American Journal of Agriculture and Forestry
2018; 6(6): 162-168
http://www.sciencepublishinggroup.com/j/ajaf
doi: 10.11648/j.ajaf.20180606.11
ISSN: 2330-8583 (Print); ISSN: 2330-8591 (Online)

Application of *Aspergillus sydowii* NRRL250 to Degrade Caffeine in Pu-erh Tea

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To cite this article:

Binxing Zhou, Cunqiang Ma, Hongzhen Wang, Tao Xia, Xiaohong Li, Yang Wu. Application of *Aspergillus sydowii* NRRL250 to Degrade Caffeine in Pu-erh Tea. *American Journal of Agriculture and Forestry*. Vol. 6, No. 6, 2018, pp. 162-168. doi: 10.11648/j.ajaf.20180606.11

Received: August 30, 2018; **Accepted**: September 17, 2018; **Published**: October 15, 2018

Abstract: Pu-erh tea is produced by a solid-state fermentation. The natural microbiota presented in pu-erh tea influence caffeine level. In previous study, one effective fungi was selected from pu-erh tea and identified as *Aspergillus sydowii* NRRL250, which could lead caffeine degradation. In this paper, *A. sydowii* NRRL250 was inoculated into a liquid medium with different initial caffeine concentrations (600, 1200 and 1800 mg/L of caffeine, respectively) to explore caffeine degradation products. The solid-state fermentation of sun-dried tea leaves and submerged fermentation of tea infusion were carried out to investigate the application of *A. sydowii* NRRL250 through an inoculation. Samples were collected periodically, and the contents of caffeine, theophylline, 3-methylxanthine and theobromine were determined by HPLC. In the substrate tests, caffeine degraded drastically, theophylline and 3-methylxanthine were detected and increased obviously with the degradation of caffeine, and theobromine was not detected. In the solid-state and submerged fermentation, caffeine decreased radically ($p<0.05$), only about 4.14±0.771 mg/g and 157.8±10.21 mg/L of caffeine were remained, respectively. And theophylline had a dramatic increase ($p<0.05$), 28.29±2.463 mg/g and 501.2±13.55 mg/L of theophylline were produced in the end of the fermentation. 3-Methylxanthine also increased significantly ($p<0.05$) in the fermentation. Theobromine remained stable without significant change ($p>0.05$). Compared with the submerged fermentation without caffeine addition, the extra addition of caffeine could enhance the productions of theophylline and 3-methylxanthine significantly ($p<0.05$). Therefore, theophylline and 3-methylxanthine were the main degradation products from caffeine, caffeine concentration had a significant ($p<0.05$) effect on the productions of theophylline and 3-methylxanthine. And *A. sydowii* NRRL250 had great application potential to produce decaffeinated and high-theophylline tea through an inoculation.

Keywords: Pu-erh Tea, Fungi, Caffeine, Theophylline, Fermentation

1. Introduction

Pu-erh tea, a well-known traditional Chinese tea, is produced by a natural solid-state fermentation process with sun-dried green tea leaves (*Camellia sinensis* var. *assamica*) as raw material, which has been produced and drank for hundreds years in Southwestern China [1]. Based on recent researches, pu-erh tea has definite efficacy on reduction of waist fat, anti-oxidation, reduction of atherosclerosis probability, antibiotic, and anticancer [2-4]. Microorganisms, involved in pu-erh tea solid-state fermentation, have been mainly studied using culture-based approaches and culture-independent approaches, which includes *Aspergillus niger, A. tubingensis, A. fumigatus, A. acidus, A. awamori, Rhizomucor pusillus, R. tauricus, Blastobotrys adeninivorans, Arxula adeninivorans, Pichia farinose* and *Candida tropicalis* [5-7].

Caffeine (1, 3, 7-trimethylxanthine) is a key flavor

substance in many popular drinks, especially in tea and coffee. Caffeine remains stable in the processing of general tea (green tea, black tea, oolong tea and white tea) [8]. Due to the participation of microorganisms, especially the various fungi, caffeine content is changeable in pu-erh tea. In recent studies, caffeine content has a significant ($p<0.05$) decline in a natural solid-sate fermentation [9, 10]. And, an effective strain was selected from pu-erh tea solid-state fermentation and identified as *Aspergillus sydowii* NRRL250, which could lead caffeine biodegradation [11, 12].

In this paper, *A. sydowii* NRRL250 was used as the experimental strain and inoculated into a liquid medium with different initial caffeine concentrations to explore caffeine degradation products. The application of *A. sydowii* NRRL250 in solid-state fermentation and submerged fermentation were investigated through inoculation. This report found that theophylline and 3-methylxanthine are the main degradation products from caffeine in *A. sydowii* NRRL250 metabolism. In addition, the inoculation of *A. sydowii* NRRL250 is an appropriate method to produce decaffeinated and high-theophylline tea.

2. Materials and Methods

2.1. Materials

Sun-dried green tea leaves (*C. sinensis* var. *assamica*) with moisture content 6.25% by weight were provided by Yunnan Agricultural University (China). Caffeine (≥99%), theophylline (≥99%), 3-methylxanthine (≥99%) and theobromine (≥99%) were purchased from Sigma-Aldrich (USA). Acetonitrile was purchased from Fisher (USA). *A. sydowii* NRRL250 (EF652450), selected from pu-erh tea, was identified by and stored at Yunnan Institute of Microbiology (China).

2.2. Inoculation in a Liquid Medium

Spore solutions of *A. sydowii* NRRL250 were prepared by growing the fungi in dishes containing solid culture medium with glucose at 30°C for 5 d [13]. Two loopfuls of strain were transferred aseptically from a dish slant into 25 mL of a sterile liquid medium (per liter: potato starch 4 g, dextrose 20 g, chloramphenicol 0.1 g) with different initial caffeine concentrations (600, 1200 and 1800 mg/L of caffeine, respectively) in a 125 mL Erlenmeyer flask. The flask was incubated aerobically on an incubator shaker (250 rpm) at 30°C for 48 h. The volume of the seed was 10 % (v/v) of total initial volume [14]. The flask was incubated in an orbital shaker for 3, 6, 9, 12 and 15 d (130 rpm, 30°C), respectively. Biodegraded products of caffeine were analyzed by HPLC [15].

2.3. A Solid-State Fermentation Inoculated by A. Sydowii

Two loopfuls of strain were transferred aseptically from a dish slant into 25mL of sterile tea infusion in a 125 mL Erlenmeyer flask. The flask was incubated aerobically on an incubator shaker (250 rpm) at 30°C for 48 h as the seed for inoculation. Sun-dried green tea leaves (20 g) was mixed with distilled water (12.25 mL) to have a solid content of 62% (w/w). After sterilization at 121°C for 5 min, 1 mL seed was inoculated into per bottle. Inoculated bottles were cultivated in an incubator (85% of humidity, 30°C) to a solid-state fermentation. Samples were collected every 5 days. Caffeine, theophylline, 3-methylxanthine and theobromine were determined by HPLC [15].

2.4. A Submerged Fermentation Inoculated by A. Sydowii

Sun-dried green tea leaves (1.0 g) were infused for 15 min in boiling distilled water (30 mL) and the tea infusion was made up to 30 mL with distilled water after filtration [14]. The seed was inoculated into sterile tea infusion with a volume of 10 % (v/v). 1000 mg extra caffeine was added into 1000 mL tea infusion to enhance caffeine concentration as caffeine-added group for the submerged fermentation. The flasks were incubated in an orbital shaker for 3, 6, 9, 12 and 15 d (130 rpm, 30°C), respectively. Caffeine, theophylline, 3-methylxanthine and theobromine were determined by HPLC [15].

2.5. Determination of Caffeine and Other Related Purine Alkaloids by HPLC

Caffeine, theophylline, 3-methylxanthine and theobromine were determined by Agilent 1200 HPLC equipment (USA) using Agilent C_{18} Chromatogram column (250 mm×4.6 mm, 5 μm) (USA) with solvent A (100% acetonitrile) and solvent B (0.2% v/v acetic acid water solution) as mobile phase [15]. The gradient was programmed as follows. The mobile phase (at o min) consisted of 92% (v/v) solvent A (100% acetonitrile) and 8% (v/v) solvent B (0.2% v/v acetic acid water solution). And then, solvent A was decreased linearly to 69% (v/v) at 50 min, whereas solvent B was increased linearly to 31% (v/v) at 50 min. The flow rate was 1.0 mL/min and 10 μL was injected. The column temperature was set at 30°C and the monitored wavelength was 280 nm.

2.6. Statistical Analysis

Three identical experiments were repeated to obtain reliable results. The mean value and standard deviation of analytic was calculated using SPSS 20.0 for Windows. The significant differences ($p<0.05$) was analyzed using one-way analysis of variance (ANOVA) by Duncan`s multiple-range test.

3. Results

3.1. Caffeine Degradation Capability in a Liquid Medium

To investigate caffeine degradation capability, *A. sydowii* NRRL250 was inoculated into a liquid medium with different caffeine concentrations. Caffeine content was determined by HPLC and the results were showed in Figure 1. Caffeine degraded obviously and caffeine removal rate enhanced drastically in the inoculation, which confirmed again that *A. sydowii* NRRL250 could lead caffeine biodegradation. As

shown in Figure 1, caffeine removal rate had a significant difference in the inoculation. Caffeine degraded completely in a low caffeine concentration (600 mg/L of caffeine). However, caffeine removal rate was only 64.2±2.10% in a high caffeine concentration (1800 mg/L of caffeine), which showed that caffeine degradation capability of *A. sydowii* NRRL250 was limited by caffeine concentration.

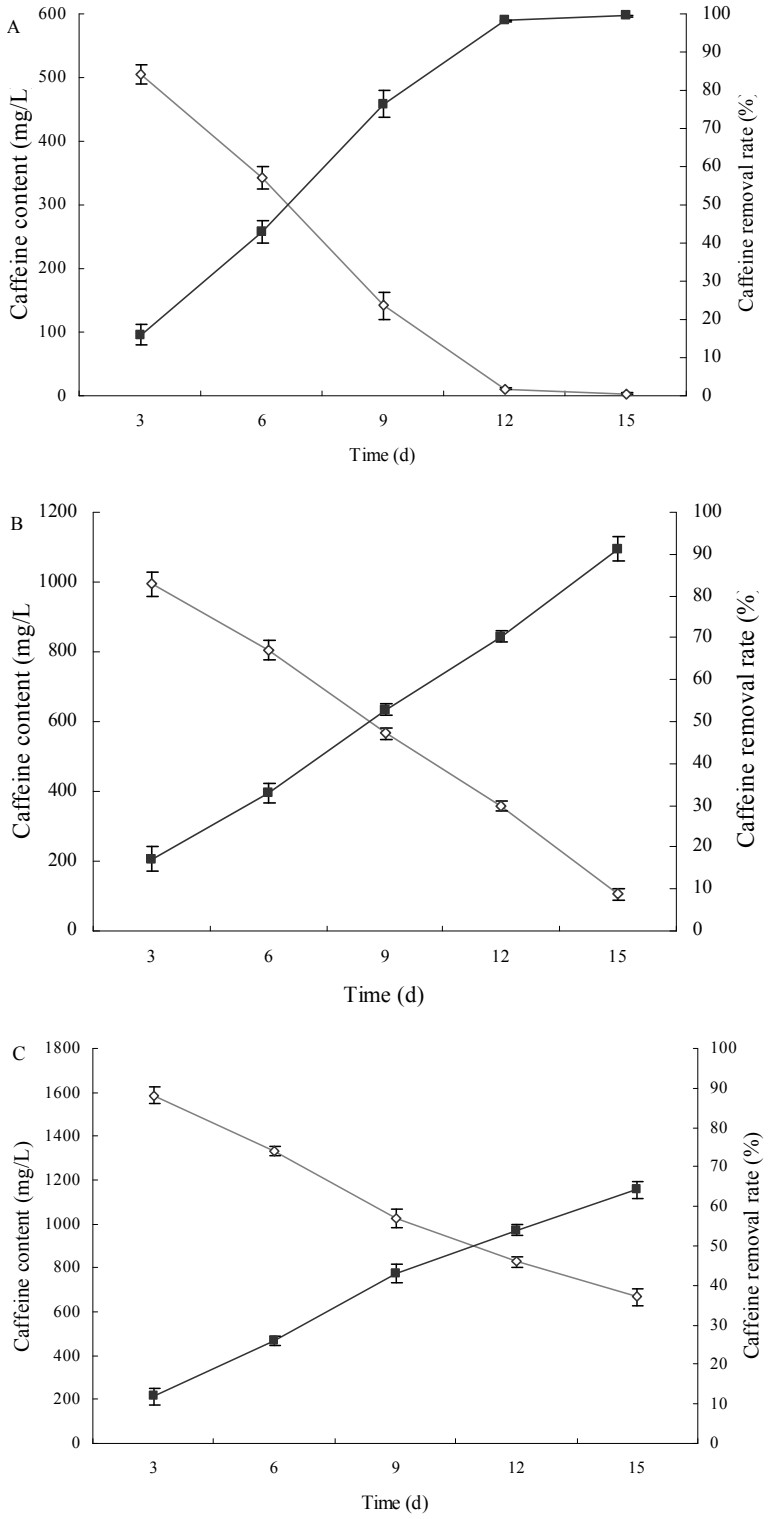
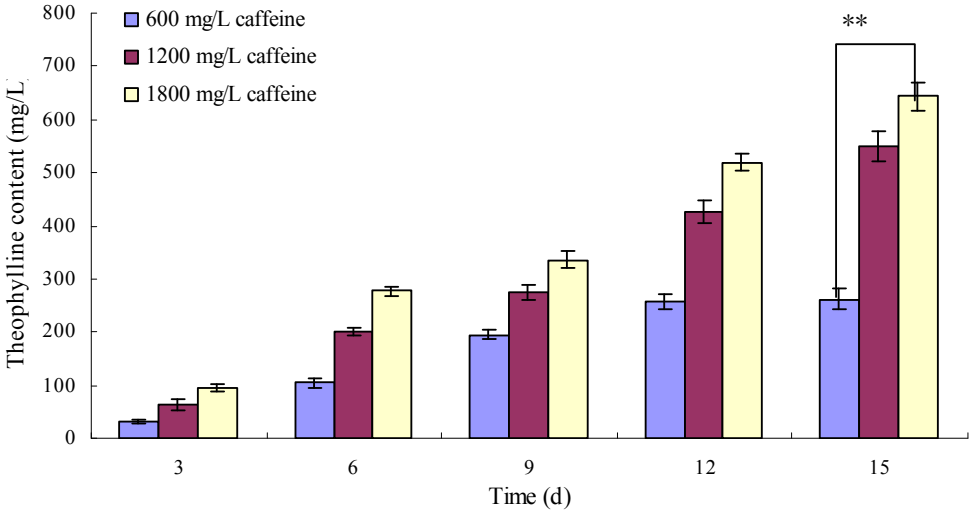


Figure 1. *Changes in caffeine content and caffeine removal rate in a liquid mediums with 600 (A), 1200 (B) and 1800 mg/L of caffeine (C), respectively.*

Open rhombus stands for caffeine content and shaded square stands for caffeine removal rate. Data are presented as mean values ± SD.
Caffeine content was determined by HPLC. Caffeine removal rate was estimated as follow:
Caffeine removal rate (%) = $(C_0-C_t)/C_0*100\%$ (1)
In Eq. (1) C_0 was the initial caffeine concentration (mg/L), C_t was the final caffeine concentration (mg/L) after the fermentation.

3.2. Caffeine Degradation Products Analysis of A. Sydowii NRRL250

In the physiology of tea tree (*Camellia sinesis* (L.) *O. Kuntze*), theophylline (1, 3-dimethyxanthine) and 3-methylxanthine are the main degradation products from caffeine [16]. And theobromine (3, 7-dimethyxanthine) participates in the caffeine anabolism as the direct precursor [17]. In the secondary metabolism of specific microorganism, the catabolism and anabolism of caffeine was similarity that in the physiology of tea tree [18, 19]. To explore caffeine degradation products, apart from caffeine, theophylline, 3-methylxanthine and theobromine were determined by HPLC in the inoculated liquid medium, and the results were showed in Figure 2 and Table 1. respectively.



Data are presented as mean values ± SD. ** shows the significant difference levels ($p<0.05$) between a liquid medium with 600 and 1800 mg/L of caffeine.

Figure 2. *The change of theophylline content in a liquid mediums with 600, 1200 and 1800 mg/L of caffeine, respectively.*

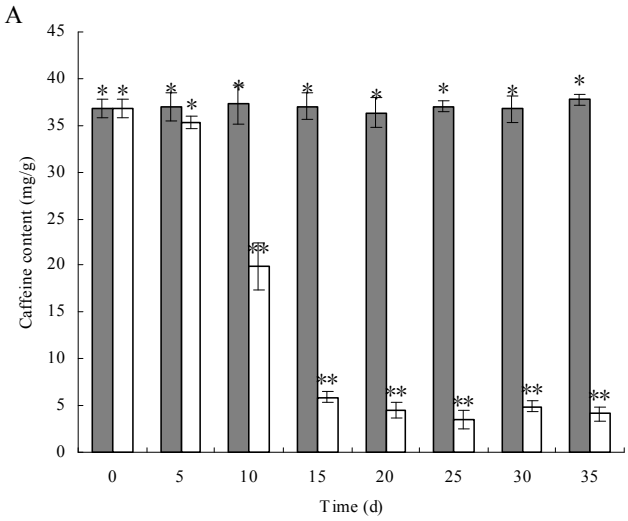
Table 1. *3-Methylxanthine contents in a liquid medium with different concentrations of caffeine.*

Time (d)	600 mg/L caffeine	1200 mg/L caffeine	1800 mg/L caffeine
3	NF	NF	14.3±2.6
6	12.9 ±2.4	28.4±1.5	32.8±2.3
9	19.4 ±2.1	38.4±3.4	57.5±5.6
12	52.2 ±5.7	68.1±6.9	129.5±5.7
15	115.8 ±10.1	178.7±10.8	191.2±4.5

All data are presented as mean ±SD. NF= not found.

As shown in Figure 2, theophylline was detected consecutively on 3 d and increased radically in the inoculation. Within 15 d, 262.2±20.7, 549.4±29.3 and 643.8±25.3 mg/L of theophylline were produced, respectively. Compared with the low caffeine concentration, the production of theophylline increased significantly ($p<0.05$) in a liquid medium with 1800 mg/L of caffeine, which showed that theophylline was from caffeine degradation and higher caffeine concentration could improve the production of theophylline to a certain extent. 3-Methylxanthine content was far below theophylline. Only about 115.8±10.1, 178.7±10.8 and 191.2±4.5 mg/L of 3-methylxanthine were produced within 15 d (Table 1). Considered that 3-methylxanthine was first detected on 6 d in low caffeine concentration (600 and 1200 mg/L of caffeine), 3-methylxanthine might be from a direct degradation product from theophylline through demethylation. Theobromine was not detected in the inoculated liquid medium, which indicated that theobromine might not participate in caffeine degradation.

3.3. Application of A. sydowii NRRL250 in a Solid-Sate Fermentation



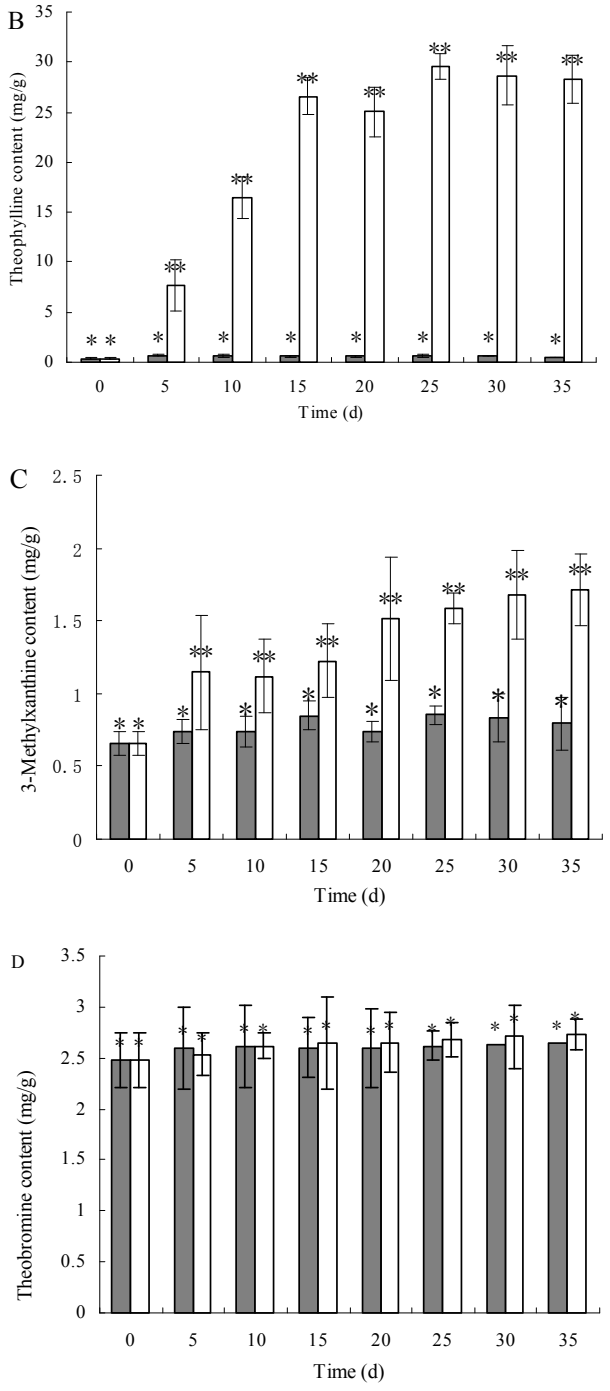
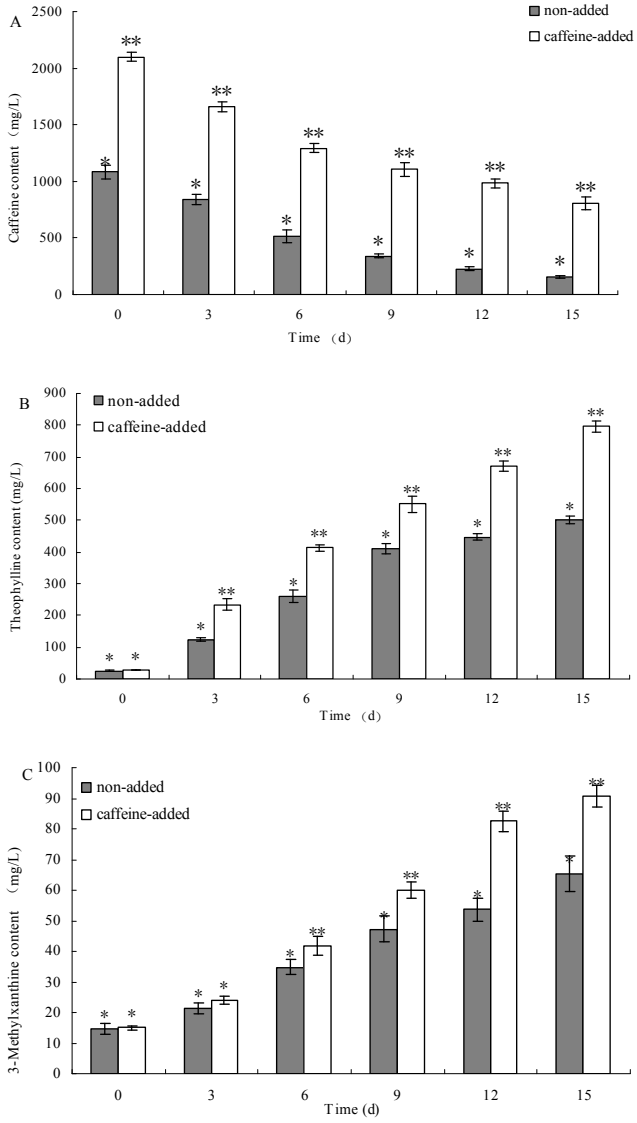


Figure 3. *Changes in caffeine (A), theophylline (B), 3-methylxanthine (C) and theobromine (D) during sterilization treatment (control) and inoculated fermentation, respectively.*

Shaded histogram stands for sterilization treatment and open histogram stands for inoculated fermentation. Data are presented as mean values ± SD. ** shows the significant difference levels ($p<0.05$) between sterilization treatment and inoculated fermentation.

Due to caffeine degradation characteristic, *A. sydowii* NRRL250 was suitable to produce decaffeinated and high-theophylline tea through an inoculation. In this paper, with sterilization treatment as control, *A. sydowii* NRRL250 was inoculated into sterilized tea leaves for solid-sate fermentation. Caffeine, theophylline, 3-methylxanthine and theobromine contents were determined by HPLC every 5 d. The results were showed in Figure 3. Compared with the sterilization treatment, caffeine content had a significant decrease ($p<0.05$) in the inoculated fermentation (Figure 3A). Meanwhile, the contents of theophylline and 3-methylxanthine had a significant increase ($p<0.05$) (Figure 3B and 3C). However, theobromine content remain stable without significant change ($p>0.05$) (Figure 3D). In the end of the fermentation, only about 4.14±0.771 mg/g of caffeine was remained. And 28.29±2.463 mg/g of theophylline and 1.71±0.243 mg/g of 3-methylxanthine were produced, respectively. Therefore, the solid-state fermentation inoculated by *A. sydowii* NRRL250 was an appropriate method to produce decaffeinated and high-theophylline tea.

3.4. Application of A. Sydowii NRRL250 in a Submerged Fermentation



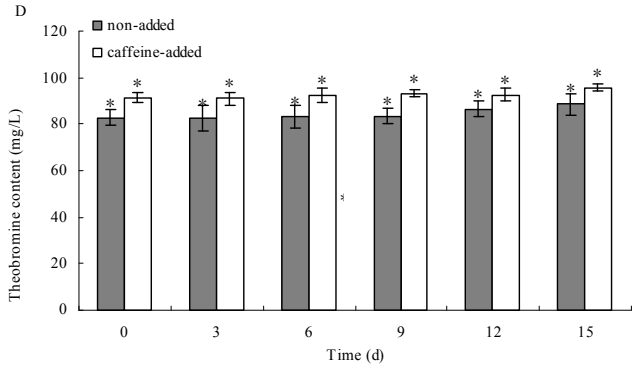


Figure 4. *Changes in caffeine (A), theophylline (B), 3-methylxanthine (C) and theobromine (D) during tea infusion submerged fermentation with and without caffeine-added, respectively.*

Data are presented as mean values ± SD. ** shows the significant difference levels ($p<0.05$) between caffeine-added group and non-added group.

A. sydowii NRRL250 was inoculated into the sterile tea infusion to investigate the application of *A. sydowii* NRRL250 in the submerged fermentation. And the caffeine-added group was carried out to explore the effect of caffeine on the productions of theophylline and 3-methylxanthine. Caffeine, theophylline, 3-methylxanthine and theobromine contents were determined by HPLC at 0, 3, 6, 9, 12 and 15 d, respectively, and the results were showed in Figure 3. *A. sydowii* NRRL250 could degrade caffeine obviously (Figure 3A) and enhance the productions of theophylline (Figure 3B) and 3-methylxanthine (Figure 3C) in the submerged fermentation. Theobromine does not participate in caffeine degradation, so theobromine content remained stable without significant ($p>0.05$) change in the submerged fermentation (Figure 3D). Compared with the non-added group, the extra addition of caffeine could enhance the productions of theophylline and 3-methylxanthine significantly ($p<0.05$). However, the caffeine degradation capability of *A. sydowii* NRRL250 was limited, not all extra caffeine could be degraded in caffeine-added group. The optimal concentration for caffeine degradation in tea infusion deserve further study.

4. Discussion

Caffeine and related methylxanthines are toxic to most bacteria and invertebrates [20]. However, several bacteria and fungi have the ability to metabolize caffeine, such as *Pseudomonas putida* CBB5 [21], *Pseudomonas* sp. GSC1182 [22] and *A. tamari* [23]. Fungi, involved in pu-erh tea solid-state fermentation, have certain effect on caffeine and other purine alkaloids [24, 25]. Previous study [11, 12] found that caffeine content decreased significantly ($p<0.05$) in a natural solid-sate fermentation and *A. sydowii* NRRL250 was selected from pu-erh tea, which could lead caffeine degradation.

A. sydowii NRRL250 was used in this paper and inoculated into a liquid medium with different caffeine concentration to explore caffeine degradation products. Theophylline and 3-methylxanthine were detected along with the degradation of caffeine, which confirmed the caffeine catabolism that theophylline and 3-methylxanthine were degradation products from caffeine through demethylation [18, 19]. Plenty of theophylline was produced in the inoculated liquid medium, which showed that theophylline was the main degradation product from caffeine and *A. sydowii* NRRL250 could be used in the production of theophylline.

Considered that *A. sydowii* is an important industrial and medical microorganism [26, 27, 28], *A. sydowii* NRRL250 had application potential in tea. Due to the caffeine degradation characteristic, *A. sydowii* NRRL250 was suitable to produce decaffeinated and high-theophylline tea through an inoculation. In a solid-state fermentation and a submerged fermentation inoculated by *A. sydowii* NRRL250, caffeine degraded drastically, and theophylline and 3-methylxanthine were produced massively, which showed that the inoculated fermentation was an appropriated way to produce decaffeinated and high-theophylline tea. And the extra addition of caffeine could enhance the productions of theophylline and 3-methylxanthine significantly ($p<0.05$).

5. Conclusions

The purpose of this research was to analyze the degradation products from caffeine and investigate the application of *Aspergillus sydowii* NRRL250 through an inoculation. The results show that *A. sydowii* NRRL250 could degrade caffeine, and convert caffeine to theophylline and 3-methylxanthine massively. During solid-sate fermentation of sun-dried tea leaves and submerged fermentation of tea infusion, the contents of theophylline and 3-methylxanthine increased significantly ($p<0.05$) along with caffeine degradation. Especially, 28.29±2.463 mg/g and 501.2±13.55 mg/L of theophylline were produced in the end of the fermentation, respectively. Therefore, *A. sydowii* NRRL250 had great application potential in the production of theophylline. In addition, the extra addition of caffeine could enhance the productions of theophylline and 3-methylxanthine significantly ($p<0.05$) in the submerged fermentation.

Acknowledgements

This work was supported by Modern Agricultural Tea Industry System of Yunnan Province, China (2017KJTX007), Modern Agricultural Industry Technology System of China (CARS-23) and Natural Science Foundation of China (C161104).

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