BIOCHEMICAL AND MICROBIAL CHANGES DURING

FERMENTATION OF TEA FUNGUS (*KOMBUCHA)*

By

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Dedication

To my father

To my mother

And to the soul and memory of my beloved brother

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ABSTRACT

This research was carried out to study the biochemical and microbial changes that occur during fermentation of tea fungus. Tea fungus, also known as Kombucha, is a fermented beverage resulting from the heavy gelatinous growth of a consortium of microorganisms in tea extract and is a popular drink in many parts of the world.

The study revealed that yeast is dominant over the bacteria at the beginning of fermentation; this microbial balance is changed during fermentation and later adjusted to what it was at the beginning. Microbial investigation indicated that during the fermentation process bacteria dominate and that *Acetobacter xylinum* is the primary organism in Kombucha, associated with some yeast species of the genera *Pichia, Candida and Debaryomyces.*

Proximate analysis revealed that Kombucha is composed of 57.59% fiber and 30.21% other carbohydrates, in addition to proteins, fats, and ash, that together make 12.20% of Kombucha dry weight. The study shows that the pH of tea extract dropped from 6.5 to 3.5 immediately after inoculation and remained around it during fermentation, while both total titratable acids and total volatile acids increased.

Increasing sugar or tea concentration in the sugared tea extract is always associated with an increase in Kombucha dry weight.

Kombucha responded variously when it was incubated at different temperatures, which affected the rate of production of total titratable acids (as lactic acid) and total volatile acids (as acetic acid).

The study also showed that Kombucha can grow to various degrees in different growth media such as cow milk, guava juice, coffee bean extract, karkadeh (roselle) extract, potato starch suspension, gum arabic solution, in addition to tea extract.

Kombucha formation was affected negatively when it was shaken at a velocity of 150 rpm; only few suspended Kombucha-like granules were formed.

Both Kombucha and unfermented tea extract exhibited antibacterial activity against *Klebsiella sp., Salmonella typhi, Staphylococcus aureus* and *Pseudomonas sp.* This ability

seems to be more efficient in Kombucha than in unfermented tea.

ملخص الأطروحة

أجري هذا البحث لدراسة مجمل التغيرات الكيميائية و الميكروبية التي تحدث في فطر الشاي(كمبوشا) أثناء التخمير، انعكاس ذلك على البنية الكيميائية و الميكروبية للكمبوشا. حيث تبين من الدراسة أن قيمة الأس الهيدروجيني للشاي قد انخفضت من 6.5 إلى 3.5 من هذه القيمة خلال أسبوع واستقرت عند التخمير،وأستمر إنتاج الأحماض المعايرة الكلية مثل حمض اللاكتيك في الزيادة، أيضا استمرت نسبة الأحماض المتطايرة الكلية مثل حمض الخليك في الزيادة. أما الميكروبات ففي بداية التخمير كانت السيادة للخمائر على حساب بكتريا حمض الخليك، هذا التوازن يستمر في التغير حتى يعود إلى نفس التوازن بعد انقضاء دورة التخمير. كما أظهر التحليل الميكروبي أن بكتريا *Acetobacter xylinum* هي الميكروب الأساسي المكون للكمبوشا بمساعدة بعض الخمائر *Candida sp, Pichia sp, Debaromyces sp.*

أما الكربوهيدرات فتشكل ما نسبته 57.59% كما وأنه قد تبين من التحليل الكيميائي أن الألياف تشكل (12.20% من تركيب الكمبوشا بينما تتألف النسبة الباقية(30.21% من الدهون والبروتين بالإضافة إلى الرماد.

أتضح أيضا أن الوزن الجاف للكمبوشا يزداد طرديا بزيادة تركيز السكر أو أوراق الشاي في محلول الشاي المحلى، كما أنه يزداد أيضا بزيادة قيمة الأس الهيدروجيني حتى يصل إلى pH 9 كحد أقصى،و مع ذلك فقد أثر التحضين في درجات الحرارة المختلفة على معدلات إنتاج كلا من حمض الخل وحمض اللبن(اللاكتيك).

ومن خلال بعض التجارب أظهرت الكمبوشا مقدرة على التكون والنمو في مستخلصات وأوساط غذائية مختلفة مثل حليب الأبقار، عصير الجوافة ، مستخلص بذور البن، محلول الكركديه،،محلول نشاء البطاطس، محلول الصمغ العربي بالإضافة ألي الشاي، بمستويات نمو مختلفة.

هذا وقد أظهرت الكمبوشا تأثرا واضحا بعملية الهز المتواصل للوسط بسرعة(150 RPM)

حيث لم تنجح عملية تكونها بالشكل المعتاد في وسط غير مستقر بل تكونت على شكل حبيبات كثيرة معلقة في محلول الشاي.

تمت أيضا دراسة الأثر المضاد للبكتيريا المحتوى في الكمبوشا وأظهرت الدراسة أن لكل من الكمبوشا والشاي غير المخمر مقدرة على تثبيط نمو كلا من:

Klebsiella sp, pseudomonas sp, salmonella typhi ,*بالإضافة إلي Staphylococcus aureus*

وهذه المقدرة تظهر في الكمبوشا بدرجة أكبر منها في الشاي غير المخمر .

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CHAPTER ONE

INTRODUCTION

Fermentation is an ancient food technology; nobody knows when it exactly started. Perhaps microbial action started before mankind was created. It is clear that the art of food fermentation is thousands of years old. In many communities today, fermentation of food is not simply a supplier of nutrients to the body but in many ways more nourishment to the soul (Dirar, 1995).

The term “fermented beverage” is always associated with alcoholic drinks but it actually covers a considerable number of non-alcoholic drinks. There are special Sudanese drinks, which are by all means non-alcoholic fermented beverages such as *Hulumur, Abreh, and Hussuwa.*

Kombucha (tea fungus) is a non-alcoholic beverage, which has been mentioned in the local media reports lately, and it had a big publicity as a new Asian miracle remedy which cures from chronic diseases.

In the 1960s, Sudanese women began to deal with Kombucha as it was introduced to them as a useful fermented beverage that, if used by nursing mothers, could protect them from evil eye (Hamad Elneil, 2000).

Recently (about three years ago) Kombucha featured once more in housewife chats as a miracle therapy recommended in a vision by a holy man to an Arabian princess suffering from cancer. And she was allegedly cured.

On the other hand, most of Kombucha consumers in Sudan are: diabetics, hypertensives, barren women, people infected with malaria, depressed individuals and those who suffer from gastritis. Moreover, most of Kombucha consumers in the Sudan

do not like its taste and odor but they take it to benefit from its alleged medicinal effects.

Therefore, the problem of Kombucha biochemical and microbial composition is a very interesting problem to study.

The objectives of the present study are: -

1-To conduct research work on Kombucha so as to establish a base for future studies.

2-To study the chemical composition of Kombucha

3-To investigate the microbiological aspects of Kombucha in the Sudan

4-To study the factors that affect the development of the symbiotic relationship between the microbes involved and their effects on the biochemical products.

5-To see if there is any antibacterial effect in Kombucha.

CHAPTER TWO

LITERATURE REVIEW

Kombucha /Combucha (tea fungus) is a fermented tea beverage that has been consumed worldwide so that it is known by many names: gout jelly fish, Honogo, Indian tea mould, Indian tea fungus, Indian tea mushroom, " it imparts immortality" (Frank, 1994), teeschwamm, Japanese or Indonesian tea fungus, wunderpilz, cajnjj, fungus japonicus, and teekwass (Hesseltine 1965). Abadie (1962) and Stone, (1998) cited another list of names such as miracle fungus, fungus of charity, fungus of a long life, Ma-Gu, Chinese or Japanese fungus, teekwasspilz, Manchurian tea or Kargasok tea.

Kombucha derives its name from a Korean physician, Kombo, who recommended it to the Japanese emperor Inkyo, who suffered from an inflammation of the stomach, as an effective remedy (Frank, 1994).

There are references that show that Kombucha tea fungus was used before two thousand years ago by the Tsin dynasty of the Chinese Empire (Frank, 1994).

2.1.The nature of Kombucha

The Kombucha colony/mat represents a symbiotic relationship between bacteria and yeast (Reiss, 1994). *Acetobacter xylinum* has been shown to be the primary bacterium in the colony (Mayser *et al*., 1995). Hesseltine (1965) reported that Kombucha is the result of the presence of *Acetobacter sp*. (NRRL B-2357) and two yeasts: *Pichia* and *Zygosacchromyces* (NRRL Y-4810 and 4882) together. Recently, Kombucha was defined by Karamadin *et. al.* (2001) as a combination of bacteria and yeasts living together symbiotically in a matrix of mycelium-like threads. Moreover, according to Steinkraus (1983), several types of yeast were isolated from Kombucha: *Saccharomyces sp.*, *Torulopsis famata, Pichia membranaefaciens* and *Candida guilliermondii*. He also stated that Formosan tea fungus contained *Candida obtuse* and *Kloeckera opiculata*.

Tea fungus/ Kombucha, an acetic acid-flavored fermented tea beverage, is widely consumed in various parts of the world. This is due in part to the fact that it can be produced at home and it is reported to be a medicinal element.

Mayser *et. al*. (1995) noted the low rate of contamination with harmful microorganisms and concluded that Kombucha can safely be prepared at home without pathogenic health risk. Moreover, in 1998, Stone found no evidence of pathogen contamination in Kombucha products fermented under sterile conditions.

2.2 Biochemical and microbial changes during fermentation of Kombucha.

Kombucha fermented for a week or more contains a number of acids commonly found in some foods such as vinegar, and smaller quantities of ethyl alcohol. Because the acid could leach harmful quantities of lead and other toxic elements from certain types of containers, some ceramic and painted containers should not be used for storing tea fungus (Stone**,** 1998).

Throughout the fermentation, yeast breaks down sucrose into glucose and fructose (Greenwalt *et.al.,* 1998). The yeasts use glucose to produce ethanol and carbon dioxide. The primary Kombucha bacterium *Acetobacter sp.* initially oxidizes ethanol to acetaldehyde and then to acetic acid (Asai, 1968). The secondary biochemical activity of *Acetobacter sp.* is the oxidation of glucose to gluconic acid. The glucose is also used by acetic acid bacteria to synthesize microbial cellulose (Asai, 1968).

The enzymatic synthesis of cellulose has been performed with various preparations obtained from *Acetobacter acetigenum* and *A. xylinum.* (Krieg and Holt, 1984).

A single cell of *Acetobacter xylinum* may polymerize up to 200, 000 glucose residues per second into β-1,4-glucan chains (Haigler and Benziman, 1982). The advantage of using bacterial system for production of cellulose is that the bacterium grows rapidly under controlled conditions and produces cellulose from a variety of carbon sources including glucose, ethanol, sucrose, and glycerol (Haigler and Benziman, 1982). The biosynthetic pathway for cellulose synthesis is very well understood in *A. xylinum*. The pathway from the substrate glucose to cellulose involves a number of reactions in which glucose is first converted to glucose-6-phosphate by the enzyme glucokinase. In the second step, glucose-6-phosphate is converted to glucose-1-phosphate by the enzyme phosphoglucomutase. In the next step, glucose-1-phosphate is converted to UDP-glucose in the presence of UTP and the enzyme UDPG pyrophosphorylase. The UDP-glucose produced is used as a substrate by the enzyme cellulose synthase. Spires and Brown (1996) stated that cellulose synthase is an enzyme with a diameter between five and 10nm. It is found most often associated with cellulose microfibrils even when cellulose is not being produced indicating an affinity for cellulose as well as glucose. In addition, in *A*. *xylinum*, this enzyme is activated by the cyclic nucleotide, c-di-GMP. Fructose, acetic acid and gluconic acid are primary constituents of the fermented sweetened black tea (Greenwalt *et. al.,* 1998). Also this author noted that the vitamin content of the fermented tea was not sufficiently concentrated to assist human health.

This investigation also revealed that no gluconic acid is present in the sample tested. Moreover, in the year 2000, Petrovska and Tozi reported that high content of group B vitamins, plus vitamin C and minerals make the Kombucha drink an excellent remedy for improving the health of human beings.

Guttapadu (2000) stated that the pH decreased steadily from 5 to 2.5 during fermentation while the weight of the tea fungus and OD of the broth increased

through 4 days of fermentation and remained fairly constant thereafter. The count of acetic acid bacteria and yeasts in the broth increased up to four days of fermentation and decreased afterward in a sample which was prepared using tea broth (0.5%W/V) supplemented with sucrose (10%W/V), using a commercially available starter culture. In addition to the results above, Chen (1997) reported that, after 2 weeks incubation at room temperature, Kombucha was composed of two portions, floating cellulose pellicle layer and the sour liquid broth. Acetic acid bacteria and yeast were isolated from 12 collections of Kombucha. The count of acetic acid bacteria in Kombucha liquid is raining from $1.4x10^5$ to10^3cfu/ml. On the contrary, count of yeast flora ranged from $3.5x10^6$ to $2.1x10^6$ cfu/ml.

Hoffmann (2000) concluded that proteins found in the Kombucha tea must consist of various enzymes secreted by the yeast and bacteria to break down the large molecules of different nutrients in the tea, which cannot enter the cells directly, for example sucrose (white sugar) and caffeine. The growth medium of Kombucha contains according to Frank (1994), black tea, sugar, several types of bacteria, yeast and compounds resulting from the fermentation and other metabolic processes, namely acetic acid, glucuronic acid, vitamins and ethanol. Since the culture continuously forms new layers of a zooglea on the surface of the liquid, a certain zooglea amount of cellulose would also be expected in the liquid. Schlegel (1986) stated that many bacteria can grow on a large variety of nutrients. This requires the capacity to produce the necessary enzymes for utilization of the nutrients, and therefore the possession of the appropriate structural genes.

2.3 Kombucha antimicrobial activity

Many studies on Kombucha investigated its antimicrobial activity against a wide spectrum of organisms. Among 264 references from 1852 to 1961, there are reports of antibiotic activity against *Agrobacterium tumefaciens* and medical value against a variety of diseases (Steinkraus *et al*., 1996). In 1965 Hesseltine reported the antimicrobial activity of neutralized Kombucha broth against *Agrobacterium tumefaciens*. Also Kombucha had an antimicrobial activity against *Bacillus cereus, Salmonella choleraesuis* serotype *typhimurium, Staphylococcus aureus and Escherichia coli (*Greenwalt *et al*., 1998). *Shigella sonnei, Aeromonas hydrophila, Yersinia enterocolitica, Pseudomonas aeruginosa, Enterobacter cloacae, Staphlyococcus epidermidis, Camplylobacter jejuni, Salmonella enteritidis, Helicobacter pylori and Listeria* are also affected (Guttapadu*, 2000*). Kombucha proved to exert antimicrobial activities against *E. coli, S. sonni, S. typhimurium, S. enteritidis*, and *Campylobacter jejuni* even at neutralized pH and after thermal denaturation (Guttapadu, *2000*).

On the other hand, Steinkraus *et al. (*1996) reported that there was no antibiotic activity observed when Kombucha was tested against *Escherichia coli, Helicobacter pylori, Staphlyococcus aureus and Agrobacterium tumefaciens*. The acidity of the product, at around 33g/l total acid, is relatively high which limits the ability of many other organisms to grow (Greenwalt *et al*, 1998). Although tea fungus at its normal acidic pH had antibacterial activity against *Staphylococcus sp.* and *Streptococcus sp.* at non-diluted concentration, 2- fold dilution tea fungus and 3-fold dilution it had no antibacterial effect when it was neutralized to pH 7 (Karamadin *et al*, 2001). Both acetic acid and lactic acid inhibit *Salmonella typhimurium*. The bactericidal effect on *Salmonella typhimurium* of these two acids together was stronger than each acid alone. Also fermentation reduced *Salmonella* in pig feed (Van Winsen *et al*., 1999). Toda *et*

al. (1989*)* demonstrated that unfermented tea (20%dry tea) might inhibit the growth of *Staphlyococcus aureus*, *Staphylococcus epidermidis*, *Salmonella typhi*, *Salmonella typhimurium*, *Shigella dysenteriae*, and *Vibrio spp*. At a concentration of 200g/l tea, Diker and Hascelik (1994) showed that extracts of black and green tea had antibacterial activity against *Campylobacter jejuni, Campylobacter coli* and *Helicobacter pylori.* Yokihiko and Watanabe (1989) found that *Clostridium botulinum* spores were killed when inoculated into tea drinks. This investigation demonstrated that the inhibitory effects observed could have been due to the catechin content in the tea. Later, it was determined that most of the bactericidal activity of tea itself may be attributed to the polyphenols, specifically catechin (Ahn *et al*., 1991;Das, 1962; Kawanamura and Takeo, 1989**).**

Moreover, *Acetobacter xylinum* is one of the few procaryotes that synthesize large amounts of the polysaccharide cellulose. This substance is formed as a result of growth at the expense of glucose and certain other sugars, and is deposited outside the cells in the form of a loose, fibrillar mesh. In liquid culture this organism forms a tough, cellulosic pellicle, which can attain a thickness of several centimeters (Stanier *et al,* 1987).

Acetobacter xylinum is a characteristic species of Kombucha (Mayser *et al*., 1995). Steinkraus *et al*. (1977) stated that *A. xylinum* was the principal organism in Kombucha and Leisinger *et al.,* (1966) found that less cellulose was formed by *A. xylinum* in shaken cultures than in static liquid cultures. The authors observed mutations of cellulose synthesizing to cellulose-free cells. In static cultures cellulose-forming cells were favored whereas shaken cultures favored cellulose-free mutant cells.

Rainbow and Rose (1969), describing work with *Acetobacter acetigenum* and *A. xylinum*, stated that although agitation increased the rate of cellulose production, as compared with that in static cultures, the total yields realized from invert sugar were less in the shaken cultures.

However, relatively slow rotation velocity (4 rpm) and large surface area enabling effective cell attachment are optimal parameters for cellulose production. Physical properties of bacterial cellulose samples synthesized either in stationary cultures or in shaken cultures revealed that cellulose from stationary cultures demonstrated a much higher value of Young's modulus (Kombucha), but a much lower value of water-holding capacity (Krystynowicz *et al*., 2002).

CHAPTER THREE

MATERIALS AND METHODS

Raw materials

3.1.1 Tea leaves

To prepare Kombucha, only one trademark, locally known as Abu Ghazaltain, was secured from the local market and used.

3.1.2 Sugar

The type of sugar used was white refined sugar, available in the local market and originally produced by Kenana factory.

3.1.3 Kombucha starter

Kombucha, which is a pancake-like zoogloea, is used as starter of the tea fungus. Among eleven collections of Kombucha, only one was used as the Kombucha starter in this study. The selected sample was brought from Halfa El-Gadeeda, in the eastern Sudan. The sample had a coherent gel matrix, and no fungal growth appeared on the Kombucha mat, while fungal growth appeared, when other samples were used.

3.2. Preparation of tea extract

100g sugar were boiled in a liter of distilled water and then 0.4% tea leaves were added; after three minutes the tea leaves were removed.

3.3 Preparation of Kombucha

Kombucha`s zoogleal mat usually grows in layers. Young layers are formed on the top and older layers remain at the bottom of the mat. The layers can easily be peeled from one another.

In starting a new Kombucha growth in this research a layer of Kombucha was fully immersed in the sugar-sweetened tea extract of 10% sugar and 0.4% tea leaves in a liter of distilled water. The solution was covered with cheesecloth, and incubated at room temperature (25°C-28°C) for a period of 7 days when it duplicated itself.

3.4 Microbiological methods

3.4.1 Sterilization of glassware

Before sterilization, glassware was washed thoroughly and left to dry, then they were sterilized at 160°C for three hours using air oven (Harrigan and McCance, 1976).

Dipping in alcohol and flaming was used to sterilize other instruments such as glass rod spreaders, forks and scissors. A combination of alcohol and UV light for fifteen minutes was used in case of plastic containers, such as blender flask.

3.4.2Sterilization of media

All media that were used in this study were autoclaved at 121°C for 15 min except when sugar-sweetened solution was used which was autoclaved at 110°C for 10 mints.

3.4.3 Viable count of bacteria

Oxoid Plate Count Agar was used for enumeration of bacteria, using the pour-plate technique as described by Harrigan and McCance (1976). Ten grams of homogeneous Kombucha layer and broth were added to ninety milliliters of peptone water to give 1/10 dilution. One ml from suitable dilutions was transferred aseptically into sterile Petri

dishes. To each dilution 10-15ml of melted and cooled (42°C) plate count agar were added. The inoculum was mixed with the medium and allowed to solidify. The plates were incubated at 37°C for 48 hours. The result was reported as the viable bacterial count Per 1 gm of sample.

3.4.4 Yeast and mold count

Standard spread-plate count technique, which was described by Harrigan and McCance (1976) was used and repeated for each sample with different growth media. From suitable dilutions of sample, 0.1 ml was aseptically transferred onto solidified Difco Malt Extract Agar, Wort Agar and Oxoid Potato Dextrose Agar each containing 0.1g of cholraphenicol per liter to inhibit bacterial growth. The sample was distributed all over the surface of plate using sterile bent glass rod distributor. The plates were incubated at 28°C for 72hours.The results were presented as CFU/g.

3.4.5 Tests for the tentative identification of bacteria.

3.4.5.1.Gram stain

A discrete colony was picked carefully with sterile wire loop. The colony was emulsified in a drop of sterile saline, placed on a clean slide and spread evenly to make a thin film. The slide was allowed to dry. The smear was fixed by using a flame. Then the smear was stained as described by Harrigan and McCance (1976).

3.4.5.2 Oxidase test

A piece of filter paper was impregnated with tetramethyl-p-phenylene diamine dihydrochloride (oxidase test solution). Then a loopful from a 24-hour culture was streaked onto the filter paper. A positive reaction was indicated by purple color after 10-15 second.

3.4.5.3 Catalase test

One drop of 3% hydrogen peroxide solution was placed on a clean slide. A loopful from a 24-hour culture was added. The release of bubbles of oxygen indicated the presence of catalase.

3.4.5.4 Motility test

The organism to be tested was grown for 24 hours at 37°C in a liquid medium containing (g/l): 10 yeast extract, 30 $CaCO_3$ and 20 ml ethanol (Frateur, 1950) and pH adjusted to 6.7. A drop of the culture was transferred to a cavity slide and the motility was examined using a light microscope.

Identification tests of bacteria were repeated three times for each microorganism.

3.4.6 Isolation of Acetic Acid bacteria

Frateur`s ethanol medium (Frateur, 1950) containing (g/l): 10 yeast extract, 30 $CaCO_3$,20g agar and20 ml ethanol and pH adjusted to 6.7. was used in isolating acetic acid bacteria. Initial attempts were not successful as streaking the broth of Kombucha or placing part of the solid mat of growth on plates of their medium gave no bacteria to isolate.

It was only when a 1:1(w/w) mixture of the liquid part and the solid part of Kombucha were blended together under aseptic condition and plating on the above media that the bacteria were easily isolated. The medium was supplemented with 1% nystatin to suppress yeast growth.

3.4.7 Purification of Yeast

Different yeast colonies were selected from the plates used for counting yeast. The colonies were selected on the basis of the differences in shape, color and size. The colonies were purified by the streaking method on plates containing solidified Potato Dextrose Agar, Malt Extract Agar and Wort Agar. The process was repeated until the

pure yeast cultures were obtained. The pure cultures were then transferred to slants of the same medium. Then they were kept in the refrigerator.

3.4.8 Tentative Identification of yeast

Selected yeast isolates were identified according to the methods described by Kreger van-Rij (1984), Barnett *et al*. (1983) and Lodder (1970) as described below.

3.4.8.1 Microscopic appearance of non-filamentous vegetative cells

Yeasts from young growing cultures were inoculated into 10 ml sterile liquid culture medium composed of 20 g glucose, 5 g yeast extract, 10 g peptone and 1000 ml distilled water, and the culture was examined microscopically after incubation at 28°C for 72 hours. The shapes of the yeast cells and the mode of budding were observed and registered.

3.4.8.2 Spore Formation

According to Kreger van-Rij (1984), the following media were used:

1 - Yeast extract - malt extract-glucose peptone agar.

2- acetate agar containing (g/l) 9.8 potassium acetate, 1.0 glucose,1.2 sodium chloride, 0.7 magnesium sulphate, 2.5 yeast extract and 20 agar.

3 - Potato dextrose agar (PDA).

Each of the isolates was inoculated in each of the above media and incubated at 28°C for one to four weeks, and then they were examined microscopically for ascospore formation.

3.4.8.3 Utilization of Carbohydrates Anaerobically

Small test tubes containing 10 ml of sterile medium composed of 0.5% peptone water, 4% glucose (or other test sugar) were inoculated with the isolate. A Vaspar

layer, 2 cm deep, was added on top of the medium. The culture was incubated at 28°C for four to five days. Fermentation was detected by the lifting of the Vaspar layer.

3.4.8.4 Utilization of Carbohydrates Aerobically

Nitrogen Base Agar medium composed of 5 gm ammonium sulphate, 1g potassium hydrogen sulphate, 0.5 g magnesium sulphate, 20 g agar and 1000 ml distilled water, was inoculated at 45°C with yeast sample and poured into Petri dishes and was allowed to solidify. Five ml of each test sugar (4%) were placed on the agar surface and incubated at 28°C, with the growth being observed every two days for about a week.

3.4.8.5 Utilization of Nitrogen Compounds for Aerobic Growth

The method is similar to the method described above except for using Carbon Base Agar (CBA) and nitrogen sources instead of carbon sources. The CBA consisted of (per 1000 ml distilled water), 5 g magnesium sulphate, 1g potassium hydrogen phosphate, 0.5g magnesium sulphate, 20 g glucose and 20 g agar.

3.4.8.6 Growth on High Concentration of D-Glucose

Fifty and sixty grams of D-glucose were dissolved each in 100 ml of 1% yeast extract solution to finally give 33.3% and 37.5% glucose solution, respectively. 1.5g agar were added and the medium was dispensed into plugged tubes, then autoclaved for 10 min at 100°C and then slanted. The slopes were inoculated lightly with each selected yeast isolate and then incubated at 28°C for four weeks and growth recorded as a positive test.

3.4.8.7 Starch Hydrolysis

Yeast isolates were grown on plates containing PDA medium. The plates were inoculated at the center and then they were incubated at 37°C. after 3-7 days of

incubation the plates were immersed in Lugol`s iodine solution. A clear zone around the area in which the microorganisms grow indicates hydrolysis of starch.

3.4.7.8.Formation of Extra-Cellular Starch- Like Compounds

Selected yeast isolates were grown in yeast extract medium composed of 20g glucose, 5gyeast extract, 10 g peptone and 1000 ml distilled water.

Fifty ml of medium were dispensed into cotton-plugged flasks and after inoculation, they were incubated at 28°Cfor 72 hours, and a few drops of dilute Lugol`s iodine solution were added .The occurrence of a blue or green color indicated that the test was positive (Van der Walt and Yarrow, 1984).

3.5 Chemical Analysis of Kombucha

3.5.1 pH

pH was measured using glass electrode, Model AMEL pH meter.

3.5.2 Moisture Content

The moisture content of each sample was determined using the method cited in AOAC (1985). Five gm of Kombucha mat was introduced in air oven at 105°C over-night. The moisture content was calculated as follows:

$$\frac{(\text{Sample wt} - \text{dry wt}) \times 100}{\text{Sample wt}}$$

3.5.3 Ash Content

The total ash content was determined by the AOAC (1985) method. Constant weight was obtained after igniting the sample in an electric muffle furnace at 600°C for 2hrs.

3. 5. 4 Crude Fiber

The crude fiber percentage was determined according to AOAC (1985). Dehydrated Kombucha mat was used. The results were calculated on dry weight basis.

3.5.5 Total Protein

The total protein content in Kombucha mat was determined using Kjeldahl method. One gram of dehydrated Kombucha mat was digested with conc. H_2SO_4. The digest was diluted by adding 100 ml distilled water and then titrated against 2% boric acid, using methyle red indicator. Total protein was expressed as total nitrogen multiplied by the factor 6.25 (AOAC, 1985).

3.5.6 The fats

3.5. 7 The Carbohydrates

The carbohydrates were determined by difference as follows:

100- (protein + ash +fiber +fat) = carbohydrates (AOAC, 1985).

The proximate chemical composition was determined in Kombucha mat, which was one month old, and another, which was one year old (no sugar or nutrients beyond the initial were added during the fermentation time; only distilled water was added).

3.5.8Total Titratable Acidity

The total titratable acidity was determined according to AOAC (1975) .One gram of Kombucha broth was added to 100ml of distilled water and the solution titrated with

0.1N sodium hydroxide to phenol-phthalein end point. The titratable acidity was expressed as percentage of lactic acid; =

$$\frac{\text{ml (.1n)NaoH x.006 x100}}{\text{Sample wt}}$$

3.5.9 Total Volatile Acid

The total volatile acids were determined according to the method adopted by AOAC (1975). Ten grams of Kombucha broth were transferred to microkjeldahl steam distillation unit and the first 200ml of distillate titrated against 0.1N sodium hydroxide to phenol-phthalein end point; the volatile acids were expressed as percentage of acetic acid; =

$$\frac{\text{ml (.1n)NaoH x.006 x100}}{\text{Sample wt}}$$

3.5.10 Determination of Alcohol

Alcohol was measured using *Veritable Ebulliometre, DUJARDIN- Salleron (Paris)* method. Fifty ml of distilled water were boiled and the water boiling point was used to adjust the Ebulliometre chart; at this point the ethanol concentration is zero. Then 50ml of sample were boiled, and the boiling point was recorded. Concentration of alcohol was read from the adjusted chart.

3.6 Formation of Kombucha using pure microbial isolates

Pure acetic acid bacterial isolates were inoculated into sugar-sweetened tea extract and the formation of Kombucha was registered after a week. Also pure yeasts were inoculated in a similar extract and observed for Kombucha formation. In addition,

both bacteria and yeast isolates were inoculated together in the tea extract and Kombucha formation is studied.

3.7 Factors affecting Kombucha formation and growth

3.7.1 Effect of shaking

Sugar-sweetened tea extract containing 10% sugar and 0.1%tea leaves was inoculated with 10% Kombucha broth in a conical flask and it was shaken at a velocity of 150 RPM and kept incubated for 4 days at room temperature (range, 25°C-28°C). After that period, the formation of Kombucha was checked.

3.7.2 Effect of Blending

Equal amounts of Kombucha mat and broth (from ten days-old culture) were blended for five minutes using domestic blender (*Moulinex*), which had un-specified one speed and incubated at room temperature (range 25°C-28°C). For 5 months Kombucha formation was examined.

3.7.3 Effect of Anaerobic Condition on Kombucha Growth.

A flask that contained ten days-old Kombucha was placed in a large sealed jar containing a lighted candle, to consume oxygen. The candle stopped burning when the atmosphere in the jar had a low concentration of oxygen. After 7days incubation at room temperature (range 25°C-28°C) alcohol was measured and Kombucha was examined.

3.7.4 Temperature Effect

Eight Flasks containing tea extract with 10% sugar and 0.1%tea leaves were inoculated with 10% Kombucha starter, they were then incubated at different

temperatures as follows: 25°C, 30°C, 37°C and 40°C. Total volatile acids, total titratable acids and pH were measured every 24 hours for five days.

3.7.5 Initial pH

Kombucha was grown in conical flasks containing 250 ml of sugar-sweetened tea extract with initial pH adjusted to the following values: 1.2, 2.5, 7 and 9. Then the cultures were incubated for a week at 30°C. Kombucha dry weight and final pH were registered at the end of the incubation period.

3.7.6 Effect of Inoculum Percentage

To study the effect of inoculum percentage on both Kombucha thickness and dry weight, the Kombucha was grown, as usual, in flasks of 500 ml capacity. Each of the flasks contained 250ml of sugar-sweetened tea extract. However, they were inoculated with Kombucha broth (7 days old) as starter using various levels of concentrations as follows: 3%, 7% 11.25%, and 16%. After 7days incubation at 28°C Kombucha thickness and weight of dry matter were examined and registered.

3.7.7 Concentration of sugar

To study the effect of sugar concentration on Kombucha, it was grown, as usual, in flasks of 500 ml capacity; each of the flasks contained 250ml of tea extract. However, the number of treatments was four and the concentration of sugar varied in them as follows: 1.25%, 2.5%, 5% and 10%. After 7days incubation at 28°C, formation of Kombucha was examined and its dry weight was determined.

3.7.8 Concentration of tea

Sugared tea extracts were prepared using different concentrations of tea leaves, namely, 0 .38%, 0 .75%, 1.5% and 3%. The sugared tea extracts were grown, as usual,

in flasks of 500ml capacity, containing 250ml, after incubation for 7 days, at 28°C, formation of Kombucha was examined and its dry matter determined.

3.7.9 Effect of different natural materials as growth media for Kombucha

Fresh cow milk, guava, gum arabic, cane sugar and potato starch, each was used in a concentration of 10% dissolved or suspended in distilled water. Also solutions containing 10% each of coffee, karkadeh, grapefruit, or black tea were sugared using 10% white sugar. Ten percent Kombucha starter was inoculated in all solutions named above. After incubation for a week at 30°C, formation of Kombucha and production of alcohol were examined.

3.8 Biochemical and microbial changes during fermentation of Kombucha

Sugared tea extract containing 0.1% tea leaves and 10% sugar and inoculated with 10% Kombucha starter, was incubated for 7days at room temperature. The pH, total volatile acids, total titratable acids and alcohol were all measured every day. Also yeast and bacteria were counted daily.

3.9 Kombucha antibacterial activity

The antibacterial activity of Kombucha was tested against four genera of bacteria: *Klebsiella*, *Salmonella*, *Pseudomonas* and *Staphylococcus*, which were kindly obtained from The Faculty of Veterinary Medicine, University of Khartoum, and the antibacterial activity of Kombucha was examined using absorbent disc method and four different solutions, namely:

1-Tea extract

2-Tea extract in addition to 33g/l lactic acid and 7g/l acetic acid

3- 7g/l acetic acid and 33g/l lactic acid dissolved in distilled water

4- Kombucha extract

A disc of filter paper (diameter 4 mm) was fully immersed in each test solution. All bacterial species were cultivated on Oxoid Nutrient Agar plates for 48 hours at37°C. Sterile cotton applicator swabs were used to inoculate the surface of the agar with the test organisms rather than adding a constant volume of culture because the swab method yielded a uniform mat of growth more consistently. A 2.5 cm disc was used as the antimicrobial disc, of inhibition (clearing). The 2.5 cm disc was saturated with Kombucha extract or other test solution and placed on the freshly inoculated agar with sterile forceps. All plates were incubated at 37°C for 24 hours. The anti-microbial activity of each test solution was estimated by measuring the zone of inhibition around the disc.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Morphology of Kombucha

Under the microscope the body of Kombucha consists of distinct structural subunits. The subunits consist of a gelatinous material produced by acetic acid bacteria, which are concentrated inside the subunits, and yeast which is concentrated on the outside (Fig.1). The most important of these organisms is *Acetobacter xylinum.* As Kombucha grows, a coherent gel matrix is formed on the surface of the tea extract (Plates 1and 2). This is the result of accumulation of both *Acetobacter spp.* and Kombucha yeast metabolites, especially cellulose,. To use Kombucha as a new starter or to use it for specific tests, a sharp pair of scissors can be used to cut the coherent gel matrix (Plate 3). The gel matrix consists of layers, which can be peeled apart easily (Plate 4). The association of easy peeling with quick formation (7-10days) in Kombucha layers perhaps helped conserve Kombucha since ancient time.

4.2 Kombucha`s microorganisms

4.2.1The Bacteria

At first, some difficulties were encountered in isolating bacteria from Kombucha. The difficulty arose from the excessive sliminess that hampered the isolation of bacteria. But it was possible to isolate the organisms by blending the sample in order to break down the cellulose mat in which the bacteria were embedded. According to Table 1, a number of the isolated bacteria belonged to the genus

Bacillus. These are gram positive, endospore-forming, motile cells that are single or in long chains.

Figure 1: Kombucha structural unit under the light microscope

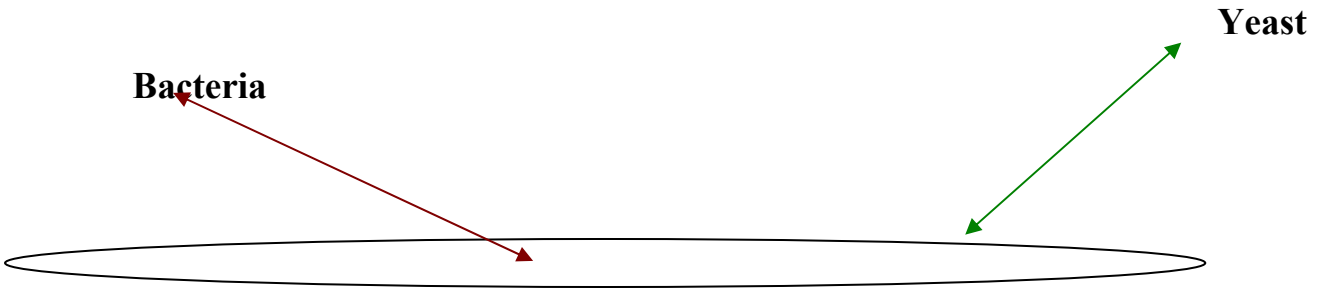


Plate 1

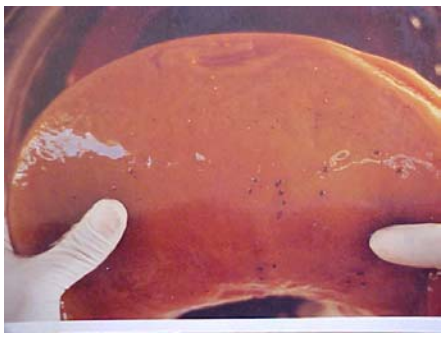


Plate 2

1:A coherent gel matrix is formed on the surface of the tea extract.

2:Kombucha mat from tea.

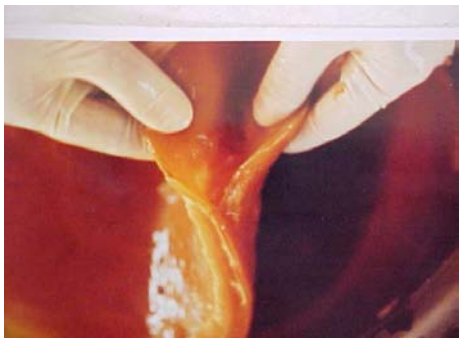


Plate 3 **Plate 4**

3: A coherent Kombucha gel matrix being cut with sharp scissors.

4: Kombucha layers being peeled apart.

But most of the bacteria were identified as *Acetobacter sp* that is a gram-negative, non-spore-forming, catalase positive, oxidase negative, motile or non-motile, single rods.

The presence of *Bacillus* in Kombucha indicates that perhaps bacteria other than *Acetobacter sp* may play an important role in Kombucha. Although it has not been reported as an essential organism in Kombucha*, Bacillus* is expected to be commonly found in dust and dust storms in Sudan and its presence in Kombucha in this country may be due to contamination with dust. We do not know the exact role played by *Bacillus* in Kombucha in Sudan. Perhaps later research will reveal this.

Acetobacter xylinum is the most important organism in Kombucha and it is responsible for the production of the major products of Kombucha (acetic acid and cellulose) (Akerstrand, 1996). The *Acetobacter sp*. isolated in our research is assumed to be *A. xylinum* in the light of the facts that it was isolated from Kombucha and that it produces a zoogleal mat.

Acetobacter xylinum has been isolated previously in Sudan from *hussuwa*, a fermented food product (EL Nour *et .al*, 1999). Appearance of *A. xylinum* in Kombucha culture depends on the production of alcohol by yeasts; that is the basis of symbiotic relationship between microorganisms in Kombucha. The bacterium oxidizes the alcohol to acetic acid in the presence of air.

4.2.2.The Yeast

According to Tables 2 and 3, most of the isolated yeasts from Kombucha were *Debaryomyces spp,* but there were also*, Candida sp* and *Pichia sp*. All of the

yeasts show ability to hydrolyze starch. These results mean that *Zygosacchromyces rouxii* and *Saccharomyces cerevisiae* reported in the literature *(*Siever*, et al.,* 1996; Blanc, 1996; Greenwalt et al, 2000 and Kurtzman *et al,* 2001*)* were not involved in Kombucha production in our case. Moreover, The relatively high concentrations of acetic acid (1.5g/l) and lactic acid (32g/l), possibly inhibit the growth of *S. cerevisiae* (Kolothumannil *et al*., 2001). Many researchers mentioned the association of *Candida sp* and *Pichia sp* with *Acetobacter xylinum* in Kombucha (Andre, 1998; Mayser, 1995). Specifically, *Debaryomyces sp* was not reported for Kombucha in the literature as far as we know. Likewise, there is no research on microbiology of Kombucha in Sudan to confirm or reject our results.

4.3 The proximate chemical composition of Kombucha mat

The proximate chemical composition of Kombucha mat after 30 days of fermentation was determined as shown in Fig. 2. The results obtained are 49.5% crude fiber, 43.2% carbohydrates, 4.8% crude protein, 1.5%ash and 1%fats.

This result can be compared with same parameters after one year of fermentation (Fig. 3) during which period no sugar or tea was added. Composition after one year was: 57.59% crude fiber, 30.21% carbohydrates 8.7% crude protein, 3% ash and 0.5% fats. These changes were obtained in all parameters and this indicates a continuity of metabolite production although no nutrients, other than distilled water, were added during a whole year! It seems that the Kombucha lived at the expense of the energy sources such as the carbohydrates and fats and converted some of these to proteins and fiber. The increase in protein after one year of fermentation may be explained according to Hoffmann (2000) that the protein found in Kombucha mat consisted of various enzymes secreted by both yeast and bacteria to break down the

large molecules of the different nutrients in the media, which cannot enter the cell directly.

Also Schlegel (1986) stated that many bacteria can grow on a large variety of nutrients. This requires the capacity to produce the necessary enzymes for utilization of the nutrients, and therefore the possession of the appropriate structural genes. That indicates the possibility that the proteins of Kombucha mat consist mainly of the enzymes of its microorganisms. Moreover, in the long term fermentation possibly increased both acetic acid and lactic acid concentrations, and that could possibly cause damage in the cell wall and membrane of some organisms so their cellular proteins were pumped out the cell (Kolothumannil and Ingledew, 2001).

However, *Acetobacter xylinum,* a characteristic species of Kombucha, continued producing its usual amount of cellulose, which increased by time. The increase of fiber associated with the decrease in carbohydrates possibly resulted from polymerization of carbohydrates to fiber by the released enzymes.

4.4 Determination of alcohol in Kombucha

There was no sign of the presence of alcohol in any experiment in this study, except when coffee beans were used to prepare a growth medium for Kombucha when 1% alcohol was registered under aerobic conditions. Also 2.5% alcohol was produced when aeration was reduced in the growth environment of Kombucha-fermented tea.

4.5 Formation of Kombucha from pure cultures of microbial isolates

Only a viscous slime layer was formed on the top of the tea extract when only pure culture of acetic acid bacteria was inoculated in the sugared tea extract. On the

contrary to that, the tea liquid became turbid and some materials were observed in the bottom of the test tube when pure yeast isolates were used.

Kombucha was formed only in the case when yeast and bacteria were used together. This result is in close agreement with Hesseltine (1965).

4.6 Factors affecting Kombucha formation and growth

4.6.1Temperature

4.6.1.1.The effect of temperature on total volatile acid production

According to Fig.4, Kombucha incubated at 25°C produced its highest concentration of total volatile acids (0.09 and 0.11g/l) on the 2^{nd} and 5^{th} days of fermentation. This means the curve of total volatile acid production had two peaks at 25°C. The highest production of total volatile acids at 30°C (0.15g/l) was registered on the 2^{nd} day of fermentation and dropped suddenly to 0.04g/l and remained around it. Also on the 3^{rd} day of fermentation the highest concentration of total volatile acids (0.09g/l) was observed at 37°C. Moreover, the highest concentration of total volatile acids at 40°C was registered after 4 days of fermentation. However, the highest concentration (0.11g/l) of the total volatile acids at the end of the fermentation period was observed at both 25°C and 40°C but the highest concentration and fastest rate of volatile acid production in this experiment were produced at 30°C.

The two peaks at 25°C can be explained as a result of a succession of the organisms. At the beginning a certain organism grew well and produced the volatile acids. When this organism consumed its energy source, the curve of total volatile acids possibly dropped due to consumption by some other organisms like over-oxidizer acetic acid bacteria. The next rise that followed may be due to production of new acetic acid.

The temperature of 30°C could be an optimum temperature for a acetic acid production. The production at 37°C is more stable. At 40°C organisms other than Kombucha organisms possibly produced the volatile acids. These new organisms grew well, and produced their metabolite at 40°C. In fact these results were registered in a short period, so an optimum temperature for Kombucha to produce its volatile acids can hardly be determined.

4.6.1.2 Effect of temperature on total titratable acids

Fig. 5 shows that the highest production of total titratable acids was observed on the first day of fermentation at 40°C. Although the highest concentration at the end of fermentation was registered for 37°C, generally there was no noticeable difference between the various temperatures.

4.6.1.3 Effect of temperature on the pH value of Kombucha

No noticeable change in the fermented tea pH was observed, all of the pH values ranged between 3.5to 3.7. Perhaps the rate of acid production in Kombucha and the rate of its consumption by microorganisms were equal. But of course the buffering action of these organic acids is an important factor is stabilizing the pH value.

4.6.2.1 The effects of initial pH on Kombucha dry weight

According to Fig. 6 the highest dry weight was observed at pH7, pH 9 and pH 2.5 in that order. No growth was observed at both pH 1.2 and pH 13. That may indicate that Kombucha had a pH range roughly from 2.5 to 9, and also indicates that the majority of Kombucha organisms grow best at neutral media (pH 7).

4.6.2.2 The effects of initial pH on Kombucha final pH

Fig.7 shows that the highest initial pH value gave the highest final pH value and vise versa, but the final pH values are relatively similar than initial values. The final pH values ranged from 3.5 to 1.2 while the initial values ranged from 9 to 1.2.

4.6.3 The effects of the inoculum percentage on Kombucha growth

Fig.8 shows the positive relation between the inoculum percentage and Kombucha dry weight. The highest inoculum percentage produced the highest Kombucha dry weight, and vice versa. In this case, when the organisms are concentrated in media that can encourage the production of its metabolites. Also in this case the thickness of Kombucha (Fig.9) and its dry weight increased while a new layer is produced on the surface of the tea extract. That can be explained as: when the essential organisms are concentrated, that can prevent Kombucha from contaminant organisms by its buffering action of pH value, and that may also enhance its antimicrobial activity.

4.6.4 The effects of tea concentration on Kombucha dry weight

Fig.10 shows that the concentrated tea (3%) gave the highest Kombucha dry weight and vice versa. The concentration of 75% gave the lowest dry weight but the general treatment indicates that the more tea added the more Kombucha produced. Increase in Kombucha dry weight when the tea concentration was increased can probably be explained by: addition of tea enriches the medium with nitrogen and other nutrient sources that enhance the building up of Kombucha.

4.6.5 The effects of sugar concentration on Kombucha dry weight

As shown in Fig.11 the highest concentration of sugar (10%) resulted in the highest dry weight of Kombucha (1.4g). The lowest concentration of sugar (1.25%)resulted in the lowest dry weight of Kombucha (0.8g). The positive relationship between sugar concentration and increase in Kombucha dry weight reflects the role played by

sugar as a carbon source for all organisms involved in Kombucha, in spite of the possible difference in their favorite carbon source.

It seems to be that the positive relationship between sugar concentration and increase in Kombucha dry weight, theoretically, refers to sucrose being converted to alcohol by Kombucha yeasts (Siever *et al*, 1996). Alcohol was used in *Acetobacter* metabolism, as a favorite carbon source while *Acetobacter* cellulose was produced.

4.6.6 Effect of different growth materials as growth media for Kombucha

Kombucha was grown in guava, milk, gum arabic, cane sugar, coffee, karkadeh, grapefruit, black tea and potato starch.

All media tested for Kombucha formation gave positive results, except grapefruit and sugar solution. The extent to which Kombucha grew differed from material to material (Table 4). Kombucha grows well in milk, which can be considered as one of the best media to grow Kombucha (Plate 6). This is probably attributed to milk being a complete medium, containing most of the growth requirements: lactose, as carbon source, protein as nitrogen source and pH, which is suitable to grow a wide spectrum of organisms, including Kombucha organisms. But for tea it is a different matter, tea is the original source of Kombucha which is consumed worldwide, which means that there are strong links between Kombucha and tea, although other media, such as milk and guava, gave better results (Plate 6).

Nevertheless, tea is the best medium to grow Kombucha to date. Tea gives a good taste and flavor, and seems to keep the symbiotic state by preventing establishment of contaminants. The various fermentation metabolic products in milk may be different from those in the case of tea or other materials.

Karkadeh also gave a positive result, giving a purple-pink Kombucha but it did not form a coherent film because it contained many air pockets. That made it spongy.

Table 4: The extent of growth of Kombucha on different materials

Media	Kombucha formation
Guava suspension	+++
Milk	+++
Gum Arabic	+
Sugar solution	-
Coffee	++
Karkadeh	+
Grapefruit	-
Black tea	++
Potato starch	+

Coffee forms a greenish gray Kombucha that looks like a coherent gel matrix. This good growth may be due to the storage proteins and carbohydrates in coffee seed, which must be a good source for Kombucha growth requirements. In addition to carbon and nitrogen sources, pH plays an important role in Kombucha growth. That explains the absence of Kombucha film in grapefruit medium (pH around 2.6 to 3.2) The lower degree of quality in Karkadeh medium (pH 3.2) was possibly due to the low pH of the medium.

Guava suspension produced a distinct pearly white Kombucha that may refer to the chemical composition and natural color of the medium (Plate 5). Osmotic pressure plays an important role in the success or failure of Kombucha formation.

High osmotic pressure was probably behind the inhibited growth of Kombucha when Kombucha starter was added to sugar solution; foam appeared on the surface yet the regular Kombucha film, which is only produced by *Acetobacter xylinum,* didn't appear. This and the appearance of ethanolic odor indicated yeast growth. It may be that yeast is more osmotolerant and the sugar solution lacked nutrients needed for the bacteria.

In the gum arabic medium, slime layer was formed by the bacterium. Many bacteria can grow on gum (Obeid, 2002). Yeast did not develop well in this case.

All the treatments above have been completed in short periods, ranging from a week to ten days. Time is a very important factor in fermentation. Many changes are expected to occur as time goes on. Microbial balance, metabolite mix and pH variations could decide which medium is to be considered the best for formation and quality of Kombucha.

4.7 Effects of physical treatments on Kombucha formation and growth.

4.7.1 Shaking

After shaking for four days at a velocity of 150 rpm, irregular Kombucha granules were formed and suspended in the tea. Acetic acid odor was observed but no noticeable change in pH occurred. Regular Kombucha was formed in the control sample. However, the optimal conditions for the production of Kombucha mat (*Acetobacter* cellulose) as described by Krystynowicz *et al*. (2002) includes rotation at a rate of 4 rpm and a large surface area.

4.7.2 Blending

Kombucha microfibers, the building units of Kombucha mat, were liberated by blending and were suspended for approximately five months. During this time a new Kombucha mat is produced on the surface while most of the matter remained suspended. In this case many changes in the arrangement of Kombucha microorganisms are expected. Considering that the distribution of Kombucha organisms depends on the types of its nutrient metabolism (fermentation/oxidation), if any change happened in this environment, it can be reflected on the types and characters of Kombucha`s metabolites.

4.7.3 Effect of anaerobic conditions on Kombucha growth

Kombucha`s coherent gel matrix was damaged when Kombucha was grown under anaerobic conditions. Also a content of 2.5% alcohol was registered in these anaerobic conditions. This can be explained by the assumption that fermentation was more suitable type of microbial metabolism. In addition to this, microbial enzymes may affect Kombucha mat, while the role of its essential organisms is absent.

Kombucha liquid

Kombucha gel **Kombucha gel**

Plate 5: Both Kombucha gel and liquid from guava.

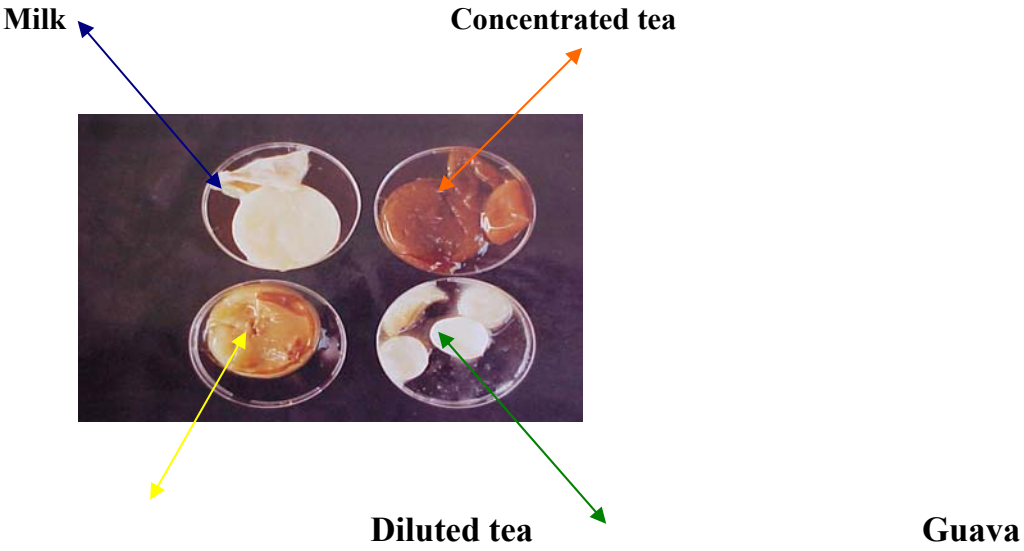
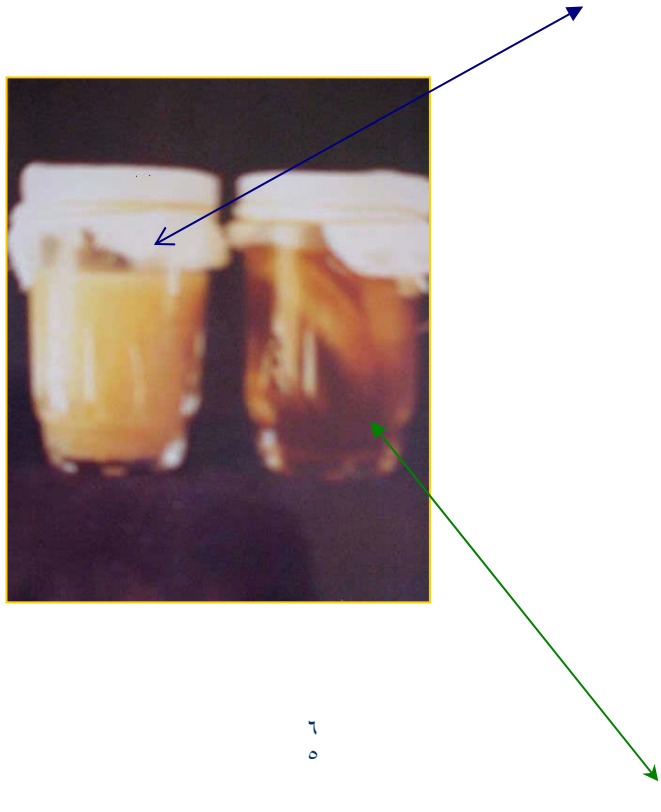


Plate 6 (a): A look of Kombucha and how the growth media affected it.



Kombucha from tea

Plate 6 (b): A look of Kombucha and how the growth media affected it.

4.8 Biochemical and microbial changes during fermentation of Kombucha.

During fermentation of Kombucha the population of bacteria increased at a variable rate until the 6th day and decreased after that. Conversely, yeast count decreased at a variable rate until the 5th day and increased after that. During this time no noticeable decrease in pH value was observed and total titratable acids, as lactic acid, increased during the first three days of fermentation to (0.01g/l)at the end of fermentation. Total volatile acids, as acetic acid, increased to (0.36g/l) during the first 7 days of fermentation at a variable rate.

As shown in Fig.12, the changes in Kombucha microbial balance (yeast is dominant over *Acetobacter sp.*) appeared from the 2nd day to the 6th day of fermentation. These changes were not associated with recognizable biochemical changes. However, biochemical changes may occur later in time.

4.9 Antibacterial activity of Kombucha and its products.

In this experiment, all tested genera and species of bacteria (*Klebsiella sp., Salmonella typhi. Staphylococcus sp and Pseudomonas sp.)* responded variously to the preparation, depending on the genus of bacteria. The antibacterial efficiency of the various preparations, in descending order, is as follows: Acetic acid +lactic acid+ tea, acetic acid + lactic acid, Kombucha extract, and tea extract. Fig.13. The preparation, which contained: acetic acid (7g/l), lactic acid (33g/l) and tea leaves (4g/l) gave the highest antibacterial effect. This effect declined in the absence of tea leaves.

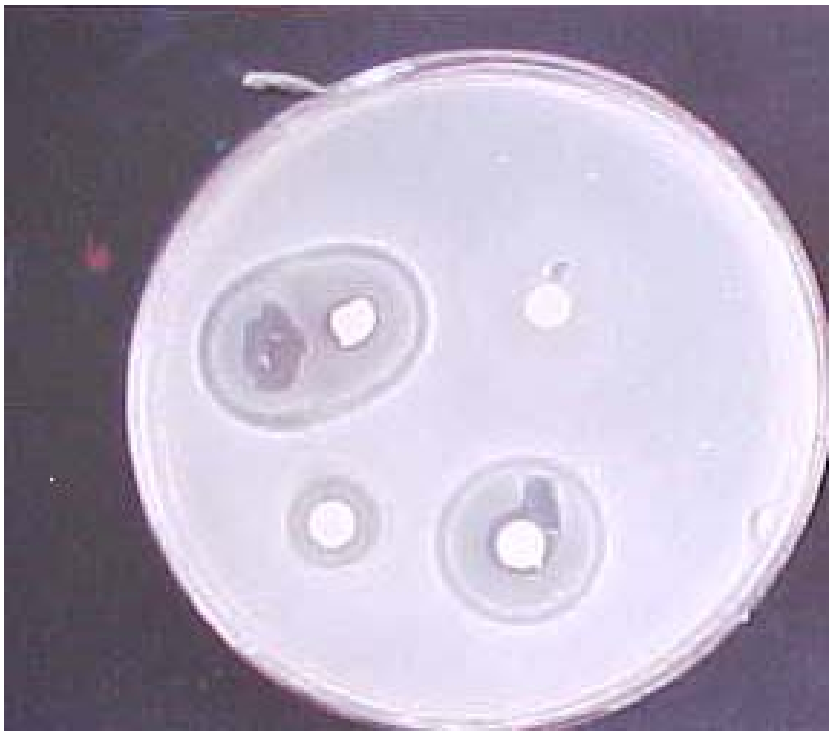
The sensitivity of bacteria to Kombucha, in descending, order was as follows: *Staphylococcus sp.* (Plate 7), *Pseudomonas sp.*, *Klebsiella sp.* and *Salmonella typhi* (Plate 8;Fig.15).

For tea extract the order is: *Staphylococcus sp., Salmonella typhi, Pseudomonas sp.*, and *Klebsiella sp.* (Fig.16). However, Kombucha had a wider clear zone.

Kombucha extract inhibited the growth of all tested bacteria: *Klebsiella sp* (Fig. 17), *Salmonella typhi* (Fig. 18), *Pseudomonas sp.* (Fig. 19), and *Staphylococcus sp.* (Fig.20). These results are in agreement with Greenwalt, *et al* (1998) who reported that Kombucha had an antimicrobial activity against *Staphylococcus sp, Pseudomonas sp* and *Salmonella sp.* Also these results confirm the findings by Guttapadu (2000) and Karamadin, *et al* (2001) who stated that Kombucha had antimicrobial activity against *Staphylococcus aureus*.

The results of this experiment show that tea and organic acids play an important role in the antibacterial activity of Kombucha.

Tea + lactic acid+ acetic acid	tea extract



Kombucha extract lactic acid+ acetic acid

Plate: 7 Kombucha and its products inhibit the growth of *Staphylococcus sp.*

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lactic acid+ acetic acid Kombucha extract

Tea extract Tea + lactic acid+ acetic acid

Plate: 8 Kombucha and its products inhibit the growth of *Pseudomonas sp.*

Lactic acid+ acetic acid Kombucha extract

Tea+ lactic acid + acetic acid tea extract

Plate: 9 Kombucha and its products inhibits the growth of *Salmonella typhi*

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