OPTIMIZATION OF SUBMERGED FERMENTATION PARAMETERS FOR INSTANT DARK TEA PRODUCTION BY EUROTUM CRISTATUM

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ABSTRACT

In this study, green tea extracts was used as material to produce a kind of instant dark tea by Eurotium cristatum via submerged fermentation. Response surface methodology was implemented with desirability function approach for optimization of fermentation parameters of instant dark tea product. The effects of inoculum size (6–12%), liquid–solid ratio (10–20 mL/g) and fermentation time (132–204 h) on mycelia dry weight, redness and turbidity of fermented tea infusion were investigated. The optimum condition was as follows: inoculum size of 9.35%, liquid–solid ratio of 14.50 mL/g and fermentation time of 169.00 h. At this optimum point, mycelia dry weight, redness and turbidity of fermented tea infusion was 0.579 g/100 mL, 41.35 and 76.96 nephelometric turbidity units, respectively. Inoculated Eurotium cristatum by submerged fermentation changed the content of main bioactive compounds in tea extracts. The tea extracts formed the unique dark tea taste, aroma and color.

PRACTICAL APPLICATION

Using waste green tea leaves as raw materials, we developed a new strategy based on submerged fermentation to process instant dark tea product by Eurotium cristatum. A dark tea infusion was achieved with good taste, aroma and color at very short time and in mild manufacturing conditions compared with traditional solid state fermentation technology. Fermentation of tea extracts by Eurotium cristatum could combine the function of tea and the fungus and develop a new product with more health benefits. The submerged fermentation method increases the process efficiency to achieve similar product quality, which will be a breakthrough for instant dark tea industry.

INTRODUCTION

Dark teas are one of the most popular commercial teas in China. Chinese dark teas (CDTs) belong to post-fermented teas, including Fu-brick tea, Puer tea, Kang-brick tea, Qing-brick tea, Liubao tea, etc. CDTs are produced mainly in Hunan, Shanxi, Sichuan and Yunnan province, China (Zhang et al. 2013). Different from the manufacturing methods of green tea, oolong tea or black tea, CDTs need to go through a special microbial post-fermented process, i.e., a “pile process”, which allows enzymatic oxidation and non-enzymatic autoxidation to complete the process. The microorganism involved in the “pile process” have been proved to be an important factor in the formation of color, aroma and taste of dark tea.

Traditional Fu-brick tea is a kind of unique Chinese dark tea with brick form compressed from the coarse leaves of Camellia sinensis. Fu-brick tea possesses varieties of beneficial health effects, including antioxidant (Cheng et al. 2013), antibacterial (Li et al. 2013), anti-obesity and hypolipidemic (Keller et al. 2013). Eurotium cristatum, a unique yellow fungus, was found to be abundant during the traditional manufacturing process of Fu-brick tea (Mo et al. 2008; Xu et al. 2011; Zhang et al. 2013). E. cristatum from Fu-brick
tea is not only strongly correlated to the quality of the tea, but also has varieties of potential benefits for human health (Xu et al. 2011; Peng et al. 2014).

The manufacturing process of traditional Fu-brick tea involves panning, rapid pile fermentation, rolling, drying, softening with steam, piling, tea brick pressing, fungal fermentation and drying (Mo et al. 2008), which is a solid-state fermentation technology (SSF). Compared with the SSF, making dark tea products by *E. cristatum* using submerged fermentation (SF) has a bunch of advantages, such as a shorter fermentation time, mild manufacturing conditions and avoiding mixed microbial contamination, and most especially, SF can simplify the process and make it easy to obtain a large-scale continuously automatic production (Hsu et al. 2002; Carnevali et al. 2007).

Instant dark tea products have been accepted by an increasing numbers of consumers all around the world in recent years. Ready-made dark tea as a conventional material used to be processed to instant dark tea, i.e., the ready-made dark tea was crushed and extracted by boiling water with gentle stirring, then filtered through ceramic membrane to remove the debris, and the filtered solution was concentrated by vacuum thickener and dried to obtain instant dark tea. Compared with dark tea, Chinese green tea resources are very abundant, and the yield of green tea in China ranks as the highest level in the world. Around 30% of green tea waste leaves (including trimming leaves, tea dust and mature leaves) was produced annually, which contained abundant active ingredients. These tea waste leaves can be used as high quality and cheap materials to produce instant dark tea products.

Response surface methodology (RSM) is a mathematical and statistical technique for exploring the relationship between the response and the independent variables and to optimize experimental variables in various processes (Panda et al. 2010; Chae and Ahn 2013; Xi and Wang 2013). However, in many cases, the production quality characteristics would be described by several responses and their relative importance is different. There are several extensions for RSM to find the best compromise among multiple responses. One of the most popular methodologies is the desirability function approach (Eren and Kaymak-Ertekin 2007; Li et al. 2007; Erbay and Icier 2009; Karazhiyan et al. 2011).

In the present study, we reported a novel protocol to produce instant dark tea products by *Eurotium cristatum* in SF. Mycelia dry weight (MDW), redness (RED) and clarity were positively and significantly correlated with dark tea total quality. The objectives of the present study were to systematically investigate the influence of SF conditions on the quality of instant dark tea and to optimize the producing conditions for instant dark tea with high level mycelia, high redness and low turbidity.

**MATERIALS AND METHODS**

**Material**

Butyl alcohol, oxalic acid and ethanol (analytical grade) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Catechin (C), Epicatechin (EC), Epigallocatechin (EGC), Epigallocatechingallate (EGCG), Epicatechingallate (ECG), Acetonitrile, Acetic acid and ethylene diamine tetraacetic acid (EDTA), high-performance liquid chromatography (HPLC) grade, were purchased from Sigma-Aldrich (Sigma, St. Louis, MO). Green tea for processing research (broken green tea, moisture content at 5.7%, tea polyphenol content at 26.3%) was provided by Damin Foodstuff (Zhangzhou) Co., Ltd. (Fujian, China). The green tea was extracted by water at 80°C for 20 min (Green tea : Water = 1 g : 15 mL) with gentle stirring, then filtered through a ceramic membrane to remove the debris. The filtered solution was concentrated into green tea extracts (GTE, liquid-to-solid ratio of 10, 15 and 20, respectively) by vacuum thickener and stored at 4°C until use. Commercial instant dark tea product made from Yiyang Fu-brick dark tea (Yiyang) and commercial instant dark tea product made from Jinwei Fu-brick dark tea (Jinwei) were provided by Damin Foodstuff (Zhangzhou) Co., Ltd.*E. cristatum* CGMCC10610 strain, which was isolated from the ripened Fu-brick tea and identified according to their morphological characteristics and internal transcribed spacer (ITS), was preserved in the China General Microbiological Culture Collection Center.

**Production of Instant Dark Tea**

In the present study, fermentation process was carried out in a shaking incubator (ZHWY-2102C; Labwit Scientific, Shanghai, China). Fungus inoculums were inoculated into 100 mL GTE with 3 × 10^6 cfu/mL for pure cultures at 28°C and 150 rpm for 169 h and culture broth was 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; Osterode, Christ, Germany) to get instant dark tea product.

**Determination of MDW**

Mycelia were collected by filtration and dried at 80°C to a constant weight (~2 h), then weighed.

**Determination of Color Difference**

Culture broth was filtrated and cooled to room temperature before the color was measured. The white plate was used as background. Colors of dark tea infusion (filtrated culture
broth) were measured using Hunter Lab Color Measuring System (Color Quest XE, Hunter Associates Laboratory, Reston, VA). Values were measured in terms of lightness (L*) and color (+a*: redness, −a*: greenness, +b*: yellowness, −b*: blueness).

**Determination of Turbidity**

Culture broth was filtrated and cooled to room temperature. Turbidity was measured with a turbidity metre (Turb555/Turb555IR, WTW, Munich, Germany) and expressed in nephelometric turbidity units (NTU). It is the measurement of optical clarity based on a light scattering technique. The greater the intensity of the scattered light, the higher the turbidity.

**Determination of Catechins**

The HPLC analytical method was used to determine catechins. Separations were carried out using an Agilent C18 reverse phase column (250 × 4.6 mm i.d., 5 μm) protected with a security guard cartridge (Gemini C18, 4 × 2.0 mm i.d., Phenomenex, Torrance, CA). A binary solvent system was utilized: Phase A, water, acetonitrile, acetic acid and EDTA (888/80/20, 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 (R², 0.9997–1.0000). Integrations of each compound were conducted at 280 nm.

**Determination of Theabrownin**

The determination of theabrownin (TB) was performed as previously reported (Gong et al. 2006). A total of 0.100 g instant tea powder was dissolved with 50 mL distilled water (60C) and then diluted to 100 mL. An amount of 15 mL of tea infusion was shaken with 15 mL of butyl alcohol for 3 min and the layers were separated after equilibration. A 2 mL sample of the aqueous layer (second) was diluted to 25 mL with 2 mL of saturated oxalic acid solution, 6 mL of distilled water and 15 mL 95% ethanol. The absorbance was measured in a 1-cm cuvette at 380 nm with a UV759S spectrophotometer (Shanghai Precision Instruments Co., Ltd., Shanghai, China). Total concentrations of TB were calculated from the following equation:

\[
TB = \frac{2E \times 16.9}{1-m} \times 100\%
\]

where \(E\) is the absorbance readings from the spectrophotometer of the above solution and \(m\) (% by weight) is the moisture content of the instant dark tea powder.

**Sensory Evaluation**

Tea samples were examined and scored by a tea tasting panel consisted of six persons (three male and three female with ages from 19 to 47 years). Tea tasters were teachers or postgraduates who had expert knowledge of dark teas. The grading system was based on a maximum total score of 100, of which 40% was awarded for taste, 30% for aroma and 30% for liquid color. The final sensory score of a sample was obtained by calculating a mean value of scores given by the six individual tasters. Yiyang and Jinwei Fu-brick tea, both traditional Chinese fermented dark teas, were used as controls.

**Optimization Design**

Firstly, a three level, three-variable Box–Behnken Design (BBD) was employed in generating a total of 17 experiments for the optimization of fermentation parameters. The parameters and levels employed were IZ (inoculum size; 6–12%), LSR (liquid–solid ratio; 10–20 mL/g) and FT (fermentation time; 132–204 h). The coded and original values for the optimization of fermentation parameters. The parameters and levels employed were IZ (inoculum size; 6–12%), LSR (liquid–solid ratio; 10–20 mL/g) and FT (fermentation time; 132–204 h). The coded and original values of independent variables were listed in Table 1, which were based on the results of preliminary experiments. The MDW, RED and TBD (turbidity) from the BBD were analyzed using response surface regression procedure of the statistical analysis system.

Secondly, desirability function approach was used to find the best compromise between the three responses (MDW, RED and TBD) based on the mathematical models constructed in RSM. In the desirability function approach, the multicriteria problem was reduced to a single criterion problem of \(D\) (desirability) optimization. The measured properties of each predicted response \(y\) were transformed to a dimensionless desirability value \(d\). The scale of the desirability function ranges between \(d = 0\), which suggests that the response is completely unacceptable, and \(d = 1\), which suggests that the response is exactly the target value. The

**TABLE 1. VARIABLES AND THEIR LEVELS IN RESPONSE SURFACE DESIGN**

<table>
<thead>
<tr>
<th>Independent Variables</th>
<th>Symbols</th>
<th>Coded levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>IZ (%)</td>
<td>(x_1)</td>
<td>6 9 12</td>
</tr>
<tr>
<td>LSR (mL/g)</td>
<td>(x_2)</td>
<td>10 15 20</td>
</tr>
<tr>
<td>FT (h)</td>
<td>(x_3)</td>
<td>132 168 204</td>
</tr>
</tbody>
</table>

FT, fermentation time; IZ, inoculum size; LSR, liquid–solid ratio.
TABLE 2. DESIGN OF DESIRABILITY FUNCTION

<table>
<thead>
<tr>
<th>Responses</th>
<th>Symbols</th>
<th>L_i</th>
<th>H_i</th>
<th>t_i</th>
<th>w_i</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDW</td>
<td>y_1</td>
<td>0.3</td>
<td>0.6</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>RED</td>
<td>y_2</td>
<td>32</td>
<td>43</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>TBD</td>
<td>y_3</td>
<td>55</td>
<td>390</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

MDW, mycelia dry weight; RED, redness; TBD, turbidity.

The value of d increases as the “desirability” of the corresponding response increases (Derringer and Suich 1980). The one-sided transformation was used to transform the individual response into corresponding desirability value. In this study, the transformations of MDW and RED are larger-the-better, the transformation of turbidity is smaller-the-better problem. The responses were transformed into d_i following the equations below:

Larger-the-better,

\[ d_i = \begin{cases} 0, & y_i \leq L_i \\ \left(\frac{y_i - L_i}{H_i - L_i}\right)^{w_i}, & L_i \leq y_i \leq H_i \\ 1, & y_i \geq H_i \end{cases} \]  

(1)

Smaller-the-better,

\[ d_i = \begin{cases} 1, & y_i \leq L_i \\ \left(\frac{H_i - y_i}{H_i - L_i}\right)^{w_i}, & L_i \leq y_i \leq H_i \\ 0, & y_i \geq H_i \end{cases} \]  

(2)

where d_i is the desirability value of the i-th response; y_i is the response value of i-th response; t_i is the parameter that determined the shape of desirability function and L_i, H_i = The lower and upper targets of the i-th response, respectively.

L_i of every response (MDW, RED and TBD) was chosen to be a little smaller than the minimum of its experimental values in Box–Behnken experiments, and H_i of every response was chosen to be a little larger than the maximum of its experimental values; In this study, because it was thought that the desirability of those responses increased in a linear manner, we set t_i = 1 for every response (Table 2).

All functions were combined into a single criterion D, where each single function could be equally treated as:

\[ D = \left(\prod d_i^{w_i}\right) \frac{1}{w_i} \]  

(3)

where w_i is the relative weight of the i-th response, which reflects the difference in the importance of different responses (Derringer 1994).

w_i of MDW was determined to be “2”, w_i of RED and TBD was determined to be “1” (Table 2) because MDW was expected to be more important than RED and TBD responses.

Statistical Analysis

The experimental data were fitted to quadratic regression models by Design-Expert version 8.0 (Stat-Ease, Minneapolis, MN). The generalized quadratic regression model was shown as Eq. (4). The coefficients of the model were represented by a_0 (constant term), a_1, a_2 and a_3 (linear coefficient), a_12, a_13 and a_23 (interactive term coefficient), a_11, a_22 and a_33 (quadratic term coefficient). Statistical significances of the terms in the regression equations were examined by Analysis of Variance. Model adequacies were checked by R^2, adjusted determination coefficient (Adj-R^2) and Adeq Precision; the model was not adequate unless its lack of fit P value > 0.05, R^2 > 0.9 and Adeq Precision > 4.

\[ y = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_{12}x_1x_2 + a_{13}x_1x_3 + a_{23}x_2x_3 + a_{11}x_1^2 + a_{22}x_2^2 + a_{33}x_3^2 \]  

(4)

RESULTS AND DISCUSSION

The submerged fermentation process of dark tea production by E. cristatum was optimized through the RSM approach. In order to obtain the highest MDW and more desired RED and TBD, the Box–Behnken design with 17 experiments was employed to optimize parameters including IZ, LSR and FT (Table 3). Results showed that the MDW ranged from 0.349 to 0.536 g/100 mL and the RED ranged from 32.4 to 40.18. The maximum values of both MDW and RED (0.536 g/100 mL, 40.18) were found with experimental conditions of IZ = 9%, LSR = 15 mL/g and FT = 168 h. At the same conditions, the minimum point of (59.33 NTU)
was obtained. The results could be explained that fungi (E. cristatum) had a significant influence on the biochemical profiles in Fu-brick tea and consequently contributed to its unique quality (Xu et al. 2014).

Model Fitting

The examination of every model adequacy was shown as the $F$ value, $P$ value, $R^2$, Adj- $R^2$ and Adeq Precision in Table 4. All models had a high $F$ value (81.65 for MDW, 154.41 for TBD and 62.39 for RED) and low $P$ value ($P < 0.01$), which indicated that all models were highly significant. The $R^2$ of predicted models for MDW, RED and TBD in dark tea infusion were 0.9906, 0.9877 and 0.9950, respectively; while the Adj. $R^2$ values were 0.9784, 0.9719 and 0.9885 for MDW, RED and TBD, respectively, which indicated a high degree of correlation between the experimental and predicted values. The fitness of the model was studied through the lack-of-fit test. The $P$ value of 0.1075 for MDW, 0.1075 for RED and 0.1658 for TBD indicated the suitability of models to accurately predict the variations. These results suggested that all models were adequate for predicting within the range of the variables employed.

The coefficients of the variables in the regression models and their significance were shown in Table 5.

### Table 5. The Coefficients of the Variables in the Regression Models and Their Significance

<table>
<thead>
<tr>
<th>Coefficients</th>
<th>MDW</th>
<th>RED</th>
<th>TBD</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>$a_0$</td>
<td>$-1.401$</td>
<td>$-61.419$</td>
<td>$2.965.678$</td>
<td>$-9.309$</td>
</tr>
<tr>
<td>$a_1$</td>
<td>$0.067^+$</td>
<td>$9.874^+$</td>
<td>$-115.495^+$</td>
<td>$0.521^+$</td>
</tr>
<tr>
<td>$a_2$</td>
<td>$0.062^*$</td>
<td>$2.951^*$</td>
<td>$-131.676^*$</td>
<td>$0.421^*$</td>
</tr>
<tr>
<td>$a_3$</td>
<td>$0.012^*$</td>
<td>$0.423^*$</td>
<td>$-18.749$</td>
<td>$0.054$</td>
</tr>
<tr>
<td>$a_{12}$</td>
<td>$1.352E-3^+$</td>
<td>$-0.068^+$</td>
<td>$0.061$</td>
<td>$-3.171E-3^*$</td>
</tr>
<tr>
<td>$a_{13}$</td>
<td>$1.375E-4^*$</td>
<td>$-0.012$</td>
<td>$0.177^*$</td>
<td>$-7.552E-5$</td>
</tr>
<tr>
<td>$a_{23}$</td>
<td>$1.217E-4$</td>
<td>$9.722E-5$</td>
<td>$0.048$</td>
<td>$-6.151E-5$</td>
</tr>
<tr>
<td>$a_{11}$</td>
<td>$-5.514E-3$</td>
<td>$-0.364^+$</td>
<td>$8.356^+$</td>
<td>$-0.025^+$</td>
</tr>
<tr>
<td>$a_{12}$</td>
<td>$-2.941E-3$</td>
<td>$-0.076^+$</td>
<td>$4.780^+$</td>
<td>$-0.013^+$</td>
</tr>
<tr>
<td>$a_{13}$</td>
<td>$-4.128E-5$</td>
<td>$-1.038E-3^+$</td>
<td>$0.059^+$</td>
<td>$-1.558E-4^+$</td>
</tr>
</tbody>
</table>

* Significant at 5% level.
† Significant at 1% level.

### Table 4. Analysis of Variance for Examination of Every Regression Model Adequacy

<table>
<thead>
<tr>
<th>Model</th>
<th>Responses</th>
<th>$F$ value</th>
<th>$P$ value</th>
<th>$R^2$</th>
<th>Adj-$R^2$</th>
<th>Adeq precision</th>
<th>Lack of fit</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDW</td>
<td>81.65</td>
<td>&lt;0.0001</td>
<td>0.9906</td>
<td>0.9784</td>
<td>21.277</td>
<td>0.00057</td>
<td>0.1075</td>
</tr>
<tr>
<td>RED</td>
<td>62.39</td>
<td>&lt;0.0001</td>
<td>0.9877</td>
<td>0.9719</td>
<td>22.564</td>
<td>0.97000</td>
<td>0.1075</td>
</tr>
<tr>
<td>TBD</td>
<td>154.41</td>
<td>&lt;0.0001</td>
<td>0.9950</td>
<td>0.9885</td>
<td>33.663</td>
<td>713.22000</td>
<td>0.1658</td>
</tr>
<tr>
<td>D</td>
<td>79.95</td>
<td>&lt;0.0001</td>
<td>0.9904</td>
<td>0.9780</td>
<td>21.674</td>
<td>0.00765</td>
<td>0.0565</td>
</tr>
</tbody>
</table>

Adj-$R^2$, adjusted determination coefficient; D, desirability value; MDW, mycelia dry weight; RED, redness; TBD, turbidity.

The $MDW$ of E. cristatum in dark tea is considered to be an important factor to judge the fermentation progress and quality of Fu-brick tea (Zhang et al. 2013). In terms of $MDW$, it could be observed that the independent variables ($x_1$, $x_3$, $x_4$), the quadratic terms ($x_1^2$, $x_3^2$, $x_4^2$) and the interaction terms ($x_1x_2$, $x_1x_3$, $x_2x_3$) were all significant ($P < 0.05$). The linear and quadratic terms of $IZ$ ($x_1$, $x_2$), $FT$ ($x_1$, $x_2^2$) and interaction terms of $x_1$, $x_2$, $x_3$, $x_4$ gave the largest effect ($P < 0.01$) followed by the interaction term of $x_1x_3$. According to Table 5, the quadratic regression model for $MDW$ was shown as Eq. (5):

$$MDW = -1.401 + 0.067x_1 + 0.062x_1 + 0.012x_1 + 1.352 \times 10^{-3}x_1x_1 + 1.375 \times 10^{-4}x_1x_3 + 1.217 \times 10^{-4}x_1x_3 \times 5.514 \times 10^{-3}x_1^2 - 0.068x_1x_1 - 0.012x_1x_3 + 9.722 \times 10^{-4}(6)$$

The $a^*$ value, which indicated the redness of tea infusion, can distinguish effectively different teas. High-quality fermented tea was deeper in redness color than that of low quality tea (Liang et al. 2005). As to $RED$ (Table 5), it could be observed that the independent variables ($x_1$, $x_3$, $x_4$), the quadratic terms ($x_1^2$, $x_3^2$, $x_4^2$) and the interaction terms ($x_1$, $x_3$, $x_1x_3$) were all significant ($P < 0.05$). However, the interaction term $x_1x_3$ was not significant ($P > 0.05$). The variables with the largest effect ($P < 0.01$) on $RED$ were the linear and quadratic terms of $IZ$ ($x_1$, $x_2$), $FT$ ($x_1$, $x_2^2$), quadratic term of $LSR$ ($x_3$) and interaction terms of $x_1$, $x_2$, $x_3$, $x_4$. According to Table 5, the quadratic regression model for $RED$ was shown as Eq. (6):

$$RED = -61.419 + 9.874x_1 + 2.951x_1 + 0.423x_3 - 0.068x_1x_1 - 0.012x_1x_3 + 9.722 \times 10^{-4}x_1x_3 - 0.364x_1 - 0.076x_3 - 1.034 \times 10^{-3}(6)$$

The appearance of clarity is a very important factor to confirm high-quality instant dark tea. In terms of TBD (Table 5), it could be observed that the independent variables ($x_1$, $x_3$), the quadratic terms ($x_1^2$, $x_3^2$, $x_4^2$) and the interaction term ($x_1x_3$) were all significant ($P < 0.05$). However, the independent variable of $x_3$, interaction terms of $x_1$, $x_2$ and $x_1x_3$ were not significant ($P > 0.05$). The variables with the largest effect ($P < 0.01$) on TBD were the linear and quadratic terms of $IZ$ ($x_1$, $x_2^2$), $LSR$ ($x_3$, $x_4^2$), quadratic term of $RED$.
FT \( (x_1^i) \), followed by the interaction term of \( x_1 x_3 \). According to Table 5, the quadratic regression model for TBD was shown as Eq. (7):

\[
TBD = 2965.678 - 115.495x_1 - 131.676x_3 \\
- 18.749x_1 + 0.061x_1x_2 - 0.177x_1x_3 \\
+ 0.048x_2x_3 + 8.356x_2^2 + 4.780x_3^2 + 0.059x_3^3
\]  

(7)

**Optimization of Procedure**

The three-dimensional (3D) response surface plots simulated by the Design-Expert software were the graphical representations of regression equation, which illustrated the relationship between independent and response variables. Two variables within the experimental range were depicted in 3D surface plots when the third variable was kept constant at the coded zero level. As shown in Fig. 1, three independent variables all have a positive impact on the MDW, RED and TBD.

Figure 1A showed the response surface plot for effect of LSR and IZ on the MDW at a FT of 168 h. At an increase of LSR from 10 mL/g to 20 mL/g, with an increase in IZ from 6 to 12%, MDW could reach a peak value at 15 mL/g LSR and 9% IZ and then gradually decline. The combination of FT and IZ (Fig. 1B) and the combination FT and LSR (Fig. 1C) also has a similar effect on MDW when the LSR and IZ were fixed at 15 mL/g and 9%, respectively.

Figure 1D showed the response surface plot for effect of LSR and IZ on the RED at a FT of 168 h. With the increase in IZ and LSR, RED could reach a peak value and then decline slightly. Figure 1E showed the response surface plot for effect of FT and IZ on the RED at a LSR of 15 mL/g. At an increase in FT from 132 to 204 h, with an increase IZ from 6 to 12%, the value of RED enhanced first and then declined. The results indicate that the effect of IZ is significant than FT on the RED. Figure 1F showed the response surface plot for effect of LSR and FT on the RED at a IZ of 9%. At an increase in LSR from 10 to 20 mL/g, with the
decrease FT from 204 to 132 h, the value of RED enhanced first and then declined. The effect of FT is significant than LSR on the RED.

Figure 1G showed the response surface plot for effect of LSR and IZ on the TBD at a FT of 168 h. At a decrease in LSR from 20 to 13 mL/g, with an increase IZ from 6 to 9%, the TBD decreased. The effect of LSR is significant than IZ on the TBD. The combination of LSR and FT also has a similar effect on TBD when the IZ was fixed (Fig. 1I). Figure 1H shows the response surface plot for effect of FT and IZ on the TBD at a LSR of 15 mL/g. Increasing the FT to 166 h and the IZ to 9% resulted in minimal TBD. With an increase of FT over 166 h and an increase of IZ over 9%, the TBD increased.

A lower inoculum level resulted in longer lag phases and reduced the mycelial yield (Baert et al. 2008; Bumbak et al. 2011). The contents of tea pigments had a positive impact on \( a^* \) value (Zou et al. 2014). Tea cream had a negative impact on the clarity of dark tea infusion. Tea extracts with a low inoculum cannot secrete enough enzymes to degrade tea cream pigment and synthesis of tea pigments. High inoculum size also has inevitable negative effects on cell culture with cell stress and nutrient limitation (Li et al. 2008). High inoculum size would bring much lysis and had a negative impact on the redness and clarity (Wu et al. 2003).

Low level LSR cannot provide nutritional support for the microorganism’s growth; a high concentration of GTE formed a high osmotic pressure fermentation system which seriously limits the microbial growth.

The mycelia yield changes with cultivation time periods. Appropriate harvest time selection is also an important factor to obtain the maximum fungal production in submerged culture (Chandra and Shoji 2007). In this study, we use GTE as the fermentation medium; a decrease in yield in this fungus on 184 h may have been caused by fungal cell lysis (Wu et al. 2003). At the beginning period of fermentation, the activities of the enzymes were relatively low (Wang et al. 2011); color of green tea extracts cannot present the characteristic of redness and clarity. Because of the fungal cell lysis in the late fermentation, the value of \( a^* \) and clarity decreased gradually.

**Desirability Function Approach**

Based on the parameters in Table 2, the \( D \) values of all experiments were calculated according to the Eqs. (1)–(3) and were shown in Table 3. Results indicated that the model was highly significant (\( P < 0.01 \)). The coefficients of determination (\( R^2 \)), adjusted determination coefficient (Adj. \( R^2 \)) value and lack of fit \( P \) value were 0.9904, 0.9780 and 0.0565, respectively (Table 4). The results suggested that the model was adequate for predicting within the range of the variables employed.

In terms of \( D \), it could be observed that the independent variables \((x_1, x_3)\), the quadratic terms \((x_1^2, x_2^2, x_3^2)\) and the interaction term \((x_1x_3)\) were all significant (\( P < 0.05 \)) (Table 5). However, the independent variable of \( x_3 \), interaction terms of \( x_3x_1 \) and \( x_2x_3 \) were not significant (\( P > 0.05 \)). The variables with the largest effect (\( P < 0.01 \)) on \( D \) were the linear and quadratic terms of IZ \((x_1, x_1^2)\), quadratic terms of LSR \((x_3^2)\), FT \((x_1^3)\), followed by the linear of LSR \((x_3)\) and interaction term of \( x_1x_3 \). According to Table 5, the quadratic regression model for \( D \) was shown as Eq. (8):

\[
D = -9.309 + 0.521x_1 + 0.421x_2 + 0.054x_3 \\
- 3.17 \times 10^{-8} x_1 x_3 - 7.55 \times 10^{-7} x_2 x_2 - 6.15 \times 10^{-6} x_2 x_3 \\
- 0.025x_1^2 - 0.013x_2^2 - 1.558 \times 10^{-4} x_3^2
\]

(8)

Figure 2A showed the response surface plot for the effect of LSR and IZ on the \( D \) at a FT of 168 h. A decrease of LSR...
from 20 to 15 mL/g, with an increase in IZ from 6 to 9% enhanced the D. The D gradually declined with a decrease in LSR below 15 mL/g and increase in IZ over 9%. In a word, D could reach a peak value at 15 mL/g LSR and 9% IZ. The combination of FT and IZ (Fig. 2B) and the combination of FT and LSR (Fig. 2C) also has a similar effect on D when the LSR and IZ were fixed at 15 mL/g and 9%, respectively. But the effect of LSR was less significant than that of IZ. Compared with IZ and LSR, FT had a slightly influence on D.

**Optimization and Verification**

Fermentation processing conditions on dark tea infusion were optimized for three responses, including MDW, RED and TBD, so the optimization was based on the regression model for D:

\[
\begin{align*}
0.52133 - 0.04933 x_1 - 3.17 \times 10^{-3} x_2 - 7.55249 \times 10^{-3} x_3 &= 0 \\
0.42102 - 3.17 \times 10^{-3} x_1 - 0.026 x_2 - 6.15081 \times 10^{-3} x_3 &= 0 \\
0.05429 - 7.55249 \times 10^{-4} x_1 - 6.15081 \times 10^{-5} x_2 - 3.11648 \times 10^{-4} x_3 &= 0
\end{align*}
\]

(9)

To zero three partial derivatives of Eq. (8), an equation set of three liner equations of three variables was obtained as Eq. (9). The equation set was solved and its solutions were the optimal fermentation processing conditions. The solutions were \( x_1 = 9.37, x_2 = 14.65 \) and \( x_3 = 169.03 \). The optimal processing conditions were IZ of 9.37%, LSR of 14.65 and FT of 169.03 h. Calculated by the predicted models of all responses (Eqs. (5)–(7)), the estimated value of MDW, RED, TBD and D was 0.575 g/100 mL, 41.44, 77.42 NTU and 0.9093, respectively. Every response value of MDW, TBD, RED and D was very close to the maximum (or minimum) of the Box–Behnken experiments. The result of the study indicated that the desirability function was an efficient tool to optimize the fermentation processing conditions of GTE for three responses.

To verify the reliability of the models under the optimal conditions, an experiment was performed at IZ of 9.35%, LSR of 14.50 and FT of 169.00 h. The experimental values for MDW, RED and TBD were 0.579 g/100 mL, 41.35 and 76.96 NTU, which were well matched with the predicted values. The errors between the predicted and experimental value were smaller than 1%. Thus, the regression models obtained by RSM could accurately predict MDW, RED, TBD and D for any combination of IZ, LSR and FT.

**Changes of Main Bioactive Components during Submerged Fermentation**

A batch of instant dark tea product had been made under the optimal fermentation conditions, and dynamic changes of main bioactive components (catechins and theabrownin) of tea infusion were determined during submerged fermentation (Fig. 3). Changes of catechins at 0, 7, 20, 48, 72, 120 and 169 h were shown in Fig. 3A,B. Figure 3A showed that contents of EGCG and ECG appeared a dramatic decrease in early fermentation period, and after 24 h, the level of EGCG and ECG were only 0.46 and 0.50 mg/g, respectively. Because of existing sensitive galate ester bond in the molecular structure, EGCG and ECG were easier to be degraded by enzymes from fungus (Qin et al. 2012). C, EC and EGC belong to flavanols, i.e., simple catechins, thus there is no ester bonds of gallic acid in their molecular structures. These simple catechins were accumulated from the decomposing of EGCG and ECG. However, the contents of simple catechins were slowly decreased after 20 h of fermentation (Fig. 3B). This is due to the generation an oxidative product of catechin on the B-ring of flavanols after long-term fermentation. A similar phenomenon was reported by other authors (Keller et al. 2013; Zhu et al. 2015).

TB is the main pigment composition and it is an important component in dark tea infusion that determines the color and quality of dark tea. The level of TB can reflect the degree of fermentation. The contents of TB at 0, 7, 20, 48, 72, 120 and 169 h were shown in Fig. 3C. Results indicated that TB content in tea infusion was decreased from 13.81 to 6.18% at the first 48 h of fermentation, and then the level of TB gradually increased (48–169 h) from 6.18 to 21.17%. Wang et al. (2014) have shown that TB content continuously increases during the traditional dark tea fermentation. The reason for the difference might be due to under an acidic condition of SF; chemical compositions of tea infusion are more likely to react complexly with each other. TB contains polyphenols, proteins and polysaccharides, and proteins are prone to be degraded in acidic conditions, which leads to reduce the solubility of settlement (He et al. 2012). Microorganisms can produce a variety of enzymes, including polyphenol oxidase (PPO), peroxidase (POD), cellulase and pectinase. Activities of PPO and POD were relatively low during the first 48 h of fermentation and catechins were converted into thearubigins mainly in this period (Wang et al. 2011). The activity of PPO and POD enzymes appeared to increase after 48 h fermentation accompanied rapidly increase of TB content (Fig. 3C).

**Quality Evaluation**

The instant dark tea product (sample) was evaluated, which was designed to compare the sensory score, color difference and turbidity with two commercial instant dark tea products, i.e., commercial instant dark tea product made from Yiyang Fu-brick dark tea (Yiyang) and commercial instant
dark tea product made from Jinwei Fu-brick dark tea (Jinwei). The detailed scores of the dark tea quality evaluation were shown in Table 6. A spider plot was shown in Fig. 4A; the taste, color, lightness ($L^*$) and redness ($b^*$) were similar in the three instant dark teas. However, compared with Yiyang and Jinwei, the sample had a higher score in items of turbidity and special fungal aroma. The product photo of three instant dark tea products was shown in Fig. 4B. As shown in Fig. 4B, both instant tea power and according tea infusion of the three instant dark tea products possesses the characteristics of Chinese dark tea.

CONCLUSION

In the present study, response surface methodology was successfully implemented with desirability function approach for the optimization of fermentation parameters of instant dark tea product. The estimated values of $MDW$, $RED$ and $TBD$ were 0.579 g/100 mL, 41.35, and 76.96 NTU, respectively, at optimized submerged fermentation conditions of $IZ$ (9.35%), $LSR$ (14.50 mL/g) and $FT$ (169.00 h). A batch of instant dark tea product had been obtained under the optimal fermentation conditions, the main bioactive compounds in green tea extracts significantly changed during submerged fermentation and tea infusion had the characteristics of dark tea after fermentation. The instant dark tea product via fermentation possessed the unique dark tea color and taste. This study presents a new strategy to use submerged fermentation method for processing instant dark tea products and hence increase the process efficiency by simple processing to achieve similar product quality, and this novel finding makes it easy to obtain a large-scale continuously automatic production. The method developed in this work may provide an attractive solution for

### TABLE 6. MEANS FOR QUALITY EVALUATION OF DIFFERENT INSTANT DARK TEA

<table>
<thead>
<tr>
<th>Quality attribute</th>
<th>Means</th>
<th>Sample Yiyang</th>
<th>Jinwei</th>
</tr>
</thead>
<tbody>
<tr>
<td>Color attribute</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lightness</td>
<td>$71.82 \pm 0.07$</td>
<td>$69.51 \pm 0.05$</td>
<td>$75.86 \pm 0.04$</td>
</tr>
<tr>
<td>Redness</td>
<td>$17.57 \pm 0.02$</td>
<td>$19.67 \pm 0.03$</td>
<td>$14.88 \pm 0.01$</td>
</tr>
<tr>
<td>Turbidity (nephelometric turbidity units)</td>
<td>$14.09 \pm 0.62$</td>
<td>$23.64 \pm 1.12$</td>
<td>$22.55 \pm 1.20$</td>
</tr>
<tr>
<td>Sensory attribute</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Color</td>
<td>$26.2 \pm 2.1$</td>
<td>$28.7 \pm 1.5$</td>
<td>$25.1 \pm 3.2$</td>
</tr>
<tr>
<td>Aroma</td>
<td>$28.5 \pm 1.4$</td>
<td>$26.1 \pm 2.6$</td>
<td>$25.3 \pm 2.3$</td>
</tr>
<tr>
<td>Taste</td>
<td>$35.1 \pm 3.6$</td>
<td>$38.0 \pm 1.1$</td>
<td>$34.8 \pm 1.9$</td>
</tr>
<tr>
<td>Total quality</td>
<td>$89.8$</td>
<td>$92.8$</td>
<td>$85.2$</td>
</tr>
</tbody>
</table>

Note: The values of lightness and redness were determined with colorimeter, Turbidity was measured with a turbidity meter; the scores of color, aroma and taste were obtained by sensory evaluation.
optimization of fermentation conditions and simultaneous optimization of several response variables.

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