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# THE AVAILABILITY OF A LACTOSE MEDIUM FOR TEA FUNGUS CULTURE AND KOMBUCHA FERMENTATION

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*Abstract* - Kombucha is a traditional beverage that is prepared by fermenting sucrose-sweetened black tea. A medium is inoculated with a cellulose pellicle (popularly known as a "tea fungus") or fermentation brought from previous cultivation process. Our aim was to test the possibility of obtaining a Kombucha beverage using different concentration of lactose as an alternative source of C-atoms. A traditional medium sweetened with sucrose or without sugar was used as control. Without lactose-fermenting yeast strains in tea fungus, lactose is not an adequate alternative source of the C-atom for Kombucha fermentation because it is not possible to obtain Kombucha with an appropriate acidity during a seven-day fermentation. Compared with the traditional medium, fermentation is significantly slower with high differences in acid content. In unsweetened tea inoculated with the beverage obtained from a previous traditional process, Kombucha fermentation processes and produces a beverage without sugar and alcohol.

*Key words:* Kombucha, tea fungus, lactose, unsweetened tea

## INTRODUCTION

Kombucha is a fermented beverage with a history of several thousand years in the East and is very popular today in the West (Kaufman, 1995). The fermentation product is a slightly sweet, carbonated, acidic tea (*Camellia sinensis* L.), which is beneficial to human health as a diuretic, for edemas, arteriosclerosis, sluggish bowels, weight loss, treating cancer etc. (Janković and Stojanović, 1994; Frank, 1995). Experience has also shown that Kombucha regulates the intestinal flora, strengthens the cells, harmonizes the metabolism, acts as a natural antibiotic and helps maintain the pH, i.e. the acid-alkaline balances in the body (Dufresne and Farnworth, 2000). However, many of these effects have not been scientifically proven (Greenwalt et al., 2000; Hartmann et al., 2000).

Kombucha is typically prepared by fermenting black tea (1.5-5 g $l^{-1}$), sweetened with sucrose (50-100 g $l^{-1}$) and inoculated with a previously fermented beverage containing 10-20% of tea fungus pellicle (Teoh et al., 2004). Recommendation can be found in some prescriptions to perform inoculation with a tea fungus pellicle with 5-10% of a beverage from a previous process. The substrate is incubated statically under aerobic conditions for 10-12 days at 20-28$^0$C (Sievers et al., 1995; Chu and Chen, 2006). Fermentation length can differ, even rising to 60 days (Chen and Liu 2000), and in this case the obtained beverage has a mild vinegary taste. However, according to longtime consumers of the Kombucha beverage, to obtain a pleasantly sour beverage, fermentation should be terminated when the titratable acidity content reaches 4-4.5 g $l^{-1}$ (Cvetković, 2008). Therefore, titratable acidity can be taken as a parameter that determines the end of the fermentation process (Cvetković et al., 2008).

The tea fungus is a symbiosis of acetic acid bacteria (*Acetobacter xylinum* is primary bacteria, and

there are also isolated strains of *Acetobacter aceti* and *Gluconobacter oxydans*) (Kersters et al., 2006) and yeasts (*Saccharomyces* sp., *Zygosaccharomyces kombuchaensis, Torulopsis* sp., *Pichia* sp., *Brettanomyces* sp. Etc.) (Jarrell et al., 2000; Liu et al. 1996; Kurtzman et al. 2001). Sucrose as the only carbon source in the cultivation medium is hydrolyzed to glucose and fructose by the extracellular enzyme invertase from tea fungus yeasts. The yeasts uptake and ferment both monosaccharides (glucose and fructose) to ethanol. Excretory ethanol is then oxidized by acetic acid bacteria to acetic acid. In addition, liberated glucose is transformed into ketogluconic acid by acetic acid bacteria. This is the main metabolic path of Kombucha fermentation (Sievers et al. 1995) and the main products are acetic acid, ethanol and gluconic acids (Blanc 1996). Other components that are present in Kombucha beverage are fructose, ethyl-gluconate, oxalic, saccharic and carbonic acids (Roussin, 1996), as well as tea components (catechins, theaflavins, flavonols etc.) and other minor microbial metabolites (invertase, other oxidative enzymes etc.) (Greenwalt et al., 2000).

Studies of some alternative cultivation media have shown that green tea has a more stimulating effect on Kombucha fermentation than black tea, yielding the fermentation product in a shorter time (Greenwalt et al., 1998). The stimulating effect of green tea on Kombucha culture was explained by a higher caffeine content compared to black tea (Hoffmann, 1998). The sweetened tea of *Echinacea purpurea* L. can also be used for Kombucha fermentation and the obtained beverage has outstanding antioxidant properties (Cvetković, 2008). As an alternative medium for Kombucha fermentation, lemon balm tea (*Melissa officinalis* L.) is chosen because of its antibacterial, antispasmodic, sedative and tonic effects. Fermentation lasts a similar time as with the traditional substrate, and the obtained beverage exhibits some antimicrobial activity (Velićanski et al., 2007). The same beverage possesses certain antiproliferative, genotoxic, and antigenotoxic potential (Četojević-Simin et al., 2010).

To the author's knowledge, the number of examined substrates as sources of C atoms in Kombucha fermentation is small. Petrović et al. (1997) investigated the biosynthesis of vitamin C during Kombucha fermentation on polysaccharide inulin obtained from the tubers of the Jerusalem artichoke (*Helianthus tuberosus* L.). The possibility of the application of a malt extract as a source of carbohydrate in a medium for tea fungus was investigated. The presence of glucose and fructose as the dominant sugars in the malt medium results in a very effective fermentation that gives a much sourer beverage in the same amount of time and makes possible the reduction of the fermentation period (Cvetković and Markov, 2003). Reiss (1994) has examined the content of ethanol and lactic acid in Kombucha beverages obtained by the biotransformation of black tea sweetened with different sugars such as sucrose, lactose, glucose and fructose. A different initial content of lactose does not affect the level of formed ethanol, which after an initial increase in the extension of incubation, decreases. Similar results have been obtained for lactic acid because its content is at the highest level after two days of cultivation, falling below the detection limit afterwards. Furthermore, as far as lactose fermentation by tea fungus is concerned, only a few investigations have been reported. Malbaša et al. (2009) investigated the metabolic activity of different tea fungus starters on pasteurized milk. Manufactured milk-based products were compared with traditional products – commercially available yoghurt and kefir. Belloso-Morales and Hernandez-Sanchez (2003) published a paper on the manufacture of the beverage from cheese whey and their conclusion was that one could obtain a product that is a sour and salty non-sparkling beverages.

The aim of the present study was to explore the possibility of expression of the physiological activities of a local tea fungus through the process of Kombucha fermentation, and to obtain black tea Kombucha, using lactose as a replacement for sucrose. During the biotransformation, we followed the parameters relevant to the bioprocess when using alternative and traditional media using standard methods for microbiological and chemical analysis. These re-

sults could be helpful in explaining the mechanism of Kombucha fermentation using this kind of substrate and the production of a beverage from different natural lactose substrates.

## MATERIALS AND METHODS

### *Tea fungus*

Fermentation was performed using the pellicle of a local tea fungus and a Kombucha beverage obtained from the pellicle by using the traditional procedure of cultivation. Our previous investigations (Markov et al., 2001) showed that it contained at least five yeast strains (*Saccharomycodes ludwigii, Saccharomyces cerevisiae, Saccharomyces bisporus, Torulopsis* sp. and *Zygosaccharomyces* sp.) and two bacterial strains of the *Acetobacter* genera.

### *Fermentation conditions*

The medium for Kombucha fermentation was prepared separately for each different commercially available carbon source by dissolving 20, 40 and 70 g/L of lactose or 0 and 70 g/L sucrose in tap water. After boiling the water, black tea (Fructus, Bačka Palanka, Serbia) was added (3 g/L) and removed by filtration after 15 min. After cooling to room temperature, the tea was inoculated with tea fungus pellicle or 10% of fermentation broth from the previous fermentation (on sucrose-sweetened tea) obtained under the same conditions. Small bioreactors (volume 0.72 L, diameter 8 cm) were filled with 0.3 or 0.33 L of the inoculated liquid phase. The bioreactors were covered with cheesecloth and fermentation at 28±1°C was monitored. All experiments were preformed twice under the same conditions, while each quantity was measured three times.

### *Sampling*

Sampling of the fermentation broth was performed periodically; each bioreactor was sampled only once in order to avoid potential contamination. During fermentation the pH value, titratable acidity, number of yeasts and acetic acid bacteria, as well as ethanol and L-/ D-lactic acid contents, were measured.

### *Methods of analysis*

The pH values were measured using an electronic pH meter (HI 9321, HANNA Instruments) calibrated at pH 4.0 and 7.0.

The titratable acidity was determined according to OIV (1990). After removing $CO_2$ from the fermentation broth, a 20-ml aliquot was taken and titrated with 0.1 mol $l^{-1}$ of NaOH.

The titratable acidity was expressed in grams of acetic acid per liter of the sample.

Ethanol and lactic acid content was determined using ethanol and D-/L-lactic acid enzymatic kit (Megazyme, CO. Wicklow, Ireland, K-ETOH 08/11 and K-DLATE 08/11).

Qualitative analysis of the sugar in the medium was performed by thin-layer chromatography on silica gel G, in a solvent system of chloroform:acetic acid:water (1:6:3, v/v/v) (Vrbaški et al., 1992). Spots were detected by spraying with a solution of 50% sulfuric acid in ethanol, then heating for 10-15 min at $120^0$C.

Total cell counts of yeasts and acetic acid bacteria in the fermentation broth were determined by the plate count method. For yeasts, the medium was Sabouraud-4% Maltose Agar (Merck, Darmstadt, Germany) with the addition of 50 mg $l^{-1}$ of chloramphenicol (Sigma-Aldrich, St. Louis, USA) as an antibiotic. The plates were incubated for 72 h at 28°C. The medium for determining the count of acetic acid bacteria was Yeast Peptone Mannitol Agar (Difco, Detroit, USA), containing 500 mg $l^{-1}$ cycloheximide (actidione; Sigma-Aldrich, St. Louis, USA) to inhibit yeast growth. Incubation at 28°C lasted 5-7 days.

## RESULTS

Change of basic parameters during biotransformation of a lactose-sweetened medium

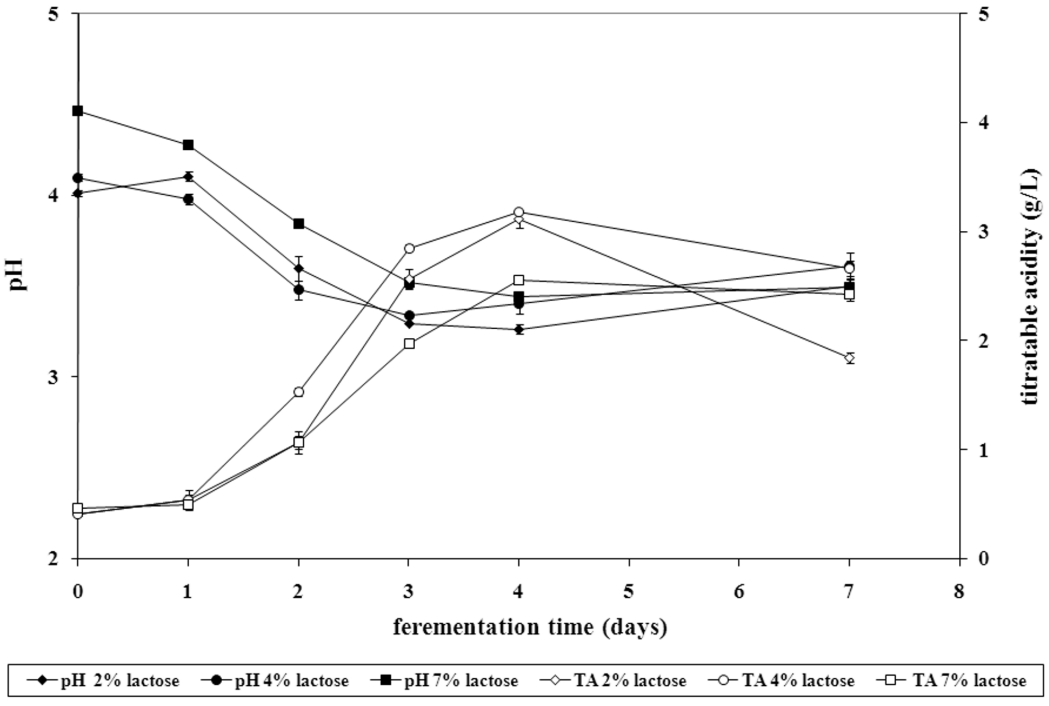


**Fig. 1.** Rate of change in pH value and titratable acidity during the Kombucha fermentation of media sweetened with lactose.

**Table 1.** Changes in number (mean value ± SD) of "tea fungus" cells during the process, in media with different lactose concentrations.

| Fermentation time (days) | Yeast (log CFU/mL) | | | Acetic acid bacteria (log CFU/mL) | | |
|---|---|---|---|---|---|---|
| | 2% | 4% | 7% | 2% | 4% | 7% |
| 1 | 6.65±0.02 | 6.65±0.10 | 6.54±0.25 | 5.83±0.03 | 5.93±0.03 | 5.98±0.11 |
| 2 | 6.67±0.08 | 6.65±0.11 | 6.68±0.11 | 6.66±0.04 | 6.67±0.11 | 6.79±0.33 |
| 3 | 6.42±0.06 | 6.42±0.08 | 6.45±0.31 | 6.38±0.03 | 6.36±0.04 | 6.42±0.40 |
| 4 | 6.42±0.07 | 6.33±0.20 | 6.33±0.20 | 6.31±0.04 | 6.13±0.11 | 6.22±0.05 |
| 7 | 6.63±0.16 | 6.41±0.12 | 6.41±0.09 | 5.53±0.14 | 5.46±0.15 | 5.68±0.52 |

The average changes in pH and titratable acidity with time in the fermentation media inoculated with Kombucha beverages (10%) are presented in Fig. 1 and the number of tea fungus cells in the same samples are presented in Table 1.

The pH value of the sweetened black tea was approximately 7, and it dropped to about 4.1-4.5 immediately after the inoculation with the fermentation broth. In the first four days, the pH value decreased by a maximum 1 unit and in the next three days the increase was only 0.2 units, reaching about 3.7 pH units at the end of process. The changes in pH were similar for all used concentrations of lactose.

For all applied concentrations of lactose during Kombucha fermentation, clear differences were found in three parts for the content of titratable acidity. During the first 24 hours there was no sign of acid production, but in the next three days in media with 2 and 4% of lactose there was similar trend of increase in acid (for 3g/L) and in medium with 7% of

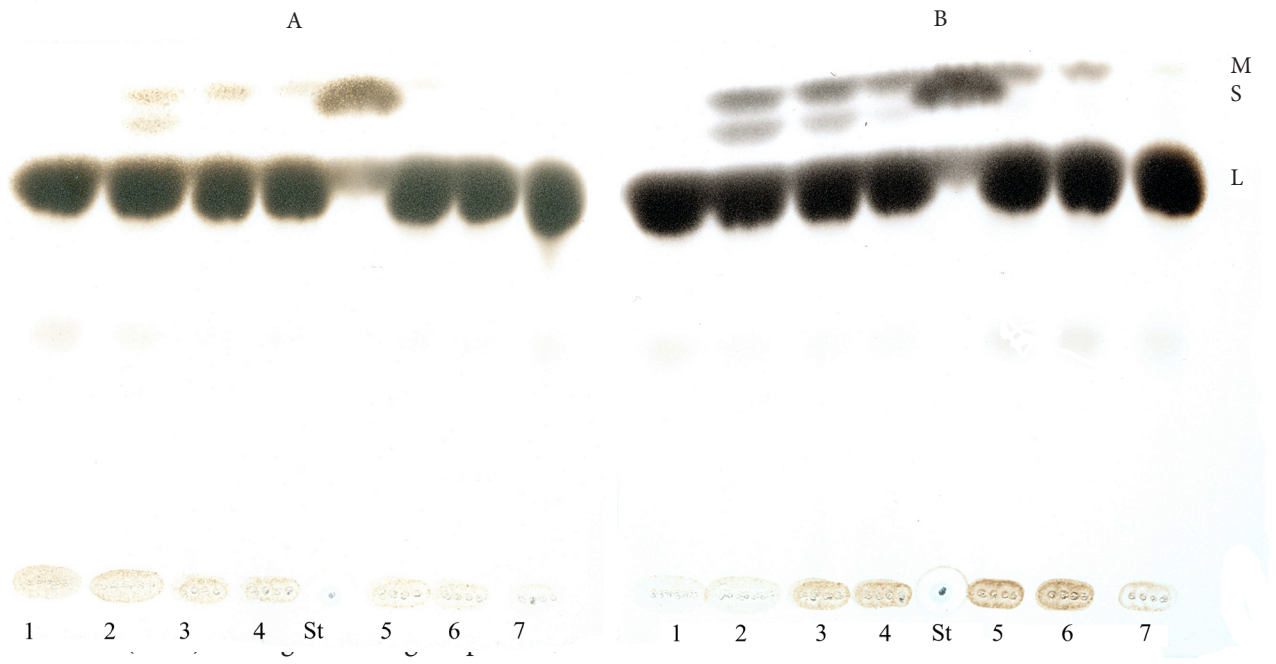


**Fig. 2.** TLC chromatogram of carbohydrates in the fermented medium sweetened with 4% lactose. **A** - inoculated with pellicle; **B** – inoculated with 10% of Kombucha beverage; M – monosaccharide; S – sucrose; L – lactose; 1 – prepared medium; 2 – inoculated medium; 3 – 7: fermented liquid after 1, 2, 3, 4 and 7 days; St - standard mixture: glucose, fructose, lactose (c= 1 mg/ml).

it was similar in all media. During the first day, the number of AAB increased by about 2 log units and then in the next two days it fell by about 0.5 units. By the end of the process, the number of AAB in the fermentation media decreased by 0.6-0.8 log units. The number of yeast cells and AAB was smaller than 2 log units during the whole process in all media inoculated with pellicle.

The selected chromatograms (Fig. 3) show that in the pellicle-inoculated medium there was no observed presence of monosaccharides.

*Change of basic parameters during biotransformation of unsweetened medium*

During four days of process, the pH values decreased, and had a similar course of change as the in control (traditional) process (Fig. 3). However, in the follow-up of the process a difference occurred because in the control process the pH continued to decrease and in the unsweetened medium it increased by 0.3 units. The inoculated media did not vary in the level of titratable acidity in the first 24 h, and then the value of titratable acidity in the control sample suddenly increased after 96 h, after which it slowly increased (Fig. 3). In the unsweetened medium, the initial titratable acidity value was same for 48 h, and in the next 48 h it increased by about 1g/L until the end, when it fell a little.

In the beginning of the process, an increased number of yeast cells could be noticed for about 0.8 log units; then until the end of the process it insignificantly fell; this was more expressed in the unsweetened medium (Table 2). The change in number of AAB was similar in both cases; in the beginning (in the first 48 h) a higher increase, by about 2 log units, and then a decrease of about 1 log unit.

The change in the qualitative profile of carbohydrates in the control Kombucha followed by chroma-

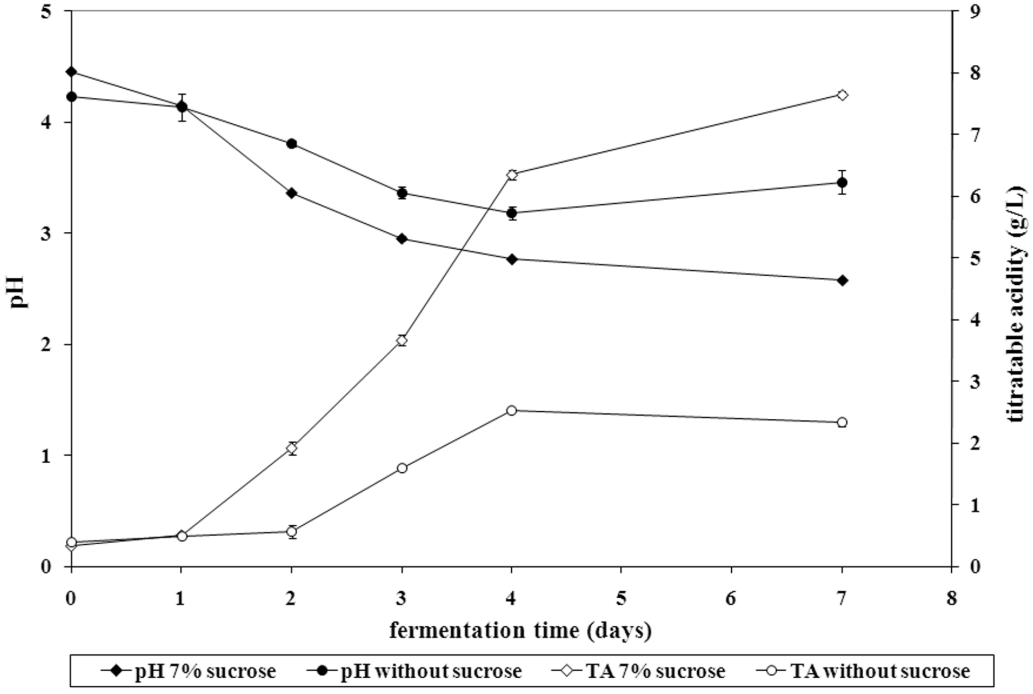


**Fig. 3.** Rate of change in pH and titratable acidity during the Kombucha fermentation of traditional medium and unsweetened tea.

**Table 2.** Changes in number (mean value ± SD) of "tea fungus" cells during process in control medium and unsweetened tea.

| Fermentation time (days) | Yeast (log CFU/mL) | | Acetic acid bacteria (log CFU/mL) | |
|---|---|---|---|---|
| | 7 % sucrose | Without sucrose | 7 % sucrose | Without sucrose |
| 1 | 6.65±0.02 | 6.65±0.10 | 6.54±0.25 | 5.83±0.03 |
| 2 | 6.67±0.08 | 6.65±0.11 | 6.68±0.11 | 6.66±0.04 |
| 3 | 6.42±0.06 | 6.42±0.08 | 6.45±0.31 | 6.38±0.03 |
| 4 | 6.42±0.07 | 6.33±0.20 | 6.33±0.20 | 6.31±0.04 |
| 7 | 6.63±0.16 | 6.41±0.12 | 6.41±0.09 | 5.53±0.14 |

**Table 3.** Ethanol and lactic acid content in different Kombucha fermented media.

| Fermented medium | Ethanol (g $L^{-1}$) (mean value±SD) | Lactic acid (mg $L^{-1}$; mean value±SD) | |
|---|---|---|---|
| | | D- | L- |
| 2% lactose | ≤ 9.3 x $10^{-5}$ | ≤ 0.2 | ≤ 0.2 |
| 4% lactose | ≤ 9.3 x $10^{-5}$ | ≤ 0.2 | ≤ 0.2 |
| 7% lactose | ≤ 9.3 x $10^{-5}$ | ≤ 0.2 | ≤ 0.2 |
| Unsweetened tea | ≤ 9.3 x $10^{-5}$ | ≤ 0.2 | ≤ 0.2 |
| Control medium | 4.53 ± 0.4 | 7.7 ± 0.5 | 38.4 ± 0.1 |

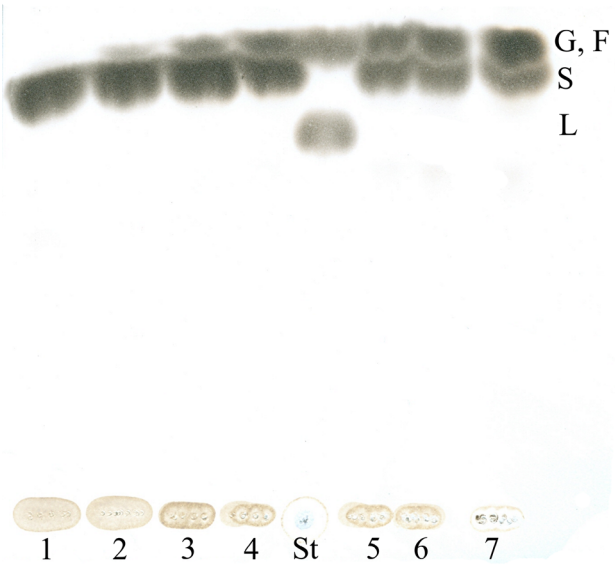


**Fig. 4.** TLC chromatogram of carbohydrates in common Kombucha fermentation
G i F – glucose and fructose; S – sucrose; L – lactose; 1 – prepared medium; 2 – inoculated medium; 3 – 7: fermented liquid after 1, 2, 3, 4 and 7 days; St – standard mixture: glucose, fructose, lactose (c = 1 mg/ml).

tography showed (Fig. 4) an increase in monosaccharide content, and at the end of the process the liquid had sucrose and its constituents.

*Ethanol and lactic acid in Kombucha beverages*

The ethanol content in the products obtained from media sweetened with lactose, as well as from the unsweetened medium, was below the detection limit of the enzyme method. In contrast, in the control Kombucha, the content of ethanol was over 100 times higher (Table 3). Similar results were obtained for lactic acid content. In the control Kombucha, D- and L-lactic acid were detected and in other samples, their content was near the detection limit.

## DISCUSSION

The changes in pH value during the process of biotransformation in the control Kombucha sample showed a strongly manifested physiological activity of yeast and bacteria. The trend of pH decrease was same as in other investigations (Cvetković, 2008) from cultures of tea fungus of the same origin. The same pH trend was observed by some other authors who used bioreactors with the same substrates for C and energy source, and similar liquid volumes. Thus, Blanc (1996) obtained a pH of about 3 after five days, Sreeramulu et al. (2000) and Veličanski et al. (2007) after four days. Increasing the titratable acidity content for obtaining optimal consuming acidity is four days, which is consistent with previous investigations (Četojević-Simin et al., 2010). In the case of the above-mentioned authors, the time was six days (Blanc, 1996), eight days (Sreeramulu et al., 2000) and four days (Veličanski et al., 2007).

The change in the number of yeast and AAB during the process is consistent with previous investigations and is comparable to other similar fermentation media. For example, Sreeramulu et al. (2000) obtained 4.48 log units of yeast cells in fermentation liquid after six days of process, while Chen and Liu (2000) obtained 7.55 log units. Teoh et al. (2004) found the count of individual yeast species on the sixth day of the process to be between 5 and 7 log units, depending on the species used.

The number of acetic acid bacteria at the end of the process (about 5.5 log units) is same as that determined by Belloso-Morales and Hernández-Sánchez (2003) or was higher than in similar fermentation media. Thus, Sreeramulu et al. (2000) obtained 5.3 log units, and Chen and Liu (2000) 4.5 log units. The differences in the number of cells of tea fungus in liquid can be explained by differences in the applied cultures and by the fact that part of bacteria are immobilized in the pellicle, same as in the sampling procedure.

According to our knowledge, the inoculation of an unsweetened base liquid has not been examined. The increase of titratable acidity and decrease of pH value, as well as the appearance of a thin pellicle, points out the viability and physiological activity of cells in these conditions (the number of yeasts and AAB is similar to the control liquid). With the

tea fungus, cells were transferred to a medium with a higher pH and lower osmotic pressure where they could use the available sugar, i.e. the monosaccharide and sucrose (Fig. 4). The content of residual sugar was determined by Sievers et al. (1995) and Blanc (1996). They deduced that in the beverage after 10 days about 1.5% of residual sucrose and fructose and 3% of glucose can be found. According to our investigation (unpublished data), the Kombucha beverage contains about 5% of dry matter, which is consistent with the literature. The process of Kombucha fermentation of culture tea fungus is finished by the fourth day and then strains of *Acetobacter* start to use the created acetic acid, which results in a decrease in the titratable acidity and an increase in the pH value (Kersters et al., 2006) versus trends in the control Kombucha. This beverage, which is slightly acidic, has a special quality because of its low caloric value. Consumers who cannot consume traditional Kombucha because of their health can consume this one.

The changes of all parameters during the process with different initial contents of lactose are similar, which leads us to conclude that there is no limit in relation to added sugar. More precisely, lactose quantity in a medium does not significantly affect the course of Kombucha fermentation. These results can be compared with the research of Reiss (1994) because of the similarity of the substrate and conditions of Kombucha fermentation. However, the results cannot be compared with those of Malaša et al (2009) because of the complexity of their substrate (pasteurized milk) with the initial contamination. Our results for the change in pH value, ethanol content and lactic acid are similar to the results of Reiss (1994). It is important to point out that in Reiss' research, media were inoculated with a layer of local tea fungus and 20% fermented Teakwass. The initial pH value decreased by about 1.5 units and fell until the end of the process (17 day), when it was close to the initial value. In other words, changes do not depend on the initial content of lactose (30, 50, 70 and 100 g/L). In addition, the level of ethanol in these types of media increased until the 6th day to 0.4 g/L and afterwards it fell to 0.01 g/L at the end of the process. Similar changes were noticed for the lactic acid content. The highest value was noticed on the second day of fermentation, and afterwards it fell and had a value of about 0.01 g/L. The whey that was used by Belloso-Morales and Hernández-Sánchez (2003) has some similarity with our substrate. The whey was inoculated with a local tea fungus culture. However, after a period of adaptation, about 80% yeast cells belonging to the species *Kluyveromyces marxianus* and *Brettanomyces bruxelensis*, known for their lactose fermentation abilities, appeared in the mentioned tea fungus. On the contrary, in our tea fungus, no lactose-positive yeasts species were detected (Markov et al., 2001). During the growing of tea fungus on whey, Belloso-Morales and Hernández-Sánchez (2003) noticed a change in AAB population.

In light of the similarities between the parameters (pH, TA, number of yeast and bacteria cell) obtained for the process in media with different contents of lactose and the parameters of the process on an unsweetened medium, the following assumptions can be made. The applied tea fungus is indifferent towards lactose and does not participate in the metabolism of yeast and acetic acid bacteria. All the reported changes are caused by the main path of Kombucha fermentation using carbohydrates introduced to the inoculum. Using this process, the obtained beverage was very similar to the original Kombucha beverage, but without ethanol and with a lower lactic acid content.

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