effect of temperature for *S. cerevisiae* wine strain in the interval of 15°C to 30°C. The observed increase in extract utilization with temperature for Anchor Brewers' Yeast and Munich Wheat Beer Yeast is also in agreement with the findings of Egharevba *et al.,* (2014) in their study of fermentation kinetics of *S. cerevisiae* in cane sugar in which extract utilization, hence fermentation rate increases with temperature within the temperature optimum of the enzyme catalysing the fermentation. For both yeasts, extract utilization was not significantly affected by the initial pH of the wort. This may probably be due to fact that the pH levels investigated lie within the optimal range for the growth of *S. cerevisiae*, hence fermentation, which is known to be between 4 and 6 (Narendranath and Power, 2005). The interactions of time with temperature and initial pH also did not have any significant effects.

From the main effects plots in Figures 16, 17 and 18 it was observed that extract decreased with time for both yeasts in a similar pattern and in accordance with normal extract fermentation profile with time. The extract dropped steeply with time and then commenced to level out after 48 hours when the fermentable sugars were exhausted, i.e. attenuation was reached. After the attenuation, there was a higher level of residual extract with Anchor Brewers' Yeast (7%) than for Munich Wheat beer yeast (6%) indicating its higher capacity of fermentation in terms of ability to ferment sugars. This observed difference may be attributed to strain differences. For both yeasts, the lowest residual extract was achieved at fermentation temperature of 30°C and the highest at 20°C while fermentation at 25°C gave intermediate results. Fakruddin *et al.*, (2012) also found 30°C and pH between 5 and 6 as the optimum for maximum yield of ethanol by a strain of S. cerevisiae (IFST-072011).

The results of the regression analysis for both showed that the starting pH did not have a significant effect ($p > 0.05$) on extract utilization. In the main effects graph, Figure 18, only marginal differences were exhibited by both yeasts at an intermediate stage of the fermentation for the three starting pH levels of fermentation, i.e. 4.0, 4.5 and 5.0. Anchor Brewers' Yeast had 8.80% while Munich Wheat Beer Yeast registered 8.34%.

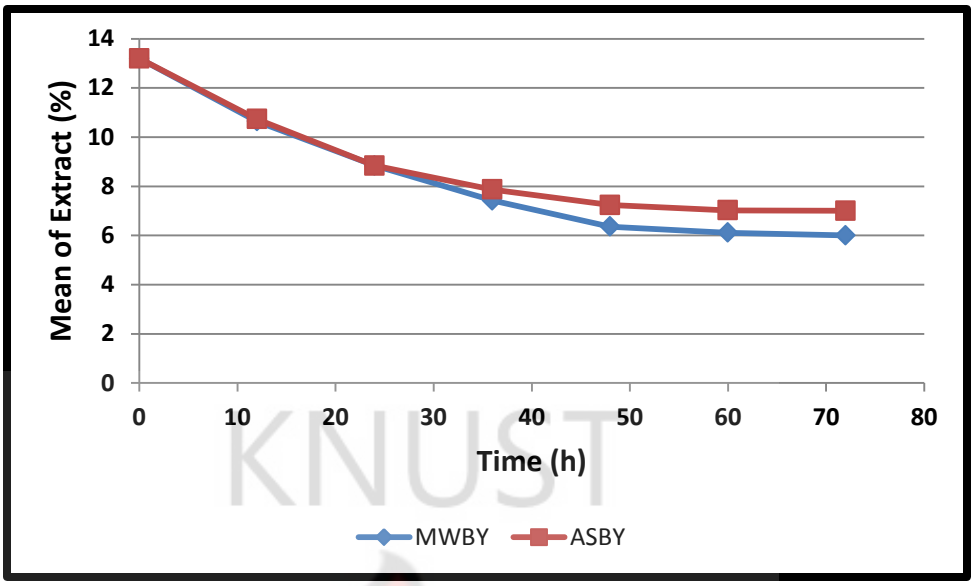

Figure 16: Extract utilization of Anchor Sorghum Beer Yeast and Munich Wheat Beer Yeast with time.

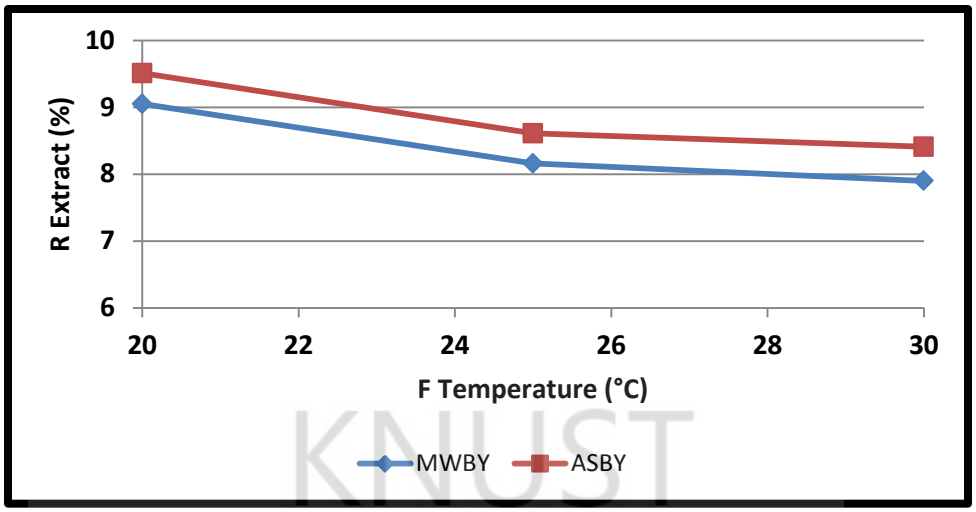

Figure 17: Extract utilization of Anchor Brewers' Yeast and Munich Wheat Beer Yeast with temperature.

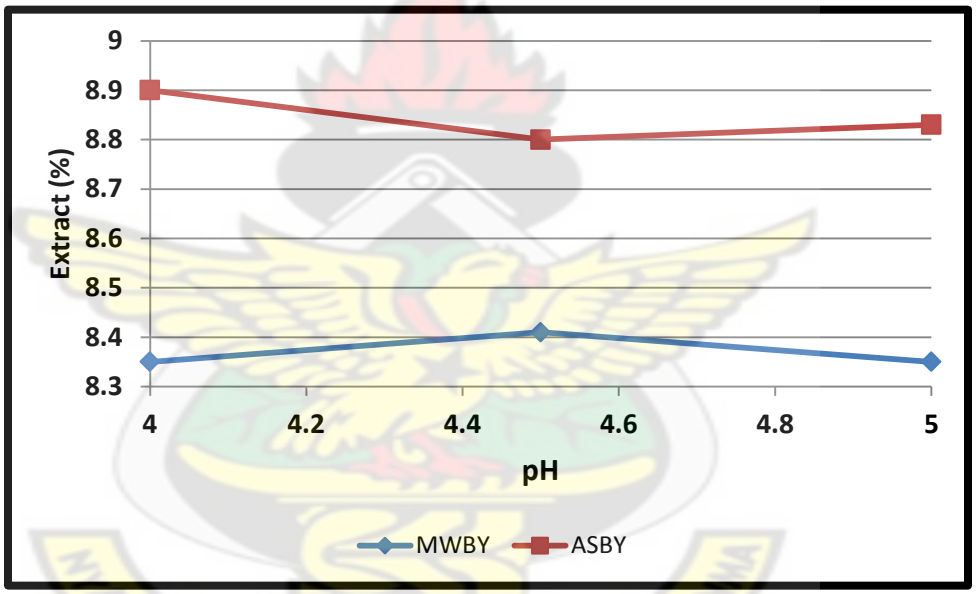

Figure 18: Extract utilization of Anchor Brewers' Yeast (A) and Munich Wheat Beer Yeast (B) with pH

The rate of extract utilization by both yeasts was faster at 30°C and 25°C than at 20°C as indicated in Figure 19. Munich Wheat Beer yeast exhibited the fastest average rate of extract utilization of 0.150% per hour at 25°C in the first 48 hours of fermentation followed by 0.146 % per hour at 30°C. The least rate

of utilization was exhibited by Anchor Brewers yeast at 20°C (0.125 % per hour). Utilization of extract (ethanol production) by a strain of *S. cerevisiae* was reported by Fakruddin *et al.*, (2012) to be fastest at 30°C.

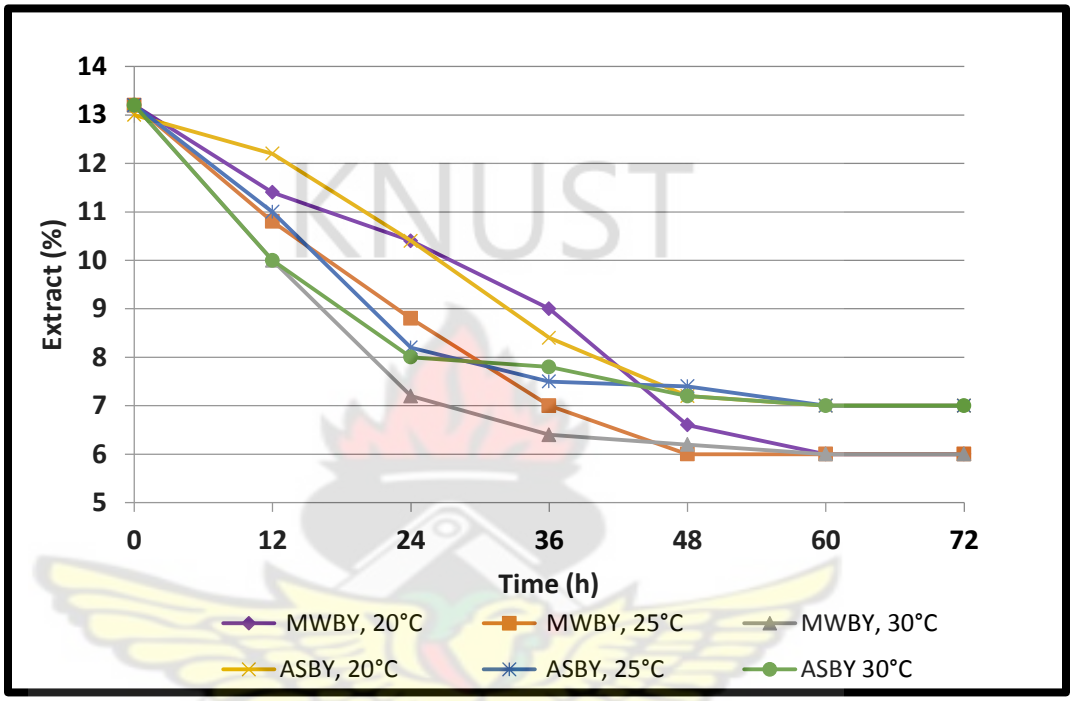

Figure 19: Comparison of the utilization of extract with time for Anchor Brewers Yeast (ABY) and Munich Wheat Beer Yeast (MWBY) at 20°C, 25˚C and 30˚C.

In Figure 20 to Figure 25, the relation between the factors and responses are depicted in the response surface plots. Again here, the similarities in the performance of the two yeasts are demonstrated by the shapes of the plots.

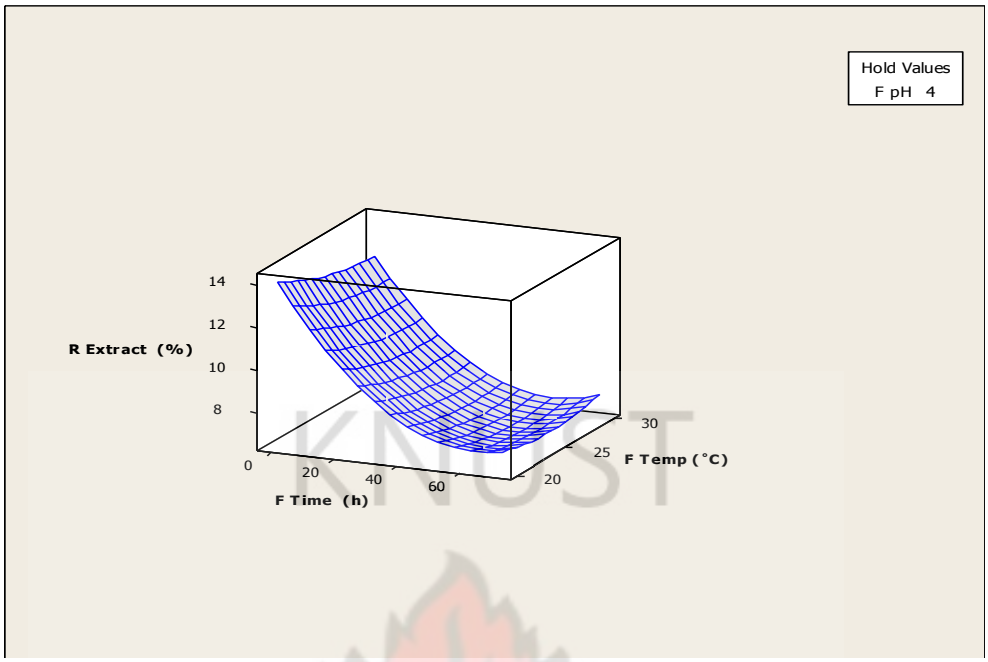

Figure 20: Utilization of Extract with Time and Temperature for ABY.

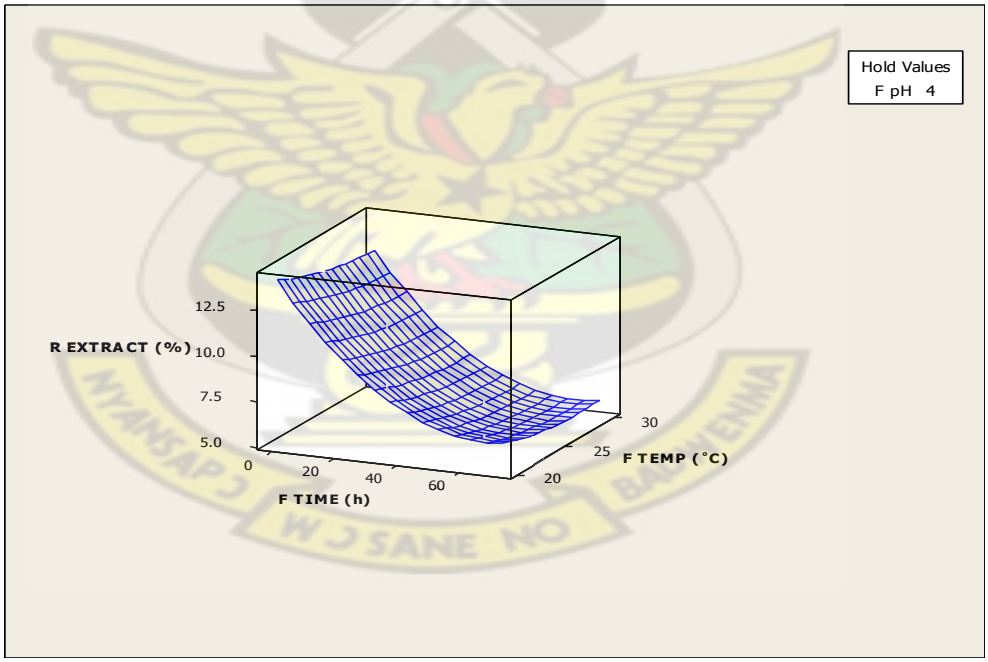

Figure 21: Utilization of Extract with Time and Temperature for MWBY.

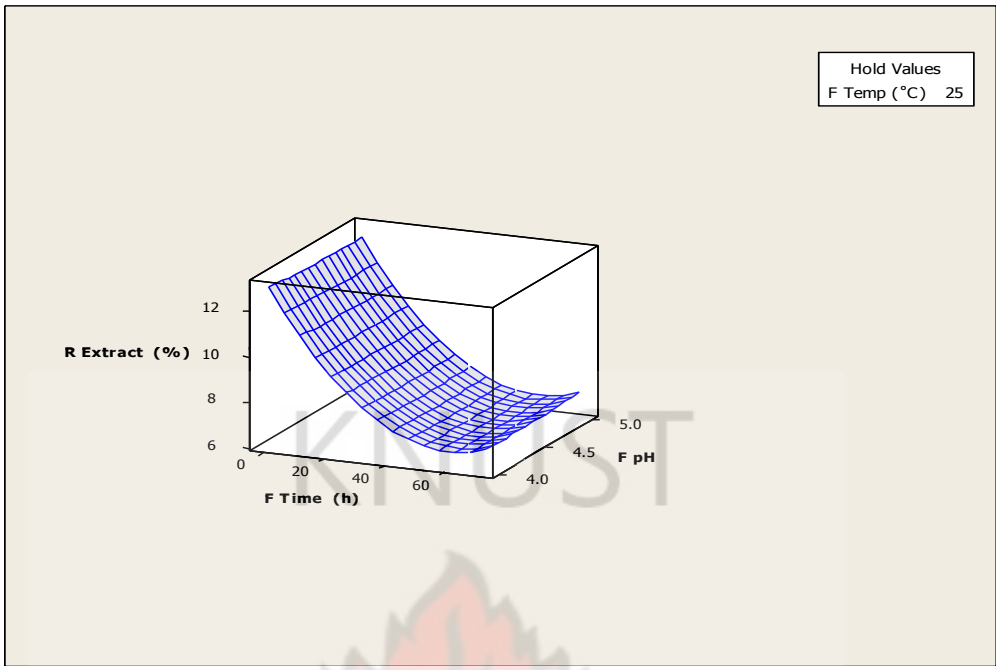

Figure 22: Utilization of Extract with Time and Factor pH for ASBY.

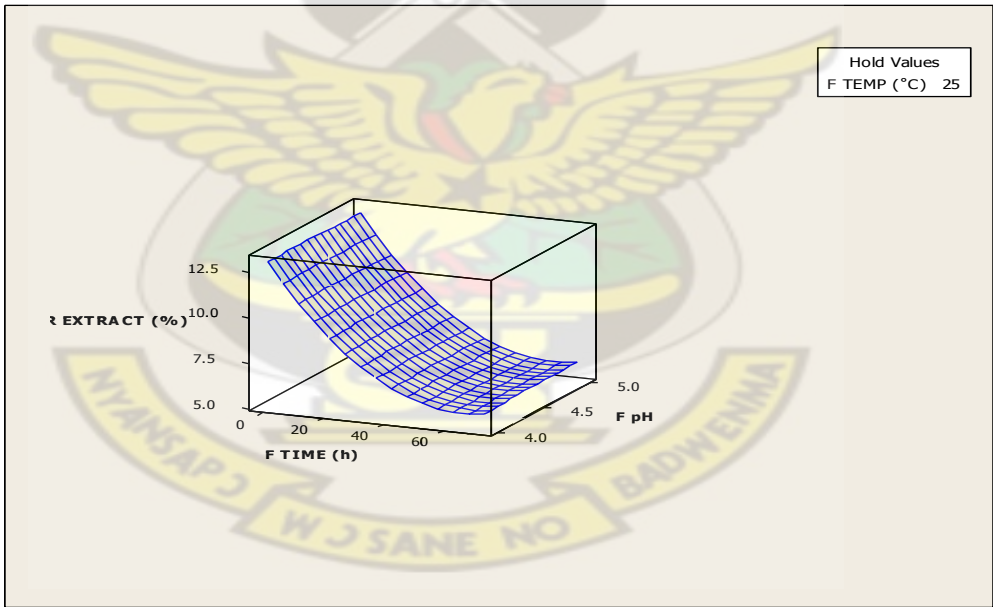

Figure 23: Utilization of Extract with Time and Factor pH for MWBY.

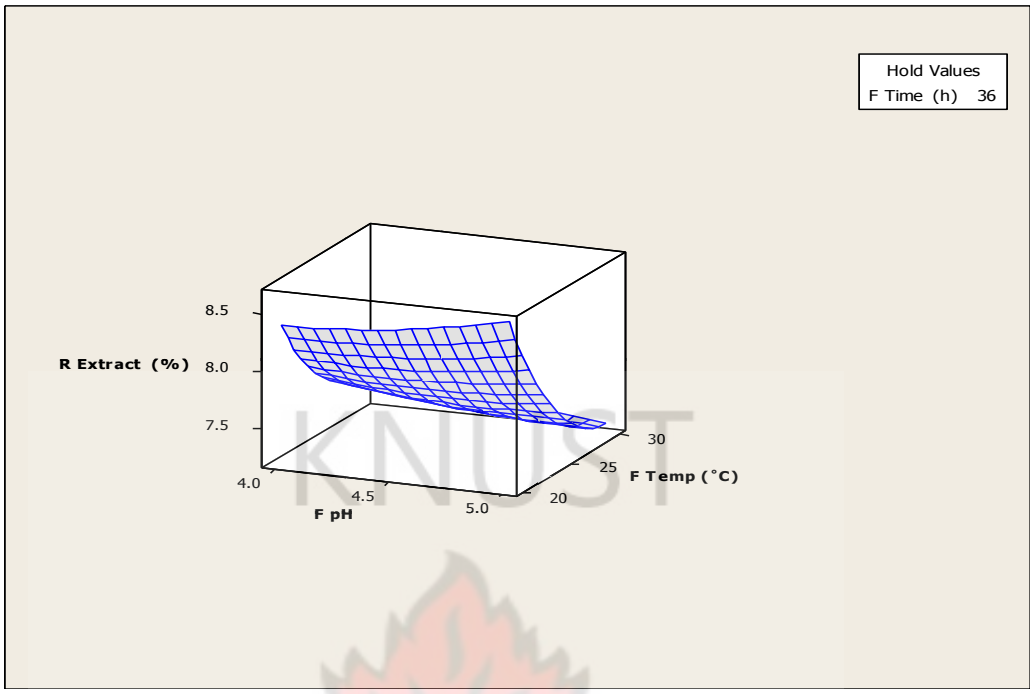

Figure 24: Utilization of Extract with Temperature and Factor pH for ASBY.

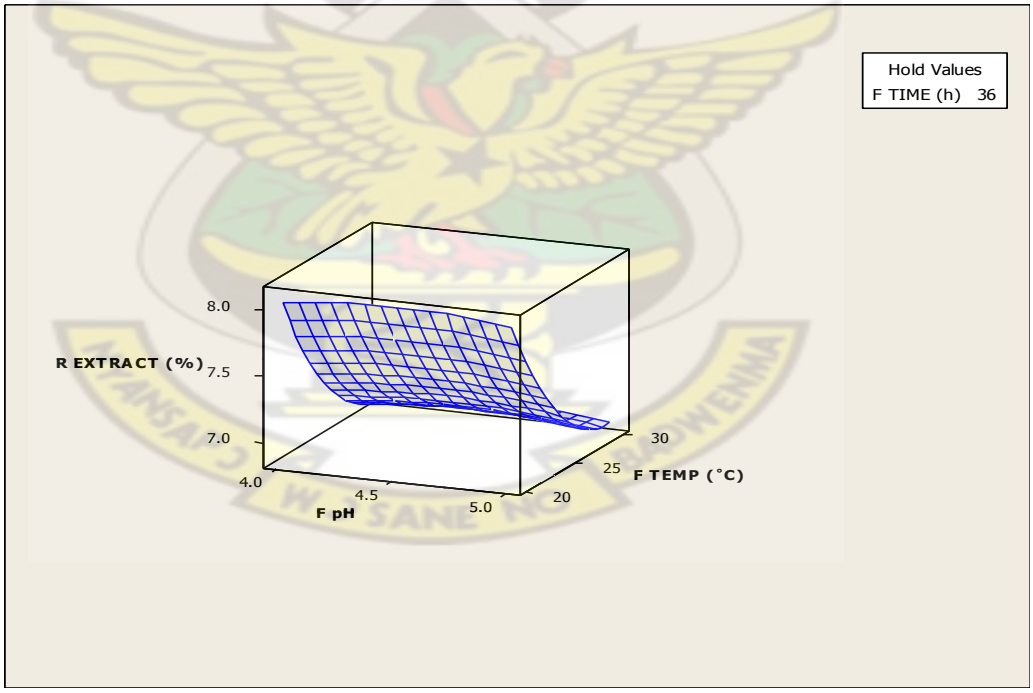

Figure 25: Utilization of Extract with Temperature and Factor pH for MWBY.

4.3.2 Effect of fermentation temperature, time and initial pH on pH of fermenting wort.

The results of the regression analysis are reported in Tables 25 and 27. The analyses of variance for both yeasts are reported in Tables 26 and 28. For both yeasts, pH of the fermenting wort (R pH) was significantly ($p < 0.001$) affected by fermentation temperature, time and initial pH, their linear interactions and the square interaction of time. The R^2 and R^2 -adjusted values of 92.7% and 91.4% respectively for Anchor Brewers' Yeast and 92.4% and 91.1% respectively for Munich Wheat Beer Yeast indicated the adequacy of fit of the model.

Table 25: Estimated Regression Coefficients for R pH Munich WBY

Term	Coef	SE Coef	T	P
Constant	3.94201	0.03125	126.128	<0.001
F Temp, (˚C)	-0.12643	0.01521	-8.312	<0.001
F pH	0.23024	0.01521	15.137	<0.001
F Time, (h)	-0.29429	0.01863	-15.798	<0.001
F Temp, (˚C) *F Temp,(˚C)	0.03833	0.02634	1.455	0.152
F pH*F pH	-0.02310	0.02634	-0.877	0.385
F Timw,(h)*F Time,(h)	0.21333	0.03227	6.612	<0.001
F Time,(˚C)*F pH	-0.05714	0.01863	-3.067	0.003
F Temp, (˚C)*F Time,(h)	-0.02125	0.02282	-0.931	0.356
F pH*F Time,(h)	-0.13607	0.02282	-5.964	<0.001
S = 0.09857 R-Sq = 92.4% R-Sq(adj) = 91.1%				

Table 26: ANOVA for R pH Munich Wheat Beer Yeast

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	6.22094	6.22094	0.6912	71.14	<0.001
Linear	3	5.32265	5.32265	1.77422	182.59	<0.001
Square	3	0.45281	0.45281	0.15094	15.53	<0.001
Interaction	3	0.44548	0.44548	0.14849	15.28	<0.001
Residual Error	53	0.51499	0.51499	0.00972		
Total	62	6.73593				

Table 27: Estimated Regression Coefficients for R pH Anchor Brewers Yeast

Term	Coef	SE Coef	T	P
Constant	3.72952	0.03625	102.882	<0.001
F Temp, (˚C)	-0.16690	0.01764	-9.461	<0.001
F pH	0.21048	0.01764	11.930	<0.001
F Time, (h)	-0.38762	0.02161	-17.940	<0.001
F Temp, (˚C) *F Temp,(˚C)	0.02595	0.03056	0.849	0.400
F pH*F pH	0.00238	0.03056	0.078	0.938
F Time,(h)*F Time,(h)	0.31143	0.03742	8.322	<0.001
F Time,(˚C)*F pH	-0.06821	0.02161	-3.157	0.003
F Temp, (˚C)*F Time,(h)	-0.07786	0.02646	-2.942	0.005
F pH*F Time,(h)	-0.14339	0.02646	-5.419	<0.001

S = 0.1143 R-Sq = 92.7% R-Sq(adj) = 91.4%

Table 28: ANOVA for R pH, Anchor Brewers Yeast

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	8.77955	8.77955	0.97551	74.63	<0.001
Linear	3	7.23757	7.23757	2.41252	184.56	<0.001
Square	3	0.91473	0.91473	0.30491	23.33	<0.001
Interaction	3	0.62726	0.62726	0.20909	16.00	<0.001
Residual Error	53	0.69281	0.69281	0.01307		
Total	62	9.47237				

The pH level of finished *pito* is important for its taste and together with the lactic acid content determines the degree of sourness and hence taste. The main effects plots of Figures 26, Figure 27 and Figure 28 indicate similar fermentation profiles for the two yeasts. The drop in pH was however faster for Munich Wheat Beer Yeast between 25°C and 30°C and initial pH of 4.5 and 5.0. This implies that the formation of organic acids which are responsible for lowering the pH of *pito* and contribute to the sourness would be fastest at higher temperatures of fermentation and at higher initial pH value of the wort. Low pH values tend to inhibit the activity of the enzymes involved in the biochemical processes as the structure of proteins of which enzymes are made, become distorted at the low pH levels. The optimal pH range for yeast growth can vary from pH 4.0 to 6.0 depending on temperature, the presence of oxygen and the strain of yeast. Optimum pH value is very important for the activity of plasma membrane-bound proteins, including enzymes and transport proteins (Narendranath and Power, 2005).

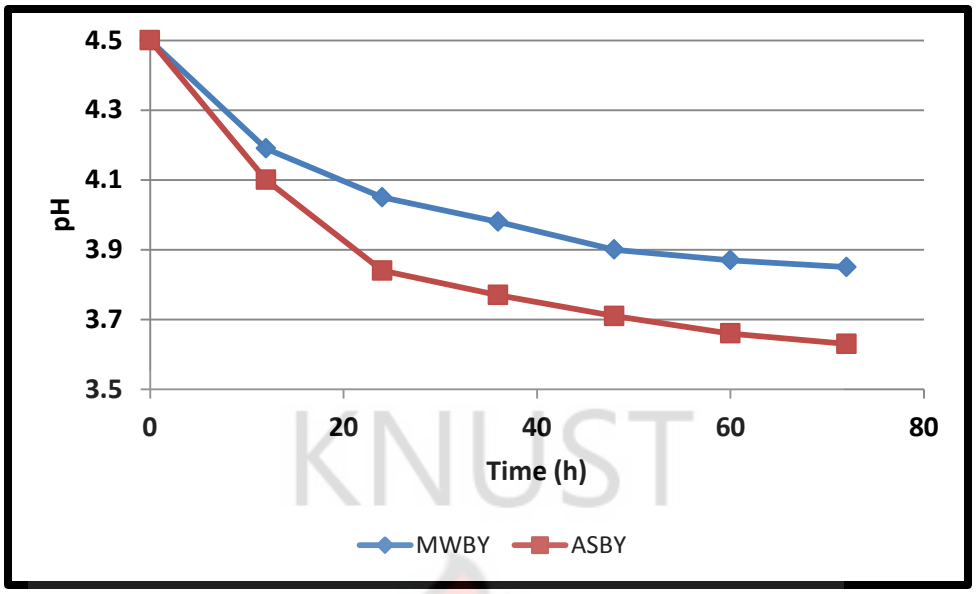

Figure 26: Variation of fermenting wort pH (R pH) with Time for Anchor Sorghum Beer Yeast and Munich Wheat Beer Yeast.

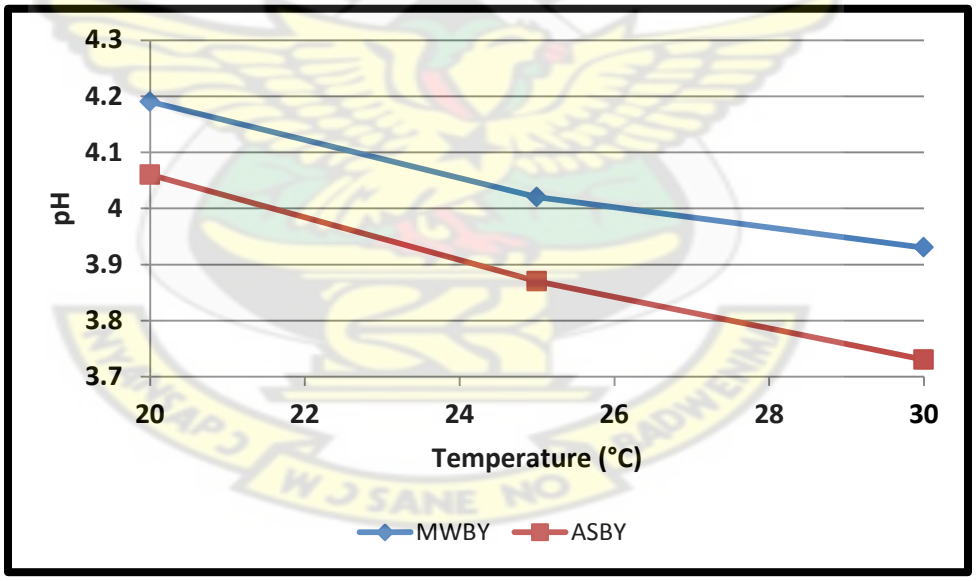

Figure 27: Variation of fermenting wort pH (R pH) with Temperature for Anchor Brewers' Yeast and Munich Wheat Beer Yeast.

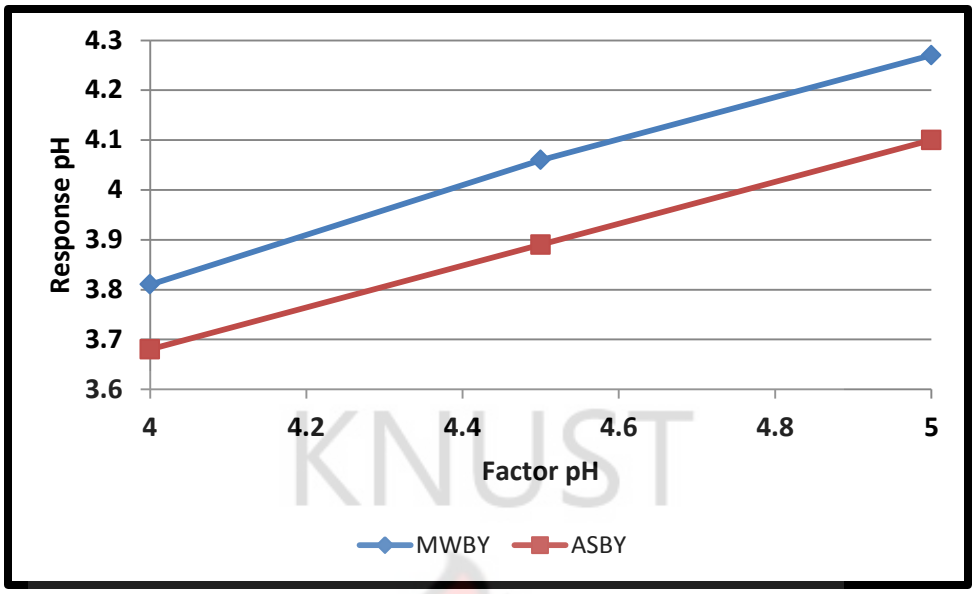

Figure 28: Variation of fermenting wort pH (R pH) with initial wort pH (F pH) for Anchor Brewers' Yeast and Munich Wheat Beer Yeast.

The variation of wort pH at different fermentation temperatures are compared in Figure 4.14. The rate of decrease in pH was fastest for both yeasts at 30˚C and slowest at 20˚C. The decrease was however faster for Anchor Brewers' Yeast than for Munich Wheat Beer Yeast at 30˚C. A faster decrease in pH would be desirable to provide an acidic environment which is unfavourable for the growth of many pathogenic and spoilage microorganisms (Phumkhachorn *et al.,* 2010). For both yeasts the pH change was fast in the first 24 hours at 30 ˚C and then declined.

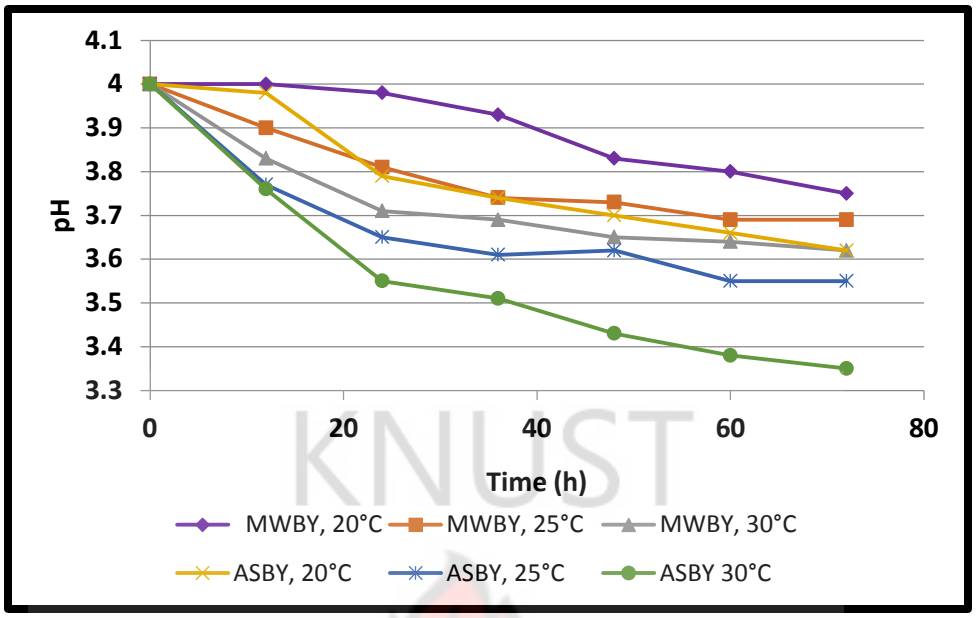

Figure 29: Variation of pH of fermenting wort at 20˚C, 25˚C and 30˚C for Munich Wheat Beer Yeast (MWBY) and Anchor Brewers' Yeast (ABY)

4.3.3. Derivation Of Optimum Fermentation Performance Conditions Of Yeasts For Alcoholic Fermentation Of *Pito* At Industrial Level.

The experimental data obtained were used to prepare overlaid contour plots, Figure 30 and Figure 31 to derive the optimal conditions for the fermentation factors time and temperature at the sour point pH of *pito* required to obtain typical end product parameters of *pito*, namely apparent extract (A.E.) between 6 and 7% and pH between 3.6 and 3.8. The derived conditions for Munich Wheat Beer Yeast were Temperature: 22.6°C and Time: 71.6 hours to achieve the end product pH of 3.8 and end product Apparent Extract, (AE) of 6.02 %. For Anchor Brewers'Yeast, the derived conditions for Temperature and Time were 24.2°C and 71.5 hours respectively to achieve the end product pH of 3.6 and end product Apparent Extract (AE) of 6.8 %. Using the initial extract at

the begining of fermentation, i.e. Original Gravity, O.G of 13.2%, the end product quality parameters were calculated with the listed formulae and reported in Table 29 based on 15 litres of fermenting wort.

ADF = (OG (%w/w) – AE (%w/w))100/OG (%w/w),

Alcohol (%Vol) = (OG (%w/w) – AE (%w/w))/2.062,

Rate of fermentation = (OG (%v/w) – AE (%v/w))150/T_{Opt}

Where,

ADF is Apparent Degree of Attenuation, OG is Original Gravity, AE is Apparent Extract and T_{Opt} is the derived optimal duration of fermentation time, h.

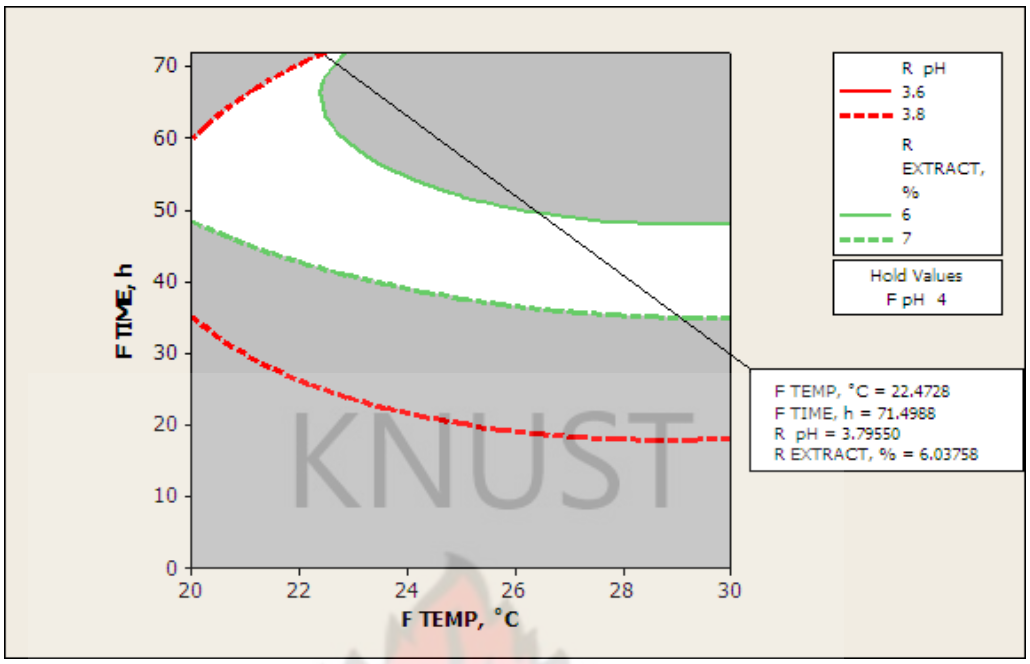

Figure 30: Overlaid contour plot indicating conditions for obtaining end product specifications for pH and Extract for Munich Wheat Beer Yeast.

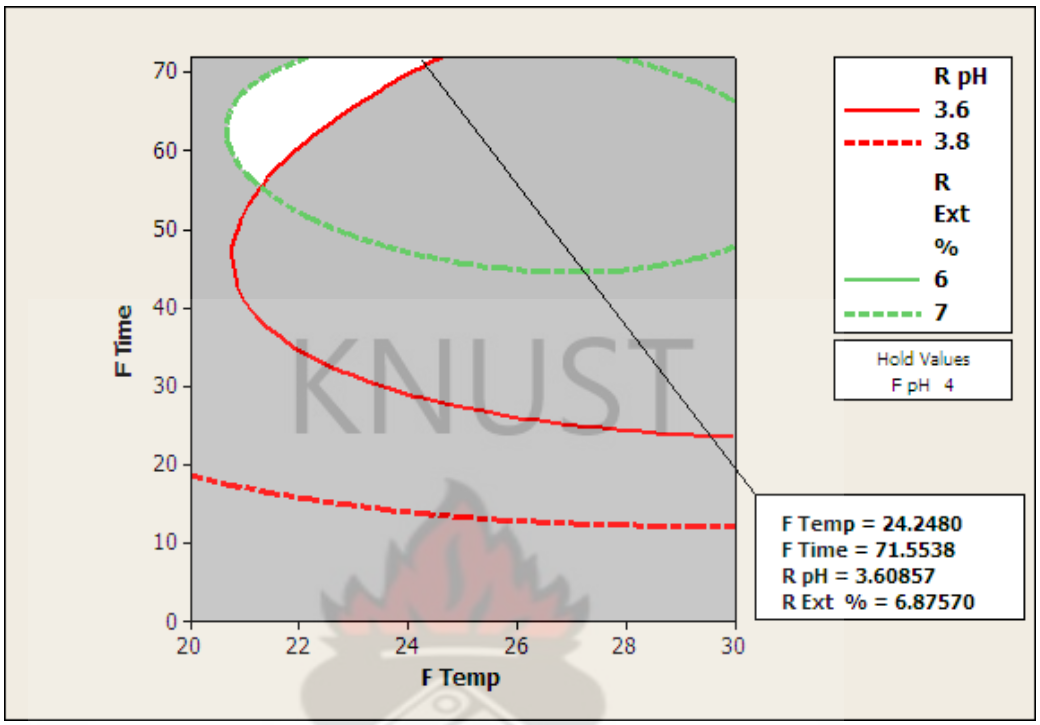

Figure 31: Overlaid contour plot indicating conditions for obtaining end product specifications for pH and Extract for Anchor Brewers' Yeast.

Table 29: Optimal conditions derived for attaining the target quality parameters of the experimental *pito* brews and the conditions for brewing *pito* with the traditional process.

	Fermentation Conditions For Experimentally Brewed Traditional *Pito*	Derived Optimal Fermentation Conditions for Munich Wheat Beer Yeast	Derived Optimal Fermentation Conditions for Anchor Brewers' Yeast
Fermentation Temp (˚C)	30	22.6	24.2
Initial Extract (OG)	13.2 % wt/wt 13.88 % vol/vol	13.2 % wt/wt 13.88 % vol/vol	13.2 % wt/wt 13.88% vol/vol
Wort pH	5.0 – 5.6	5.0 – 5.6	5.0 – 5.6
Sour Point pH	4.0	4.0	4.0
End Product Extract, (AE)	5% wt/wt 5.09 % vol/vol	6.0 % wt/wt 6.13 % vol/vol	6.9 % wt/wt 7.08 % vol/vol
Apparent Degree of Fermentation (%)	62.1	54.6	47.7
Fermentation Time (h)	72	71.6	71.5
Rate of fermentation (gExtract/h)	18.3	16.2	14.3
End Product pH	3.6	3.8	3.6
Alcohol, vol % (calc)	3.98	3.5	3.1

The fermentation rate of the traditionally brewed *pito* was higher than those derived for the yeasts under investigation. The higher temperature, (ambient ranging from 28°C to 32°C) at which traditional *pito* was fermented will result in a faster rate of fermentation as long as the temperature is not high enough to denature the enzyme catalyzing the fermentation (Hough *et al.*, 1982a). The traditional *pito* also has a higher apparent degree of fermentation which is reflected in the calculated alcohol level. The alcohol level calculated for the yeasts under investigation fall within the normal values for *pito* of 3% which is typical for sorghum beers.

4.4 Validation of derived optimal fermentation performance conditions of yeasts for alcoholic fermentation of *Pito* at ndustrial level

Three *pito* brews of 15 litres each with OG of 13.2% were made and the wort fermented using a starter culture of *L. delbrueckii* for the souring process and Anchor Brewers' yeast for the alcoholic fermentation process. The predicted fermentation conditions of temperature and time for achieving the target end product specifications were used to ferment the wort. The mean value of Apparent Degree of Fermentation and Fermentation Rate obtained were very close to the specified end product values thereby confirming the predictability of the model. The conditions for the confirmatory brews and the results obtained are given in Table 30. The experimental data for the confirmatory brews are given in Appendix 6.

Table 30: Conditions for confirmatory brews to validate predicted values and results achieved.

Optimal Fermentation Conditions Derived For Anchor Yeast		**Fermentation Conditions Applied In Confirmatory Test Brew With Anchor Yeast**	
Temp, ˚C	24.2	Temp, ˚C	24.0
Time, h	71.5	Time, h	71.5
pH	4.0	pH	4.0
Predicted Values For End Product		**Values Obtained For End Product**	
Extract, %	6.9	Extract, %	7.2
Apparent Degree of Fermentation, %	47.7	Apparent Degree of Fermentation, %	45.5
Rate of Fermentation, g Extract/h	14,0	Rate of Fermentation, g Extract/h	13.6
pH	3.6	pH	3.6

4.5 Selection of yeast of choice for use as starter culture

The experimental results have indicated that the two types of yeast investigated for their use as pure strain starter culture for *pito* fermenting sorghum wort are both capable of producing *pito* to meet the end product fermentation characteristics of traditional *pito*. However, the use of Anchor Brewers' Yeast will have economical advantages over the use of Munich Wheat Beer Yeast in terms of production cost as fermentation will take place at a higher temperature of 24°C. The fermenting temperature of Munich Wheat Beer Yeast is 22°C and it will require more energy to cool down the wort after lactic acid fermentation is completed at 48°C to 22°C and maintaining it over the period of fermentation. Additionally, the lower degree of fermentation achieved by Anchor Yeast will also result in fuller body i.e. palatefulness and a

milder *pito* in terms of alcohol level which was found to the preference of *pito* consumers (Djameh, 2010). The slower rate of fermentation by Anchor Yeast could also be an advantage over Munich Yeast as the slower rate will yield lower concentrations of higher alcohols which above their threshold levels could adversely affect flavour and consumer acceptance.

4.6 Outcomes and Conclusion

The use of *L. delbrueckii* for the lactic acid fermentation (souring) at 48°C to a sour point pH of 4.0 followed by alcoholic fermentation with Anchor Brewers' Yeast at 24°C for 71.5 hours are proposed for industrial production of *pito* subject to positive consumer acceptance evaluation of the product. Modifications of the process conditions may be necessary during large scale production where the effects of mass and temperature gradients may come into play. The application of these starter cultures shall exclude the effects of undesirable microbes that are responsible for early spoilage and also contribute to standardization, control and predictability of the *pito* production process and ultimately consistency of product quality.

CHAPTER FIVE

Comparison of Lactic Acid fermentation (Souring) and alcoholic fermentation profile of traditionally fermented *Pito* and *Pito* fermented with pure strain starter cultures.

5.1 Introduction

The production of *pito* involves two fermentations; lactic acid fermentation by lactic acid bacteria followed by an alcoholic fermentation by yeasts. Both fermentation processes proceed along the steps of Embden-Meyerhof-Parnas pathway of fermentation (glycolysis) up to the formation of pyruvic acid from where in lactic acid fermentation lactate dehydrogenase converts pyruvic acid into lactic acid and in alcoholic fermentation two enzymes from the yeast, pyruvate decarboxylase and alcohol dehydrogenase, convert pyruvic acid into carbon dioxide and ethanol. In homolactic fermentation, one molecule of glucose is ultimately converted to two molecules of lactic acid but in heterolactic fermentation carbon dioxide and ethanol are produced in addition to lactic acid (Kockova *et al.,* 2011).

The use of starter cultures has been proposed as an appropriate approach for the control and optimization of the fermentation process in order to alleviate the problems of variations in organoleptic quality and microbiological stability observed in African traditional fermented beverages (Holzapfel, 1997; Achi, 2005; Viera-Dalodé *et al.,* 2007; M u g u l a *et al.,* 2003).

The starter cultures investigated in studies so far have been isolated from traditional beers and have not been developed as stable, active and viable

commercial cultures which can be used for commercial applications at up-scaled

industrial production of the traditional sorghum beers. According to Haggblade and Holzapfel, (1989d), the body, alcohol content and taste which are determined by the fermentation process, could be controlled through the application of commercial starter cultures.

It is recommended that starter cultures for indigenous fermented foods and beverages should be isolated from the products they are supposed to be used for (Jespersen, 2003; Togo *et al*., 2002; van der Aa Kuhle, 2001; Glover *et al.*, 2005). However, the commercial cultures available on the market have not been isolated from *pito* and may not be able to adapt to its environment to ensure their successful fermentation performance. The red sorghum varieties commonly used in *pito* brewing are rich in polyphenols which tend to inhibit enzyme activities in sorghum wort (Daiber, 1978) and also influence the metabolic activities of microorganism during fermentation (Haggblade and Holzapfel, 1989e).

From the studies in chapter 3 and chapter 4, *L. delbrueckii* and Anchor Brewers'yeast (*S. cerevisiae*) were found to possess adequate fermentation capacity to meet the end product fermentation characteristics of *pito* as commercial starter cultures in the industrial production of *pito* and the conditions of their optimal performance were also derived. In this study their lactic acid and alcoholic fermentation profiles of *pito* wort under the derived

optimal conditions were evaluated alongside those of traditionally fermented *pito* to determine how they compare.

5.2 Materials and Methods

Pito samples were brewed with 4 kg of sorghum malt prepared according to the malting procedure described by Djameh *et al.,* (2015) from *kadaga* red sorghum variety commonly used in brewing *pito* in Ghana. The malt was milled using a hammer mill equipped with 0.4 mm sieve. The milled malt (about 4 kg) was mashed in 20 litres of water at ambient temperature (28°C-32°C). The mash was left to sediment for 60 minutes after which the clear supernatant liquor into which the amylolytic enzymes were extracted was decanted and kept aside. The sediment of the mash was boiled for 30 minutes and then recombined with the supernatant (enzyme rich) liquor. The temperature of the mixed mash was adjusted to 62°C, and held there for 60 minutes and then raised to 72°C for another 60 minutes to undergo saccharification at these temperatures. After saccharification (or starch conversion), the mash was divided into two portions for fermentation with the traditional *pito* process and with single cell starter cultures respectively.

5.2.1 Traditional *Pito* fermentation process

The saccharified mash was left to undergo spontaneous lactic acid fermentation at ambient temperature (28°C-32°C). After a sour level of pH 4.25 and 0.33% lactic acid level was attained, the mash was filtered and boiled for 90 minutes. It was cooled to ambient temperature and left to stand for 120 minutes to sediment and clarify. The clarified wort (5 L) was filled into a glass

fermentation jar and 5 g of dried yeast, (locally known as *dambile*) from a commercial *pito* brewer's brew was added to it to initiate alcoholic fermentation at ambient temperature of 28°C -32°C.

5.2.2 Starter culture *Pito* fermentation process

The other half of the saccharified mash was filtered immediately after the starch conversion and the wort was boiled for 30 minutes to sterilize and then cooled to 45˚C. A 5 litre portion was filled into a glass jar and inoculated with a starter culture of *L. delbrueckii*, (500 ml, with cell population density of 2.2 x 10^7 / ml) and fitted with a fermentation lock. The bottle was left to stand for 12 hours at 45˚C in a thermostatic water bath to undergo lactic acid fermentation. Part of the wort (1 Litre) was drawn for continued monitoring of pH and lactic acid formation beyond the 12 hours. The remaining lot was then cooled to 24˚C and pitched with 5 g of Anchor Brewers' Yeast to undergo alcoholic fermentation at a controlled temperature of 24˚C.

5.2.3 Analysis

Samples of *pito* from both processes were drawn off every 4 hours for the measurement of pH, Lactic acid content and Extract during the lactic acid fermentation process and every 24 hours during the alcohol fermentation process until the fermentation limit was attained when extract remained constant. pH was measured with a Hanna digital pH meter HI 98190,

manufactured by Hanna Instruments, USA and Extract was measured with Anton Paar DM 35N Densimeter, manufactured by Anton Paar, Graz, Austria. The amount of lactic acid produced by the lactic acid bacteria during the fermentation was determined by titration of 25 ml of sample against 0.1N NaOH to a colour change from yellow to green at the endpoint at pH 7 using Bromothymol blue (0.1 bromothymol blue in 100 ml 20 % ethanol) according to the procedure described by Kunze (2004a). The measurements were carried out three times and average values obtained. Apparent Degree of Fermentation (ADF) and alcohol levels after fermentation were determined as previously described.

5.3 Results and Discussion

5.3.1 Evaluation of Lactic Acid Fermentation Profiles

The experimental data for lactic acid fermentation during the fermentation of *pito* by the traditional process and by the commercial starter cultures are reported in Tables 31 and 32 respectively.

Table 31: Experimental data for lactic acid fermentation of *pito* by the traditional process

Traditional *Pito*						
Time, h	pH, (Mean)	pH, (STDev)	Extract %, (Mean)	Extract %, (STDev)	Lactic Acid %, (Mean)	Lactic Acid%, (STDev)
0	5.22	0.04	11.2	0	0.13	0.008
4	5.1	0.07	11.2	0	0.137	0.003
8	5.04	0.03	11.2	0	0.158	0.006
12	4.25	0.03	11.2	0	0.331	0.023
16	3.95	0.07	11.2	0.1	0.428	0.014
20	3.82	0.13	11.2	0.1	0.526	0.016
24	3.76	0.08	11	0.1	0.489	0.033
30	3.75	0.04	11	0	0.489	0.004
34	3.78	0.03	11	0	0.482	0.014
38	3.78	0.04	11	0.1	0.482	0.013

Table 32: Experimental data for lactic acid fermentation of *pito* with starter cultures

Starter Culture *Pito* Process						
Time, h	pH, (Mean)	pH, (STDev)	Extract %, (Mean)	Extract %, (STDev)	Lactic Acid, % (Mean)	Lactic Acid %, (STDev)
0	5.1	0.1	11.2	0	0.13	0.011
4	5.09	0	11	0.1	0.137	0.004
8	4.99	0.0	11	0	0.144	0.006
12	4.54	0.6	10.4	0.3	0.27	0.057
16	4.01	0.3	10	0.3	0.565	0.023
20	3.97	0.1	9.8	0.3	0.57	0.037
24	3.91	0.1	9.4	0.6	0.63	0.017
30	3.9	0.2	9	0.6	0.655	0.023
34	3.89	0.2	9	0.4	0.695	0.010
38	3.75	0.0	9	0.6	0.709	0.001

The formation of lactic acid during lactic acid fermentation was at a faster rate and to a higher level for the pure single strain culture fermented *pito* (0.015 % per hour on average) than for traditional *pito* (0.009 % per hour on

average) as indicated by the plots in Figure 32. This observation is however contrary to the findings of Dumeyakor and Ohta (1993), who carried out the lactic acid and alcoholic fermentations in one step and reported a faster rate of lactic acid formation in mixed cultures. In a single culture of lactic acid bacteria, in this case using *L. delbrueckii* which is homofermentative, lactic acid is the only end product of the fermentation. However, in a mixed culture of traditional *pito*, there may be hetereroferementative lactic acid bacteria and other microorganisms which utilize the substrate, sugar, to form other products apart from lactic acid (Lindsay, 1985). Additionally, in the mixed culture fermentation, there is competition for nutrients with other microorganisms whose primary metabolites may be not acids.

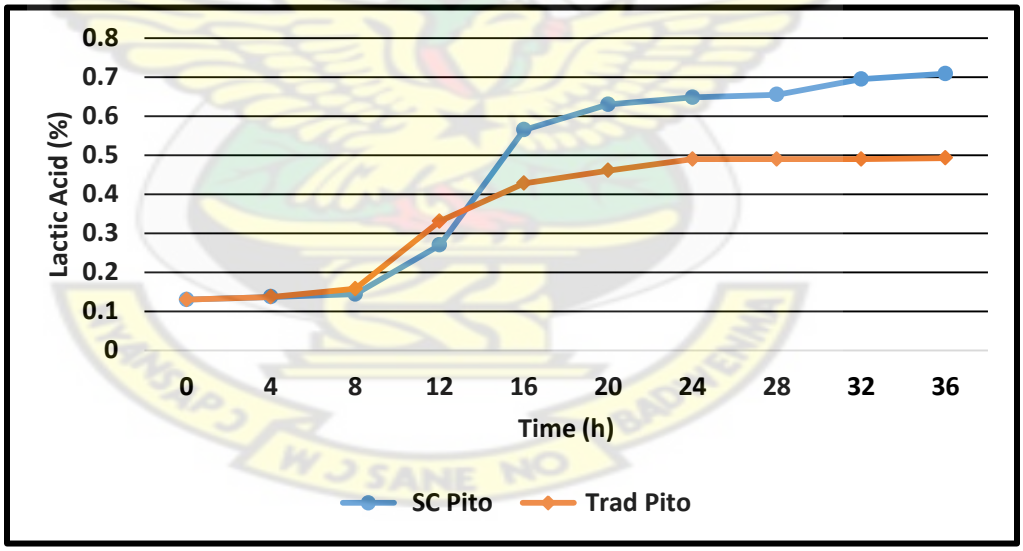

Figure 32: Formation of lactic acid during the lactic acid fermentation of pito wort with single strain starter culture of L. delbrueckii (SCP) and with mixed population of lactic acid bacteria in the traditional process (TP).

There was an observed difference in the variation of pH in the two *pito* brews as indicated in Figure 33. There was a drop of 1.35 units for starter culture *pito* during the period of 38 hours and 1.44 units for traditional *pito* during the same period. The decrease in pH was faster for traditional *pito* with 0.038 units per hour against 0.036 units per hour for starter culture *pito*. This observation was similar to that of Dumeyakor and Ohta (1993) in which the pH of mixed culture *pito* dropped faster than that of *pito* brewed with single culture, suggesting that there may be other metabolites such as nitrogenous products contributing to the drop in pH in the traditionally fermented *pito*. For both brews however, pH levelled out after 15 hours. During the steady level of pH, nutrients may have been depleted or the fermenting environment may have become toxic from the lactic acid formed (Kashket, 1987). The trend of the pH decrease was a reflection of the formation of lactic acid.

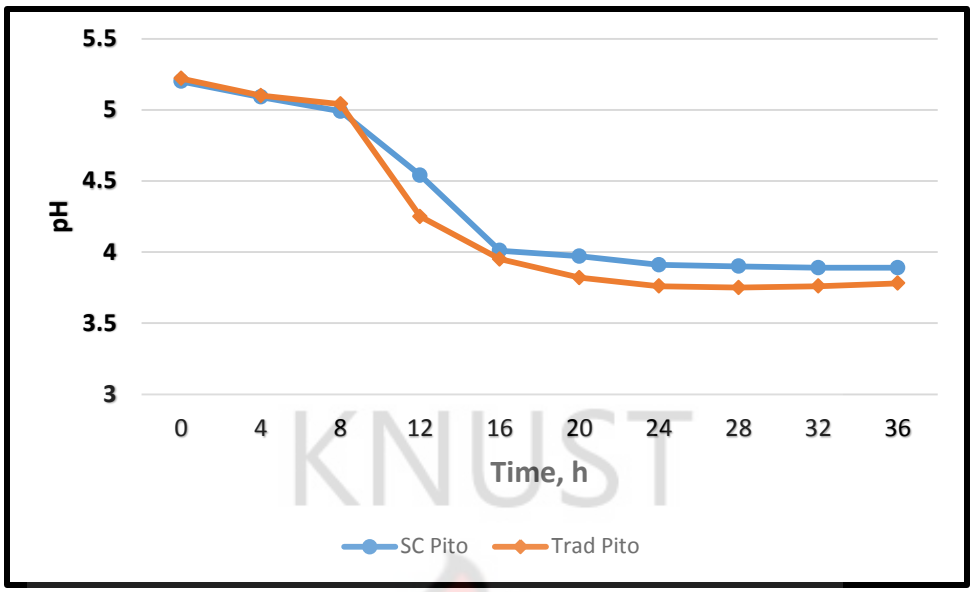

Figure 33: Variation of pH during the lactic acid fermentation of pito wort with single strain starter culture of L. delbrueckii (SC) and with mixed population of lactic acid bacteria in the traditional process (TP).

Extract utilization during lactic acid fermentation is shown in Figure 34. The level of extract, mainly sugar, remained relatively unchanged during the lactic acid fermentation in the traditional process (0.28g/h) compared to the starter culture process (2.50g/h) which may be because traditional *pito* contained amylolytic lactic acid bacteria which may be converting starch and dextrins in the wort to replace the sugar being used to form lactic acid as observed for *L. amylolyticus* in Chapter Three. Some strains of *L. fermentum* and *L. plantarum* commonly found in spontaneously fermented sorghum worts exhibit amylolytic activity (Fossi and Tavea, 2013). *L. delbrueckii* used as the starter culture in starter culture (SC) *pito* is not an amylolytic lactic acid bacterium and hence the decrease in extract exhibited during the process when it is carrying out lactic acid fermentation as indicated in Figure 5.3.

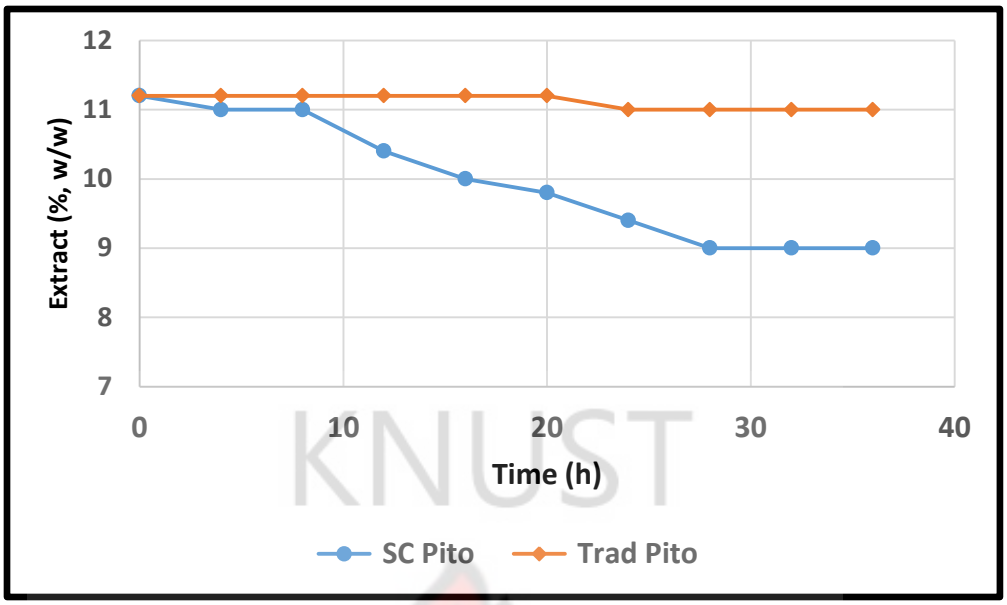

Figure 34: Utilization of Extract during lactic acid fermentation of pito by single strain starter culture of L. delbrueckii (SC) and with mixed population of lactic acid bacteria in the traditional process (TP).

5.3.2 Evaluation of Alcoholic Fermentation Profiles

The experimental data for fermentation profile during the alcoholic fermentation of *pito* by the traditional process and by the pure single strain commercial starter cultures are reported in Tables 33 and 34 respectively.

Table 33: Experimental data for alcoholic fermentation of *pito* by the traditional process

TRADITIONAL *PITO* PROCESS				
Time, h	Extract %, Avg	Extract %, STDev	pH, Avg	pH, STDev
0	11.2	0.00	5.22	0.04
4	11.2	0.00	5.1	0.10
8	11.2	0.10	5.04	0.07
12	11.2	0.10	4.25	0.15
16	11.2	0.00	3.95	0.04
20	11.2	0.10	3.82	0.05
24	11	0.20	3.76	0.02
30	11	0.20	3.75	0.03
34	11	0.10	3.78	0.04
38	11	0.10	3.78	0.05
62	9	1.21	3.75	0.05
86	5.9	0.26	3.71	0.04
110	5.6	0.20	3.64	0.04
134	5.2	0.30	3.59	0.04
158	5	0.20	3.57	0.06

Table 34: Experimental data for alcoholic fermentation of *pito* by the starter culture process

STARTER CULTURE *PITO* PROCESS				
Time, h	Extract %, Mean	Extract %, STDev	pH, Mean	pH, STDev
0	11.2	0.00	5.1	0.06
4	11	0.10	5.09	0.00
8	11	0.26	4.99	0.01
12	10.4	0.53	4.54	0.41
16	10	0.20	4.01	0.20
20	9.8	0.35	3.97	0.09
24	9.4	0.17	3.91	0.05
30	9	0.44	3.9	0.11
34	9	0.20	3.89	0.13
38	9	0.40	3.72	0.05
62	8.4	0.20	3.7	0.02
86	8.2	0.30	3.68	0.03
110	8	0.20	3.66	0.06
134	8.2	0.35	3.64	0.06
158	8	0.00	3.6	0.01

The alcoholic fermentation profile of both Starter culture *pito* and Traditional *pito,* Figure 35, followed the typical pattern of sugar fermentation with a lag phase in which the yeasts adapt to the wort environment, an active log phase in which the wort nutrients are used for cell growth (as long as oxygen is available) and anaerobic glycolysis when oxygen is exhausted and a final stationary phase of inactivity when all the fermentable sugars have been used up (Kunze, 2004b).

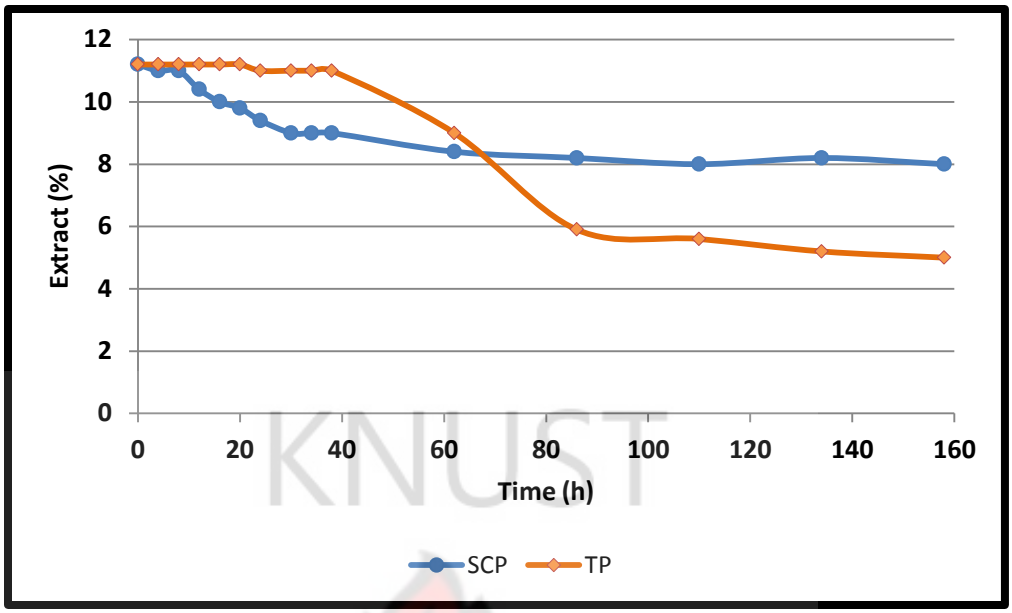

Figure 35: Utilization of Extract by single strain culture of *L. delbrueckii* and Anchor Brewers' Yeast (SCP) and by mixed population of microflora of the traditional process (TP) in alcoholic fermentation of *pito* wort.

It was observed that extract utilization, hence decrease in extract was faster for traditional *pito* in the first 80 hours of fermentation with an average of 4.12g/h compared with 1.72g/h for starter culture *pito*. The total extract utilization was also higher for traditional *pito*. Demuyakor and Ohta (1993) obtained similar findings in their study of fermentation profile of *pito* by single and mixed cultures of yeasts in which higher utilization of extract by the mixed culture of yeasts also translated into higher alcohol content of *pito* fermented by mixed cultures of yeasts. The observation however was in contrast with the findings of Avicor *et al.* (2015) who found a faster rate of utilization of extract with *pito* brewed with single culture of yeasts. It was however also noted by Demuyakor and Ohta (1993) that the utilization of extract was affected by the type of sorghum used for the wort extraction which may be on account of

different levels of polyphenols in different varieties of sorghum. Polyphenols are known to inhibit enzyme activity in sorghum wort (Daiber, 1978). The trend in decrease of pH followed the same rate and pattern for both *pito* types as indicated in Figure 36. This indicates that the microorganisms from the starter culture and from the mixed population involved in the fermentation are either taking up phosphate ions or excreting organic acids and nitrogenous compounds at a similar rate.

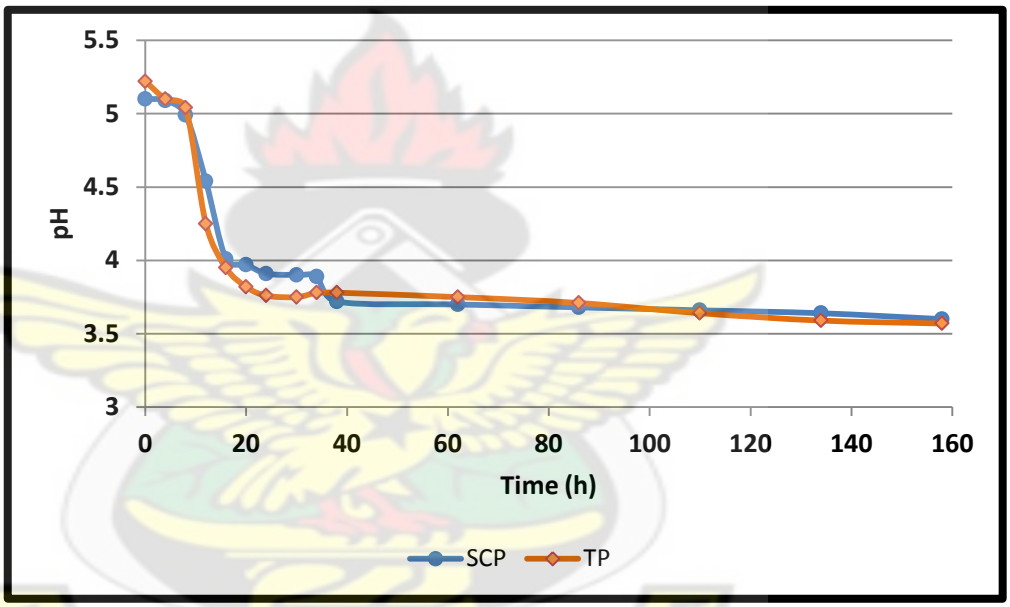

Figure 36: Change in pH during alcoholic fermentation of pito wort by single strain starter culture of L. delbrueckii and Anchor Brewers' Yeast (SCP) and by mixed population of microflora in the traditional process (TP).

A higher apparent degree of fermentation was observed for the traditional *pito* from its lower residual extract than the *pito* fermented with starter cultures. This implied lower alcohol content and a fuller body or palate fullness for the starter culture *pito*. *Pito* is a food drink taken by many consumers to

sustain them hence a fuller body which is more satiating may be more desirable than a highly attenuated *pito* with thinner body and less palate fullness. This view is in line with the finding that consumers of traditional sour sorghum beers in South Africa regard the sourness and body or viscosity of the beer as the most important quality characteristics (Novellie, 1968). A study in Ghana has also indicated that a milder *pito* with less alcohol content, e.g Dargarti *pito* is more popular than the stronger alcohol Frafra *pito* (Djameh, 2010). It would therefore appear that *pito* brewed with pure single strain starter cultures of lactic acid bacteria and brewers' yeast *S. cerevisiae* will find acceptability with consumers.

5.4 Conclusion

The lactic acid fermentation and alcoholic fermentation profiles of *pito* wort fermented by single strain commercial starter culture of lactic acid bacteria *L. delbrueckii* and *S. cerevisiae*, Anchor Brewers'Yeast respectively differ from those of worts fermented by mixed culture of microflora of the traditional *pito* process. The differences are on account of the specific characteristics of the microorganisms in utilizing nutrients in the fermenting medium and in synthesizing enzymes that are required for the bioconversions. The commercial starter cultures can however be applied for the souring process and alcoholic fermentation of *pito* to achieve souring and alcohol levels in processing *pito* that are comparable to the levels obtained by the use of mixed population cultures in the traditional method of brewing *pito*. Their application will facilitate the up-scaling of *pito* production to industrial level where a controlled

and predictable process that will eliminate variation is a prerequisite. Variations in aroma and flavour profiles usually associated with traditional food and beverage products produced with single strain starter cultures not isolated from them will however require sensory evaluation of the product from the application of the commercial starter cultures to determine its acceptability to customers.

CHAPTER SIX

Evaluation of product quality of traditionally fermented *Pito* and *Pito* fermented with pure strain starter cultures.

6.1 Introduction

Beer quality is generally considered to be the sum of all characteristics which allow the product to meet the market requirements so that consumers to whom it is directed are attracted and satisfied. Beer quality then can be considered in the general sense to be in the eyes, nose, mouth and mind of the consumer (Broderick, 1977). Beer, like any product however, must meet its analytical and taste specifications and deviations from these must be considered as defects. Quality is also considered as absence of defects. In home–brewed traditional sorghum beers, however, specific statements about quality requirements are virtually meaningless as beer recipes differ across the continent of Africa and variations occur even between brewers in the same village (Novellie and De Schaepdrijver, 1986, Haggblade and Holzapfel, 1989d).

The quality of beer can be classified into measurable and descriptive characteristics. Chemical and physical analyses provide measurable parameters. These cover constituents that make up the character of the beer and may be derived from the raw materials unchanged or as a result of chemical and biochemical transformation of the raw materials during the processing (Hough *et al.,* 1982b). The beer constituents are also grouped generally into

volatile and non-volatile components. The descriptive parameters are covered by sensory evaluation in which flavour profile is assessed by consumers.

The traditional sorghum beers are drunk while still fermenting and have limited shelf life. The application of starter cultures in the fermentation process is expected to improve the shelf life and quality consistency of the beers. Shelf life evaluation is therefore important in the quality assessment of the beers produced by the use of starter cultures with traditionally produced beer as a control.

Beer quality in the broadest sense is the sum of all those characteristics which allow a product to meet the market requirements, i.e. to attract and satisfy consumers to whom it is directed. Sensory evaluation is one method by which the reaction of consumers of a product to a change in processing method can be obtained.

In this study, physicochemical analytical parameters relating to beer quality, shelf life, volatile fermentation by-products and the sensory characteristics of the *pito* brewed with starter cultures were evaluated.

Physical and chemical analysis of beers involves the measurement of analytical parameters commonly used to describe a beer and which can be conveniently measured in Quality Assurance work (Broderick, 1977). They are the terms used to define the requirements of regulatory bodies and generally provide an assessment of the fermentation performance and an indication of the quality of the product. The values of these parameters are specific to the type of beer and largely influenced by the nature of the raw material and

processing method. Sorghum malt is known to be low in beta amylase (Taylor and Robbins, 1993; Ijasan *et al.,* 2011; Palmer, 1989; Welborn, 1991) which splits maltose off the non-reducing end of the starch chain (Kunze, 2004a). It also produces glucose and maltotriose which together with maltose constitute the fermentable sugars. The wort produced from sorghum malt consequently has a lower percentage of fermentable sugars and attenuation limit (degree of fermentation) compared to barley malt beers which are very rich in beta amylase. Traditional sorghum beers are therefore generally characterized by low alcohol levels, averaging 3% by volume. The soluble materials extracted into the wort from the malt during mashing are known as extract and is made up of mostly sugars and protein. The amount of extract before fermentation begins is the original extract, generally designated as OG, meaning original gravity. In the course of fermentation its value decreases as it is utilized by yeast to produce alcohol. In the case of *pito* or sour beers, extract material, i.e. sugars are also used by lactic acid bacteria to produce lactic acid. The final residual extract in the fermented beer is denoted by AE, meaning apparent extract and influences the mouthfeel or palatefulness of the beer. The fermentable extract is split into almost equal parts alcohol and carbon dioxide during fermentation. The carbon dioxide provides sparkle and liveliness to the beer and induces a foamy head on the beer. Beers with low carbon dioxide content taste flat (Kunze, 2004c). The alcoholic content of the beer is usually regarded as a measure of its strength (Hough *et al.,* 1982b). The higher the

original gravity, OG, of the beer the more full-bodied it tastes and this is due to the alcohol content and residual extract, AE (Kunze, 2004c).

The colour of the beer is influenced by the type of malt used. Pale malts produce pale beers and dark or roasted malts including caramel malts produce dark beers. There are two main types of sorghum used in brewing *pito* in Ghana, namely red and cream cultivars. They contain varied levels of tannins and condensed polyphenols in the peripheral tissues of the testa as described by Rooney *et al.* (1980). There are however some varieties which do not contain tanins. The tannin and condensed polyphenols impact on the enzyme activity of the malt and colour of the finished beer as well as imparting astringency or bitterness to the taste.

The taste and stability of beer is also influenced by its pH. *Pito* as a sour beer, is characterized by low pH values between 3.1 -3.8 (UNIDO, 2007).

According to Narzis (1980), a normal full beer contains 3.5 – 5.0 % extract, 3.4 – 4.5% alcohol, 0.35 – 0.55 % carbon dioxide, and 90 – 92 % water. These values vary for different beer types or styles.

Pito is a relatively clear beer compared to other traditional frican sorghum beers known collectively as opaque sorghum beers for their high levels of turbidity. Turbidity of beer may be of biological or non-biological origin. Biological turbidity is often caused by yeasts which sediment very slowly and remain in suspension. Among these are some species of wild yeasts, *Torulopsis*, which comprise very small cells and are therefore slow in sedimentation (Hough *et al.*, 1982c). Non –biological turbidity is caused by

colloidal systems formed by protein-polyphenol complexes which are an instrinstic part of beer. These complexes coagulate in time to form larger and larger masses which appear as haze (turbidity) and eventually as a precipitate and finaly as sediment (Broderick, 1977). For traditional sorghum beers, particles of unconverted starch may also contribute to the turbidity.

The determination of extract and alcohol in beer were generally carried out by density measurements with pycnometer or density hydrometer and distillation procedures. These methods have been replaced in breweries by high precision analyzing machines such as the Anton Paar Beer Analyzer which can perform a large number of high precision analysis in a very short time (Kunze, 2004c).

According to Fu and Labuza (1993), the shelf life of a food can be defined as the time period within which the food is safe to consume and/or has an acceptable quality to consumers. Shelf life testing consists basically of selecting the quality characteristics which deteriorate most rapidly in time and the mathematical modeling of the change. Piergiovanni and Limbo (2003) reported that food products can deteriorate due to microbial spoilage, loss of nutrients and pigments, production of undesirable components, physical changes and that although several deterioration modes may occur simultaneously, it is the most sensitive one that limits shelf life. The selected quality factor should be a good indicator of this sensitive deterioration mode, and its critical limit can be determined based on legal and marketing considerations.

According to Waltzeko and Labuza (1976) and Gacula (1975), Hedonic scoring, which indicates acceptance on a numerical scale, e.g. a 1-9 point scale labeled from "dislike extremely" to "like extremely", is typically used for shelf-life evaluation. The shelf life determined in this way is called the practical shelf life (PSL). Polhemus (2005) described a method by which a reasonable shelf life of a product can be established in which samples of the product are at different lengths of time after production and a statistical model constructed for one or more critical variables to predict that point in time after which the probability that the product will still be sound falls below some specified threshold, the Spoilage Level.

Traditionally produced sorghum beers are drunk while still fermenting. They are unstable and their organoleptic characteristics continue to change, and within 72 h it becomes too acidic to be consumed. The short shelf-life of this traditional brewing method limits its consumption to within a day of its production and hence its availability is limited to the seasonal availability of the sorghum grain (Osseyi *et al.,* 2011). It was also reported by Kutyauripo *et al.,* (2009) that most traditional, African cereal-based fermented foods deteriorate rapidly and become unacceptable to consumers within one to four days of production and that the deleterious changes are

primarily due to the objectionable off-flavour or over-souring induced by continued icrobial activities after production. The limited shelf life (stability) of sorghum beers has been reported as the major problem confronting commercial brewers in Sudan (Dirar, 1978), in Tanzania (Tisekwa, 1989), in

Nigeria (Sanni *et al.*, 1999) in Rwanda (Lyumugabe *et al.*, 2010) and in Benin (Konfo *et al.,* 2012). Manzocco and Lagazio, (2009) used Hydrogen ion concentration (pH) and sourness as indices to correlate with the percentage of consumers rejecting the product during storage to determine the shelf life of brewed coffee. In this study the level of sourness was used as the index to determine the spoilage level of *pito* for shelf life studies.

Beer is a complex mixture of several different compounds some of which are derived from the raw materials and survive the brewing process unchanged while others are the result of chemical and biochemical transformations of the raw materials during the brewing process (Hough *et al.,* 1982b). These constituents together make up the character of the beer with different beers containing the same compounds in different proportions rather than new constituents. One of the key factors in the quality assessment of a beer is its flavour which is determined largely by a complex mixture of volatile chemical constituents in varying composition and concentrations (Stewart, 2004). According to Horak (2011), beer flavour, a combination of odour and taste is a very important factor in the consumer's perception of the quality of the beer. The total sensory profile of beer is the main contribution to the success of the product on the market with the exception of price. Aroma is one of the most important identity signals of alcoholic beverages as well as an indicator of quality (Fan *et al.,* 2006). The flavour and aroma compounds of the beer are metabolic by-products from yeast which are formed during fermentation and are passed into the beer. Some of these fermentation by-products react with

each other or change in their amount and composition in the course of the fermentation (Kunze, 2004b).

The formation and partial degradation of fermentation by-products is closely related to the metabolism of the yeast. The fermentation by-products have a decisive influence on the taste and aroma of beer (Kunze, 2004b). An overview of yeast fermentation of wort into beer is given in Figure 37.

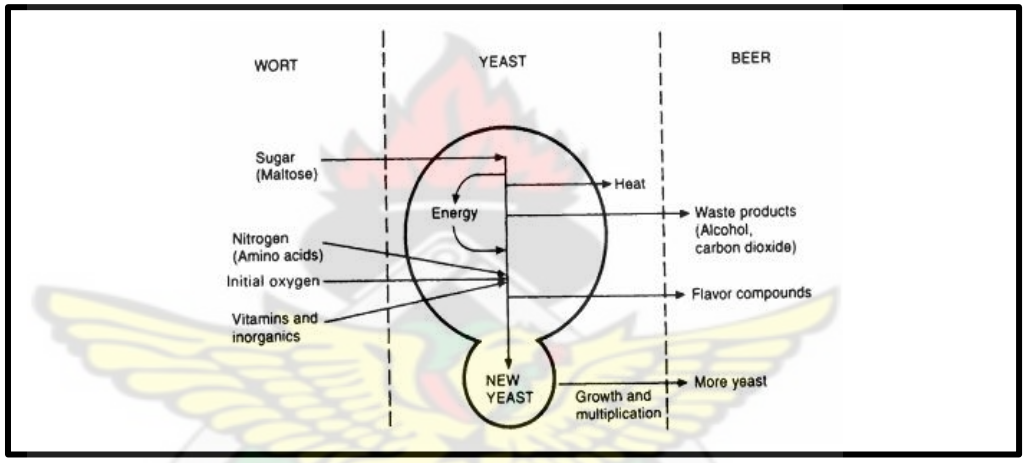

Figure 37: Overview of yeast fermentation. Source: Lewis and Young (2002)

The flavour-active fermentation by-products may be present at levels just below the threshold level at which they can be perceived. They exhibit specific flavour notes at their threshold levels. These notes may be characteristic of the beer and therefore deemed desirable. They could however also be uncharacteristic of that beer type and therefore considered undesirable or a taint. There is a distinction between the aroma substances; green beer aroma substances and mature beer aroma substances. The green beer aroma substances are diacetyl, aldehydes and sulphur compounds. In the classic

European type lager beers brewed from barley malt, these impart to the beer an unclean, immature and unbalanced taste and aroma (Kunze, 2004b). They impair beer quality in high concentrations. The mature beer aroma substances are higher alcohols and esters. These are the ones that determine the aroma of the finished beer and their levels in specific ranges are requirements for a high quality beer. The relationship between the levels of these volatile aroma compounds and the quality perception of the beer may not be the same for the classic European lager type beers and the traditional African beers brewed from sorghum as they are different beer types.

The profile of the volatile aroma compounds is determined by the yeast strain, the composition of the wort that is pitched, level of free amino nitrogen, the extract content and the fermentation conditions such as temperature, pitching rate and aeration level (Deželak *et al.*, 2015, Erten *et al.*, 2007). The flavour profile and hence quality perception of *pito* brewed with a single strain pure culture starter yeast at defined fermentation conditions is therefore expected to be different from that of traditional *pito* brewed with a wide combination of yeasts of varying strains and fermentation characteristics. During the alcoholic fermentation of wort by the yeast *Saccharomyces cerevisiae*, aldehydes, organic and fatty acids, esters and higher alcohols (fusel oils) are excreted as by-products in addition to the major products ethyl alcohol and carbon dioxide (Erten *et al.*, 2007). Acetaldehyde is the most important aldehyde of beer. It usually lies in the range 2–20 mg/l. Concentrations of 20–25 mg/l acetaldehyde causes green apple flavour, green vegetation or vegetable

flavour (green beer flavour) and levels over 150 mg/l may cause an aldehydic character of high astringency (Šmogrovičová, 2014).

The vicinal diketones, diacetyl and 2,3- pentane dione are produced when acetohydroxy acids formed from pyruvic acid are excreted by yeast cells into the fermentation medium and converted by non-enzymatic oxidative decarboxylation (Hough *et al.*, 1982b; Kunze, 2004b). The conversion is favoured by pH decrease, increasing temperature and introduction of oxygen. The maximum acceptable level of total diacetyl in a matured lager type beer is 0.1mg/l. Higher alcohols are formed from the catabolic route, i.e. Ehrlich pathway in the presence of amino acids and from the anabolic route from sugars via biosynthesis (Erten *et al*., 2007) as illustrated in Figure 38. The keto acid produced in both pathways is decarboxylated to the corresponding aldehyde and then the resultant aldehyde is reduced to the corresponding higher alcohol. The formation of higher alcohols is increased by increasing fermentation temperature, reducing amino acid content of the pitching wort, intensive aeration of the pitching wort, pitching temperatures above 8°C, and increasing the wort concentration above 13% (Kunze, 2004b). Full pale beers have higher alcohol content of 60-90mg/L. Levels above this range damage the flavour and acceptability of the beer (Kunze, 2004b).

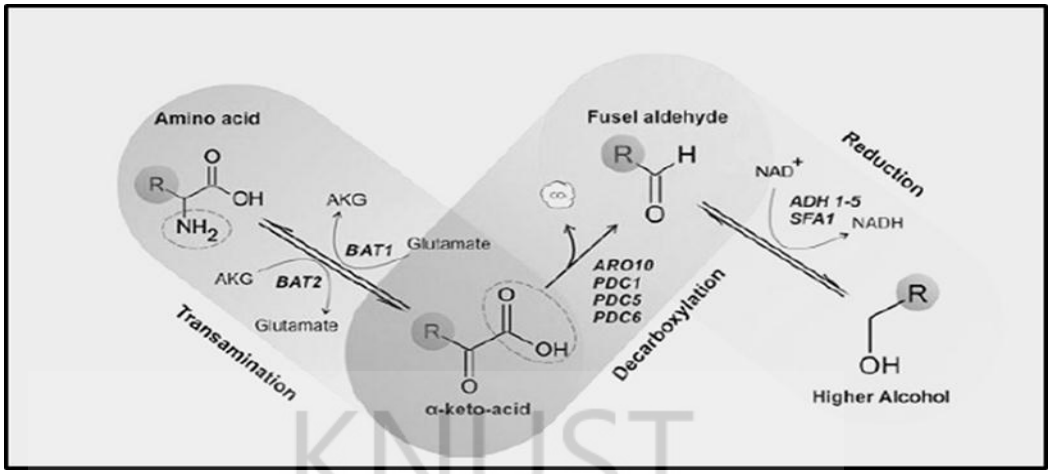

Figure 38: The Ehrlich pathway for higher alcohol synthesis. Source: Pires *et al*.,(2014)

Esters are the most important aroma compounds in beer; in higher concentrations however, they can impart an unpleasant bitter and fruity taste to the beer (Kunze, 2004b). They are classified into two different groups based on their chemical structure, i.e. the acetate esters and the medium-chain fatty acid (MCFA) ethyl esters (Mascia, 2014). Acetate esters are formed by condensation reaction of activated

acetyl-coenzyme A (acetyl-CoA) with ethanol or a higher alcohol while MCFA ethyl esters are formed by reaction of ethanol and long chain acyl-CoA as illustrated in Figures 39 and Figures 40 respectively. The formations are catalyzed by alcohol transferase (Erten *et al.,* 2007). All alcohols and carboxylic acids present in beer are theoretically capable of esterification reaction potentially forming almost 4000 esters (Hughes, 2009). The ester content depends on the beer type and the original gravity of the wort. Top fermenting beers contain up to 80 mg ester/litre and bottom fermenting beers

contain up to 60 mg ester/litre (Kunze, 2004b). Ester production is increased among others by increasing the attenuation limit and attenuation achieved, increasing the aeration of the wort and higher fermentation temperatures (Kunze, 2004b).

According to Smogrovicova (2014), the flavour of a beer depends not only on the content of its compounds but more on their ratio. The optimum higher alcohols -to- esters ratio for lager beers range from 4.1 to 4.7:1.

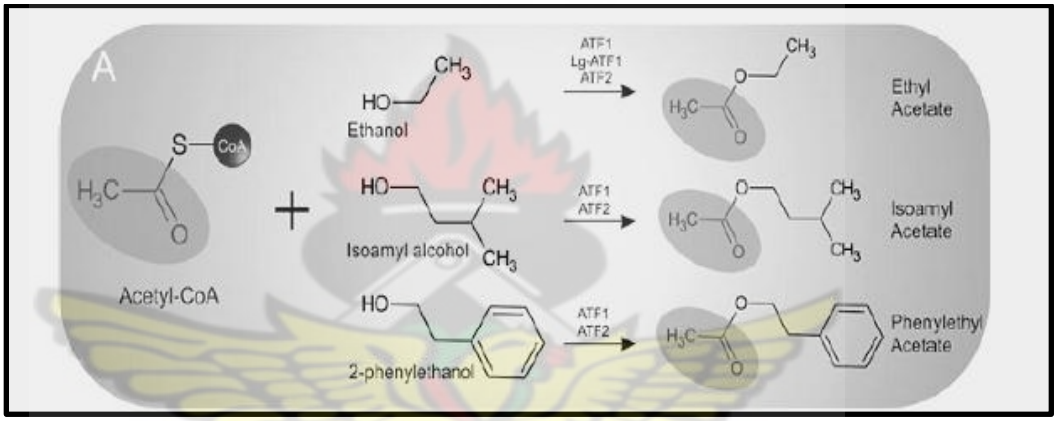

Figure 39: Scheme of reactions leading to the formation of acetate esters.

Source: Pires *et al.*, (2014)

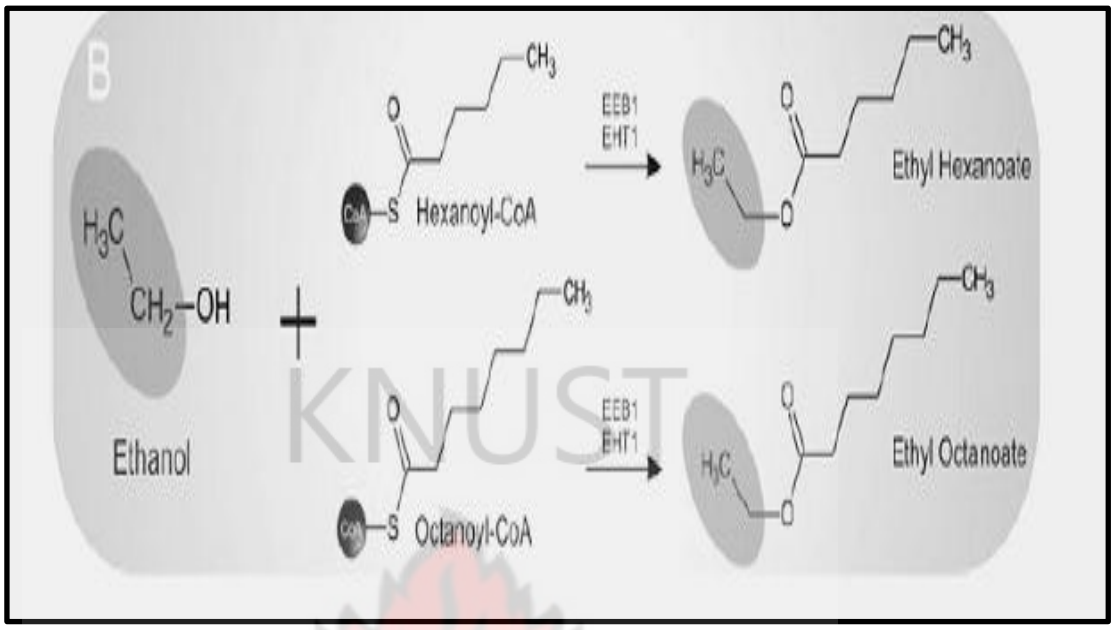

Figure 40: Scheme of reactions leading to the formation of MCFA ethyl esters.

Source: Pires et al., (2014)

Sensory evaluation is defined by Institute of Food Technologists (IFT), Sensory Division as a scientific discipline used to evoke, measure, analyze and interpret reactions to those characteristics of foods and materials as they are perceived by the senses of sight, smell, taste, touch and hearing and finds application in product development (Gengler, 2017; Nasirpour, 2017). When developing a new process for a product, the new process should maintain or improve the product. If the products differ, affective tests are required to establish whether the experimental product is liked as well as or more than the control product (IFT, 1981). According to Smythe *et al.*, (2002) alcoholic content, clarity, colour, formation and retention of good head of foam among other parameters contribute to the enjoyment of beer but it is the flavour, the

taste and aroma, which really determine the acceptability and drinkability of the beer.

The flavour of a beer is one of its important quality characteristics. It must be suitable for the type of beer and is often influenced by elements depending on country of origin and on fashion but it must however satisfy customers' expectation in order to win them from similar products made by competitors (Kunze, 2004c). Flavour has been described as a complex sensation comprising taste, odour, roughness or smoothness, hotness or coolness, pungency or blandness (Hough *et al.*, 1982d). Harrison (1970) however described it as a combination of two sensations, odor and taste. Taste is conveyed by aroma, palatefulness and liveliness i.e. sparkle (Kunze, 2004c). Besides ethanol and carbon dioxide, there are a multitude of other minor products of yeast metabolism formed during fermentation many of which contribute to beer flavour and aroma (Briggs *et al.*, 2004). The metabolic activities of mesophilic lactic acid bacteria along with other undesirable bacteria (Acetobacter), however, produce acetic acid, volatile off-flavours, fruity odors, and pellicles which render the taste, odor and texture of the beer unacceptable to consumers (Lyumugabe *et al.*, 2012).

Traditional or natural fermentation methods are initiated by endogenous flora to yield products that have unique or single quality attributes (Obire, 2005). However, according to Jimoh *et al.,* (2012) when indigenous traditional technologies are adopted for industrial application, the methods of preparation are altered and this leads to a product of altered flavour and unacceptability.

The 9-point hedonic scale has been the most commonly used scale for testing consumer preference and acceptability of foods since its development (Lim, 2011). This study was carried to determine if *pito* brewed with starter cultures will be perceived to be of equal or higher quality than *pito* brewed with the traditional process of spontaneous fermentation with a complex population of lactic acid bacteria and yeasts from the malt and brewing environment.

6.2 Sample Preparation

The samples of *pito* for the comparative evaluation of product quality were prepared using the processing methods described in Chapter Five.

6.3 Physicochemical Analysis.

6.3.1 Method

The samples, 400 ml of each of the test *pito* brews starter culture and traditional, were attemperated to 20°C and decarbonated according to the method described by American Soceity of Brewing Chemists, ASBC by transferring into 1L Erlenmeyer flask and shaking, gently at first and then vigorously, until gases no longer escape from it. The decarbonated sample was clarified by filtering through Whatman No.12 fluted filter paper. The temperature of the filtrate was readjusted to 20°C and the analyses were carried out using an Anton Paar DMA 4500M Beer Analyser. For turbidity measurement, the sample was unfiltered and measurement carried out with a Hanna turbidity meter model H193703C, Hanna Instruments, USA according

to the procedure described in Analytica-European Brewing Convention, 1987. Lactic acid % was measured as described previously in Chapter 3.6.3. Colour and Bitterness were measured by the spectrophotometric method of European Brewing Convention described in Analytica EBC, 1987. Samples were drawn from three replicate brews for the analytical determinations and mean values were reported.

6.3.2 Results and Discussion

The measured parameters in both *pito* brews compared favourably except for turbidity where the traditionally brewed *pito* had a much higher value. The analytical values are given in Table 35. In a similar study of *pito* by Oriji *et al.* (2003) *pito* was brewed in the laboratory with pure cultures of *L. plantarum* in combination with *S. cerevisiae* and *Pediococcus halophilus* in combination with *Candida tropicalis* isolated from a local brew. pH, colour, titrable acidity, alcohol content, specific gravity were also found to compare favourably with that produced by the traditional method.

Table 35: Analytical values of traditionally fermented *pito* (Trad *Pito)* and *pito* fermented with single strain Starter Cultures (SC *Pito*)

QUALITY PARAMETER	TRAD *PITO*	SC *PITO*	RANGE, (UNIDO, 2007)
Original Extract, (%)	13.0±0.2	13.0±0.2	8.0 — 14.0
Apparent Extract, (%)	5.20±0.17	6.40±0.17	2.0 — 7.0
Real Extract, (%)	6.80±0.17	7.80±0.10	3.0 — 8.0
Alcohol, (Vol %)	4.20±0.28	3.60±0.10	2.0 — 5.0
pH	3.50±0.10	3.60±0.20	3.1 — 3.8
Lactic Acid, (%)	0.76±0.02	0.61±0.04	0.4 — 0.9
Colour, (EBC)	50.70±0.02	45.55±0.87	15 — 80
Turbidity, (FTU)	225.0±5.6	39.1±1.2	N/A

The comparatively high turbidity of 225 FTU measured for traditionally brewed *pito* could be due to the presence of slow sedimenting yeast strains or wild yeasts. Turbidity level of 39.1 FTU for the starter culture *pito* is considered as just slightly hazy which is exceptionally clear for a traditional African sorghum beer most of which are generally opaque because the beers are not subjected to cold storage which induces sedimentation of the yeasts. The Anchor Brewers'Yeast used in fermenting the starter culture *pito* (Appendix 5) is known to have the characteristics of both top fermenting and bottom fermenting yeasts. The yeasts can therefore be expected to sediment towards the end of the fermentation and leave the beer partially clarified.

Sefa-Dedeh *et al.* (1999) also reported that quality indices of *pito* produced using *S. cerevisiae* as a single-starter organism compared favourably with the traditional brew. Similar to the findings of Lyumugabe *et al.*, (2014) and Demuyakor and Ohta (1993) where traditional sorghum beer *ikigage* and *pito* respectively fermented with mixed culture of microorganisms produced more alcohol than in the fermentation with single strain starter culture, in this study, a slightly lower quantity of alcohol was produced in the single strain culture fermentation. This is also reflected in the higher apparent extract of the starter culture *pito* with its implication of lower degree of fermentation as both brews started with the same original extract of 13%. Again, in agreement with their findings, the mixed culture fermentation produced more titrable acid (lactic acid) and had lower pH.

In the absence of the use of hops to provide bitterness in *pito* brews, the measured bitterness can be attributed to polyphenol bitterness, protein bitterness and yeast bitterness (Kunze, 2004c). As the source of yeast bitterness is the only difference between the two brews, it can be concluded from the same value for bitterness that the yeasts have no significant effect on the bitterness of *pito*.

The colour of the *pito* brewed with single strain starter cultures of lactic acid bacteria and yeast was lower (45.55 EBC) than of the traditional process *pito* brewed with mixed cultures (50.70 EBC) but both values were higher than the values (17 ±3 and 18 ±2 EBC respectively) obtained by Avicor *et al.*,(2015) in a similar study. In their work, malt from *chireh*, a white non-tannin sorghum

variety was used as against the red tannin sorghum, *kadaga*, used in this study. The red pigment in the testa of the grain must have been leached into the mash accounting for the comparatively high colour of the beer.

6.3.3 Outcomes and Conclusions

The measured analytical parameters of *pito* brewed with starter cultures of single strain lactic acid bacteria, *L. delbrueckii* and brewers' yeast, Anchor Brewers' Yeast were similar to those of *pito* brewed with the traditional method of fermentation with mixed population of lactic acid bacteria, yeasts and molds. The starter culture *pito* was however less turbid and had a higher colour from the variety of malt used. The measured parameters of both experimental beers were all well within the range normally known for traditionally brewed *pito*. Pure single strain starter culture of *L. delbrueckii* together with pure single strain starter culture of brewers' yeast Anchor Brewers' Yeast can ferment *pito* wort adequately to yield a product that conforms to the physicochemical quality of traditional *pito*.

6.4 Shelf Life Evaluation

6.4.1 Method

6.4.1.1 Establishment of spoilage level of *Pito*

Twelve tasters who regularly drink traditional *pito* and were familiar with its taste were recruited and introduced to the use of the hedonic scale in evaluating taste. They were then asked to taste and evaluate a sample of traditionally brewed *pito* (obtained from a local commercial brewer) daily for

10 days using the hedonic scale. The *pito* sample was filled into a 5 litre glass fermentation bottle which was fitted with a fermentation lock to vent carbon dioxide from the continuing fermentation but prevent ingress of air whose presence will lead to rapid over-sourness due to accumulation of acetic acid. The taste and analytical samples were drawn from a tap at the bottom of the fermentation bottle. The criterion for finding the sample undrinkable was chosen to be over-sourness, the characteristic that limits the shelf life of *pito*. This is the point on the scale where the sample will be disliked for being too sour. The development of lactic acid in the sample was measured alongside the taste testing for the establishment of the Spoilage Level i.e. the lactic acid content yielding the over-sourness. The lactic acid content was determined by titration of 25 ml of sample against 0.1N NaOH to a colour change from yellow to green at the endpoint at pH 7 using Bromothymol blue (0.1 bromothymol blue in 100 ml 20% ethanol) as indicator according to the procedure described by Kunze (2004a). The determinations were carried out in triplicate and average values obtained.

6.4.1.2 Establishment of shelf life

Lactic acid content of the experimental *pito* samples fermented traditionally with a mixed population of natural microflora as control and of *pito* fermented with pure single strain starter cultures of *L. delbrueckii* and Anchor Brewers' Yeast (*S. cerevisiae*) was measured for 10 days and done all over again three times. Both samples were filled into 1.5 litre empty natural mineral water plastic bottles (Voltic Mineral Water) in a similar manner in which *pito* is

usually packaged for sale, with the cap loosely closed on to avoid build-up of carbon dioxide gas inside which would result in the bursting of the bottle. The bottles were kept at ambient temperature (28°C – 32°C) and the daily analytical samples taken by pouring through the mouth of the bottle. The regression analysis method was used to plot a fitted model for the data, lactic acid content against time, and the established Spoilage Level was used to determine the shelf life of the two *pito* brews, i.e. number days it will take each of them to reach the established Spoilage Level as determined by over-sourness (Polhemus, 2005). The regression analysis was carried out using Statgraphics Centurion XVI.I from StatPoint Technologies Inc., Warrenton, Virginia, USA.

6.4.2 Results and Discussion

In the 9 point scoring scale used to evaluate the acceptance of the samples score 6 to 9 are the positive scores indicating liking for the sample. Score 1 to 4 are the negative scores indicating dislike. Score 5 is a neutral score indicating neither like nor dislike for the sample. In the taste tests carried out by the consumers, the score for liking decreased progressively daily with the increasing level of lactic acid in the drink as indicated Table 36.

Table 36: Raw scores (Mean and Standard Deviation) of panellists evaluating taste of *pito* to determine the spoilage level

DAY	LACTIC ACID LEVEL (%)	HEDONIC SCORE ON THE SCALE OF 9
1	**0.48±0.03**	**7.7±0.8**
2	**0.51±0.01**	**7.4±0.5**
3	**0.54±0.01**	**6.9±0.5**
4	**0.58±0.02**	**6.4±0.5**
5	**0.74±0.01**	**6.2±0.4**
6	**0.86±0.02**	**4.5±0.5**
7	**0.94±0.03**	**3.8±0.8**
8	**0.99±0.03**	**2.8±0.6**
9	**1.01±0.02**	**2.3±0.5**
10	**1.08±0.03**	**1.8±0.5**

Key: 9 = Like Extremely, 8 = Like Very Much, 7 = Like Moderately, 6 = Like Slightly, 5 = Neither Like Nor Dislike, 4 = Dislike Slightly, 3 = Dislike Moderately, 2 = Dislike Very Much, 1 = Dislike Extremely.

In the pooled scores given in Table 37, it is observed that the neutral score indicating an uncertainty of having a liking for the sample commenced after day 5, i.e. below score 5 (Neither Like nor Dislike). Up to Day 5 (0.74 % Lactic acid content), none of the consumers expressed a dislike for the sample, both in the pooled Score of 1 to 5 and of Score 1 to 4. At Day 6, (0.86 % Lactic acid content), 50 % of the panelists disliked the sample at Score of 1 to 4 and 100 % at Score of 1 to 5.

Table 37: Pooled scores (Mean and Standard Deviation) for evaluation of spoilage level by taste panel

DAY	LACTIC ACID %	SCORE 4 TO 1		SCORE 5 TO 1	
		COUNT	%	COUNT	%
1	0.48±0.03	0	0	0	0
2	0.51±0.01	0	0	0	0
3	0.54±0.01	0	0	0	0
4	0.58±0.02	0	0	0	0
5	0.74±0.01	0	0	0	0
6	0.86±0.02	6	50	12	100
7	0.94±0.03	10	83	12	100
8	0.99±0.03	12	100	12	100
9	1.01±0.02	12	100	12	100
10	1.08±0.03	12	100	12	100

It can be concluded that spoilage of the *pito* on account of over-sourness is perceived at a lactic acid level of 0.86 %. However as according to Polhemus (2005), trade shelf life is reached when 90 % of the degrading parameter is reached, the spoilage level is therefore fixed at a lactic acid content of 0.77 % in *pito* for the determination of its shelf life. The 0.77 % spoilage level is in line with the range of 0.13 % to 0.61 % reported by Sawadogo-Lingani *et al* (2007) in normal comsumable *pito* and *dolo*. This level of lactic acid is however higher than the 0.5 % reported by Mashanda (1997) for traditional sorghum beer *chibuku* in Zimbabwe to become unacceptable. This difference may be on account of local palates developed over the years for various traditional sour sorghum beers in the different communities they are produced.

The daily variation of lactic acid content in the tested experimental samples of *pito* brewed with pure single strain starter cultures of *L. delbrueckii* and

Anchor Brewers' yeast and with mixed population microflora of the traditional process is given in Table 38.

Table 38: Daily lactic acid levels (Mean and Standard Deviation) of *pito* samples for the determination of shelf life.

DAY	LACTIC ACID (%) TRADITIONAL *PITO*	LACTIC ACID (%) STARTER CULTURE *PITO*
1	0.49±0.05	0.36±0.03
2	0.66±0.05	0.40±0.02
3	1.06±0.06	0.55±0.03
4	1.23±0.05	0.71±0.04
5	1.35±0.06	0.76±0.03
6	1.58±0.04	0.89±0.03
7	1.76±0.06	1.06±0.05
8	1.95±0.06	1.37±0.08
9	2.05±0.04	1.60±0.05
10	2.20±0.05	1.80±0.04

For the starter culture (SC) fermented experimental *pito*, a squared-X model was fitted to describe the relationship between LACTIC ACID % and DAY which yielded the equation:

$$\text{LACTIC ACID \% SC} = 0.390366 + 0.0145359*\text{DAY}^2$$

The Analysis of Variance (ANOVA) showed a significant relationship ($p < 0.05$) between LACTIC ACID % SC and DAY at 95.0% confidence level. The R-Squared value from the regression analysis indicated that the fitted model explained 99.1% of the variability in LACTIC ACID % SC. A Pearson correlation coefficient of 0.995394 also indicated a relatively strong relationship between the variables.

The plot of fitted model of the variation of lactic acid level in Starter Culture brewed *pito* (SC) is illustrated in Figure 41

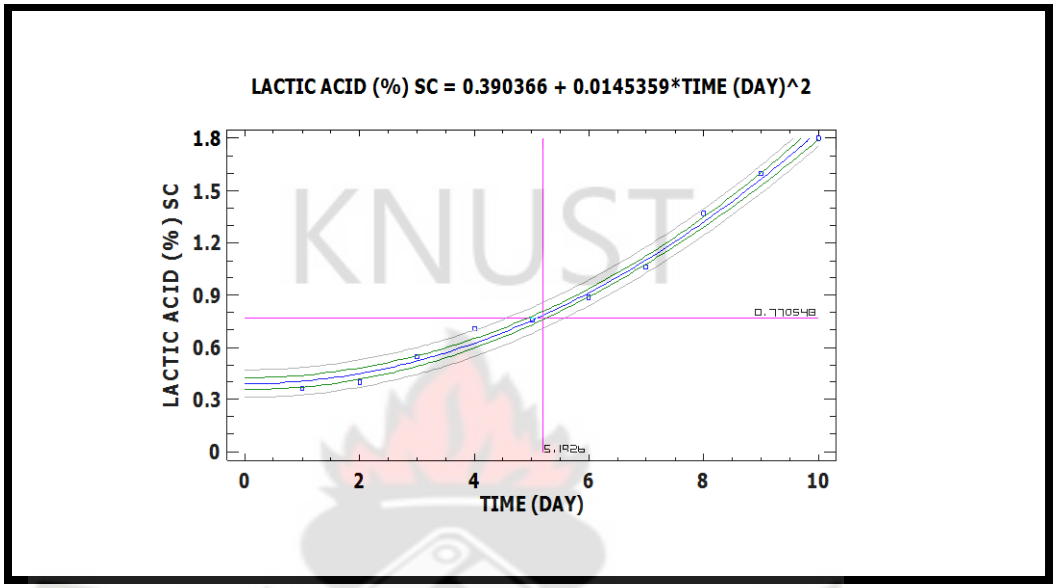

Figure 41: Plot of a fitted model of the relationship between lactic acid content of starter culture pito and days of storage.

From the fitted model, the established Spoilage Level of *pito* of 0.77 % Lactic acid, will be reached in 5 days. Similarly, regression analysis of the data for traditionally brewed *pito*, (TP) yielded a square root-X model for the variation of lactic acid with day as the best fit with equation:

LACTIC ACID %, TP = -0.402283 + 0.816833*sqrt(DAY),

where Sqrt is square root (√)

The Analysis of Variance (ANOVA) showed a significant relationship ($p < 0.05$) between LACTIC ACID %, TP and DAY at 95.0% confidence level with

a Pearson correlation coefficient of 0.996. The R-Squared value from the regression analysis indicated that the fitted model accounted for 99.2% of the variability in LACTIC ACID %, TP. The plot of the fitted model is illustrated in Figure 42.

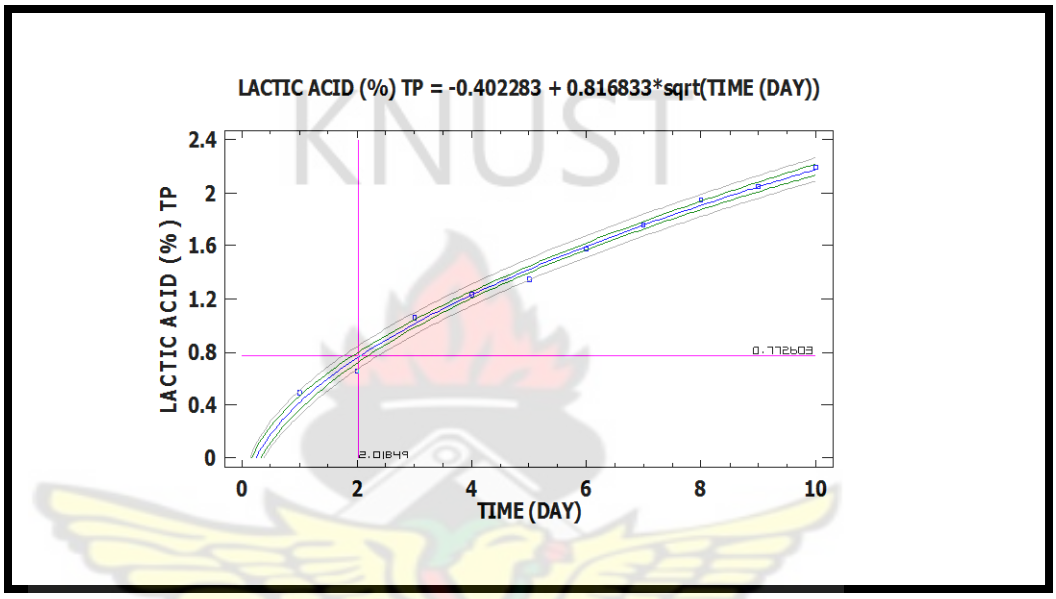

Figure 42: Plot of fitted model indicating the relationship between lactic acid level of traditionally brewed pito and days of storage.

The tables of coefficient for the fitted models and ANOVA tables are given in Appendices 7, 8, 9, and 10.

From the plot, it would take 2 days to reach the Spoilage Level of 0.77 % lactic acid for the traditionally brewed *pito*. The measured shelf life of 2 days is close to the 3 days shelf life of *pito* reported by Ellis *et al.* (2005) and in agreement with the reported limited shelf life of 1 to 3 days for traditional African sorghum beers due to over sourness (Novellie and De Schaepdrijver,

1986; Tisekwa, 1989; Maoura *et al.,* 2009; Lyumugabe *et al.,* 2010). The observed shelf life of 5 days for *pito* brewed with pure single strain starter cultures of *L. delbrueckii* and *S. cerevisiae* conveyed an improvement of shelf life over traditionally brewed *pito* for the experimental test beers. The commercial *pito* sample brewed traditionally by an indigenous *pito* brewer and used for the establishment of spoilage level was not rejected by the taste panellists until the 6^{th} day because the exclusion of air ingress into the fermenting *pito* by the use of the air lock prevented the metabolic activities of acetobacter which convert ethanol into acetic acid in the presence of air and are known as the major cause of spoilage by over-sourness (Blandino *et al.,* 2003; Briggs *et al.,* 2004; Haggblade and Holzapfel,1989b; Kutyauripo *et al*., 2009; Konfo *et al.*, 2015). The experimentally brewed test samples were kept in their normal conditions of sale, i.e. exposure to air ingress and ambient temperature. The improved shelf life of 5 days achieved for *pito* brewed with the starter cultures is in agreement of the view of Briggs *et al*, (2004) that the shelf life of traditional African sorghum beers brewed in modern scrupulously cleaned, dust controlled plants, using pure cultures of yeast and perhaps of Lactobacilli, may still be only five days and that various attempts to stabilize these beers met only with limited success.

6.4.3 Conclusion

Fermenting *pito* with pure single strain commercial starter cultures of *L. delbrueckii* and Anchor Brewers' Yeast extends its shelf life by two days over that of *pito* fermented with the traditional process with mixed population of

microflora by prolonging the duration after which the product becomes undrinkable on account of becoming too sour. The application of this process will therefore enable the sales period of *pito* to be extended from the usual 3 days to 5 days. Preventing the ingress of air into fermenting *pito* has also been shown to extend the shelf life by slowing down the rate of acid production and development of sourness. The study has supported the view that the maximum shelf life of traditional African sour beers brewed with sorghum may not exceed 5 days even when fermented with starter cultures when the product is kept in active state of fermentation at tropical and sub-tropical warm ambient temperatures for sale and consumption. The method of using over-sourness as spoilage level indicator to determine shelf life by regression analysis has proven to be an effective means of predicting the shelf life of *pito* as the results correlate closely with reported literature values.

6.5 Comparison of volatile fermentation aroma by-products

6.5.1 Method

Samples of the *pito* fermented with the pure cultures and with the traditional process were filled into 330 ml bottles and pasteurized at 62 °C for 25 minutes. The determination of volatile aroma compounds was carried out according to the Headspace method for fermentation by-products of MEBAK III, WBBM 2.21.1, 1996 at Research Center Weihenstephan for Brewing and Food Quality of the Technische Universitat Munchen, Germany.

Three bottles of 330 ml pasteurized starter culture *pito* and 3 bottles of 330 ml pasteurized traditional *pito* were analysed as fresh samples and then again

after 4 weeks of storage at 28°C – 30°C to check the storage stability of the fermentation by-products. The samples were analyzed on a Perkin Elmer Clarus 580 gas chromatograph GC-FID system with a flame ionization detector, a Turbo Matrix 40 (HS) autosampler (20 ml vial and 2 ml sample volume) and an INNOWAX Column (dimension 60 m long, 0.25 mm i.d. and 0.5 µm thickness). The carrier gas was helium 5.0, ECD quality, split 20 ml/min. Vials containing the samples were equilibrated to 60°C for 25 mins. 1 min after injection at 50°C the temperature was increased at 7°C /min to 85°C. The internal standard was p-cymene and the software was TotalChrom. The concentration of compounds in the samples was calculated by the software from calibration curves created from peak areas of compounds and internal standards.

6.5.2 Statistical Analysis

The analyses were carried out in duplicate because of the high reproducibility of the method after which means and standard deviations were calculated. One-way Analysis of variance (ANOVA) with Statgraphics Centurion XVI Version 16.1.11 was used to analyze the volatile components in the starter culture *pito* and traditional *pito* samples followed by Fisher's least significant difference (LSD) procedure to determine statistically significant differences between the means at the 95% confidence level. Principal Component Analysis was also carried out using Minitab (Release 14) software to reduce the measured volatile compounds into two main components which would explain the total variation in the data.

6.5.3 Results and Discussion

The volatile fermentation by-products measured in the *pito* samples were acetaldehyde, esters, higher alcohols and diacetyl. These were present at different intensities and as aroma compounds, would contribute to the aroma of the samples according to the levels present and their interactions (Hough *et al.*, 1982b; Kunze, 2004b). The *pito* fermented with pure strain of brewer's yeast (*Saccharomyces cerevisiae)*, i.e. Anchor Brewers' Yeast had a higher level of total volatile compounds (353.13 mg/L) than the traditionally *pito* which was fermented with several different yeasts (229.04 mg/L). The concentration of the various fermentation by-products measured are given in Table 39. A significant difference was found to exist between all the aroma compounds determined in *pito* fermented with starter culture and the traditional *pito*. Analysis of volatile fermentation compounds in single and mixed culture fermentation of *pito* by Demuyakor and Ohta, (1993) also revealed differences in the fermentation.

Table 39: Concentration of volatile fermentation aroma compounds (Mean and Standard Deviation) in fresh and 4-week stored traditional *pito* (Trad *Pito*) and *pito* produced with starter cultures of *L. delbrueckii* and Anchor Brewers'Yeast (SC *Pito*)[*]

COMPONENT (mg/l)	TRAD *PITO*, FRESH	SC *PITO*, FRESH	TRAD *PITO*, 4 WEEKS STORAGE	SC *PITO*, 4 WEEKS STORAGE
Acetaldehyde	1.96±0.13[a]	32.65±2.07[b]	1.17±0.16[a]	34.43±1.16[b]
n-Propanol	23.91±0.23[a]	134.17±1.36[b]	23.76±0.73[a]	135.43±3.42[b]
i-Butanol	33.11±0.46[a]	75.84±0.11[b]	33.16±0.69[a]	76.89±1.17[b]
Iso-Amylalcohol	140.05±1.90[a]	100.87±0.16[b]	141.43±5.71[a]	101.72±0.85[b]
Ethylacetate	28.93±1.5[a]	9.37±0.54[b]	29.18±1.89[a]	9.66±0.23[b]
i-Amylacetate	1.09±0.06[a]	0.24±0.00[b]	1.08±0.08[a]	0.25±0.01[b]
Diacetyl, total	0.18±0.01[a]	0.21±0.00[b]	0.17±0.01[a]	0.23±0.01[b]
Total Volatiles	229.04±4.33[a]	353.13±1.20[b]	229.77±9.26[a]	358.37±6.84[b]

Values are means of two determinations. Mean values with same superscript in a row are not significantly different ($p>0.05$).

The differences in the levels of volatile fermentation compounds produced in the two types of *pito* can be explained by the diversity of intrinsic properties of the different strains of yeasts to produce the compounds as was reported by Zhu *et al.*, (2016). Yeast strains differ greatly with regard to their formation of fermentation by-products, in particular higher alcohols and esters and the ratio of the floral smell esters to the higher aliphatic alcohols (Kunze, 2004b). Romano *et al*., (2003) have reported that in the fermentation of wine, the growth of each wine yeast species is characterized by a specific metabolic activity which determines the concentrations of flavour compounds in the final wine and have noted that the significant strain variability in the species may

cause a loss of characteristic aroma and flavour determinants when starter cultures are used for fermentation.

There was no significant difference between the level of volatile fermentation by-products in the fresh *pito* samples and samples stored for 4 weeks at 28-30°C to accelerate ageing. This implied aroma stability of at least 4 weeks for the samples. This observation can be explained by the absence of any further metabolic activity of yeast in the samples as they were pasteurized.

Acetaldehyde is the most important aldehyde in beer and is a normal intermediate product in alcoholic fermentation (Kunze, 2004b). It can either be reduced to ethanol or oxidized to acetic acid in the finished beer (Hough *et al.,* 1982b). The measured value in Starter Culture *pito* of 32.65 mg/L is very high compared to the 8 to 10 mg/L (Kunze, 2004b) found in matured lager beers and much higher still than the 0.019mg/L obtained in experimental traditional sorghum beer *ikigage* of Rwanda brewed with a starter culture of *S. cerevisiae* and *L. fermentum* (Lyumugabe *et al.,* 2014). The traditional *ikigage* brewed by the peasants themselves contains 0.076 mg/L acetaldehyde. However, the 1.95 mg/L measured in traditionally brewed *pito* while lower than the range given by Kunze (2004b) is consistent with the range of 0.5 to 10 mg/l given by Hough *et al.,* (1982b) for Irish stout. The higher fermentation temperature of Traditional *pito*, 30°C compared to 24°C for Starter culture *pito* also favours reduction of aldehydes and can explain its lower value in traditional *pito* compared to starter culture *pito*. The effect of the warmer fermentation temperature can explain the lower level of total diacetyl in traditional *pito*

whose reduction in beer is also favoured by higher fermentation temperatures. The level of acetaldehyde in the starter culture *pito* is also beyond the threshold value of 2–20 mg/L given by Ma *et al*., (2016) for ales in which class of beers *pito* falls by its fermentation conditions.

The higher alcohols determined were n-propanol, i-butanol and iso-amylalcohol (2-, 3- methylbutanol). These made up 310 mg/l, i.e. 88.0 % of the total volatile compounds measured in the *pito* brewed with starter cultures in comparison to197.07 mg/L, i.e, and 86 % in traditionally brewed *pito*. These high concentrations are consistent with the observations of Ashraf *et al.,* (2010), that of all the secondary metabolites formed by yeasts, the higher alcohols are produced in the highest concentrations during fermentation, where propanol, isobutyl alcohol and isoamyl alcohol are the predominant aroma compounds. The high concentration of higher alcohols in the *pito* brews is also in agreement with the observation of Hough *et al.,* (1982b) that the concentration of higher alcohols in home-brewed beers and wines is at least 10 times higher than those in the commercial products. The high concentrations of i-propanol (134.2 mg/L) and amylalcohol (100.87 mg/L) in the starter culture *pito* are however quite high in comparison with the values of 10.95 mg/L for propanol in pure culture *S. cerevisiae* and 53.1 mg/L for isoamyl alcohol in mixed culture of *S. cerevisiae* and *C. tropicalis* obtained by Coulibaly *et al.,* (2016) in their study on the influence of freeze dried starter cultures on volatile compounds in *tchakpalo*, the Ivorian sorghum beer equivalent of *pito*.

Higher alcohols are formed through the catabolic Ehrlich pathway in presence of amino acids and from anabolic route from sugars by way of biosynthesis. Their formation is dependent upon the fermentation temperature; an increase in temperature results in increased concentrations of higher alcohols, except for n-propanol. Brown and Hammond (2003) gave the taste thresholds for typical higher alcohols to be in the order of 2–100 mg/L. Typical levels of amyl alcohol has been found to be 38.0–100.0 mg/L. (Cortacero-Ramırez *et al.*, 2003). The value of amyl alcohol measured in starter culture *pito* falls within these established ranges.

Esters contribute to the overall flavour of beer and are the most important aroma compounds in beer but abnormally high levels may be regarded as off-flavours. According to Hough *et al*. (1982b) wild yeasts *Hansenula* and *Pichia* produce large quantities of ethyl acetate by aerobic fermentation and reported that Lambic and gueuze beers contain 33.4 – 167.0 mg/L of ethyl acetate. These beers are sour beers produced by spontaneous fermentation similar to traditional *pito*. They are fermented by a boutique of microorganisms. The value of ethyl acetate 29.2 mg/L measured in the traditional *pito* stored for 4 weeks fell close to the lower limit of the range but lambics are stored for one to three years before consumption during which period the ester content increases by esterification of alcohols. The level of esters in the starter culture *pito* are relatively low compared to the levels in traditional *pito* but much higher than the 0.338 mg/L and 0.068 mg/L respectively for ethyl acetate and iso amylacetate obtained in the *pito* style Rwandan sour sorghum beer *ikigage*

brewed with starter cultures reported by Lyumugabe *et al.* (2014). Esters are formed during fermentation by esterification of fatty acids and also in small amounts by esterification of higher alcohols (Kunze, 2004b). The ester content depends on beer type. Bottom fermentation beers contain up to 60 mg/l and top fermentation beers contain up to 80 mg/L ester (Kunze, 2004b). Wei *et al.*, (2007), reported that the contents of ethyl acetate and isoamyl acetate in extruded white sorghum beer ranging 8.0–50.0, and 0.86–6.00 mg/L respectively gave the beer a typical aroma. The value of ethyl acetate in the starter culture *pito* fell within this range.

According to Meilgaard (1975), the concentration of isoamyl acetate found in lambic sour beers is much lower than the concentrations in classic beers and reported that isoamyl acetate can range anywhere from 1.2 – 2.8 mg/L in lagers and 0.7 – 3.3 mg/L in ales. The value of 0.24 mg/L and 1.085 mg/L measured in starter culture and traditional *pito* respectively can therefore be assessed to be consistent with the levels in this class of sour beers. The lower level of higher alcohols found in traditional *pito* in conjunction with the higher level of esters found in it compared to the higher level of higher alcohols and lower level of esters in starter culture *pito* suggests that the various different yeasts present in the traditional *pito* collectively possess a higher ability to convert higher alcohols into esters than the single strain lactic acid bacteria and yeast used in fermenting the starter culture *pito*.

A graphical comparison between volatile fermentation by-products in the experimental *pito* brews, a similar type sour German wheat beer and a *pito*

brewed by Demuyakor and Otha, (1993) with mixed culture microflora obtained from traditional *pito* brewers is presented in Figure 43. The experimental starter culture *pito* (SC *Pito*) showed higher concentrations of propanol and iso-butanol than the other three beers while the experimental traditional *pito* (Trad *Pito*) showed the highest concentration of amylalcohol. The level of acetaldehyde was outstandingly high in the mixed microflora culture brew of Demuyakor and Ohta (1993). The level exceeded the 150 mg/L which according to Smogrovicova (2014) may cause an aldehydric character of high astringency values. Diacetyl and isoamylacetate levels were very low in all three beers and characteristic of sour type beers.

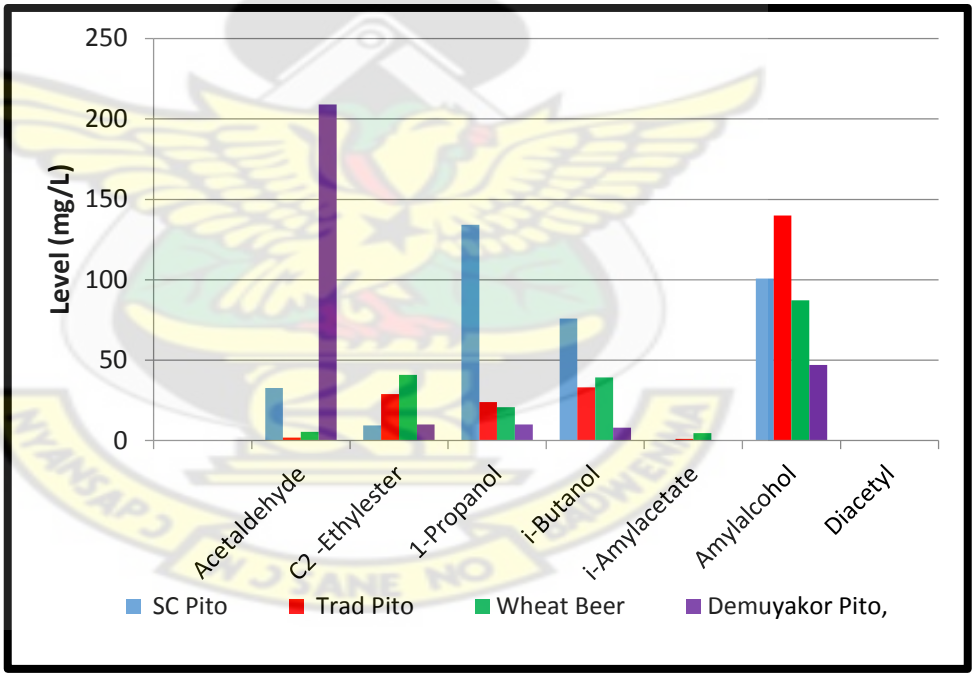

Figure 43: Levels of volatile fermentation by-products in fresh starter culture *pito* (SC *Pito*), traditional *pito* (Trad *Pito*), a sour German wheat beer (Wheat Beer) and *pito* brewed by Demuyakor and Otha, (1993) with mixed culture microflora (Demuyakor *Pito*).

It is noteworthy that the varying conditions of fermentation for beers, e.g. fermentation temperature and yeast dosage affect the level of fermentation by-products and this could account for some of the differences in the values cited for the various beers.

The volatile compounds in the fresh *pito* brews were reduced to two main components by Principal Component Analysis in Figure 44. The two components accounted for 100% of the total variation in the data (Component 1, 77.1 % and Component 2, 22.9%). The two brews were very much separated from each other by the predominant volatile compounds.

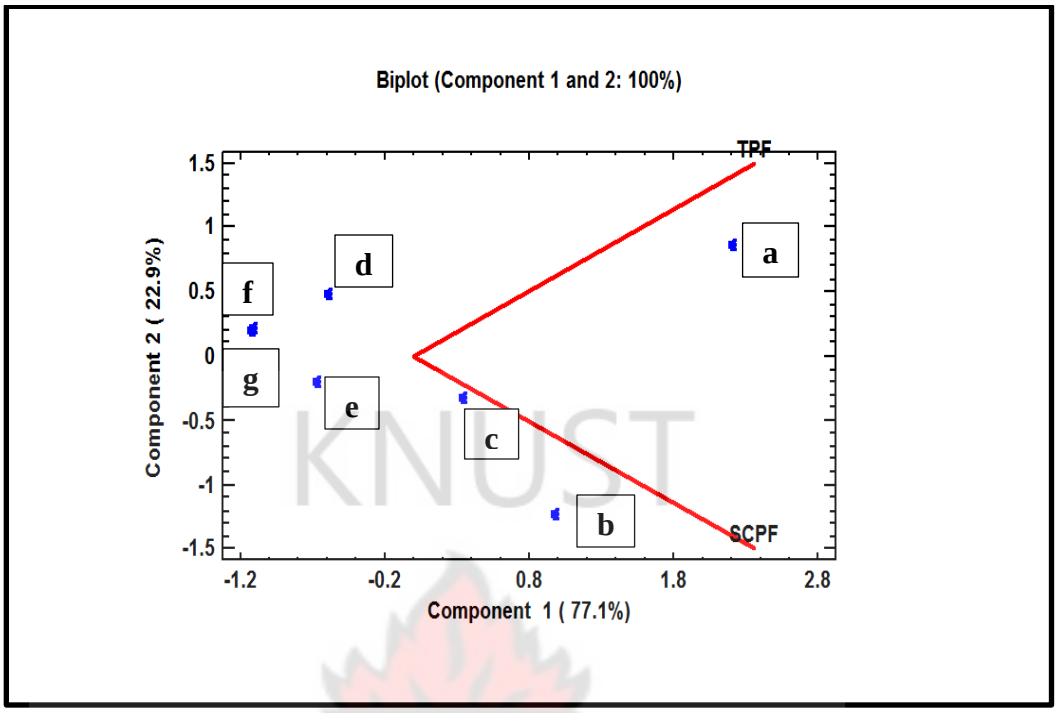

Figure 44: Principal Component Analysis loadings for volatile fermentation compounds in fresh Starter Culture *pito* (SCPF) and fresh Traditional *pito* (TPF)

Key: **a** = iso amylalcohol, **b** = n-propanol, **c** = i-butanol, **d** = ethyl acetate, **e** = acetaldehyde, **f** = diacetyl, **g** = isoamyacetate

The predominant higher alcohol in the starter culture *pito* was n-propanol and in traditional *pito* was iso-amylalcohol. Iso butanol also contributed positively to Component 1. Component 2 was characterized by ethyl acetate and acetaldehyde. The chromatograms for the analysis of the volatile fermentation by-products are presented in Appendices 11 to 18.

6.5.4 Outcomes And Conclusion

The volatile fermentation by-products that contribute to the flavour profile of *pito* have been identified and their levels determined. A significant difference was found to exist in the levels of all the aroma compounds determined in *pito* fermented with single strain commercial starter culture of lactic acid bacteria and brewer's yeast and traditional *pito* fermented with a boutique of bacteria, yeasts and moulds. In the brew with single strain starter cultures, the level of the fermentation by-products (metabolites) was accounted for by the specific intrinsic fermentation characteristics of the single strain microorganism whereas in the brew with the varied population of microorganism the level of the fermentation by-products was influenced by the characteristics of the individual microorganism. There was altogether a higher level of total volatile aroma components in the starter culture *pito* suggesting an enhanced aroma over the traditional *pito*. The concentration of the volatile compounds in both types of *pito* however was consistent with those found in sour type spontaneous fermented beers. There was no significant change in the concentration of compounds after forced aging for a period of four weeks suggesting flavour stability for that duration.

6.6 Sensory evaluation of *Pito* brewed starter cultures and *Pito* brewed with traditional process.

6.6.1 Method.

The *pito* samples were prepared as described in Chapter Five and filled into kegs and sent to a *pito* drinking bar where *pito* drinkers regularly come over to drink traditionally brewed *pito*. Permission was previously obtained from the *pito* brewer to use her customers for the test. The test was based on consumer acceptability of the products through a modified 9 point hedonic evaluation at 95 % confidence level. As most of the consumers were not literate, a pictorial scale (shown in Appendix 19) using denominations of the local currency, Ghana Cedi (GHS) with which they are very familiar was used as an equivalent to the conventional word categories ranging from "like extremely" to "dislike extremely". In the pictorial scale, pictures of GHS50, GHS20, GHS10, GHS5, GHS2, GHS1, GHS50p, GHS20p and GHS10p were equated respectively to Like Extremely, Like Very Much, Like Moderately, Like Slightly, Neither Like nor Dislike, Dislike Slightly, Dislike Moderately, Dislike Very Much, Dislike Extremely. The participants were asked to taste the samples and indicate which currency denomination best represented how much they liked or disliked the sample based on the value of the currency denomination. The samples were scored on Taste, Aroma and Overall Liking. The samples were served from the tap at the bottom of the fermenting vessel into 100 ml disposable plastic cups for the test participants. The first sample was served, evaluated and taken away before the second sample was served.

The scores which were reported verbally by the participants and were recorded directly on a score sheet by the test administrator. A total number of 89 consumers participated in the test.

6.6.2 Statistical Analysis

A two-way analysis of variance (panel–product) with interaction effect was done using XL-Stats® (Addinsoft, France). The best model using R^2 was used in modelling the data. This was followed by Fisher's LSD post hoc analysis to show how samples differ from each other when a significant model effect is obtained. Agglomerative Hierarchical Clustering in XL-Stats using Euclidean distances based on dissimilarity matrix and Ward's agglormerative method was also done to show if there were consumer groups with similar liking patterns.

6.6.3 Results and Discussion

There was no statistical significant difference between the two *pito* products for overall liking or taste liking. There was however a significant difference between the two products for aroma liking as indicated in Table 40. The scores are illustrated graphically in Figure 45.

Table 40: Sample means and significance for consumer acceptance

	Overall Liking	Taste Liking	Aroma Liking
SC *Pito*	6.663[a]	6.629[a]	6.955[b]
Trad *Pito*	6.449[a]	6.775[a]	6.225[a]
Pr>F	0.142	0.05	0.003
Significant	No	No	Yes

The values with the different superscripts in a column are significantly different ($p< 0.05$)

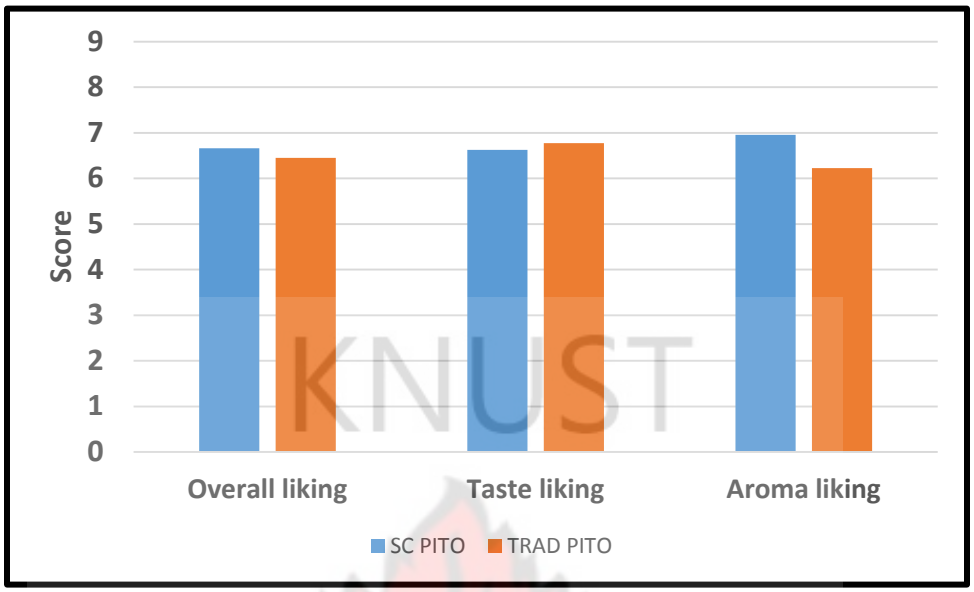

Figure 45: Product mean liking scores for *pito* brewed with single strain starter culture (SC *Pito*) and traditionally brewed *pito* with a mixed population of microflora (Trad *Pito*)

Based on the overall liking results, Cluster analysis revealed three clusters of consumers. Group one consumers (65%) liked both TRAD *PITO* and SC *PITO* about the same, the centroid being 7.4 and 6.9 respectively. Group two consumers (19%) liked SC *PITO* (centroid = 7.5) but did not like TRAD *PITO* (centroid = 4.3). Group three consumers (16%) liked TRAD and SC *PITO* about the same but their level of acceptance for both products were low (centroids = 5.2 and 4.7 respectively). The profile plot in Figure 46 shows the pattern for overall liking for the two products across the different groups.

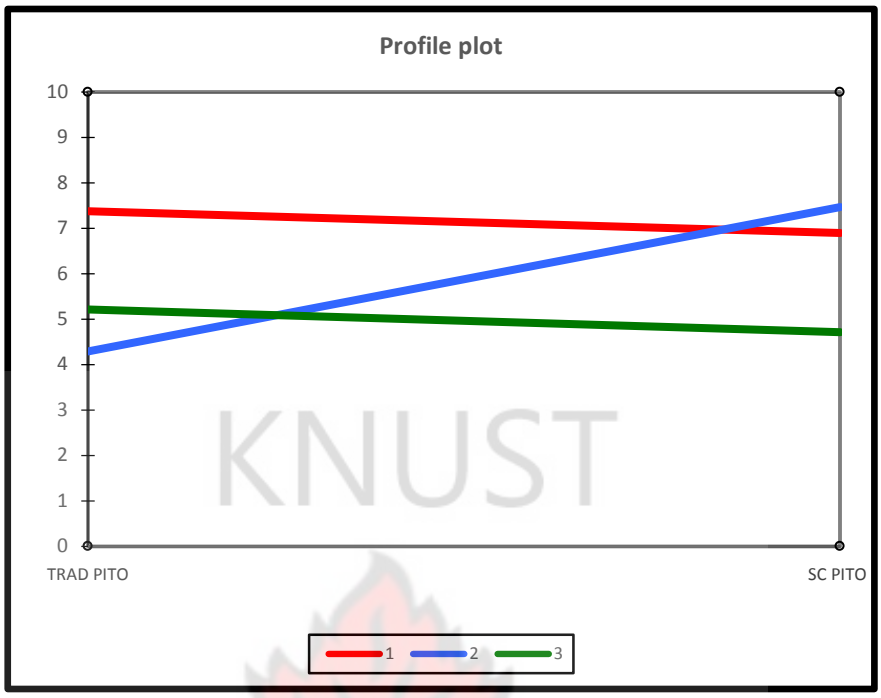

Figure 46: Profile plot showing overall liking patterns for traditional process *pito* brewed with a mixed population of microflora (Trad *Pito*) and for starter culture *pito* brewed with single strain starter cultures of *L. delbrueckii* and ASBY for three groups of consumers labelled red for group 1, blue for group 2 and green for group 3.

In a similar sensory evaluation of *dolo*, the Burkina Faso equivalent of *pito* fermented with starter cultures of one strain of *L. fermentum* and two strains *S. cerevisiae* isolated from *dolo* and *pito* respectively, Glover *et al.*, (2009) found taste and aroma not significantly different from the commercial *dolo* produced traditionally. Oriji *et al.*, (2003) also reported taste and flavour of '*pito*' produced with pure cultures of *L. plantarum* in combination with *S. cerevisiae* and *Pediococcus halophilus* in combination with *Candida tropicalis*, all isolated from *pito*, to compare favourably with that of *pito* produced by the traditional method. Similar results were obtained by Lyumugabe (2013) where

the association of *S. cerevisiae* with *I. orientalis* and *L. fermentum* produced *ikigage* sour sorghum beer with taste, aroma and mouth feel similar to *ikigage* beers brewed locally by peasants.

Concentrations of volatile fermentation compounds above their threshold levels damage flavour and acceptability of beer. The threshold level for n-propanol, iso-butanol and iso-amyalcohol are 600 mg/l, 160 mg/l and 110 mg/l respectively (Meyer, 2015). The preference of aroma of starter culture *pito* over traditional *pito* can therefore be linked to the higher content of isoamylalcohol of 140.05 mg/l in traditional *pito* which has exceeded its threshold value of 110 mg/l. The starter culture *pito* in comparison has a level of 100.9 mg/l which is below the threshold level. The level of the other higher alcohols in both *pito* brews are 23.91 mg/l and 33.11 mg/l for n-propanol and iso-butanol respectively for traditional *pito* and 134 mg/l for n-propanol, 75.84 mg/l for iso-butanol in starter culture *pito* are all below the their threshold levels.

The ratio of higher alcohols to esters which also affects aroma ranges from 1:2.5 to 3.1 for European-type pale beers. It is very much higher, 1:32.3 for the starter culture *pito* compared to the traditional *pito*, 1:6.6. According to Hough *et al.*, (1982b), esters undoubtedly contribute to the overall flavour of beer but abnormally high values may be regarded as off flavours. Ethyl acetate level in Traditional *pito* of 28.93, mg/l obtained from the analysis of fermentation by-products in Chapter 6.5, is much higher than the level of 9.37 mg/l in starter culture *pito*. Its threshold level is given as 25-30 mg/l (Kunze, 2004b) again

leading to the possibility that its level falling at the higher end of the threshold level in the traditional *pito* may have contributed to the aroma preference of starter culture *pito*.

6.6.4 Outcomes and Conclusion

The sensory evaluation found no difference in overall liking of *pito* brewed with starter cultures compared to the control traditionally brewed *pito*. Overall there was no significant difference in sensory scores for the consumers in the study. The quality of both products was positively perceived. However, a small percentage of consumers (4%) liked Starter Culture *pito* more than Traditional *pito* on overall liking. The application of starter cultures enhanced the aroma of *pito*. The improvement in aroma may be linked to the higher levels of volatile aroma compounds observed to have been formed in the *pito* brewed with starter cultures but which had not exceeded their threshold levels. There is a high chance of success for *pito* fermented with pure single strain starter cultures of lactic acid bacteria, *L. delbrueckii* and *S. cerevisiae*, Anchor Brewers' yeast on the market. This can be concluded from the fact that the test participants constitute consumers who patronize *pito* and having perceived no significant difference in the two products in overall liking would patronize the starter culture *pito* as much as they patronize the traditional *pito.*

CHAPTER SEVEN

General Conclusion and Recommendations

This study has investigated the application of commercial starter cultures in the fermentation of the traditional West African sour sorghum beer known as *pito* in Ghana and Nigeria, *dolo* in Burkina Faso, Niger and Mali, and *tchakpalo* in Ivory Coast, Benin and Togo towards industrial production. The study has demonstrated that the application of a starter culture of *L. delbrueckii* for souring a 12.3 % *pito* wort at 45˚C with a cell population density of 2.2 x 10^7 cells/ml for 12 hours followed by fermentation with a single strain starter culture of brewer's yeast *Saccharomyses cerevisiae*, Anchor Brewers' Yeast, at 24 °C for 71.5 h, is a viable process for industrial production of *pito*. The fermentation profile of *L. delbrueckii* and Anchor Brewers' Yeast in *pito* wort followed typical lactic acid and alcoholic fermentation indicating that their performance is not inhibited by the polyphenol levels in the red sorghum variety, *kadaga*, used in the study. This implies that this variety of sorghum which ranks among the popular varieties malted in Ghana for *pito* brewing can be recommended for use in brewing *pito* at the industrial scale. *Pito* brewed with the commercial starter cultures of *L. delbrueckii* and Anchor Brewers' Yeast with the fermentation conditions derived for their optimal performance will not differ significantly from traditionally brewed *pito* in sensory and physicochemical qualities. The starter culture brewed *pito* will have an improved shelf life over that of traditionally brewed *pito*. Bottled pasteurized products will have flavour stability extending over 4 weeks. The starter culture

process has been shown to be predictable and controlled rendering it suitable for standardization and applicable for the production of improved and consistent quality *pito* at industrial level with a high chance of success on the market.

Further tests at pilot plant level are suggested to determine if any modifications to the derived process parameters will be needed during up-scaling where the effects of mass and temperature gradients come into play. *L. amylolyticus* which is an amylolytic LAB and which has been found in the study to convert residual starch and dextrins in *pito* wort into sugars during the souring step should be investigated for its suitability of use to produce stronger brands of *pito* like Frafra *pito* without the usual need for increase in material input i.e. malt. *L. amylolyticus* also has a potential application for the production of a sweet and sour non- alcoholic malt drink when the fermentation process is terminated after the lactic acid step and not continued with the alcoholic fermentation with yeast. This potential also needs to be investigated for its realization.

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APPENDICES

Appendix 1: Supplier's Instruction for propagating *L. delbrueckii*

Lactobacillus strain „H1"is cultivated "in congress wort (brewer`s wort without hops) for 4 days at 48 °C. The inoculation amount is 5 ml from a base culture to 150 ml of congress wort.

Dr. Johannes Hinrichs

Source: Dr. Johannes Hinrichs

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Geschäftsführung: Dr.-Ing. Josef Fontaine
Vereinsregister 24043 Nz, Amtsgericht Charlottenburg

Appendix 2: Supplier's Instruction for propagating *L. amylolyticus*

The optimum propagation-temperature for Lactobacillus Amylolyticus is 45°C; max temperature 48°C.

The storage time in fridge suppose to be not longer than 2-3 weeks

Propagate in 10-times multiplication-rate 24 max 48hours at 45°C.

1ml of the liquid culture in 9ml non hoped wort (8-12°P), 24h at 45°C

=> +90ml non hoped wort (8-12°P), 24h at 45°C =>+ 900ml non hoped wort (8-12°P), 24h at 45°C =>=>+ 9000ml non hoped wort (8-12°P), 24h at 45°C and so on.

Careful with oxygen! Make sure, that you have low/no oxygen pick up.

Source: Ulrich Peise

Labor Hefebank <labor@hefebank-weihenstephan.de>

Appendix 3: Product Data Sheet for Munich Wheat Beer Yeast

MUNICH

WHEAT BEER YEAST

SACCHAROMYCES CEREVISIAE

NATURAL
KOSHER
GMO FREE

1. Origin

Munich German Wheat Beer yeast originated in Bavaria and is used by a number of commercial breweries to produce German-style wheat beers. The propagation and drying processes have been specifically designed to deliver high quality beer yeast that can be used simply and reliably to help produce wheat beers of the finest quality. No colours, preservatives or other unnatural substances have been used in its preparation. The yeast is produced in ISO 9002 certified plants.

2. Microbiological Properties

- Classified as Saccharomyces cerevisiae .
- Top fermenting yeast.
- Typical analysis of Munich active dried yeast:

Percent solids	93% –95%
Living yeast cells	$\geq 5 \times 10^{9}$ per gram of dried yeast
Wild yeast	< 1 per 10^{6} yeast cells (Lysine method)*
Bacteria	< 1 per 10^{6} yeast cells*

- Finished product is released to market only after passing a rigorous series of tests.

*According to ASBC and EBC methods of analysis.

3. Brewing Properties

- Quick start and vigorous fermentation, which can be completed in 4 days above 17°C.
- Medium to high attenuation.
- Fermentation rate, fermentation time and degree of attenuation are dependent upon inoculation density, yeast handling, fermentation temperature and nutritional quality of the wort.
- Munich is a non flocculent strain. In classic open fermentation vessels, the yeast can be skimmed off the top. Some settling can be promoted by cooling and use of fining agents and isinglass.
- Aroma is estery to both palate and nose with typical banana notes. Does not display malodours when properly handled. Munich yeast has found widespread use in the production of German Weizen and Hefeweizen.
- Munich yeast is best used at traditional ale temperatures after rehydration in the recommended manner.

4. Usage

- When 100 g active dried yeast is used to inoculate 100 litres of wort, a yeast density of 5-10 million cells per millilitre is achieved. The pitching rate may be adjusted to achieve a desired beer style or to suit processing conditions.
- Sprinkle the yeast on the surface of 10 times its weight of clean, sterilized (boiled) water at 30 - 35°C. Do not use wort, or distilled or reverse osmosis water, as loss of viability may result. **DO NOT STIR**. Leave undisturbed for 15 minutes then stir to suspend the yeast completely, and leave it for 5 more minutes at 30-35°C. Adjust the temperature to that of the wort and inoculate without delay.
- Attemperate in steps of 10°C at 5-minute intervals to the fermentation temperature by mixing aliquots of wort. Do not allow attemperation to be carried out by natural heat loss. This will take too long and could result in loss of viability or vitality.
- Temperature shock, at greater than 10°C, may cause formation of petite mutants leading to long-term or incomplete fermentation and possible formation of undesirable flavours.
- Munich German Wheat Beer yeast has been conditioned to survive rehydration. The yeast contains an adequate reserve of carbohydrates and unsaturated fatty acids to achieve active growth. It is unnecessary to aerate wort.

5. Storage

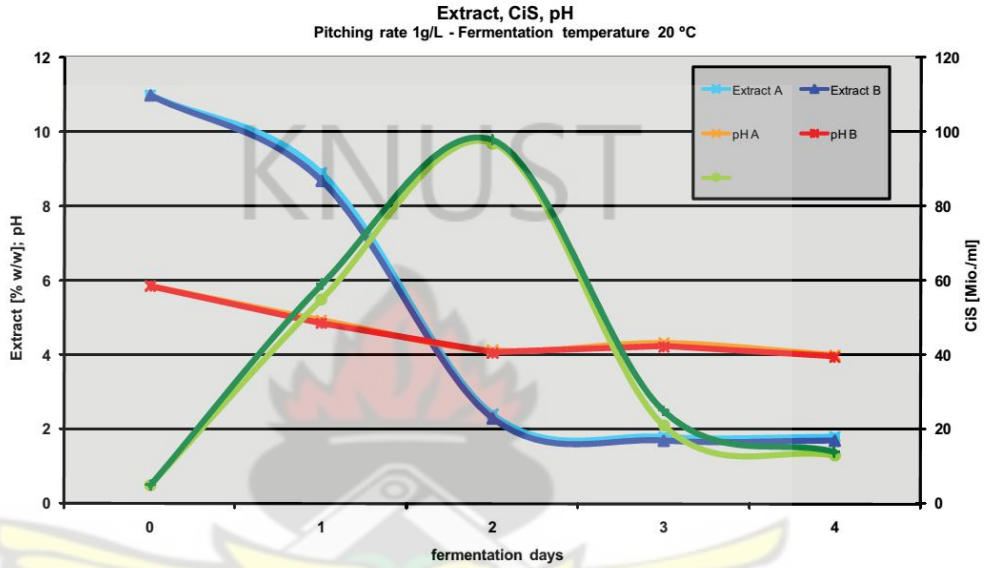
- All active dried yeast should be stored dry and below 8°C. Packaging should remain intact.
- Activity loss is about 25% per year at 8°C and 50% per year at 22°C in unopened sealed packs.
- Munich will rapidly lose activity after exposure to air. Do not use 500 g or 10 kg packs that have lost vacuum. Opened packs must be re-closed, stored in dry conditions below 4°C and used within 3 days; 11 g sachets are not vacuum packed but are flushed with nitrogen gas to protect the yeast.
- Do not use yeast after the expiry date printed on the pack.

MAY 2010

MUNICH

WHEAT BEER YEAST

SACCHAROMYCES CEREVISIAE



Ideal for German and Belgian wheat beers and banana and clovy flavours. High attenuation and fast complete fermantations. And, that's just for starters!

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The information herein is true and accurate to the best of our knowledge, however, this data sheet is not considered as a guarantee expressed or implied, or as a condition of sale of this product.

DISTRIBUTED BY:

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Appendix 4: Product Data sheet for Anchor Brewers' Yeast

TEMPERATURE RANGE: This strain ferments well over the range of 20 to 33°C. ALCOHOL TOLERANCE: This strain displays a good alcohol tolerance of up to at least 15% v/v.

SPECIFICATIONS

Physical Specifications

Test / Parameter	Specification	Test Method
Particle shape	Beads/particulate	Visual

Chemical Specifications

Test / Parameter	Specification	Test Method
Dry matter	>92%	Internal method

Microbiological Specifications

Test / Parameter	Specification	Test Method
Viable Yeast count / cell count	>1.0E10 CFU/g	Internal method
Total Bacterial count	<1.0E6 CFU/g	Internal method
Lactobacillus count	<1.0E6 CFU/g	Internal method
Wild Yeast	<1.0E5 CFU/g	Internal method
Mould	<1.0E3 CFU/g	Internal method

Analytical results

Test / Parameter	Typical batch sample	Units
E. coli	<10	CFU/g
Coliforms	<10	CFU/g
Total Bacteria	1.70E+03	CFU/g
Wild Yeast	<10	CFU/g
Mould	<10	CFU/g
Viable Yeast Count	1.48E+10	CFU/g
Lactic Acid bacteria	2.00E+03	CFU/g
Dry Matter	95.92	%
Protein	45.2	%
Foam head	250	>mm
Sugar Foam	10	<min

Appendix 5: **Classification of Anchor Brewers' Yeast, Strain BDY.**

The BDY strain, given its wide range of fermentation temperature (20-30degC), it is neither a classic top or bottom fermenting strain. Lager yeasts (bottom fermenters) are classically better suited to low temperature fermentations, while ale yeasts (top fermenters) are better suited to higher temperatures.

The recommended dosage is 0.1 – 0.2% m/v.

Source: **Shawn Cummings** <SCummings@anchor.co.za>

Appendix 6: Results of Confirmatory Tests To Validate Predicted Conditions For Fermentation

Parameter	Test Conditions	Test 1	Test 2	Test 3	Mean	Stdev
Extract, % (OG)	13.2	13.2	13.2	13.2	13.2	0
Extract, % (AE)	N/A	7.4	7	7.2	7.2	0.2
Ph	4	3.56	3.66	3.58	3.6	0.1
Temperature, °C	24	24	24	24		
Time, h	71.5	71.5	71.5	71.5		

Appendix 7: Table of Coefficients for fitted model for Starter Culture Pito

Parameter	Least Squares Estimate	Standard Error	T- Statistic	P-Value
Intercept	0.390366	0.0249121	15.6698	< 0.001
Slope	0.0145359	0.00049495	29.3681	< 0.001

Appendix 8: **ANOVA table of fitted model for Starter Culture Pito.**

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Model	2.2208	1	2.2208	862.49	<0.001
Residual	0.02059	8	0.00257488		
Total (Corr.)	2.2414	9			

Appendix 9: Table of Coefficients for fitted model for Traditional Pito

Parameter	Least Squares Estimate	Standard Error	T-Statistic	P- Value
Intercept	-0.402283	0.0611385	-6.57987	0.0002
Slope	0.816833	0.0260695	31.3329	<0.001

Appendix 10: ANOVA Table for fitted model for Traditional Pito

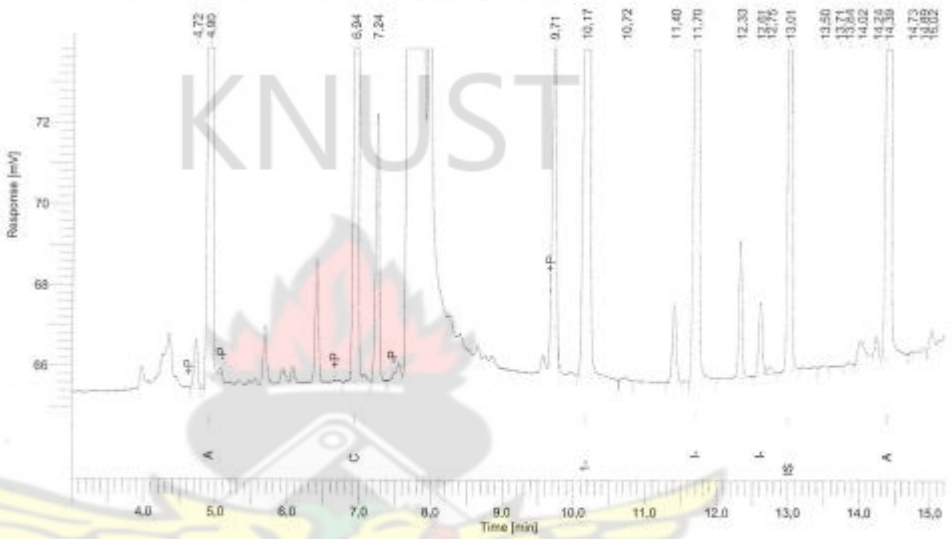
Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Model	3.01425	1	3.01425	981.75	0.0000
Residual	0.02456	8	0.003070		
Total (Corr.)	3.03881	9			

Appendix 11: Chromatogram of Fermentation By-Products of Pito Brewed with Starter Culture and Aged for 4 weeks

Software Version : 6.3.2.0646
Sample Name : 99902-1
Instrument Name : G-17_ECD_FID
Rack/Vial : 0/0
Sample Amount : 1,000000
Cycle : 7

Date : 30.06.2016 09:54:29
Data Acquisition Time : 24.05.2016 18:09:16
Channel : A
Operator : Analyst
Dilution Factor : 2,000000

Result File : \\serv\tcprocess\TC_Daten\G-17_ECD_FID\daten\gnp\kw_21_16\di_007.rst
Sequence File : \\serv\tcprocess\TC_Daten\G-17_ECD_FID\methoden\GNP_VDK.seq



GNP

Time [min]	Height [µV]	Area [uV*sec]	Area/ Amount	Component Name	mg/l
4,902	101100,77	326752,56	19446,4492	Acetaldehyd	33,61
6,941	26657,34	90589,24	19091,6235	C2-Ethylester	9,49
10,166	33806,46	149103,48	2241,9374	1-Propanol	133,01
11,700	50374,86	169657,65	4461,3166	i-Butanol	76,06
12,611	1806,52	6523,06	54808,9238	i-Amylacetat	0,24
13,012	21770,71	64457,65	---------	IS_N-Butanol	---------
14,394	68112,90	210283,91	4159,2684	Amylalkohole	101,12
	303629,55	1017367,56	1,0421e+05		353,52

Appendix 12: Chromatograph of Fermentation By-Products of Fresh Traditional Pito.

Software Version : 6.3.2.0646
Sample Name : 99900-1
Instrument Name : G-17_ECD_FID
Rack/Vial : 0/0
Sample Amount : 1,000000
Cycle : 3

Date : 30.06.2016 09:53:43
Data Acquisition Time : 24.05.2016 16:29:18
Channel : A
Operator : Analyst
Dilution Factor : 2,000000

Result File : \\serv\tcprocess\TC_Daten\G-17_ECD_FID\daten\gnp\kw_21_16\di_003.rst
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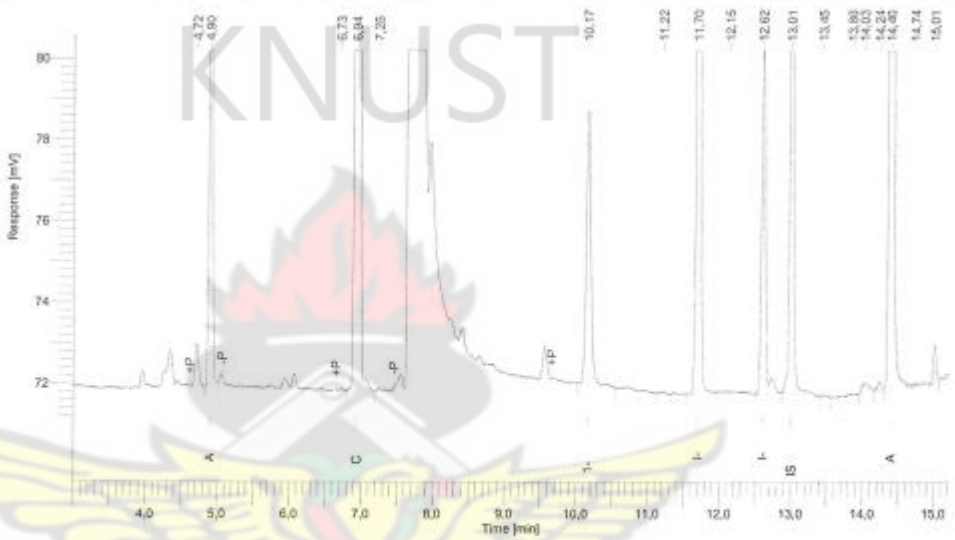

GNP

Time [min]	Height [µV]	Area [uV*sec]	Area/ Amount	Component Name	mg/l
4,902	11381,94	36815,41	39574,2445	Acetaldehyd	1,86
6,944	86363,08	289323,01	20795,1116	C2-Ethylester	27,83
10,169	6742,96	30999,81	2610,4772	1-Propanol	23,75
11,702	24788,09	84705,33	5168,5443	i-Butanol	32,78
12,616	8550,83	31165,61	60109,0851	i-Amylacetat	1,04
13,014	23993,83	73336,47	---------	IS_N-Butanol	---------
14,400	102864,56	313984,68	4527,2123	Amylalkohole	138,71
	264683,29	860330,31	1,3278e+05		225,96

Appendix 13: Chromatogram of Fermentation By-Products of Fresh Pito brewed with Starter Culture.

Software Version	: 6.3.2.0646	Date	: 30.06.2016 09:54:41
Sample Name	: 99903-1	Data Acquisition Time	: 24.05.2016 18:59:15
Instrument Name	: G-17_ECD_FID	Channel	: A
Rack/Vial	: 0/0	Operator	: Analyst
Sample Amount	: 1,000000	Dilution Factor	: 2,000000
Cycle	: 9		

Result File : \\serv\tcprocess\TC_Daten\G-17_ECD_FID\daten\gnp\ikw_21_16\di_009.rst
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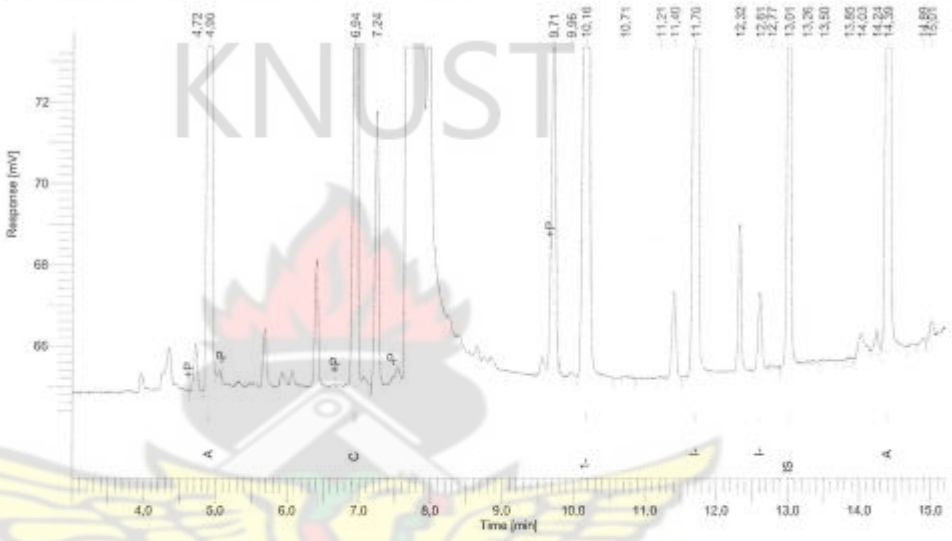

GNP

Time [min]	Height [uV]	Area [uV*sec]	Area/ Amount	Component Name	mg/l
4,901	100123,77	326612,05	20949,1606	Acetaldehyd	31,18
6,939	26848,89	91799,27	20418,8177	C2-Ethylester	8,99
10,162	36513,22	159512,19	2380,9462	1-Propanol	135,13
11,695	53354,09	180667,50	4769,6840	I-Butanol	75,76
12,608	1909,58	6807,89	57542,8245	I-Amylacetat	0,24
13,008	23147,26	69115,84	----------	IS_N-Butanol	----------
14,390	72320,22	223399,26	4424,7799	Amylalkohole	100,98
	314217,04	1057914,00	1,1047e+06		352,27

Appendix 14: Chromatogram of Fermentable By-Products of 4-Weeks Aged Traditionally brewed Pito.

Software Version : 6.3.2.0646
Sample Name : 99901-1
Instrument Name : G-17_ECD_FID
Rack/Vial : 0/0
Sample Amount : 1,000000
Cycle : 5

Date : 30.06.2016 09:54:21
Data Acquisition Time : 24.05.2016 17:19:17
Channel : A
Operator : Analyst
Dilution Factor : 2,000000

Result File : \\serv\tcprocess\TC_Daten\G-17_ECD_FID\daten\gnp\kw_21_16\dl_005.rst
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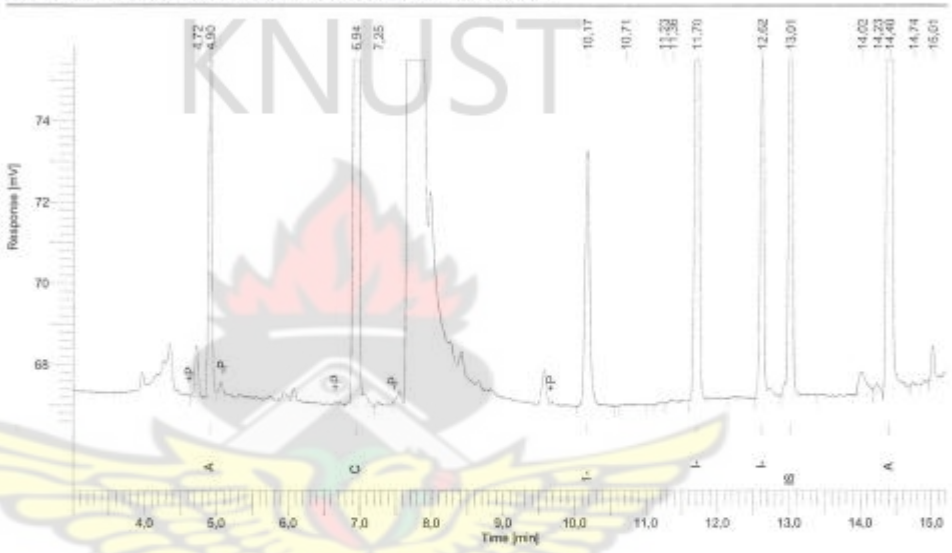

GNP

Time [min]	Height [µV]	Area [uV*sec]	Area/ Amount	Component Name	mg/l
4,902	8732,19	28416,48	44285,9239	Acetaldehyd	1,28
6,942	86476,10	289424,26	18973,0080	C2-Ethylester	30,51
10,167	6289,87	29392,97	2422,1602	1-Propanol	24,27
11,702	23200,81	79552,21	4727,8811	i-Butanol	33,65
12,615	8588,68	31016,85	54364,5076	i-Amylacetat	1,14
13,012	21909,91	65663,37	----------	IS_N-Butanol	----------
14,396	98489,48	296108,44	4071,2745	Amylalkohole	145,46
	253687,05	819574,56	1,2884e+05		236,32

Appendix 15: Chromatogram of Vicinal Diketones in Fresh pito brewed with Traditional Process.

Software Version	: 6.3.2.0646	Date	: 30.06.2016 09:55:29
Sample Name	: 99900-1	Data Acquisition Time	: 24.06.2016 22:09:13
Instrument Name	: G-17_ECD_FID	Channel	: B
Rack/Vial	: 0/0	Operator	: Analyst
Sample Amount	: 1,000000	Dilution Factor	: 1,000000
Cycle	: 16		

Result File : \\serv\tcprocess\TC_Daten\G-17_ECD_FID\daten\vdk\kw_21_16\dl_2_002.rst
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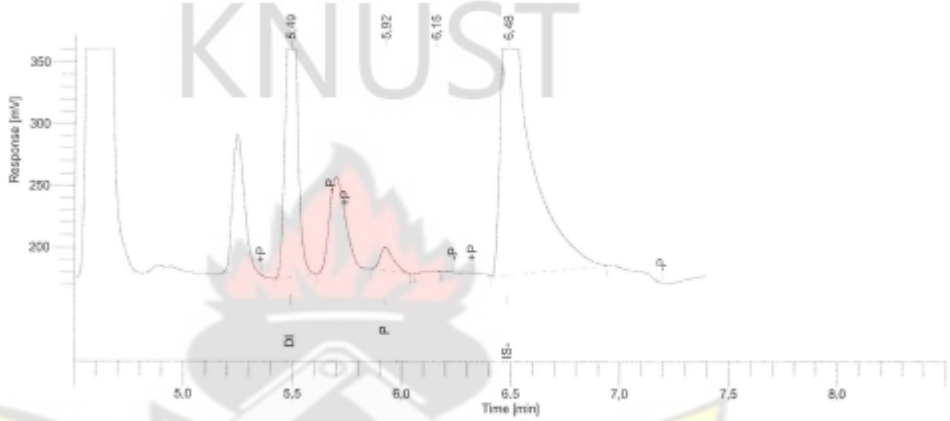

VDK

Time [min]	Height [µV]	Area [µV*sec]	Area/ Amount	Component Name	mg/l	mg/l
5,487	307038,93	1009881,49	3,6364e+06	Diacetyl	0,278	0,2777
5,922	19021,74	79197,84	-5,506e+08	Pentandion	-0,000	-0,0001
6,483	431246,90	2682458,14	----------	IS-Hexandion	----------	----------
	757307,57	3771537,47	-5,469e+08		0,278	0,2776

Appendix 16: Chromatogram of Vicinal Diketones in 4 Weeks Aged Pito Brewed with Starter Culture.

Software Version : 6.3.2.0646
Sample Name : 99902-1
Instrument Name : G-17_ECD_FID
Rack/Vial : 0/0
Sample Amount : 1,000000
Cycle : 20

Date : 30.06.2016 09:55:42
Data Acquisition Time : 24.05.2016 22:40:12
Channel : B
Operator : Analyst
Dilution Factor : 1,000000

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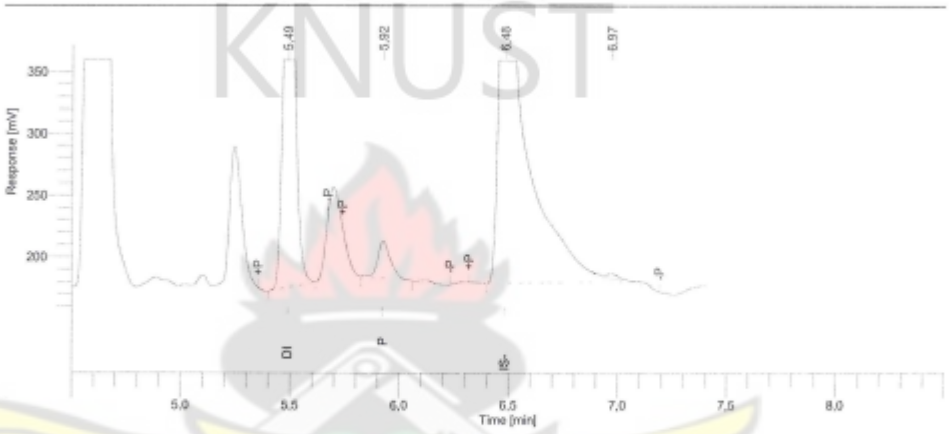

VDK

Time [min]	Height [µV]	Area [uV*sec]	Area/ Amount	Component Name	mg/l	mg/l
5,489	391064,82	1316876,51	3,9099e+06	Diacetyl	0,337	0,3368
5,924	30353,47	123709,31	1,3029e+07	Pentandion	0,009	0,0095
6,483	457224,78	2902338,43	----------	IS-Hexandion	----------	----------
	878643,07	4342924,25	1,6939e+07		0,346	0,3463

Appendix 17: Chromatogram of Vicinal Diketones in 4 weeks Aged Pito Brewed with Traditional Process

Software Version	: 6.3.2.0646	Date	: 30.06.2016 09:55:36
Sample Name	: 99901-1	Data Acquisition Time	: 24.05.2016 22:24:43
Instrument Name	: G-17_ECD_FID	Channel	: B
Rack/Vial	: 0/0	Operator	: Analyst
Sample Amount	: 1,000000	Dilution Factor	: 1,000000
Cycle	: 18		

Result File : \\serv\tcprocess\TC_Daten\G-17_ECD_FID\daten\vdk\kw_21_16\di_2_004.rst
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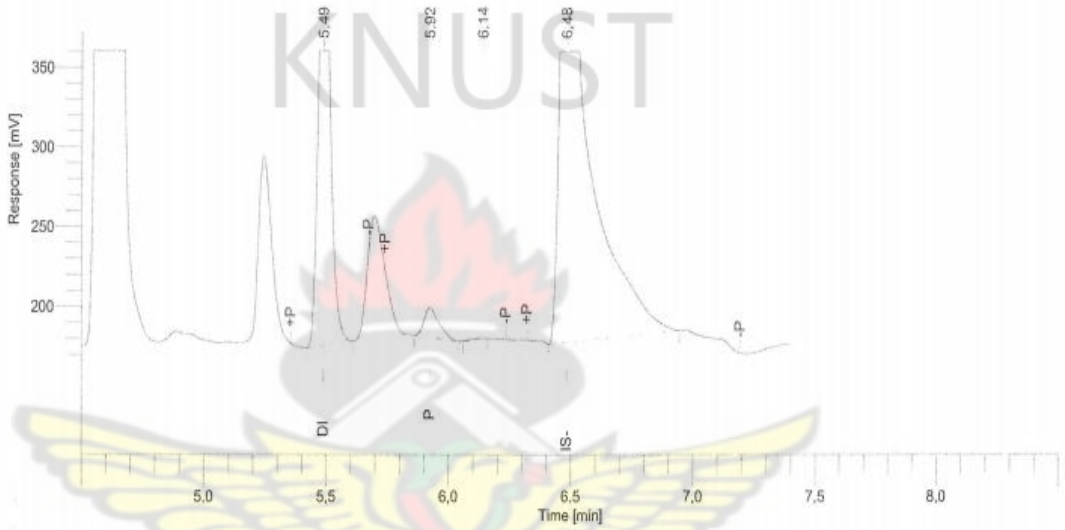

VDK

Time [min]	Height [µV]	Area [uV*sec]	Area/ Amount	Component Name	mg/l	mg/l
5,487	291919,85	961676,26	3,7913e+06	Diacetyl	0,254	0,2537
5,922	18404,83	76461,16	-5,467e+07	Pentandion	-0,001	-0,0014
6,483	446625,94	2812612,97	----------	IS-Hexandion	----------	----------
	756950,62	3850750,39	-5,088e+07		0,252	0,2523

Appendix 18: Chromatogram of Vicinal Diketones in Fresh Pito Brewed with Starter Culture.

Page

Software Version	: 6.3.2.0646	Date	: 30.06.2016 09:55:50
Sample Name	: 99903-1	Data Acquisition Time	: 24.05.2016 22:55:42
Instrument Name	: G-17_ECD_FID	Channel	: B
Rack/Vial	: 0/0	Operator	: Analyst
Sample Amount	: 1,000000	Dilution Factor	: 1,000000
Cycle	: 22		

Result File : \\serv\tcprocess\TC_Daten\G-17_ECD_FID\daten\vdk\kw_21_16\di_2_008.rst
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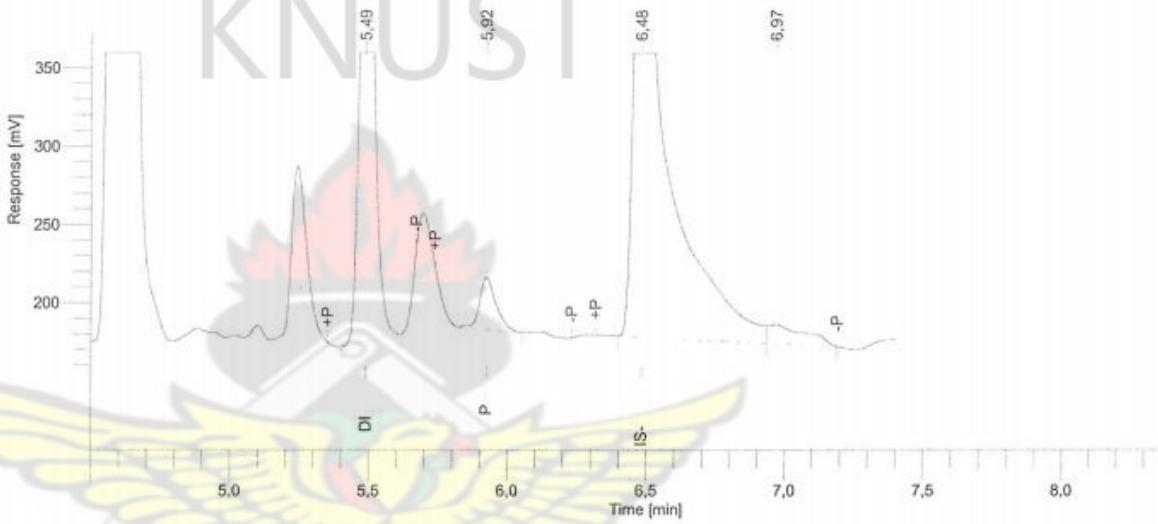

VDK

Time [min]	Height [µV]	Area [uV*sec]	Area/ Amount	Component Name	mg/l	mg/l
5,489	378538,98	1280581,96	3,9104e+06	Diacetyl	0,327	0,3275
5,924	33467,42	137002,81	1,0852e+07	Pentandion	0,013	0,0126
6,483	454599,20	2939083,91	----------	IS-Hexandion	----------	----------
	866605,60	4356668,67	1,4763e+07		0,340	0,3401

Appendix 19: Pictorial Scale of 9- Point Hedonic Scale for sensory evaluation of pito.

LIKE EXTREMELY = 9 ☐

LIKE VERY MUCH = 8 ☐

LIKE MODERATELY = 7 ☐

LIKE SLIGHTLY = 6 ☐

NEITHER LIKE NOR DISLIKE = 5 ☐

DISLIKE SLIGHTLY = 4 ☐

DISLIKE MODERATELY = 3 ☐

DISLIKE VERY MUCH = 2 ☐

DISLIKE EXREMELY = 1 ☐