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Study on the increase mechanism of the caffeine content during the fermentation of tea with microorganisms

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Abstract

The tea caffeine content had previously been shown to increase reasonably after being treated with mixed microorganisms for a period of time. In this study, single microorganisms were used in the fermentation of black and green teas in order to find which microorganism has the best effect on increasing the caffeine content. The results demonstrated that molds fermentation increased the caffeine content, but yeasts fermentation decreased the caffeine content. Among the three molds in this study, *Aspergillus niger* van Tieghem has the most remarkable effect, and the caffeine content in dry green tea increased from an initial 3.47% to 9.63%. The increase rate was 177.5% on the 16th day. Furthermore, the changes of caffeine and theophylline were of a similar trend. Possibly theophylline instead of theobromine is the precursor of caffeine in the living microorganisms. The new biosynthetic route is different from that in tea plants.
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Keywords: *Aspergillus niger* van Tieghem; Caffeine; Mechanism; Microbial fermentation; Tea

1. Introduction

Caffeine is an attractive compound because of its extensive applications in pharmacological preparations, including analgesics, diet aids and cold/flu remedies. In addition, it can be applied as an additive in many popular carbonated drinks. About 120,000 tonnes of caffeine is consumed worldwide every year. According to a publication in 1983 of the Scientific Committee for Food (SCF) of the Commission of the European Communities (1983), normal quantities of caffeine consumption have no carcinogenic, teratogenic, or mutagenic effects in humans.

Caffeine exists widely in the leaves, seeds and fruits of a large number of plants. Among them, cocoa beans, tea, coffee, cola and guarana are the best known. One method to obtain caffeine is extracting it from tea or coffee (Feng, 1991; Yue & Wu, 2002), an other method is synthesizing from urea or uric acid in several steps (The Merk Index, 1996; Zubair, Hassan, & Al-Meshal, 1986). 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 application in food and drink because it contains harmful chemical residues, such as dimethyl sulphate, chloroacetic acid and sodium cyanide, resulting from the synthetic process. Pure caffeine used in the food and drink originates 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) (Yang & Wang, 1999), a worthy project is 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 in the process (Matthew, Anthony, Mark, & Saraswathi, 2003), up to now, much attention has been concentrated on the improvement of methods in order to reduce the cost of natural caffeine. There are several methods to

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obtain 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 would be to increase the caffeine content in tea or coffee by biosynthesis. Recent metabolic studies on tea or coffee have elucidated the biosynthetic pathway of caffeine. The major route begins with xanthosine and proceeds via three successive *N*-methylations of 7-methylxanthosine, 7-methylxanthine and theobromine (Ashihara, Gillies, & Crozier, 1997; Ashihara, Monteiro, Gillies, & Crozier, 1996; Suzuki, Ashihara, & Waller, 1992). In addition, a number of other pathways have also been suggested (Kato, Kanehara, Shimizu, Suzuki, & Gillies, 1996; Schulthess, Morath, & Baumann, 1996). Traditional method for increasing caffeine content is variation of the growing conditions or cultivation of new species with high 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.

In the manufacturing of dark green tea, the increase of caffeine content in tea leaves in comparison with fresh leaves has been observed for a long time (He & Lin, 1986; Wang, Tan, & Shi, 1991). However, the mechanism for caffeine increase has rarely been addressed; perhaps much attention is paid to improve the quality of dark green tea by altering the chemical composition of tea leaves during manufacturing. Our previous study demonstrated that the caffeine content in tea leaves increased reasonably after treating leaves with mixed microorganisms for a period of time (i.e. orthodox pile-fermentation). However, the caffeine contents varied slightly under the same temperature and humidity conditions with no microorganisms growing (i.e. sterile pile-fermentation). This illustrates that the increase of caffeine depends significantly on the growth and reproduction of microorganisms (Wang, Hu, Wan, & Pan, 2005). However, which microorganism growth in the fermentation system is responsible for the greatest contribution to the caffeine increase has not been identified. In this study, three molds and two yeasts, which are the dominant microorganisms during the processing of dark green tea, were inoculated, respectively, into dry tea or tea infusion. The most important microorganism responsible for caffeine increase was observed from the variation characteristics of caffeine content during the fermentation of tea.

2. Materials and methods

2.1. Materials

Dried green and black teas (producing area, Huangshan, China) were purchased from specialized tea outlets in Hefei City, Anhui Province, China. Caffeine, theobromine and theophylline (analytical grade) were purchased from Sigma Chemical Co. (St. Louis, MO). Magnesium oxide, basic lead acetate, concentrated sulphuric acid and acetonitrile were obtained from Shanghai Chemical Reagents Co, Shanghai, 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-μm membrane filter.

2.2. Microorganism culture

Aspergillus niger van Tieghem ACCC 30005, *Rhizopus arrhizus* Fisher AS 3.2893, *Mucor circinelloides* van Tieghem AS 3.2484, *Candida albicans* (Robin) Berkhout ACCC 2100, and *Candida famata* (Saito) Lodder ACCC 2052 were all bought from China General Microbiological Culture Collection Center (CGMCCC), Institute of Microbiology, Chinese Academy of Sciences, Beijing, China. They were maintained on PDA medium slants (CGMCCC medium No. 14) or malt agar medium slants (CGMCCC medium No. 13) and preserved at 2–8 °C. Actively growing microorganism cells grown in the above medium slants were used for inoculation.

2.3. Microbial fermentation of tea leaves

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 placed, respectively, 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 flasks were inoculated with two loops full of actively growing mold or yeast cells from slant culture. Subsequently, these flasks were put into a constant temperature and humidity incubator maintained at 30 °C and 85% humidity for reproduction of microorganisms. Every 4 days, one of the Erlenmeyer flasks was taken out for analysis of purine alkaloids, including caffeine, theobromine and theophylline.

2.4. Microbial fermentation of tea juice

Tea (50 g) was infused in 500 mL of distilled water and was boiled for half an hour. The residual solid was separated by filtration, and the clear filtrate was cooled to room temperature. The procedure was repeated, and then the extracts were combined. The resultant clear tea juice was adjusted to 1000 mL with distilled water, and then eight portions of the tea infusion (100 mL each) were placed, respectively, into eight 250-mL Erlenmeyer flasks with cotton plugs. After being wrapped with kraft paper, the Erlenmeyer flasks were put into an autoclave and sterilized at 15 psi for 20 min. After cooling to room temperature, the flasks were inoculated with two loops full of actively growing mold or yeast cells from slant culture. The flasks were incubated at 30 °C in an orbital shaker incubator at 150 rpm. Every 4 days, one of the Erlenmeyer flasks was taken out, and the tea ferment was centrifuged. The super-

natant was transferred into a 100-mL volumetric flask and the volume was adjusted with distilled water. The solution obtained was used for analysis of purine alkaloids, including caffeine, theobromine and theophylline.

2.5. Determination of caffeine, theobromine, and theophylline

The solid aliquots (5 g) obtained at various stages of different treatments were dried at 103 ± 2 °C for 16 h, and then the resultant solids were crushed into powder. The dried tea powder (1 g) 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. The residues were collected by filtration and washed with hot water obtained from washing beaker. This procedure was repeated two times, 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 of it or 2 mL of treated solution of fermented tea juice was transferred into another 100-mL volumetric flask. An aqueous solution (2 mL) of saturated basic lead acetate was added into the tea infusion for removal of 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 sulphuric acid solution (4.5 mol L^{-1}) were mixed with 49.8 mL of water in a 100-mL volumetric flask. The mixed solution was allowed to stand for 30 min and was then filtered. Two milliliters of the filtrate was filtered through a 0.45-μm membrane filter, and the final measurement solution was obtained. The essential application features of the high-performance liquid chromatography (HPLC) are the same as those described by Matissek and Kordsmeyer (1994). Analysis was carried out on a HPLC apparatus (Model 600, Waters Corporation, Milford, MA, USA) equipped with a Waters 6000A pump, a Waters Model 440 fixed wavelength detector at 272 nm, a C18 reverse phase column (15 cm × 4.6 mm id, particle size 5 μm in diameter, Merck, Darmstadt, Germany) and a sample injector system (Model 7125, Rheodyne, Rohnert Park, CA, USA) with a 5-μl sample loop. Acetonitrile–water (12:88, v/v) was used as mobile phase 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).

3. Results and discussion

For investigating the effects of microorganisms on the caffeine increase in the green and black teas, dry tea and tea soup were fermented by different microorganisms, and then the caffeine and other alkaloids contents in the tea at different times were measured by a HPLC method. As shown in Fig. 1, the alkaloids, caffeine, theobromine and theophylline can be separated fully in the HPLC chromatogram.

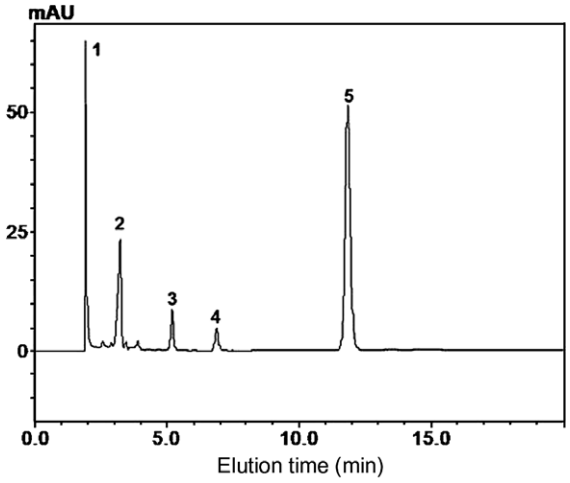

Fig. 1. HPLC chromatogram of alkaloids in tea: 1-unknown; 2-purine; 3-theobromine; 4-theophylline; 5-caffeine.

Fig. 2a shows variation of caffeine content in the dry green tea with different fermentation times. The results illustrate that fermentation with the three molds, *A. niger*

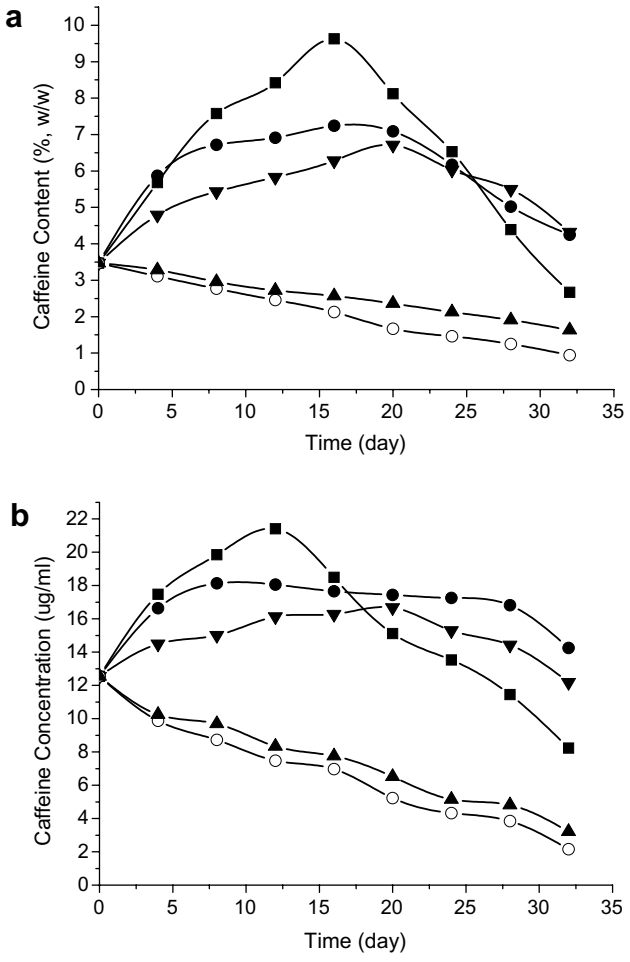

Fig. 2. Variation of caffeine in dry green tea (a) and green tea infusion (b) during fermentation with different microorganisms: ■*Aspergillus niger* van Tieghem; ●*Rhizopus arrhizus* Fisher; ▼*Mucor circinelloides* van Tieghem; ○*Candida albicans* (Robin) Berkhout; ▲*Candida famata*(Saito) Lodder.

van Tieghem ACCC 30005, *R. arrhizus* Fisher 3.2893 and *M. circinelloides* van Tieghem AS 3.2484, could increase the caffeine content of tea, while fermentation with the yeasts, *C. albicans* (Robin) Berkhout ACCC 2100 and *C. famata* (Saito) Lodder ACCC 2052, decreased the caffeine content of tea. Among the three molds, *A. niger* van Tieghem had the most positive effect on the increase of caffeine content, and on the 16th day of fermentation, the caffeine content reached a maximum value of 9.63%. The increased rate of caffeine content was 177.5% relative to the initial content of 3.47%. However, on the 28th day, the caffeine content in green tea was lower than that of those fermented with the other two molds.

The green tea infusion was also applied as a fermentation material under the same fermentation conditions, and the results are shown in Fig. 2b. The variation characteristic of caffeine content during the fermentation of tea infusion was similar to that of dry green tea. The highest increase of caffeine concentration was observed for the infusion fermented with *A. niger* van Tieghem, and the maximum increase reached 70.7% in comparison with the initial concentration in the infusion. The increasing rate at the maximum (70.7% on 12th day) of caffeine content in the infusion was lower than that (177.5% on 16th day) in the dry green tea. Furthermore, the time for the maximum value of the caffeine concentration in green tea infusion was on the 12th day, 4 days sooner than that for dry green tea. During the fermentation of green tea infusion with *A. niger* van Tieghem, the caffeine concentration decreased more quickly and was lower than that fermented with the other molds after 20 days. Such data indicate that the components in tea leaves, which are assimilated and then utilized to produce caffeine by microorganisms, are poorly soluble in water.

Based on Fig. 2, we can conclude that the three molds, *A. niger* van Tieghem ACCC 30005, *R. arrhizus* Fisher 3.2893 and *M. circinelloides* van Tieghem AS 3.2484, can enhance the caffeine content in green tea through fermentation, whereas the two yeasts had no effect. Accordingly, in our further investigation, only the molds were used to ferment the tea.

The dry black tea was fermented with three molds and the results are shown in Fig. 3a. Apparently, the caffeine content in dry black tea also increased with fermentation times. Among the three molds, *A. niger* van Tieghem had relatively significant effect in raising the caffeine content, and the maximum increase rate of 46.4% relative to initial caffeine content is much lower than that in the green tea. On comparing the data of green and black teas, we may conclude that the essential components for the microorganisms to produce caffeine in the black tea were lower than those in the green tea.

The black tea infusion used as a fermentation material with the variation of caffeine concentration in tea infusion with fermentation time is shown in Fig. 3b. The results show that the caffeine concentrations remained almost constant using *A. niger* van Tieghem and *R. arrhizus* Fisher in

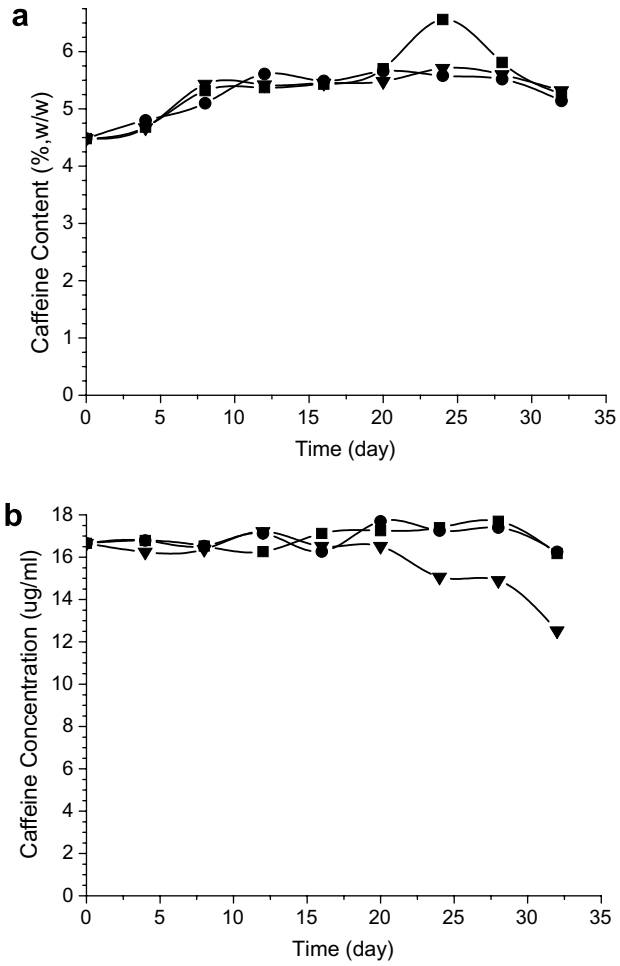

Fig. 3. Variation of caffeine in dry black tea (a) and black tea infusion (b) during fermentation with different microorganisms: ■*Aspergillus niger* van Tieghem; ●*Rhizopus arrhizus* Fisher; ▼*Mucor circinelloides* van Tieghem.

fermentation, while fermentation with *M. circinelloides* van Tieghem lowered the caffeine content in tea infusion. Pasha and Reddy (2005) reported that fermentation of black tea infusion by yeast resulted in reduction of caffeine. Therefore, black tea infusion is unsuitable for the production of caffeine by microorganisms.

To investigate why the caffeine content of dry green tea increased during fermentation, the content variations of theobromine which is the the precursor of caffeine during the biosynthesis in tea plant were recorded. The results are shown in Fig. 4a. All the three molds increased the theobromine content. Among them, *A. niger* van Tieghem had the greatest influence on the increase of the theobromine content, and its maximum value was 52.6% relative to the initial content in the dry green tea, which was much lower than the increased rate of caffeine. Additionally, the maximum content of theobromine during fermentation with *A. niger* van Tieghem appeared on the 8th day and then fell sharply. However, the maximum of caffeine appeared on the 16th day, 8 days ahead of the time of theobromine.

We also followed the theobromine content in the black and green tea infusions obtained from fermentation with

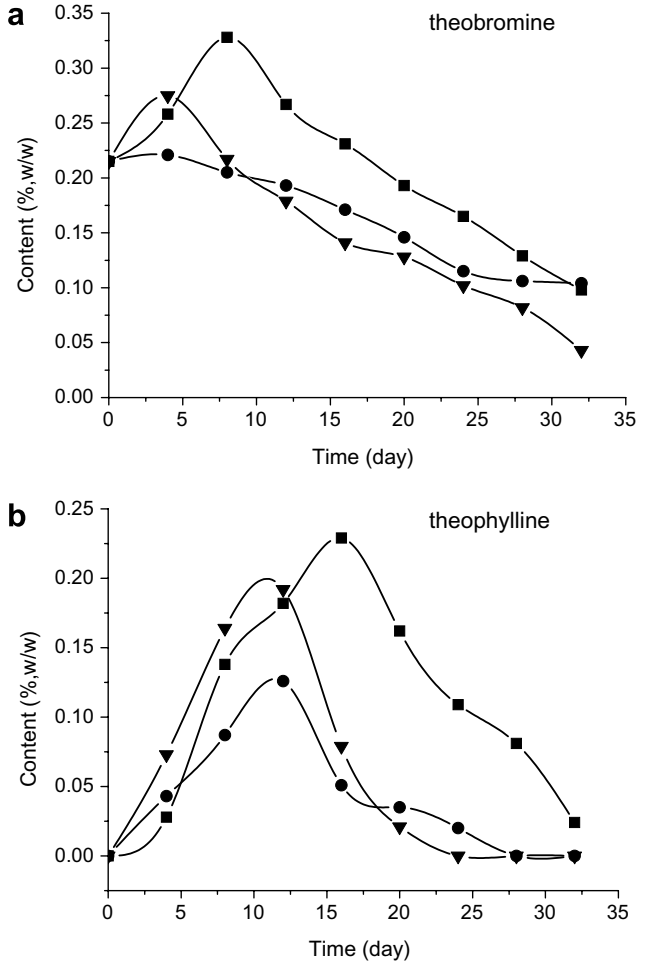

Fig. 4. Variation of theobromine (a) and theophylline (b) in dry green tea with fermentation times during fermentation with various microorganisms: ■*Aspergillus niger* van Tieghem; ●*Rhizopus arrhizus* Fisher; ▼*Mucor circinelloides* van Tieghem.

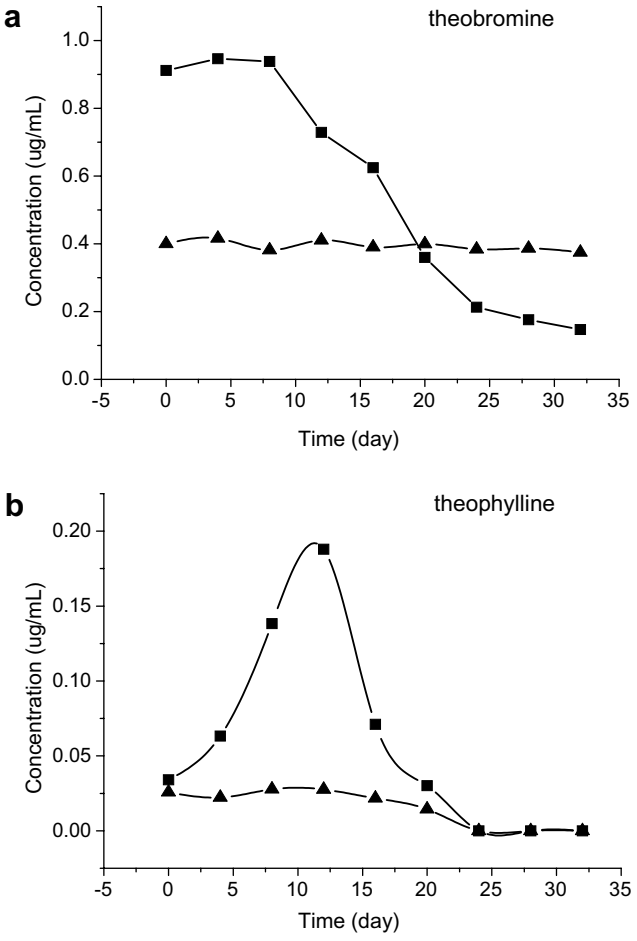

Fig. 5. Variation of the theobromine (a) and theophylline (b) in black (▲) and green tea (■) infusions with fermentation times during fermentation with *Aspergillus niger* van Tieghem.

A. niger van Tieghem. The results illustrate that the theobromine content in green tea infusion increased slightly during earlier stage of fermentation, and then decreased daily, while almost no variation of the concentration in black tea infusion was observed (Fig. 5a). According to Figs. 2, 4a and 5a, we can find that the variation of caffeine and theobromine contents was not at the same pace in the same fermentation system. In other words, probably there was no relation between caffeine and theobromine. Thus the biosynthetic route from theobromine to caffeine, which occurs in tea plant, may be excluded from the fermentation system. During the fermentation of dry green tea with *A. niger* van Tieghem, we detected the content change of theophylline which is a potential precursor of caffeine. The result is shown in Fig. 4b.

Fig. 4b shows that caffeine variation is similar to the variation of theophylline during the fermentation of dry green tea. The highest content of theophylline appeared on the same day as that of caffeine. Because of the determination method, the initial theophylline content in dry green tea could not be detected. Therefore, the content of theophylline on the 4th day fermentation had to be used as the initial content. In comparison with this value, the maximum content of theophylline increased by 717.9%. The infusions of green and black teas with *A. niger* van Tieghem were performed and the content change of theophylline was measured. The result is shown in Fig. 5b. The maximum content of the theophylline in the green tea infusion increased by 451.8% on the 12th day, and on the same day the caffeine content reached maximum (Fig. 2b). However, the theophylline concentration in black tea infusion varied slightly (Fig. 5b). On comparison of Fig. 2b with Fig. 5b, we can observe that the changes of caffeine and theophylline concentrations in green tea infusion are similar in the same fermentation system. Therefore, the increase of caffeine content in the green tea leaves may be via the methylation of the theophylline in the microbial metabolism; this is different from the biosynthetic route of caffeine in the tea plant, which is via methylation of theobromine. Based on the method of Kato et al. (1999), we also tried to determine the activity of caffeine synthase in the fermentation system. The result showed that no caffeine synthase existed in the fermentation system. This demonstrates that microorganisms established a new biosynthetic route to

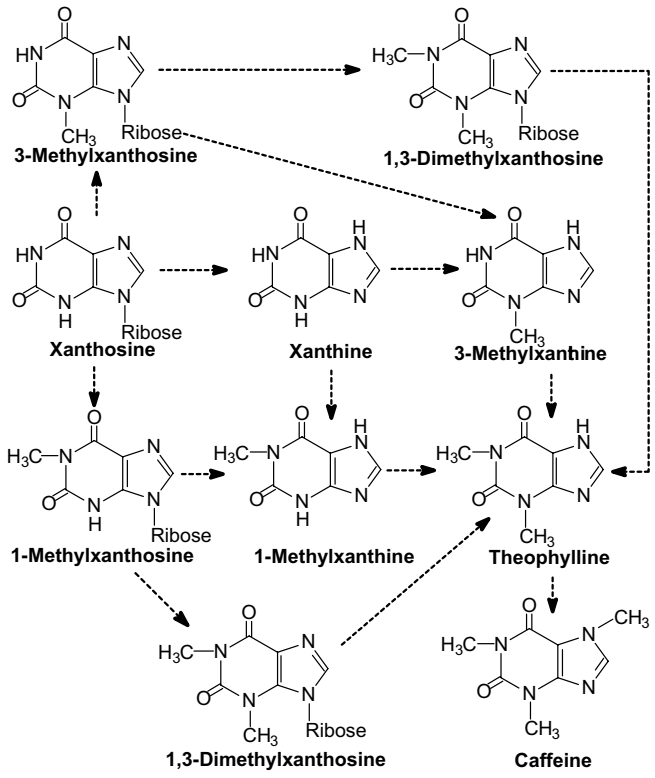

Fig. 6. Possible biosynthetic pathways of caffeine in the fermentation of *Aspergillus niger* van Tieghem.

synthesize caffeine which was different from that of tea plant when the microorganisms were stimulated by some tea components. In tea leaves, the biosynthetic pathway of caffeine is considered to start with the first methylation of xanthosine yielding 7-methylxanthosine. After de-ribosylation the resultant 7-methylxanthine is further methylated to theobromine and finally to caffeine. Whereas the sequential methylations are different in the fermentation of *A. niger* van Tieghem, the possible biosynthetic pathway of caffeine is proposed in Fig. 6. The caffeine biosynthesis in microorganism may begin with the xanthosine that is converted to 3-methylxanthosine or 1-methylxanthosine catalyzed by *N*-3-methyltransferase or *N*-1-methyltransferase. Their dephosphoribosylation yields 3-methylxanthine or 1-methylxanthine, which are methylated to produce theophylline in the presence of an *N*-1-methyltransferase or *N*-3-methyltransferase. The final methylation step in the pathway is the conversion of theophylline to caffeine catalyzed by an *N*-7-methyltransferase. The other biosynthetic route may start with two successive methylations of xanthosine yielding 1,3-dimethylxanthosine, and then its de-ribosylation produces theophylline. Finally, the caffeine is formed by methylation at *N*-7 of theophylline.

4. Conclusions

Fermentation of dry green tea with three molds, *A. niger* van Tieghem, *R. arrhizus* Fisher and *M. circinelloides* van Tieghem, enhances its caffeine content, but the two yeasts decrease the caffeine content of tea. Among the molds, *A. niger* van Tieghem has the greatest influence on the increase of the caffeine content. The change of caffeine and theophylline with fermentation time is similar in the fermentation process. Possibly, theophylline is the biosynthetic precursor of caffeine in living microorganisms.

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