

## Research Article

### Study of Bioactive Components and Color Properties of Dark Tea Infusion Manufactured by *Eurotium cristatum* Using Submerged Fermentation

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**Abstract:** The use of green tea infusion as culture broth for production of Dark Tea Infusion (DTI) with *Eurotium cristatum* was investigated. Fermentation process of produced DTI was monitored for 120 h, bioactive components and color properties were studied. After 120 h of fermentation, the levels of caffeine and flavonoids increased while contents of tea polyphenols, free amino acids and carbohydrates decreased. Thearubigins (TRs) and Theabrownin (TB) were the representative component in DTI, which increased in amount during Submerged Fermentation (SF). Turbidity of cultured broths decreased while lightness increased. The cultured broths formed the typical dark tea color characteristics of “redness” and “maroon” after SF and color properties were obviously improved.

**Keywords:** Bioactive components, color properties, dark tea infusion, *Eurotium cristatum*, submerged fermentation

## INTRODUCTION

Fu-brick tea is a kind of unique dark tea with brick form compressed from the older, coarse and rough leaves and small branches of *Camellia sinensis*, mainly distributed in Hunan, Sichuan, Shaanxi and Yunnan provinces in China (Zhang *et al.*, 2013). *E. cristatum* is a safe and healthy fungi used during the traditional manufacturing process of Fu-brick tea (Malloch and Cain, 1972; Xu *et al.*, 2011a). Studies showed that, corresponded with stimulation of enzymes, *E. cristatum* fermentation can result in changes of catechins, free amino acids, proteins and polysaccharides (Wu *et al.*, 2010; Zhang *et al.*, 2014). These changes not only formed the unique color, aroma and taste of Fu-brick tea (Xu *et al.*, 2007), but also endow Fu-brick tea varieties of beneficial health effects, including antioxidant (Cheng *et al.*, 2015), antibacterial (Keller *et al.*, 2013), anti-obesity and hypolipidemic (Li *et al.*, 2013; Fu *et al.*, 2011). Accordingly, Fu-brick tea has been accepted and loved by growing numbers of consumers throughout China, Korea and Japan.

The process techniques of traditional Fu-brick tea in solid-state fermentation involve panning, rapid pile fermentation, rolling, drying, softening with steam, piling, tea brick pressing, fungal fermentation and drying (Mo *et al.*, 2008). However, the production process remains lengthy. Compared with the Solid-State Fermentation (SSF), making dark tea products by

*E. cristatum* using SF have a bunch of advantages, such as short fermentation time, mild manufacture conditions and avoid mixed microbial contamination. Above all, SF simplified the process and easy realization of large-scale production (Hsu *et al.*, 2002; Carnevali *et al.*, 2007).

Recent studies have focused on the biological activities of dark tea (Luo *et al.*, 2013; Zhu *et al.*, 2015). In this study, the SF of *E. cristatum* by using the Green Tea Infusion (GTI) was studied. The changes of bioactive components and color properties of cultured broths were investigated.

## MATERIALS AND METHODS

**Materials:** The green tea material (4<sup>th</sup> to 7<sup>th</sup> tea leaves) for processing research was provided by Damin Foodstuff (Zhangzhou) Co., Ltd. (Zhangzhou, Fujian, China). The green tea was broken up manually and extracted by water at 80°C for 20 min (Green tea : water = 1:15, w/v) with gentle stirring, then filtered through 0.2 μm ceramic membrane (Pall, USA) to remove the debris. The filtered green tea infusion was concentrated to as culture broth (6%, w/w) and stored at 4°C until use. *E. cristatum* strain, which was isolated from the ripened Fu-brick tea and identified according to their morphological characteristics and ITS, was deposited in the China General Microbiological Culture Collection Center (CGMCC) under accession number 10610. Folin-Ciocalteu phenol, Ninhydrin, Rutin,

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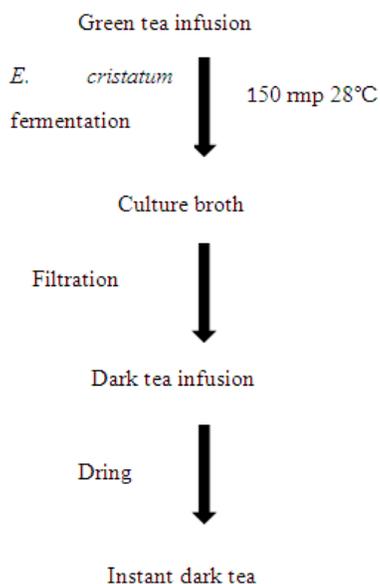


Fig. 1: Simplified flow diagram of processing procedures for instant dark tea

Aluminum chloride, Anthrone, Glucose, Glutamic, Phosphoric acid, Sodium carbonate, Sodium hydroxide, Ethyl acetate, Normal butanol, analytical grade, were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and Catechin (C), Epicatechin (EC), Epigallocatechin (EGC), Epigallocatechin Gallate (EGCG), Epicatechin Gallate (ECG), Caffeine, HPLC grade, were purchased from Sigma-Aldrich (Sigma, USA). Other reagents were analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd.

**Instant dark tea process:** In the present study, fermentation process was carried out in a shaking incubator (ZHWHY-2102C; Labwit Scientific, Shanghai, China). Fungus inoculums were inoculated into 100 mL GTE with  $3 \times 10^6$  CFU/mL for pure cultures at 28°C and 150 rpm for 120 h, culture broth were obtained. Then the culture broth was filtered by filtrate paper to remove mycelia and freeze-dried using a vacuum freezing dryer (GAMMA 1-16 LSC; Christ, Germany) to get instant dark tea product. The processing technic of instant dark tea process was demonstrated in Fig. 1.

**Determination of chemical composition:** Cultured broths were collected at different fermentation time of 0, 12, 24, 36, 48, 60, 72, 84, 96, 108 and 120 h, respectively. The collected cultured broths were filtrated and then freeze-dried for all compositions determination. The contents of total polyphenols, flavonoids, free amino acids and total carbohydrates were determined by a colorimetric method (Li *et al.*, 2013; Lin *et al.*, 2014). Total polyphenol contents were determined by a method with Folin-Ciocalteu phenol reagent using gallic acid as a standard. Total flavonoids were measured using a method with 10%  $\text{AlCl}_3$  solution with rutin as a standard. Free amino acids were

measured using a method with Ninhydrin solution with glutamic as a standard. Total carbohydrates were analyzed using anthrone-sulphuric acid assay with glucose as a standard. The total contents of tea pigments were detected with the method as Wang *et al.* (2011). The HPLC analytical method was used to determine catechins. Separations were carried out using an Agilent  $\text{C}_{18}$  reverse phase column (250×4.6 mm i.d., 5 μm) protected with a security guard cartridge (Gemini  $\text{C}_{18}$ , 4×2.0 mm i.d., Phenomenex). A binary solvent system was utilized: Phase A, water, acetonitrile, acetic acid and EDTA (888/90/20/2, v/v); Phase B, water, acetonitrile, acetic acid and EDTA (18/800/80/2, v/v). For catechins analysis 10 μL of each sample was injected. Samples were separated at a flow rate of 1.0 mL/min with a linear gradient elution from 0 to 100% A over 10 min, followed by decreasing A to 68% within 15 min. The elution with 68% A remained 10 min followed by increasing A to 100%. Catechins were verified by the elution time of standards and were quantitated by the corresponding standard curves ( $R^2$ , 0.9997-1.0000). Integrations of each compound were conducted at 280 nm.

#### Measurement of the color difference and turbidity:

Cultured broths were collected at different fermentation time of 0, 12, 24, 36, 48, 60, 72, 84, 96, 108 and 120 h, respectively. Then these samples were filtrated by filtrate paper and cooled to room temperature before the color was measured. The white plate was used as background. Colors of culture broth were measured using Hunter Lab Color Measuring System (Color Quest XE; Hunter Associates Laboratory, USA). Values were measured in terms of Lightness ( $L^*$ ) and color (+a\*: red, -a\*: green, +b\*: yellow, -b\*: blue). Turbidity was measured with a turbidity metre (Turb555/Turb555IR; WTW, Germany) and expressed in Nephelometric Turbidity Units (NTU).

**Data analysis and statistics:** All data were expressed as means±standard deviations of triplicate determinations. Origin (Version 8.0; Origin Lab, USA) and Excel (Version 2010, Microsoft, USA) were used for statistical analysis.

## RESULTS AND DISCUSSION

**Tea polyphenols analysis:** Fermentation is the most important procedure for forming the special flavor of dark tea which derived from polyphenols by oxidation. It also influences the contents of major catechins in teas. As a result, dark tea develops a taste that is wonderfully complex, silky smooth and mellow. Total Tea Polyphenols (TTP), flavonoids and catechins content change were illustrated in Fig. 2. Results showed that the levels of TTP and catechins have changed significantly during SF. TTP decreased by 22.89%, while flavonoids increased by 11.16% (Fig. 2A). There is a dramatic decrease in EGCG and

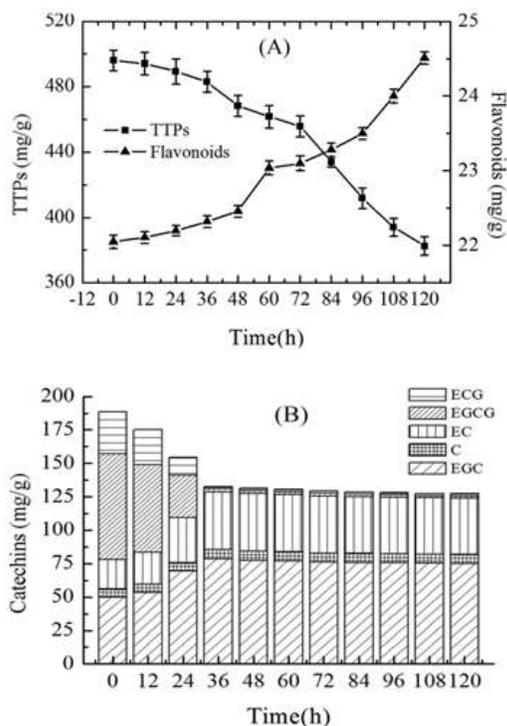


Fig. 2: Changes in the tea polyphenols; (A): Catechins; (B): Content over 120 h

ECG during SF (Fig. 2B). Since the gallate ester bond of EGCG and ECG are sensitive chemical group with respect to fungal enzymes, while C, EC and EGC are simple catechins, the molecular structure of which are flavanols without the ester bond of gallic acid. During beginning period of the fermentation (0-36 h), these simple catechins were accumulated from the decomposing of EGCG and ECG. But the contents of simple catechins were still decreased due to an oxidative product of catechin on the B-ring of flavanols after long-term fermentation (Qin *et al.*, 2012). Similar behavior has been reported by other authors (Zhang *et al.*, 2014; Keller *et al.*, 2013; Zhu *et al.*, 2015).

**Total free amino acid, carbohydrates and caffeine analysis:**

Total free amino acid and total carbohydrates contents were shown in Fig. 3A. Results showed that the contents of total free amino acid were slightly increased from 71.13 to 77.32 mg/g at the first 24 h of fermentation and then there was a gradually decrease after 24 h, final total free amino acid contents were determined to be 65.96 mg/g, which were highly decreased as compared with at the beginning of fermentation. The levels of total free amino acids were accumulated at the first 24 h of fermentation because of the decomposing of protein by fungus protease and then they were utilized subsequently due to the need of the microbial nitrogen metabolism (Wan, 2003).

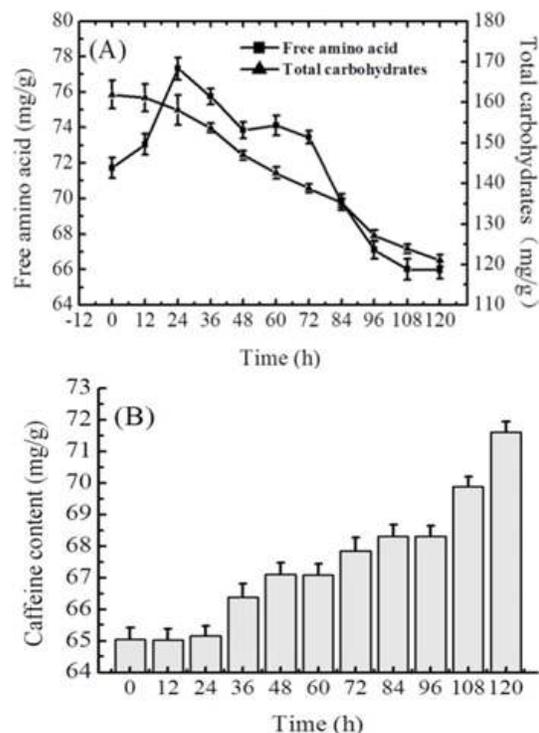


Fig. 3: Changes in the total free amino acid carbohydrates; (A): Caffeine; (B): Content over 120 h

Total carbohydrates can be used as main carbon and energy source during the microorganism fermentation. Total carbohydrates continue to be consumed during SF. It dropped from 153.67 to 121.01 mg/g (Fig. 3A).

The change of caffeine content was illustrated by Fig. 3B. The results indicated that caffeine content increased apparently during SF. The phenomenon that the content of caffeine in tea leaves can increase during the traditional manufacturing of dark tea has been observed (Wang *et al.*, 2005). Wang *et al.* (2008) have indicated that *A. niger* van Tieghem has a significant influence on the increase of caffeine content, the change of caffeine is similar to theophylline during fermentation. Theophylline might act as a biosynthetic precursor of caffeine in living microorganisms.

**Tea pigments analysis:** Tea pigments, including Theaflavins (TFs), Thearubigins (TRs) and Theabrownin (TB), are important component in dark tea infusion and can determine the color and quality of the dark tea. During the fermentation, the contents of TRs levels increased continuously, from 11.63 to 19.81% (Fig. 4A). Wang *et al.* (2010) have shown that TB content continuously increases during the traditional dark tea fermentation. However, our results indicated that content of TB was decreased from 13.81 to 6.12% at the first 48 h of fermentation and then the TB content

Table 1: Three color coordinates ( $L^*$ ,  $a^*$  and  $b^*$ ) and turbidity (NTU) of cultured broths at different time

Time (h)	$L^*$	$a^*$	$b^*$	Turbidity (NTU)
0	23.68±0.55	37.14±0.29	40.80±0.62	776.38±36.38
12	24.87±0.53	39.83±0.21	42.87±0.71	603.20±31.20
24	27.95±0.50	40.55±0.22	48.16±0.65	512.39±21.39
36	29.37±0.51	40.60±0.37	50.58±0.58	449.73±18.61
48	29.50±0.51	41.28±0.26	50.82±0.71	323.27±15.73
60	30.72±0.53	42.16±0.30	52.91±0.46	253.62±8.62
72	32.30±0.52	42.31±0.25	53.91±0.51	205.35±8.55
84	35.18±0.51	42.43±0.33	55.42±0.66	166.20±4.84
96	37.83±0.50	42.06±0.27	56.51±0.49	136.15±4.77
108	38.45±0.54	41.09±0.35	57.01±0.54	121.49±3.35
120	39.62±0.53	40.21±0.26	59.99±0.52	96.15±2.87

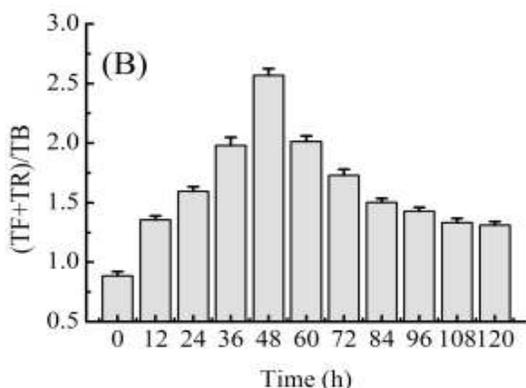
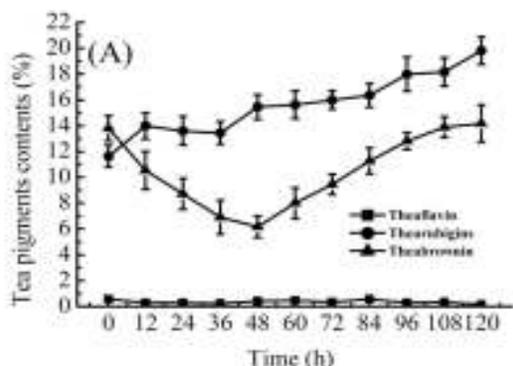


Fig. 4: Changes in the tea pigments; (A): Content and the index of (TF + TR)/TB; (B): Over 120 h

was gradually increased (48-120 h) from 6.12 to 14.17% (Fig. 4A). This might be because SF had an acidic condition. TB contains polyphenols, proteins and polysaccharides, proteins under acidic condition variability, reducing the solubility of settlement (He *et al.*, 2012). TFs have no obvious change during the fermentation (Fig. 4A). Microbes can produce a variety of enzymes during SF, including Polyphenol Oxidase (PPO), Peroxidase (POD), cellulase and pectinase. Activities of the enzymes involved in PPO and POD were relatively low during the first 48 h of fermentation and catechins were converted into TRs mainly in this period. The activity of PPO and POD enzymes appeared to increase after 48 h fermentation accompanied rapidly increase of TB content (Wang *et al.*, 2011, 2014).



Fig. 5: Changes in tea infusion color during fermentation; a, b, c illustrate the color of cultured broths of 0, 84 and 120 h

**Lightness and turbidity:** Turbidity and  $L^*$  Change were shown in Table 1. Lightness significantly increased from 23.68 to 39.62 during the fermentation. Turbidity sharply decreased from 776.38 to 96.15 NTU. Tea cream not only has an unattractive appearance but also damages quality of tea infusion. Catechins, minerals and polysaccharides are important chemical components in the formation of tea cream (Xu *et al.*, 2011b, 2012). Tea cream is a precipitate formed as tea cools and is assumed to be due to complexation between polyphenols and various other compounds (Aurélié *et al.*, 2010). Using submerged fermentation process, polyphenols, free amino acids and carbohydrates showed a substantial decrease in cultured broths, interaction between different chemical composition and polyphenols were weakened, therefore, lightness and clarity of cultured broth had improved significantly.

**Redness and yellowness:** The values of  $a^*$  ( $+a^*$ : red,  $-a^*$ : green) and  $b^*$  ( $+b^*$ : yellow,  $-b^*$ : blue) were positively and significantly correlated with dark tea appearance and total quality and the  $b^*$  was also significantly correlated with quality of tea infusion, high quality fermented tea was deeper in redness and yellowness color than that of low quality tea (Liang *et al.*, 2005). Changes of  $a^*$  and  $b^*$  were reported in Table 1.  $a^*$  showed an increasing trend during 0-84 h fermentation and began to decline after 84 h.  $b^*$  increased from 40.81 to 59.99 (Table 1). This phenomenon indicated that *E. cristatum* could

considerably improve the color of green tea infusion by submerged fermentation.

The contents of TFs, TRs and TB significantly correlated with the color parameters of tea infusion, the index of (TF + TR) /TB, which represented the redness of dark tea infusion, strongly correlated to the corresponding color parameters of dark tea infusion (Zou *et al.*, 2014). The index of (TF + TR) /TB in our study were shown in Fig. 4B. The index of (TF + TR) /TB sharply increased with time up to a maximum value and then decreased. The variation trend was similar to a\* value (Table 1). The color of tea leaf and tea infusion changed constantly during the traditional dark tea solid-state fermentation and the color of dark tea leaves changes to “maroon” and the tea infusion color becomes to “redness” or “maroon” (Xie *et al.*, 2009). As shown in the picture (Fig. 5), the color of tea infusion presented light-yellow at the beginning of fermentation. After 84 h, the color of culture broth showed the characteristics of “redness” and “maroon”.

## CONCLUSION

The main bioactive compounds and color of tea infusion can distinguish effectively different tea. Inoculate *E. cristatum* by SF can change the content of main bioactive compounds in tea infusion. The culture broth formed the unique dark tea color characteristics of “redness” and “maroon”. The results provided us the feasibility of using green tea material for manufacturing the instant dark tea product by the submerged fermentation.

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