MICROBIOLOGICAL AND CHEMICAL CHARACTERISATION OF THE TRADITIONAL MANUFACTURE OF MURATINA WINE //

by

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A thesis submitted in partial fulfilment

of the requirements for the degree of

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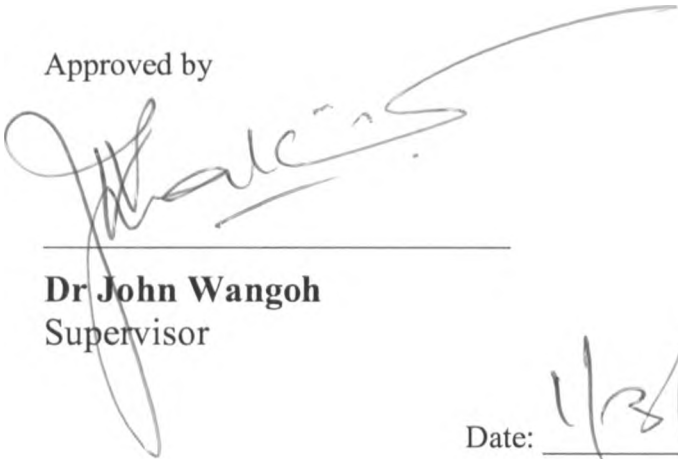## FOOD SCIENCE AND TECHNOLOGY

UNIVERSITY OF NAIROBI

2003

DECLARATION

I declare that this is an original piece of work, which has not been presented to any university or other institution of higher learning.

....

James Njuguna Wahome

Date:........30/10/03............

Approved by

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DEDICATION

To my Parents.

ACKNOWLEDGEMENT

DEFINITIONS AND ABBREVIATIONS OF TERMS

Muratina Fruit: Fruit of the sausage fruit tree.

Muratina sponge: The fibrous tissue that is obtained after pre-conditioning the Muratina fruit.

Sponge pre-conditioning: The set of treatments accorded to matured Muratina fruits including, halving them to expose the fibrous insides, boiling in water and sun drying them before the main conditioning treatment.

Sponge conditioning: The set of treatments accorded to sponges including their incubation in cane juice or any appropriate media for the purposes of colonizing them with fermentative yeasts that naturally occur in these materials.

Sponge re-conditioning: The treatment that is given to sponges from active brewing units after they have participated in several brewing cycles. This treatment is meant to dispose the old culture from them, and free them for re-colonisation with a new culture.

Traditional brewing unit: The vessel that carries an aggregate of Muratina sponges and in which fermentation is carried out.

Brewing cycle: The span between the events of introducing raw brews into brewing units and harvesting the matured brew.

Fermentation process: The biochemical progression through which raw Muratina brews are transformed into alcoholic concoctions by the activity of yeasts, which are immobilized by the sponges within brewing units.

Muratina yeast: The principal yeast that caused Muratina fermentation and constituted the Muratina culture.

Muratina Brew: The raw liquid for Muratina fermentation.

Muratina Wine: The fermented alcoholic liquor.

Brewing sponges: Sponges extracted from brewing units during fermentation.

MEA: Malt Extract Agar
MRS: Mayeux, Rogosa and Sandine Agar
PCA: Plate Count Agar
PDA: Potato Dextrose Agar
MYPG: Malt Yeast Extract Peptone Glucose Agar
ADY: Active Dry Yeast
CFU: Colony Forming Units
RPM   Rotations per minute
AOAC: Association of Official Agricultural Chemists

ABSTRACT

The microbiological and chemical aspects of the traditional manufacturing process of Muratina (a traditional Kenyan wine) were investigated. The objective was to elucidate the scientific basis of the process. Various aspects of the process were investigated in traditional breweries and in experiments. The wine was obtained by fermentation of sweetened sugarcane juice in the presence of Muratina sponges. These sponges were the fibrous tissues of Muratina [Fruit of the sausage fruit tree (*Kigelia africanus*)].

It was revealed that colonisation of sponges with yeasts that occurred in naturally fermenting cane juice formed the basis of Muratina manufacture. Sponges were colonized by yeasts in concentrations of 10^5-10^6 CFU per gram of their dry matter. This was achieved when sponges were incubated in the juice to *condition* them to the brewing process. Yeasts were then concentrated to a biomass with a concentration of 10^9-10^{11} Colony Forming Units per gram of sponge dry matter, during fermentation. Therefore, sponges were used as inoculants in Muratina fermentation. In raw brews they achieved inoculation in the range of 10^5 - 10^8 CFU /ml. This ensured that yeasts dominated the processes from the beginning. Alcoholic fermentation was therefore predetermined. Processes were hence successful irrespective of contamination that came with fresh brews.

Fermentation processes required between 1 and 7 days to complete. The matured product contained 2 – 8 %(vol) ethanol, and 0.5 - 1.4 % lactic acid, while its pH lay between 2.7 and 3.2.The use of acidic ingredients and production of acids during fermentation ensured that fermentation processes progressed in acidic environments in between pH 3.0 and 4.0. This limited participants in the fermentation to yeasts and lactic acid bacteria. Due to evolution of ethanol, acids and accompanying changes in pH, bacteria were eliminated so that yeasts dominated the process almost as pure cultures.

The maintenance of great yeast numbers in brewing units ensured process successfulness. The capacity of sponges to immobilize the yeasts facilitated this. Recycling sponges between processes ensured adequate inoculation was achieved in every process. On average this was done in twenty-seven brewing cycles before they caused flavour defects in the end product. Consequently, the old culture required disposal by washing sponges in water and then sun drying them. This prepared sponges for re-*colonisation* in future brewing processes. These aspects formed the basis of Muratina manufacture.

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1.0. INTRODUCTION

Muratina is a traditional wine made by the fermentation of sweetened sugarcane juice. Its fermentation is carried out in traditional brewing units, which, comprise old winery barrels, and an aggregate of Muratina sponges; carries of the yeast culture. These sponges are fibrous tissues of the sausage fruit tree *(Kigelia africanus)*. The wine, which is brewed for commercial purposes in the city of Nairobi, is consumed in great quantities. It is the traditional brew of the people of east and central Kenya, and is consumed while actively fermenting. Its composition varies widely. On average, it contains 0.5 -1.2 % lactic acid, 2 – 8 % [vol] ethanol.

Muratina is one of the traditional Kenyan beverages, which have considerable social, cultural and economic significance. It is brewed alongside other main indigenous beverages, which include Busaa (an opaque beer made from maize grits and finger millet *(Eleucine coracana)* malt](20), Chang'aa [a distillate of Busaa] (21), and Miti [a concoction of honey and herbal extracts] (42). These products are widely consumed by the low-income earners because they are cheap in comparison bottled beers and wines. The middle and high-income segments however, perceive them as unhygienic and despise them.

The government regulates the production of indigenous alcoholic beverages, also known collectively as "pombe za kienyeji" in Kiswahili. Thus, commercial brewing is restricted to the city of Nairobi, while small-scale brewing but for specified functions such as dowry rituals and circumcision ceremonies in the rural areas requires authorization by the village administration. Restrictions not withstanding, illegal production is widespread in rural and urban centres. These beverages are socially indispensable as alternative refreshments due to the ever-increasing prices of lager beers and falling standards of living. Their production therefore, is an important economic activity that is a source of government revenue, income to brewers and small-scale growers of crops that find use as raw materials. Brewing activities also provide invaluable employment.

Traditional beverage production may be considered an art because, despite the applicable rudimentary technology, poor hygiene of brewing environments, raw material contamination and lack of scientific knowledge by the brewers, the processes are accomplished with great success.

However, this not withstanding, there is an obvious need to upgrade the traditional production procedures in line with modern technological advances, and with the aim of enhancing their general acceptability and value. In this country, little has been done towards the improvement of such traditional processes as has been done elsewhere.

Examples of the envisaged improvements include, minimizing their production costs, shelf life enhancement, quality control and generally addition of value and acceptability. Examples of traditional processes that have been studied and developed with great success are, The Kaffir beer of South Africa (22), Pulque in Mexico (11), Palm wine of Nigeria (8), and the banana wines Ugwagwa and Mbege in Tanzania (34,35).

In Kenya, Muratina brewing has remained a traditional activity that is accomplished with great success. However, the scientific basis of its manufacturing process had not been elucidated.

Therefore, the main objective of this study was to characterize the microbiological and chemical aspects of the traditional process of Muratina manufacture.

The specific objectives thus, were: -
- To investigate the technological status of the traditional process
- To investigate the microbiological and chemical aspects of the fermentation process
- To investigate the roles of Muratina sponges and cane juice in the fermentation
- To isolate and characterize the principal yeast that caused Muratina fermentation, and asses the possibility of using it as a pure culture inoculum in brewing experiments.

2.0. LITERATURE REVIEW

2.1. Muratina Definition

Muratina is a sweet - sour alcoholic beverage that is obtained by the fermentation of brews made of sugar cane juice, crystalline cane sugar, and water. Their fermentation is accompanied in the presence of Muratina sponges that carries the starter culture for the process.

2.2. Patterns of Muratina Production

Through selective licensing, the Government regulates production of Muratina and other traditional alcoholic liquors. In doing so, brewing is restricted to certain areas, considerations being security and social status of the inhabitants. Licenses are not restrictive to any specific beverage, but Chang'aa brewing is outlawed. A licensee is entitled to a maximum production of six thousand litres per annum according to the Traditional Liquor Act of 1971. The stipulated production thus, is equivalent to forty-two litres a day. This quantity is appropriate for domestic refreshment but unrealistic in commercial terms. Consequently, the Act is largely ignored and illegal brewing is widespread. Subsequently, the production of traditional liquor was difficult to quantify (42).

The main beverages in commercial production were Busaa, Muratina, Miti and the outlawed Chang'aa. Their brewing was confined to slums and other areas where low-income earners, who were their major consumers, lived. The types of liquors brewed in a locality were determined by the ethnic composition of the inhabitants in relation to their ethnic origins.

2.3. Raw Materials for Muratina Brewing

(i) Sugar Cane Juice

This is the basic raw material. It is obtained by crushing matured stalks of the sugar cane plant (*Saccharum Officianarum*) using various means. The useful and millable portion of the cane stalks contains $73 - 76$ % (w/w) water and $24 - 27\%$ (w/w) solids. In the solids fraction, those soluble constitute $10 - 16\%$ (w/w) by weight of the cane, while the insoluble fibre constitute 16% of weight of cane. The milling operation extracts part of the water fraction together with the dissolved solids while the inextractable portion is discarded in the bagasse.

Composition of the juice is quite complex and varies according to cane variety, age and numerous factors (24). The average composition is illustrated on Table 1. Juice pH range between 4.73 and 5.63 and the average lies between 5.2 and 5.4. Badly stored juice develop very low pH. Consequently, this and its titratable acidity are the common indicators of it's quality. Due to deterioration during storage, it's composition changes (24).

In Muratina brewing, the juice was normally diluted by addition of water, and then sweetened with crystalline cane sugar or honey to make the raw Muratina brew. Dilution was made necessary, firstly by the scarcity of sugar cane and secondly, the need to reduce on costs associated with cane sourcing and milling. In the brewing process, the juice played several roles. It was an ingredient, and the source of yeasts that constituted the Muratina starter culture. As an ingredient, it provided sugars and other substances that were substrates for the fermentation.

The juice also contains higher plant pigments including chlorophyll A and B, carotenoids, xanthophylls, flavonoids and polyphenols, which were extracted from green parts of cane during the milling process (18). They imparted a desirable yellow colouration in the Muratina drink. This was an important quality attribute (9).

TABLE I. COMPOSITION OF SUGARCANE JUICE

Constituent	Percentage of soluble solids by weight	
Sugars	75-92	
-sucrose		70 - 88
-glucose		2 - 4
-fructose		2 - 4
Salts	3.0-4.5	
-Of organic acids		1.5 - 4.5
-Of inorganic acids		1.5 - 4.5
-Of carboxylic acids		1.1 - 3.0
-Of amino acids		0.5 - 2.5
Organic non-sugars	0.5-0.6	
-Proteins		0.001 - 0.005
- Starch		0.050
-Gums [Waxes, fats and others]		0.3 - 0.6
-Others		0.01 - 015

Source: Irvine (12)

(ii) Crystalline Cane Sugar

This is crystalline sucrose, which is an extract of sugarcane juice. It was the main substrate in Muratina brewing and therefore, determined the maximum ethanol content that was realizable in wine. It enhanced pigmentation in brews due to its content of coloured compounds. These include flavonoids, polyphenols, and products of hydroxymethylfurfural degradation (18). These are illustrated in Table 2.

TABLE 2. COLOURANTS AND OTHER MINOR CONSTITUENTS OF CANE AND REFINED SUGAR

Source	Main colourant[s]	Minor colourants
Cane rind Raw sugar	quercetin	
	Kaempferol Umbelliferone	Aspartic acid Caffeic acid Oleic acid Quinic acid Syringic acid
Cane leaves	Coniferin Coumarin	ρ-Hydroxybenzaldehyde ρ-hydroxbeynzoic acid Rutin Vannilic acid Vanillin
Refined sugar	Chlorogenic acid Hydroxymethyl furfural Meraconic acid Ferulic acid	Aconitic acid ρ -coumarin Glycolic acid Lactic acid Levulinic acid Malic acid Oxalic acid Palmitic acid Sinapic acid Succinic acid

Source: Irvine (12)

Crystalline sugar is contaminated by mineral elements that are mainly derived from sugar processing equipment. Some of these metals are essential bio-factors in yeast metabolism and hence are desirable to the brewing process (13). These minerals are illustrated in Table 3.

TABLE 3. METALS IN RAW SUGAR

Metal	Amount in ppm
Iron	12.71
Manganese	3.58
Copper	1.22
Zinc	0.50
Lead	0.20
Nickel	0.12
Chromium	0.07
Cobalt	0.04
Cadmium	0.005
Silver	0.002

Source: Irvine (12).

(iii) Honey

This was an optional ingredient. It was a suitable alternative to sugarcane juice in Muratina brewing, but its prohibitive cost limited this (9). It is a rich source of fermentable sugars and other substances as illustrated in Table 4.

TABLE 4. COMPOSITION OF HONEY

Constituent	Percentage by weight
Water	17.70
Glucose	32.29
Fructose	39.29
Maltose	7.11
Dextrins and gums	1.03
Ash [silicon, potassium, copper etc]	Trace
Plant pigments	Trace
Enzymes	Trace
Organic acids	Trace

Source: Abayo (2)

It's golden colour enhanced wine appeal (9). It has antibiotic properties and acidic too [pH 3.6 – 4.2]. These aspects were beneficial to the fermentation process by limiting bacteria growth and enhancing yeast activity (4, 30).

2.4. The Muratina Sponges

These are fibrous tissues of the sausage fruit that occur in different sizes. They weigh between 200 and 500g, measure 20cm to 60cm in length, and 10 to 15 cm in width. To make sponges out of the fruits, the following was done. Fruits were halved longitudinally and boiled repeatedly, while changing water every time. This exposed the rough fibrous insides [Figure 1] and their vast capacity that immobilized yeasts in great numbers during the brewing process. Boiling extracted bitter tannic substances, loosened sponge fibres, and facilitated seed removal. This enhanced exposure of sponge insides. These treatments *pre-conditioned* sponges to the brewing process.

The boiled halves were then sun-dried, and subsequently *conditioned* to the brewing process in two stages. The first stage entailed their incubation in naturally fermenting sugarcane juice; diluted honey; or fermenting Muratina brews for 4-8 days. Finally, they were sun-dried and ready for use in brewing operations (9).

Conditioning entailed sponge colonization with yeasts that occurred in the conditioning media. This process made sponges carriers of yeasts and therefore was a fundamental aspect of Muratina brewing.

After participating in predetermined brewing cycles, sponges were washed in water and then sun dried. This was to dispose the old yeast biomasses from their cavities and free them for *re-colonisation*. The accumulated yeast matter spoiled the wine flavour (9). The dried sponges were then directly usable in brewing processes or where necessary, required enhanced colonisation to boost the yeast populations. The microbiological aspects of the *conditioning* process were therefore, investigated.

The primary role of sponges as yeast carriers was obvious. However, they played other secondary roles in the fermentation. For example, when they were lifted towards the liquid surface as carbon dioxide escaped during the turbulent period of fermentation, they promoted yeast dispersion. This facilitated uniform fermentation (4).

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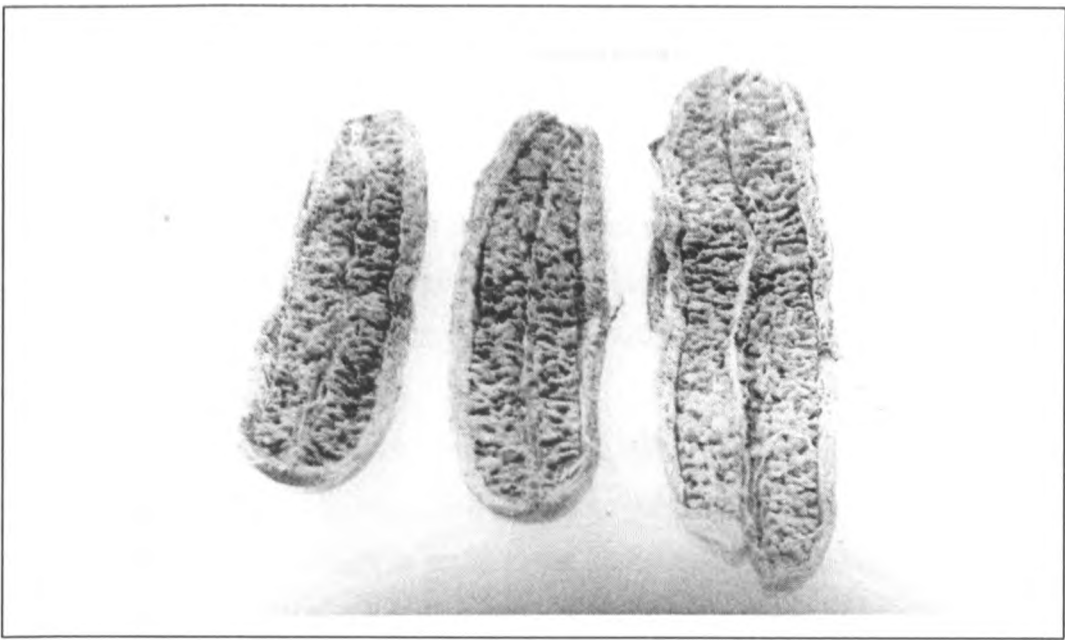
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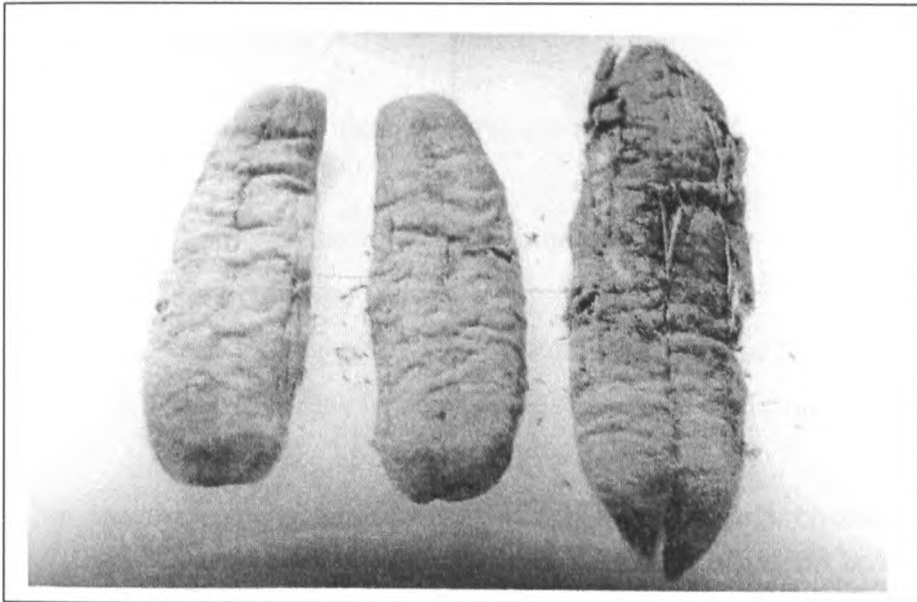
(b) Smooth outer surface

FIGURE 1. PHOTOGRAPH OF CONDITIONED MURATINA FRUIT HALVES

2.5. Steps in Muratina Wine Manufacture

The Muratina brewing process began with the preparation of a brewing unit. This involved packing and fastening a bunch of *conditioned* sponges at the bottom of a selected container. A preferred quantity of brew was then formulated from a predetermined mix of ingredients, and filled into this unit. Sponges provided inoculation and initiated fermentation. This process was completed in 1-7 days and brews were ready for consumption (9).

Matured wine was harvested by emptying the units. They were immediately re-filled with fresh quantities of brew to begin new brewing cycles. Ordinarily, brewing was carried out at ambient temperature though in some cases, units required incubation near heat sources to hasten the processes (9). After participating in numerous brewing cycles however, sponges were washed in water and sun dried. This was to *re-condition* them. Such treated sponges were re-assembled in new units for continued brewing.

2.6. Muratina Brewing in Relation to Spontaneous Fermentation of Plant Juices

Harkishor (10) briefly described the microbiology of Muratina fermentation and reported that:

(i)   Yeasts, lactic acid bacteria and the bacterium *Sarcina* were involved in the fermentation.

(ii)   The process involved a predominant alcoholic fermentation caused by yeasts.

(iii)   The Muratina fruits were the starters for the fermentation process.

He however, never reported the microbiological and chemical aspects of the entire manufacturing process and hence this study.

From literature sources, the natural fermentation of plant juices involves yeast and lactic acid bacteria, which colonize sugary parts of the source plants (8, 23). While studying the microbiology of oil palm sap and Sudanese date wine fermentations, Faparusi (23) and Mohammed (19), traced the origin of causative microorganisms to the trees. Similarly, the source of microorganisms that caused natural fermentation in cane juice was traced to the cane plants (24). The Muratina brewing process thus, was related to the natural fermentation process that sugarcane juices undergo during storage. This is because the juice was the basic ingredient and a medium from which yeasts that colonized sponges originated.

Numerous authors have studied the microbiology of naturally fermenting sugarcane juices. They reported the greatest sources of contamination as the milling equipment, and traced the origin of microorganisms to the sugar cane plant. The level of contamination in freshly extracted juice was therefore considerable and hence their fermentation was initiated at the mill. Subsequently, juices deteriorate rapidly in a process that is greatly influenced by storage temperature (24).

The dominant contaminant in freshly extracted juice was reported to be the bacterium *Aerobacter areogenes*. It occurred in concentrations of 4.5×10^7 colony forming units per millilitre of juice, and caused deterioration by increasing viscosity through the production of slimy polysaccharides from sucrose (34). Other important microorganisms were the lactic acid bacteria. These produced lactic and acetic acids, which caused sourness and enhanced sucrose inversion into constituent reducing sugars.

Examples of these and other bacteria were: - *Lactobacillus plantarum, pseudomonads, Bacillus cereus, acid producing Streptomyces and Zymomonas mobilis.* The fungi included *invertase positive yeast, Penicillium* and other moulds (24). Levels of juice contamination as reported by different authors are illustrated in Table 5.

TABLE 5. MICROORGANISM COUNTS OF FRESHLY EXTRACTED CANE JUICE

Colony forming units	Count/ml	Author
Bacteria	8×10^6-7.5×10^8	Millstein et. al (1941)
Bacteria	6×10^5	Pederson & Hucker (1946)
Bacteria	4.0×10^5-8.4×10^6	Smith (1956)
Bacteria	4.2×10^8-5.1×10^8	Mayeux (1960)
Bacteria	9.1×10^6-6.7×10^7	Duncan & Colmer (1964)
Coliform bacteria	1.7×10^5-1.3×10^7	Mayeux (1960)
Coliform bacteria	3.5×10^4-3.5×10^6	Duncan & Colmer (1964)
Yeast	4.6×10^6-5.7×10^6	Mayeux (1960)

Source: Tilbury (34)

In a study on the microbiology of cane plants and juice in Brazil, over fifty different species of microorganisms were isolated (24). Of these, yeasts were prevalent. The dominant species were *Saccharomyces cerevisiae, Pichia membranefaciens, Candida krusei, Torulopsis stellata, Pichia fermentans, Saccharomyces carlsbergenses var, alcoholphila (n, var), Candida guilliermondi and Candida intermedia var, ethanolphilia.*

In a related study in The United Kingdom, Tilbury (34) investigated microbiological changes in aseptically extracted and stored cane juice over a 15-day period, and made the following observations.

(i)   *Aerobacter aerogenes* dominated freshly extracted juice at counts of 4.5×10^7 CFU/ml.

(ii)   *Leuconostoc mesenteroides* increased and reached 10^5-10^6 CFU/ml in 3-6 days and dominated in this period

(iii)   The Homo-fermentative lactic acid bacteria *Lactobacillus plantarum* and *Lactobacillus casei* counts reached 10^7 CFU/ml after 6-10 days.

(iv)   The *Coliforms* and members of the genus *Bacillus* did not exceed 10^4 CFU/ml during the entire storage period.

(v)   Other gram negative, catalase positive rod shaped bacteria that were capable of growing at low pH and, which were not identified reached high numbers (10^7 CFU/ml) after 3-10 days.

(vi)   The mesophilic yeast count increased gradually from 10^3 CFU/ml to 10^7CFU/ml after 7-10 days.

(vii)   Moulds did not increase significantly during the period.

Juice analysis revealed that deterioration occurred progressively after 2 days. This was characterized by loss of sucrose, rise in reducing sugar content, falling pH, increased content of gum and juice viscosity. These changes were accompanied by a significant increase in lactic acid producing bacteria. Mesophilic yeasts that caused sucrose inversion and produced acid belonged to the genera *Torulopsis* and *Candida*. Members of these yeast genera had also been isolated in Brazilian cane and juice (24).

In yet another study, Swings et.al (33) reported the involvement of bacterium *Zymomonas mobilis* in fermenting sugarcane juice. This bacterium was responsible for the alcoholic fermentation of sugarcane juice to obtain *Caldo-de-cana-picado,* a drink made in Northeast Brazil.

According to literature sources, spontaneous fermentation of sugarcane juice is influenced mainly by three factors namely: - temperature, pH, and contamination. Depending on the prevalent influence, it undergoes either of two types of lactic acid fermentation. At storage temperatures lower than 20^0C, fermentation is characterized by increased sourness and viscosity due to production of slimy polysaccharides from sucrose. The bacteria *Leuconostoc mesenteroides, Aerobacter aerogenes* and *Bacillus mesentericus* cause this. At temperatures between 20^0C and 37^0C, fermentation is characterized by increased souring with no change in viscosity. Homofermentative lactic acid bacteria cause this. These conditions were favourable to the growth of yeasts that occured in juices and which, are capable of causing alcoholic fermentation (13, 24). Solid contaminants influenced the course of fermentation as sources of microorganisms and also by facilitating their dispersion in juice. In Muratina brewing, sponges increased the solid matter content and were sources of microorganisms too. Consequently, they determined the course of fermentation to be alcoholic and yeast dominated.

Ripening honey is contaminated by a diversity of microorganisms that originate with bees and nectar sources (4). According Ruiz-Argueso et.al (26), yeasts and lactic acid bacteria were prevalent in ripening honey in Spain. The yeasts, which belonged to various fermentative genera, were *Saccharomyces mellis, Saccharomyces cerevisiae, Torulopsis stellata, Torulopsis apicola and Torulopsis magnoliae. Torulopsis magnoliae* was prevalent in ripening honey syrups. The bacteria *Gluconobacter* and *Lactobacillus* dominated the ripening syrups but disappeared when the moisture contents went below 17% in ripened honey. The bacteria of the *Zymonomas* species *were* also isolated from ripening honey.

It is evident from literature sources that similar fermentative microorganisms contaminated sugarcane juice and honey. These were the raw materials for Muratina brewing. This implied their common roles as sources of these microorganisms. In Muratina brewing, sources of microorganisms were quite varied. These included human contamination, brewing equipment, ingredients and the sponges. Despite this, however, brewing processes and particularly the fermentation stages were reproduced with great success and repeatability. Thus, factors that enabled the Muratina starter to overcome contamination in every brewing process and bring about successful fermentation were investigated.

Studies have shown that lactic acid bacteria and yeast cultures commonly bring about indigenous fermentations of plant materials. These microoganisms are naturally associated with these materials. They adopt specific symbiotic relationships to create complex cultures that are starters for the processes. Examples of such cultures exist in Tibi grains, which are used as inoculants in the fermentation of sucrose solutions to make the Belgian Tibi, a mildly alcoholic beverage (40). The Tibi culture is comprised of *Lactobacillus brevis, Streptococcus lactis* and *Saccharomyces cerevisiae*. In the grains, microorganisms are bound in a polysaccharide matrix that is produced by the bacteria. Similar associations between *Lactobacillus brevis* and *Saccharomyces fragilis*, have been demonstrated in Kefir grains, the inoculants for Kefir fermentation. Kefir is a Russian acid-alcoholic beverage made by fermentation of various milks (40). Whether similar associations existed in Muratina sponges during fermentation would be an interesting study option in future.

Despite the ability of the Muratina starter to cause repeatable fermentations, it was recycled in limited brewing cycles and changed. This was occasioned by flavour deterioration in the product. To change the starter, sponges were washed and sun-dried. This *re-conditioned* them for repeat fermentations.

From literature sources, perpetual recycle of microbial cultures is limited by many factors. These include culture death through contamination, competition, phage attack and mutation (17, 25). The Muratina culture is subject to these limitations too. Thus, the possibility that the Muratina starter culture was rendered ineffective by contamination upon its recycling in fermentations was investigated.

The bacterium *sarcina sp.*, which is a spoilage agent in alcoholic beverages, was reported to be involved in Muratina fermentation (10). It produced copious amounts of the compound diacetyl in beer and spoilt the beer if present. Thus, this compound was assayed in Muratina wines.

3.0. MATERIALS AND METHODS

3.1.0. Investigations on the Technological Status of Traditional Manufacture of Muratina

3.1.1. Survey on Traditional Muratina Manufacturing

A survey was carried out with the objective of collating pertinent information regarding the traditional manufacturing process of Muratina wine from experienced brewers. Technological implications of the various processing aspects were then analysed.

The format of the questionnaire that was used in the exercise is illustrated below.

Interview Schedule intended for Traditional Muratina Brewers

Brewer's address...........................

Location.................................

Date......................................

1. What materials do you use to manufacture Muratina?....................................

2. Where do you get these materials?...

3. How do you prepare them for the actual process?...

4. At what ratios do you combine the ingredients?..

5. How then do you prepare Muratina?...

6. How long is the brewing cycle?..

7. How do you determine the end of fermentation?..

8. How do you ensure fermentations were completed on time?...........................

9. What factors caused delay or failure of fermentation?................................

10. How then, do you address these causes?...

11. What do you consider as important elements of Muratina quality?.................

12. What are the main quality defects of Muratina and what caused them?...........

13. Can you relate the origin of these defects to specific stage(s) of the manufacturing
    process ?...

14. How do you ensure that defects never occurred?....................................

15. Can you account for the role of each ingredient in the brewing process?.........

16. How do you harvest and package the wine?..

17. How long is the shelf life and could it be extended?...............................

18. After how many brewing cycles do you change the sponges?

19. Why must sponges be changed?..

20. Did you treat the sponges in any manner apart from sun drying them?.............

21. What constraints do you face in this business?...

22. How much wine do you produce and sell on daily basis?............................

Thank you.

3.1.2. Assessing the Influence of Manufacturing Method on Muratina Composition

To assess the influence of different manufacturing methods on composition, 500ml samples of the ready to drink beverage were purchased from selling counters. These were taken to the laboratory and deep-frozen pending chemical analysis. Samples were assayed for ethanol, reducing sugar, dissolved solids and diacetyl contents. Their pH and colour intensities were also measured.

3.2.0. Characterization of Muratina Fermentation

During the survey, it was established that the wine was brewed in cycles, using the traditional brewing units. Durations of the fermentation processes varied from one brewery to another, but the range was between 1 and 7 days. Thus, in 60% of the breweries, average process length was 24 hrs; in 35% - 48 hours, while in the remaining 5%, this was beyond 48-hours. The processes were categorized for the sake of this exercise according to the applicable duration of fermentation. Three categories were created, thus, rapid fermentations that required 24-hours or less, Moderate processes that required between 24 and 48-hours, and lastly The Extended processes that required more than 48 hours to complete. At least one process was investigated in each of these categories to establish whether they involved similar microbiological and chemical phenomena.

3.2.1. Sampling during fermentation

Samples of fermenting material were taken at different times during fermentation. Thus, 500ml quantities of material were drawn from brewing units and aseptically filled in sterile bottles. These were packed in picnic boxes chilled with a frozen gel refrigerant, and delivered to the laboratory within an hour after collection.

In the laboratory, samples were subjected to microbiological examination and the remaining portions deep-frozen pending chemical analysis. Samples of cane juice and Muratina sponges were collected and treated similarly.

In the rapid (24-hours) processes, samples were taken at 0, 6, 12 and 24 hours. In the moderate (48-hours) processes at 0, 6, 12, 36, and 48 hours, while in Extended (7-days) processes, at 0,6,12,24,48,72,96,120,144 and 168 hours. Rapid processes required frequent sampling. Since brewing experiments were carried out in traditional breweries away from the laboratory, it was only practical to sample them during daytime. Therefore, sampling times were selected appropriately. At the breweries, selected fermenting units, brew formulae (raw material measurements), room temperature, start-up time and time of last sampling were noted and recorded.

3.3.0. Investigations on the Muratina Starter

3.3.1. Determining the Sources of Yeast

To determine the sources of yeasts that colonized sponges, the traditional method of *conditioning* them to the brewing process was simulated in the laboratory. This entailed incubation of sterilized sponges in sugarcane juice for 4 days, and then drying them in the sun. Microbiological aspects of this process were investigated.

Method

Sponges were sliced to convenient sizes, submerged in water held in Erlenmeyer flasks and sterilized at $121^{0}C$, 15-psi for 15 minutes in a batch autoclave. Sterilized sponges were then oven-dried to constant weight. The pieces were aseptically handled and submerged in fresh cane juices that were held in sterilized flasks. Flasks and contents were sealed with cotton plugs, and incubated at $25^{0}C$ for 4 days. The juice and sponges were re-examined for constituent flora after this period. The juice was discarded after the exercise and sponges sun-dried for four days. Their constituent flora was then re-examined. In the control experiments, sponges were sterilized and sun-dried without incubation in cane juice.

3.3.2. Assessment of Sponges as Yeast Carriers

The results of Section 3.3.1 confirmed that cane juice was the origin of yeasts that colonized sponges. However, it was important to establish how this colonization influenced Muratina fermentation. Thus, two sets of experiments were performed. In one case, brews were incubated at 25^0C and left to stand for 4-days and without the inclusion of sponges in them.

In the other case, sterile sponges were placed in brews as in normal Muratina brewing and similarly incubated at 25^0C for 4-days. The brews were examined for attendant microbiological and chemical changes during this period. Thus, in both cases, brews were left to undergo natural fermentation.

3.3.3. Determining the Basis of Sponge Re-conditioning Process

In order to determine what factors limited the performance of Muratina starters in fermentations upon their continuous recycle in brewing processes, a series of brewing experiments were performed. To start these experiments thus, a local brewer kindly provided fresh starters in form of freshly *re-conditioned* and sun-dried sponges. These were used to set experimental brewing units as follows: -

Method

A pair of gourds were prepared for the duplicate experiments. To each set of a pair, eight large sponges were fastened to make experimental brewing units. Eight-litre quantities of brews were then filled to each of the sets to commence experiments. Units were then incubated at 25^0C in separate cabinet incubators. A fermentation cycle lasted 96-hours [4 days], and during this time, attendant chemical and microbiological changes were determined. After a particular brewing experiment, fermented brews were decanted from the units and replaced with fresh ones to start a new process. This was repeated in seven brewing cycles. In the fourth cycle however, the brews were simple solutions of crystalline cane sugar in water. Such concoctions were usually fermented, when complete brews could not be constituted due to unavailability of cane juice or any other reason (9).

Analysis was done for all processes, except the fifth and sixth, to save on material costs. After the seventh brewing run, sponges were examined to determine possible changes in microbial composition of the culture.

Analytical methods described in Section 3.5 applied. Duplicate experiments were carried out in each case and results presented are average of these. Experimental brews contained cane juice, 18.85% (v/w): crystalline cane sugar, 5.30% (v/w); and water, 75.85% (v/w). This was the average formulation of Muratina brews as determined from the survey findings [Table 7]. Gourds, the traditional brewing vessels were selected to simulate barrels that are widely used in commercial brewing.

3.4.0. Experimental Brewing with Pure Cultures of Selected Microorganisms

Experimental brewing with pure cultures of yeast and lactic acid producing bacteria that were isolated at different stages of traditional Muratina fermentation was carried out.
Experimental brews were prepared as described in section 3.3.3. These were heat preserved at 121^0C, 15psi for 15 minutes and then cooled to 25^0C in running water. Sponges were equally sterilized at 121^0C, 15-psi for 15 minutes and then oven-dried. In these experiments, sponges aided yeast dispersion in the brews as opposed to being inoculants. To sanitize them, gourds were retained in boiling water for 30 minutes before use.

3.4.1. Experiments with Muratina Yeast Cultures

Muratina yeasts of characteristics described in section 4.2.3, were propagated in incremental steps as follows: - Actively growing yeast cells were cultured on Malt-yeast extract – peptone-glucose [MYPG] agar slants, and incubated at 28^0C for 3 days. Yeast cells were inoculated in tubes containing 10-ml of MYPG broth, and incubated under agitation at 50 r.p.m in a shaking incubator at 28^0C for 3 days. After 3 days, the realized growth was quantitatively transferred to 100-ml of MYPG broth contained in indented Erlenmeyer flasks, and incubated similarly.

The resultant yeast suspension was again transferred into 350-ml of sterilized Muratina brew and incubated as above. The resultant culture suspension was applied as the starter inoculum in the experiments. Culture purity was ascertained by microscopic examination of gram stained smears. Yeast concentration in the starter suspension was $2.5 \times 10^8 \pm 0.5 \times 10^8$ CFU/ml. Thus, 1.05 litres of this suspension was added to 16.0-litres of experimental brews and provided initial yeast concentrations of $6.0 \times 10^6 \pm 0.2 \times 10^6$ CFU/ml.

The application rate of the starter was five percent (5% (v/w), which is recommended in conventional batch brewing (17). Comparable rates of inoculation were determined in traditional and experimental brewing processes.

Experiments were carried out in open and sealed vessels, which enabled the creation of aerobic and anaerobic conditions during fermentation. Sealed vessels were equipped with tubes that facilitated outward leakage of carbon dioxide due to the creation of positive pressure during fermentation. The attendant changes during fermentation were tracked by regular analysis of samples.

3.4.2. Experiments with Yeast and Lactic Acid Bacteria Cultures

Yeast cultures were propagated as described in section 3.4.1. Lactic acid bacteria cultures were obtained by incremental propagation just like in the case of yeasts but in a different medium. Bacteria isolates were maintained on yeast-glucose-chalk agar slants (section 3.5.3 iii). Actively growing cells were transferred into 10-ml quantities of sterilized MRS broth and incubated at 30^0C for 48 hours. The resultant growth was then quantitatively transferred into 100-ml quantities of MRS broth. These were then incubated under agitation at 50 r.p.m in a shaking incubator maintained at 25^0C for 48 hours. The resultant growth was then transferred into 350-ml of sterilized Muratina brews and incubated under similar conditions. The bacteria concentration in the resultant suspensions was $9.5 \times 10^8 \pm 0.2 \times 10^8$ CFU/ml. The bacterium, which was coccoid, produced much acid and isolated on most occasions at the early stages of fermentation, probably belonged to the genus *Leuconostoc* (25).

To obtain the composite inoculum, equal proportions of the yeast and bacteria culture suspensions were combined. The mixtures contained $2.5 \times 10^7 \pm 0.2 \times 10^7$ CFU of yeast, and $9.0 \times 10^7 \pm 0.2 \times 10^7$ CFU of lactic acid bacteria per ml of suspension. Thus, 1.0 litres of the composite inoculum was added to 8.0-litre quantities of sterilized Muratina brews. The suspensions provided: $1.9 \times 10^6 \pm 0.2 \times 10^6$ CFU of yeast and $7.9 \times 10^7 \pm 0.2 \times 10^7$ CFU of the lactic acid bacteria per millilitre of Muratina brew. Changes due to fermentation were tracked as earlier explained. Duplicate experiments were carried out under aerobic and anaerobic conditions.

3.5.0. Analytical Methods

3.5.1. Methods of Chemical Analysis

a) Alcohol content

This was determined by measuring specific gravity of wine distillates according to AOAC Method 11.005. The alcohol content equivalent to specific gravity was read from conversion Tables 52.003(1).

b) Dissolved solids

De-alcoholised wine samples from (a) above were made to 100ml with distilled water in a Volumetric flask. The soluble solids content was measured at 20^0C using a refractometer.

c) Reducing sugars

These were determined according to Lane and Eynon Method 31.034 of the AOAC (1).

d) Total acidity

This was determined according to AOAC Method 11.042 (1). Acidity was expressed as lactic acid, the dominant acid in soured cane juices (34). Twenty-five millilitres of samples were heated to incipient boiling on a hot plate to de-gas them by driving off carbon dioxide.

In a separate flask, 200-ml of hot boiling water was measured and neutralized to a distinct pink end point with 0.1N NaOH, using phenolphthalein indicator solution. Five millilitres of the degassed wine sample was added to the neutralized water, and the mixture titrated against standardized 0.1N NaOH solution to a pink end point. Acidity was calculated as follows: -

Grams lactic acid/100ml wine=ml NaOH x Normality x 0.09 x 100/5.

e) Volatile acidity

This is normally analysed in wines to determine oxidation by acetic acid bacteria (17). Volatile acids were extracted from wine samples using an improvised distillation apparatus. This was in place of the 'cash' still apparatus, which is recommended by the AOAC Method 11.043 as the standard steam generator. The improvised apparatus comprised of a 1-litre capacity Erlenmeyer flask fitted with a rubber bung that carried a pair of glass tubing.

One tube was the water feed pipe and the other, a steam conduit. In its operation, water in the Erlenmeyer flask was heated to generate steam, which was then directed to a round-bottomed flask containing 25ml of wine sample. The steam volatilised acids from the sample, which were then extracted via the side tubing and condensed in a vertically mounted tap water cooled condenser column. Three hundred millilitres of distillate was collected and rapidly neutralized with 0.1N NaOH to a distinct pink end point using phenolphthalein as indicator. The percentage volatile acidity was calculated as follows: -

Grams acetic acid/100ml of wine =ml 0.1N NaOH x 0.006 x 4

(f) Diacetyl content

This was determined according to the colorimetric Method that is adopted for its determination in beers (39). Diacetyl concentration was read from a calibrated standard curve [Figure 2].
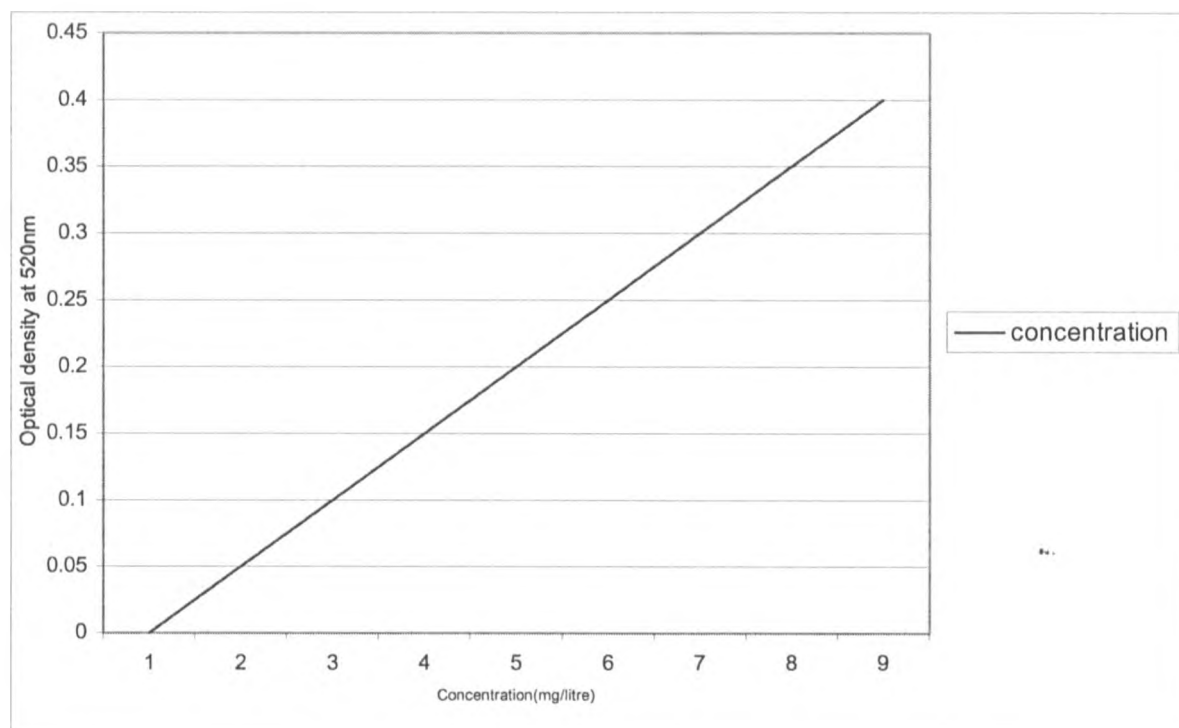
FIGURE 2: STANDARD CURVE FOR THE DETERMINATION OF DIACETYL CONCENTRATIONS

(g) Determination of pH

This was measured using a PYE-UNICAM pH meter according to AOAC Method 11.041 (1).

(h) Quantification of Wine Colour Intensity

The wine had a yellowish colour that had a maximum absorbance of light at 420nm wavelength. Intensity of this colour was measured as the percentage transmittance when light at this wavelength was passed through the wine sample. Absorbance was measured in a spectrophotometer, using 1-cm cuvettes. To read the absorbance, samples were clarified by centrifugation at 4500r.p.m in a Beckman T56 centrifuge followed by vacuum filtration through filter paper no.4. Measurements were done according to ICUMSA Method No.4 (18). This method is recommended for the measurements of colour in liquors, syrups and cane juices to track the progress of sugar refining operations (18). Colour intensity was then expressed as the percentage transmittance. Clean distilled water was used as the standard zero colour.

3.5.2. Isolation and Enumeration of Microorganisms

Microorganisms were enumerated in sugar cane juice, brews at different stages of fermentation, crystalline cane sugar and extracts of Muratina sponges. The colony forming units were enumerated in plate cultures of these materials. Thus, the total viable microorganisms, yeast and moulds, lactic acid producing bacteria and acetic acid bacteria populations were determined.

(i) Liquid Samples

These were diluted before enumeration. Thus, 1-ml aliquots of the samples were diluted in 9-ml of 0.85% sterile saline solutions, and appropriate decimal dilutions prepared. In highly contaminated materials such as cane juice, fermenting brews and sponge extracts, the 10^{-4} to 10^{-8} dilutions were cultured. In cane sugar solutions, the 10^{0} to 10^{-3} dilutions were cultured due to their minimal contamination.

(ii) The Sponges

These constituted a microbial habitat and the resident microorganisms required to be extracted for their enumeration. Thus, sponges were sliced to convenient sizes that weighed between 10 and 15 grams.

These were then placed in baffled flasks, and covered in 85-90 ml of 0.85% sterile saline solutions. To facilitate microbial extraction into the diluent, flask and contents were agitated at 250 r.p.m in the laboratory shaker for five minutes. The resultant extract was diluted as above. In sun-dried and *re-conditioned* sponges, the 10^0 to 10^{-4} dilutions were cultured, while the higher 10^{-7} to 10^{-9} dilutions were cultured in the case of brewing sponges. After extraction, sponges were dried to constant weight and their weights recorded. The microbial numbers were expressed as CFU/gram of sponge dry matter and were determined as below.

$$CFU/gram\ dry\ matter = \frac{No.\ of\ colonies\ \times\ dilution\ factor\ \times\ volume\ of\ saline\ diluent}{Weight\ of\ dry\ sponges\ (grams)}$$

(iii) Crystalline Cane Sugar

Fifteen-gram quantities of sugar were dissolved in 85-ml sterilized distilled water, and decimal dilutions prepared. These were cultured as above, and microbial contaminants expressed in terms of colony forming units per gram of sugar.

3.5.3. Enumeration Methods

(i) Total Viable Microorganisms

These were enumerated on plate count agar (Oxoid). 1-ml aliquots of the 10^{-5}-10^{-8} dilutions were cultured and incubated at 30^0C for 48 hours (24).

(ii) Yeasts and Moulds

These were enumerated on acidified Potato Dextrose Agar (PDA). 1-ml aliquots of appropriate dilutions were cultured as follows: $10^0 - 10^{-4}$ dilutions of sun-dried sponge extracts, $10^{-4} - 10^{-8}$ dilutions for cane juices, raw and fermenting brews. The $10^0 - 10^{-1}$ dilutions for the crystalline cane sugar solutions were plated. Plates were incubated at 28^0C for 3-days and enumerated. Plates containing cultures from brewing sponges were refrigerated at 4^0C pending isolation and characterization of the predominant yeast.

(iii) Lactic Acid Bacteria

These were cultured on media that was composed of yeast extract, 10.0g; peptone, 10.0g; D-glucose, 20g; $CaCO_3$, 10g; agar, 20.0g per litre of distilled water (25). Cultured plates were incubated at 30^0C for 48-hours and colonies enumerated. On this media, clear areas were formed around the lactic acid producing bacteria colonies due to chalk dissolution.

Media selectivity was enhanced by anaerobic incubation in BBL [Baltimore Biological Laboratories, Cockeysville, Maryland] gas Pak systems. The bacteria caused extreme media clarification, while yeasts formed large colonies and were easily identified. A negative catalase reaction and the gram stain further identified the Lactic acid bacteria. Isolates were maintained on a medium composed of Yeast extract, 6g; glucose, 50g; agar, 5g, $CaCO_3$; 10g per litre of distilled water and stored at 28^0C (25).

(iv) Acetic Acid Bacteria

These were enumerated on spread plates of a solidified medium composed of: D-glucose, 20.0g; yeast extract (Difco); 10.0g, agar, 20.0g per litre of distilled water. Sample aliquots [0.1ml] of the $10^{-1} - 10^{-4}$ dilutions of brewing sponge extract and fermenting brews, were cultured on solidified media plates and incubated at 30^0C for 48-hours (7). The tiny Acetic acid bacteria colonies were distinguished from *Muratina yeasts* by the star-shaped colonial configuration of the latter. Presumed acetic acid bacteria colonies were purified on the same medium after its modification by the addition of calcium carbonate and ethanol. The modified medium was composed of yeast extract (Difco) 10.0g; $CaCO_3$, 20.0g; Agar 20.0g per litre of distilled water (7). The molten medium was made alcoholic by addition of a solution containing 15-vol.% ethanol. This was sterilized through a 0.20um membrane filter before addition. The ethanol content of modified medium was made to 3.0% (v/w) (7).

Fifteen millilitres of the modified medium was dispensed in plates and allowed to set. Presumed isolates were then streaked on the medium, and incubated aerobically at 30^0C for 48-hours. Positive acetic acid bacteria isolates caused oxidation of ethanol to acetic acid, which then dissolved chalk at the points of growth.

3.5.4. Characterisation of "Muratina Yeast"

The yeast that was cultured from brewing sponge extracts was named the *Muratina yeast*. It also dominated in cultured extracts of sun-dried and conditioned sponges, cane juice and fermenting brews. It formed distinct star-shaped colonies as shown in the photograph Figure 3. It was purified through repeated culturing on PDA agar, and was maintained on Malt Extract Agar. To obtain actively growing cultures, the yeast was cultured on MYPG agar slants, and incubated at 28^0C for 3 days. The MYPG agar, medium was composed of Malt extract; 3.0g, Yeast extract; 3.0g, Peptone; 5.0g, Glucose; 10.0g, Agar; 10.0 g per litre of distilled water (37). To explain the behaviour of the *Muratina yeast* during fermentation, sponge colonization and during sun drying, several of its growth characteristics were studied.

(i) Determination of Growth Characteristics

These characteristics were studied in liquid and solid growth media. Actively growing cells cultured on MYPG agar, were inoculated into 30-ml of sterilized MYPG broth and incubated at 28^0C for 3 days. This culture was studied for growth characteristics, cell morphology, dimensions, and mode of reproduction. These were also studied in 3-day old cultures, which were raised on MYPG agar under similar conditions. The cultures were left on a laboratory bench for 4-weeks and examined weekly for the same characteristics.

(ii) Ability to Form Ascospores

The Muratina yeast was expected to survive desiccation at the sun-drying stage of sponge *re-conditioning* by forming spores. Its ability to do so was tested as follows: Actively growing cultures were raised on MYPG agar, and sub-cultured twice on Malt-Extract-Agar, the presporulation media. Growth from this medium was then cultured on Mc'lary's acetate agar and Adam's agar (37). The latter media contained glucose and sodium acetate to induce spore formation. Cultures were then incubated at 28^0C for 3 days and examined for existence of spores. These cultures were then left on the laboratory bench for 4-weeks and examined weekly for the presence of spores by microscopic examination of stained smears.

Composition of Sporulation Media

Malt Extract Agar: malt extract; 20g, and agar; 12.0g per litre of distilled water (37). Mc'lary's acetate agar: Glucose; 1.0g, potassium chloride; 1.8g, yeast extract; 2.5g, sodium acetate; 8.2g, and agar; 15.0 per litre of distilled water (37).
Adam's agar: Glucose; 0.4g, sodium acetate; 2.5g and agar; 20.0g per litre of distilled water. The media were sterilized at 121^0C, 12-psi pressure for 15 minutes (37).

(iii) Fermentation of Sugars

The ability of yeast to ferment sucrose, glucose, fructose, galactose, maltose, lactose and raffinose was tested. Test solutions containing 6% (v/w) of test sugar were prepared by dissolving the sugars in 0.5% [v/w] solutions of yeast extract in water. Raffinose was made to 12% [v/w]. These solutions were sterilized through 0.20μm membrane filters. Then, 1-ml aliquots of the sterile solutions were put in Durham tubes containing invert tubes and 2-ml aliquots of a basal fermentation medium.

The sugar concentration in basal medium was made to 2-%(v/w) for all sugars except in the case of raffinose, where this was made to 4 % (v/w)(37). The basal fermentation medium was composed of Yeast extract; 4.5g and peptone; 7.5g per litre of distilled water (37).

Actively growing yeast cells were transferred from MYPG agar slants and suspended in 4.5ml of sterilized distilled water. Then, 0.1 ml aliquots of this suspension were added to tubes containing the basal medium and test sugar solutions, and incubated at 28^0C for 3 days. Positive fermentation was indicated by gas collection in invert tubes. The volume of gas collected indicated the ease of fermentation for the test sugar.

(iv)   Acid Production

This was tested by culturing actively growing cells in an opaque medium, which was composed of yeast extract, 5.0g; calcium carbonate, 5.0g; sucrose, 50.0g; agar 20.0g per litre of distilled water (37). Cultures were incubated at 28^0C for 3 days. Positive acid production was indicated by clarification of the opaque medium around the margins of colonial growth following chalk dissolution by the acids produced. Acid production was classified as weak or strong depending on the degree of media clarification.
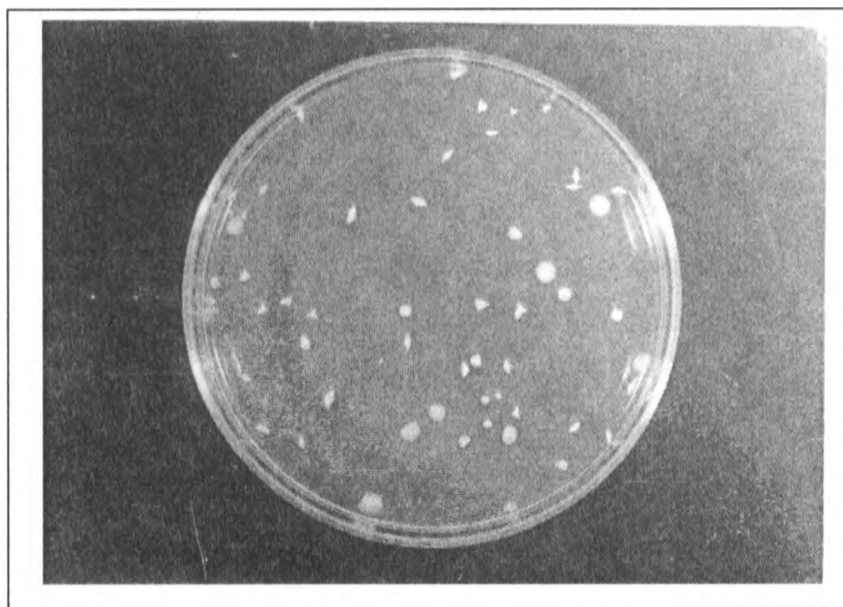
FIGURE 3:PHOTOGRAPH SHOWING THE DISTINCT STAR-SHAPED MORPHOLOGY OF 3-DAY OLD *MURATINA YEAST* COLONIES GROWING ON POTATO DEXTROSE AGAR PLATES AFTER INCUBATION AT 28°C (X 0.5).

4.0. RESULTS

4.1.0. The Traditional Manufacture of Muratina Wine

The technological status of the traditional process of Muratina manufacture was investigated through site visits and interviews with traditional brewers. The findings were analysed and reported.

4.1.1. Brewing Equipment and Materials

The traditional Muratina brewer employed both rudimentary and modern equipment depending on the level of production. The equipment pool included the common, specialized, and those improvised for specific operations such as juice extraction, brewing and filtration among others. These equipment and materials are described according to the unit operations in which they found use.

(a) Extraction of Cane Juice

In large scale Muratina production, modern single stage cane-mills were used for this purpose. The mills were mainly electric powered though manually operated ones were encountered in a few cases. The latter type found use where electricity was not available. In the milling operation, cane was fed to the mill and recycled at least twice to optimise juice yields. The juice was then collected in troughs placed under the mill, and filtered through porous fabrics to remove solid debris. The clarified juice was ready for immediate use. However, would the juice require storage, this was done in open containers that allowed escape of gas that was produced by it's spontaneous fermentation. This process, which started soon after extraction, limited its shelf life to a few hours. Hence, it demanded immediate use.

In domestic brewing where smaller quantities of juice were required, the equipment and extraction procedures were different. Thus, juice extractors were improvised devices made of iron sheets. In their construction, one side of a sheet was perforated with nails to create sharp and rugged projections. After perforation, the sheets were fixed onto wooden frames to complete the devices.

To use them, cane stalks were chopped into convenient sizes and peels removed from most of the round face to expose the flesh. The naked face was then placed against the rugged projections of the perforated sheets and rubbed back and forth. The sharp edges macerated the cane into fine shreds, which were collected on a jute cloth. Shreds were then soaked in small amounts of water, and wrapped into a ball using sisal twines. The balls were then manually pressed to expel the juice. This is a tedious procedure that was hence confined to home brewing.

(b) Brewing.

In commercial production, Muratina was brewed in drums made from iron, fibreglass, plastic, wood or other acceptable materials. However, the most popular vessels were old wine barrels with a holding capacity of between 200 and 400 litres of water. Their preference was based on several attributes. These were the ability to retain warmth and resistance to corrosion. The first attribute was important because it reduced the need of warming brewing environments, while the second aspect guaranteed durability. Wooden vessels are resistant to corrosion by acids unlike metallic ones. In addition, indentations that existed at joints between individual staves that made up the barrels, provided variable sites upon which sponge fastening devices were hooked during fermentation.

In small-scale and domestic brewing, vessels of smaller capacities (10-20 litres) were used. These were earthen pots, gourds and plastic jerricans among others.

(c) Weight and Volume Measurements.

Where Muratina was brewed for commercial purposes, brewers had developed economic raw material formulation, which from their experiences yielded acceptable products. Raw materials were used rationally and measured according to applicable ratios. Thus, spring balances and weighing scales were used to measure ingredients like crystalline cane sugar and honey. Liquids including cane juices, water and ready brews were measured in containers of standard measure of between 0.5 and 50 litres capaciites. These containers were usually plastics.

(d) Filtration.

Foreign matter consisting of small bits of cane, leaves, soil and other solids, gained access to the juice during the milling process. These were removed by filtering the juice through porous cloths or ordinary household strainers. The ready brews were equally strained to remove suspended yeast matter and detached sponge fibres. This process however, did not remove colloidal solids and microorganisms so that ready brews were turbid by the time of consumption.

(e) Mixing.

In the brewing process, crystalline cane sugar was firstly dissolved in water and then mixed with cane juice to constitute the raw brew. To ensure homogeneity, cane sugar was vigorously stirred to dissolution using strong wooden spatulas. Cane juice was then added to this solution, and the mixture thoroughly agitated. Sometimes it was necessary to agitate slowly fermenting units, but without dislocating sponges from their hooked positions. Dipping spatulas into the units and disturbing the sponges tactically, accomplished this.

(f) Wine Handling.

The ready wine was tapped from brewing units using either of two methods. In the first method, the brew was scooped using buckets or any appropriate vessels, while in the other option; brews were emptied by suction using plastic hosepipes. These choice methods ensured only the supernatant brew was drawn, while the flocculent matter that settled on sponges at the vessel bottoms was not agitated. The settled yeast matter caused cloudiness and bitterness in the drink. The wine was then filtered and served for consumption. At the selling counters, it was stored in temporary reservoirs from where it was measured and served to waiting customers. This was done in non-breakable implements such as used cooking fat tins, plastics and common jugs. These were available in standard capacities in the range of a quarter to two litres. Alternatively, the drink was packed in half-litre beer bottles to ease retailing. In wholesaling, it was measured in any quantities between 10 and 50 litres, and packed in plastic jerricans for distribution.

(g) Modification of Brewing Environments

Muratina brewing was carried out in designated rooms that were constructed with common building materials such as wood, concrete blocks or earth. The floors were either cemented or bare. The buildings were go-downs, stores or ordinary living rooms with adequate ventilation. They remained locked and only accessed by the brewers.

In spacious rooms that accommodated up to thirty barrels, brewing units were set and left to undergo fermentation at ambient conditions. In some cases and for smaller rooms at that, open fires or firewood burners were set at central points to modify the warmth status during the brewing process. This was particularly so in the cold season. Thus, fires were left to burn throughout the brewing period or partly and at night, in order to maintain room temperature between 24^0C and 30^0C. This practice, which was still adopted by less than fifty percent of Muratina brewers, seemed to be dying away due to discovery of alternative methods of achieving rapid fermentation. These methods entailed using more sponges in brewing units, and the mandatory use of cane juice and at optimum levels in brew formulations. In home brewing, units were placed next to kitchen fires for the same reasons.

(h) Drying the Sponges

After participating in predetermined brewing cycles, sponges were appropriately dried and preferably in the sun. In sunny weather, they were spread at the rooftops of brewing houses to dry. In the rainy season, this was impossible. Therefore, to ensure continuity in brewing during such periods, drying beds were constructed inside brewing houses. Here, sponges were spread to dry naturally. Under these circumstances, sponges that have participated in numerous brewing cycles and collected considerable biological matter in their cavities required to be washed before they were spread. This biological matter was the yeast biomass and a rich medium for mould growth (29). Non-removal of this material made sponges liable to infection by moulds during the drying process. Washing sponges prior to sun drying emptied their cavities and kept off the moulds (9).

4.1.2. Raw Material Preparation

Sugar cane was purchased from commodity traders and small-scale farmers in the regions surrounding the city of Nairobi. After procurement, cane was stored and milled according to demand for juice. The possibility of storing extracted juice was rightly avoided because of its poor shelf life. When this was inevitable, the juice required storage in open containers and inoculation with numerous sponges to promote alcoholic fermentation. This effectively counteracted lactic acid fermentation, which was characterized by extensive sourage of juice (24). Soured juice was not desirable because it made a bitter product. Honey and crystalline sugar were purchased from local dealers and were ready for immediate and direct use. Though cane juice was the basic raw material, honey was a suitable alternative. In commercial brewing, its usage was limited by prohibitive cost. When honey was used in the process, the product was a premium brand that retailed at a higher price (9).

The various ingredients were combined at predetermined ratios that varied from one brewer to another as illustrated in Table 6. To interpret these rations, it was assumed that: The average sucrose content of cane juice was 13%[w/v](12); when dissolved in water, one and a half units of crystalline sugar increased volume by one unit (4), and that crystalline cane sugar was pure i.e. 100% sucrose. Ingredients were either combined to constitute the complete brew before they were exposed to the unit or were introduced piecemeal. In the latter case, undiluted juice was introduced first in order to directly wet the sponges. This enhanced contact between the resident culture and the juice, the latter being a rich source of nutrients (9). Crystalline cane sugar was then dissolved in water making part of the ration, and poured into the unit to complete the formulation.

TABLE 6. INGREDIENT RATIONS IN MURATINA BREWING [IN TWENTY BREWERIES]

Cane juice] [%v/v]	crystalline cane sugar [w/v]	Water (v/v)	Estimated sugar content %[w/v]
40.0	9.0	51.0	14.0
16.0	7.0	77.0	9.0
46.0	8.0	46.0	13.0
100.0	-	-	13.0
11.0	7.0	82.0	8.0
4.0	9.0	87.0	10.0
11.0	7.0	80.0	10.7
16.0	7.0	77.0	9.0
15.0	9.0	76.0	11.6
8.0	11.0	81.0	8.0
7.0	5.0	88.0	7.0
8.0	5.0	87.0	6.8
21.0	4.0	75.0	7.1
9.0	9.0	82.0	11.3
7.0	11.0	82.0	11.3
6.0	8.0	86.0	7.0
6.0	5.0	81.0	10.0
11.0	8.0	81.0	10.0
11.0	7.0	82.0	8.2
4.0	8.0	88.0	8.9

The computed average composition of fresh brews [Table 7] showed that though the juice was the basic ingredient, its role as a source of sugar was secondary in Muratina brewing.

TABLE 7. AVERAGE COMPOSITION OF RAW MURATINA BREWS

Component	Proportion in brew [%]	Sugar content [%]	Contribution to sugar pool [%]
Sugarcane juice	18.85	13.0	22.4
Crystalline cane sugar	5.30	100	77.6
Water	75.85	None	None
Brew	100	9.85	100

4.1.3. Conditioning the Muratina Sponges

Muratina brewers maintained large stocks of dry and *conditioned* sponges. This was to ensure continuity in brewing operations when other sponge lots were being subjected to the *re-conditioning* process. Conditioning prepared sponges for brewing roles. It involved incubating them in Muratina brews for 4-8 days, and then sun-drying them. The brews were however, discarded after the exercise. In home brewing, sponges were incubated in diluted cane juices, honey or ready wine for a similar duration. The yeasty material, which accumulated in brewing units after several fermentation cycles was also useful in this regard. The aim of this activity was to colonize sponges with yeasts that occurred in these materials. Colonized sponges were then used to inoculate large quantities of Muratina brews.

4.1.4. The Muratina Brewing Process

This started with the selection of appropriate containers preferably barrels, with capacities ranging between 200 and 400 litres. Appropriately conditioned sponges were then selected and laid at the barrel bottoms to make the Muratina brewing units. It is in such units where fermentations were carried out.

The units carried between 50 and 100 pieces of the sponges piled systematically with their rough insides facing upwards. The pile, which occupied the lower quarter of the barrel, reduced the effective holding capacity to between 150 and 300 litres.

Above the pile, a twig or several wooden pieces were hooked at various points on the inner barrel wall, to confine sponges at these depths. This prevented them from coming out of the liquid through floatation and particularly so when fermentation was characterized by vigorous gas production. After sponge confinement, the units were ready for brewing. This was started up by filling units with measured quantities of brew. In doing so, a foot-deep headspace was maintained above the liquid level to allow frothing during fermentation.

(i) Inoculation

This was achieved when brews were exposed to sponges that were carried in brewing units. The degree of inoculation that was achieved in any process was uncontrollable but could be influenced. This was by increasing the number of sponges in the unit or doubling the amount of cane juice in brew formulations. In brewing units utilizing freshly *conditioned* or *re-conditioned* sponges, the degree of inoculation was critical to the success of fermentation. In this case it had to be improved by either of the two methods.

(ii) Incubation

Brewing was carried out at ambient temperature, which ordinarily lay between 24^0C and 30^0C. The temperature of freshly prepared brews lay between 23^0C and 25^0C, and was greatly influenced by water, which made up to seventy five (75%) percent of brew volume. The fermentation process itself and other external heat sources where provided, improved the warmth status of the brew to about 26^0C. These conditions were within the optimum range for yeast growth and therefore promoted alcoholic fermentation (13, 24). These conditions were however, subject to weather variations and draughts created by regular disturbances in brewing rooms.

After inoculation, brews were left to ferment without undue interference. In poorly fermenting units however, this became necessary. Thus, part of the brew was replaced with an equivalent amount of fresh cane juice and the sponges turned without being dislocated from their positions in the units (9). The juice enhanced fermentation by providing more nutrients to the culture, while agitation aroused the immobilized yeasts into suspension. The net effect was an enhanced suspended yeast concentration and growth. Similar activities are performed in conventional brewery fermentations to increase yeast activity in poorly inoculated and nutritionally deficient wort (3).

Muratina fermentation processes were completed in 1-7 days depending on several factors. These were the nutritional adequacy of brews in supporting microbial growth, viability and concentration of the yeast inoculum immobilised by the sponges among others. Experienced brewers confirmed maturity of brews by organoleptic assessment. Matured brews were mildly sweet, sour and astringent. Cessation of gas evolution complemented tasting in maturity determination.

(iii) Harvesting of Matured Wine

The wine was harvested soon after confirmation of maturity. This was necessary because the product acquired bitterness upon prolonged contact with sponges thereafter. This flavour defect may be akin to what is described as *yeast bite* in beers. This occurred when separation of matured beer and yeast was delayed after completion of primary fermentation (6). Matured wine was extracted from the units by scooping or through suction. It was then filtered through porous cloths and was ready for consumption. Normally, the wine was turbid due to the presence of suspended microoganisms and effervescent due to continuing fermentation and gas evolution.

In the two extraction methods, brewing units were left intact so that simply refilling them with fresh material, started new brewing cycles. Numerous fermentation cycles were repeated in the same unit until acceptable products could not be guaranteed. Sponges were then removed from the units, washed and sun-dried to dispose the old culture.

The vessels similarly were cleaned, sun-dried and re-equipped with fresh lots of *re-conditioned sponges* and the cyclic processes repeated *as* depicted in Figure 4.

Sugar cane — Crystalline cane sugar (6 parts) — Matured Muratina fruits

Juice extraction (milling) — Addition of water (76 parts) — Split into halves

Juice filtration — Mix thoroughly — Boil in water (Several times)

Clarified juice (18 parts) — Homogenous solution — Dry in the sun

Mix — Incubate in cane juice (4 – 8 days) → Discard juice

Muratina brew — Dry sponges in the sun

Assemble appropriate number in choice container (Brewing unit)

Fill unit (Inoculation) — Wash in water

Fermentation (25°C – 30°C, 1 – 7 days) → Change sponges (after 27 brewing cycles)

Muratina wine

FIGURE 4: FLOW DIAGRAM OF THE TRADITIONAL MANUFACTURING PROCESS OF MURATINA WINE.

4.1.5. Sustenance and Change of Sponges

In order to sustain brewing operations, a viable starter culture was maintained. This was ensured by: -

    (i)   Supplying adequate growth factors in fermenting material through regular and sustained use of sugarcane juice in brew formulations.

    (ii)   Maintaining sponges in submersion and particularly at the intervals between the end and start of new fermentation cycles. In the absence of complete brew formulations to start new fermentation cycles, concoctions made by dissolving cane sugar in water were filled in the units to submerge and engage the sponges. This kept off the opportunistic oxidative bacteria and yeasts, which could infect exposed sponges, and cause death of the useful culture (17).

    (iii)   Changing sponges regularly.

On average, Muratina brewing units had an operational span of 5.6 weeks before sponges were changed or *re-conditioned*. They produced 27 wine crops in this time while the average span of a brewing cycle was 45.6 hours [Table 8]. In this time, sponges were choked by a viscous creamy sludge, which was mainly composed of yeast matter that has been generated over time. This flocculent matter was referred to as *Mairiti* in Kikuyu. Its generation was rightly attributed to the presence of cane juice in the brews. This material caused haziness and made the drink harsh to the palate. This was one reason why sponges required to be changed regularly. Washing sponges in water, and then drying them preferably in the sun disposed the material. The rate of generation of yeast matter was influenced by composition of brews in relation to juice percentage. It provided vital substrates and therefore influenced the operational life of brewing units.

In fermentation of brews containing substantial proportions of juice, vibrant yeast growth occurred. Consequently, sponge cavities filled rapidly and required to be emptied at short brewing intervals. Conversely, in fermentation of brews containing small juice percentages, poor growth occurred. Fermentations were slow and hence, fewer brewing cycles were accomplished in the operational span of units.

Variations in schedules of changing the sponges were expected due to differences in brewing rations. After washing and sun drying them, sponges were directly re-usable in new brewing units.

TABLE 8. SCHEDULE FOR CHANGING MURATINA SPONGES [FROM 20 BREWERIES]

Brewing life of unit [weeks]	Length of brewing cycle [hours]	No. of cycles before change of sponges
4	24	30
8	24	56
4	24	30
1	24	42
7	24	30
9	24	56
4	24	30
4	24	30
6	48	30
2	48	7
4	48	15
8	48	28
8	48	30
12	48	42
4	48	15
9	48	31
9	48	31
8	48	11
4	72	10
2	7 [g]	2
Average 5.6	45.6	27

g) Days

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4.1.6. Composition of Muratina Wine

The composition of Muratina varied considerably. On average, it contained 4.5-vol.% ethanol, 1.0 %[w/v] lactic acid, and 0.24%[w/v] acetic acid and average pH lay between 3.0 - 3.1.

TABLE 9. CHEMICAL COMPOSITION OF MURATINA WINES

Ethanol content [%vol.]	Total acidity [a] (%)	Volatile acidity [b] [%]	pH	Dissolved solids [%]	Reducing sugars [%]	Diacetyl [ppm]	Optical density [c] (%)
6.9	1.4	0.18	3.0	4.5	1.1	0.1	45.8
4.1	1.05	0.28	3.3	2.5	-	0.12	63.4
8.0	1.4	0.18	3.0	4.5	1.2	0.1	52.7
8.9	1.4	0.34	3.3	4.5	0.14	0.09	49.5
3.3	0.82	0.17	3.2	2.5	0.6	0.10	79.2
5.8	1.32	0.22	3.1	2.0	0.1	0.06	80.5
3.7	0.09	0.34	3.3	2.5	0.44	0.06	80.5
4.1	1.07	0.32	3.2	3.0	0.44	0.06	80.5
6.1	1.44	0.39	3.0	4.0	0.9	0.07	61.3
2.7	1.12	0.29	3.2	2.0	-	0.08	76.8
2.4	0.2	0.25	3.2	1.5	0.04	0.02	73.8
3.7	0.09	0.18	3.0	2.5	0.44	0.06	72.8
2.6	0.95	0.27	3.2	1.5	0.2	0.07	60.2
5.8	1.4	0.26	3.0	2.5	-	0.06	68.2
4.3	1.5	0.09	3.1	3.2	0.79	0.09	84.2
2.9	1.05	0.12	3.0	2.5	1.2	0.14	85.2
4.0	1.14	0.23	3.1	2.0	0.1	0.09	72.7
4.5	1.08	0.22	3.0	3.0	0.6	0.09	55.7
3.5	1.4	0.18	2.9	2.0	0.2	0.15	72.3
2.8	1.23	0.30	3.0	4.5	0.9	0.09	69.5

a- as lactic acid, b- as acetic acid, c- as transmittance [%] of monochromatic light at 420-nm wavelength

The beverage had a yellowish colouration of variable intensity as determined by the content of juice in brew formulations. On average, the optical density [measured as the absorption percentage of monochromatic light at 420nm- wavelength] was above 69%.

Brewing techniques, deterioration of juices and other uncontrollable factors, influenced the final product composition as illustrated in Table 9. These results showed that a lacto-alcoholic fermentation process obtained Muratina. To assess the influence of process variables on composition, some cause-effect relationships were examined by calculating correlation coefficients between presumed relationships.

4.1.7. Influence of Manufacturing Method on Muratina Composition

(a) Ethanol content

This was influenced by the sugar concentration that was attained in different brewing rations, as the following correlation coefficients confirmed.

(i) Sugar content of brew versus ethanol content of wine

The correlation coefficient was determined as r = + 0.96, P<0.05, C.1 (0.3, 0.96). The high and positive correlation showed that sugar concentration in the brew, determined the ethanol content of wine. This was expected in alcoholic fermentations.

(ii) Proportion of cane juice in brew versus alcohol content of wine

The average proportion of cane juice in raw brews was eighteen percent, and this fraction contributed twenty two percent of the total fermentable sugar[Table 7]. Despite this percentage, the juice played a significant role in the determination of ethanol content as confirmed by the high and positive correlation. The coefficient thus was determined as, r=+0.79 ± 0.03 (p<0.05).

Besides its sugar content, the juice was a significant source of vital substrates and yeast. It boosted yeast numbers numerically and by enhanced growth. This promoted alcoholic fermentation.

(iii) Percentage of crystalline cane sugar in brew versus alcohol content in wine

On average, crystalline cane sugar constituted seventy eight percent (78%) of the total sugar content. It is this fraction therefore that principally determined the amount of sugar available for fermentation. The correlation between crystalline sugar and ethanol content was however, less intense than in the case of cane juice. This was because the metabolic conversion of sugar to ethanol was dependent on factors other than sugar concentration. A positive correlation coefficient was determined as, r=0.53±0.02) P< 0.05).

(b) Wine Acidity

Acidity of raw and fermented brews was influenced mainly by cane juice. This is because it was used while at advanced stages of natural fermentation. Soured juice contains appreciable quantities of lactic and acetic acids that are produced mainly by the homofermentative and heterofermentative lactic acid bacteria (24). Depending on the degree of sourage and its proportion in brew formulations, the juice influenced wine acidity. The correlation was positive. Thus, $r = 0.37 \pm 0.03$ ($P<0.05$). This represented a moderate association that was suggestive of the occurrence of limited lactic acid fermentation.

A positive relationship was established between cane juice percentage and volatile acidity in wine. Thus, $r = 0.24 \pm 0.01$ ($P < 0.05$). This was a weakly positive relationship. This implied that cane juice had a minor influence in this regard.

In alcoholic fermentations, yeasts produce considerable amount of acids too, depending on the intensity of their activity (13).

(c) Dissolved Solids content

The dissolved solids content in wine roughly indicated the degree of sugar utilization in the fermentation. This varied between 2.0 and 4.5% suggesting that wine was harvested before all the sugar was utilised.

(d) Intensity of Wine Colouration

An intense yellowish colour, which was preferred in Muratina, was obtained by ensuring a rational presence of cane juice in brew formulations. Thus, $r = -0.64 \pm 0.03$ ($P < 0.05$). A moderate and negative relationship existed between juice percentage in brew and intensity of the yellowish colour. Colour intensity and light transmittance values of solutions are inversely related hence the negative coefficient. The association confirmed that intensity of wine colouration was influenced the juice percentage in brew.

(e) Diacetyl content

Diacetyl was assayed as a potential flavour compound in Muratina. This compound imparted the butterscotch flavour in fermented dairy and alcoholic products, but was only desirable in the former. In dairy products, the bacteria *Streptococcus diacetylactis* produced it. The common brewing yeast *Saccharomyces cerevisiae* produced it in alcoholic beverages (27). In beers infected by the bacteria *sarcina sp,* it occurred in high concentrations (38). This bacterium was reported to participate in Muratina fermentation (10).

The average diacetyl content in Muratina was 0.085 ppm. This compared favourably with its threshold concentration [0.1-0.2mg/litre] in lager beers, wines and whiskies (38). Its influence on Muratina flavour was not determined.

4.1.8. Quality and Shelf life Aspects of Muratina wine

Muratina composition varied widely as were the brewing techniques. In addition, its shelf life was limited to a day due to the content of microorganisms. Spoilage was characterized by increased sourness. This was caused by exhaustion of any residual sugar due to continued microbial activity and possible oxidation by acetic acid bacteria. Spoilt products were not discarded but modified for palatability.

Beverage quality was defined subjectively in terms of sourness, colour and palatability. Objective measurements of alcohol content and other attributes did not apply. The attributes of a good drink were astringency, mild sweetness and an intense yellowish colouration. The drink was preferred lukewarm at the time of consumption.

The common quality defects, their causes and possible corrections are summarized below.

Sourness

This occurred in brews made with a high proportion of soured juice. In matured drinks, souring occurred during storage due to continued post-harvest microbial activity. To improve palatability, drinks were sweetened with small doses of crystalline cane sugar or honey.

Light coloured drinks

These occurred if brews contained low proportions of cane juice. Adding doses of honey enhanced the colour.

Sweet drinks

These were either harvested prematurely, or resulted from failing brewing units that required immediate replacement of the sponges.

Harsh and bitter drinks

These were obtained in later fermentation cycles of a brewing unit. Yeast biomasses that accumulated over-time caused this. Such units required immediate replacement of the sponges too.

4.1.9. Status of Traditional Liquor Manufacturing

Traditional liquors were manufactured in cottage-size factories located in low-income residential areas of the city. The government through selective licensing regulated production. Thus, Table 10 shows the allocation of 569 brewing licenses among administrative divisions in the city of Nairobi in 1991/92(42).

The main liquors in production were Muratina, Busaa, Miti and Chang'aa. Their total production could not be quantified due to lack of valid statistics from the regulating authorities and widespread illegal brewing. From the findings of the survey and eyewitness accounts, the author estimated daily production at 150- 2000 litres per brewer, depending on the size of operation. Subsequently, annual production was estimated at between 50,000 and 1090,000 litres per brewer. At a retail price of Kshs. 10.00 per litre, this production translated to sales revenues of between Kshs. 0.5 and 10.9 million per brewer. This was commendable from an informal manufacturing enterprise.

In addition, traditional manufacturing ventures offered cheap refreshment to those who may not afford bottled lager beers and wines. They were a source of government revenue through licences, created employment opportunities and added value to crops, which ordinarily had no commercial use.

TABLE 10. THE DISTRIBUTION OF TRADITIONAL LIQUOR BREWERIES IN THE CITY OF NAIROBI[H] (1991/1992).

Administrative zone	No. of breweries
Pumwani	85
Makadara	72
Kasarani/Embakasi	189
Kibera	107
Parklands/Dagoretti	116

[H]- information courtesy of provincial liquor licensing office, Nairobi (42).

4.2.0. Characterisation of Muratina Fermentation

The Muratina fermentation process was studied in four traditional breweries. The fermentation processes were carried out in traditional brewing units and in cycles. The Matured wine crops were therefore harvested and the units refilled with fresh concoctions to begin new brewing cycles. The microbiological and chemical aspects of such brewing cycles and in different locations were investigated.

4.2.1. Characteristics of Raw Materials

a) Sugar Cane Juice

Juices were used when actively fermenting and therefore exhibited wide variations in pH and acid content [Table 11]. These are the common indicators of microbial caused deterioration (24).

TABLE 11. pH AND COMPOSITION OF CANE JUICE

Total acidity[a] [%]	Volatile acidity[b] [%]	Sucrose content[^{0}Brix]	pH
0.55-1.40	0.06-0.19	13.4-14.5	3.5-3.7

a) as lactic acid, b) as acetic acid

Juices were infected by a diversity of microorganisms [Table 12], and were the main sources of contamination in brewing processes. They boosted the concentration of yeasts in brewing units, and were sources of bacteria that caused limited lactic acid fermentation.

TABLE 12. MICROBIAL CONTENT OF CANE JUICES [AVERAGE OF EIGHT SAMPLES]

Colony forming units	counts per ml
Total microorganisms	$2.9 \times 10^8 \pm 0.6 \times 10^8$
Yeasts and moulds	$3.7 \times 10^7 \pm 0.2 \times 10^7$
Lactic acid bacteria	$2.9 \times 10^8 \pm 0.6 \times 10^8$
Acetic acid bacteria	$3.0 \times 10^4 \pm 0.5 \times 10^4$

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(b) Crystalline Cane Sugar

On average, this constituted 5.3% [w/v] of brews but contributed 77.6% [w/v] of fermentable sugars. Considering the level of contamination [Table 13], and subsequent dilution in formulating the brews [Table 7], it was an insignificant source of microbial contamination. The sugar was dissolved in water that was part of the ration. This made 81% of the brew volume. Ultimately, the solutions contributed insignificant contamination and acidity. They were neutral in pH.

TABLE 13. MICROBIAL CONTAMINATION IN CRYSTALLINE CANE SUGAR

Colony forming units	Colony forming units/g
Total microorganisms	$5.0 \times 10^3 \pm 0.5 \times 10^3$
Yeast and moulds	$4.0 \times 10^3 \pm 0.2 \times 10^3$
Lactic acid bacteria	$2.2 \times 10^2 \pm 0.2 \times 10^2$
Acetic acid bacteria	$1.5 \times 10^1 \pm 0.2 \times 10^1$

(c) Muratina Sponges

Yeasts in variable concentrations colonized them and therefore were the yeast carriers. Depending on their immediate condition, they carried variable yeast loads. For example, freshly *conditioned* ones were colonised by fewer yeasts because they were desiccated during sun drying. Those in active brewing, immobilized yeasts in greater numbers. This is why yeasts dominated the flocculent matter that was extracted from such sponges. Sponges and hence Muratina brewing units, immobilized great yeast populations that caused alcoholic fermentation. Levels of sponge colonization are illustrated in Table 14.

TABLE 14. MICRO FLORA OF MURATINA SPONGES

Total viable microorganisms [a]		Yeasts and moulds [a]
Conditioned sponges	$2.7 \times 10^4 \pm 0.5 \times 10^4$	$2.7 \times 10^4 \pm 0.5 \times 10^4$
Brewing sponges	$6.4 \times 10^9 \pm 0.5 \times 10^9$	$6.4 \times 10^9 \pm 0.5 \times 10^9$
Muratina floc [c]	$3.3 \times 10^6 \pm 0.5 \times 10^6$	$3.3 \times 10^6 \pm 0.5 \times 10^6$

a] –CFU per gram of sponge dry matter, c] –CFU per millilitre of extract suspension.

Sponge Extract

Brewing sponges were soaked in thick flocculent slurries that consisted of yeast biomasses suspended in wine residues. The material was acidic as shown in Table 15. Its composition showed that the brewing unit, and hence the Muratina culture thrived in acidic conditions.

TABLE 15. ACIDITY AND PH OF FLOCCULENT SPONGE EXTRACT [AVERAGE OF EIGHT SAMPLES]

Total acidity [a] [%]	Volatile acidity [b] [%]	pH
1.19 ± 0.17	0.23 ± 0.07	3.0 ± 0.1

a)-as lactic acid, b)as-acetic acid

4.2.2. The Muratina Fermentation Process

The microbiology of Muratina fermentation was investigated in eight complete brewing processes, which were carried out in different breweries.

(a) Rapid Brewing Processes.

These processes guaranteed high turnovers because they were completed within 24-hours. Consequently, they were encountered in 40% of visited breweries. Two such processes were investigated. In the first case, brews with a sugar content of 14.0%, were composed of cane juice; 41%, crystalline cane sugar; 6.3%, and 52.7% water (all on weight per volume basis) were fermented.

These were brewed at ambient temperature [25^0C], and fermentation processes completed in 24-hours. Yeasts and lactic acid bacteria, which were the main contaminants in cane juice, dominated freshly prepared brews at concentrations in the range of 10^6-10^7CFU/ml. After brews encountered sponges in brewing units (inoculation), the lactic acid bacteria population remained at 2.8×10^7 CFU/ml, but yeasts were boosted to 9.0×10^7CFU/ml by the culture that resided in the units.

With the onset of gas production and hence alcoholic fermentation, lactic acid bacteria decreased steadily and disappeared altogether in 12 hours. Probably carbon dioxide and ethanol production aided this.

It has been suggested that lactic acid bacteria of plant origin do not survive alcoholic fermentation (32). Yeasts increased gradually in this period, and so was the ethanol content. After disappearance of bacteria, yeasts dominated almost as pure cultures as illustrated in Figure 5.
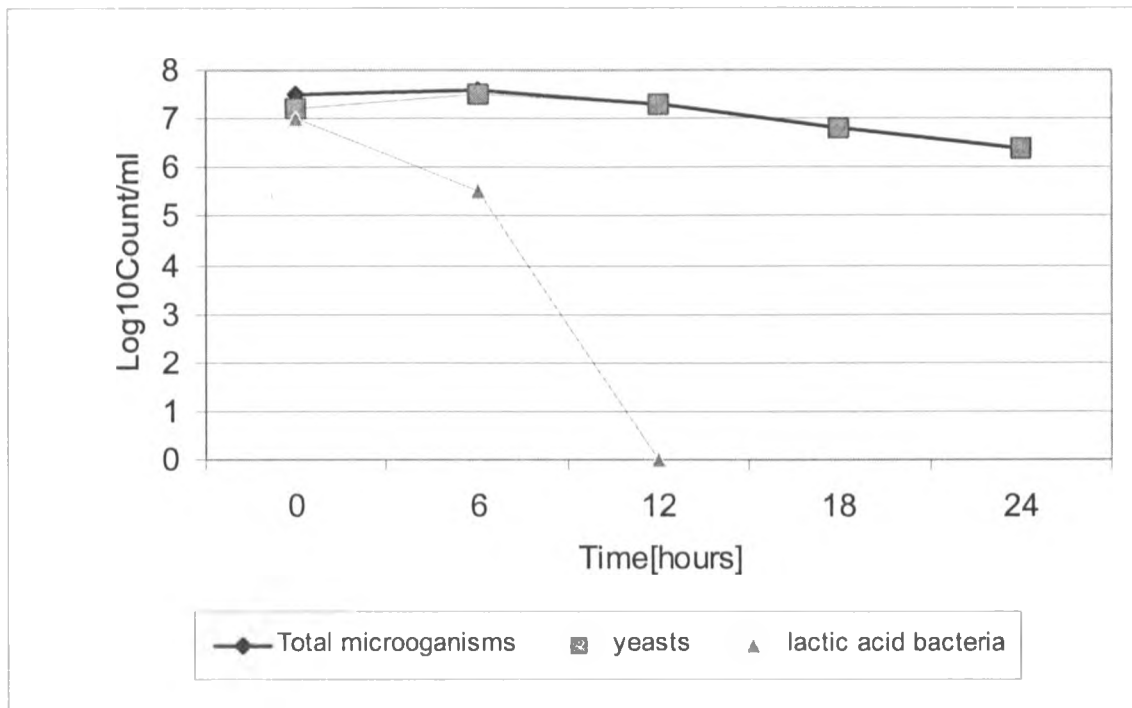
FIGURE 5. CHANGES IN MICROBIAL POPULATIONS DURING A 24-HOUR MURATINA BREWING PROCESS.

In 24 - hours, the ethanol content had risen to 6.1 % vol.; acidity to 1.08%[w/v] as lactic acid; while the pH of fermenting brews had fallen from 3.7 to 3.0. These changes are presented in Table 16.

TABLE 16. CHEMICAL CHANGES IN A 24-HOUR MURATINA BREWING PROCESS

Fermentation Time [hours]	Temp (^{0}C)	Total acidity[a]	Volatile acidity [b] [%]	pH	Ethanol [vol.%]	Dissolved solids[%]
0	24.0	0.29	0.15	3.7	0.9	13.8
6	25.0	0.36	0.16	3.3	2.2	10.8
12	26.0	0.60	0.17	3.2	3.7	9.5
24	25.5	1.08	0.18	3.0	6.1	3.7

a) as lactic acid, b) as acetic acid

In a second case, pure cane juice was brewed at ambient temperature [25^0C]. The juice was actively fermenting by the time of use. It's contamination consisted of: Total viable microorganisms, 5.2 x 10^8 CFU/ ml yeasts and moulds, 3.8 x 10^8 CFU /ml and lactic acid bacteria, 2.0 x 10^6CFU/ml. After the juice was filled in the brewing units, the resident inoculum boosted the microbial counts. Thus, the total count of viable microorganisms and yeast, increased to 8.2 x 10^8 CFU/ ml and 6.9 x 10^8 CFU /ml respectively as shown in Figure 6.
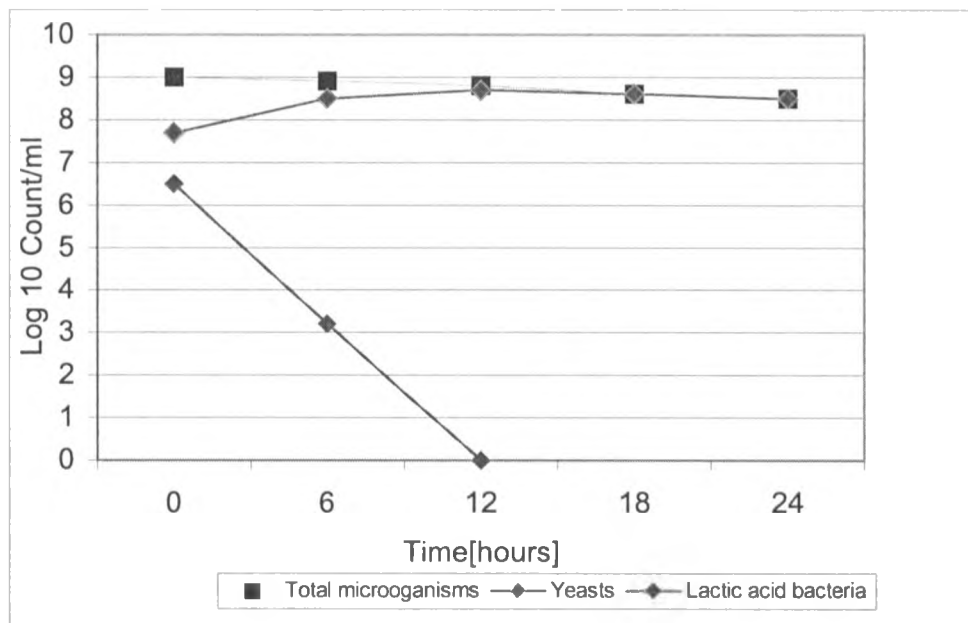
FIGURE 6: CHANGES IN MICROBIAL POPULATIONS DURING THE FERMENTATION OF SUGAR CANE JUICE IN MURATINA BREWING UNITS.

Inoculation did not affect the lactic acid bacteria numbers. Hence, they remained at 2.5 x 10^6 CFU/ml. They however, decreased steadily with the commencement of alcoholic fermentation and disappeared altogether in 12 hours. These bacteria caused limited lactic acid fermentation in this period, and boosted the acid content from 0.53% to 0.89% lactic acid. Yeast dominated the process after 6 hours and in numbers beyond 10^8 CFU/ml, and almost as pure cultures.

Fermentation was characterized by vigorous gas evolution, ethanol production and increased acidity. The fermented brew contained 8.0% vol. ethanol and 1.4% lactic acid as illustrated in Table 17.

TABLE 17. CHEMICAL CHANGES DURING THE FERMENTATION OF SUGAR CANE JUICE IN MURATINA BREWING UNITS.

Fermentation time[hours]	Temp [^{0}C]	Total acidity [a)] [%]	Volatile acidity [b)] [%]	pH	Ethanol [vol.%]	Dissolve solids [^{0}Brix]
0	25	0.53	0.12	3.70	2.0	13.3
6	25	0.60	0.15	3.30	3.0	9.8
12	26	0.89	0.16	3.25	5.4	6.8
24	26	1.40	0.27	3.05	8.0	4.3

a)-as lactic acid, b)-as acetic acid

(b) Brewing Processes of Moderate Speed

These processes were completed in more than 24 - hours but in less than 48 - hours. They were considered to be of moderate speed. In a studied case of processes in this category, brewing was carried out at ambient [25°C] temperature and fermentation completed in 48 hours. The brews were composed of cane juice, 4.5%; crystalline cane juice sugar, 16.5%; and water 78.8% and on weight per volume terms. The average sugar content was 9.8%. Due to the low juice percentage, brews contained less contamination thus: Total count $1.1x10^6$ CFU/ml, yeasts $9.0x10^5$ and lactic acid bacteria $1.0x10^6$CFU/ml. After inoculation, yeasts increased and peaked at $1.0x10^7$CFU/ml in 24 hours. Finally they decreased to $4x10^6$CFU/ml at the end of process. After the bacteria disappeared in 24 hours, yeasts dominated once again as pure cultures as shown in Figure 7.
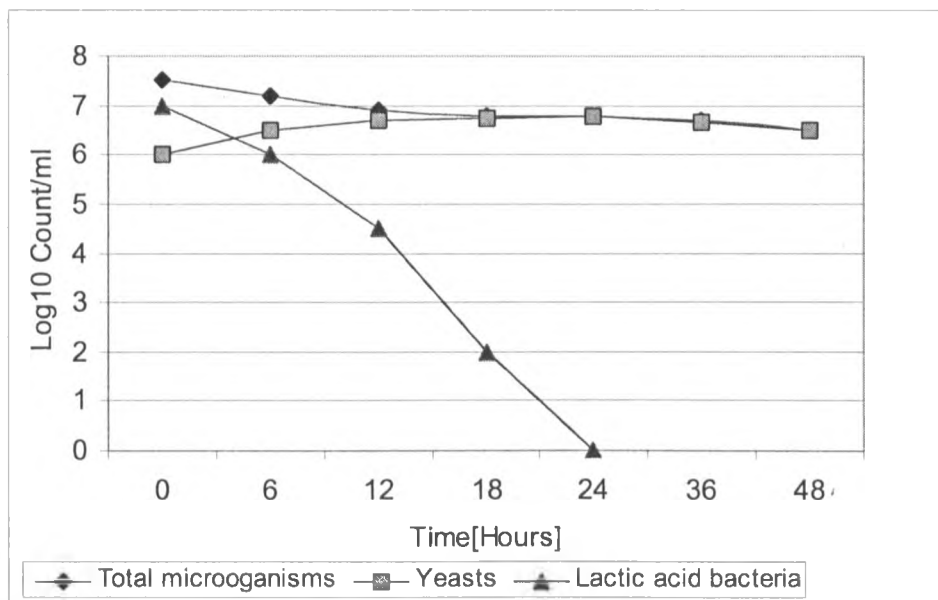

FIGURE 7: MICROBIAL CHANGES IN A 48-HOUR MURATINA BREWING PROCESS

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This process was slow in relation to the studied 24-hour processes because it enjoyed lesser inoculation. This was attributed to the small percentage of juice in brews. The fermented brew contained 5.3 vol.% ethanol and 0.44% lactic acid. Its pH had dropped to 2.7 despite the low acid content. Theses changes are illustrated in Table 18. This was attributed to poor buffering capacity of the brews owing to the small percentages of cane juice (24).

TABLE 18. CHEMICAL CHANGES IN A 48-HOUR MURATINA BREWING PROCESS

Time [hours]	Total acidity [a] [%]	Volatile acidity [b] [%]	Temp. [^{0}C]	pH	Alcohol [vol.%]	Dissolved solids[^{0}Brix]
0	0.10	0.12	24.0	3.3	.087	9.8
6	0.37	0.02	24.5	3.25	1.60	9.2
12	0.37	0.02	24.5	2.75	2.80	8.3
24	0.41	0.05	24.5	2.7	3.3	4.3
36	0.42	0.06	24.5	2.7	4.8	3.5
48	0.44	0.064	25.0	2.7	5.3	2.7

a) as lactic acid, b) as acetic acid

(c) Extended Brewing Processes

Processes that required more than 48 hours to complete were considered exceptions in commercialised Muratina brewing. They represented slow fermentation processes, which occurred in poorly constituted brews that were also brewed in poorly colonized brewing units. This was particularly the case in units that were utilizing *freshly conditioned* sponges. These were poorly colonized and carried less concentrated yeast inoculum. Subsequently, the processes had to experience gradual yeast growth to build numbers that were sufficient to cause significant alcoholic fermentation.

Thus, the studied case was a 7- day fermentation process. Like in other cases, fermentation progressed at ambient temperature [25^0C]. The brews were made from cane juice; 14.4%, crystalline cane sugar; 8.3% and water, 77.3% all expressed in weight per volume basis. Contamination in fresh brews consisted of yeasts; 1.0×10^7 CFU/ml, bacteria; 1.2×10^7 CFU/ml. Inoculation boosted the yeast population to 8.0×10^7 CFU/ml. This then increased to concentrations beyond 10^8 CFU/ml in the remaining process time. After the bacteria disappeared in two days, yeasts dominated the process as illustrated in Figure 8.
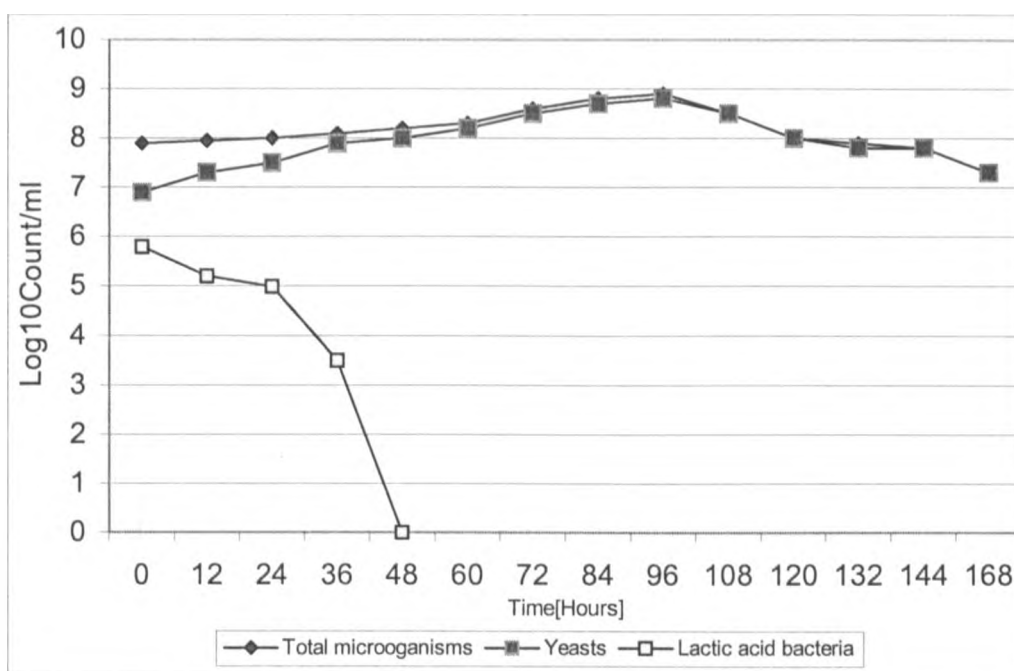
FIGURE 8: MICROBIAL CHANGES IN A 7-DAY MURATINA BREWING PROCESS.

This process registered the highest and consistent yeast concentrations during fermentation, but was the slowest among the studied cases. The end brews contained 5.5 vol.% ethanol and 0.59% lactic acid and pH was 2.9 as illustrated in Table 19.

TABLE 19. CHEMICAL CHANGES IN A 7-DAY MURATINA BREWING PROCESS.

Time [Hours]	Total acidity [a] [%]	Volatile acidity [b] [%]	pH	Ethanol [vol.%]	Dissolved solids[^{0}Brix]
0	0.13	0.05	3.7	-	10.0
6	0.14	0.06	3.5	0.20	9.5
12	0.20	0.65	3.2	1.30	9.0
24	0.24	0.07	3.1	2.50	7.8
48	0.37	0.08	3.1	3.80	7.2
72	0.44	0.08	3.1	4.10	6.4
96	0.49	0.09	3.0	4.20	6.0
120	0.56	0.11	3.0	5.10	5.5
144	0.58	0.11	2.9	5.40	5.4
168	0.59	0.12	2.9	5.50	5.00

a)-as lactic acid, b)-as acetic acid

These findings indicated that although Muratina was brewed under similar circumstances, obvious differences in relation to constitution of raw brews and fermentation rates existed. The processes displayed similar trends in terms of microbiological and chemical changes. However, although the determined yeast concentrations were in the same range ($10^6 - 10^8$ CFU/ml), fermentation rates were different. This was because brewing units differed in the effective yeast concentrations, which however, were not quantified.

The reported counts therefore, represented yeasts that were suspended in the fermenting liquid. Those that were immobilized in the sponges were not enumerated. The suspended counts however, reflected yeast growth patterns during fermentation.

The immobilized yeast population determined fermentation rates and therefore, duration of brewing processes. The composition of brews influenced this. Fermentations of brews with high juice percentages were bound to be faster. This was so because the juice boosted yeast populations in brewing units through contamination and by enhanced growth. The opposite was true.

4.2.3. Some Characteristics of the Muratina Yeast

The prevalent yeast in Muratina brewing process formed distinct star-shaped colonies on different culture media. It dominated dry-conditioned and brewing sponges, sugarcane juice and fermenting brews. It was thus referred to as the *Muratina yeast*.

Eight isolates from each of the mentioned sources were purified, and their fermentative and cultural behaviour investigated. Some of the characteristics are illustrated in Tables 20 and 21. These isolates caused alcoholic fermentation and brewing experiments confirmed this.

TABLE 20. FERMENTATIVE UTILIZATION OF SUGARS BY THE *MURATINA YEAST.*

Test sugar	Result
Glucose	+++
Fructose	+++
Sucrose	+++
Maltose	++
Galactose	++
Raffinose	+
Lactose	-

KEY
+++   Positive fermentation with much gas production
++   Positive fermentation with moderate gas production
+   Positive fermentation with mild gas production
-     Negative fermentation

TABLE 21. CULTURAL CHARACTERISTICS OF MURATINA YEASTS

Culture media and growth conditions	Growth description
Malt-yeast-extract-peptone-glucose broth after incubation at 28^0C for 3 days	• Heavy flocculent growth at the base of fermentation tube • Cell dimensions: 2-3 μm wide and 4-6 μm long, ovoid and globose • No pellicle formation in 4 weeks
Malt-yeast-extract-peptone-glucose-agar and potato dextrose agar after incubation at 28^0C for three days.	• Colonies distinctively star shaped, creamy-white • Colonies produced a fragrance typical of Muratina wine and conditioned sponges.
Mc'Lary's acetate agar, Malt agar and Adams agar	• No ascospore formation
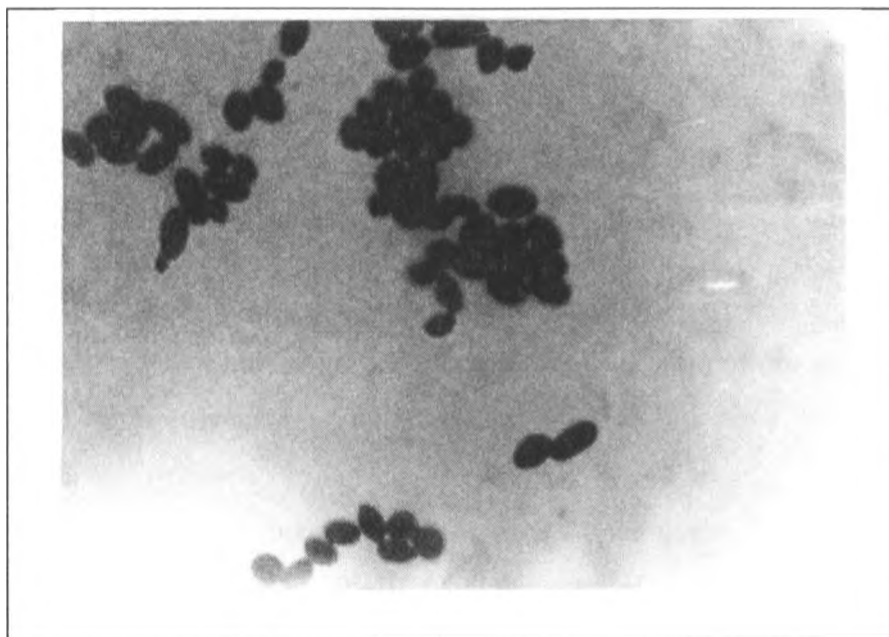
FIGURE 9: PHOTOGRAPH OF MULTILATERALLY-BUDDING *MURATINA YEAST* CELLS [1000-X MAGNIFICATION OF 48-HOUR OLD CULTURE IN MALT-YEAST-EXTRACT-PEPTONE-GLUCOSE BROTH]

4.3. Sponges and the Muratina Culture

4.3.1. Sponge Conditioning

Incubating halved Muratina fruits in sugarcane juice and in fermenting Muratina brews, conditioned them to the brewing process. The yeasts that dominated in these media colonized them. Conditioning was a four-stage process. The first stage involved boiling sponges in water several times while changing water every time. This sterilized them by killing vegetative microorganisms and extracted the bitter tannic compounds inherent to the fruit. At the second stage, sponges were sun-dried and in the process, trapped some aerobic microorganisms. These were inconsequential to the brewing process. These two stages were simulated in experiments by sterilizing sponges and then sun drying them. The microbial counts of sponges after sterilization and subsequent sun-drying were insignificant, but dominated by moulds [Table 22]. In the third stage, sun-dried sponges were incubated in active brewing units, sugar cane juice or diluted honey. Incubating sterilized and sun-dried sponges in cane juice at 25^0 C simulated this stage. Natural fermentation of juice was enhanced in the process as confirmed by the increased yeast percentage [Table 22].

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TABLE 22. MICROBIAL CHANGES IN SUGARCANE JUICE IN THE PROCESS OF SPONGE CONDITIONING

	Total viable microorganisms[d]	Yeasts[d]	% Yeasts
Day 1	$4.6 \times 10^6 \pm 0.5 \times 10^6$	$1.6 \times 10^5 \pm 0.5 \times 10^5$	3.5
Day 4	$5.8 \times 10^8 \pm 0.2 \times 10^8$	$2.4 \times 10^8 \pm 0.2 \times 10^8$	41

d)-colony forming units per ml juice

Sponges were colonized at intensities of 10^{10}-10^{11} CFU per gram of dry matter, with yeasts comprising 27% of the colonizing population [Table 23].

In the fourth and last stage, sponges were sun-dried once again. This event reduced the colonizing microbial population to 10^5-10^6 CFU per gram of sponge dry matter, and boosted the yeast percentage from twenty-seven percent (27%) to eighty-eight percent (88%). The surviving yeasts were then propagated in subsequent brewing processes to create a biomass within sponges, the Muratina starter culture.

TABLE 23.   MICROBIAL CHANGES IN SPONGES DURING THE CONDITIONING PROCESS

	Total microorganisms [e]	Yeasts and moulds[e]	% Yeasts
(i) Sterile sponges	<1	<1	Nil
(ii) After incubation	$1.8 \times 10^{11} \pm 0.1 \times 10^{11}$	$4.8 \times 10^{10} \pm 0.5 \times 10^{10}$	27
(iii) After sun- drying	$4.8 \times 10^5 \pm 0.1 \times 10^5$	$4.2 \times 10^5 \pm 0.1 \times 10^5$	88

e) CFU per gram of sponge dry matter

In the control experiments, sponges were sterilized and sun-dried without prior incubation. Moulds dominated them. This observation confirmed that in the conditioning process, fermentative yeasts, which are common contaminants of cane juice, colonized the sponges.

4.3.2. Impact of Sponge Colonization on Fermentation

The impact of sponge colonization on the brewing process was demonstrated in experiments that were controls for the experimental brewing processes. In one case, brews were left to undergo natural fermentation i.e. no sponges were involved. The results showed that microbial loads in fresh brews were as high as 10^6-10^8 CFU per ml, and mainly emanating from the contamination of cane juices. However, this changed with time due to fermentative activity.

The total counts decreased initially following the disappearance of Lactic acid bacteria, which were the dominant bacteria group. In this time they caused limited lactic acid fermentation thus increasing brew acidity, which then depressed brew pH to 3.0. These conditions in turn limited bacteria survival hence their disappearance in the first 48 hours. Yeasts thrived under these conditions, and boosted the total count. Eventually, they dominated brews at concentrations in the range of 10^7-10^8 CFU per ml as illustrated in Figure 10.
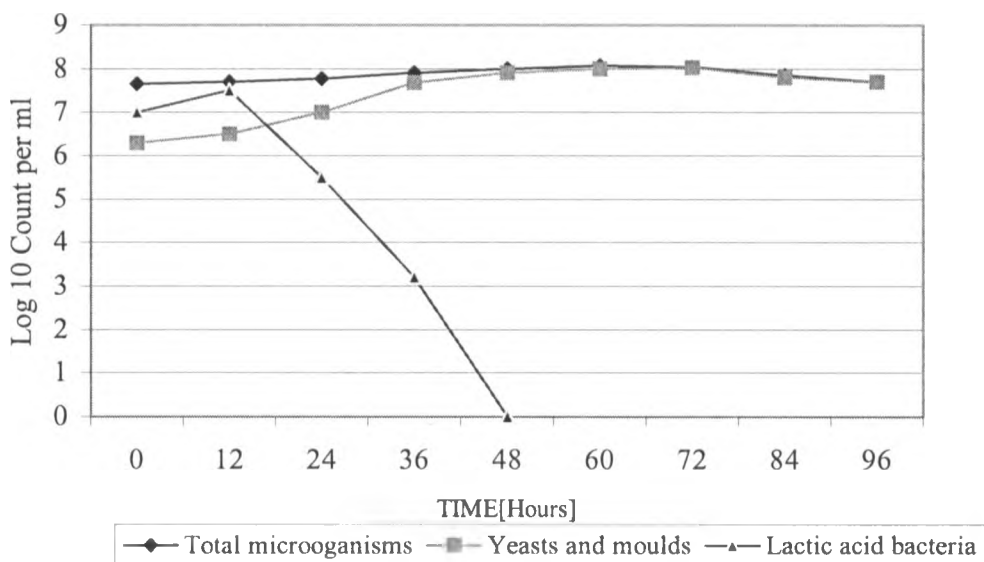
FIGURE 10: MICROBIAL CHANGES IN NATURALLY FERMENTING MURATINA BREWS

Despite the enhanced yeast populations, minimal alcoholic fermentation occurred. Consequently, reducing sugars accumulated in this period, and probably aided by acid induced inversion of sucrose [Table 24]. In the absence of enhanced yeast activity, lactic acid fermentation dominated. Therefore, though the concentration of yeast in this experiment was as high as determined in traditional fermentations, the latter enjoyed greater inoculation.

TABLE 24. CHEMICAL CHANGES IN NATURALLY FERMENTING MURATINA BREWS

Time [hours]	Total acidity[a] [%]	Volatile acidity[b] [%]	pH	Ethanol [vol.%]	Reducing sugars [%]
0	0.27	0.07	3.7	-	0.2
12	0.49	0.08	3.3	-	0.78
24	0.52	0.13	3.2	0.90	2.5
36	0.55	0.16	3.0	1.00	2.5
48	0.68	0.20	3.0	1.10	2.8
60	0.94	0.21	2.95	1.10	3.3
96	1.10	0.28	2.90	1.30	5.0

a) as lactic acid ,b) as acetic acid

In another set of experiments, numerous sterile sponges were incubated in similarly prepared brews. These were then incubated at ambient temperature [25^{0}C] for an equivalent duration.

As in the previous experiments, yeasts dominated after the persistence of lactic acid bacteria that increased total acidity to beyond 1.0 % and volatile acidity to 0.28 %. This suggested the activity of heterofermentative lactic acid bacteria. In the absence of alcoholic fermentation by yeasts, lactic acid fermentation dominated in this time as illustrated in Figure11.

FIGURE 11: MICROBIAL CHANGES IN NATURALLY FERMENTING BREWS DOSED WITH STERILIZED SPONGES.
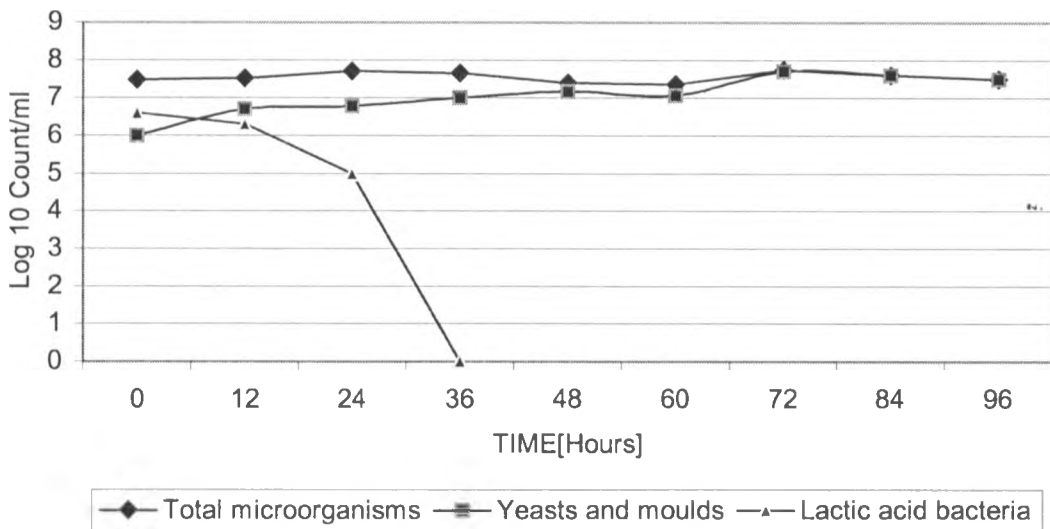
The level of microbial contamination in the brews remained at $10^6 - 10^8$ /ml in the entire duration. After the disappearance of lactic acid bacteria in 36-hours, yeasts dominated but caused limited alcoholic fermentation as illustrated in Table 25.

TABLE 25. CHEMICAL CHANGES IN NATURALLY FERMENTING BREWS DOSED WITH STERILIZED SPONGES

Time [hours]	Total acidity[a] [%]	Volatile acidity [b] [%]	pH	Ethanol [vol.%]	Reducing sugar [%]
0	0.21	0.07	3.7	-	0.1
12	0.40	0.08	3.3	-	0.5
24	0.45	0.12	3.2	0.9	0.6
36	0.54	0.15	3.1	1.0	0.81
48	0.63	0.19	3.0	1.10	1.46
60	0.87	0.21	3.0	1.15	2.4
96	1.00	0.27	2.95	1.40	3.3

a)-as lactic acid , b) -as acetic acid

These control experiments demonstrated that: -

(i)   Yeasts and lactic acid bacteria dominated the natural fermentation of Muratina brews.

(ii)   The bacteria caused lactic acid fermentation, which dominated at the early hours of fermentation. However, by producing acid, the pH approached 3.0 and limited their growth. Rather they were self limiting. Fermentative activity by yeasts further boosted acidity.   These events ensured that brewing units were maintained under very acidic conditions.

(iii)   Although yeast concentrations that were determined in brews ranged from $10^6 - 10^8$ CFU/ml, higher concentrations existed in ideal brewing units to warrant alcoholic fermentation within 1-4 days. Thus, the purpose of colonizing sponges was to boost yeast populations in brewing units. Yeasts that contaminated brews further enhanced the population. Conditioning sponges before their integration in brewing processes predetermined fermentation to be alcoholic and yeast dominated as opposed to the alternative lactic acid type of fermentation that was caused by bacteria.

4.3.3. The Functioning of Muratina Culture in Brewing Processes

Experimental brewing was performed with the objective of investigating how the Muratina starter culture performed upon repeated use in brewing processes. Thus, a complete brewing unit was set up with dry, *re-conditioned* sponges so that experiments were started with *new cultures*. The culture was used in 7 - brewing cycles, each limited to 4 days. Therefore it was retained in brewing units for 28-days.

(A) The First Brewing Cycles

The initial fermentation cycles were initiated with fresh cultures in the form of freshly *re-conditioned* sponges. Contamination in fresh brews was 6.0×10^7 CFU per ml. This was composed of yeasts; 6.0×10^6 and lactic acid bacteria; 1.0×10^7 CFU per ml among others. These changes are shown in Figure 12.
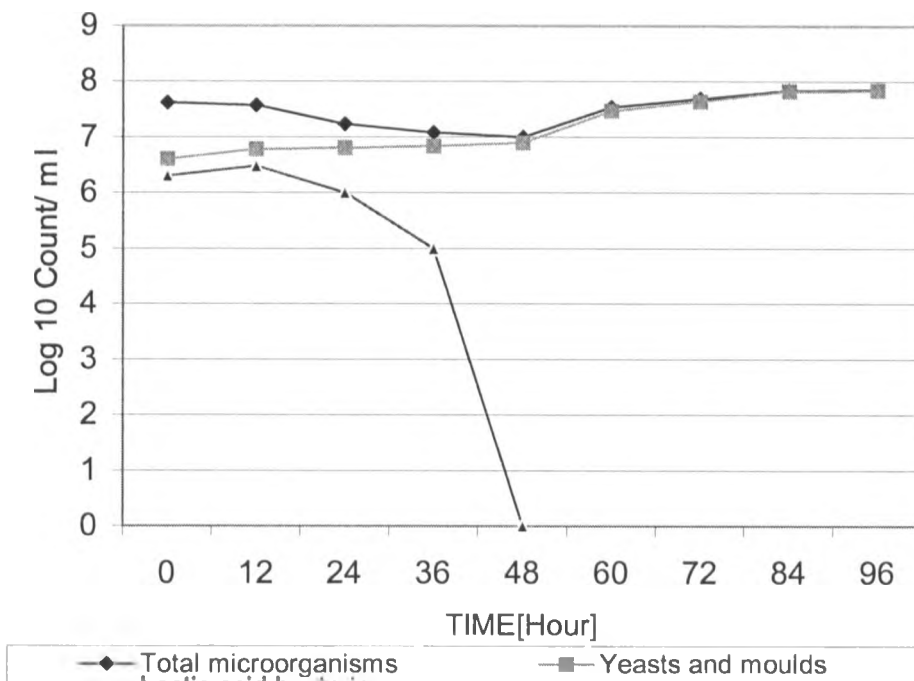

FIGURE 12. CHEMICAL CHANGES DURING THE FIRST FERMENTATION CYCLE IN A TRADITIONAL MURATINA BREWING UNIT.

Lactic acid bacteria dominated the early hours and caused increased acidity. They disappeared in 48-hours and hence the reducing total count at this time. This trend was counteracted by increased yeast growth and their eventual dominance. After 4-days, the brews had accumulated 2.10 % [vol.] ethanol. The attendant chemical changes are illustrated in Table 26.

In the first brewing cycles therefore, the resident yeast populations of freshly re-*conditioned* sponges were increased. Such fermentations were an extension of sponge colonisation. They boosted yeast populations in brewing units. Due to limited yeast activity, these and the natural processes [section 4.3.2] were comparable. They were characterised by increased acidity and minimal alcoholic fermentation.

TABLE 26. CHEMICAL CHANGES DURING THE FIRST FERMENTATION CYCLE IN A TRADITIONAL MURATINA BREWING UNIT.

Time [hours]	Total acidity[a] [%]	Volatile acidity[b] [%]	pH	Ethanol [vol.%]
0	0.07	0.03	3.6	-
6	0.13	0.05	3.5	-
12	0.15	0.05	3.3	-
24	0.44	0.08	3.2	-
36	0.57	0.18	3.15	-
48	0.65	0.19	3.10	0.2
60	0.72	0.19	3.05	1.5
72	0.74	0.25	3.0	1.7
84	0.95	0.25	3.0	1.8
96	1.15	0.34	3.0	2.1

a)-as lactic acid , b)-as acetic acid

b) The Second Brewing Cycles

In these cycles, brews encountered the boosted inoculum. The contaminant yeasts of incoming brews boosted the resident inoculum of brewing units. Thus, yeasts increased from 2.0×10^6 CFU per ml, to numbers in excess of 2.0×10^7 CFU per ml in 24 hours [Figure13].
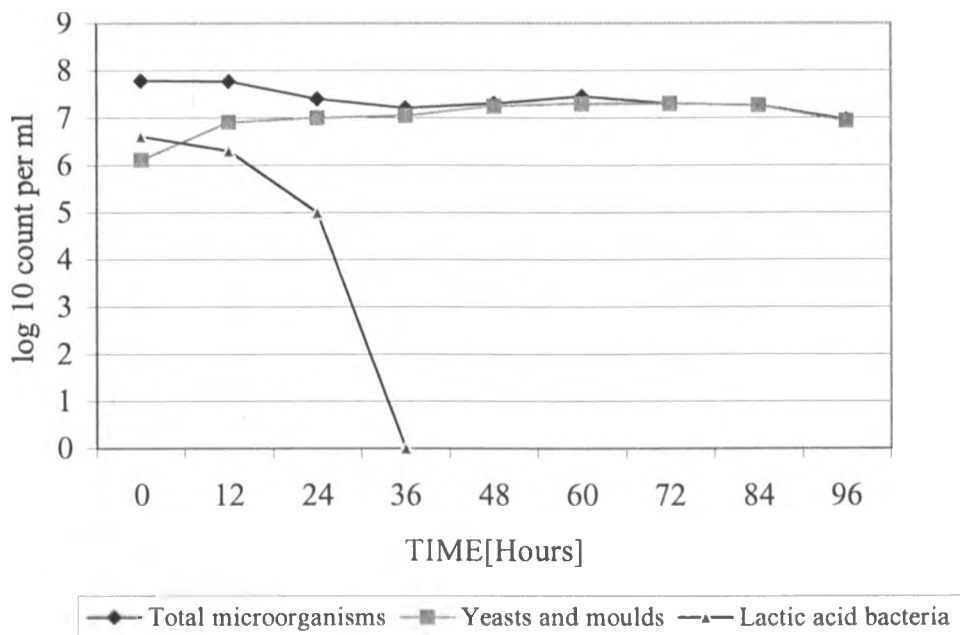
FIGURE 13: MICROBIAL CHANGES DURING THE SECOND FERMENTATION CYCLE IN A TRADITIONAL MURATINA BREWING UNIT.

The enhanced yeast numbers ensured successful processes. After 4-days, the alcohol content of the brews had increased to 5.9 %[vol.], 1.2 % lactic acid, pH 3.0. The attendant changes are shown in Table 27.

TABLE 27. CHEMICAL CHANGES DURING THE SECOND FERMENTATION CYCLE IN A TRADITIONAL MURATINA BREWING UNIT.

Fermentation time [hours]	Total acidity[a] [%]	Volatile acidity[b] [%]	pH	Ethanol [vol.%]
0	0.20	0.06	3.4	0.13
6	0.52	0.08	3.3	0.87
12	0.60	0.12	3.2	1.10
24	0.73	0.16	3.1	2.20
36	0.81	0.23	3.1	2.70
48	0.91	0.27	3.0	3.10
60	1.00	0.34	3.0	4.00
72	1.10	0.36	3.0	5.00
96	1.20	0.39	3.0	5.90

a)-as lactic acid ,b)-as acetic acid

Like in the first cycle, yeasts dominated after the disappearance of lactic acid bacteria. These results confirmed that Muratina brewing units were yeast reservoirs. They operated in acidic environments in the pH range 3.0 - 4.0.

c) The Third Fermentation Cycles

The results of these experiments were similar to the preceding cases in terms of microbiological changes. Initial brew contamination was 1.0×10^6 CFU/ ml yeasts, 8.0×10^6 CFU / ml lactic acid bacteria. Upon their exposure to resident inoculum of brewing units, yeast counts increased from 10^6 to 10^7 CFU /ml. Limited lactic acid fermentation occurred in the first 48-hours when lactic acid bacteria existed. Thereafter, yeasts dominated the processes [Figure 14].

These experiments once again demonstrated the dominant presence of yeasts at the beginning of every process. Irrespective of initial contamination, processes were successful.
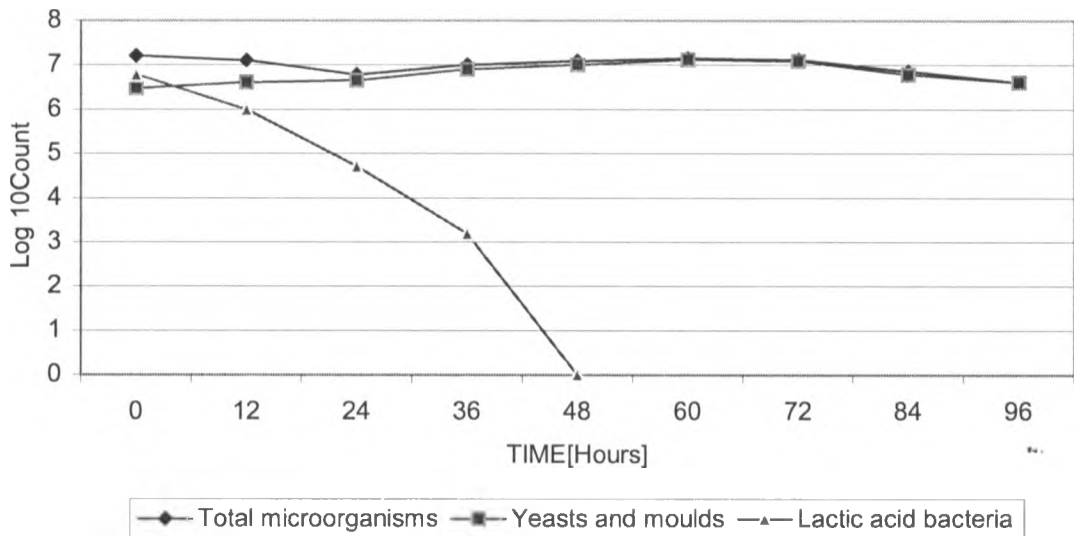

FIGURE 14: MICROBIAL CHANGES DURING THE THIRD FERMENTATION CYCLE IN A TRADITIONAL MURATINA BREWING UNIT.

Similarities of results demonstrated repeatability of Muratina fermentations. The profiles of acid development, alcohol evolution, yeast growth and elimination of bacteria were reproduced in these experiments.

TABLE 28. CHEMICAL CHANGES DURING THE THIRD FERMENTATION CYCLE IN A TRADITIONAL MURATINA BREWING UNIT.

Time [hours]	Total acidity[a] [%]	Volatile acidity[b] [%]	pH	Ethanol [vol.%]
0	0.22	0.08	3.4	1.2
6	0.51	0.11	3.35	1.9
12	0.62	0.12	3.3	2.8
24	0.64	0.14	3.2	3.5
36	0.77	0.16	3.1	4.2
48	0.87	0.19	3.0	5.3
60	0.9	0.20	3.0	6.7
72	0.9	0.21	3.0	7.0
96	1.1	0.24	3.0	8.0

a)-as lactic acid , b)-as acetic acid

At the end of these cycles, the alcohol content of brews had increased to 8.0 %[vol.], lactic acid to 1.10%, acetic acid to 0.24% and pH had settled to 3.0.

d) The Fourth Fermentation Cycles.

In these cycles and unlike others, brews were mixtures of water and crystalline cane sugar. Such mixtures were commonly used to engage brewing units at intervals between wine harvesting and start of new brewing cycles. The mixtures were also brewed when complete formulae could not be constituted due to unavailability of cane juice. In presence of properly colonised sponges, the mixtures were fermented into alcoholic concoctions.

Due to the absence of cane juices, the greatest sources of contamination in Muratina brews, the experimental mixtures contained insignificant contamination (4.2×10^2 CFU per ml). Wholesome brews were highly contaminated i.e 10^6-10^8 CFU/ml. After mixtures were exposed to sponges inside brewing units, their microbial content was boosted to 4.6×10^6 CFU per ml. The lactic acid bacteria were hardly detected [Figure 15].

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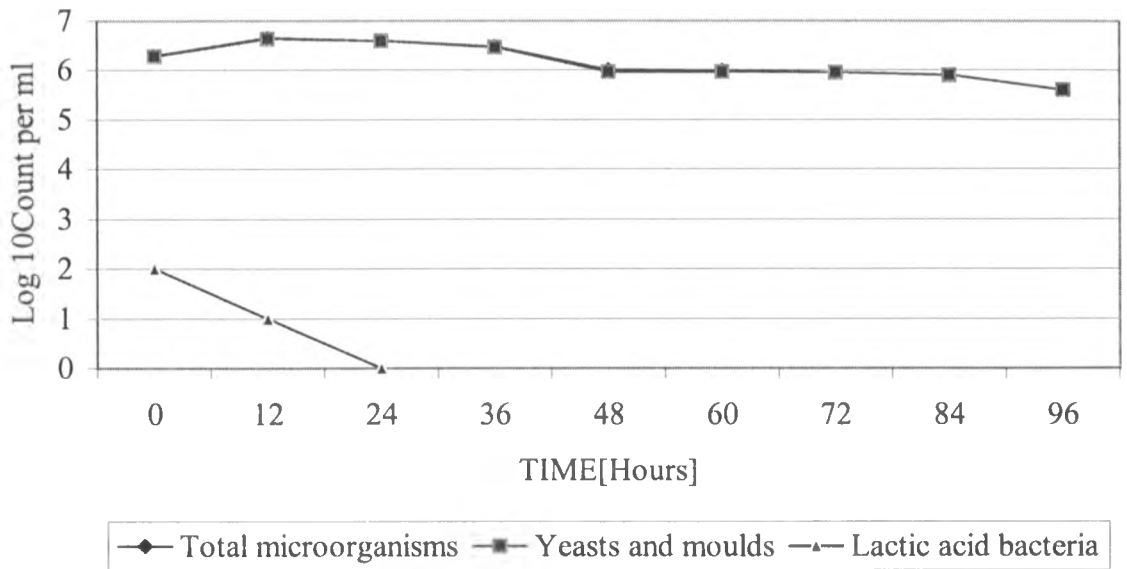
FIGURE 15. MICROBIAL CHANGES DURING THE FERMENTATION OF CANE SUGAR SOLUTION IN A TRADITIONAL MURATINA BREWING UNIT.

The mixtures were fermented into alcoholic concoctions with 8.0%[vol] ethanol content, 0.86 % lactic acid, 0.26% acetic acid, and pH 2.90. The attendant changes are illustrated in Table 29.

TABLE 29. CHEMICAL CHANGES DURING THE FERMENTATION OF CANE SUGAR SOLUTIONS IN A TRADITIONAL MURATINA BREWING UNIT.

Time [hours]	Total acidity[a][%]	Volatile acidity[b] [%]	pH	Ethanol [vol.%]
0	0.20	0.10	3.20	1.2
6	0.21	0.12	3.10	1.8
12	0.41	0.16	3.05	2.4
24	0.51	0.18	3.00	3.2
36	0.61	0.20	3.00	4.5
48	0.69	0.21	3.00	5.2
60	0.71	0.22	2.95	5.7
72	0.77	0.24	2.90	6.0
96	0.86	0.26	2.90	6.8

a)- as lactic acid , b) as acetic acid

It was obvious that consistent fermentation of such mixtures could not be sustained in brewing units for long. This is because the mixtures were poor in nutrient composition except for the sucrose content. They were deficient in growth factors, which were provided by cane juice in ideal Muratina brews. These results once again confirmed that sponges and hence brewing units accumulated yeasts. Their numbers ensured fermentations were successful irrespective of contamination from freshly constituted brews.

The fifth and sixth brewing cycles were carried out in these experimental units. However, the attendant chemical and microbiological changes were not determined because of cost considerations. The brews were analysed after the designated times. They contained between 7 and 8 %[vol.] ethanol, $1.0 - 1.20$ % lactic acid. Their composition was comparable to what obtained in the earlier cycles.

e)   The Seventh Fermentation Cycles

These were the last brewing experiments to be inoculated with the particular lot of the sponges. Initial contamination of fresh brews consisted of 5.0×10^6 CFU per ml of yeasts and 1.0×10^6 CFU per ml, lactic acid bacteria. The bacteria decreased and disappeared in 60-hours, while the yeasts dominated to the end. These changes are illustrated in Figure 16].
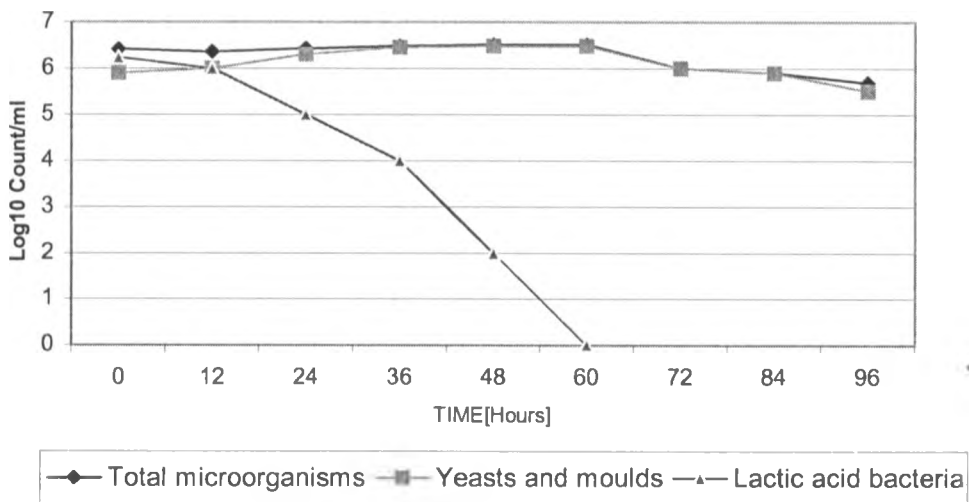

FIGURE 16. MICROBIAL CHANGES DURING THE SEVENTH FERMENTATION CYCLE IN A TRADITIONAL MURATINA BREWING UNIT.

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The fermented brews ethanol content was 8.3%[vol.]. This confirmed successful fermentations. The starter had remained effective in the 28 days of continuous participation in brewing processes. In these last cycles, no significant events were noted with reference to composition of product brews. This was particularly with regard to alcohol and acid contents which compared favourably with the earlier cycles [Table 30].

TABLE 30. CHEMICAL CHANGES DURING THE SEVENTH IN ROW OF FERMENTATION CYCLE IN A TRADITIONAL MURATINA BREWING UNIT.

Time [hours]	Total acidity[a] [%]	Volatile acidity[h] [%]	pH	Ethanol [vol.%]
0	0.30	0.10	3.5	0.27
6	0.46	0.16	3.35	0.17
12	0.56	0.18	3.25	2.50
24	0.59	0.19	3.20	3.50
36	0.67	0.20	3.15	5.10
48	0.82	0.22	3.10	5.80
60	0.99	0.25	3.05	7.10
72	1.04	0.26	3.00	8.00
96	1.2	0.32	3.00	8.30

a) as lactic acid , b) as acetic acid

These brewing experiments revealed the functioning of Muratina brewing units and the conditions under which the starter culture operated.
These conditions were:

(i)   Yeast accumulation in brewing units. Fresh brews encountered concentrated yeast inoculum whose intense metabolic activity determined fermentations to be alcoholic. Consequently, fermentations succeeded irrespective of initial level of brew contamination. This was the reason why Muratina fermentations displayed great repeatability.

(ii)   Range of pH. Cane juices that have undergone variable degrees after extraction and before use made fresh brews acidic. They were the main sources of lactic acid bacteria that caused limited lactic acid fermentation in the juice itself, and at the early stages of Muratina fermentation. The vigorous metabolic activity of yeasts that was warranted by their high concentrations produced significant acidity too. By retaining sponges in the units, they recycled wine residues that enhanced the existing acidity too. Acidic conditions were thus maintained throughout the brewing process. Consequently, brewing units operated within a narrow range of pH thus, between 2.7 and 4.0. Yeasts thrived in this environment and dominated the fermentation processes almost exclusively as pure cultures.

4.3.4. Condition of Sponges After Seven Fermentation Cycles.

After 28-days of continuous brewing, a creamy suspension commonly referred to as *Mairiti* filled the sponge cavities and submerged them. This suspension consisted of flocculent yeast biomass that had been generated over time. It, exuded an odour reminiscent of putrefying organic matter, caused cloudiness and imparted a harsh, bitter taste to the beverage. The wine harvesting methods therefore, ensured that it was only the supernatural brew that was decanted. The material was not disturbed in the process in order to minimize the mentioned effects. The presence of this material in the ready beverage was not desired. Its microbial composition is presented in Table 31.

TABLE 31. MICROBIAL COMPOSITION OF MURATINA SPONGE EXTRACT AFTER SEVEN FERMENTATION CYCLES.

	Viable count/ $g^{f)}$.
Total microorganisms	$2.1 \times 10^{10} \pm 0.5 \times 10^{10}$
Yeasts and moulds	$2.0 \times 10^{10} \pm 0.5 \times 10^{10}$
Lactic acid bacteria	<1
Acetic acid bacteria	<1

f) -on dry matter basis

In prolonged operations of brewing units as simulated in these experiments, a definite microbiological phenomenon was envisaged to provide a scientific justification for changing the sponges and *"reconditioning"* them. The hypothesis was that: upon repetitive use of sponges in fermentation, acetic acid bacteria infected them, and caused death of the yeast culture. The ineffective culture was then liable to opportunistic infections by lactic acid bacteria, owing to accumulation of yeast and yeast autolysis products such as B-vitamins (16, 31). Following this therefore, products from the infected units would become extremely sour due to enhanced acid content. These events then, would have been accompanied by changes in microbial groups that dominated fermentation. This would be the justification.

In real brewing and in experiments, sponges remained submerged during fermentation and consequently, were maintained anaerobically. This eliminated the possibility of their infection by oxidative microorganisms such as the acetic acid bacteria. Furthermore, fermentation enhanced anaerobiosis by gas production. Thus, acetic acid bacteria being oxidative were kept off. However, they could thrive in stuck fermentations or as spoilage agents in matured wine, but not in properly conducted Muratina fermentations. Lactic acid bacteria, which were incidental participants in Muratina fermentation as common contaminants in cane juice, did not feature in sponges after the lengthy brewing experiments. They are the most common contaminants in recycled yeast cultures [15].

The results showed that the culture remained effective during the 4 - weeks of continuous brewing. This duration was reasonable though shorter than 5.6 weeks, the average operational span of Muratina brewing units as was determined in the survey [section 4.10]. So, the microbiological factors that prompted the change of sponges were not elucidated by the limited data. In the short run however, change was prompted by flavour deterioration that emanated from accumulated yeasty matter. When to do so was determined by flavour deterioration beyond threshold levels, which were determined by the brewers themselves. As a suggestion, the culture should be changed after say three to four brewing cycles before the said matter accumulated to taint the brew taste.

From literature sources, microbial cultures cannot be recycled ad infinitum, as they are liable to contamination by opportunistic microorganisms (6). Thus, soon or later, the Muratina culture would have suffered the same fate. Sponges must be changed at a point. The purpose of changing and *reconditioning* sponges was to dispose the used yeast biomass that they immobilised within their cavities. This opened them for fresh re- colonization by new and fresh yeast generations.

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4.4.0. Experimental Brewing with Pure Cultures of Selected Microoganisms

These experiments were designed to assess the possibility of reproducing Muratina fermentations through conventional brewing methods. Sterilizing experimental brews, containers and sponges controlled the levels of microbial contamination.

4.4.1. Brewing with Muratina Yeast

In processes that were carried out in aerated conditions, initial yeast concentrations were 4.0×10^6 CFU per ml. They increased to 1.0×10^8 per ml by day 2, and subsequently stagnated in this range for the remaining duration of experiment [Figure 17]. This level of inoculation compared favourably with what obtained in brewing experiments [section 4.3.3].
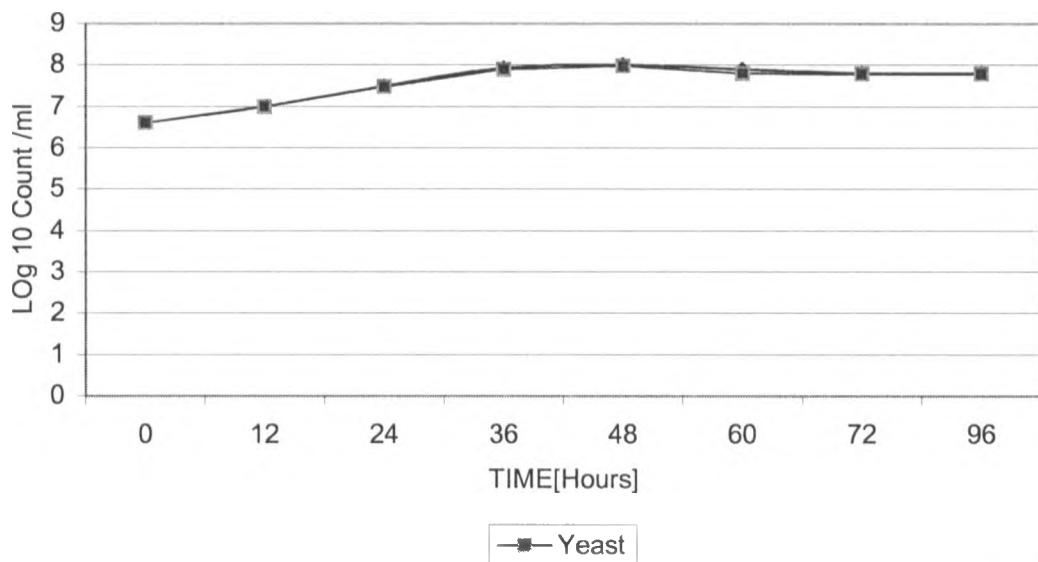
FIGURE 17. GROWTH OF MURATINA YEAST IN AEROBIC CONDITIONS

After 4 days, significant alcoholic fermentation had occurred. This had increased the ethanol content to 4.0 vol.% and decreased dissolved solids from 13.4 to 4.5%. In this time, brew pH had fallen from 5.2 to 3.5 [Table32]. The lactic acid content did not exceed 0.37 % (w/v). This implied that limited lactic acid fermentation was necessary in Muratina fermentations. It boosted brew acidity to concentrations beyond 1.0%. The presence of ethanol in the resultant brews confirmed that the said *Muratina yeast* indeed caused alcoholic fermentation.

TABLE 32.CHEMICAL CHANGES DUE TO MURATINA YEAST GROWTH IN AEROBIC CONDITIONS.

Time [hours]	Total acidity[a] [%]	Volatile acidity[b] [%]	pH	Ethanol [Vol. %]	Dissolved solids [°Brix]
0	0.13	0.07	5.25	-	13.4
6	0.19	0.11	4.05	0.8	11.0
12	0.23	0.12	3.80	1.0	10.4
24	0.25	0.12	3.70	2.4	9.7
48	0.35	0.13	3.65	3.3	8.0
72	0.36	0.14	3.65	3.8	6.6
96	0.37	0.16	3.65	4.0	6.0

a)-as lactic acid b)- as acetic acid

In non-aerated processes, yeasts increased from 4×10^7 CFU per ml which was the initial concentration, to 6.0×10^7 CFU per ml in 36-hours. Thereafter they decreased to 6.0×10^6/CFU ml by day 4. This was due to their flocculation and sedimentation. Yeast growth patterns in these experiments are illustrated in Figure18.
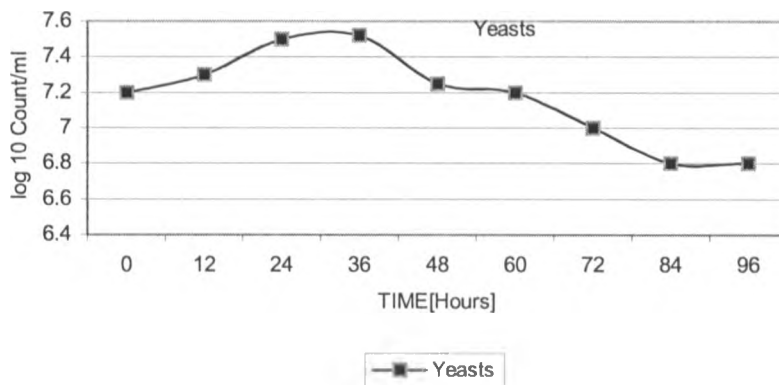

FIGURE 18: GROWTH OF MURATINA YEAST IN ANAEROBIC CONDITIONS

After 4-days, the ethanol content of brews had increased to 4.5 %[vol.], total and volatile acid contents were 0.36 % and 0.15 % respectively. These changes caused the pH to drop from 5.2- 3.6. Analytical results were almost a replica of the aerobic case [Table 33].

Time [Hours]	Total acidity[a] [%]	Volatile acidity[b] [%]	pH	Ethanol [vol.%]	Dissolved solids [°Brix]
0	0.14	0.08	5.2	-	13.4
6	0.19	0.10	4.5	0.1	11.0
12	0.24	0.11	4.0	1.1	10.4
24	0.25	0.12	3.8	2.5	9.4
48	0.30	0.13	3.7	3.5	7.8
72	0.35	0.14	3.6	4.0	6.0
96	0.36	0.15	3.6	4.5	5.0

a) as lactic acid ,b) as acetic acid

These results showed that: -

(i) Muratina yeasts thrived in both aerated and non-aerated situations. These conditions existed at different stages of fermentation. In open vessel fermentations, brews were naturally aerated at the surface before the onset of Carbon dioxide production created a blanket above the liquid surface. This limited natural aeration and created anaerobiosis. Yeast activity regulated the degree of aeration during fermentations [41].

(ii) Yeasts, through their metabolic activity produced acids and enhanced acidity in brewing units.

4.4.2. Brewing with Mixed Cultures of Yeast and Lactic Acid Bacteria.

In these experiments, equal parts of Muratina yeast and lactic acid producing bacteria cultures were used as sources of inoculation. The composite inoculum provided 1.9×10^6 and 7.9×10^7 CFU per ml, of yeast and lactic acid bacteria respectively. Under aerobic conditions, lactic acid bacteria increased steadily in the first 12 days and reached 2.2×10^8 CFU per ml. Thereafter, they decreased with the rising acidity and falling pH. They decreased and disappeared altogether in 72-hours. Lactic acid fermentation dominated in this period. The fermented brew contained 1.5 % lactic acid and 0.46 % acetic acid. This suggested that these bacteria were heterofermentative. Increased acidity promoted sucrose inversion and hence the increased reducing sugar content.

The growing yeast population could not assimilate these sugars as fast as they were produced. Consequently, reducing sugars accumulated. Similar events were observed in naturally fermenting brews [Tables 24 and 25]. At the same time, yeasts gradually increased. Thus, starting with 4.0×10^6 CFU per ml, they reached 1.0×10^8 CFU per ml in 72-hours and dominated thereafter [Figure 19]. This coincided with the start of discernible alcoholic fermentation. After 96- hours, the brews' alcohol content had increased to 2.3 %[vol.].
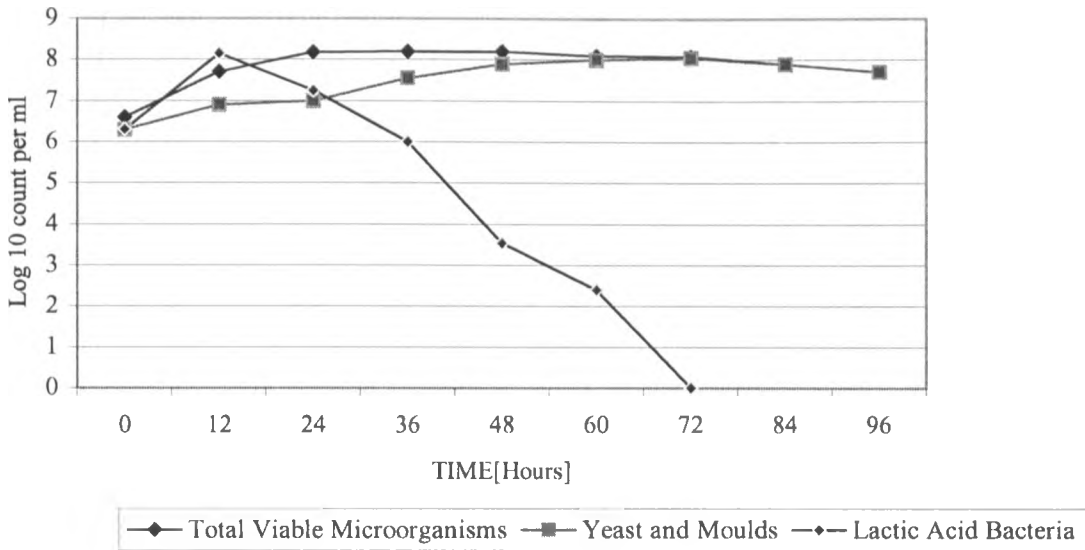
FIGURE 19: GROWTH OF THE MURATINA YEAST AND LACTIC ACID BACTERIA IN AEROBIC CONDITIONS

These results showed that in the absence of high yeast concentrations to cause alcoholic fermentation, aerobic conditions favoured lactic acid bacteria growth. In real Muratina fermentations, bacteria that originated with cane juices were prevalent at the early stages. They caused limited lactic acid fermentation before yeasts dominated and caused alcoholic fermentation. Aerobic conditions that existed at this time appeared to influence bacteria growth and enhanced acid development.

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Time [hours]	Total acidity[a] [%]	Volatile acidity[b][%]	pH	Ethanol [vol.%]	Reducing sugar [%]	Dissolved solids [°Brix]
0	0.16	0.02	4.2	0	0.35	12.5
6	0.22	0.04	3.85	0.2	0.70	12.0
12	0.45	0.11	3.50	0.3	0.90	11.5
24	0.49	0.13	3.40	0.5	1.10	11.2
36	0.63	0.19	3.35	0.9	1.70	10.5
48	0.67	0.21	3.30	1.1	1.90	10.1
60	0.72	0.23	3.25	1.3	1.90	9.8
72	1.03	0.31	3.20	1.9	2.50	9.2
84	1.3	0.38	3.15	2.1	2.40	8.7
96	1.5	0.46	3.10	2.3	2.30	8.4

a) as lactic acid , b) as acetic acid

Under anaerobic conditions, lactic acid bacteria steadily decreased from 2.0×10^6 CFU per ml initially, and disappeared altogether in 24-hours. However, yeasts thrived in these circumstances and dominated the process as illustrated in Figure 20.
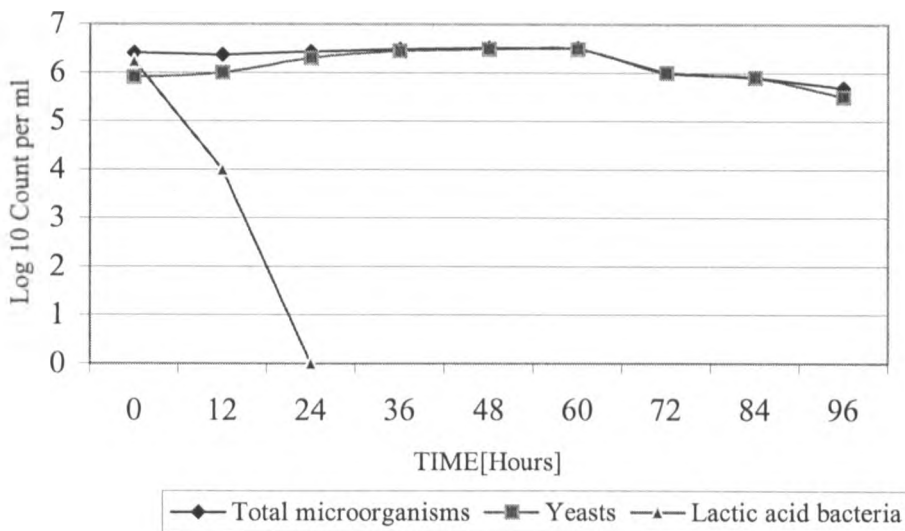

FIGURE 20: GROWTH OF YEASTS AND LACTIC ACID BACTERIA IN ANAEROBIC CONDITIONS.

Alcoholic fermentation prevailed. The fermented brews contained 5.6 % [vol.] ethanol, and 0.42 % lactic acid [Table 35]. Anaerobic conditions were created during periods of carbon dioxide production. In open vessel fermentations, a gas blanket that was created above the liquid surface created anaerobiosis by limiting natural aeration.

TABLE 35. CHEMICAL CHANGES DUE TO GROWTH OF MURATINA YEAST AND LACTIC ACID BACTERIA IN ANAEROBIC CONDITIONS

Time [hours]	Total acidity[a] [%]	Volatile acidity[b] [%]	pH	Ethanol [vol.%]	Reducing sugars [%]	Dissolved solids [°Brix]
0	0.18	0.05	4.05	-	2.50	10.0
6	0.30	0.05	3.80	0.80	2.00	10.0
12	0.32	0.06	3.70	1.20	1.65	9.0
24	0.33	0.06	3.65	2.10	1.70	8.5
36	0.37	0.06	3.60	2.40	1.65	8.0
48	0.37	0.07	3.55	2.60	1.62	7.1
60	0.39	0.07	3.35	3.30	1.60	6.2
72	0.41	0.07	3.32	5.00	1.50	5.6
96	0.42	0.08	3.30	5.60	1.32	4.8

a)-as lactic acid , b)as acetic acid

This was enhanced by saturation of the fermenting material with carbon dioxide that was produced by the fermentation. These conditions were selective against bacteria, enhanced yeast growth and promoted alcoholic fermentation. The prevalence of reducing sugar quantities suggested that the Muratina yeasts were also invertase positive.

These results demonstrated the necessary preconditions for successful Muratina fermentations. These were, but, not limited to:

(i)   The presence of high yeast concentrations in brewing units at the beginning of every process. By their intense metabolic activity, alcoholic fermentation prevailed. Sponges ensured high yeast concentrations were retained in brewing units.

(ii)   The presence of lactic acid bacteria at the early stages of fermentation. These caused limited lactic acid fermentation. They acidified brews and ensured the prevailing pH was below 4.0. Therefore, they limited their own growth. Yeasts thrived in these conditions.

These results confirmed that the possibility of using yeast and bacteria cultures as an improvement of Muratina brewing existed. In order to obtain an acid-alcoholic product while using these cultures, the following was suggested

* ❖ Brewing should be carried out in aerated or open vessels that allowed limited lactic acid bacteria growth. This enabled the production of more than 1.0 % lactic acid in the brews. The real Muratina brews contained more than the mentioned percentage. The acidity made Muratina sour, an important attribute of its quality (9).

* ❖ The composite culture should have high Yeast: Lactic acid bacteria ratio. This was to allow limited lactic acid fermentation by the bacteria before the main yeast dominated phase of fermentation eliminated them. The fermentative activity of yeasts further boosted acidity, both in the wine and brew.

5.0. DISCUSSION

5.1. Roles of Raw Materials in the Brewing Process

(i) Sugar Cane Juice

The raw brew formulations for Muratina manufacture were based upon this basic raw material. It was the source of fermentation substrates sugars, nitrogenous compounds and mineral elements. In formulating brews, the juice was diluted with water and subsequently sweetened by the addition of crystalline cane sugar. Consequently, cane juice and the brews had similar chemical substances, but differed in concentrations due to these additions. Alterations in juice composition were necessary to save on raw material costs and to boost the product yield. The juice, which was used while actively fermenting, was the greatest source of contamination in Muratina brewing. It was important as a source of microorganisms that comprised the Muratina culture. Honey, as a source of sugars and yeasts, played similar roles in the process.

(iii)   Muratina Sponges

The preparation of sponges from matured fruits entailed the removal of degenerated pulp to create a fibrous frame. The fibrous insides of the frame have an extensive capacity of immobilizing yeasts in great numbers. This attribute was the main asset in Muratina manufacturing and formed the basis of their use.

5.2. Basis of Sponge Conditioning

Before sponges were used in brewing processes, they were boiled in several changes of water. This process killed vegetative microorganisms, so that sponges were considered *sterile* after the treatment. Sterilized sponges were then incubated in naturally fermenting sugarcane juice to be colonized by yeasts. The intensity of colonization, which was expressed as yeast concentration per unit of their dried weight, lay between 10^9 - 10^{11} CFU. Since colonization was not selective, sponges acquired bacteria too. These however, were eliminated in subsequent sun drying. The colonizing yeast population was reduced considerably in the process, but still, they dominated in dried sponges. Before their application in brewing processes, sponges were further incubated in brews in order to propagate the surviving yeasts into a concentrated inoculum. This was the Muratina starter culture. The yeast concentration lay between 10^9 and 10^{11}/CFU per gram of dry matter. The conditioning process was meant to colonise sponges with Muratina yeasts.

5.3. Characteristics of Muratina Fermentation

Muratina was fermented in brewing units, which were common vessels that carried an aggregate of sponges, carriers of starter inoculum. Thus, filling raw brews into these units achieved their inoculation. The inoculum, which was carried by the sponges, became resident in brewing units and was routinely boosted by yeasts that contaminated raw brews. Thus, a high concentration of yeasts in the range of 10^6 -10^8 CFU/ml of brew, was attained at the beginning of every process. A vibrant fermentation process that was characterized by exuberant frothing and sustained gas evolution was thus, determined. During fermentation, the attendant yeast counts fluctuated between 10^6 - 10^8 CFU/ml of brew, and caused alcoholic fermentation.

Yeast populations that were immobilised by the sponges influenced fermentation rates. This was the reason why despite enjoying comparable yeast concentrations, the experimental and actual brewing processes differed in fermentation rates. It was the resident yeast population of brewing units that determined fermentation rates. This was related to the duration of brewing cycles. Though the determined counts reflected growth patterns during fermentation, they were not representative of total yeast populations that existed in brewing units. This was so because, yeasts that settled out of the brew as flocculent clusters and got immobilized by sponges during fermentation, were not enumerated. The reported counts therefore represented suspended yeasts while those immobilized were not enumerated.

In conventional brewing, yeast concentrations during fermentation are expressed in terms of *suspended yeast cells* because cells that settle out of the fermenting liquid as sediments are inaccessible for enumeration (3, 27,28). The content of immobilised yeasts thus, determined the course and success of Muratina fermentation. By ensuring that sponges carried sufficient inoculation, rapid fermentation was predetermined. In turn, this created conditions that stimulated yeast activity. The use of sponges as yeast carriers made the Muratina process comparable to other conventional fermentation processes that involved inoculation with yeast preparations.

According to Irvine (12), spontaneous fermentation of stored cane juice is greatly influenced by three factors namely:- Temperature, solid matter content and pH. Since sugar cane juice and Muratina brews were chemically similar, their fermentation processes will be influenced by the same factors. Their manipulation by traditional brewers ensured successful fermentations.

(i) Temperature

Sugar cane juice may undergo either of two types of fermentation depending on storage temperature. Below 20^0C, a lactic acid fermentation that is characterized by increased viscosity prevailed. At storage temperatures of 20^0C-30^0C, a lacto-alcoholic fermentation that is caused by mesophilic yeast and lactic acid bacteria is promoted (24).

In Muratina brewing, brews were made and fermented at ambient temperature, which ordinarily lay between 23°C and 30°C. Temperatures in this range promoted the latter type of fermentation. Yeast populations carried by the sponges further enhanced this. The alcoholic type of fermentation was thus, predetermined.

(ii) pH

Sugar cane juice and generally solutions, which are encountered in the sugar industry, exhibit buffering capacity to a high degree (24). This attribute that is derived from the natural content of nitrogenous matter and organic acids in juice, was diminished by dilution during the formulation of Muratina brews. On average, pH of juice lies between 5.2 and 5.4.This however, falls to lower values due to development of organic acids by natural fermentation. The juice, which is the only ingredient that is acidic and has buffering attributes, constitutes eighteen-percent of the brew volume after its dilution. Because of this, its acid content and buffering attributes were greatly reduced. Consequently, raw Muratina brews were mildly acidic and weakly buffered. Thus upon acid production during fermentation, pH of brew dropped towards 3.0. Thus, from the onset, fermentations occurred in a very acidic environment. Acidity in brewing units was boosted by the residual ferment, which was carried over from preceding brewing cycles via the sponges. These conditions restricted participant microorganisms to lactic acid bacteria and yeasts (30). The bacteria were eliminated with the onset of alcoholic fermentation so that yeasts dominated thereafter almost as pure cultures. It has been suggested that lactic acid bacteria of plant origin do not survive alcoholic fermentation (32).

(iii) Solid Matter Content

In sugar cane juice, the solid matter content consisted of minute shreds of cane flesh, rinds, leaves and particles of adhering soil. These solids were incidental contaminants that promoted natural fermentation in stored juices as sources of microorganisms (24). In Muratina brewing, the juice was filtered to reduce the solid matter content while the residual colloids were reduced by subsequent dilution. Muratina brews were therefore, clarified liquids.

According to literature sources (3,14,27), clarified liquids fermented poorly because they lacked sites upon which, yeasts could attach. In their situation, yeasts do not remain in suspension. They settle out of the liquid after the inoculation stage and do not participate in fermentation. Fermentability of such clarified liquids however, was improved by dispersing inert colloidal matter in them. This provided surfaces for yeast adsorption and hence dispersion in the medium for effective fermentation (14). In Muratina brewing, this handicap was overcome by the integration of Muratina sponges to the brewing process. They were not only carriers of yeasts, but also provided vast surfaces for yeast adsorption. During fermentation, they were buoyed as carbon dioxide escaped towards the liquid surface. In the process, they facilitated yeast dispersion and uniform fermentation. They effectively increased the solid matter content and accelerated fermentation. Their presence predetermined the dominant type of fermentation to be alcoholic.

5.3.1. Yeast Characteristics and Sponge Structure

Colonizing sponges with yeasts that occurred in naturally fermenting cane juice created the Muratina starter. The colonization process was then explained on the basis of studied cultural characteristics of the yeast concerned, and the conduct of the brewing process. Firstly, the Muratina yeast reproduced by multilateral budding, which occurred at different sites of the vegetative cell. In liquid growth media, aggregates of budding cells formed clusters that settled out of the media as sediments. During the sponge conditioning or brewing processes, sedimenting clusters of yeasts settled on sponges, which were normally fastened at the bottom of brewing containers. Consequently, the settling clusters were immobilized in the extensive fibrous capacity of the sponges. Ensuring that the rough sponge insides, their vast surface areas and holding capacity were exposed to the settling cells optimised this.

This was ensured when setting up brewing units. Thus, sponge insides were oriented towards the liquid surface to maximize exposure of immobilization sites to the settling cells. Through this process, sponges were effectively colonized and made yeast habitats. Growth and sedimentation in brewing processes continuously boosted the immobilised yeast population. It is this resident yeast biomass that inoculated Muratina brews.

Since sponges remained submerged during the brewing process, the yeast that caused Muratina fermentation was classified as a *bottom fermenting* strain (17,31). Its sedimentary and fermentative characteristics were exploited in sponge conditioning and setting up of brewing units. Yeast characteristics and the sponge structure therefore, enabled the creation of Muratina starter and formed the basis of Muratina fermentation.

5.3.2. Identity of Muratina Yeast

The Muratina yeast was not identified because this was not an objective. However, it shared several fermentative and cultural characteristics with *Torulopsis stellata* (37), the prevalent yeast in naturally fermenting cane juice, ripening honey and molasses (16,26,34). These characteristics were the fermentative ability, star-shaped colonial morphology, inability to form spores, resistance to desiccation, reproduction by multilateral budding, flocculation and sedimentation tendencies of undetached cell aggregates (37). However, this did not preclude species of other fermentative genera such as *Saccharomyces, Candida, Pichia, and Torula,* which are common in these materials (24,26).

5.3.3. Performance of Muratina Culture in Brewing processes

Brewing processes were started with new cultures that were carried in either *re-conditioned* sponges or freshly *conditioned* ones. At this stage, low yeast populations colonized the sponges due to their reduction by solar desiccation. Their yeast loads were increased in the first brewing cycles. These enhanced colonisation but were rarely successful in terms of alcoholic fermentation (9). Due to enhanced yeast concentrations in the sponges, ensuing brewing processes were accomplished with great success and repeatability. This was the reason why brews with an alcohol content of 8 % [vol.] were obtained in periods as short as 24-hours. Similarly, it was possible to ferment brews of minimal contamination like the crystalline cane sugar solutions, to alcoholic concoctions in the same brewing units.

The sponge yeast biomass was continuously enhanced by growth in every brewing cycle and then retained during the operational span of a brewing unit. Consequently and overtime, the degenerate yeast matter choked the sponge insides and effectively displaced them for occupation by new and viable yeast generations. The yeasty matter altered the product flavour by imparting a harsh and bitter taste. This flavour defect was related to that described as *yeast bite* in lager beers and reputedly caused by delayed separation of matured beer and yeast after the end of primary fermentation (17). Upon detection of these defects, the yeasty matter required to be disposed and sponges washed, sun dried and then subjected to fresh *re- colonisation.*

A minor objective of this study was to determine the biological factors that arose from recycling sponges in fermentations. In carrying out brewing experiments, the hypothesis was that with increasing number of brewing cycles, sponges were infected by bacteria that caused flavour defects in wine. The occurrence of bacteria in overwhelming numbers would be the justification for culture contamination and hence disposal by *re-conditioning* the sponges. From literature sources, lactic acid bacteria are major contaminants in recycled brewing yeast cultures and compressed Active Dry Yeast (ADY) (6,29). These were not detected in the sponges after the seven brewing cycles of experiments. It has been reported (15,30) that, products of yeast autolysis particularly the B-vitamins stimulated bacteria growth in recycled cultures. These were abundant in the brewing sponges. Therefore, it was a matter of time before the anticipated phenomenon had set in.

Certainly, this would also occur in processes that did not enjoy sufficient inoculation, a prerequisite for rapid fermentation. Slow fermentations are liable to bacteria infections (15). In commercial Muratina brewing, profit was the main concern and beverage quality had to be safeguarded. Consequently, sponges were changed when the mentioned flavour defects occurred in order to avoid product rejection. The anticipated biological phenomenon need not have arisen by then.

5.3.4. Purpose of Sponge Re-conditioning

Washing sponges in water followed by their desiccation in the sun, disposed the old culture from their insides. This freed their vast network for *re-colonization.* Washing physically removed the yeast matter and prevented their infection by moulds during subsequent sun drying. The yeasty matter is a suitable culture medium for mould growth (29).

During sun drying, the remaining yeast populations were greatly reduced by desiccation. The survivors managed this stage as vegetative cells because the *Muratina yeast* failed to demonstrate spore-forming abilities when cultured in the laboratory.

5.4. Suggestions for Process Improvement

The pure culture experiments demonstrated that the Muratina brewing process could be improved as has been done for other traditional African beverages. Possible process improvements would include:-

- Using composite cultures of Muratina yeast and selected lactic acid bacteria in place of the sponges and their conditioning processes.

- Controlling microbial contamination in brews by heat processing or any appropriate measure. This would minimize variation in product composition by limiting participants in the fermentation to the cultured microorganisms.

- Adoption of forced agitation systems to ensure yeast redistribution in the milieu during fermentation. This would replace the sponges as tools that performed this role in traditional brewing.

6.0. CONCLUSION

Muratina wine was obtained primarily by alcoholic fermentation of brews made of sugar cane juice, crystalline sugar and water. The brews underwent a mixed fermentation that was caused by yeasts and lactic acid producing bacteria. These were the normal contaminants of sugar cane juice. The process involved limited lactic acid fermentation, which occurred at the early stages of brewing processes and a main alcoholic fermentation that was yeast dominated. The wine was sour and alcoholic as a result.

The manufacture of Muratina revolved around adoption and integration of Muratina sponges in the brewing process. Integration involved their colonization with fermentative yeasts that were extracted from naturally fermenting sugar cane juice or the Muratina brewing process itself. The extensive fibrousnesses of the sponges made them suitable yeast habitats. They immobilized yeasts and concentrated them in great numbers so that when placed in brewing units, they provided sufficient inoculation and caused alcoholic fermentation.

In the brewing process, culture integrity was maintained by conditions, which were created by the fermentative activity. These were the creation of anaerobic conditions by saturation of fermenting material with carbon dioxide, acid production and accompanying reduction of pH, and alcohol production. These conditions limited possible contaminants so that Muratina yeast dominated later stages of fermentation as in pure culture situations. The inoculum that was retained in brewing units within the sponges ensured successful fermentations despite the presence of heavy and frequent contamination by fresh brew formulations.

After several brewing cycles, the yeast biomass, which accumulated in sponge insides, spoiled wine's flavour due to continued autolysis. Washing and sun-drying sponges disposed the old culture and emptied their insides for *re-colonisation* with a new culture. Such treated sponges were then used in new brewing cycles.

The Muratina brewing process exploited the natural fermentation of cane juice as a primary source of yeasts. These were immobilised by sponges in great numbers so that colonised sponges were used as inoculants in Muratina brewing. Therefore, they initiated fermentations of large quantities of Muratina brew. This was the basis of Muratina manufacture.

6.1.0. Suggestions for Further Study

Though it was demonstrated that Muratina could be produced with pure cultures, detailed work should be done to: -

- Determine the most economic recipe
- Fully identify Muratina yeast and the prevalent lactic acid bacteria
- Determine the appropriate ratios of microorganisms in composite mixed cultures and their possible biochemical interactions during Muratina fermentations.

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