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Modelling ozone-based process for decontamination of *Opisthorchis viverrini* in cyprinid fish

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Abstract Result indicated that the quadratic model with a correlation coefficient (R^2) of 0.9376 was significantly predicted the responses ($p<0.05$). RSM model showed that optimal condition refers to 0.50 ppm ozone for 20 min, resulting in 100% decontamination of *Opisthorchis viverrini* metacercariae (OVMC). These findings demonstrated the high efficiency and practical applicability of ozone-based treatment as a food safety intervention to reduce opisthorchiasis transmission through cyprinid fish, with potential impact on public health in endemic regions.

Keywords: Response surface methodology, Cyprinid fish, Ozone

Introduction

Opisthorchiasis is a parasitic infection by the liver fluke *Opisthorchis* spp., which is commonly found in the far East and South East Asia. *O. felineus* is the prominent fluke in Eastern Europe, while *C. sinensis* is endemic in south China, Japan, Korea and Taiwan. In Thailand *O. viverrini* is the only parasite of opisthorchiasis (Kaewpitoon *et al.,* 2008). It is known that freshwater fish, especially cyprinid fish, are important intermediate hosts of *O. viverrini*.

Moreover, in Thailand, the tradition of eating raw or uncooked fish products, such as a raw fish salad (koi pla), salt-fermented fish (pla-ra) and sticky rice-fermented fish (pla-som), as the main reason that liver flukes are a problem. To control *O. viverrini,* consuming cooked freshwater fish will reduce the risk of liver fluke. However, some local people in Thailand believe that food tastes better when raw or undercooked than when well-cooked. These beliefs are

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important risk factors of foodborne infection, including that with parasites. Therefore, finding a way to destroy *O. viverrini* without using heat is a challenging research area.

The use of ozone is another interesting alternative for destroying *O. viverrini* in freshwater fish, because ozone is a method to kill pathogenic microorganisms such as *Escherichia coli*, *Salmonella*, and *Listeria monocytogenes*; fungi (*Alternaria* and *Aspergillus flavus*); protozoa, fungal spores, bacteria, and virus in the general food industry, such as meat, plants, and grain. Ozone breaks microbial cells from the cell membrane and the DNA structure inside the cell. Ozone has the good properties of being extremely oxidative and easily decomposed, with no residue in food. Using ozonated water at a concentration of 6 mg/L for 90 sec can inhibit microbial contamination in beef for steaks during slaughter and the extension of meat shelf life at 4°C for 15 days. The result showed that the ozone treatment effectively reduced the growth of microorganisms that develop in refrigeration, including *Lactobacillus sakei*, *Leuconostoc gasicomitatum*, and *Lactococcus piscium* (Botta *et al*., 2018).

A report on strawberries, tomatoes, cabbage, and green beans using ozone at concentrations of 126-136 ppm for 3 and 15 min showed that the development of bacteria and fungi decreased. An average ozone concentration of 128.7 ppm for 3 min inhibited the growth of bacteria and fungi (*Enterobacter* sp., *Chrysobacter* sp., *Xanthomonas*, *Chromobacter*, *Aspergillus* sp., and *Fusarium* sp.) by at least 5 logs, which could extend the storage life of the produce at least 2 times (Roy *et al*., 2021).

In fresh-cut bell peppers (Zamboni variety), ozone at concentrations of 0-9 ppm for 0-24 h at 18-20°C and 95% relative humidity is used to control pathogens in food, including *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* Typhimurium. The result found that 9 ppm of ozone for 6 h decreased the maximum pathogen and can be used as an alternative method to reduce pathogens in fruits (Alwi and Ali, 2014).

Nevertheless, ozone is environmentally friendly and has been recognized as safe (GRAS) and can be used directly with food. The efficiency of ozone use in each food industry is different. Whether it is the level of ozone concentration used and the contact time, along with the lack of knowledge on the application of ozone in the freshwater fish, it is therefore necessary to develop a process system for using ozone to destroy *O. viverrini* in freshwater fish that can be practically applied at the laboratory and field levels to increase the safety of fermented fish products.

Materials and methods

Sample preparation

Cyprinoid fish that could likely be contaminated with *O. viverrini* metacercariae (OVMC) were included and purchased from retail markets in Sa Kaeo Province, Thailand. All Cyprinoid fish were preserved on ice and transferred to the laboratory within 30 min. Fresh fish was scaled and rinsed with running water to remove the blood and debris. Finally, the samples were stored at 25±2 °C until used.

Preparation of aqueous ozone

Aqueous ozone or ozonated water was prepared by an ozone generator (OZONER®-013-30G, Pro-Tech-Sci Co., LTD, Chiang Mai, Thailand). Ozone saturated water was obtained by bubbling ozone gas using air diffuser into 5-L of tap water in plastic tank at 25±2 °C. Ozone system was at a controlled air flow rate of 60 L hr^{-1} which led to the ozone concentration (ozone dosage) of about 30 g hr^{-1}. The ozone concentration was semi-quantitatively measured by Ozone test kit (HI38054, HANNA instruments inc., USA).

Decontamination experiment

Decontamination was done using various condition including ozone dosages and contact times as per experimental design. Using each experiment, about 2-kg fish samples were placed in plastic tank containing 5-L of aqueous ozone at 20-25 °C for an adequate time. Water was included as a control treatment. After treatment, the presence of OVMC in fish was determined using a standard pepsin-HCl digestion method (Pinlaor *et al.,* 2013; Sithithaworn *et al.,* 1997). Briefly, the individual fish were chopped and digested with 0.25% pepsin-1.5% HCl in 0.85% NaCl solution at ratio of 1:1. The digestion was incubated at at 37 ºC for 1 hours, then OVMC in fish were captured using the following series of sieves with 1000, 850, 300 and 106 µm mesh sizes, respectively. The debris obtained by filtering with the 106 µm mesh was washed and precipitated with 0.85% NaCl solution in sedimentation jar. Finally, OVMC were examined and counted under a stereomicroscope and the intensity of OVMC to be calculated using Equation (2).

$$I = N/G \tag{2}$$

where I is the intensity of OVMC (OVMC/g), N is number of OVMC and G is weight of fish (gram).

Egg reduction rate (EER) was calculated using Equation (3).

$$ERR = \left[\left(\frac{I^*}{I}\right) \times 100\right] - 100 \quad (3)$$

where ERR is the egg reduction rate (%), I* is intensity of OVMC in each treatment (OVMC/g) and I is intensity of OVMC in control treatment (OVMC/g).

Experimental design

The experiments were designed using the Design Expert software version 10 (Stat-Ease, USA) using central composite design (CCD) of RSM for modelling process of *O. viverrini* decontamination in cyprinid fish using ozone. Two independent variables were ozone dosages (0.10–0.50 ppm) and contact times (10.00–30.00 min) with five different levels (-α, -1, 0, +1, +α) shown in Table 1. The total experimental runs conducted are computed by Equation (4) (Owolabi *et al.,* 2018).

$$N = 2^n + 2n + n_c \quad (4)$$
$$N = 2^2 + 2(2) + 6 = 4 + 4 + 5 = 13$$

where n is number of independent variables (factors), n_c is number of center points and N is the overall total of experimental treatment.

RSM suggested total of 13 experimental treatments to predict response as shown in Table 2. Using each experiment, about 2-kg fish samples were dipped into an aqueous ozone at various ozone dosage (X_1) and contract times (X_2). EER were designed as the response variable (Y). The response variable was analysed using quadratic regression model presented in Equation (5) (Hazbawi and Safaeinezhad, 2023).

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_1X_2 + a_4X_1^2 + a_5X_2^2 \quad (5)$$

Where Y is the response variable, a_0, a_1, a_2, a_3, a_4 and a_5 are the regression coefficients of model, and X_1 and X_2 are the independent variables.

To evaluate the adequacy of the model, the coefficient of determination (R^2), adjusted R^2 (Adj. R^2), predicted R^2 (Pred. R^2), the percentage of coefficient of variation (%CV), lack-of-fit test were used. The statistical significance of the model was with a level of $p < 0.05$.

Table 1. Independent variables for optimization

Variables	Unit	Symbols	Levels				
			-1.68	-1	0	+1	+1.68
Ozone Dosage	ppm	X_1	0.05	0.10	0.40	0.50	0.80
Contact time	min	X_2	6.00	10.00	20.00	30.00	34.00

Table 2. Experimental design and values of response of response surface methodology for ozone decontamination of OVMC in fish

Treatments	Factors		ERR (%)
	X_1	X_2	
1	0.30	20.00	-100.00
2	0.40	20.00	-100.00
3	0.40	20.00	-100.00
4	0.40	20.00	-85.85
5	0.50	10.00	-100.00
6	0.40	34.00	-100.00
7	0.80	20.00	-100.00
8	0.05	20.00	-45.31
9	0.40	6.00	-100.00
10	0.10	10.00	-51.26
11	0.10	30.00	-73.80
12	0.40	20.00	-100.00
13	0.50	30.00	-91.24

Results

RSM modeling

This study was carried out to optimize the aqueous ozone decontamination viz. ozone dosage and contact time for fish using response surface methodology and central composite design. Independent variables were an ozone dosage (X_1) and contract times (X_2), while the response variable was the egg reduction rate (EER) of OVMC in fish. The experimental data was shown in Table 2. EER of 13 treatments exhibited the highest value of EER at -100.00%, while the lowest value of EER was at -45.31%. To identify the most suitable regression model for predicting EER, the linear, two factor interaction (2FI), quadratic and the cubic models were compared (Table 3). From the model summary statistics, quadratic model showed a non-significant lack-of-fit p-value (0.3669) and the higher values of correlation coefficient (R^2) of 0.8931 and predicted R^2 of 0.7258. Therefore, quadratic model was selected as the best fit model.

Table 3. Fit summary of egg reduction rate

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	0.0142	0.0278	0.4874	0.1815	
2FI	0.2833	0.0269	0.5025	0.0301	
Quadratic	0.0019	0.3669	0.8931	0.7258	Suggested
Cubic	0.6879	0.1561	0.8712	-0.5319	Aliased

Analysis of variance (ANOVA) was used to evaluate the model significance (Table 4). The result showed that the model was significant with the *p*-value below 0.05 ($p < 0.05$). X_1, X_1X_2, and X_1^2 parameters were found significant effect at value of $p < 0.05$ if added to the models. While X_2, and X_2^2 were found not significant effect at value of $p > 0.05$, and therefore had to be removed from the models. Additionally, the non-significant lack-of-fit value ($p = 0.3669$) confirmed that there was no discrepancies found in the model. The R^2 and adj. R^2 of 0.9376 and 0.8931 was obtained, representing the good correlation between the predicted data and the experimental data. A linear distribution is observed that indicates the well-fitting model (Figure 1).

Table 4. Analysis of variance (ANOVA) of quadratic model of Egg reduction rate (EER) of OVMC in fish

Source	Sum of Squares	Mean Square	F Value	p-value Prob > F	
Model	4253.34	850.67	21.05	0.0004	significant
A-X_1	*2574.87*	*2574.87*	*63.72*	*< 0.0001*	
B-X_2	*23.74*	*23.74*	*0.59*	*0.4685*	
AB	*244.92*	*244.92*	*6.06*	*0.0433*	
A^2	*1396.92*	*1396.92*	*34.57*	*0.0006*	
B^2	*1.73*	*1.73*	*0.043*	*0.8421*	
Residual	282.85	40.41			
Lack of Fit	*144.51*	*48.17*	*1.39*	*0.3669*	*not significant*
Pure Error	*138.34*	*34.58*			
Cor Total	4536.19	40.41			

Std. Dev.	6.36	R^2	0.9376
Mean	-88.34	Adj R^2	0.8931
C.V. %	7.20	Pred R^2	0.7258
PRESS	1243.79	Adeq Precision	14.609
-2 Log Likelihood	76.93	BIC	92.32
		AICc	102.93

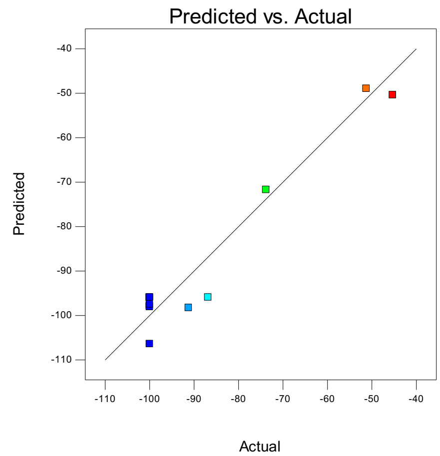

Figure 1. Correlation of predicted and experimental (actual) data by the model

The quadratic equation for predicting the optimal point for the response viz. EER of OVMC in fish. Has been presented as follow:

$$Y = -9.66297 - 38051146X_1 + 3.91250X_1X_2 + 354.26563X_1^2 \qquad (6)$$

Based on Equation (6), EER value will decrease with increase of ozone dosage. The three-dimensional response surface and related contour plots was illustrated in Figure 2.

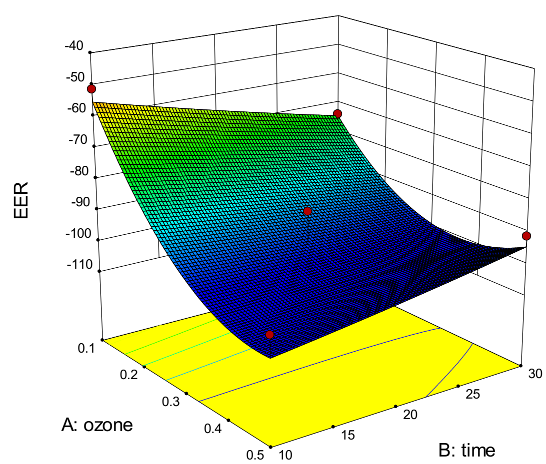

Figure 2. Three-dimensional surface plot and contour plot of EER

The last step in this investigation was to find the optimal solution using a numerical method. According to Figure 3, the model of predicting the optimum conditions for maximum EER of OVMC in fish were an ozone dosage of 0.33 ppm and contract times at 25.30 min. The predicted EER value was -100%.

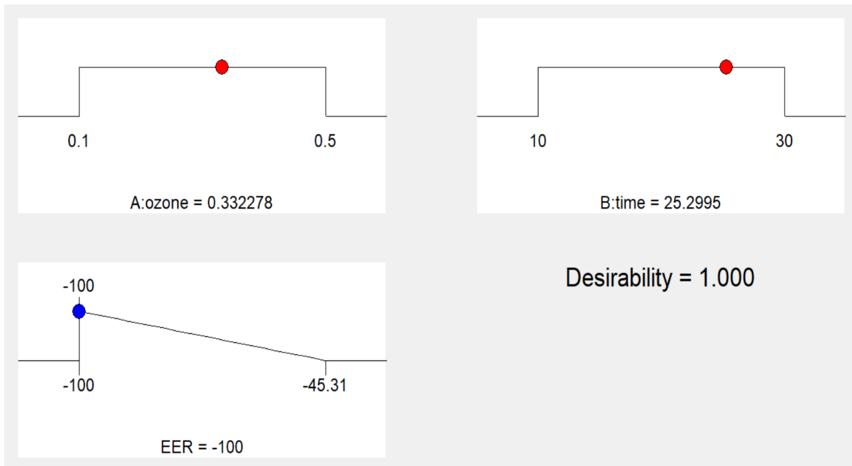

Figure 3. Ramps graphs of optimized process

Model validation

To evaluate the predictive performance of the RSM-derived quadratic model, the experimental runs using the predicted optimized conditions were conducted. The experimental and predicted results of the response is shown in Table 5. The experimental value was found to be same to the predicted value. The percentage error was 0.00%. Thus, the model was useful in predicting the optimal conditions for ozone decontamination of OVMC in fish. EER strongly decreased in 10 min contact time while after 15 min contact time was enough to inactivate 100% of OVMC in fish (Figure 4).

Table 5. Experimental and predicted results

Ozone dosage (ppm)	Contract times (min)	Predicted EER (%)	Experimental EER (%)	Error (%)
0.33	25.30	-100	-100	0.00

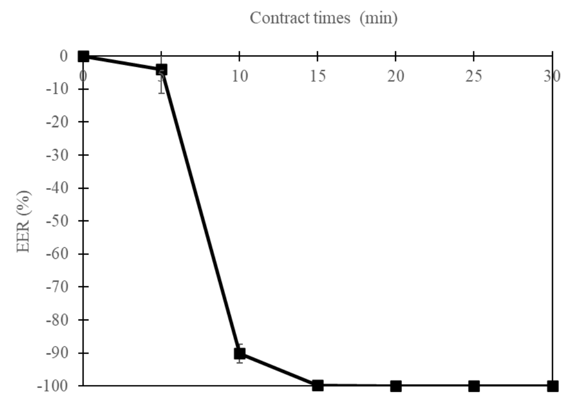

Figure 4. Egg reduction rate of OVMC in ozone dosage of 0.33 ppm at various contact time

Discussion

In recent years, ozone can be used as an alternative to control the growth of spoilage and pathogenic microorganisms in food industry. In Food and Drug Administration (FDA) of the U.S., ozone is recognized as a Generally Recognized as Safe (GRAS) and has now been approved for use as a disinfectant in foods since 1997 (Guzel-Seydim *et al.,* 2004). It is effective in destroying microorganisms such as bacteria, fungi and virus (Epelle *et al*., 2023). However, few studies have investigated the influence of ozone in parasites, and no studies have reported its effect on *O. viverrini*.

The elimination efficiency of microorganism by ozone is affected by intrinsic factors (type of food, characteristics, food weight and water activity) and extrinsic factors (temperature, contact time, ozone concentration and application method) (Xue *et al*., 2023). In traditionally, optimization of decontamination process usually exhibited "one-factor-at-a-time (OFAT) depending on contact time and concentration of ozone, which was tedious, time-consuming and uneconomical (Alexander *et al*., 2016; J. Chen *et al*., 2022). Response surface methodology (RSM) can avoid the limitations of conventional methods and is commonly used in many fields. The analysis of interaction between the independent variables including ozone dosages (0.10–0.50 ppm) and contact times (10.00–30.00 min) was done by considering the response variable as EER of OVMC in fish. RSM result show that ozone dosages significant effect on the efficacy of ozone decontamination of OVMC in fish. In contrast, contact time did not significantly affect on the efficacy of ozone decontamination of OVMC in fish. Efficiency of aqueous ozone in decontamination of OVMC in fish increased with increase of ozone dosage. The finding of this study correlates with previous studies (Baumann *et al*., 2024; Khalifa *et al*., 2001; Rajabi *et al*., 2015). The major target of ozone treatment is the cell wall, which under stress leads to cell wall damage, leakage of intracellular content, and cell lyses (Sripong *et al*., 2022). Moreover, ozone can also destroy protein, enzyme and DNA (Xue *et al*., 2023). Although, ozone dosages in the range of 0.20 - 0.50 ppm have significant effect on the efficacy of decontamination of OVMC in fish. The RSM result indicated that ozone dosages at 0.33 ppm was the optimal dosage for decontamination of OVMC in fish.

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Conflicts of interest

The authors declare no conflict of interest.

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