

RESEARCH PAPER

Withdrawal of Stimulants from Tea Infusion by SCOBY During Kombucha Fermentation: A Biochemical Investigation

Soumya Majumder, Arindam Ghosh, Sourav Chakraborty and Malay Bhattacharya*

Molecular Biology and Tissue Culture Laboratory, Department of Tea Science, University of North Bengal, Siliguri, Darjeeling, Pin.: 734013, India

*Corresponding author: malaytsnbu@gmail.com

Paper No.: 3

Received: 17-02-2020

Revised: 21-05-2020

Accepted: 27-05-2020

ABSTRACT

Caffeine content in kombucha is very much lower than that of normal tea infusion. Though this stimulant plays an important role in kombucha fermentation as it regulates the starter or SCOBY (symbiotic colony of bacteria and yeasts) to produce the cellulose network on broth to accelerate the fermentation process. This research was designed to investigate the aftermath of caffeine and related tea alkaloids in kombucha through preliminary biochemical tests and chromatographic analysis where both the broth (beverage) and SCOBY (the cellulose layer) were taken as individual samples. The beverage clearly revealed negative in all the tests where the SCOBY extract showed richness in alkaloid content. Moreover, GC-MS analysis revealed presence of caffeine (8.7%); guanosine (12.01%), the precursor of caffeine; thymine (4.08%); and some undesirable components which has confirmed that SCOBY has an ability to capture a huge amount of stimulants from tea during fermentation.

Keywords: Caffeine, stimulants, tea, kombucha, SCOBY

It is widely accepted that tea leaf and its infusion contain caffeine and other stimulants or alkaloids in significant quantities. Consequently, it is also reported that these stimulants (especially caffeine) are either absent or found in a very low quantity in the probiotic fermented version of tea beverage, called kombucha. According to Malbaša *et al.* (2006), quantity of these alkaloids gets decreased during fermentation of tea to kombucha. Interestingly, reports have confirmed that caffeine and other compounds can stimulate the ability of *Acetobacter xylinum* (primary bacterium of SCOBY) to produce a cellulose network, the base of kombucha's microflora (Fontana *et al.* 1991). According to Greenwalt *et al.* (2000), methylxanthines and other stimulants can also inhibit the normal switch-off mechanism of cellulose

synthase which may also help to accelerate the formation of that cellulose network. However, there are no report that can clarify the existence of those stimulants in kombucha; whether as by-products of microbial metabolism during fermentation or its involvement into some other microbial pathways inside the SCOBY. This throws a doubt on the fate of caffeine and other alkaloids of tea infusion in its fermented form. So, to find out the aftermath of those components in kombucha, we prepared and carried out preliminary biochemical tests, followed

How to cite this article: Majumder, S., Ghosh, A., Chakraborty, S. and Bhattacharya, M. (2020). Withdrawal of Stimulants from Tea Infusion by SCOBY During Kombucha Fermentation: A Biochemical Investigation. *Int. J. Food Ferment. Technol.*, **10**(1): 21-26.

Source of Support: None; **Conflict of Interest:** None



by GC-MS analysis to put up a scientific explanation for better understanding. The results have been described here in this communication.

MATERIAL AND METHODS

Preparation of samples

A strong tea infusion was prepared with CTC tea (1% w/v) for fifteen minutes so as to obtain alkaloids in the infusion as much as possible. Sucrose (6% w/v) was used as nutrient for the microbial culture inside the tea broth. Following protocols of Greenwalt *et al.* (2000) and Zhu *et al.* (2014), a total of five batches of kombucha batches were prepared and incubated under a sterile condition. Healthy batch of kombucha was selected after 30 days of incubation. The broth or kombucha (KB) and the thick floating SCOBY were taken out separately as different samples for further experiments. For obtaining extract, SCOBY was squeezed hard to separate SCOBY extract (SE) and the dry and hard remaining cellulose was discarded (Fig. 1 a, b, c).

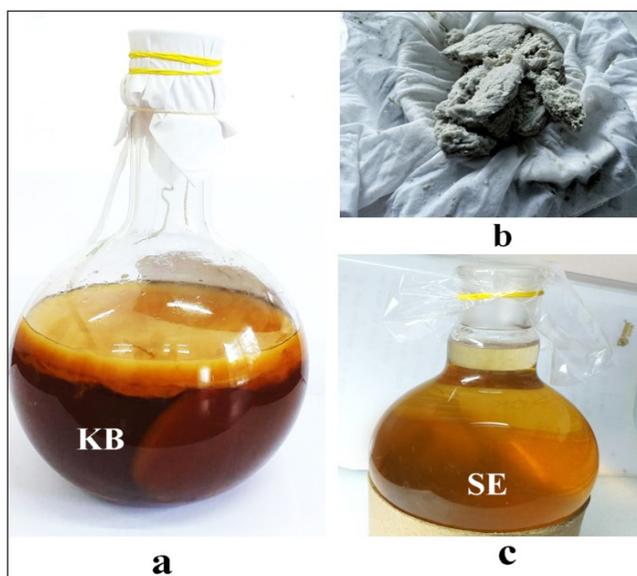


Fig. 1: Image of (a) selected broth containing SCOBY, (b) squeezed cellulose and (c) SCOBY extract

Preliminary biochemical tests

Tests were performed with both KB (Kombucha broth) and SE (SCOBY extract) to detect alkaloids

and caffeine. Alkaloids were tested by Mayer's, Wagner's and Marme's tests (Santra *et al.* 2006) where formation of precipitate confirms the presence of alkaloids. Mayer's reagent is used to detect alkaloids in natural products. Mayer's reagent was freshly prepared by dissolving mercuric chloride (1.36 g) and of potassium iodide (5 g) in 100 ml of distilled water. Most alkaloids are precipitated by Mayer's reagent and give a cream coloured precipitate. For Wagner's test, 1 ml of diluted HCl and Wagner's reagent (solution of iodine in potassium iodide) were added to 2 ml of sample and mixed. Formation of reddish-brown precipitate showed the presence of alkaloids. In 3 ml of each sample, a few drops of Marme's reagent (20 g of KI in 20 ml of distilled water; 10 g of CdCl₂ in 50 ml distilled water) were added and diluted H₂SO₄ was then added to the mixture. Yellowish white precipitate shows presence of alkaloids. Caffeine detection was carried on following murexide test (Santra *et al.* 2006) where appearance of purple colour confirms the presence of caffeine.

Following the technique of column chromatography (Bhattacharya *et al.* 2009), we separated caffeine and related alkaloids from both KB and SE through a silica gel (200-400 mesh size) packed column. As analyte, 10 ml of each KB and SE were used and for mobile phase, two solvents (chloroform and dichloromethane) were selected on the basis of affinity of targeted compounds towards solvents. Each fraction was collected individually, dried and tested for caffeine confirmatory test (the murexide test).

Gas chromatography mass spectrometry analysis

KB and SE (1 ml of each) were taken in individual test tubes and left for complete air drying. The dried samples were finally dissolved in 1 ml of ethanol prior to GC-MS analysis. The analysis was performed by GCMS-QP2010 Plus (Shimadzu Corporation, Kyoto Japan) attached with a Rxi-5 fused-silica capillary column (0.25 μm film thickness, 0.25 mm internal diameter and 30 m of length) following the protocol of Majumder *et al.* (2020).

RESULTS AND DISCUSSION

Preliminary biochemical tests

The results of preliminary tests (alkaloid and caffeine detection tests) show the absence of any alkaloid in beverage or KB. Moreover, the murexide test for caffeine detection gave a positive result for SE only. These results clearly showed that SE contains the alkaloids of tea and one of the major components was caffeine unlike the komucha (KB). Furthermore, it confirmed the earlier reports of Greenwalt *et al.* (2000) and Malbaša *et al.* (2006) as they stated that caffeine and other alkaloids of tea get decreased during kombucha fermentation.

In column chromatographic separation of caffeine and related components, chloroform and dichloromethane were used as mobile phase because our targeted compounds are already reported to be highly soluble in those two organic solvents (Jaber-Vazdekis *et al.* 2006; Shalmashi and Golmohammad, 2010). Presence of caffeine was simply recognized in the fractions which showed white crystalline structures. Furthermore, the murexide test was again

assessed to confirm presence of caffeine and related compounds and to check whether KB contains any traces of such compound or not. But, the murexide test also resulted negative for KB.

Meanwhile, results of the above experiments confirmed that sample SE contains caffeine and related alkaloids in it unlike the beverage. These result have not only proven that SCOBY needs caffeine and other stimulants for growing and stimulating the fermentation process but also suggested that alkaloids or stimulants (like caffeine), a major part of tea may get removed from the infusion as SCOBY picks all of them during kombucha fermentation. Further, to evaluate and judge the results of these tests, GC-MS analysis was performed as described earlier.

Gas chromatography mass spectrometry analysis

GC-MS chromatograms are given in Fig. 2 and peak reports of both KB and SE are represented in Table 1 and Table 2 respectively.

Expectedly, SCOBY extract (SE) showed richness in caffeine content with an amount of 8.70% while, in

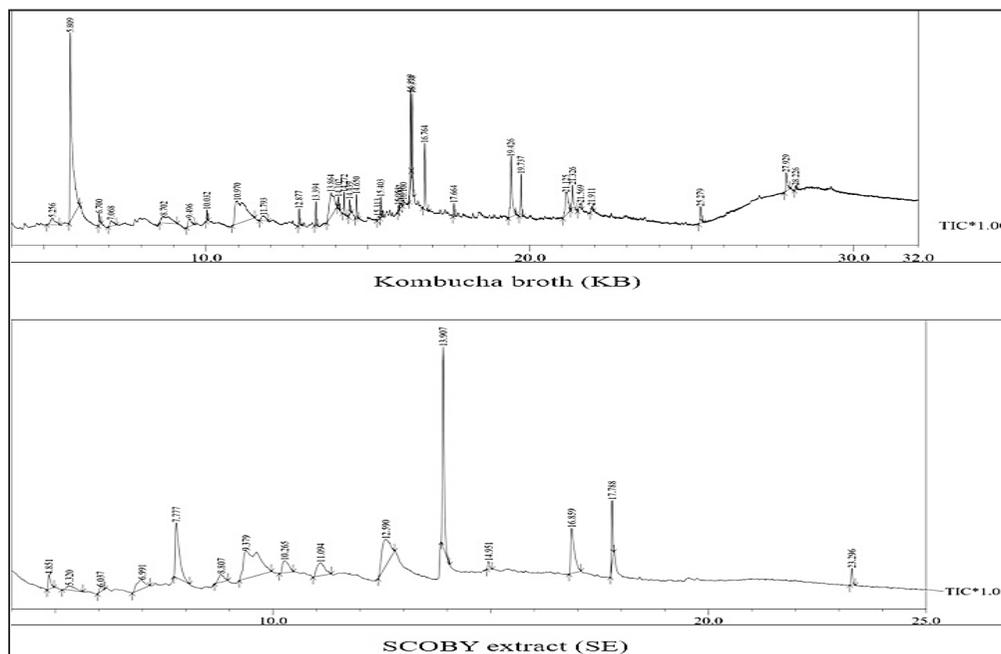


Fig. 2. GC-MS chromatograms

Table 1: GC-MS peak report of kombucha beverage (KB)

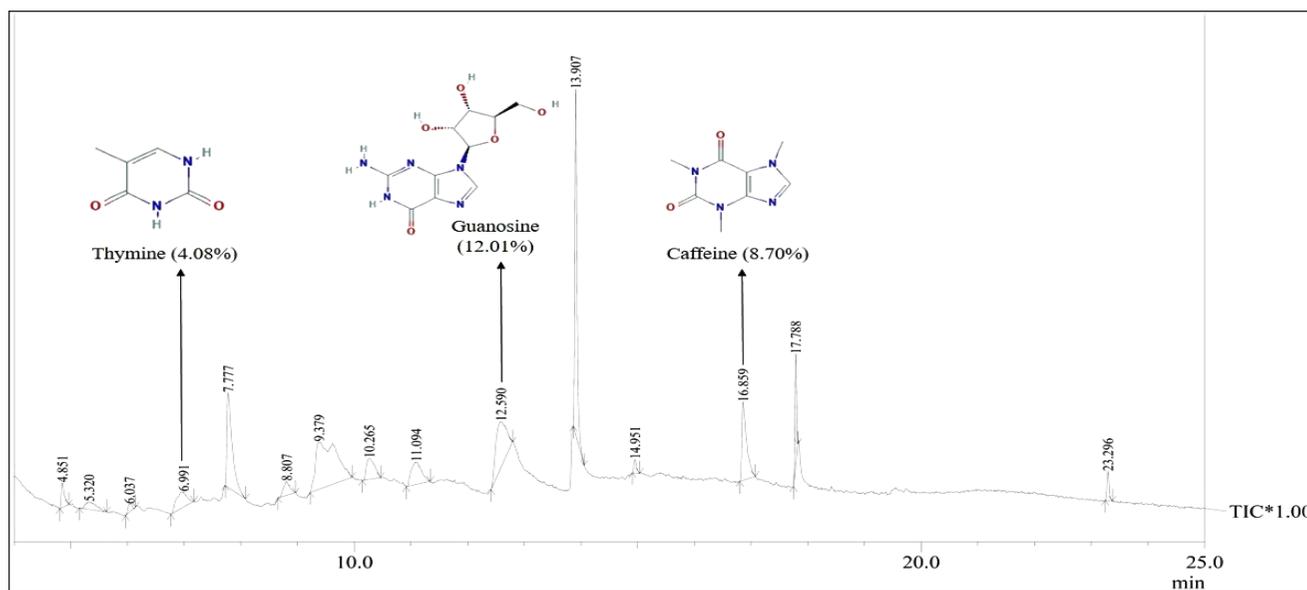
Peak#	R.Time	Area	Area%	Compounds of KB
1	5.256	205343	1.9	Acetamide, N,N'-carbonylbis-
2	5.809	2847707	26.41	2-Cyclopenten-1-one, 2-hydroxy-
3	6.7	80876	0.75	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one
4	7.068	118295	1.1	2H-Pyran-2,6(3H)-dione
5	8.702	423879	3.93	Glycine, N-(trifluoroacetyl)-, 1-methylpentyl ester
6	9.496	150019	1.39	1,5-anhydro-6-deoxyhexo-2,3-diulose
7	10.032	51040	0.47	1-Heptanol, 6-methyl-
8	10.97	1547557	14.35	5-Hydroxymethylfurfural
9	11.793	140443	1.3	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-
10	12.877	97534	0.9	1-Tridecanol
11	13.394	118862	1.1	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8
12	13.864	739740	6.86	1,8-Nonadien-3-ol
13	14.102	66317	0.61	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methy
14	14.272	124985	1.16	(1S,5S)-2-Methyl-5-((R)-6-methylhept-5-en-2-yl)bicyclo[3.1
15	14.439	87835	0.81	Phenol, 3,5-bis(1,1-dimethylethyl)-
16	14.65	139087	1.29	(1R,5R)-4-Methylene-1-((R)-6-methylhept-5-en-2-yl)bicycle
17	15.313	26808	0.25	1-Isopropylbenzocyclobuten-1-ol
18	15.403	107623	1	Pentafluoropropionic acid, undecyl ester
19	15.953	29755	0.28	2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl-
20	16.007	40408	0.37	2-Oxadamantane-1-carboxamide, N-(1-phenylethyl)-
21	16.1	46858	0.43	1-Methyl-1-isopropoxy-1-silacyclohexane
22	16.319	503249	4.67	ar-Turmerone
23	16.378	464133	4.3	Tumerone
24	16.764	424480	3.94	Curlone
25	17.664	63489	0.59	1-Hexadecanol
26	19.426	675715	6.27	n-Hexadecanoic acid
27	19.737	266947	2.48	Oxalic acid, monoamide, N-(3,4-dimethylphenyl)-, heptyl est
28	21.125	444757	4.12	cis-9-Hexadecenal
29	21.326	233163	2.16	Octadecanoic acid
30	21.569	91559	0.85	(Z)-Ethyl heptadec-9-enoate
31	21.911	49358	0.46	Unknown compound
32	25.279	151135	1.4	1,2-benzenedicarboxylic acid, diisooctyl es
33	27.929	196112	1.82	9-octadecenamide
34	28.226	29515	0.27	(3,7-dimethyl-octa-2,6-dienylsulfanyl)-benz
		10784583	100	

the beverage or KB, there was no traces of caffeine and related compounds. Moreover, besides caffeine, another alkaloid and major purine nucleotide of tea, guanosine (as guanosine hydrate) was detected as the major compound of SE with a peak area of 12.01%. Interestingly, the compound guanosine is also known to be present in tea leaves and that too as a precursor of caffeine (Negishi *et al.* 1992). Now, if we accept the fact that SCOBY has captured all the stimulants from broth, then presence of another

SE component; e.g. thymine (4.08%), a pyrimidine plant stimulant (Šormová *et al.* 1960), should also be taken into consideration along with caffeine and guanosine. However, in KB, there were neither any traces of above mentioned compounds nor any other stimulants. So, this is definitely a remarkable confirmation to our interpretation that microflora of SCOBY biofilm has picked up all the tea stimulants from kombucha which have been detected by GC-MS (Fig. 3). Besides alkaloids, presence of an infamous

Table 2: GC-MS peak report of SCOBY extract (SE)

Peak#	R.Time	Area	Area%	Compounds of SE
1	4.851	193897	1.89	2,4-dihydroxy-2,5-dimethyl-3(2h)-furan-3-one
2	5.32	200609	1.96	1,4-diazabicyclo[2.2.2]octane
3	6.037	74123	0.72	1-propanol, 2-methyl-2-[(2-methyl-2-propenyl)oxy]-
4	6.991	418123	4.08	Thymine
5	7.777	1223451	11.93	4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-
6	8.807	214122	2.09	1,3-Dioxolane, 4-methylene-
7	9.379	2324289	22.66	5-Hydroxymethylfurfural
8	10.265	415246	4.05	2,4(3H,5H)-furandione, 3-propyl-
9	11.094	559322	5.45	Isovaleric acid
10	12.59	1231914	12.01	Guanosine
11	13.907	1821421	17.75	Hexadecane
12	14.951	89934	0.88	1,6-methanonaphthalen-1(2h)-ol, octahydro-4,8a,9,9-tetramethyl-,
13	16.859	892196	8.7	Caffeine
14	17.788	448012	4.37	Dibutyl phthalate
15	23.296	152277	1.48	1,2-Benzenedicarboxylic acid
		10258936	100	

**Fig. 3:** Chromatogram of SCOBY extract showing peaks of caffeine, guanosine and thymine

flaw compound of wines, isovaleric acid (5.45%); a toxic compound, 1,3-Dioxolane, 4-methylene- or DABCO (1.96%) and a susceptible oxidative agent, 1,3-dioxolane, 4-methylene (2.09%) in SE is also a good sign as these substandard and unacceptable components were completely absent in KB or might have been removed by the biofilm in the same way like the stimulants. However, isovaleric acid is

also reported as a metabolite of *Brettanomyces* sp. (Licker *et al.* 1998), a common yeast found in SCOBY (Greenwalt *et al.* 2000).

The GC-MS analysis and other biochemical tests have helped to discover that both the beverage and the biofilm extract, may be a part of the same broth, but, are distinct to each other regarding their chemical characters. Moreover, the chemical profile

has also been unfolded for SCOBY extract through this set of experiments. *Acetobacter xylinum* has been previously reported to produce the cellulose network stimulated by caffeine and other stimulants (Fontana *et al.* 1991; Greenwalt *et al.* 2000). But, through a biochemical analysis, it is very hard to identify responsible and specific organism(s) involved in picking up stimulants like caffeine and its precursor guanosine. Moreover, caffeine is also known to exhibit antimicrobial activity. So, chances may be less for other microorganisms to capture this big amount of caffeine from tea infusion. Furthermore, microbiological and metabolic pathway studies on SCOBY in future are needed to reveal the same.

CONCLUSION

Biochemistry of kombucha has already been well explored by various scientific communities where SCOBY extract remained untouched in the field of metabolomics. Removal of caffeine and other alkaloids by SCOBY have been explored by this study and through which we have come into a simple but effective conclusion that SCOBY of kombucha, not only helps in fermentation but also carry on some unexpected metabolic processes where it takes up all the stimulants and unpleasant compounds from kombucha to make the beverage completely decaffeinated and healthy as well.

REFERENCES

- Bhattacharya, M., Mandal, P. and Sen, A. 2009. *In vitro* detection of antioxidants in different solvent fractions of Ginger (*Zingiber officinale* Rosc.). *Indian Journal of Plant Physiology*, **14**(1): 23-7.
- Fontana, J.D., Franco, V.C., De Souza, S.J., Lyra, I.N. and De Souza, A.M. 1991. Nature of plant stimulators in the production of *Acetobacter xylinum* ("tea fungus") biofilm used in skin therapy. *Applied biochemistry and biotechnology*, **28**(1): 341-51.
- Greenwalt, C.J., Steinkraus, K.H. and Ledford, R.A. 2000. Kombucha, the fermented tea: microbiology, composition, and claimed health effects. *Journal of Food Protection*, **63**(7): 976-81.
- Jaber-Vazdekis, N.E., Gutierrez-Nicolás, F., Ravelo, Á.G. and Zárate, R. 2006. Studies on tropane alkaloid extraction by volatile organic solvents: dichloromethane vs. chloroform, *Phytochemical Analysis: An International Journal of Plant Chemical and Biochemical Techniques*, **17**(2): 107-13.
- Licker, J.L., Acree, T.E. and Henick-Kling, T. 1998. What is "Brett" (Brettanomyces) flavor?: A preliminary investigation, <https://doi.org/10.1021/bk-1998-0714.ch008>.
- Majumder, S., Ghosh, A. and Bhattacharya, M. 2020. Natural anti-inflammatory terpenoids in *Camellia japonica* leaf and probable biosynthesis pathways of the metabolome. *Bulletin of the National Research Centre*, **44**(1): 1-4.
- Malbaša, R., Lončar, E., Djurić, M., Klašnja, M., Kolarov, L.J. and Markov, S. 2006. Scale-up of black tea batch fermentation by kombucha. *Food and Bioproducts Processing*, **84**(3): 193-9.
- Negishi, O., Ozawa, T. and Imagawa, H. 1992. Biosynthesis of caffeine from purine nucleotides in tea plant. *Bioscience, Biotechnology, and Biochemistry*, **56**(3): 499-503.
- Santra, S.C., Chatterjee, T.P. and Das, A.P. 2006. College Botany Practical. Vol. 1. New Central Book Agency (P) Ltd., pp. 221.
- Shalmashi, A. and Golmohammad, F. 2010. Solubility of caffeine in water, ethyl acetate, ethanol, carbon tetrachloride, methanol, chloroform, dichloromethane, and acetone between 298 and 323 K. *Latin American Applied Research*, **40**(3): 283.
- Šormová, Z., Melichar, O. and Šorm, F. 1960. Some pyrimidine derivatives as new types of plant stimulants. *Collection of Czechoslovak Chemical Communications*, **25**(11): 2889-98.
- Zhu, C., Li, F., Zhou, X., Lin, L. and Zhang, T. 2014. Kombucha-synthesized bacterial cellulose: Preparation, characterization, and biocompatibility evaluation. *Journal of Biomedical Materials Research Part A*, **102**(5): 1548-57.