Optimization of gaseous ozone for decontamination process of black pepper (*Piper nigrum* L.)

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**Abstract.** Indonesia is one of the largest pepper producers in the world with high export value. Processing of pepper in Indonesia oftenly does not meet the hygiene and quality standards that desired by importers, one of the standard quality required is total plate count (TPC) which shown total microorganism. In this study, whole black pepper exposed with gaseous ozone to reduce total microorganism. The purpose of this study was to determine the optimum flow rate and exposure time of ozonation as a treatment for decontamination of black pepper to reduce maximum reduction of total microorganism with minimum changes in piperine content. The optimization is done by using Response Surface Method (RSM) with Central Composite Design (CCD) with flow rate of gaseous ozone (1–4 L/min) and exposure time of ozonation (10–40 minute). The responses of the ozonation process were total microorganism and piperine content. This research shown that ozonation process of black pepper reduced the total microorganism of 3–4 log CFU/g. The optimum condition of flow rate and exposure time provided by Design Expert were flow rate of 1 L/min and exposure time of 10 minutes that resulted a model validity value on the response of piperine levels with high to perfect accuracy.

1. **Introduction**

Pepper (*Piper Nigrum* L.) is spices commodity with high economic value, which produce second mostly in Indonesia, where most of it is exported. According to the International Pepper Community (IPC) [1], Indonesia is the second largest pepper producing country in the world after Vietnam with 18% share of production in 2016. Despite being one of the largest pepper producers, producing pepper in Indonesia is still done traditionally where hygiene and quality aspects are the main problems that oftenly does not meet the standards required. It was found that black pepper produced by farmers in Lampung contained a total microorganism contamination of 6 log CFU/g while black pepper from collectors contained a total microorganism contamination of 9 log CFU/g [2]. This number exceeds the desired standard, which according to USFDA, total microorganism contamination in spices should be less than 6 log CFU/g.

In order to provide pepper to meet the standard required, a microorganism reduction treatment can be applied in spice industry, such as ozone treatment. Ozone is an effective sanitizer with very high oxidation reduction potential, which approved by the FDA for direct application on food products. Compared to other decontamination technique such as fumigation, ozone treatment has advantage, which is, it lack of residual components on products, as it liberates unharmed oxygen as the major end product [3]. The mechanism of ozone destroys microorganisms is by the progressive oxidation of vital cellular component, first it oxidizes sulfihydryl groups and amino acids of enzymes, peptides, and
proteins to shorter peptides. Then, ozone reacts with the double bonds of unsaturated lipids in the cell envelope that caused a leakage of cell contents and lysis [4]. The effect of gaseous ozone as microorganism reduction treatment has been studied on some dried products such as black pepper [5], figs [6], peanuts [7], and oregano [4]. It was found that gaseous ozone treatment reduced total microorganism population by 3–4 log CFU/g but resulted in oxidation of certain volatile oil constituents [5]. As mentioned before, ozone is a reactive compound that imposes oxidative stress that can result in a variety responses such as physiological changes of commodities that may effected some compounds in the product [8]. One of the main compound that determined the quality of pepper is piperine, an alkaloid contributes for the pungency of black pepper.

Therefore, it is necessary to study the optimization of gaseous ozone treatment (flow rate and exposure time) as decontamination process of whole black pepper that can reduce maximum total microorganism population with minimum changes in piperine content. This study presents flow rate of gaseous ozone and exposure time of ozonation at optimized conditions using Central Composite Design under Response Surface Methodology (RSM).

2. Materials and methods

2.1. Material treatment

Black pepper was harvested in Bangka, then handled by PT Cinquer Agro Nusantara (Tegalluar, Kabupaten Bandung, Indonesia). The process that done are double-washed (sorting), quality evaluation, and packaging. The sample that taken for this study is packaged in a double layer sack bag of 250 grams black pepper then transferred to the laboratory and stored at room temperature.

2.2. Design of experiments, central composite design (CCD)

The Central Composite Design (CCD) under Response Surface Methodology (RSM) embedded in the design expert software (10.0.0) was employed. The experimental design generated 13 experimental runs are presented in table 1 with the results of responses. These were used to investigate the effects of flow rate (1–4 L/min) and exposure time (10–40 min) of the ozonation treatment on black pepper. Black pepper was ozoned in the airtight chamber for 5 minutes at various flow rates and kept inside the chamber for various times suggested by the software for each run. The resulting ozone-treated black pepper for each run then analyzed for 2 responses input in the software, TPC (CFU/g) and piperine content (%).

| Table 1. Experimental design. |
|--------------------------------|
| Run | Flow rate (L/min) | Exposure time (min) | TPC (10^5 CFU/g) | Piperine content (%) |
|-----|-------------------|---------------------|-----------------|---------------------|
| 1   | 2.5               | 25                  | 12.00           | 6.62                |
| 2   | 4                 | 40                  | 21.00           | 6.74                |
| 3   | 2.5               | 25                  | 0.50            | 6.33                |
| 4   | 0.38              | 25                  | 3.00            | 6.84                |
| 5   | 2.5               | 46.21               | 32.00           | 6.32                |
| 6   | 2.5               | 25                  | 0.48            | 6.38                |
| 7   | 2.5               | 25                  | 0.52            | 6.69                |
| 8   | 2.5               | 25                  | 7.80            | 6.3                 |
| 9   | 1                 | 40                  | 29.00           | 6.99                |
| 10  | 4                 | 10                  | 16.00           | 7.15                |
| 11  | 4.62              | 25                  | 0.60            | 7.01                |
| 12  | 2.5               | 3.78                | 0.60            | 7.27                |
| 13  | 1                 | 10                  | 0.97            | 6.84                |
2.3. **Ozone treatment**

The ozonation device consisted of oxygen tank, ozone generator (LUSO OZ-5 G) and an airtight chamber. Ozone was produced by providing dry oxygen to the ozone generator. Flow rate of gaseous ozone was adjusted by microcontroller with interface through an integrated ozonizer software. The data input was flow rate (1–4 L/min) and exposure time (10–40 min). The range of the flow rate and exposure time was selected based on results of scientific literature and preliminary research. The samples of 25 grams black pepper were placed on the perforated metal tray inside the sterilized airtight chamber then treated with gaseous ozone for 5 minutes. After 5 minutes, the samples kept inside the airtight chamber to let the ozone reacted with the surface of the sample for 10–40 minutes of exposure time.

2.4. **Microbiological analysis**

A gram sample were diluted in 9 ml of BPW and homogenized for 1 min in a vortex mixer. After homogenization, 1 ml aliquots of homogenized sample were serially diluted in 9 ml of BPW to 10⁻⁴. 1 ml aliquots of each diluents is taken onto three plates then pour the medium (PCA). All plates were incubated at 37 °C for 40 hours and then the colonies were counted with colony counter.

2.5. **Piperine analysis**

The principle of piperine analysis is thermal extraction with ethanol followed by reading the absorbance using UV Vis spectrophotometer at a wavelength of 343 nm. Piperine was extracted from 0.5 grams of ground sieved black pepper in 50 ml of ethanol by reflux for 3 hour in the dark. After extraction, the cooled solution filtered into a 100 ml volumetric flask with a filter paper. The flask and filter paper was washed with ethanol and the volume the made up the volume in the flask to the mark. 5 ml aliquot of the solution then transferred to a 50 ml volumetric flask and made up to the mark with ethanol. 5 ml of this solution then transferred into a 25 ml volumetric flask and again made up the mark with ethanol. The absorbance (A) of this solution then measured at 343 nm and the piperine content expressed as a percentage that determined using Eq. (1)

\[
\% \text{ Piperine} = \frac{A_s}{A_{1\%}} \times \frac{50}{5} \times \frac{25}{5} \times \frac{100}{m} \times \frac{100}{100 - H}
\]

where \( m \) is the mass, \( H \) is the moisture content, \( A \) is the absorbance of the final test solution, \( A_{1\%} \) is the absorbance at 343 nm of a 1% solution of piperine in a cell of optical path length 1 cm which equals 1238 nm [9].

3. **Result and discussion**

3.1. **Analysis of responses**

Optimization of ozone treatment for decontamination of black pepper was conducted by using runs as listed in table 1. The aim of this optimization was to obtain the correlation between the measured responses and the corresponding parameters. Parameters that considered in this study were flow rate (A) and exposure time (B). These parameters were chosen as the independent variables while total microorganism and piperine content as the responses (dependent variables) of the study. The final central composite design obtained for both total microorganism count and piperine content of ozonized black pepper with significant terms was quadratic as suggested by the software, and is given as:

\[
\text{Tot. Microorganism} = -4.63351 + 5.15994(A) - 0.48011(B) - 0.255889(AB) + 0.30806(A^2) + 0.035303(B^2)
\]

\[
\text{Piperine content} = +7.54381 - 0.37052(A) - 0.038474(B) - 6.22222 \times 10^{-3}(AB) + 0.11022(A^2) + 8.13333 \times 10^{-4}(B^2)
\]
Equation (2) and (3) shows how the individual variables (quadratic) or double interaction affected total microorganism and piperine content in ozonized black pepper. The negative coefficient values indicate that individual or double interactions factors negatively affected total microorganism or piperine content, whereas positive coefficient values mean that factors increase total microorganism or piperine content for each equation. In this case, all the variables, A, B, AB, A², B², are significant model terms. The Lack of Fit F-Value for both responses (total microorganism and piperine content of 0.3004 and 0.3030, respectively) > 0.05 implies the lack of fit is not significant relative to the pure error (table 2, 3). The adequacy of the models was justified by the analysis of variance (ANOVA) (table 2 and 3). The Prob > F value is the probability of F-statistics value and is used to test the null hypothesis. The parameters having F-statistics probability value less than 0.05 are said to be significant. Both parameters model have the p-value of 0.0117 and 0.0308 (p-value < 0.05) meant that the model are significant. The number of standard deviation separating actual and predicted values can be measured by internally studentized residuals. The normality assumption was satisfied as the residual plot approximated along a straight line as shown in both responses graph (figure 1). The analysis of diagnostic case statistics of data shows that the model fits well to optimize the independent variables for both total microorganism and piperine content.

Table 2. Anova of total microorganism response.

| Source          | Sum of Squares | df | Mean Square | F Value | p-value | Prob > F |
|-----------------|----------------|----|-------------|---------|---------|----------|
| Model           | 1323.54        | 5  | 264.71      | 7.05    | 0.0117  | significant |
| A-flow rate     | 1.65           | 1  | 1.65        | 0.044   | 0.8398  |           |
| B-exposure time | 749.55         | 1  | 749.55      | 19.95   | 0.0029  |           |
| AB              | 132.60         | 1  | 132.60      | 3.53    | 0.1024  |           |
| A²              | 3.34           | 1  | 3.34        | 0.089   | 0.7742  |           |
| B²              | 438.91         | 1  | 438.91      | 11.68   | 0.0112  |           |
| Residual        | 262.96         | 7  | 37.57       | 1.72    | 0.3004  | not significant |
| Lack of Fit     | 148.11         | 3  | 49.37       | 1.72    | 0.3004  | not significant |
| Pure Error      | 114.85         | 4  | 28.71       | 1.72    | 0.3004  | not significant |
| Cor Total       | 1586.50        | 12 |             |         |         |          |
| R-Squared       | 0.8343         |    |             |         |         |          |
| Adj R-Squared   | 0.7159         |    |             |         |         |          |

Table 3. Anova of piperine content response.

| Source          | Sum of Squares | df | Mean Square | F Value | p-value | Prob > F |
|-----------------|----------------|----|-------------|---------|---------|----------|
| Model           | 1.00           | 5  | 0.20        | 4.86    | 0.0308  | significant |
| A-flow rate     | 0.011          | 1  | 0.011       | 0.27    | 0.6167  |           |
| B-exposure time | 0.32           | 1  | 0.32        | 7.81    | 0.0267  |           |
| AB              | 0.078          | 1  | 0.078       | 1.91    | 0.2099  |           |
| A²              | 0.43           | 1  | 0.43        | 10.40   | 0.0145  |           |
| B²              | 0.23           | 1  | 0.23        | 5.66    | 0.0489  |           |
| Residual        | 0.29           | 7  | 0.041       |         |         |          |
| Lack of Fit     | 0.16           | 3  | 0.054       | 1.68    | 0.3070  | not significant |
| Pure Error      | 0.13           | 4  | 0.032       |         |         |          |
| Cor Total       | 1.29           | 12 |             |         |         |          |
| R-Squared       | 0.7764         |    |             |         |         |          |
| Adj R-Squared   | 0.6166         |    |             |         |         |          |

A high value of the adjusted determination coefficient of 0.7159 and 0.6166 (total microorganism and piperine content, respectively) was estimated. This result means that 71.59% of total microorganism and
61.66% of piperine content affected by the variables or combination of the variables and can be
described by the selected model. This means the quadratic model can be used to explore design and to
find the optimum conditions of this process.

Figure 2 shows the equivalent 3-dimensiol view for the interaction of factors as they influence the
responses. Figure 2a shows that exposure time is more significantly affected total microorganism than
flow rate, which shows that the longer exposure time applied, the higher the total microorganism
resulted. This might happened because ozone in the system has reacted and decomposed into oxygen
[10]. In this study, gaseous ozone generated only for five minutes, then samples were kept inside the
airtight chamber to let them exposed to the ozone. Ozone inside the system might already reacted with
the surface of black paper and decomposed into oxygen when the exposure time was longer.

Figure 2 b shows that flow rate and exposure time of ozonation treatment affected piperine content of
black pepper, but not as much total microorganism affected. Piperine content of ozonized black pepper
is smaller than piperine content of black pepper without ozonation treatment (control), which is this
might happen because ozone reacted with piperine, an organic compound classed with the alkaloids
that presented in black pepper. Degradation of organic compounds reported due to various intermediate
radical formations, leading to electrophilic and nucleophilic reactions occurring with aromatic
compounds. Various organic compounds present in food product such as carotenoids, anthocyanins,
ascorbic acid, and amino acid were found to be oxidised with the complex reactions of radical
transformation during ozonation with simultaneous degradation and formation of new compounds [11].
However, the effect and reactions of ozone with specifically alkaloids has not been studied yet.

3.2. Validation of optimized ozonation treatment model
Optimization is done to get a combination of flow rate and exposure time which results in the ozonation
treatment with maximum reduction of total microorganism and minimum changes in piperine content.
The optimization criteria is minimize. The most important response is total microorganism. The
optimization is carried out with single objective optimization of total microorganism response. The
output obtained from the optimization is the solution of ozone treatment criteria (flow rate and exposure
time) with the desired responses. The most optimum ozonation treatment solution given the greatest
desirability value, which a value that show the ability of the program to meet the specified optimization
criteria. The desirability given from the solution is 0.912.
Table 4. Validation of optimum ozonation treatment.

| Optimum criteria | Responses | Prediction | Actual | Validity (%) |
|------------------|-----------|------------|--------|--------------|
| Flow Rate (L/min) | Exposure Time (min) | TPC (log CFU/g) | 2 | 3.11 | 44.50 |
| 1.86 | 10 | Piperine content (%) | 6.82 | 6.84 | 99.71 |

Figure 2. 3D and contour plate for ozonation treatment interaction of flow rate and exposure time on (a) total microorganism; (b) piperine content.

The optimum factors given by Design Expert is validated by experiment, the analyzed responses data results then compared with the predicted responses given. The results of validation responses in optimum ozonation treatment can be seen in table 3. The response of total microorganism has a low reliability of 44.50%. The actual response shown higher total microorganism than the prediction. The result was affected by the morphology of black pepper. Surface area is an important factor in food decontamination process where smaller particles and larger surface areas require higher ozone concentrations [4]. Black pepper has a wrinkly surface that could cause the ozone molecule did not hit the surface evenly. Therefore, higher flow rates which practically produce higher concentration should be given so the ozone could hit the surface of black pepper evenly. Commodities with smoother surface could be more effectively exposed to gaseous ozone [12]. The smooth surfaces increases O3 mass transfer, therefore O3 molecule diffusion to microorganism is more efficient and faster and eventually caused penetration to the surface and microbial destruction more effective [13].

The ozonation process of black pepper reduced total microorganism by 3-4 log CFU/g, where the total microorganism of black pepper before exposed with gaseous ozone is 4.4 x 10^9 CFU/g. The result shows that gaseous ozone can be applied as a decontamination process of black pepper. At least 2 log CFU/g of total microorganism should be reduced so it can be applied in the food industry [14].

4. Conclusion
Statistical analysis, which shown in the equation given by Design Expert, reported that both flow rate and exposure time of ozonation treatment of black pepper were significant factor that affected the total microorganism and piperine content of black pepper. However, the reliability of the total microorganism responses on an optimum gaseous ozone treatment for black pepper that given by Design Expert is
considered low of 44.50%. The effective process of ozonation for decontamination of black pepper can be achieved with higher flow rate of gaseous ozone that produce high ozone concentration.

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Acknowledgment
Supports by PT Cinquer Agro Nusantara (Tegalluar, Bojongsoang, Kabupaten Bandung) are greatly acknowledged.