Optimization of the factors affecting BT-2 black tea fermentation by observing their combined effects on the quality parameters of made tea using Response Surface Methodology (RSM)

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ABSTRACT

This research work aimed to optimize the fermentation time, temperature, and relative humidity of the black tea produced from Bangladesh Tea 2 (BT-2) variety by observing their quality parameters. Total theaflavin (TF), thearubigin (TR), the ratio of TF: TR, total liquor color (TLC), high polymeric substances (HPS), and total phenolic content (TPC) were evaluated for quality measurements of BT-2 black tea. Response Surface Methodology (RSM) with Box-Behnken design (BBD) was applied to optimize fermentation time, temperature, and relative humidity as well as evaluate the effects of optimized conditions on the quality of made tea. The results obtained from the response surface optimization affirmed that under the optimum conditions of time (80.14 min), temperature (28.76°C), and relative humidity (92.30%), the model showed the value of TF (0.69%), TR (5.57%), HPS (8.61%), TLC (3.05%), and TPC (7.95 GAE g/100g tea). Moreover, the optimized model found that the TF:TR value was 1:9.13, which is close to black tea's optimum quality. The values observed in experiments were highly congruent with the predicted value by the regression model. The Analysis of Variance (ANOVA) test revealed that the model was significant for TF, TR, HPS, TLC, TPC, and TF/TR values of prepared BT-2 black tea at different levels (p < 0.001 to p < 0.01). The composite desirability of the model was 0.93, which suggests that the developed model could be utilized effectively to maintain the quality parameters of BT-2 black tea during fermentation.

1. Introduction

The cheapest and most consumed beverage after water is black tea, which is consumed worldwide mainly for its distinct flavor, taste, aroma, and briskness. The young leaves and buds of Camellia sinensis (L) O Kuntze plants are used to make black tea. Currently, more than 58 countries worldwide produce tea. Bangladesh is one of the world’s top tea-producing countries (Ali et al., 2014). The internal consumption of tea in Bangladesh increases daily, but the current yield per hectare is relatively low compared to other tea-producing countries. Thus, several researchers are still improving tea plants’ productivity by different organic and inorganic treatments and developing new varieties (Ahmad and Hossain, 2013; Hossain et al., 2013, 2021). Bangladesh Tea Research Institute (BTRI) has been already released 18 clones in the Bangladesh tea (BT) series. Among all the varieties, the BT-2 variety is known to give good quality black tea with higher productivity (Yasin et al., 2020). However, very few studies investigated the variations in BT-2 varieties in terms of biochemical and quality parameters.

The black tea manufacturing process involves the following six steps: green leaf handling, withering, rolling, fermentation, drying, and sorting (Qu et al., 2019). Albeit all the processing steps significantly affect the quality of the black tea, the fermentation step is by far the most critical stage due to the various crucial biochemical events during this process (Rahman et al., 2020). Tea leaves’ endogenous flavonols are subjected to several oxidative enzymatic reactions. The main biochemical components of tea leaves are catechins. During fermentation, catechins oxidized into theaflavins (TF) followed by thearubigns (TR) through enzyme-catalyzed reactions (Chen et al., 2010; Bisen et al., 2017; Das et al., 2019). The quality of the black tea (e.g., TLC-total liquor color) and the amount of TF and TR pigments are strongly correlated because these compounds are accountable for the briskness, astringent taste, color, and brightness of the black tea (Samanta et al., 2015). Besides, the total
phenolic content (TPC) in the tea leaves is also considered as the quality parameter of tea due to their antioxidant activities and medicinal potential. Moreover, the proteolytic enzymes during fermentation hydrolyze phenolic complexes into soluble-free phenols and more biologically active phenols. The bioconversion of phenolic compounds from their conjugated to free forms during fermentation enhances their antioxidant activity (Tiku et al., 2017). However, depending on the fermentation process, TPC in black tea can vary significantly (Zareef et al., 2019). On the other hand, during the oxidation process, the formation of high polymerized substances (HPS) also plays a significant role in the quality of black tea since the higher amount of HPS makes the tea liquor cloudy and implies that the oxidation process is not functioning properly (Palanivel et al., 2020).

Several factors that can influence tea fermentation, such as temperature, time, relative humidity, oxygen availability, and pH, are responsible for forming high levels of desired products. Among these, time, temperature, and relative humidity are important factors for black tea’s quality maintenance. For example, the TF:TR may change with different fermentation times. In a study, Asl and his co-workers have demonstrated a decrease in total TF, briskness, and liquor color with increased fermentation temperature and time (Asl et al., 2012). In contrast, short-time and medium fermentation temperatures favor the production of dark-colored and thicker black tea (Teshome, 2019). Accordingly, maintaining a low fermentation time and temperature ensured the formation of simple theaflavin by limiting the conversion of polyphenol compounds (Somsong et al., 2020). Moreover, Hafezi et al. (2006) found that the HPS content of the same variety of tea also varied from 8.0% to 11.0% under the same manufacturing process differences in fermentation time. Moreover, the relative humidity near saturation is favorable for better fermentation. Jolvis Pou (2016) described that there were no changes in the color of green foliage and aroma formation when kept in a vacuum condition. Therefore, it is important to find out a suitable optimal condition of fermentation time, temperature, and relative humidity to maintain the better quality of the black tea. Though numerous studies showed the combined effect of duration and temperature of fermentation on the quality of black tea, the combined effects of time, temperature, and relative humidity have not been fully recognized yet. However, no previous studies have been found on the processing conditions optimization to maintain the quality parameters of BT-2 varieties. It will help the manufacturers to obtain a good quality tea, and the consumers can easily select the best quality tea from different varieties.

In this context, Response Surface Methodology (RSM) is an efficient statistical technique for design, which has been extensively applied to optimize a process that affects desired response by several factors and their interactions. The experimental design simultaneously considers several factors and attempts to characterize the relationship of an independent variable with one or more response or dependent variables. RSM applies the least square technique to fit a mathematical model using an experimental design such as the Box-Behnken design (BBD), central composite design (CCD) (Bezzerra et al., 2008; Hossain and Hossain, 2021; Maran et al., 2015; Hossain et al., 2020). BBD typically have fewer design points than CCD, making them less expensive to operate with the same number of factors. In contrast to CCD, which can have up to five levels per factor, BBD always have three levels per factor. As a result, it is less time-consuming and labor-intensive. Additionally, unlike CCD, BBD never feature runs with all factors set to their extreme values, such as all the low values (Yukada et al., 2018).

Therefore, this study aimed to investigate the effects of optimized fermentation conditions on BT-2 black tea’s quality parameters. Temperature, time, and relative humidity (RH) were selected as the fermentation parameters affecting the quality of BT-2 black tea. Moreover, RSM with BBD was used to optimize fermentation temperature, time, and RH for the quality parameters of BT-2 black tea such as total theaflavin (TF), thearubigin (TR), the ratio of TF:TR, total liquor color (TLC), high polymeric substance (HPS), and total phenolic content (TPC).

2. Materials and methods

2.1. Substrates and chemicals

BT-2 tea leaves were collected from the experimental tea garden of Department of Food Engineering and Tea Technology, Shahjalal University of Science and Technology, Sylhet-3114, Bangladesh. All the chemicals used in this study were of analytical grade and collected from Sigma-Aldrich (US) and Merck (Germany).

2.2. Tea manufacturing

Tea leaves of BT-2 (Bangladesh Tea-2) variety for miniature manufacture were plucked from the experimental tea garden of Department of Food Engineering and Tea Technology, Shahjalal University of Science and Technology, Sylhet, Bangladesh (24° 91’ N latitude, 91° 83’ E longitude, and 7.25 m altitude). Plants were grown under natural conditions. The leaves mainly consisted of two young leaves and a bud. The plucked leaves were withered in natural conditions for 16 h. The withered leaves were rolled in a pilot-scale rolling machine (Model: TZ-50, Zhengzhou Tairy Trading Co., Ltd., China). The macerated leaves were then fermented for 70–100 min at 25–35 °C, maintaining 75–95% relative humidity (RH). The entire fermentation process was conducted in the humidity chamber (Model: 2500, Thunder Scientific Corporation, U.S.A.). To achieve black tea with roughly 3% moisture, fermentation was terminated by drying for 120 min at 90 °C (Lee et al., 2016).

2.3. Determination of theaflavin (TF), thearubigin (TR), total liquor color (TLC), and high polymerized substances (HPS)

The quality parameters of BT-2 black tea such as TF, TR, TLC, and HPS were evaluated by solvent extraction method described by Angayarkanni et al. (2002). At first, tea brew was prepared by infusing 2 g of tea sample granule with 90 mL boiling water in a water bath (Model: FSPGD28, Fisher Scientific, Canada) for 10 min. The brew was then filtered into a 100 mL volumetric flask and distilled water was added to bring it up to volume while it was still hot. In 9 mL of double-distilled water, 1 mL of tea brew was added to get solution ‘A’. At the same time, the extraction solvents and reagents were prepared. A separating funnel was used for the rest of the experiment. Approximately 25 mL brew was added to 25 mL of isobutyl methyl ketone (IBMK). In the separating funnel, IBMK (L1) and aqueous (L2) layers were formed. From the IBMK layer (L1), 1 mL of the solution was mixed with 45% ethanol to get solution ‘B’. Again, 10 mL from the IBMK layer was added to 10 mL 2.5% of Na2HPO4 solution. From this mixture, 1 mL IBMK layer was mixed with 45% ethanol to get solution ‘C’. On the other side, 10 mL of aqueous layer (L2) was added to 10 mL n-butanol to get another two layers formed. From the butanol layer, 1 mL was added to 9 mL of 45% ethanol (solution ‘D’), while 1 mL from the aqueous layer was mixed with 9 mL 45% ethanol (solution ‘E’). The Ba, Ca, Da, and Ea are the absorbance of the fraction B, C, D, and E solution at 380 nm against 45% ethanol. The absorbance (A) of solution A was measured against distilled water as a blank. Values of TF, TR, TLC, and HPS were calculated according to Eqs. (1), (2), (3), and (4):

\[\text{TF} (\%) = \left(4.313 \times C_a \times 200\right) \div \left(\text{Sample wt.} \times \text{DMC}\right)\]  
\[\text{TR} (\%) = \left(13.643 \times \left(B_a + D_a - C_a\right) \times 2 \times 100\right) \div \left(\text{Sample wt.} \times \text{DMC}\right)\]  
\[\text{HPS} (\%) = \left(13.643 \times E_a \times 2 \times 100\right) \div \left(\text{Sample wt.} \times \text{DMC}\right)\]  
\[\text{TLC} (\%) = \left(10 \times A_d \times 2 \times 100\right) \div \left(\text{Sample wt.} \times \text{DMC}\right)\]

where, DMC is the dry matter content. The TF and TR multiplication coefficients of pure substances and the dilution factor (Roberts and Smith, 1963; Ahmad et al., 2016). Value 10 is the dilution factor for TLC (Thanaraj and Seshadri, 1990).
2.4. Determination of total phenolic content

The total polyphenols were estimated according to the modified Folin-Ciocalteu reagent method described by Zazman et al. (2021) and Rahman et al. (2016). About 1 g of tea sample was powdered with mortar and pestle to mix with 96% ethanol. The components were filtered, and the filtrate was diluted with ethanol to a volume of 50 mL. The extract was then diluted 50 times with water. About 4 mL of Folin-Ciocalteu’s reagentwater (1:1) and 2 mL of 35% Na₂CO₃ were mixed with 2 mL diluted vigorous extract in a 10 mL test tube. Then, another 2 mL double-distilled water was added to the solution to make up to 10 mL. After that, the solution was mixed by a vortex mixer (Model: K-550-G, Fisher Scientific, Canada) and kept for 30 min at room temperature to form blue color. The absorbance of the solution was measured spectrophotometrically (Model: UV-1601, Shimadzu Corporation, Japan) at 700 nm against the reagent as blank. The total phenolic content in the solution was measured from the standard curve of gallic acid (GAE g/100g tea).

2.5. Statistical analysis and experimental design

The Design-Expert® (version 12.0.3), and Minitab® (version 14) software was applied for the experimental design and the analysis of variance (ANOVA) for the data. Box-Behnken design (BBD) in the form of three-level three-factor was used to optimize the experimental fermentation process by Response surface methodology (RSM). After a factorial screening test, three factors, such as fermentation time (X₁), temperature of fermentation (X₂), and relative humidity (X₃), were identified as the most influencing independent variables. Every variable was encoded as +1, 0, and -1, which corresponded to the actual high, medium, and low values, respectively (Table 1), and accordingly total of 15 experiments were performed (Table 2). Data were analyzed by multiple regressions using the least-square method, and the Box-Behnken design was used to fit the experimental data with the second-order quadratic equation expressed by Eq. (5):

$$y = a_0 + \sum_{i=1}^{n} a_i x_i + \sum_{i=1}^{n} a_{ii} x_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} a_{ij} x_i x_j$$

where, y is the measured response variables, xᵢ and xⱼ represent the levels of independent variables. a₀ is a constant (predicted response at the center), aᵢ, aᵢᵢ and aᵢⱼ are the linear, quadratic, and two factors interaction coefficient of the model, respectively. All the statistical significance tests were based on the total error criteria, and data were significant at 95% level of confidence.

2.6. Model validation

Additional experiments in triplicate under optimum fermentation conditions were done in order to validate the RSM-derived model. for the data. Box-Behnken design (BBD) in the form of three-level three-factor was used to optimize the experimental fermentation process by Response surface methodology (RSM). After a factorial screening test, three factors, such as fermentation time (X₁), temperature of fermentation (X₂), and relative humidity (X₃), were identified as the most influencing independent variables. Every variable was encoded as +1, 0, and -1, which corresponded to the actual high, medium, and low values, respectively (Table 1), and accordingly total of 15 experiments were performed (Table 2). Data were analyzed by multiple regressions using the least-square method, and the Box-Behnken design was used to fit the experimental data with the second-order quadratic equation expressed by Eq. (5):

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3. Results and discussion

3.1. Box-Behnken design and Response Surface Methodology analysis

The present study considered temperature, time, and relative humidity as independent variables for optimization study based on the previous research works. Box-Behnken Design (BBD) was applied to optimize fermentation time, temperature, and relative humidity for the quality parameters of BT-2 made black tea, such as TF, TR, TF:TR, HPS, TLC, and TPC. The statistical model was achieved using BBD, with the three independent variables (time, temperature, and humidity) according to their high (+1), medium (0), and low (−1) levels (Table 1). The experimental values for responses (TF, TR, TF:TR, HPS, TLC, and TPC) under a set of 15 experiments are demonstrated in Table 2. The results exhibited that the yield of TF, TR, HPS, TLC, and TPC ranged from 0.29 to 1.05%, 3.21–8.62%, 4.8–10.61%, 2.69–4.17%, and 5.11 to 14.80 GAE g/100g tea, respectively based on different fermentation conditions.

However, the obtained results were analyzed by the analysis of variance (ANOVA). The significance of each coefficient was determined using the Fisher Test (F) with a p-value at a 5% confidence level (Prob > F < 0.05) (Table 3). The higher F-value and smaller p-value reflect that the corresponding variables will be more significant. In the case of all responses, the model F-value was higher, and the probability (Prob > F) value was less than 0.05, which indicates the adequacy of the applied model to predict different responses under different fermentation conditions (Kumar et al., 2011). The effect of each independent variable on the response variables investigated in this study was calculated directly using the respective coefficient in the fitted model.

The adequacy and fitness of the model were assessed by the coefficient of determination (R²). The R² value was used to determine the degree of fit. The empirical model fits the actual data better if the R² value is close to 1 (Cornell and Berger, 1987). The R² coefficient of all the responses in the present study ensured that the quadratic model was adapted satisfactorily to the experimental data, and the suggested mathematical equations can explain more than 90% of the variations in the experimental data. The predicted models appeared to represent the observed values reasonably. Thus, the models sufficiently explained the responses. Besides, the adjusted R² value can consider as a corrected value for R² after removing the unnecessary model terms. If the model includes a large number of non-significant terms, the adjusted R² will be significantly smaller than the R². In this study, the adjusted R² value for TF, TR, HPS, TLC, and TPC were 0.9278, 0.9931, 0.9599, 0.9875, and 0.9974, respectively, which were very close to their corresponding R² value. Additionally, the high adjusted R² value indicated that the model was significant for all responses. As shown in Table 3, the coefficient of variance (CV) for TF, TR, HPS, TLC, and TPC was found to be 8.66, 2.23, 5.03, 3.44, and 2.83, respectively. The CV is a measure of the model's reproducibility as a ratio of residual variation of the data relative to the mean size. In general, if the CV of a model is less than 10%, it is considered reasonably reproducible (Granato and de Araújo Calado, 2014). The small CV value of all responses in this study (Table 3) revealed that the obtained data were accurate and dependable. The adequate precision value is a ratio of ‘signal to noise’, and all the responses in this study showed this desirable ratio greater than 4, indicating an adequate signal that can be used for design space navigation. The lack of fit parameter quantified the model's inability to describe data in the experimental domain at points that didn't include in the regression (Lenth, 2009). In the present study, the non-significance “lack of fit” value (p > 0.05) stipulated that the quadratic model is statistically significant for all the responses.

Fermentation in the manufacturing of black tea is a critical processing step. During this stage, the majority of significant chemical transformations occur. The chlorophyll of tea leaves is degraded enzymatically (i.e., peroxidase and polyphenol oxidase) during fermentation (Jolvis Pou, 2016). Temperature, time, and relative humidity are the critical process conditions that impact the tea's quality. It is well documented that temperature control is critical for producing good-quality tea, because fermentation at either low or extremely high temperatures might result in enzyme deactivation. For example, Cloughley found that the value of central African black tea raised by approximately 15–19 p Kg⁻¹ through decreasing the fermentation temperature by 20 °C from 36 °C to 16 °C (Cloughley, 1980). The author also suggested that controlling the fermentation temperature during oxidative product development would improve black tea quality. Furthermore, Samanta et al. (2015) explored the impact of temperature (20–35 °C) on the quality parameters of CTC (crush, tear, curl) black tea (Camellia sinensis var. assamica) during fermentation. They reported that at 20 °C, both the TF and TR ratios and the brightness were at their maximum values. In addition, they also concluded that low-temperature fermentation is optimal for producing high-quality black tea.

Similarly, the fermentation time is critical since it significantly influences the quality of black tea. Fermentation time is not pre-determined; it varies according to the variety of tea, plucking standard, degree of maceration, withering, and rolling. The aspects of liquor's quality, such as brightness, briskness, astringency, and strength, attain their optimum level at different times. As a result, optimization must be carried out to ensure the best possible overall effect. Several studies have demonstrated that TF and TR concentrations and acceptable quality attributes improve with fermentation time, reach optimal levels, and subsequently degrade if the fermentation duration is extended (Sanyal, 2011; Stodt et al., 2014). In contrast, Gill et al. (2011) reported that a number of desirable characteristics are missing from over-fermented tea, even if it has a body. The author also suggested that maintaining an optimal TF/TR (1:10) is necessary for a high-quality cup of tea. In 2001, Obanda and his colleagues observed the maximum levels of total TF, TR, TLC, briskness, brightness at 90, 120, 60, and 60 min of fermentation, respectively, at a 20 °C fermentation temperature (Obanda et al., 2001).

The optimal relative humidity, similar to fermentation time and temperature, is essential to maintain the quality of tea during the oxidation process. Numerous studies have suggested maintaining relative humidity at 95–98% during the fermentation process, and even a small drop in humidity level affects the quality parameters of tea. However, it was noted by Sanyal (2011) that the temperature is typically high in the afternoon with low relative humidity, and the air must be humidified under these or similar conditions to keep the rolled leaves fresh and cool during fermentation. He also suggested that dry air should not pass over the tea leaves because this causes blackening and disrupts an oxidation rate. In a separate study, Lee et al. (2009) found that low relative humidity (20%) and refrigerated condition (−4 °C) significantly increased the total flavanol contents, total phenolic contents, and ascorbic acid contents of green tea. In addition, Ning et al. (2020) found that the dandelion (Taraxacum mongolicum Hand. Mazz.) black tea exhibited a consistent and tight structure, bright-orange liquor color, mellow flavor as well as good quality, and a high level of antioxidant activity at 80% fermentation humidity. So, it is crucial to find out the optimal relative humidity for a specific variety of tea.

3.2. Effect of independent variables on response values in the RSM model

The regression analysis revealed that each of the three independent variables had a linear effect on the response values (TF, TR, HPS, TLC, and TPC). As shown in Table 3, X₁ (time), X₂ (temperature), and X₃ (relative humidity) terms for all responses were more significant at p < 0.01. It is evident from Table 3 that the interaction between time, temperature, and relative humidity significantly affected the content of TR, TLC, and TPC, while there was no significant effect on TF and HPS content (p > 0.05). Similarly, X₁² (time)², X₂² (temperature)², and X₃² (relative humidity)² were also found to be significant model terms for all responses at p < 0.01.

The three-dimensional response curves were plotted to illustrate how the variable interacts and determine each variable's optimal level to achieve maximum response. Figure 1 shows the response surface curves.
Figure 1. Response surface plots of interaction effects of fermentation time, temperature, and relative humidity on TF: Theaflavin (A, B, C); TR: Thearubigin (D, E, F); HPS: High Polymeric Substance (G, H, I); TLC: Total Liquor Color (J, K, L); TPC: Total Phenolic Content (M, N, O); TF:TR (P, Q, R).
for TF, TR, HPS, TLC, TPC, and TF: TR, respectively. Each figure illustrates the effect of two variables while holding the third variable constant at the middle level. Figure 1A, B, C exhibit the response surface curves for TF formation as a function of time (X1), temperature (X2), and humidity (X3). The interaction effect between the independent variables was not significant, but the variables individually showed a significant effect on the TF formation (Table 3). While the fermentation time, temperature, and relative humidity were at a low level, the TF production was high. This result is strongly reinforced by the results reported by Asil et al. (2012), who observed that the TF formation level increased at a low level of fermentation time and temperature. Having said that, they also did not get any significant interaction in the effects of fermentation temperature and time on the formation of TF. However, TF levels decreased with increasing fermentation temperature and time. The declination of TF with increased temperature and time is due to the conversion of TF into TR rapidly or changes in the multiple forms of polyphenol oxidase (Samanta et al., 2015). Also, Ngure et al. (2009) demonstrated that as fermentation duration increased, the TF levels decreased.

TF is a distinct compound generated when catechins are oxidized by enzymes during the manufacturing process of black tea. TF impart an orange or orange–red color to black tea and aid in the impression of mouthfeel and cream production (Lin and Sun, 2020; Wang et al., 2021). They are dimeric compounds with a benzotropolone skeleton produced by the co-oxidation of specific catechin pairs (Kosinśka and Andlauer, 2014). When catechins are subjected to an oxidative conversion in vitro, polyphenol oxidase (PPO) serves as a catalyst for the formation of theaflavins (Teng et al., 2017). PPO is deposited in the chloroplast of black tea leaves, while phenolic compounds are stored in vacuole (Yu et al., 2020). The maceration of tea leaves promotes the oxidation of catechins by oxygen, which is catalyzed by PPO and results in the creation of quinones. These quinones are formed when the B-rings of dihydroxy and trihydroxy catechins are oxidized (Deka et al., 2021). They condense to generate various theaflavins, which imparts orange color in the fusion of black tea. Furthermore, astringency is a tactile sensation felt on the tongue that is predominantly caused by flavanol polymers (Lesscheve and Noble, 2005). Sensorially, astringency is described as a puckering or drying sensation in the mouth. Flavan-3-ol monomers, dimers, and trimers, i.e., the theaflavins gallates found in black tea, have been demonstrated to evoke an astringent sensation. This may occur as a result of unprecipitated complexes forming with saliva proteins (Bhuyan et al., 2015). A higher gallate concentration in the TF indicates increased astringency (Obanda et al., 2001). Consequently, as fermentation progresses, the TF contents of black tea varies, the taste and color characteristics of black tea may also change.

The effect of varying fermentation temperature, time, and humidity on TR production is shown in Figure 1D, E, F. It is clear from the figures and Table 3 that the individual and combined interaction effects of independent factors on the formation of TR were significant (p < 0.05). It was obvious that TR production increased with high time (100 min), and temperature (35 °C), and mid-level humidity (85%). The maximum TR production under these conditions was 6.67% (Table 2). With a further increase in the humidity, the TR production decreased slowly, indicating that excessive humidity would not increase the TR production anymore. The production of TR occurs due to oxidative TF degradation as well as polymerization of the degradation products (Ansari et al., 2011). Several studies mentioned that TR content increased significantly due to high peroxidase activity at high fermentation time, temperature, and humidity (Borah et al., 2000; Samanta et al., 2015).

According to some authors, the majority of catechins found in fresh tea leaves are converted to TR during the fermentation process, and so represent the final group of compounds produced during the manufacturing of black tea (Koch, 2021). TR is a water-soluble, acidic molecule that is primarily responsible for the tea infusion’s red-brown color (Kalidass et al., 2019). TR account for up to 30%–60% of the solids in black tea infusions due to their very high-water solubility and affect the color, strength, and briskness of black tea (Kuhnert, 2010). However, its distinct flavor and aroma are owing primarily to the presence of TF (Khan and Mukhtar, 2007). Thus, in the production of bright black tea, it is important to maintain the contents of TF and TR because extended oxidation during fermentation resulted in a decrease in TF and an increase in TR (Robertson, 1992). Obanda et al. (2004) and Owuoro et al. (2006) showed in their individual studies that higher levels of TR reduced the brightness of black tea liquor. The authors found that the findings of spectrophotometric measurements corresponded to the brightness values determined by taster evaluation.

The three-dimensional plot of HPS in Figure 1G, H, I depicted that no interaction between different variables was found to be significant to the response due to a higher p-value (p > 0.05) (Table 3). Nevertheless, the individual factors had a significant effect. It could be noticed from the figures that the higher production of HPS was achieved at the mid-level of fermentation time, temperature, and humidity but decreased slowly beyond the range. This might be due to the level of TF that tended to decline with increased time, temperature, and humidity since TR forms complex, HPS in reaction with TF (Muthumani & Kumar, 2007). The maximum yield of HPS was found to be 10.61% when fermentation time, temperature, and humidity were 85 min, 30 °C, and 85%, respectively (Table 2). The result of this study indicates a decrease in the HPS levels with increasing fermentation time, temperature, and relative humidity.

HPS are the results of polymerization during the fermentation process. An increased formation of HPS during oxidation was similar to color production patterns (Hafezi et al., 2006). Usually, HPS have a negative impact on the quality of black tea. Hence, the tea liquor becomes cloudy or dark as the HPS concentration increases. Besides, a higher concentration of HPS suggests an inefficient oxidation process (Palanivel et al., 2020). In contrast, several studies also reported that HPS increases the color of the brew, and the function of HPS in color formation is more significant than other TR groups (Hafezi et al., 2006; Muthumani & Kumar, 2007). Few high polymerized compounds can determine the color and quality of black tea (Huang et al., 2019; Wang et al., 2014). To be more specific, theobromine (TB) is most probably the highly polymerized compound in black tea (Takeo, 1976). TB is a type of highly polymerized compound that is non-dialytic and water-soluble. TB is mainly produced from the oxidation of TF and TR (Dong et al., 2018). In black tea liquor, the total content of TB is 4–9% (w/w) (Gong et al., 2012). Recently, numerous studies have revealed that TB may be one of the main highly polymerized components in dark tea, contributing to its brownish-red color, distinctive smell, and full-bodied flavor (Kraujalyte et al., 2016; Liang and Xu, 2001). Interestingly, in a recent study, Alam et al. (2020) recorded the average value of the TB contents in seventeen marketed brands of black tea of Bangladesh was 7.04%. Moreover, the authors also concluded that the status of TB contents determined in all the studied brands was convincingly high and comparable with the standard of other countries. Hence, the current study put forward the idea that BT-2 black tea might have higher contents of TB like the other studied marketed brands of black tea in Bangladesh. On that account, the present study preferred the maximum value of HPS during the optimization process, considering the TB contents, and prioritized the color over cloudiness.

Correspondingly, the distinctive color of black tea is produced during the fermentation operation. During the fermentation process, two primary pigment groups, TF and TR, are produced by the oxidation of colorless catechins that are abundant in fresh tea leaves. In this present study, total color (TLC) changes in BT-2 black tea leaves were similar to the pattern of HPS (%) content. It could be inferred that the color of the BT-2 black tea was significantly influenced by the individual and interactions between the fermentation variables. From Figure 1J, K, L, it appears that the mid-level of fermentation time, temperature, and humidity produced BT-2 black tea with a high-level liquor color than the low and high level of fermentation variables. This result was in agreement with the findings of Ali et al. (2012) and Kidist et al. (2013), who observed the increase in temperature decreased the TLC.
It is commonly recognized that color, in addition to flavor and taste, is a significant characteristic of made tea. Color, the attractiveness of made tea, has long influenced consumers' purchasing decisions. Unexpected color could be an indication of low-quality tea. During fermentation, the color of the black tea leaves may change from turquoise to yellowish green, then to yellowish red, yellowish brown, and eventually to dark brown. This process of color change is referred to as “red stain” in tea manufacture (Ghosh et al., 2012; Gill et al., 2011; Sharma et al., 2015). From a biochemical standpoint, red stain occurs as a result of the pigment's dynamic transformation from polyphenols to TF, TR, and TB (Obanda et al., 2001, 2004). Additionally, it was demonstrated that there was a definite relationship between the amount of TF and TR contained in liquor and its mouth feel. Malec (1988) investigated seasonal fluctuations in the concentrations of TF, TR, and caffeine in Argentinian black teas. Having said that, the authors also stated that a positive association existed between TLC and TF, but not TR. Contrary to Malec, Whitehead & Muhime (1989) found a link between TR and TLC. Moreover, two fractions of TR, namely TRSI and TRSII, have been found to play key roles in assessing TLC. Obanda et al. (2004) designated the TR butanol-insoluble fraction as TRSII (which is most likely equivalent to TB) and the TR butanol-soluble fraction as TRSI. According to the authors, TLC can be determined by TRSI and TF. Additionally, they stated that TF has a beneficial effect on liquor brightness, whereas overall TR, particularly TRSII (but not as much TRSI), has a negative effect on liquor brightness. On the other hand, Roberts and Chandrasa (1982) described a method for monitoring fermentation by detecting color. They found that the color development in tea is primarily associated with the formation of TF, TR, and TRP (TR polymers). It is recommended to maintain the TF and TR values during the fermentation process in order to get a better TLC.

Total phenolic content (TPC) in BT-2 black tea can also be affected significantly by fermentation time, temperature, and humidity (Table 3). Kim et al. (2018) found that the amount of TPC was gradually decreased with increased temperature and reaction period. The responses for TPC also showed a similar trend in the present study (Figure 1M, N, O). This result was also confirmed by the TF contents of the present study, which decreased with increased time, temperature, and relative humidity. Besides, in a previous study, Chang et al (2020) explained that when the black tea is subjected to fermentation for more extended periods at high temperature, chemical degradation and structural changes of temperature-sensitive compounds such as (-)-epigallocatechin-3-gallate can occur. Furthermore, it is also evident from Table 2 that the highest amount of TPC (14.10 GAE g/100g tea) was observed in the 10th run with temperature (25°C), time (70 min), and relative humidity (85%), while the lowest value of TPC (5.11 GAE g/100g tea) was found under the conditions using temperature (30°C), time (100 min), and relative humidity (95%).

Phenolic compounds have long been recognized as a significant determinant of the quality of black tea (Hazarkia et al., 1984). The entire profile of phenolic compounds contained in black tea has been found to be highly correlated with its price and sensory features (Takeo, 1976). Certain phenolic compounds, for example, gallic acid, quinic acid derivatives as well as catechins and their gallates, have no effect on the color of black tea (Bailey et al., 1990). This is since these phenolic compounds can only absorb light in the ultraviolet (UV) spectrum. However, these phenolic compounds may influence the flavor and other liquor characteristics of black tea (Caffin et al., 2004). For instance, astrigency has been associated with chlorogenic acid, one of the quinic acids found in black tea (Haslam, 1989). Additionally, extractable polyphenols such as TF and TR, as well as other flavonoids, can establish hydrogen bonds with caffeine, a process known as “cream down” in black tea liquor (Roberts, 1962). The “cream down” process of black tea is related to the tea's quality since it can contribute color, flavor, and mouthfeel to the liquor (Bailey et al., 1990; Roberts, 1962). Moreover, it is also believed that the activity of oxidative enzymes converts tannins and catechins to brown-black compounds in fermented tea leaves. Several of these compounds exhibit an affinity for melanin (Sava et al., 2001). Therefore, optimizing fermentation conditions is necessary to maintain control of oxygenation processes.

The values of TF and TR in the present study are presented in the form of a ratio in Table 2. In a study, Gill and his colleagues suggested that the standard ratio of TF:TR is 1:10 (Gill et al., 2011). The data from Table 2 depicts that few experimental runs showed a higher ratio of TF:TR than the standard ratio (1:10). Therefore, additional experiments were conducted in the present study to optimize the independent variables for maintaining the standard ratio of TF:TR (1:10). It is also clear from the three-dimensional response curves (Figure 1P, Q, R) that the ratio of TF:TR varying in the present study at different fermentation times, temperature, and relative humidity.

As the concentrations of TF and TR are fundamental quality parameters, their ratio is also essential to get a good combination of briskness, strength, brightness, body, and color of the black tea liquor (Rahman et al., 2020). Tea manufacturers must produce teas with the optimal ratio of TF to TR, as required by market demand. During fermentation step, enzymatic oxidation results in chemical changes in tea leaves such as protein degradation; polyphenol oxidation; the formation of few volatile compounds from amino acids, lipids, terpenoids, and carotenoids; chlorophyll conversion to phaeophorbides and phaeophytins. This process changes the color of the leaves from green to a deep coppery red, possibly as a result of the production of two-color compounds, TF and TR, and the aroma begins to develop (Mason et al., 2015). Together, these compounds can give the tea liquor its distinctive color. If the fermentation is allowed to continue for an extended period, TF is transformed to TR. The concentration of TF achieves a maximum value at which the ratio of TF to TR approaches 1:9 or 1:10 and the color of processed tea turns coppery red. This is the optimum fermentation point. Beyond this point, the concentration of TR continues to increase as the fermentation duration increases, resulting in a thickening of the liquor body. Over fermented tea has ‘body’ and lack other desirable characteristics of good cup of tea (Owuor and Reeves, 1986).

### 3.3. Optimization and validation of the model

A simultaneous optimization method using the Derringer's desirability function (D) was carried out by Design Expert® (version 12.0.3) to optimize the fermentation conditions like time, temperature, and relative humidity to maintain the quality parameters, i.e., TF, TR, TF, TR, HPS, TLC, and TPC of BT-2 black tea. The software optimizes the responses using a second-order model. In addition, Derringer's desirability function (D) is an effective technique when multiple responses are optimized with different targets. The D value other than zero (0) indicates that all responses are concurrently within an acceptable range, and for a D value near to 1, indicates that the factors are combined in a way that the

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**Table 4. Target values and Fit values of different parameters to achieve the optimum conditions.**

| Parameters                  | TF:TR | TPC (GAE g/100g tea) | TLC (%) | HPS (%) | TR (%) | TF (%) |
|-----------------------------|-------|---------------------|---------|---------|--------|--------|
| Target                      |       |                     |         |         |        |        |
| Max.                        | 9.38  | 7.89                | 3.17    | 8.69    | 5.41   | 0.76   |
| Predicted value             | 9.13  | 7.95 ± 0.51         | 3.05 ± 0.08 | 8.61 ± 0.25 | 5.57 ± 0.42 | 0.69 ± 0.07 |
| Actual value                | 9.13  | 7.95 ± 0.51         | 3.05 ± 0.08 | 8.61 ± 0.25 | 5.57 ± 0.42 | 0.69 ± 0.07 |

Actual values expressed as mean ± standard deviation of the mean (n = 3).
response values are close to the target values (Lee et al., 2018). Table 4 summarizes the predicted values for various responses under optimal conditions (within the range constraint). When the range of constraints was chosen, the optimal conditions were 80.14 min time, 28.76 °C temperature, and 92.30% relative humidity. However, the recommended conditions are challenging to maintain in practice, and some deviations are expected during processing. Therefore, the optimal conditions were determined as time 80 min, temperature 28 °C, and relative humidity 92% (Table 4).

The experiments were carried out under the experimental optimum conditions to examine the variation in BT-2 black tea quality parameters. The experimental values for TF, TR, TF:TR, HPS, TLC, TPC under the optimal conditions are summarized in Table 4, demonstrating that the experimental results were very close to the predicted values, thereby validating this model. The value of composite desirability (D) was 0.93 out of 1, which indicated that the values observed in experiments were highly congruent with the value predicted by the regression model (Figure 2). So, these results further demonstrated that the response surface model with desirability function could be effectively applied to optimize the fermentation conditions for maintaining the quality parameters of the BT-2 black tea.

4. Conclusion

Fermentation has been considered as the most vital step to maintain the quality and flavor of black tea preparation. TF, TR, and other quality compounds were found to form during the fermentation period. Therefore, the fermentation environment needs to be strictly controlled for all the parameters because slight fluctuations in temperature, time, and humidity can result in loss of quality and production. The present study optimized the fermentation time, temperature, and relative humidity for the quality parameters (i.e., TF, TR, TF:TR, HPC, TLC, TPC) of the BT-2
variety. From the findings of this study, it can be concluded that the standard quality of the BT-2 black tea variety might be preserved under the optimum condition of time 80.14 min, temperature 28.76°C, and relative humidity 92.30%. Hence, tea manufacturers could implement this optimized model in their factories to ensure efficiency and better quality of BT-2 black tea in Bangladesh and the International market.

Declarations

Author contribution statement

Mohammad Alzal Hossain: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Tanvir Ahmed, Md. Sakib Hossain, Pappu Dey: Performed the experiments; Wrote the paper.

Shafaet Ahmed: Contributed reagents, materials, analysis tools or data.

Md. Monir Hossain: Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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