The Rhythmic Growth Pattern of Microbes is Antithetical to Antioxidant Activity of Kombucha: A New Finding in Food Biochemistry

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Authors' contributions

This work was carried out in collaboration among all authors. Authors SM and MB designed the study and wrote the protocol. Authors SM, AG and SC performed all the analyses of the study. Author SM managed the literature searches and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Background: Kombucha is a traditional, popular and probiotic health drink having strong antioxidant properties. Involvement of various bacteria and yeasts in kombucha fermentation have been previously described by several scientists. In this research, we aimed to determine the growth pattern of microbes involved in kombucha fermentation, using the orthodox turbidimetric method and a simultaneous determination of antioxidant activity regularly.

Methods: This experiment was designed in a simple way to evaluate the interrelation between growth of microbes involved in kombucha fermentation and the rate of release of antioxidant molecules using spectrophotometric growth study and DPPH free radical scavenging assay.

Results: In this research, some new characteristics of kombucha have been found regarding microbial fermentation. Moreover, we prepared broths using different types of sugars as carbon source for a comparative analysis.

Significance: In all the broths, it was found that there is a negative correlation between rhythmic microbial growth pattern and antioxidant activity which is definitely a new finding in food science.

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1. INTRODUCTION

Kombucha, the slightly sweet and acidic probiotic fermented tea became popular chronologically in China, Japan, Russia, Germany, the entire Europe and the Americas. The main reasons, for which it has been praised by people of different age groups, are its flavour; antioxidant activity; anti-stress and other bioactivities, lower caffeine and cool serving. Kombucha is prepared by brewing sweetened tea infusion with a "symbiotic colony of bacteria and yeasts" or, concisely the SCOBY [1,2]. Antioxidant molecules as metabolites of SCOBY actually enhance bioactive potentials of kombucha establishing a difference between the normal tea infusion and its fermented form. Formation and changes in the appearance of SCOBY denotes the rate of microbial growth which also gradually turns the broth transparent (after the growth of sufficient microbes to form the biofilm) unlike other common microbial broths. However, the lower dense liquid or broth of kombucha which is literally used for consumption also contains huge amount of microbes. So, just like the SCOBY, this liquid part is also used as a starter, exclusively or together with SCOBY, for brewing kombucha [2]. We intended to determine the growth pattern of microbes (present in both SCOBY and the broth) involved in kombucha fermentation, using the orthodox turbidimetric method. A parallel determination of antioxidant activity was done to evaluate the interrelation between growth of microbes and rate of the release of antioxidant molecules which are preliminary assumed to represent or indicate the rate of microbial metabolism. Moreover, we prepared broths which contain different types of sugars as carbon source for a comparative analysis.

2. METHODOLOGY

2.1 Sample Preparation

A strong tea infusion was made by boiling CTC black tea (fresh tea collected from local tea factory) (1% w/v) for fifteen minutes. Following protocol of Zhu et al. [1] and Majumder et al. [3], a number of kombucha batches were prepared and incubated under a sterile condition. For the starter, strong kombaucha from a single healthy previous batch was used. Three monosaccharide sugars (glucose, fructose and galactose) and three disaccharides (sucrose, maltose and lactose) were used individually in batches as carbon source or nutrient. All these batches were cultured in a mechanical shaker-incubator during the study with an intention to obstruct the cellulose network formation. Following the same procedure, a total of three jars as replicas for each broth were prepared. A representative sample from each broth was taken off at every 24 hours, carefully, to avoid the sediments (debris).

2.2 Growth Study

Turbidimetric determination is the quickest and simplest method, useful for plotting growth curves of microbes in broth or liquid media. It is used to examine trends in growth by tracking (spectrophotometrically) the changes in optical density (OD) over time. The protocol of Brown [4] was followed to study the growth pattern of microbes present in our broths by measuring OD of each sample regularly (on every 24 hours) at 660 nm. Readings were plotted on a graph with time in the X-axis and OD in the Y-axis. Data of each sample is given as mean of 3 replicates.

2.3 Free Radical Scavenging Activity (DPPH assay)

The free radical scavenging activity through DPPH assay was performed following the spectrophotometric method described by Bhattacharya et al. [5]. Data for each sample is expressed as mean of 3 replications.

3. RESULTS AND DISCUSSION

The results of microbial growth study are represented as growth curves in Fig. 1. A lag phase of approximately three days was observed in the initial part of fermentation in all the broths. Then, broths containing monosaccharides as carbon source showed an exponential growth or log phase from 3rd day to 7th day of fermentation while, in disaccharide broths, the log phase continued till 6th day only. Later, all the disaccharide broths showed a stationary phase of 48 hours (from 6th day to 8th day) before entering into the death phase gradually. In monosaccharide broths, the stationary phase was very minimal and ignorable as after showing the peak of growth on 7th day, the microbes entered into a very sharp death phase as reflected in Fig. 1. So, two distinct types of growth patterns were established in monosaccharide and disaccharide broths.
Free radical scavenging activity, in monosaccharide broths, was recorded lowest on 7th day of fermentation and thereafter increased steadily when the microbes involved in those broths actually entered into the death phase. Before that, there was a decreasing tendency in antioxidant activity when the growth was in log phase. Interestingly, this same correlation was also found in the disaccharide broths too. In both cases, between 8th and 9th day, as the death phase started, antioxidant activity exhibited a sharp increase while the growth curves inclined exponentially. Here, again two distinct types of patterns were established for monosaccharide and disaccharide broths (Fig. 2) and, interestingly, it was absolutely opposite to the microbial growth seen in each broth.

Generally one to three weeks are considered as ideal incubation period for brewing one gallon of kombucha [2]. So, the microbial activities (growth, development and metabolism) are believed to be lengthy, slow growing (compared to other microbial cultures) and active in that period whose evaluation has been done through this study. Furthermore, on aging, the broth becomes very much acidic and vinegary in flavour which indicates the end of sugar source and microbial growth as well [2]. After studying the above facts, samples were considered to be collected at every 24 hours for fifteen days which was later justified enough by the graphs depicting our results. Aiming to determine proper growth pattern as well as the changes in antioxidant property in a parallel manner, the bottom most portion or sediments (mainly cell debris) were avoided carefully during sample collection as those was nothing other than the debris of dead microbial cells which could screw up the overall study. Formation of cellulose network, biofilm and clumps are the drawbacks in turbidimetric method of growth study. So, prepared batches were left in a shaker-incubator during incubation period to determine the proper OD for obtaining the growth curve of all the bacteria and yeasts of kombucha (both broth and the biofilm) in aggregate.

In disaccharide broths, no remarkable change was observed after 10th day; neither in growth curve nor in antioxidant activity. Lactose broth showed better results in growth study and antioxidant assay by exhibiting highest growth in log phase and highest antioxidant activity in death phase compared to other disaccharides. Lactose is the disaccharide of glucose and galactose, and interestingly, this fact became relatable with the results as both of these monosaccharides exhibited the highest antioxidant activity. Both microbial growth and antioxidant activity together can be a basic parameter to judge the quality of any probiotic or fermented beverage. If one particular fermenting broth can help its microbes to reach up to the maximum level of growth, that too in a faster way, during growth phase and can exhibit the best antioxidant property at the end of fermentation, then that particular broth can surely be considered as the best among other broths incubated in a similar environment. And, in this case, the overall results established lactose (disaccharide) and glucose (monosaccharide) as best among all carbon sources; even compared to sucrose which is commonly used for brewing.

According to the previous studies, microbial cultures synthesize small bioactive molecules during the stationary and death phase which are not produced in the trophophase or log phase. Calam [6] and Ruiz et al. [7] referred these compounds as secondary metabolites which do not appear to have any obvious function in cell metabolism. It is also reported that secondary metabolism occurs in continuous cultures at low growth rates [8]. Microorganisms actually need the primary products of biosynthesis (known as anabolites or primary metabolites) for their own metabolism, growth and development during log phase which are then converted or developed into secondary metabolites in their declining phase of growth. Tea, as in its infusion form, is already known as a good source of antioxidants that are actually anabolites or primary products or part of the substrate which are used by microbes during kombucha fermentation. Meanwhile the change in antioxidant property was taken as a parameter that actually denotes the consumption of substrates (anabolites) by microbes and following occurrence of secondary metabolites produced by microorganisms of SCOBY during different stages of their growth. Moreover, our intention was to study how the release of bioactive molecules (mainly antioxidants) as secondary metabolites are dependent over the growth pattern of microbes. Interestingly, all these above facts together correlated with our results to uplift the quality of this experiment as the decreasing antioxidant activity during log phase actually denoted the usual property of microbes in that phase where primary metabolites or anabolites are up taken. Similarly, sharp increase of antioxidant activity after stationary phase simply denoted the secondary microbial metabolism. Reportedly,
acidic environment (low pH) inside a broth effects the growth and development of kombucha microbes which also upregulates the release of bioactive metabolites or antioxidants during fermentation [9]. Primarily, organic acids present in starter and tea catechins in the substrate create this acidic environment while, during progress in fermentation, the acidity of the broth increases due to production of organic acids by bacteria. It is also reported that, due to this high acidity, oxygen starvation occurs which reduces microbial cells to indicate the occurrence of stationary phase and further death phase [9,10]. Moreover, the result of this research significantly interrelate all the above hypothesis regarding growth of SCOBY’s bacteria and yeasts, metabolism and their symbiotic interactions.

Tea is generally famous for having antioxidant molecules in it. So, existence of antioxidant property in its fermented or original form was neither a wonder nor an object of this experiment. Our concern in this study was to observe the regularly changing pattern of antioxidant activity. And, through the results, this experiment exhibited an exact indicator like property to figure out the rate of primary and secondary metabolism of microbes (described earlier) inside the broths.

![Microbial growth curves in monosaccharide broths](image1.png)

![Microbial growth curves in disaccharide broths](image2.png)

**Fig. 1.** Microbial growth curves in different kombucha broths (a. monosaccharide broths, b. disaccharide broths)
Fig. 2. DPPH free radical scavenging activity in different kombucha broths (a. monosaccharide broths, b. disaccharide broths)

Being different parameters, it was difficult to put the mean results of both growth study and antioxidant activity in a single graph. So, initially both images (graphs of growth pattern and antioxidant assay) were prepared individually, then one was simply superimposed over another manually, keeping the Y axis (Day of experiment) same (Fig. 3). This process was necessary to exhibit the interrelation between microbial growth and antioxidant activity and compare our findings with previous literatures where interrelations between growth and secondary metabolism are described. A heat map is also presented in Fig. 4 to depict the growth pattern, changes in antioxidant activity and the negative correlation between them. Both Fig. 3 and Fig. 4 are distinct and expressive of all the results found through this experiment.
Fig. 4. Heat map [white (low) to red (high)] to understand the growth patterns and changes in antioxidant activity for each broth and the negative correlation between them

4. CONCLUSION

Over the past few decades, significant scientific information has been accumulated regarding microbial growth study. However, this information has not been collectively discussed together with antioxidant activity which should be essential to understand the metabolomics of kombucha or any other microbial culture. In this research, growth of microbes, use of anabolites, release of metabolites etc. were also properly evaluated using this simple, time worthy, low cost, easy and orthodox methodology. This spectrophotometric study of growth and antioxidant activity on regular interval is not a new approach at all but the cumulative use of these two parameters which has established this antithetical rhythm between growth rate and antioxidant is definitely a new finding in food microbiology as it has opened up the most ignored secret of microbial metabolism. This experiment can also be used as a time saving and easiest strategy to determine the quality of any fermented and probiotic beverage.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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TABLE 1: Growth and antioxidant activity of different sugars for each broth over 15 days

| Time Index | Glucose | Fructose | Galactose | Sucrose | Maltose | Lactose |
|------------|---------|----------|-----------|---------|---------|---------|
| Day 1      |         |          |           |         |         |         |
| Day 2      |         |          |           |         |         |         |
| Day 3      |         |          |           |         |         |         |
| Day 4      |         |          |           |         |         |         |
| Day 5      |         |          |           |         |         |         |
| Day 6      |         |          |           |         |         |         |
| Day 7      |         |          |           |         |         |         |
| Day 8      |         |          |           |         |         |         |
| Day 9      |         |          |           |         |         |         |
| Day 10     |         |          |           |         |         |         |
| Day 11     |         |          |           |         |         |         |
| Day 12     |         |          |           |         |         |         |
| Day 13     |         |          |           |         |         |         |
| Day 14     |         |          |           |         |         |         |
| Day 15     |         |          |           |         |         |         |
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