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# Effect of Microbial Fermentation on Caffeine Content of Tea Leaves

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Caffeine is widely used in the food and pharmaceutical industries. For safety concerns, natural caffeine is preferred over synthetic products despite of its high cost. To explore more economical methods of acquiring natural caffeine, we adopted a microbial fermentation technique to increase the caffeine content of tea leaves. Our studies showed that the caffeine content in tea leaves increased reasonably after treating leaves with microorganisms for a period of time (i.e. orthodox pile-fermentation), and the amount of caffeine content increase varied significantly between black and green teas (27.57% and 86.41%). These results suggested that the change of caffeine content in tea leaves during the pile-fermentation depended not only on the growth and reproduction of microorganisms, but also on the tea composition.

**KEYWORDS: Green tea; black tea; caffeine; microbial fermentation; pile-fermentation**

## INTRODUCTION

Caffeine exists widely in the leaves, seeds, and fruits of a large variety of plants. Among them, cocoa beans, tea, coffee, cola, and guarana are the most well-known. Each year, about 120 000 tons of caffeine is consumed worldwide. Caffeine can be used medically as a cardiac stimulant and a mild diuretic, and it can be applied as an additive in many popular carbonated drinks and also as a component of a number of pharmacological preparations, including analgesics (where caffeine acts as an adjuvant), diet aids, and cold/flu remedies. According to a publication in 1983 of the Scientific Committee for Food (SCF) of the Commission of the European Communities (*1*), normal quantities of caffeine consumption have no carcinogenic, teratogenic, or mutagenic effects in humans.

Caffeine is commonly obtained by two means: extracting from tea or coffee (*2, 3*) and synthesizing from urea or uric acid in several steps (*4, 5*). The cost of caffeine obtained by extraction from tea or coffee is much higher than that obtained by chemical synthesis, largely due to the low caffeine content in tea or coffee. However synthetic caffeine is generally prohibited for use in food and drink because it contains harmful chemical residues, such as dimethyl sulfate, chloroacetic acid, sodium cyanide, from the synthetic process. Pure caffeine used in the food and drink comes mostly from the process of decaffeinating coffee and tea. Since its price (500−800 dollars per kilogram) is much more expensive than that of the synthetic caffeine (10−15 dollars per kilogram) (*6*), it is a worthy project to develop methods to acquire natural caffeine at a lower cost. Although the investigation on further lowering the cost of synthetic caffeine via an inexpensive and novel method is still undergoing (*7*), up to now, much attention is concentrated on the improvement of methods in order to reduce the cost of natural caffeine. There are several methods to get caffeine from tea or coffee: extraction by chloroform or by supercritical carbon dioxide, sublimation by heating, and so on. However, none of these methods can lower the cost significantly. The most effective way to reduce the price of natural caffeine would be to increase the caffeine content in tea or coffee by biosynthesis. Recent metabolic studies on tea and coffee have elucidated the biosynthetic pathway of caffeine. The major route begins with xanthosine and proceeds through three *N*-methylations via 7-methylxanthosine, 7-methylxanthine, and theobromine (*8*−10). In addition, a number of other pathways playing minor roles have also been suggested (*11, 12*). The traditional methods for increasing caffeine content are to change the growing conditions or to cultivate new species that have higher caffeine content. Since these approaches can only improve caffeine yield by a limited amount, new strategies of increasing the caffeine content in natural products need to be developed.

Biotechnological methods employing microorganisms offer practical and economical solutions to many manufacturing processes in the food and pharmaceutical industry (*13*). Engineered fermentation processes utilize selected organisms to produce economically useful products in high yield from cheap substrates under the proper environment. So far, many products with biomedical and therapeutic applications, including antibiotics, vitamins, steroids, organic acids, amino acids, microbial enzymes, and microbial polysaccharides, have been produced by microorganisms under optimized conditions (*14, 15*). To our

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knowledge, little information has been published on the production of alkaloids and caffeine through microbial fermentation.

The phenomenon that the content of caffeine in tea leaves can increase to some extent in comparison with fresh leaves during the manufacturing of dark green tea has been observed for a long time (*16*, *17*). However, there have been few mechanistic studies on this phenomenon, perhaps because much attention is paid to improve the quality of dark green tea by altering the chemical composition of tea leaves during the manufacturing. Although it has been suggested that the changes of tea components might be partly due to the growth and reproduction of microorganisms including bacteria, yeast, and aspergillus (*18*, *19*), the reason for the caffeine increase during the processing of dark green tea has not been investigated experimentally. To find more economical methods of producing natural caffeine, we examined this phenomenon systematically. In this paper, we report our recent research results concerning caffeine increase in tea leaves.

## MATERIALS AND METHODS

**Materials.** Dried green and black teas (producing area, Huangshan, China) were purchased from specialized tea outlets in Hefei City, Anhui Province, China. Caffeine was purchased from Sigma Chemical Co. (St. Louis, Mo) as analytical standard material. Magnesium oxide, basic lead acetate, concentrated sulfuric acid and acetonitrile were purchased from Shanghai Chemical Reagents Co (China) and used as received without further purification. All reagents and solvents were of AnalaR or HPLC grade. Water used in the HPLC mobile phase was laboratory-distilled and filtered through a 0.2 $\mu$m membrane filter.

**Media.** Fungi culture medium contained 10 g of glucose, 10 g of agar, and 100 g of peeled potato in 1000 mL of deionized water; bacteria culture medium contained 1.5 g of beef extract, 5 g of peptone, and 10 g of agar in 1000 mL of deionized water; yeast culture medium contained 2.5 g of yeast extract, 10 g of glucose, 5 g of peptone, and 10 g of agar in 1000 mL of deionized water.

**Pile-Fermenting of Green Tea and Black Tea.** Tea (200 g) and water (100 mL) were fully mixed in order that the water could be absorbed and well distributed in the tea, and then eight portions of the wet tea (20 g each) were respectively placed into eight Erlenmeyer flasks with cotton plugs. Subsequently, these flasks were put into a constant temperature and humidity incubator maintained at 30 °C and 85% humidity for reproduction of microorganisms adhered to the tea sample. Every 4 days, one of the Erlenmeyer flasks was taken out for analysis of microorganisms and caffeine content.

**Sterile Pile-Fermenting of Green Tea and Black Tea.** Tea sample (200 g) and water (100 mL) were fully mixed, and then eight portions of the wet tea (20 g each) were respectively put into eight Erlenmeyer flasks with cotton plugs. After being wrapped with kraft paper, the Erlenmeyer flasks were put into an autoclave and sterilized at 15 pounds per square inch (psi) for 20 min. The cooled Erlenmeyer flask was then dealt with in the same way as pile-fermenting.

**Pile-Fermenting of Water-Treated Tea.** Tea (200 g) was infused in 1000 mL of distilled water and was boiled for half an hour in order to remove the water-soluble components, such as tea polyphenols and caffeine. The tea leaves were then recovered by filtration under vacuum through a Buchner funnel. The procedure wherein tea leaves were infused and recovered by filtration was repeated four times. The residual tea was pile-fermented and then dealt with in the same way as pile-fermenting after part of it was left for determination of caffeine.

**Analysis of Microorganisms.** Part of the tea in the Erlenmeyer flask taken from the incubator at the prescribed time intervals was used for analysis of microorganisms, and the residue was used for determination of water and caffeine. The tea used for analysis of microorganisms was weighed and then it was placed into a 100-mL sterile water blank in a sterile cabinet. The blank capped tightly was well shaken to break up any clumps of microorganisms that adhered to the tea. Then 1 mL of this 1:100 dilution of the original microorganisms was transferred into a 9-mL sterile water blank using a sterile pipet. After closing the test tube, the contents were swirled well by snapping the bottom of the tube. The procedure was repeated by transferring 1 mL of this 1:100 dilution into another 9-mL water blank. After thorough mixing, the last tube will represent a 1:10 000 000 dilution of the original microorganisms. The 1-mL samples of the last dilution were transferred into tubes of different cultural media that had been melted and cooled to 45 °C. The contents were well mixed by swirling and then put into sterile Petri plates. The media was allowed to harden, invert, and incubate for 48−72 h at 30 °C. After the incubation was complete, the colonies growing in and on the cultural medium in each Petri plate were counted. The population of microorganism was calculated according to the eq 1

$$P = \frac{N \times 10^7}{M} \quad (1)$$

where $P$ is the population of microorganism per gram of pile-fermented tea, $N$ is the number of colonies growing in and on the cultural medium in each Petri plate, and $M$ is the dry weight (g) of pile-fermented tea. All the data obtained are the average values, and two experiments and six measurements were made (three measurements each).

**Determination of Caffeine.** The aliquots (5 g) taken at various stages of different treatments were dried at 103 ± 2 °C for 16 h, and then the solids obtained were crushed into powder. One gram of the dried tea powder was mixed with MgO (0.25 g), and the mixture in a beaker was extracted with 60 mL of boiling water on a heating plate for 45 min. After filtration of the solution under vacuum through a Buchner funnel, the beaker was washed with hot water. This procedure was repeated, and the two filtrates obtained were collected in a 100-mL volumetric flask. When the solution was cooled to room temperature, the volume was adjusted with distilled water, and then 10 mL was transferred into another 100-mL volumetric flask with a pipet. An aqueous solution (2 mL) of saturated basic lead acetate was added into the tea infusion to remove the tea polyphenols, pigments, and proteins from the tea infusion, and subsequently the volume was adjusted with distilled water. The mixed solution stood for 1 h. After filtration, 50 mL of filtrate and 0.2 mL of sulfuric acid solution (4.5 mol·L$^{-1}$) were mixed with 49.8 mL of water in a 100-mL volumetric flask. The mixed solution stood for 30 min and then was filtered. Two milliliters of the filtrate was filtered through a 0.45-um membrane filter to obtain the final measurement solution. The essential application features of the high-performance liquid chromatography (HPLC) are the same as those described by Matissek (*20*). Analysis was carried out on a HPLC apparatus equipped with a Waters 6000A pump, a Waters Model 440 fixed wavelength detector at 272 nm, a C18 reverse phase column (Merck, 15 cm × 4.6 mm id, particle size 5 um), and a sample injector system (Rheodyne) with a 5 $\mu$L sample loop. The mobile phase used was acetonitrile−water (12:88, v/v) at a flow rate of 1.0 mL·min$^{-1}$. All the data obtained are the average values of six measurements (two tests and three measurements each test).

## RESULTS AND DISCUSSION

During the pile-fermenting process of dark green tea, reproduction and metabolism of microorganisms including yeast, mold, and bacteria can change the chemical composition of tea leaves, which led to formation of the characteristics of dark green tea (*19*). The increase in caffeine during the pile-fermenting stage of dark green tea has been previously observed (*17*), but this phenomenon was not explained. Thus we studied the various factors influencing the caffeine content increase.

**Effect of Microorganisms on Caffeine Content of Tea.** The analysis results of microorganisms and caffeine contents are listed in **Tables 1** and **2**. Experiments were performed twice, each in triplicate, and the maximum CV was 6.2%. The data listed in **Tables 1** and **2** indicate that, during the pile-fermentation of green and black teas, the numbers of microorganisms were changed greatly with time, and the caffeine contents in pile-fermented teas varied apparently with the variation of microorganisms, especially with the changes of

**Table 1.** Variation of Dominant Microorganisms and Caffeine Contents with Time during the Pile-Fermentation of Green Tea

| days | 0 | 4 | 8 | 12 | 16 | 20 | 24 | 28 | 32 |
|---|---|---|---|---|---|---|---|---|---|
| bacteria[a] (SD) | 0.00 (0.00) | 0.00 (0.00) | 7.27 (0.189) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 30.97 (1.291) |
| yeast[a] (SD) | 0.00 (0.00) | 3.60 (0.126) | 4.62 (0.171) | 2.25 (0.055) | 1.47 (0.029) | 2.72 (0.105) | 2.22 (0.116) | 0.87 (0.054) | 0.00 (0.00) |
| fungi[a] (SD) | 2.90 (0.032) | 13.40 (0.281) | 10.82 (0.265) | 12.96 (0.217) | 10.64 (0.196) | 10.48 (0.226) | 12.89 (0.258) | 23.29 (1.079) | 3.04 (0.106) |
| total[a] | 2.90 | 17.00 | 22.71 | 15.21 | 12.11 | 13.20 | 15.11 | 24.16 | 34.01 |
| caffeine[b] (SD) | 3.82 (0.143) | 4.13 (0.136) | 4.52 (0.129) | 4.27 (0.116) | 3.95 (0.121) | 5.05 (0.219) | 5.63 (0.174) | 5.85 (0.119) | 4.12 (0.251) |

[a] The unit of the microorganisms is $10^7$ per gram of dry matter. [b] The unit of the caffeine contents is grams per 100 g of dry matter. The values given above are averages of two experiments, each in triplicate, conducted under the same condition.

**Table 2.** Variation of Dominant Microorganisms and Caffeine Contents with Time during the Pile-Fermentation of Black Tea

| days | 0 | 4 | 8 | 12 | 16 | 20 | 24 | 28 | 32 |
|---|---|---|---|---|---|---|---|---|---|
| bacteria[a] (SD) | 0.00 (0.00) | 0.00 (0.00) | 2.34 (0.0481) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 0.00 (0.00) | 12.99 (0.771) |
| yeast[a] (SD) | 0.00 (0.00) | 2.85 (0.087) | 3.90 (0.172) | 2.01 (0.068) | 1.31 (0.021) | 0.00 (0.00) | 0.00 (0.00) | 0.70 (0.027) | 0.00 (0.00) |
| fungi[a] (SD) | 0.00 (0.00) | 4.27 (0.136) | 1.68 (0.047) | 6.73 (0.221) | 8.99 (0.461) | 1.11 (0.051) | 3.03 (0.136) | 3.20 (0.075) | 4.90 (0.141) |
| total[a] | 0.00 | 7.12 | 7.92 | 8.74 | 10.30 | 1.11 | 3.03 | 3.90 | 17.89 |
| caffeine[b] (SD) | 4.06 (0.053) | 3.77 (0.109) | 3.85 (0.074) | 4.15 (0.131) | 4.37 (0.142) | 3.82 (0.169) | 3.22 (0.162) | 2.72 (0.063) | 2.84 (0.043) |

[a] The unit of the microorganisms is $10^7$ per gram of dry matter. [b] The unit of the caffeine contents is grams per 100 g of dry matter. The values given above are averages of two experiments, each in triplicate, conducted under the same condition.

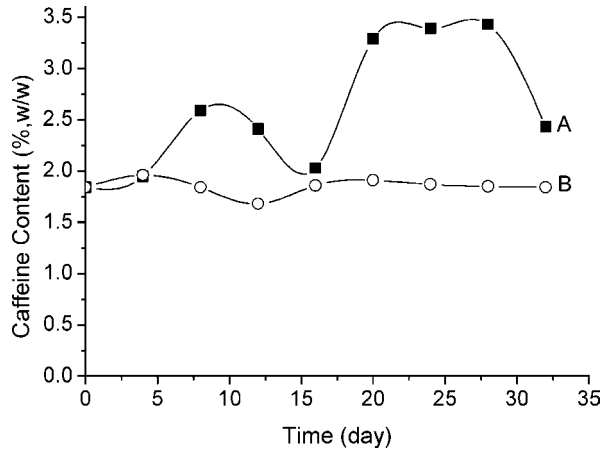


**Figure 1.** Effect of microorganisms on caffeine content in green tea: (**A**) pile-fermenting, (**B**) sterile pile-fermenting.

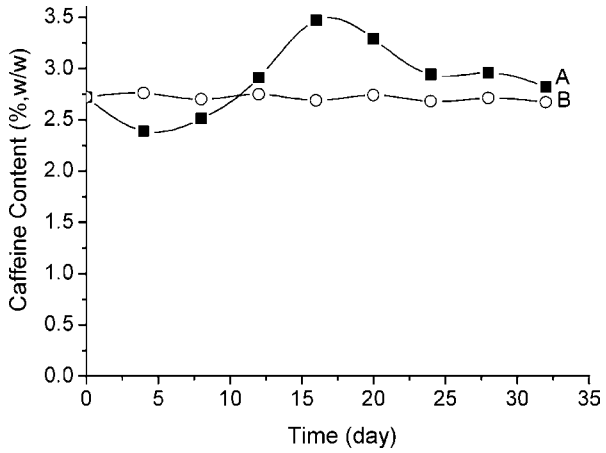


**Figure 2.** Effect of microorganisms on caffeine content in black tea: (**A**) pile-fermenting, (**B**) sterile pile-fermenting.

fungi and yeast. Furthermore, the bacteria were not detected until day 32. In other words, bacteria have little effect on the change of caffeine content during the pile-fermentation. To exclude the influence of tea material, different teas were pile-fermented. At the same time the sterile pile-fermentations were performed to confirm the effect of microorganisms on the increase of caffeine contents in pile-fermented teas. The results are shown in **Figures 1** and **2**. The caffeine contents in the green and black teas also apparently increased during the process of pile-fermenting, whereas the caffeine contents varied slightly under the same temperature and humidity conditions with no microorganisms growing (i.e. sterile pile-fermentation). This illustrated that the increase of caffeine depended significantly on the growth and reproduction of microorganisms, and it had no relation to the temperature and humidity. How do the microorganisms promote the increase of caffeine? This question could not be answered in terms of the data obtained in our experiments; it should be a function of (a) microbial enzymes that are produced by microorganisms and then excreted to the surface of tea leaves, since these enzymes will catalyze the caffeine biosynthesis to produce caffeine in vitro, or (b) the secondary metabolism of microorganisms, that is, the caffeine is biosynthesized in vivo by microorganisms after taking the essential components such as xanthosine from teas. Therefore, the caffeine increase must be related to the microorganisms, which possess the genetic information needed to produce caffeine or caffeine biosynthetic enzymes, also related to the tea components, which are absolutely necessary to the biosynthesis of caffeine. More research on exploring the reason for caffeine increase will continue in our laboratory.

**Effects of Green and Black Teas on the Increase of Caffeine.** Green and black teas were used as cultural media to pile-ferment. The data listed in **Tables 1** and **2** show that the numbers of microorganisms adhering to the pile-fermented green teas always exceed that of pile-fermented black teas. Furthermore, significant differences were observed in the rate increase, and the time for the formation of the highest caffeine content differed between green tea (86.41%, at day 28) and black tea (27.57%, at day 16) (**Figure 3**). The reason for this is unknown, but it could be related to the different chemical compositions between green and black teas, which will influence the growth and metabolism of microorganisms adhering to the surface of tea leaves. The previous study (*21*) showed that there were indeed significant compositional differences between processed products of green and black teas, which resulted from the differences in plant variety, growth conditions, and processing methods. Among these factors, the major factor is the processing method. For example, the enzymatic oxidation of catechins

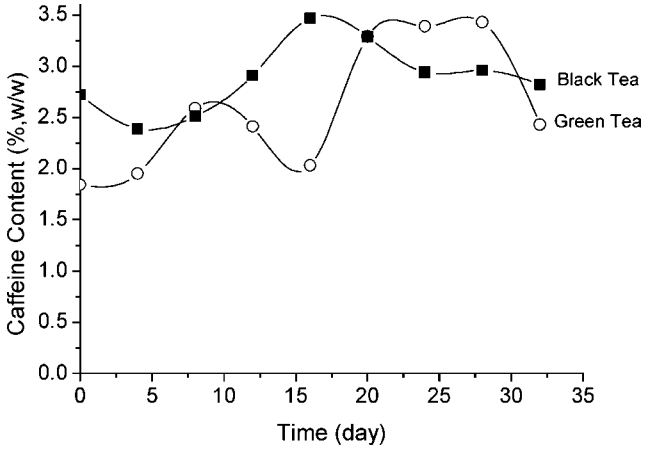


**Figure 3.** Effects of pile-fermenting on caffeine content in green tea and black tea.

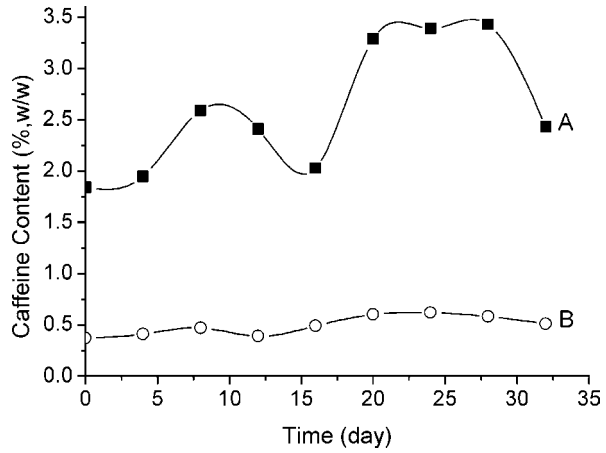


**Figure 4.** Effect of water-insoluble components on changes of caffeine content during pile-fermenting of green tea: (**A**) pile-fermenting of untreated green tea, (**B**) pile-fermenting of water-treated green tea.

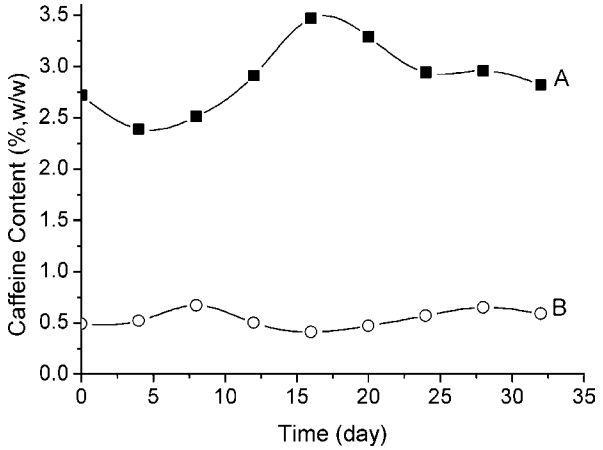


**Figure 5.** Effect of water-insoluble components on changes of caffeine content during pile-fermenting of black tea: (**A**) pile-fermenting of untreated black tea, (**B**) pile-fermenting of water-treated black tea.

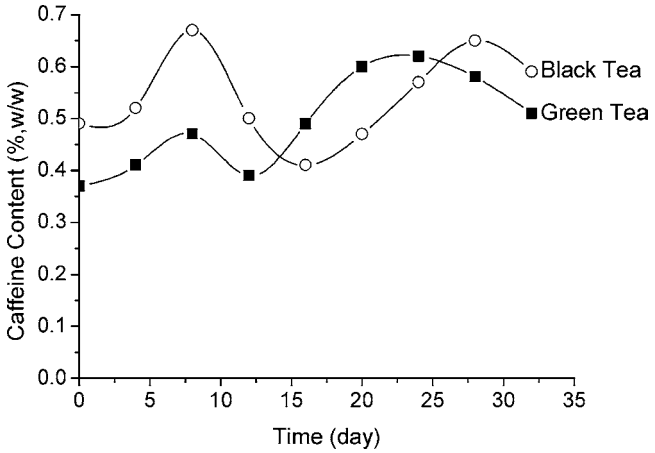


**Figure 6.** Comparison of effects of water-insoluble components in green and black teas on caffeine content during pile-fermenting.

during the fermentation stage of black tea production leads to the reduction in total catechins level. However, the deactivation of the polyphenol oxidase (PPO) enzyme by the pan-firing of the fresh leaves in the green tea processing halts this "fermentation" process and a much lower loss of catechins was observed. Furthermore, the oxidation of catechins during the fermentation stage of black tea production can cause the degradation and oxidation of other components, such as carotene and vitamins, to form the characteristic flavor of black tea by coupling oxidation, which may lower the content of essential substances to caffeine biosynthesis. As a result, the increased rate of caffeine production during the pile-fermentation of green tea is much higher than that of black tea. The different growth situations of microorganisms caused by the different inhibitory effects of black and green teas on microorganisms also result in the marked differences in the changing regularity of caffeine content of black and green teas with fermenting time, which is shown in **Figure 3**.

**Effect of Water-Insoluble Components in Teas on the Caffeine Increase.** To investigate further the effects of different parts of tea components on the caffeine increase, the untreated teas and water-treated teas were used. **Figure 4** is a comparison of the caffeine contents in the green tea before and after water-treatment during the pile-fermentation. A little reduction in the rate of caffeine increase and a similar regularity for the changes of caffeine content with time during the pile-fermentation of water-treated green tea were observed in comparison with those of untreated green tea. This can be explained in terms of (a) a much lower loss of the essential substances to caffeine biosynthesis after infusing with boiling water (i.e., these substances are almost insoluble in water) and (b) the weak inhibitory effect of tea-soluble substances, such as polyphenols, on the growth and reproduction of microorganisms which are thought to be related to caffeine biosynthesis. This will be further elucidated in the next section by comparison with the properties of black tea.

For black tea, we observed a little increase in the rate of caffeine increase during the pile-fermentation of water-treated black tea in comparison with that of untreated black tea (**Figure 5**). This provides further evidence to confirm that the essential substances for caffeine biosynthesis are almost insoluble. A similar result was obtained by Pasha and Reddy. In their experiments, the black tea extract was used to ferment with yeast and the reduction of caffeine was found out (*22*). A significant difference in the regularity for the changes of caffeine content with time was also observed during the pile-fermentation. **Figure 5** demonstrates that, during the pile-fermentation of water-treated black tea, the highest caffeine content appeared 8 days ahead of that for the untreated black tea. The probable reason is that the water-soluble components of black tea, such as theaflavins and thearubigins, have a stronger inhibitory effect on the growth and reproduction of microorganisms than those of green tea. Thus, when the soluble substances in black tea were removed by infusion and then pile-fermented, the microorganisms grew

and reproduced rapidly from the very beginning, leading to a rapid increase in caffeine, just as observed during the pile-fermentation of green tea.

**Figure 6** shows similar changing regularity of caffeine content with fermenting time during the pile-fermentation of water-treated black and green teas. This indicates that the soluble substances in black tea have stronger effect on caffeine increase than that of the green tea through affecting the growth and reproduction of microorganisms. This confirms further that the caffeine increase during the pile-fermentation was caused by the growth and reproduction of microorganisms.

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