Studii şi Cercetări	Martie 2019	Biologie	28/1	75-83	Universitatea"Vasile Alecsandri" din Bacău

OBSERVATIONS REGARDING THE ISOLATED MICROBIOTA FROM KOMBUCHA TEA

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***Key words**: Kombucha, fermented tea, microbial ecology, cultural method, biomass*

INTRODUCTION

Kombucha tea is known as the tea fungus, having the botanical name of Medusomices gisevii, given by Lindau in 1915. It is a consortium of yeasts and bacteria, which has the ability to convert the active principles of black tea and sugar into substances with remarkable medicinal actions. The history of this tea dates back more than 2000 years, being discovered in China, when talking about a fungus that could bring immortality (Dufresne C.et al. 2000, Frank G. W., 1991). It is usually prepared by fermenting black tea, sweetened with sugar, at room temperature for 10-12 days. From a biological point of view, this so-called "tea fungus" is in fact a symbiosis of yeasts and acetic acid-producing bacteria, the cellulose film that normally forms being described as "fungus" (Mayser P., Fromme C., Leitzmann C., Gruender K., 1995).

Yeasts present in Kombucha culture (Fig. 1) ferment sugar in the tea medium with ethanol, which is then oxidized by bacteria that produce acetic acid. The result of the low pH and the presence of antimicrobial metabolites reduce the competition of bacteria, yeasts and filamentous fungi (Greenwalt C.J. et al., 2000). The colony formed in this product Kombucha is based on an association (consortium) of yeasts and bacteria. This colony consists of at least 12 species of microorganisms, living in symbiosis and forming a remarkable biological system through its complexity and composition.

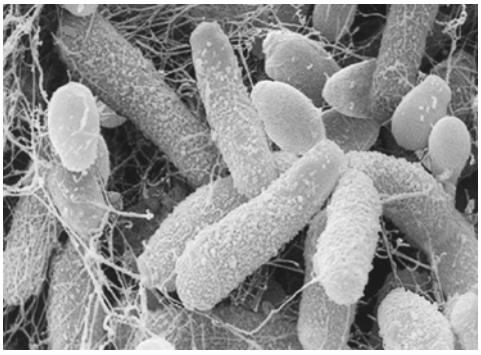

Fig. 1. SEM image of Kombucha culture (Greenwalt C.J. et al., 2000)

Tea fungus (*Medusomyces gisevii)* or Kombucha is the most commonly used name for this symbiotic growth of acetic acid-producing bacteria (*Acetobacter xylium, Acetobacter xylinoides, Bacterium gluconicum, Acetobacter aceti şi Acetobacter pasteurianus*) and osmophilic yeast strains (*Schizosaccharomyces pombe, Saccharomyces ludwigii, Kloeckera apiculata, Saccharomyces cerevisiae, Zygosaccharomyces rouxii, Zygosaccharomyces bailii, Brettanomyces bruxellensis, Brettanomyces lambicus, Brettanomyces custersii, Pichia membranaefaciens*, *Torulopsis, Candida*) in a thicker gelatinous membrane (Zooglea), which must be grown in black or green tea (Villarreal S., et al., 2018, Greenwalt C.J. et al., 2000, Jarrell J., et al. 2000). Similar to kefir derived from milk, the microbial composition of Kombucha beverage may not be accurate as it depends on the source of the inoculum for fermentation of the tea (Coton Monika et al., 2017).

The chemical composition of the Kombucha fungus has not been fully discovered; it is only known that it contains important amounts of vitamin C as well as vitamins in group B (B1, B2, B3, B6, B12), this largely attests to the antioxidant properties of fungi as well as stimulating the body's immunity. Substances with very strong antibactericidal and antiviral action have been discovered in this product, which justifies the use with remarkable results in the case of infectious diseases.

An analysis of the fermented liquid showed the presence of acetic, lactic, gluconic, gallic and glucoronic acid, these being the majority chemical compounds. Gluconic acid is considered by many researchers to be the main therapeutic agent present in Kombucha, because it functions in the liver as a detoxifying agent (Frank G. W., 1991, Jarrell J., et al. 2000).

Kombucha tea has gained substantial popularity as the beneficial effects have already been demonstrated: antimicrobial, antioxidant and anti-carcinogenic action, antidiabetic action, for treating gastric ulcer and lowering cholesterol etc. It has also been shown to have an impact on the immune response and liver detoxification (Greenwalt C.J. et al., 2000, Marsh A.J., et al., 2014).

In particular, Kombucha has been shown to have antimicrobial action on several species of microorganisms such as: *Helicobacter pylori, Salmonella typhimurium, Staphilococcus aureus, Agrobacterium tumefaciens, Bacillus cereus, Shigella sonnei, Escherichia coli* (Jankovic, 1996, Greenwalt C.J. et al., 2000).

Although Kombucha tea has been reported to have curative effects, there is some evidence on the toxicity associated with this tea. Since it greatly intensifies the digestion process, it is recommended that people suffering from hyperacid gastritis should use this product with caution.

At the same time, in the case of people presenting with fermentation colitis, the administration is initially done in small doses and if unpleasant manifestations occur the administration will be stopped (Jankovic, 1996, Greenwalt C.J. et al., 2000).

The aims of this paper are: testing the favorable media for the growth of microorganisms; measuring the pH of the drink throughout the fermentation; performing macroscopic and microscopic observations on the cultures developed on the tested media, performing simple stains and Gram stains; quantitative assessment of biomass developed on culture media.

MATERIALS AND METHODS

The following materials were used in the preparation of the Kombucha tea medium: distilled water, black tea leaves, starter Kombucha culture (Viva Nature World, Hungary) and white sugar.

In order to isolate the microorganisms present in the Kombucha product, a variety of culture media was preparated which are commonly used in microbiology. After preparation, all culture media were sterilized in autoclave at 120 °C for 15 minutes.

The culture media prepared were (Mazareanu C., et al 2003, Prisecaru M., et al 2015): *Nutrient broth medium (B) 100 mL* contain: meat extracts 0.1 g, yeast extract 0.2 g, peptone 0.5 g, NaCl 0.5 g, distilled water 100 ml,pH= 6.8; *Medium with peptone water (A) 100 mL* contain: peptone 1 g, NaCl 0.5 g, distilled water 100 ml, pH= 7.2; *Malt extract medium (M) 100 mL* contain: malt extract 3g, distilled water 100 ml, pH= 5.5; *Malt extract and agar medium (MEA) 100 mL* contain: malt extract 30g, agar 15 g, distilled water 100 ml, pH= 5.5, Oxytetracycline (10%); *Sabouraud Medium (S) 250 mL* contain: glucose 10 g, peptone 2.5 g, agar 5 g, distilled water 250 ml, pH= 5.6 *; GYC Medium 250 mL* contain: yeast extract 2.5 g, glucose 5 g, calcium carbonate 5 g, agar 3.75, pH= 6.8*; Medium for Acetobacter (Ac+Et) 250 mL* contain: dextrose 12.5 g, yeast extract 2.5 g, calcium carbonate 1.25 g, ethanol 17.5 ml, pH= 6.8.

Preparation of the Kombucha tea medium:

Kombucha culture was kept cold at a temperature of 5 ° C until the tea medium was prepared (Fig. 2a). On the day of preparation the Kombucha culture was kept at room temperature.

Fig. 2a. Preparation of the Kombucha tea medium

1000 ml of distilled water were boiled for 15 minutes and 5.4 g of black tea leaves were introduced. After 5 minutes of infusion, 100 g of sugar was added to the mixture and mixed until homogenization.

Kombucha culture was introduced (Kombucha mushroom + 10% Kombucha tea) after the mixture reached room temperature (Fig. 2b).

Fig. 2b. Preparation of the Kombucha tea medium

The obtained tea medium (MK1) was stored in a glass vessel that was covered with gauze to prevent access to insects or spores that could contaminate the tea.

Throughout the experiment, the MK1 tea medium was maintained at a temperature between 21-24 °C (Fig. 2c).

Fig. 2c. Preparation of the Kombucha tea medium

The fermentation of the Kombucha tea was followed for a period of 22 days. The formation of a new Kombucha fungi (Fig.3) from the "mother" fungus inoculated in the MK1 medium were followed, during this interval.

The MK1 medium was transferred to a new MK2 medium, being obtained by the same preparation method and with the same components, so a new sweetened tea was prepared in the same quantity, after which the Kombucha fungus and 100 mL of the old tea medium were transferred. The medium transfer was realized because the old fungus had no longer the necessary conditions to develop a new fungus, which is also specified in the speciality literature. The new fungus was formed within a few days after being transferred to the new MK2 fresh medium.

Inoculating techniques used to isolate the microbiota from Kombucha

Inoculating techniques used were: surface inoculating (Fig. 4), on solid medium with inoculating loop and Pasteur pipette, inoculating on deep solid media and inoculating with Pasteur pipette on liquid media (Fig. 5).

Fig. 3. Development of the Kombucha culture from the time of preparation until the day 22 fermentation

Fig. 4. Surface inoculating on solid medium

Fig. 5. Inoculating with Pasteur pipette on liquid media

During the experiment, pH, temperature, alcohol concentration and CO_2 formation were measured as a result of glucose fermentation. At the same time, macroscopic observations were made on the development of microorganisms on the culture media used. Also, simple stains, Gram stains as well as a quantitative assessment of biomass were made.

RESULTS AND DISCUSSIONS

Determination of pH

During the experiment it was observed that the pH value has changed, i.e. the pH has been decreasing from Day 1 of fermentation until the end of fermentation on Day 22. The pH values were recorded on different days with the help of a pH-meter (Fig. 6).

The largest differences were found in days 1-5 when the pH value decreased considerably from 6.5 to 5.4. This difference shows that acetic acid-producing bacteria by have a greater development during this period. After this interval, acetic acid-producing bacteria had a slower development, so the pH decreased from 5.4 on Day 5 to 5.2 on Day 13, and at the end of fermentation on Day 22 it reached a value of 4.8. The decreasing of pH value was also confirmed by the literature (Ai Leng et al., 2004).

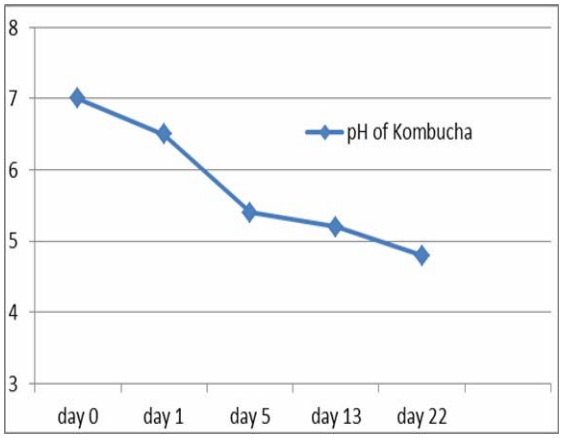

Fig. 6. Determination of pH during the fermentation

The non-alcoholic nature and the collection of the gases resulting from the fermentation process.

The non-alcoholic nature of Kombucha product obtained from the fermentation process was verified by measuring with the help of an alcohol meter (Fig.7) commonly used to measure the alcohol content of wine, vinegar and other products.

Fig.7. The non-alcoholic nature of Kombucha product

The image shows that Kombucha product did not contain alcohol at the time of measurement with the help of an alcohol meter. The measurement was performed on Day 22 of fermentation. The literature states that the alcohol is formed in this product only if the fermentation time has exceeded 30 days; also the percentage of alcohol that is formed is reduced to maximum 0.5%.

To detect the gas production resulting from the activity of microorganisms, Durham tubes were used (Fig. 8). They were introduced upside down in tubes containing culture media A, B and M and which were inoculated with Kombucha tea. It can be seen that in the Durham tubes, no CO_2 bubbles were formed, so no glucose fermentation took place.

Fig. 8. Culture media A, B and M inoculated with Kombucha tea in which Durham tubes were introduced

Macroscopic observations

The macroscopic aspect is a helpful criterion in the recognition of bacterial species, because bacteria often have characteristic aspects of the cultures on both liquid and solid media. The macroscopic examination will necessarily be completed by a microscopic examination.The macroscopic examination followed: the surface of the colonies, the shape, the profile, the optical density, the color, the consistency and the edge of the colonies (Fig. 9a- 9f.).

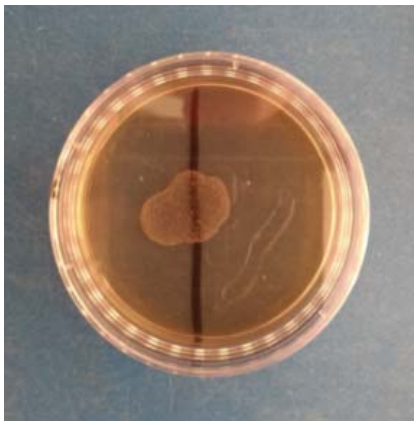

Fig. 9a.The MEA culture medium presented: rough, punctiform colonies, flat profile, transparent / semitransparent, yellowish color, uneven edge

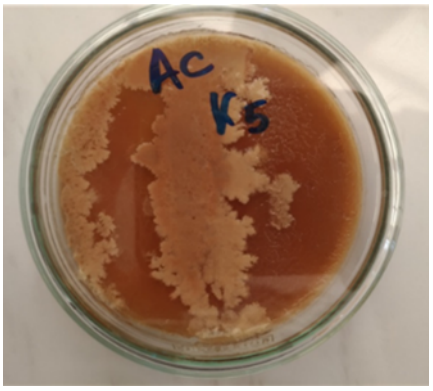

Fig. 9b. The culture medium for Acetobacter (Ac) showed: rough type colonies, high / umbonate profile, opaque, yellow-white color, uneven edge, mucosal-creamy consistency

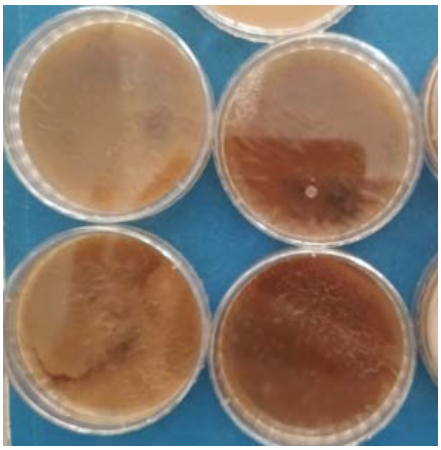

Fig. 9c. GYC culture medium presented: tangled, glossy colonies, flat profile, opaque density, brownie color, skiny consistency, uniform edge

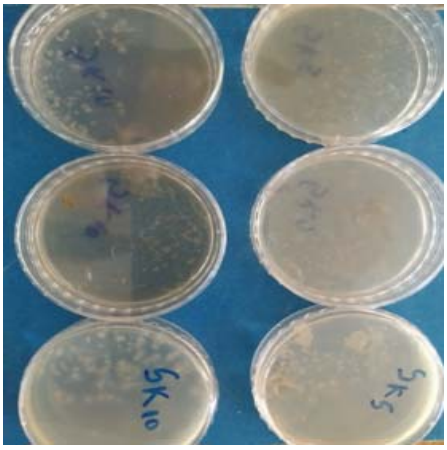

Fig. 9d. Sabouraud culture medium presented: smooth and punctiform colonies, flat profile, transparent / semi-transparent, white color, skiny consistency, uniform edge

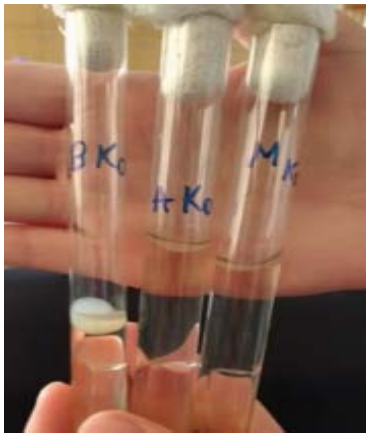

Fig. 9e. The culture media used to highlight CO_2 with B, A and M showed: transparent / semitransparent colonies, mucous consistency in depth and egg white beaten foam on the surface, white color

Fig. 9f. On the A, B and Z culture media supplemented with sugar: the surface colonies had the texture of whipped egg whites foam and in depth they had mucous consistency, transparent / semi-transparent density, cloudy

Microscopic observations

A few slides were prepared to observe the microorganisms that formed on the culture media used at microscope (Figs. 10- 12).

Following Gram staining and simple staining, we observed bacterial cells of bacillus and yeast from the culture media tested.

The simple staining with methylene blue under microscope view shows the development of dense colonies of cocci bacteria and a filamentous form. In the case of microscope visualization of the preparation after Gram staining, a good development of a filamentous form is observed in the first image, as well as dense colonies of cocci bacteria can be observed.

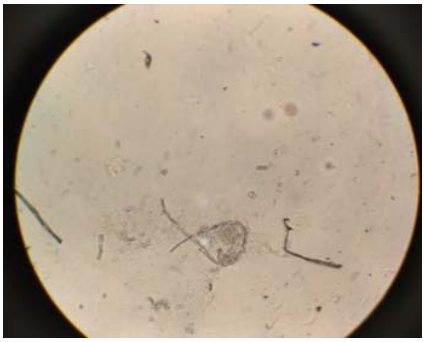

Fig. 10. Microscope observations on MEA culture medium: cocci, bacilli filamentous bacteria are observed

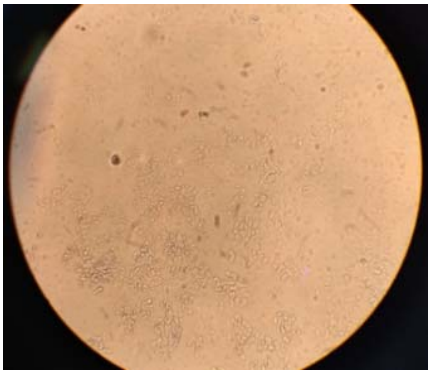

Fig. 11. Culture medium with A: dense colonies of cocci bacteria are observed

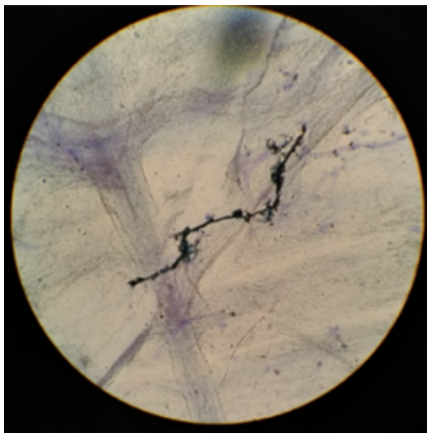

Fig. 12. Microscope view of simple staining with methylene blue

Determination of biomass

In order to follow the action of sugar (Z) and of black tea (T), three types of media were inoculated (Fig. 13), namely the medium with peptoned water (A), the medium with malt (M) and the medium with nutritional broth (B).

These culture media were simply inoculated with Kombucha tea (K) - control sample, with K tea + sugar (10 g / 100 mL medium) and K tea + leaf black tea (0.5 g / 100 mL medium). The action of these ingredients was directly determined (Fig. 14) by weighing the biomass formed on the culture media used. Thus on the liquid media biomass was removed with the aid of a clamp and spread on a piece of gauze to drain excess water. After 2-3 minutes the water was drained and the biomass was determined by weighing. Values are expressed in mg.

Fig. 13. Determination of biomass (mg)

The most favorable culture medium for the development of microorganisms was the broth medium. It is noted that sugar has a positive effect on microorganisms in all 3 culture media used, while black tea has greatly inhibited their development.

Determination of biomass on solid culture media.

On solid culture media an amount of 2-3 mL of distilled water was added so that the biomass can be easily removed, and after 2-3 minutes the biomass was taken with a scalpel and placed on a piece of gauze to drain the excess of water.

After 2-3 minutes the water was drained and the biomass was determined by weighing with an analytical balance.

We should point out that Sabouraud (S) and Gyc media were poured into small Petri dishes compared to the Ac medium which was poured into larger Petri dishes (Fig. 15).

Following the determination of the biomass developed on solid culture media we observed that the Ac medium allowed a good development of microorganisms (5566 mg) compared to the S (1064 mg) and Gyc (1428 mg) culture media.

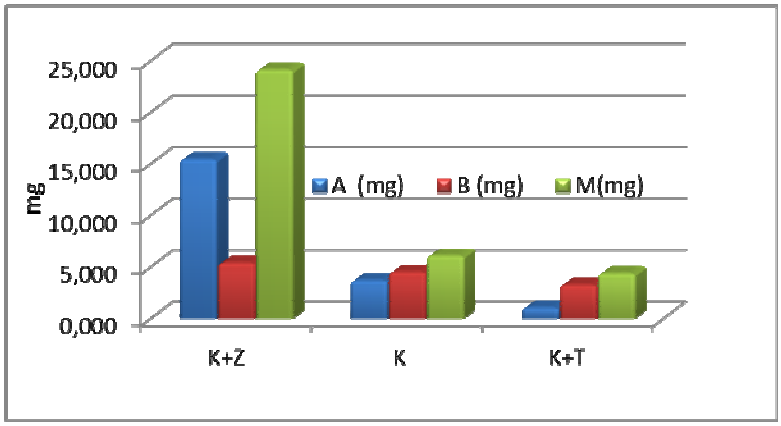

Fig. 14. Biomass variation in A, B, M culture media inoculated with K+Z, K and K+T

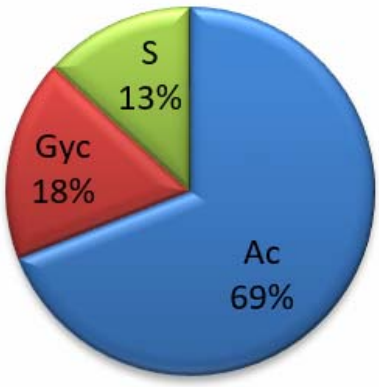

Fig. 15. Determination of biomass on Ac, S and Gyc solid culture media

CONCLUSIONS

Experimentally, it was found that the largest differences on pH were found between Days 1-5 when the pH value decreased considerably from 6.5 to 5.4. This difference shows that acetic acid-producing bacteria have a greater development during this period. After this interval the acetic acid-producing bacteria had a slower development, so the pH decreased from 5.4 on Day 5 to 5.2 on Day 13, and at the end of fermentation on Day 22 it reached a value of 4.8.

The non-alcoholic nature of the Kombucha product obtained from the fermentation process was verified (on Day 22) by measuring with an alcohol meter. The alcohol content is formed only after 30 days of fermentation.Also, the use of Durham tubes showed that no CO_2 was formed, so no glucose fermentation took place.

Macroscopic observations on solid and liquid media showed that not all media favored the development of microorganisms present in Kombucha tea. The culture medium for *Acetobacter* was the solid medium that allowed the best colony development compared to the malt and yeast culture media which allowed a reduced development or the jealous medium did not allow the development of colonies.

The most favorable liquid medium was the nutrient broth medium followed by the peptoned water medium and finally the malt extract medium.

The sugar used favored the development of microorganisms on these culture media compared to the media containing only Kombucha without added sugar, while the media containing black tea showed a very weak development of microorganisms. So the action of the black tea in a larger quantity does not allow the development of a Kombucha culture, while the sugar will favor a good development.

Following Gram staining, we observed the development of Gram positive bacteria (cocks, streptococci as well as some filamentous bacillary forms).

The determination of the biomass by weighing confirmed that the culture medium for *Acetobacter* (Ac) was the most favorable, followed by the Gyc medium and finally by the Sabouraud (S) medium. For the liquid media the most favorable was the nutrient broth culture medium (B) followed by the peptoned water medium (A) and finally the malt medium (M), which allowed a reduced biomass development.

ABSTRACT

The Kombucha product is based on 3 ingredients: sugar, black tea and a complex of yeasts and acetic acid-producing bacteria that have received the botanical name of *Medusomices gisevii*.

The objectives of this paper were: testing of favorable culture media to the growth of microorganisms in Kombucha; measuring the pH of the drink throughout the fermentation; performing macroscopic and microscopic observations on the cultures developed on the tested media, performing simple stains and Gram stains; quantitative assessment of biomass developed on culture media.

The experiments confirmed that not all culture media favored the development of microorganisms present in Kombucha tea. The culture medium for *Acetobacter* was the solid medium that allowed the best development of the colonies compared to the malt and yeast media, which allowed a reduced development. In the case of jealous medium, this did not allow the development of colonies. The most favorable liquid medium was the broth medium, followed by the medium with peptonated water and finally the medium with malt extract.

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