*Int. J. Food Ferment. Technol.*, **10**(2): 113-118, December 2020

**DOI:** 10.30954/2277-9396.02.2020.7

**RESEARCH PAPER**

# Stimulants of tea Infusion are Completely Withdrawn by SCOBY during Kombucha Fermentation: A Biochemical Investigation

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**Paper No.:** 249 **Received:** 05-10-2020 **Revised:** 25-11-2020 **Accepted:** 17-12-2020

**ABSTRACT**

Caffeine content in kombucha is very much lower than that of normal tea infusion. But, 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 replied 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 stimulants 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

**How to cite this article:** Majumder, S., Ghosh, A., Chakraborty, S. and Bhattacharya, M. (2020). Stimulants of tea Infusion are Completely Withdrawn by SCOBY during Kombucha Fermentation: A Biochemical Investigation. *Int. J. Food Ferment. Technol.*, **10**(2): 113-118.

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

fermented form. So, to find out the aftermath of those components in kombucha, we prepared and carried on preliminary biochemical tests, followed by GC-MS analysis to put up a scientific explanation for better understanding.

## MATERIALS 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 protocol of Greenwalt *et al.* (2000) and Zhu *et al.* (2014), a number 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 source for further experiments. For obtaining extract, SCOBY was squeezed hard to separate SCOBY extract (SE) and the dry and hard remaining 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 and SE 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. 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 (Bhattcharya *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 dicholoromethane) were selected on the basis on affinity of targeted compounds towards solvents. Each fractions were collected individually, dried and tested for caffeine confirmatory test (the murexide test).

### Gas chromatography mass spectrometry analysis

KB and SE (1 ml 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

Results of preliminary tests have supported previous reports as the beverage or KB resulted negative in all the alkaloid detection while biofilm extract or SE showed positive result. Moreover, the murexide test for caffeine detection also gave a positive result for SE only. These results clearly indicates that SE contains the alkaloids of tea and one of their major components caffeine unlike the komucha (KB).

In column chromatographic separation of caffeine and related components, chloroform and dicholoromethane 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 as the fractions 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 already 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. Now, to evaluate and judge the results of these tests, GC-MS study was conducted.

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

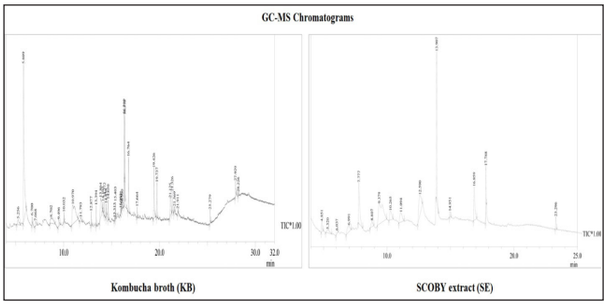


**Fig. 2:** GC-MS chromatograms

The GC-MS analysis and other biochemical tests have helped to discover that both samples, 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 secret chemical profile has also been unfolded for SCOBY extract through this set of experiments. It can be assumed that *Acetobacter xylinum* is one of them as it has previously been 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 those stimulants. Moreover, caffeine is also known to exhibit antimicrobial activity. So, chances may be less for other microorganisms to capture this big

**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-Oxoadamantane-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 | |

**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 | |

amount of caffeine from tea infusion. Furthermore, this should be left over future microbiological and metabolic pathway studies on SCOBY.

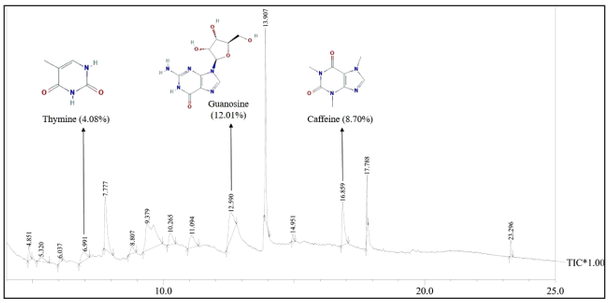


**Fig. 3.** Chromatogram of SCOBY extract showing peaks of caffeine, guanosine and thymine

## 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 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. Microbiological experiments and higher analytical chemistry should be implemented along with this study to isolate stimulants like caffeine from old, discarded, unusable or molded SCOBY in future.

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