UDC 663.88.001.53:615.89
APTEFF, 36, 1-266 (2005) BIBLID: 1450–7188 (2005) 36, 187-196
Original scientific paper

PREPARATION OF KOMBUCHA FROM WINTER SAVORY (*Satureja montana* L.) IN THE LABORATORY BIOREACTOR

Dragoljub D. Cvetković and Siniša L. Markov

The possibility of obtaining kombucha from winter savory tea has been tested in the laboratory bioreactor by applying starter cultures and traditional way of inoculation. On the basis of the obtained results, it can be concluded that applying the inoculating method with the beverage from the previous process of biotransformation yielded kombucha beverage (capacity 15 l) from winter savory tea in the laboratory bioreactor. The application of defined starter culture from the isolate of yeast and acetic acid bacteria of local tea in the glass jar (capacity 5 l) gave 3 litres of kombucha beverage, which is acceptable according to the basic parametres and sensory characteristics. However, the application of the same starter culture in the laboratory bioreactor did not result in synchronized activity of yeast and bacteria.

KEY WORD: Kombucha; winter savory; bioreactor, starter culture

INTRODUCTION

Kombucha is a traditional fermented tea that has gained popularity across the world, as it is increasingly associated with health-promoting effects (1). It is slightly sweet, carbonated, acidic tea beverage. The product comprises of sugars, organic acids, tea compounds, vitamins and minerals, and reportedly exerts a number of healing effects, but there is no solid scientific evidence available yet for its efficacy (2, 3).

Sweetened black tea is substrate that is being biotransformed into kombucha beverage by symbiotic activities of yeasts and acetic-acid bacteria. Because of its chemical structure and positive effects on the process of biotransformation there are recommendations to use green tea instead of black one (4, 5). There are very few papers concerning the application of medicinal herbs for getting kombucha beverage. Reiss prepared the substrate from

Dragoljub Cvetković, M.Sc., Assist., Dr. Siniša Markov, Assoc. Prof., University of Novi Sad, Faculty of Technology, Department of Biotechnology and Pharmaceutical Engineering, 21000 Novi Sad, Bulevar Cara Lazara 1, Serbia and Montenegro

peppermint tea and lime blossom tea and according to the low level of production of a few metabolites he claimed that we can get real kombucha beverage just by using black or green tea (6). However, our results (7, 8) show that the leaves of medicinal-spicy herb *Satureja montana* L. (winter savory, local name Rtanj tea) can be successfully used for the preparation of kombucha beverage. The process of biotransformation of winter savory tea does not differ significantly from the traditional process with black tea (9), if we consider the pH value, total acidity and the number of yeast cells and acetic acid bacteria.

In the available literature (data base SCI for the period 1990-2004) there are no papers related to the preparation of kombucha in the vessels of greater capacity (for example, a few tenths of litres). The data of such a process are the basis for the process on a semi-industrial level, which is a necessary phase in the development of a process towards the industrial scale. Kombucha has been mainly prepared in small volumes (1 to 3 l) under home conditions. For the inoculums local tea fungi are used or commercially produced tea fungi in the form of colony/math (or "mushroom"). It is interesting to note here that in the scientific papers local tea fungus are mainly used, very often without mentioning the authentic origin (2-4,6).

Hesseltine was the only one who prepared kombucha from the black tea by applying isolates from tea fungus, i.e. by using starter culture (10). Just according to sensor grades, only known to kombucha expert, without chemical indicators, he claims that the typical product can only be produced by using all three isolates (one bacteria and two yeasts). It is interesting that beside the mentioned strains of the same tea fungus, a new strain has been isolated and described as a completely new type - *Zygosaccharomyces kombuchaensis* (11).

The aim of this paper was to investigate the preparation of kombucha from winter savory tea in the laboratory bioreactor by applying the starter cultures and traditional way of inoculating (by adding 10% (v/v) of the beverage from the previous fermentation).

EXPERIMENTAL

Substrate for kombucha fermentation was prepared by adding 70 g/l of commercial sucrose and 7.5 g/l dry leaves *Satureja montana* L. (winter savory tea, Rtanj tea) in boiled tap water. The tea leaves were steeped for 10 min and removed by filtration. After cooling to about 30^0C the substrate was separated into 3 l of sterile glass jars (capacity 5 l) with diametar of 17 cm and 15 l laboratory bioreactor, previously sterilized with hot water.

The process of biotransformation of winter savory tea has been performed at 28^0C.

Laboratory bioreactor (capacity 33 l and occupational capacity of 30 l) is a nonstandardized device that has been designed and made in our project. It is of a conical cyllindric shape (diameter of 30 cm, the angle at the top of the conus is 60^0) the same as with standard tanks in brewing industry.

It has been equipped by the devices for measuring and maintaining the temperature and the possibility of blowing in the sterile air onto medium surface. At the very bottom of the conical part there is a microbiological tap for taking samples.

Strains of yeasts (marked as 2/1, 5/3 and 7/2/) and acetic acid bacteria (marked as 5 and 9) used for the preparation of starter cultures, isolated from locally tea fungus (12), were deposited in the collection of microorganisms which belong to the Laboratory for Microbiology at the Faculty of Technology. Yeast and bacterial strains were grown and

maintained on YM agar (Difco Lab.). For the preparation of the inoculi the yeasts have been cultivated onto YM slant agar, and acetic acid bacteria in De Carr broth (13).

For the traditional inoculation with 10% (v/v) of beverage, kombucha produced from the same tea was used, produced for the period of 15 days.

pH value was measured using electronic pH-meter (MA 5730, «Iskra», Kranj, Slovenia).

Total acidity was determined by conductometric titration with sodium hydroxide (14).

The determination counts of yeasts cells and acetic acid bacteria was performed following previously described methods (15).

RESULTS AND DISCUSSION

A. The application of starter cultures in preparation of kombucha beverage from winter savory tea

In order to test the process of biotransformation of sweetened winter savory tea in large vessels two independent assays had been performed. The inoculum was prepared from the isolate consisting of the yeasts and acetic acid bacteria from the local tea fungus, i.e. the substrate was inoculated by a defined starter culture (Tables 1 and 2). For the control sample the same medium was used, but in the jar that was inoculated by the same inoculum. During the process of biotransformation the pH value of the fermentation liquid was measured and determined the content of the total acids as basic parametres of the process, and their values are given in Tables 3-5.

Table 1. Number of yeast cells (ml^{-1}) in suspensions for inoculation

Isolate	**Direct method**		**CFU**	
	I assay	II assay	I assay	II assay
5/3	$7.0x10^7$	$2.3x10^8$	$2.5x10^7$	$2.4x10^8$
7/2	$1.1x10^8$	$1.15x10^8$	$3.5x10^7$	$1.2x10^8$
2/1	$4.5x10^7$	$8.1x10^7$	$1.7x10^7$	$1.1x10^8$

Table 2. Number of acetic acid bacteria cells (ml^{-1}) in suspensions for inoculation

Isolate	**CFU**	
	I assay	II assay
5	$1.5x10^7$	$1.59x10^8$
9	$4.5x10^7$	$4.0x10^8$

At the end of the process of biotransformation the counts of yeast cells and acetic acid bacteria cells were determined by Koh's method. In the beverage of the first assay of the experiment the count of yeast cells was $4x10^5$ ml^{-1}, and of acetic acid bacteria cells

$2.5x10^6$ ml^{-1}. At the end of the process of biotransformation in the second assay there were $4.4x10^6$ ml^{-1} yeast cells and $2.5x10^6$ ml^{-1}acetic acid bacteria cells. In the initial suspensions of the first assay the number of bacteria and yeast cells was almost 10 times smoller than in the second assay. It can be stated that the same relation was preserved at the end of the process of kombucha fermentation as well.

Table 3. Change of the pH value of fermentation liquid during the process of biotransformation in the first assay

Fermentation time (day)	**Sample**	
	bioreactor	control jar
4	4.34	4.01
6	4.11	3.82
8	3.98	3.69
11	3.85	

Table 4. Change of the total acidity (g/l) content during the process of biotransformation in the first assay

Fermentation time (day)	**Sample**	
	bioreactor	control
4	0.93	1.80
6	1.89	3.09
8	2.506	4.40
11	4.12	

Table 5. Changes of the pH value and total acidity during the process of biotransformation in the bioreactor from the second assay

Fermentation time (day)	**3**	**6**	**7**	**8**	**9**	**10**	**11**
pH value	5.0	4.13	3.99	3.92	3.80	3.70	3.68
Total acidity (g/l)	0.77	1.72	2.08	2.16	2.43	2.70	2.84

In both of these two assays pH values of uninoculated winter savory tea was 7.42, and of the inoculated with starter culture 6.11, whereas the total content of acid at that pH value was 0.08 g/l. The same trends of changes were present in both experiments. After the initial abrupt decrease, in the further process the pH value changed very little (Table 3). The total acidity content increased moderately from the fourth to the eleventh day in the

fermentor medium (Tables 4 and 5). The comparison of the results in respect of cell counts in the inoculi (Tables 1 and 2) and the total acidity content (Tables 4 and 5) indicates that in kombucha fermentation there is no relationship with the count of cells, but with the physiological state of the cells.

According to the change of pH value and total acidity content the process of biotransformation of winter savory tea into kombucha beverage in both types of vessels is similar to the changes that other authors have reported for preparing kombucha from black tea (16, 17).

After the eleventh day of biotransformation the beverage obtained from the bioreactors in both experiments was sensory evaluated as sweet, insufficiently sour, slightly bitter, and with significant content of ethanol. Contrary to these samples, the beverage from the jar was evaluated as acceptable. A possible explanation for disturbing the balance of the biotransformation process towards alcohol fermentation is that there are enough acetic acid bacteria, but they do not demonstrate their activity in the bioreactor as they do when they are in smaller vessel, i.e. in the jar. In addition, the yeasts, because of their high count and activity disturb the balance in the symbiotic community. Checking this hypothesis was undertaken with the beverage from the second assay. Namely, three litres of biotransformed liquid was taken from the bottom and the middle of the bioreactor, sampled and transferred to the jars and left at the temperature of 28^0C. After two days of the additional process (i.e. on the thirteenth day from the beginning of the process) the basic parametres remained almost unchanged: pH value was 3.53, and the total acidity content was 4.15 (g/l). The added time of two days did not influence significantly the activities of the yeasts and acetic acid bacteria of the tea fungus, but on the surface of the liquid there appeared thin, transparent, celuloid membrane. After prolonging the process of biotransformation for five days, the parameters of the process were as follows: pH value was 3.48, and the total acidity content raised to 5.47 g/l. Finally, after nineteen days of prolonging the process the basic parameters of biotransformed liquid from the bottom of the bioreactor were pH 3.32 and the total acidity content 10.25 g/l. According to these results it can be concluded that the biotransformed liquid from the bioreactor contains necessary number of cells of acetic acid bacteria, which can, under certain conditions, exhibit physiological activity.

On the basis of the experiment carried out in the bioreactor by applying the isolate of yeasts and acetic acid bacteria of local tea fungus – starter culture, we can conclude that the process of fermentation can be done with larger volume of the fermentation medium in relation to the volume of the substrate in the jars. In order to lead the process of biofermetation towards obtaining the kombucha beverage of desired sensory characteristics, it is necessary to change the conditions of the process in the way that would activate acetic acid bacteria, i.e. eliminate factor(s) that hinder simultaneous activity of microbial culture of tea fungus.

B. The application of traditional inoculum in the preparation of kombucha beverage from winter savory tea

The procedure of obtaining kombucha beverage from Rtanj tea in the laboratory fermentor by applying the traditional process of inoculating, by adding 10% (v/v) of the beverage from the previous fermentation, was followed in two independent (assays) repetitions. As a control, two jars of 5 litres containing 3 litres of substrate were used.

The parameters of the inoculi are given in table 6, and the changes in the process of kombucha fermentation in figures 1 and 2.

Table 6. Parameters of inoculi

	pH	Total acidity (g/l)
Assay I	3.76	2.48
Assay II	3.62	3.92

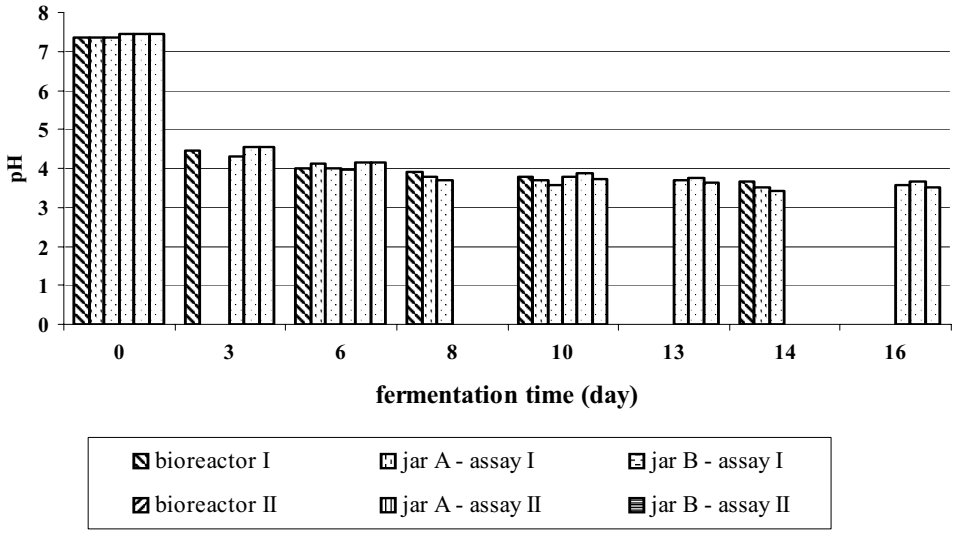

Fig. 1. Change of pH value during biotransformation process of winter savory tea to kombucha

During the process of kombucha fermentation in both experiments the pH changed almost equally, no matter which jar was used. In the first three days the process of pH value decreased by 3 units, so that in the next three days it decreased by the further 0.3 units. In the next 8 to 10 days the pH value decreased by 0.4-0.5 units. The changes of pH during these fermentations are in accordance with the results obtained during kombucha fermentations in the jars of different volumes with the different value of substrate that inoculated by pellicle (unpublished data).

The changes of total acidity during kombucha fermentation in both expriments did not differ, i.e. the same tendencies related to certain jar volumes were observed. However, there are some differences in accumulating the acid between the vessels. In the beginning of the process, the major amount of acid is created in the bioreactor. In the middle phase of the process, i.e. between the sixth and the tenth day, the content of total acidity equalized and started to increase in the smaller vessels. In the final phase of the process, this trend continued, so that we can find sourer beverage in the jars. The differences in the dynamics of acid formation can be connected to the vessel configuration. The anaerobic conditions are firstly produced in the bioreactor, which is a prerequisite for the beginning of the process of fermentation. On the contrary, in the jars, the oxygen is longer present in the base and suits the respiratory activity of the yeast, i.e. its growth and reproduction. After estab-

lishing the balance in the lower part of the jar, the anaerobic conditions are produced that support the fermentation. The ethanol formed that way is being transformed to acetic acid, so that in the beginning the total acidity is a measure for the speed of yeast performing.

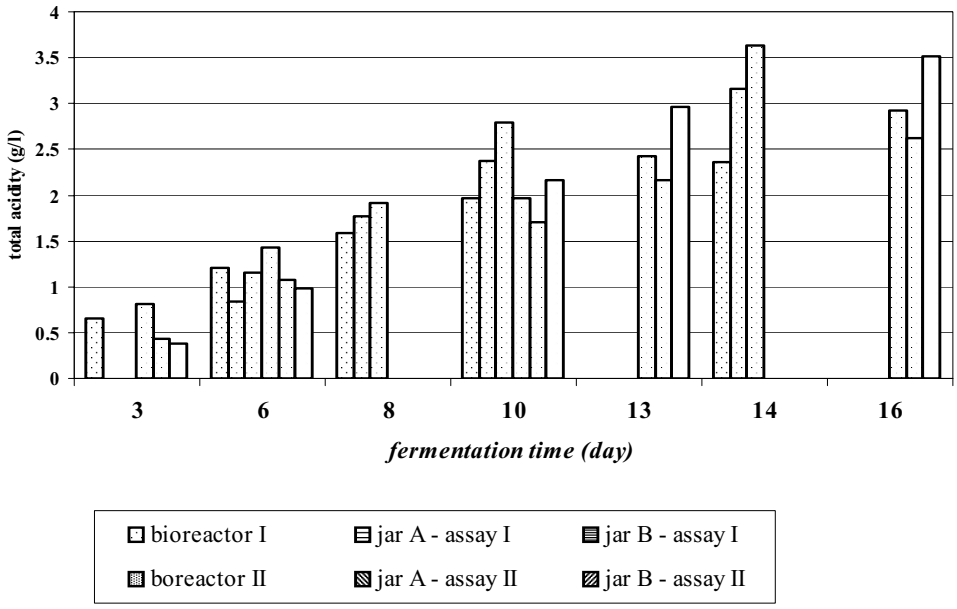

Fig. 2. Total acidity during biotransformation process of winter savory tea to kombucha

After some time, during the process, the performing speed of acetic acid bacteria is being expressed fully. It should be highlighted here that fermenting liquids in both types of vessels (both bioreactors and jars) do not differ significantly in respect of the number of viable yeasts and bacteria cells (the results are not given). Main physiological activity of acetic acid bacteria in the kombucha fermentation is the conversion of ethanol into acetic acid in the aerobic conditions of the surrounding. For the realization of this cycle of reactions, it is necessary to ensure a permanent supply of ethanol. However, if the bottom of the vessel is the main place for producing ethanol by tea fungus yeasts and the surface of the vessel the main place for producing acetic acid by acetic acid bacteria, it is obvious that the rate of the process in both types of vessels can not be the same for the relation of D/H is different (in the bioreactor the ratio diameter/height is D/H = 300/365 = 0.82, and in the jars D/H = 160/155 = 1.03). The force of diffusion moves the produced ethanol to the upper layer and becomes the substrat for bacteria. Also, the surface/volume (P/V) ratio is different in the two types of vessels (in the bioreactor P/V = 0.0047 (cm^{-1}), and in the jars P/V = 0.065 (cm^{-1})). The surface of the vessel is the only place for transmitting the oxygen from the air onto the fermentation liquid, and it is necessary for the bacteria to express their metabolic activities. With a similar cell count for the particular volume unit there is less possibility for transmitting the oxygen, and at the same time for producing acetic acid.

Sensory evaluation of kombucha beverage from the second experiment was performed by 6 appraisers-laymen, but who are consuming it for several years, are given in Table 7.

Table 7. Sensor grades for kombucha beverage from the bioreactor and the traditional jars

Sensor indicators	Traditionally prepared kombucha	Kombucha from the laboratory bioreactor
smell	6A	3A + 3B
taste	6A	2A + 4B
sourness	6A	3A + 3B
sweetness	6A	4A + 2B
general impression	6A	3B + 3A

A - perfect to minor deviations; B - slightly noticeable deviation to noticeable defect; C - strong defects to completely changed

The main defects of the beverage from the fermentor were insufficiently distinctive smell, lower acidity, i.e. major sweetness of the beverage. None of the appraisers noticed either smell or taste of alcohol, which means that the process in the bioreactor was not anomalous, but just that it was slower than the process that was going on in the jar. We can suppose that with prolonging the process until the content of total acidity above 3 (g/l), we would get the beverage of sensory characteristics that would not differ from the characteristics of the traditionally prepared beverage.

For bioreactor's kombucha beverages, from both assays, the possibility of applying them in the next process of fermentation was tested. Samples (of 3 litres) from the bottom and the middle of the fermentor were transmitted into the jars (of 5 litres) and the process of kombucha fermentation was continued. The parameters of beverages that were obtained after 8 additional days of the process are given in Table 8.

Table 8. Characteristics of the beverages obtained by the continuation of kombucha frementation in the jars

	Fermenting liquid from			
	bottom of bioreactor		middle of bioreactor	
	pH	total acidity (g/l)	pH	total acidity (g/l)
Assay I	3.50	3.32	3.44	3.81
Assay II	3.50	3.30	3.44	3.81

On the basis of the obtained results we can conclude that all the beverages contain the potential for the continuation of the process of kombucha fermentation. It is evident that there is no difference regarding the place where the samples were taken for prolonging the process. The difference in the content of total acidity of 0.5 (g/l) between the samples of the first and second assay probably caused by the initial difference (0.4 g/l) in total

acidity before the continuation of the process of kombucha fermentation. Besides, it can also be noticed that in the continuation the rate of the process was exactly the same in all vessels.

CONCLUSION

On the basis of the above it is possible to drow the following conclusions:

- By applying kombucha beverage in the amount of 10% (v/v) it is possible to produce acceptable kombucha beverage from winter savory tea from 3 to 15 litres;
- By applying the defined starter culture from the isolate of yeasts and acetic acid bacteria it is possible to obtain the acceptable kombucha beverage from winter savory tea in the glass jars of 3 litres:
- By applying the same defined starter culture, under the same conditions as in the glass jars it is not possible to obtain the acceptable kombucha beverage from winter savory tea in the laboratory bioreactor (capacity of fermentation medium is 15 litres).

ACKNOWLEDGMENT

We are thankful to the Ministry of Science and Environmental Protection of the Republic of Serbia for the financing this study as a part of Project BTN.7.2.3.0424B.

REFERENCES

1. Greenwalt, C. J., K.H. Steinkraus and R. A. Ledford: Kombucha, the Fermented Tea: Microbiology, Composition, and Claimed Health Effects. J. Food Protect. **63** (2000) 976-981.
2. Janković, I. and M. Stojanović: Microbial and Chemical Composition, Growth, Therapeutical and Antimicrobial Characteristics of Tea Fungus. Mikrobiologija **31** (1994) 35-43.
3. Janković, I. and M. Stojanović: Acidogenic and Alcohologenic Properties of Tea Fungus Microorganisms. Mikrobiologija **32** (1995) 21-27.
4. Greenwalt, C. J., R. A. Ledford and K. H. Steinkraus: Determination and Characterization of the Antimicrobial Activity of the Fermented Tea Kombucha. Lebensm.-Wiss. Technol. **31** (1998) 291-296.
5. Dufresne S. and E. Farnworth: Tea, Kombucha, and health: a review, Food Resear. Int. **33** (2000) 409-421.
6. Reiss, J.: Der Teepilz und seine Stoffwechselproducte, Dtsch. Lebensm-Rundsch. **83** (1987) 286-290.
7. Cvetković, D.: Metabolic activity of tea fungus on different medium, M.Sc. thesis, Faculty of Technology, University of Novi Sad, 2003.
8. Cvetković, D.D. and S. L. Markov: Cultivation of Tea Fungus on Sweetened Winter Savory (*Satureja montana* L.), III Međunarodna eko-konferencija, 22-25.09.2004., Novi Sad, Proceedings, Book II, p. 379-384.

9. Markov, S., D. Cvetković and B. Bukvić: Use of tea fungus isolate as starter culture for obtaining of kombucha", 8th International Symposium Interdisciplinary Regional Research Hungary-Romania-Serbia and Montenegro, Szeged 19-21.04.2005, proceedings, CD-ROOM FFP08.
10. Hesseltine, C. W.: A millenium of fungi, food and fermentation, Mycologia **57** (1965) 149-197.
11. Kurtzman, C. P., C. J. Robnett and E. Basehoar-Powers: *Zygosaccharomyces kombuchaensis*, a new ascosporogenous yeast from "Kombucha tea". FEMS Yeast Research **1** (2001) 133-138.
12. Markov, S. R. Malbaša, M. Hauk and D. Cvetković: Investigation of Tea Fungus Microbe Assotiations. I The Yeasts. Acta Periodica Technologica **32** (2001) 133-138.
13. Divies, C. and R. Cachon: Alterations par les bacteries acetiques - in Oenologie, Fondaments scientifiques et technologiques, C. Flanzy (ed.), Londres, Paris-New York (1998) 539-554.
14. OIV Recuiel des methodes internationales d' analyse des vins et des monts, OIV, Paris (1990) 155-159.
15. Cvetković, D. and S. Markov: Cultivation of tea fungus on malt extract medium. Acta Periodica Technologica **33** (2002) 117-124.
16. Sievers, M., C. Lanini, A. Weber, U. Schuler-Schmid, and M. Teuber: Microbiology and Fermentation Balance in a Kombucha Beverage Obtained from a Tea Fungus Fermentation. Syst. Appl. Microbiol. **18** (1995) 590-594.
17. Sreramulu, G., Y. Zhu and W. Knol: Kombucha Fermentation and Its Antimicrobial Activity. J. Agric. Food Chem. **48** (2000) 2589-2594.

ДОБИЈАЊЕ КОМБУХЕ ОД РТАЊСКОГ ЧАЈА (*Satureja montana* L.) У ЛАБОРАТОРИЈСКОМ БИОРЕАКТОРУ

Драгољуб Д. Цветковић и Синиша Л. Марков

Испитана је могућност добијања комбухе од ртањског чаја у лабораторијском биореактору применом стартер култура и традиционалног начина инокулисања. На основу добијених резултата може се закључити да се применом инокулисања са напитком из претходног процеса биотрансформације може добити комбуха напитак (запремина 15 лит.) од ртањског чаја у лабораторијском биореактору. Применом дефинисане стартер културе од изолата квасаца и бактерија сирћетног врења пореклом од локалне чајне гљиве у стакленим теглама (запремине 5 лит.) добија се 3 лит. комбуха напитка, прихватљивог по неким параметрима и сензорним својствима. Међутим, применом исте стартер културе у лабораторијском биореактору не долази до синхроног деловања квасаца и бактерија, односно добија се напитак са израженим дефектима.

Received 30 May 2005
Accepted 27 September 2005