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Contact information:
Nong Lam University
Room 404, Thien Ly Building
Linh Trung Ward, Thu Duc District, Ho Chi Minh City, Vietnam
Tel: (84-28)37245670
Email: jad@hcmuaf.edu.vn

The Journal of Agriculture and Development
Volume 17 - Issue 6 (2018)
pISSN 2615-9503
eISSN 2615-949X

CONTENT

Application of SARIMA model to forecasting the natural rubber price in the world market

Nhien T. Pham

Department of Economics, Nong Lam University, Ho Chi Minh City, Vietnam

ARTICLE INFO

Research Paper

Received: November 02, 2018
Revised: November 23, 2018
Accepted: December 09, 2018

Keywords

Natural rubber price
Rubber
Rubber market
Rubber price forecast
SARIMA

Corresponding author

Pham Thi Nhien
Email: ptnhien@hcmuaf.edu.vn

ABSTRACT

This study was conducted to develop a forecasting model to predict the price natural rubber in the world market by using the Seasonal Autoregressive Integrated Moving Average (SARIMA). The dataset for model development was collected from series data of average monthly closing average prices in the natural rubber - Ribbed Smoked Sheet No.3 (RSS3) on the Tokyo Commodity Exchange (TOCOM) for the period of January 2007 - September 2018. The RSS3 price on the TOCOM provided the reference price for natural rubber in the world market. It resulted SARIMA$(2,1,2)(1,1,1)_{12}$ model was selected as the best-fit model. The model achieved 0.000 for Probability value (*P-value*); 8.86 for Akaike Information Criterion (AIC) and 9.01 for Schwarz Information Criterion (SIC); 6.68% for Mean Absolute Percentage Error (MAPE) and 21.43 for Root Mean Square Error (RMSE). This model was used to forecast the world's natural rubber price during October 2018 - December 2020. This study may be helpful to the farmers, traders, and the governments of the world's important natural rubber producing countries to plan policies to reduce natural rubber production costs and stabilize the natural rubber price in the future, such as by setting suitable areas for natural rubber plantation in each country, and defining appropriate and sustainable alternative crop areas in each country.

Cited as: Pham, N. T. (2018). Application of SARIMA model to forecasting the natural rubber price in the world market. *The Journal of Agriculture and Development* 17(6), 1-7.

1. Introduction

Natural rubber (NR) is a major economic plant in the plantation sector in terms of providing income to the growers for a long-time and also it serves as raw materials for various industrial products. Over 20 million families are dependent on rubber cultivation for their livelihood in the world natural rubber market. Rubber has been one of the most essential economic plants in the world in past 10 years. According to the statistics of Association of Natural Rubber Producing Countries (ANRPC), the total global production of natural rubber increases from 10.06 million metric tons in 2007 to 13.54 million metric tons in 2017. In 2017, the largest rubber producing countries are Thailand, Indonesia, Malaysia, Vietnam, India and China which collectively account for 90% of world production. Rubber is primarily exported to China, Germany, United States and Iran to be processed to produce vehicle tires, belts, shoe soles, medical gloves and parts for electronic equipment. Approximately 70% of primary processed natural rubber is used to produce automobile tires (ANRPC, 2017).

Furthermore, farmers have made a huge investment in the initial period (5 years), switching to other crops in the middle of the economic life of their plantation would involve huge losses. Therefore, price forecasting of natural rubber is significant to help the farmers to decide upon their production by the expected prices. This results in the requirement for statistical techniques to provide accurate and timely price forecast by tak-

ing into account the information to the farmers, traders and policymakers so that they may make production, marketing and policy decisions well in advance.

There are a few studies that used Autoregressive Integrated Moving Average (ARIMA) model to forecast natural rubber prices. By using monthly data, Rani & Krishnan (2018) showed that the model ARIMA(4,1,4) was found to be the best model to predict of prices of natural rubber in India in 2017. The forecast result was suitable for explaining the fluctuation of natural rubber prices in 2017. The other study was conducted to forecast the prices of natural rubber of Thailand. It was found that the ARIMA(1,0,1) model was the most suitable that could be explained for variations of the natural rubber price of Thailand. (Cherdchoongam & Rungreunganun, 2016). The ARIMA forecasting model was developed to predict Malaysian natural rubber prices in 2014. An illustration for real-time forecasts for natural rubber prices in the Malaysians showed its ease of use. The forecast result of natural rubber prices was suitable (Khin & Thambiah, 2014).

The ARIMA forecasting model would not be really effective for seasonal time data. To develop a forecasting model consistent with the data series available seasonality, the ARIMA model is expanded by adding autoregressive and moving averages to the number of seasons as known as Seasonal Autoregressive Integrated Moving Average (SARIMA). By using the SARIMA$(1,1,1)(1,0,0)_{12}$ model, the study demonstrated the volatility of seasonal potato prices in Delhi and found that the model had the most reasonable forecast results. The forecast result of potato prices was suitable (Chandran & Pandey, 2007). Similarly, Adanacioglu & Yercan conducted a forecast of tomato prices in the Antalya city in Turkey. Their study showed that the SARIMA$(1,0,0)(1,1,1)_{12}$ model was the most appropriate for the monthly wholesale price series. The forecast result of tomato prices was roughly equal to the real ones (Adanacioglu & Yercan, 2012).

There is hardly any literature available on forecasting the prices of natural rubber by using Seasonal Autoregressive Integrated Moving Average (SARIMA) model. This study was designed to develop a Seasonal ARIMA temporal model using long-term historical prices in the world's natural rubber market. This model is selected because of the capability to correct the local trend in data, where the pattern in the previous period can be used to forecast the future. Thus, this model also supports the modeling of one perspective as a function of time. Due to the seasonal trend of time series used, the SARIMA is selected for the model development.

2. Material and Methods

The dataset for model development was collected from time series data of Ribbed Smoked Sheet No.3 (RSS3) prices on the Tokyo Commodity Exchange (TOCOM) for the period of January 2007 - September 2018 because it is Japan's largest commodity exchange and one of the largest markets in the world for the buying and selling of natural rubber. TOCOM has offered RSS3 rubber contracts for almost 66 years and it has the largest trading volumes for rubber futures. The RSS3 price on the TOCOM provides the reference price for natural rubber on the world market. The original dataset is plotted as presented in Figure 1.

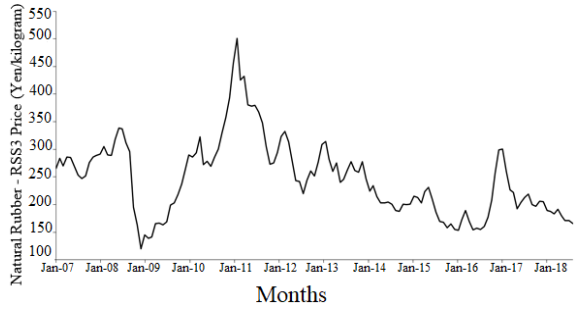


Figure 1. Natural rubber - RSS3 prices from January 2007 to September 2018 on TOCOM (TOCOM, 2018).

Since time series plot of the historical data exhibited the seasonal variations which present similar trend every year, then SARIMA was chosen as the appropriate approach to develop a model prediction.

One of time series models which is popular and mostly used is Box - Jenkins ARIMA model. Seasonal ARIMA is based on the theory of ARIMA. ARIMA models employ a combination of linear operators for the representation of a time series. The general class of ARIMA(p,d,q) comes from three parts: d is the level of differencing, p is the

autoregressive order, and q is the moving average order.

We have a time series, Z_t, t = 1, 2, . . ., n (here n is 129), the first, an autoregressive-moving average ARMA(p,q) model has the form:

$$\begin{aligned} Z_t &= \phi_1 Z_{t\text{-}1} + \phi_2 Z_{t\text{-}2} + ... + \phi_p Z_{t\text{-}p} \\ &= C + U_t - \theta_1 U_{t\text{-}1} - \theta_2 U_{t\text{-}2} - ... - \theta_q U_{t\text{-}q} \end{aligned}$$

Or

$$\begin{aligned} &Z_t - \phi_1 Z_{t\text{-}1} - \phi_2 Z_{t\text{-}2} - ... - \phi_p Z_{t\text{-}p} = \\ &C + U_t - \theta_1 U_{t\text{-}1} - \theta_2 U_{t\text{-}2} - ... - \theta_q U_{t\text{-}q} \end{aligned} \quad (1)$$

Where:

The constant is notated by C, while ϕ is an autoregressive operator, U is a random shock corresponding to time period t, and θ is a moving average operator.

If we introduce the backshift operator, B, where:

$$BZ_t = Z_{t\text{-}1}; B^2 Z_t = B(BZ_t) = Z_{t\text{-}2}$$

And so on (1) can be rewrited as:

$$\begin{aligned} &Z_t - \phi_1 BZ_t - \phi_2 B^2 Z_t - ... - \phi_p B^p Z_t = \\ &C + U_t - \theta_1 BU_t - \theta_2 B^2 U_t - ... - \theta_q B^q U_t \end{aligned} \quad (2)$$

Or

$$\begin{aligned} &(1 - \phi_1 B - \phi_1 B^2 - ... - \phi_1 B^p) Z_t = \\ &C + (1 - \theta_1 B - \theta_1 B^2 - ... - \theta_1 B^q) U_t \end{aligned} \quad (3)$$

Because the series is not stationary (i.e., has no fixed mean level), then the autoregressive portion of the ARMA(p,q) model must include a stationary inducing operator. For a non-seasonal series, this is most frequently accomplished through a differencing operator (or product of differencing operators) of the form (1-B). That is, instead of modeling the nonstationary series Zt, we model the series

$$(1 - B)Z_t = Z_t - Z_{t\text{-}1}$$

Physically this corresponds to modeling the change in the series rather than the series itself. Usually only a single differencing operator is required. On rare occasions in the modeling of nonseasonal series, the operator may need to be repeated, say d times. The model we then consider is an autoregressive-integrated moving average or ARIMA(p,d,q) model of the form

$$\begin{aligned} &(1 - \phi_1 B - \phi_1 B^2 - ... - \phi_1 B^p)(1 - B)^d Z_t = \\ &C + (1 - \theta_1 B - \theta_1 B^2 - ... - \theta_1 B^q) U_t \end{aligned} \quad (4)$$

Some situations were encountered in which a time series exhibits some periodic or seasonal pattern. For example, data recorded monthly may exhibit "similar" behavior from year to year; that is, a seasonality of period 12. Data recorded quarterly may have 4 as its seasonality, and data recorded hourly may have 24 as its periodicity. In such situations, seasonal ARIMA models need to be employed to account for any seasonal pattern present in the series.

Multiplicative seasonal ARIMA models are often described as SARIMA(p,d,q)(P,D,Q)$_s$ models, where s is the seasonality, and P, D and Q refer to the orders of the seasonal AR, seasonal differencing and seasonal MA parts of the model, and s is the length of the seasonal period (s = 12).

This multiplicative seasonal model can be expressed as:

$$\begin{aligned} &(1 - \phi_1 B - \phi_1 B^2 - ... - \phi_1 B^p)(1 - \phi_1 B^s - \\ &\phi_1 B^{2s} - ... - \phi_1 B^{Ps})(1 - B)^d (1 - B^s)^D Z_t \\ &= C + (1 - \theta_1 B - \theta_1 B^2 - ... - \theta_1 B^q)(1 - \\ &\Theta_1 B^s - \Theta_2 B^{2s} - ... - \Theta_1 B^{Qs}) U_t \end{aligned} \quad (5)$$

Where

ϕ is a seasonal autoregressive operator and Θ is a seasonal moving average operator.

A SARIMA $(p,d,q)(P,D,Q)_{12}$ model was constructed using monthly natural rubber price data from January 2007 to September 2018 and a forecast of natural rubber prices from October 2018 to September 2020, following the four steps below:

- Step 1: Identification of model
  This step focus on selection of the order of regular differencing (d), seasonal differencing (D), the non-seasonal order of Autoregressive (p), the seasonal order of Autoregressive (P), the non-seasonal order of Moving Average (q) and the seasonal order of Moving Average (Q). The number of order can be identified by observing the autocorrelations function (ACF) and partial autocorrelations function (PACF).

- Step 2: Estimation of parameters
  The historical data is used to estimate the parameters of the tentatively model in Step 1.
- Step 3: Diagnostic checking
  Diagnostic test is used to check the adequacy of the tentatively model.
- Step 4: Forecasting
  The final model in Step 3 is used to forecast the forecast values (Box et al., 2008).

3. Results and Discussion

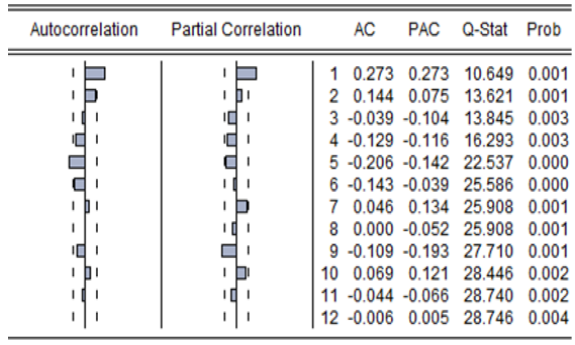
As it was earlier stated that development of SARIMA model for any variable involves four steps namely identification, estimation of parameters, diagnostic checking and forecasting. Each of these steps is explained for natural rubber prices.

3.1. SARIMA model identification

SARIMA model is estimated only after transforming the variable under forecasting into a stationary series. Stationary series is the one whose values vary over time only around a constant mean and a constant variance. A popular formal method of determining stationarity is the Augmented Dickey Fuller (ADF) test. The estimates of necessary parameters and related statistics for the time series of natural rubber prices without differencing and after first differencing are presented in Table 1.

The analysis exposed that the hypothesis of random walk that underlying process of generating the time series is nonstationary cannot be rejected without differencing, as the ADF test statistics is less than the critical value at 1% and 5% level. The ADF tests for the differenced time series of natural rubber prices revealed that the series were stationary after first difference.

The ACF and PACF values of the series from which the seasonal differences are taken are presented in Figure 2. The seasonal spikes at ACF and PACF after 1 lag are observed as being cut off after taking the seasonal difference of the series. This suggests that the seasonal order of Autoregressive equals 1 (P = 1), and the seasonal order of Moving Average equals 1 (Q = 1). In addition, the discontinuation of ACF and PACF values after 2 lags indicates that the non-seasonal



	AC	PAC	Q-Stat	Prob
1	0.273	0.273	10.649	0.001
2	0.144	0.075	13.621	0.001
3	-0.039	-0.104	13.845	0.003
4	-0.129	-0.116	16.293	0.003
5	-0.206	-0.142	22.537	0.000
6	-0.143	-0.039	25.586	0.000
7	0.046	0.134	25.908	0.001
8	0.000	-0.052	25.908	0.001
9	-0.109	-0.193	27.710	0.001
10	0.069	0.121	28.446	0.002
11	-0.044	-0.066	28.740	0.002
12	-0.006	0.005	28.746	0.004

Figure 2. ACF and PACF of natural rubber - RSS3 price at the first difference.

order of Autoregressive and the non-seasonal order of Moving Average can not be greater than 2. Therefore, this stuty choose the non-seasonal order of Autoregressive equals 2 (p = 2) and the non-seasonal order of Moving Average equals 2 (q = 2).

So, the tentative specifications were
$SARIMA(1,1,1)(1,1,1)_{12}$,
$SARIMA(1,1,2)(1,1,1)_{12}$,
$SARIMA(2,1,1)(1,1,1)_{12}$,
and $SARIMA\ (2,1,2)(1,1,1)_{12}$.

In addition, the goodness-of-fit statistics employed were the Adjusted R-squared (R_a^2), Akaike information criterion (AIC), Schwarz information criterion (SIC) and Standard error (SE). R_a^2 must be larger as better, while AIC, SIC and SE must be lower as better.

Criteria were given in Table 2 suggest that $SARIMA(2,1,2)(1,1,1)_{12}$ model as the most suitable model for forecasting. This model was selected with the lowest AIC, SIC and SE values and the largest R_a^2 value.

3.2. Estimate the parameters of the tentatively model

Parameters of the model can be estimated by using the result of regression $SARIMA(2,1,2)(1,1,1)_{12}$ model.

The Table 3 shows that the *P-values* associated with the variable coefficients are 0.000. These indicate that all the coefficients of variables in this equation is statistically significant at the level of 1% significance. There are three negative parameters such as AR(2), MA(1) and SAR(1). The positive parameters are AR(1), MA(2) and SMA(1). The particular parameters of the model were gen-

Table 1. ADF tests of natural rubber prices at levels

ADF test statistics	Critical value at 1%	Critical value at 5%	Probability	Level of Integration
-2.835	-3.478	-2.883	0.056	I(0)
-8.8675	-3.477	-2.882	0.000	I(1)

Table 2. Three model selection criteria

Models	R_a^2 (%)	AIC	SIC	SE
SARIMA(1,1,1)(1,1,1)$_{12}$	30,31	8.89	9.01	20.19
SARIMA(1,1,2)(1,1,1)$_{12}$	30,62	8.90	9.03	20.23
SARIMA(2,1,1)(1,1,1)$_{12}$	30,65	8.91	9.03	20.31
SARIMA(2,1,2)(1,1,1)$_{12}$	34,95	8.85	9.01	19.75

erated as the following results: AR(1) = 0.969; AR(2) = -0.831; MA(1) = -0.764; MA(2) = 0.834; SAR(1) = -0.591; SMA(1) = 0.946 and Constant = -1.253.

3.3. Diagnostic checking

ACF and PACF of residuals were given in Figure 3 indicated that the autocorrelation values less than to |0.2|. It implies that the residuals of the respective time series are white noise. (i.e. there is no autocorrelation).

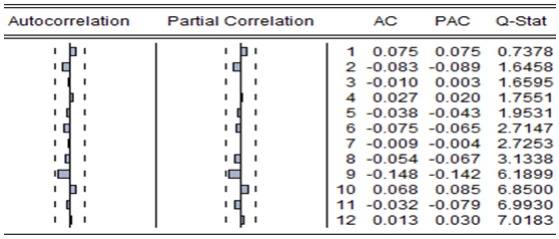


Figure 3. ACF and PACF of residuals of the selected SARIMA(1,2,1)(1,1,1)$_{12}$ model.

In Table 4, the Breusch-Godfrey Prob. Chi-square statistic is 0.525. This value is greater than 1% presented for the best selected model. It also implies that the residuals of the respective time series are white noise, implying that the model fitness is acceptable.

Moreover, Durbin-Watson statistic of the SARIMA(2,1,2)(1,1,1) equals 1.85 also showed no autocorrelation.

Otherwise, in Box-Jenkins models, the random error component plays a dominant role in determining the structure of the model. The result of heteroskedasticity test (Autoregressive Conditional Heteroscedasticity - ARCH test) with Prob. Chi-Square statistic is 0.374. This value is greater than 1%, demonstrated that there is no heteroscedasticity. This result was showed in Table 5.

Based on the results of the analysis, the SARIMA(2,1,2)(1,1,1)$_{12}$ model was selected as the best-fit model for forecasting, since it provides a reasonable fit to the highly seasonal time series data.

3.4. Forecasting

The final SARIMA(2,1,2)(1,1,1)$_{12}$ model was used to forecast the prices of world natural rubber during October 2018 - December 2020. This model was the most suitable for explaining the fluctuation of natural rubber prices on the world market prices. The result of forecasting was presented in Table 6.

The result of forecast shows that the world natural rubber prices tend to increase slightly from 173.63 yen per kilogram in October 2018 to 219.31 yen per kilogram in July 2019, and have decreasing trend to 167.45 yen per kilogram in May 2020. After that there is a slight increase and reaches 171 yen per kilogram in December 2020. Therefore, the world natural rubber price remain stable about 170 yen per kilogram from October 2018 to December 2020.

3.5. Error measures

The accuracy of the forecasting can be evaluated using error measures. It is achieved by comparing the original data and the forecast values. In this paper, Mean Absolute Percentage Error (MAPE) and Root Mean Square Error (RMSE) were used as the error measures.

Table 3. Estimated regression results of SARIMA(2,1,2)(1,1,1)$_{12}$ model

Variables	Coefficients	Standard Error	t Stat	*P-value*
C	-1.253	2.461	-0.509	0.611
AR(1)	0.969***	0.089	10.808	0.000
AR(2)	-0.831***	0.082	-10.092	0.000
SAR(1)	-0.591***	0.064	-9.117	0.000
MA(1)	-0.764***	0.087	-8.782	0.000
MA(2)	0.834***	0.078	10.665	0.000
SMA(1)	0.946***	0.013	71.209	0.000

***Corresponds to the significance level statistics, 1%.

Table 4. Breusch-Godfrey Serial Correlation LM Test

F-statistic	0.868362	Prob. F(12,109)	0.581
Obs*R-squared	11.04076	Prob. Chi-Square(12)	0.525

The result showed MAPE and RMSE values for the selected model were 6.68% and 21.43 respectively. Thus, the empirical result indicated that the model was able to accurately represent the natural rubber prices historical dataset.

The trend of the predicted natural rubber prices on the world market during October 2018 - December 2020 was showed in Figure 4.

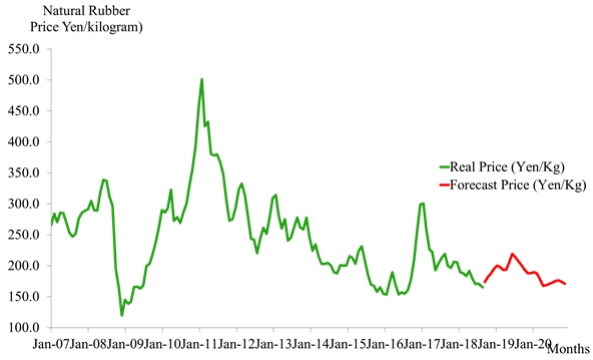


Figure 4. The forecasting results of natural rubber prices in the world market during October 2018 - December 2020 (Yen/kg).

This information is necessary and useful for the natural rubber producers and consumers as well as traders and planners for new investment decisions in the natural rubber world market. Especially, the forecasting result may be useful for the farmers because globalization and market integration, there is a huge price fluctuation where farmers can not decide upon their farming practices. Time series forecasts are generated by models based on changes over time in previously observed values or historical datasets. The SARIMA forecasting model can serve as a useful tool for the farmers to decide upon their production. However, it should be up-dated from time to time with incorporation of current data.

Beside the prices of natural rubber in the past periods, there are other factors having potential influence on natural rubber prices on the world market. The factors comprise of world economic growth, crude oil price, exchange rate (yen/$), world natural rubber consumption and world synthetic rubber consumption. . . . Oil price change by all means affects nearly everything in the world. Its byproduct is also used as one of the main content to produce synthetic rubber. As synthetic rubber is a perfect substitute goods of natural rubber so when oil price increases, synthetic rubber price increases, and then natural bubber price also increases. Conversely, when oil price decreases lead to natural rubber price also reduces. In addition, the devalued yen made rubber futures at TOCOM economically more attractive to the overseas investors so natural rubber price also increases and on the contrary. Therefore, to make the best decision, decision makers should be combine this forecasting result with these factors.

4. Conclusion

In this paper, an efficient technique was presented to accurately predict time series data of world natural rubber prices. Multiplicative seasonal SARIMA models provide an economical way to model time series whose have seasonal tendencies. Based on the results of the analy-

Table 5. Heteroskedasticity test - ARCH

F-statistic	1.077348	Prob. F(12,103)	0.387
Obs*R-squared	12.93619	Prob. Chi-Square(12)	0.374

Table 6. The forecasting results of natural rubber prices in the world market (Yen/kg)

Months	Prediction		
	2018	2019	2020
January	-	195.188	187.980
Frebruary	-	200.199	189.484
March	-	197.971	187.716
April	-	193.310	177.972
May	-	193.364	167.449
June	-	205.852	168.569
July	-	219.311	170.756
August	-	213.621	172.769
September	-	206.939	175.484
October	173.628	200.890	176.425
November	181.885	193.306	174.178
December	187.354	188.101	171.096

sis, SARIMA$(2,1,2)(1,1,1)_{12}$ model was the most suitable model that can be able to represent the historical data. The model attained 0.000 for P value; 8.86 for Akaike Information Criterion; 9.01 for Schwarz Information Criterion; 6.68% for Mean Absolute Percentage Error and 21.43 for Root Mean Square Error. This model can be used to forecast the prices of natural rubber on the world market. The world natural rubber prices have a light increasing trend during the October 2018 to July 2019 and reach 219.31 yen/kg and have decreasing trend to 171.09 yen/kg after that. Forecasting the future prices of natural rubber through the most accurate time series model can help the governments of the world's important natural rubber producing countries as well as consumers and traders to perform better strategic planning and also to help them in maximizing revenue and minimizing the loss.

Acknowledgments

The author would like to express their sincere gratitude to the Tokyo Commodity Exchange for providing time series data of natural rubber prices.

Conflict of interest statement

The author declares that there is no conflict of interest.

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Efficacy of organic acids as an alternative to antibiotic growth promoters in weaned pigs

Tung M. Che[1*], Nhan T. M. Nguyen[1], Hai T. Nguyen[1], Tai T. Le[2], & Alexandre Péron[3]

[1]Department of Animal Production, Nong Lam University, Ho Chi Minh City, Vietnam
[2]Animal Breeding Center, Long An, Vietnam
[3]Provimi South-East Asia, Singapore

ARTICLE INFO

Research Paper

Received: October 09, 2018
Revised: November 01,2018
Accepted: November 15, 2018

Keywords

Antibiotics
Diarrhea
Growth performance
Nursery pigs
Organic acids

***Corresponding author**

Che Minh Tung
Email: tung.cheminh@hcmuaf.edu.vn

ABSTRACT

The objective of the experiment was to compare effects of dietary supplementation of organic acids (ProHacid Advance, PRO) and antibiotics on growth performance, diarrhea incidence, frequency of antibiotic treatment, and *E. coli* shedding in weaned pigs. A total of 224 crossbred weaned pigs [(Yorkshire x Landrace) x Duroc; 29 days old)] were allotted to 1 of 4 treatments with 7 replicate pens of 8 pigs each according to sex, litter origin and weight in an RCBD. The 4 dietary treatments included (1) basal diet + 50 mg/kg neomycin and 10 mg/kg enramycin (positive control, PC), (2) basal diet without antibiotics (negative control, NC), (3) As 2 + 0.25% PRO, and (4) As 2 + 0.5% PRO. Over a 4-week study, the results showed that there were no differences in the average daily gain and average daily feed intake of pigs among the 4 treatments ($P = 0.328$). However, pigs fed the PC diet (1.642) and 0.25% PRO-supplemented diet (1.641) had a lower feed to gain ratio ($P < 0.05$) than those fed the NC diet (1.808). The diarrhea incidence of pigs fed the 0.25% PRO-supplemented diet (7.36%) was lower ($P < 0.01$) than that of pigs fed the PC diet (11.61%), NC diet (16.70%), and 0.5% PRO-supplemented diet (10.08%). The frequency of antibiotic treatment of pigs consuming the 0.25% PRO-supplemented diet (4.67%) was lower ($P < 0.01$) than that of pigs consuming the PC diet (7.33%), NC diet (9.53%) and 0.5% PRO-supplemented diet (7.65%). No differences were found in the number of fecal *E. coli* among the 4 treatments. In brief, 0.25% PRO added to a nursery pig diet would be considered a potential alternative to the use of antibiotics in feed, but further research on this aspect is needed.

Cited as: Che, T. M., Nguyen, N. T. M., Nguyen, H. T, Le, T. T., & Péron, A. (2018). Efficacy of organic acids as an alternative to antibiotic growth promoters in weaned pigs. *The Journal of Agriculture and Development* 17(6), 8-14.

1. Introduction

Weaning is a critical time during which piglets commonly have low feed intake, retarded growth and diarrhea. Organic acids added to diets may provide benefits through maintaining a low gastric pH that potentially enhance nutrient digestion and reduce pathogen survival. In recent decades, acidifiers have been reported as potential alternatives, among other feed additives, to antibiotics in pig diets (Partanen & Mroz, 1999; Kim et al., 2005; Kil et al., 2011). Much of this interest arises from increased public awareness and objection to the use of antibiotics as growth promoters in animal diets. Some previous studies have shown favorable effects with dietary or-

ganic acids in improving growth performance and feed efficiency, but others have found no effects or negative responses (Partanen & Mroz, 1999; Kil et al., 2011; Che et al., 2012). Hence, an evaluation of the efficacy of acid products under typical Vietnam conditions is needed to guide the use of an acid-related product. This would provide pig producers with more tools to cope with the recent in-feed antibiotic ban in Vietnam. The objective of the experiment was to evaluate effects of organic acids on growth performance, diarrhea incidence, frequency of antibiotic treatment, and *E. coli* shedding of weaned pigs from 29 to 57 days of age.

2. Materials and Methods

2.1. Experimental design, animals, and housing

Two hundred and twenty-four crossbred weaned pigs [(Yorkshire x Landrace) x Duroc; 29 days old; 8.44 ± 1.01 kg of BW] were randomly allotted to 4 treatments in a randomized complete block design. Pigs were blocked by their initial body weight within sex. Ancestry was equally distributed across treatments. The 4 experimental treatments included (A) basal diet with antibiotics (50 mg/kg neomycin and 10 mg/kg enramycin, positive control, PC), (B) basal diet without antibiotics (negative control, NC), (C) basal diet without antibiotics + 0.25% ProHacid Advance (PRO), and (D) basal diet without antibiotics + 0.5% PRO. Each pen within a block had the same number of gilts and barrows. There were 8 pigs/pen and 7 replicate pens/treatment. Pigs were housed in an environmentally controlled building. Each pen measured 2.0 m x 2.5 m in size with slatted floor and had one nipple waterer.

2.2. Experimental diets and animal feeding

The basal diet was formulated to meet or exceed the nutritional requirements of pigs during the experimental period (NRC, 1998). The experimental diets were obtained by adding antibiotics or PRO on top of the basal diet. ProHacid Advance is a blend of organic acids and their salts, consisting of citric acid, fumaric acid, benzoic acid, calcium formate, calcium lactate, and potassium sorbate. It was provided by Provimi Vietnam. Neomycin and enramycin were included in diets at levels of 50 ppm and 10 ppm, respectively. The ingredient composition of the basal diet is presented in Table 1. Pigs were fed a one-phase feeding program (29-57 d old). Diets were in mash form. Pigs had free access to feed and water throughout the experiment.

Table 1. Ingredient and nutrient composition of the basal diet (as-fed basis)

Ingredients	Percentage
Corn, ground	56.15
Soybean meal, 46%	34.20
Soybean oil	4.50
MCP (15% Ca, 23% P)	1.40
Limestone, 38%	1.50
Salt	0.30
Mineral premix[1]	0.50
Vitamin premix[2]	0.50
Antioxidant	0.10
Zinc oxide	0.33
Phytase	0.01
L-Lys, 78.8%	0.38
DL-Met, 99%	0.08
L-Thr, 98.5%	0.05
Nutrient analysis	
ME, kcal/kg[3]	3400
DM, %	89.55
Crude Protein, %	20.98
Ether extract, %	6.75
Crude fiber, %	2.29
Ash, %	6.28
Ca, %	0.90
Total P, %	0.70

[1]Provided per kg of diet: Fe (100 ppm), Cu (40 ppm), Zn (80 ppm), Mn (20 ppm), Se (0.3 ppm), I (0.3 ppm).
[2]Provided per kg of diet: vitamin A (6000 IU), vitamin D3 (600 IU), vitamin E (60 IU), vitamin K (5), vitamin B2 (9 mg), vitamin B5 (27 mg), vitamin B12 (0.05 mg), niacin (50 mg).
[3]Calculated.

2.3. Feed sample analyses

A feed sample was ground to pass through a 1-mm screen before analysis and analyzed according to the standard methods. Diet samples were analyzed for DM (EC 152/2009), CP (AOAC 2001.11), crude fat (TCVN 4331:2001), crude fiber (AOCS Ba-6a-05), ash (EC 152/2009), Ca (AAS08, reference 73/46/EEC), and P (AOAC 965.17). The nutrient analyses were performed by Upscience Vietnam in Binh Duong province, Vietnam. The analyzed nutrient composition of the basal diet is presented in Table 1.

2.4. Measurement of pig performance, diarrhea incidence, and antibiotic treatment

The initial BW of pigs in each pen was recorded at the commencement of the experiment. The subsequent pen weights and feed disappearance measurements were determined at 57 days of age. The ADG, ADFI, and F:G were calculated on a per-pen basis. Pigs with diarrhea were recorded daily by visual observations with a score from 1 to 5 (1 = normal; 2 = moist feces; 3 = mild diarrhea; 4 = severe diarrhea; 5 = watery diarrhea). Incidence of diarrhea was calculated by counting pig days with diarrhea score of 3 or greater (pasty and liquid feces) during the entire experimental period. The number of antibiotic treatments per pen was also recorded daily.

2.5. Bacterial analysis

Fecal samples were directly collected by a fecal loop from one identified pig per pen at 29 days of age for enumeration of *E. coli.*, and subsequent samples were taken from the same pigs in each pen at 43 and 57 days of age. Fecal samples were placed on ice for transportation to the lab, where analysis was immediately done. A 10-g sample of feces was added to 90 mL of buffered peptone broth, homogenized, and then serially diluted by ten-fold. From all five consecutive dilutions (from 10^{-1} to 10^{-5}), 1 mL was inoculated into each 3 lauryl sulfate broth (LSB) media and incubated at 37^0C for 24 to 48 h. Gas positive tubes of 3 consecutive dilutions were transferred into *E. coli* medium (EC) and incubated at 44.5^0C for 24 h. The EC positive tubes were used to inoculate eosin methylene blue (EMB) agar plates and incubated for 24 h at 37^0C for *E. coli* isolation. Finally, presumptive *E. coli* colonies from the EMB agar plates were confirmed by the IMViC test. Positive tubes of three consecutive tubes were used for standard MPN calculation. The number of *E. coli* is expressed as $\log_{10}$ MPN per one gram of fecal sample.

2.6. Statistical Analysis

Data were analyzed as an RCBD using the GLM procedure (SAS Inst. Inc., Cary, NC). The pen was considered the experimental unit for ADFI, BW, ADG, and FCR, whereas individual pig was considered the experimental unit for the other parameters. When a significant F value for treatment means was observed in analysis of variance, the treatment means were compared using Tukey's test. The incidence of diarrhea and frequency of medical treatments were compared by Chi-square test. Treatment effects were considered significant at $P < 0.05$.

3. Results

3.1. Growth performance

At the commencement of the experiment (29 days of age), there were no differences ($P = 0.471$) in the initial BW of pigs among the treatments (Table 2). At the end of the experiment (57 days of age), although pigs fed the diet containing 0.25% PRO (17.65 kg/pig) had greater BW than those fed the PC diet (17.48 kg/pig), NC diet (16.78 kg/pig), and 0.5% PRO-supplemented diet (17.40 kg/pig), these differences were not statistically significant ($P = 0.286$). There were no differences in ADFI and ADG of pigs among the 4 treatments ($P > 0.05$). However, there were significant differences in F:G ratios of pigs among the 4 treatments ($P = 0.021$). Particularly, pigs fed the PC diet (1.642) and 0.25% PRO-supplemented diet (1.641) had a lower F:G ratio ($P < 0.05$) than those fed the NC diet (1.808).

3.2. Diarrhea incidence and medical treatment

There were significant differences in the diarrhea incidence of pigs among the 4 treatments ($P < 0.001$; Figure 1). Pigs fed the 0.25% PRO-supplemented diet (7.30%) had a lower rate of diarrhea ($P < 0.01$) than those fed the PC diet (11.61%), NC diet (16.70%) and 0.5% PRO-supplemented diet (10.08%). The diarrhea incidence of pigs fed the NC diet was greater ($P < 0.001$) than that of pigs fed the PC diet and 0.5% PRO-supplemented diet. No difference ($P = 0.168$) in the incidence of diarrhea was found between the PC diet and 0.5% PRO-supplemented diet.

There were significant differences in the frequency of antibiotic treatments among the 4 treatments ($P < 0.001$; Figure 2). Pigs fed the 0.25% PRO-supplemented diet (4.67%) had a lower frequency of antibiotic treatments ($P < 0.01$) than those fed the PC diet (7.33%), NC diet (9.53%) and 0.5% PRO-supplemented diet (7.65%). The frequency of antibiotic treatments

Table 2. Effects of dietary supplementation of organic acids on growth performance of nursery pigs

Item	Dietary treatments[1]				SEM	*P*
	Positive control	Negative control	0.25% ProHacid Advance	0.5% ProHacid Advance		
Initial BW, kg/pig	8.45	8.45	8.44	8.41	0.05	0.909
Final BW, kg/pig	17.48	16.78	17.65	17.40	0.33	0.286
ADFI, g	530.3	524.2	533.1	528.6	18.46	0.989
ADG, g	322.6	293.5	325.3	321.2	12.91	0.297
F:G, kg/kg	1.642^{b}	1.808^{a}	1.641^{b}	1.657^{ab}	0.040	0.021

[1]7 pens/treatment and 8 pigs/pen.
[a-b]Means with different superscript letters within a row differ ($P < 0.05$).

Table 3. Effects of dietary supplementation of organic acids on fecal shedding of *E. coli* (Log_{10} MPN/g)

Age, d	Dietary treatments[1]				SEM	*P*
	Positive control	Negative control	0.25% ProHacid Advance	0.5% ProHacid Advance		
29	6.01	5.82	6.06	6.42	0.391	0.744
43	5.60	5.52	5.77	4.59	0.424	0.230
57	5.44	5.55	5.76	6.55	0.745	0.050

[1]n = 7 (7 pigs/treatment).

of pigs fed the NC diet was greater ($P = 0.027$) than that of pigs fed the PC diet but was not different ($P = 0.061$) from that of pigs fed the 0.5% PRO-supplemented diet. No difference ($P = 0.735$) in the frequency of antibiotic treatments was found between the PC diet and 0.5% PRO-supplemented diet.

3.3. Fecal *E. coli* concentration

No effects of dietary treatments on *E. coli* shedding were found at 29 days of age ($P = 0.744$; Table 3). At 43 days of age, there were no differences in the *E. coli* counts among the 4 treatments ($P = 0.230$). At the end of the experiment (57 d old), the *E. coli* counts in pigs receiving the 0.5% PRO-supplemented diet (6.55 $\log_{10}$ MPN/g) tended ($P = 0.050$) to be greater than those in pigs fed the PC diet (5.44 $\log_{10}$ MPN/g), NC diet (5.55 $\log_{10}$ MPN/g), and 0.25% PRO-supplemented diet (5.76 $\log_{10}$ MPN/g).

4. Discussion

For decades, antibiotics have been used in food animal production for disease prevention and growth promotion. However, in recent years, the use of antibiotics as growth promoters has declined due to an increasing concern about antimicrobial resistance in bacteria. Vietnam has recently banned the use of antibiotics as growth promoters in animal feeds, but some antibiotics such as neomycin and enramycin can be used at low doses. In the current study, organic acids added to the nursery diets had the same weight gain as those fed the PC diet and numerically increased the weight gain of pigs by over 9% as compared with the NC diet. Indeed, it was reported that effects of organic acids on growth rate of weaned pigs were inconsistent. Some researchers have shown positive effects with dietary supplementation of organic acids in improving growth rate (Bergstrom et al., 1996; Boling et al., 2000), but others have found nothing or negative responses (Radecki et al., 1988; Manzanilla et al., 2004; Che et al., 2012). The efficacy of organic acids on growth performance may be dependent on several factors such as complexity of diet, type of acid, inclusion level of acid, etc. For example, addition of 0.3% benzoic acid to a nursery diet did not clearly affect weight gain of pigs (Phan et al., 2014), but a diet supplemented with 0.5-1.0% benzoic acid improved growth rate of pigs (Kluge et al., 2006; Guggenbuhl et al., 2007).

Organic acids added to a diet have been shown to produce beneficial effects through reduced survival of pathogens and increased digestion of nutrients leading to better animal health and performance (Partanen, 2001; Lawlor et al., 2006; Kil et al., 2011). Especially, under stressful or dis-

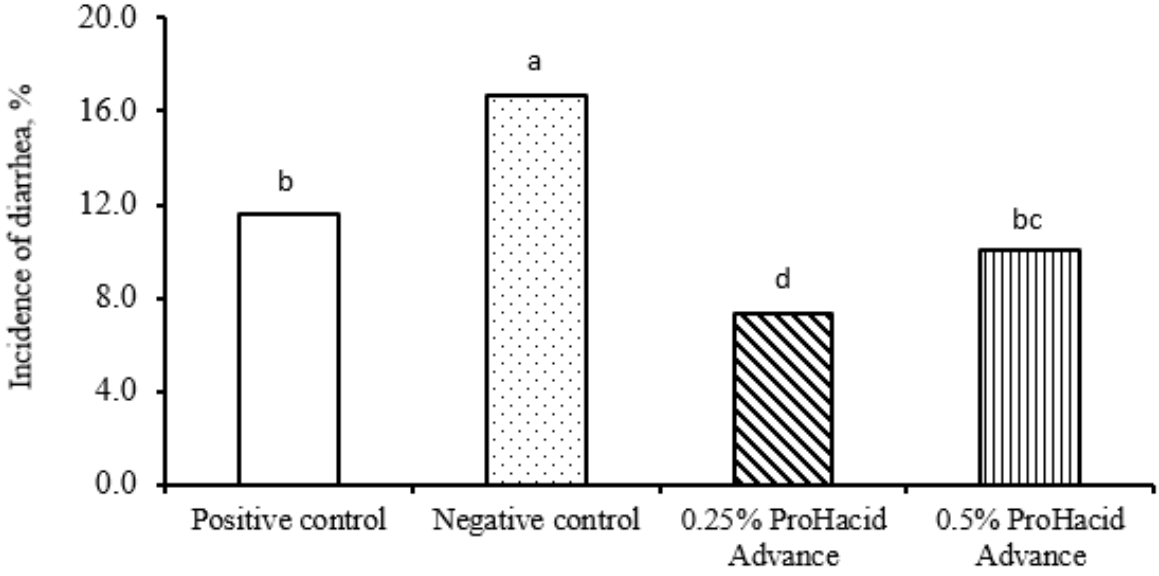

Figure 1. Effects of ProHacid Advance on the incidence of diarrhea during the experimental period. There were 56 pigs/treatment. Diarrhea incidence: Diarrhea x 100/pig days; Diarrhea: number of pig days with diarrhea; Pig days: number of pigs x the number of days of diarrhea observation. [a-d]Means with different superscript letters differ ($P < 0.05$).

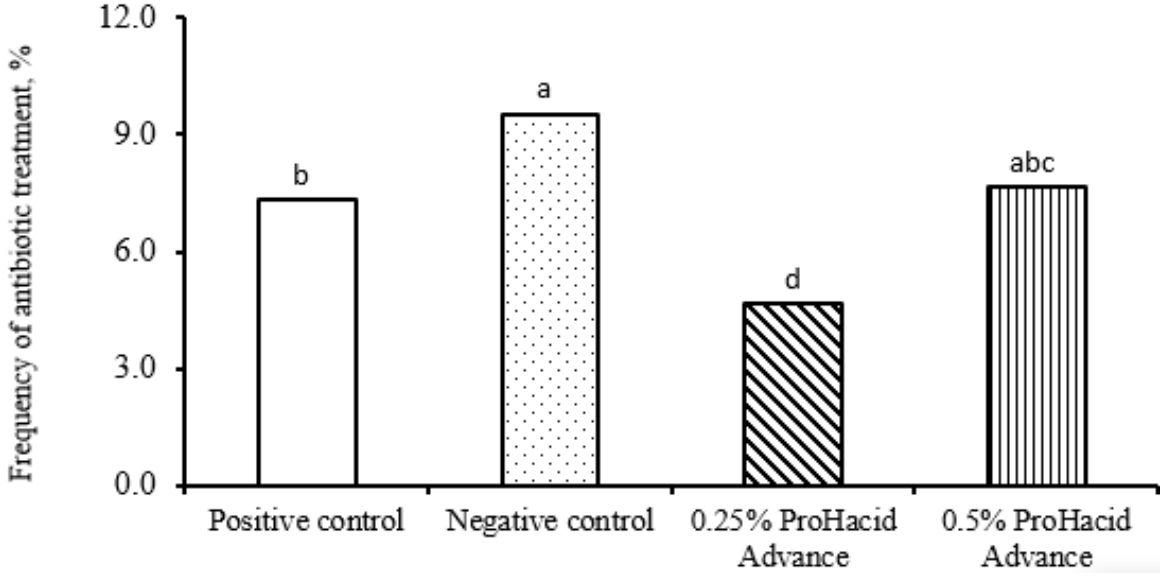

Figure 2. Effects of dietary supplementation of ProHacid Advance on the frequency of antibiotic treatment during the experimental period. There were 56 pigs/treatment. Frequency of antibiotic treatment: Medical treatment x 100/pig days; Medical treatment: number of pig days with treatment; Pig days: number of pigs x the number of days of medical treatment observation. [a-d]Means with different superscript letters differ ($P < 0.05$).

ease challenge conditions, organic acids may be a useful tool to reduce unfavorable impacts such as increased diarrhea and high mortality (Risley et al., 1993; Tsiloyiannis et al., 2001; Manzanilla et al., 2004). First, it should be noted that although there were no clear effects of organic acids on weight gain of pigs, 0.25% PRO added to the diet significantly improved the feed efficiency of pigs as compared with the NC diet (Table 2). This improvement is likely associated with the ability of organic acids in lowering gastrointestinal pH, thereby increasing nutrient digestibility (Ravindran & Kornegay, 1993; Partanen & Mroz, 1999; Partanen, 2001). In a meta-analysis of organic acids for pigs, Che & Quach (2011) reported that organic acids added to a nursery diet significantly improved the dry matter and crude protein digestibilities. In the current study, pigs fed the diet containing antibiotics (neomycin & enramycin) had the same FCR as those fed PRO-supplemented diets. In addition, feeding the 0.25% PRO-supplemented diet to pigs reduced the incidence of diarrhea compared to feeding the other diets. This improvement may be associated with the organic acids-enhanced feed efficiency which would help limit the substrates, especially protein for bacterial fermentation. In cecum and colon, it was reported that undigested proteins produced toxic compounds causing diarrhea in pigs (Makkink, 2001).

Dietary supplementation of PRO did not clearly influence the *E. coli* shedding of pigs (Table 3). These results agree with those of previous studies. Walsh et al. (2007) reported that pigs fed a blend of organic acids had the same *E. coli* shedding as those fed the control. According to Phan et al. (2014), 0.3% benzoic acid added to nursery diets did not affect the number of fecal *E. coli*. In the intestines, the balance of harmful and beneficial microbes is likely more important than the dominance of certain bacterial strain. This helps maintain a balanced microflora system leading to more healthy gut and less diarrhea. It has been shown that organic acids differently affect the microflora populations along the gastrointestinal tract, and they do not produce an environment that is favorable for potentially beneficial bacteria but harmful to coliforms and *E. coli* (Che & Quach, 2011). As shown in Table 3, pigs fed 0.5% PRO-supplemented diet had a numerically greater number of fecal *E. coli* than those fed the other diets at 56 days of age.

5. Conclusions

ProHacid Advance added to a nursery diet at an inclusion level of 0.25% improved the feed efficiency of pigs as compared with the negative control. There were no differences in growth rate and feed efficiency between pigs fed ProHacid Advance and antibiotics. The addition of 0.25% ProHacid Advance reduced the diarrhea incidence and frequency of medical treatment of pigs as compared with the positive and negative controls. Thus, ProHacid Advance would be considered a potential alternative to the use of antibiotics in nursery pig diets, but further research on this aspect is needed.

Conflicts of interest

The authors declare no conflicts of interest.

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Field assessment of the efficacy of M.B., LIBDV and Winterfield 2512 strain vaccines against infectious bursal disease in chickens

Ho M. Nguyen[1], Anh T. Quach[1*], Anh T. T. Le[2], & Hien T. Le[1]

[1]Faculty of Animal Science and Veterinary Medicine, Nong Lam University, Ho Chi Minh City, Vietnam
[2]Vetstar, Ho Chi Minh City, Vietnam

ARTICLE INFO

Research Paper

Received: October 12, 2018
Revised: November 09,2018
Accepted: November 28, 2018

Keywords

Break through MDA
Chicken
M.B. strain
Uniformity

***Corresponding author**

Quach Tuyet Anh
Email: anh.quachtuyet@hcmuaf.edu.vn

ABSTRACT

Live virus vaccines are very important parts of the prevention of Infectious Bursal Disease (IBD) in chickens. However, the successful IBD vaccination depends on IBD field pressure, vaccination technique, the immune status of the chicken, and especially IBDV strains used in the vaccines which are able to break through a higher level of maternal-derived antibodies (MDA). The objective of this field study was to compare the efficacy of a new vaccine based on M.B. strain to other commercial vaccines (LIBDV and winterfiled 2512) in terms of speed of antibody immune response and interference to Newcastle Disease (ND) vaccination. Six houses of broilers, each with 15,000 to 16,000 chickens, were divided into two groups: (1) vaccinated with M.B. strain (group A) and (2) vaccinated with LIBDV or 2512 strains (group B). Blood samples were collected prior to the 1st IBD vaccination, and at 21, 28 and 35 days of age for IBD and ND antibodies. Comparison of lesion scores and uniformity of the bursa of Fabricius (BF) at 28 and 35 days of age was carried out. Results showed that both groups had good immune responses, but group A showed significantly higher IBD antibody titers at 28 and 35 days of age. Antibody titers for ND and histopathological lesion scores of the BF were not significantly different between the 2 groups. The BF in group A was more uniform and had fewer lesions when compared with that in group B. In conclusion, the IBD vaccine with an M.B. strain can provide better immunological efficacy than LIBDV and 2512 strains.

Cited as: Nguyen, H. M., Quach, A. T., Le, A. T. T., & Le, H. T. (2018). Field assessment of the efficacy of M.B., LIBDV and Winterfield 2512 strain vaccines against infectious bursal disease in chickens. *The Journal of Agriculture and Development* 17(6), 15-23.

1. Introduction

For many years, Infectious Bursal Disease (IBD) has been a serious problem threatening the poultry industry (Berg, 2000; Alkie & Rautenschlein, 2016). In March 2018, a report from ILDEX Vietnam showed that 12/64 provinces (18.75%) in Viet Nam in 2017 had IBD disease outbreaks. Live vaccines have been proved to be a powerful tool in controlling Gumboro disease (Van den Berg et al., 2000; Eterradossi & Saif, 2008; Muller et al., 2012). Currently, There are 3 types of live IBD vaccines available for young chickens including live attenuated IBD vaccines, immune complex vaccines, and recombinant vaccines (Gardin et al., 2011; Muller et al., 2012). However, IBD live vaccine is still a good solution for high vvIBD challenge areas (Berg & Meulemans, 1991). This type of vaccine protects chickens as the vaccine viruses replicate at the bursa of fabricius and induce a strong immune reaction leading to high antibody titers, and the shedding of vaccine virus to the environment helps reduce the field virus pressure at farms (Gomes et al.,

2015). In addition, live IBD virus vaccine is very important for primary vaccination of the pullet. When stimulating memory cells, it acts as a good primer to inactivated IBD vaccination (Gardin et al., 2011). It is necessary to have high IBD antibody titers in the breeders that then pass to the offspring protecting them in the first 2-3 weeks of life (Eterradossi & Saif, 2008; Fantay et al., 2015). Before the maternal-derived antibodies (MDA) drop too low, vaccinating broilers is really the key to the continuation of the protection (Fantay et al., 2015; Jackwood, 2017). For that reason, live IBD vaccines should have the ability to break through high levels of MDA to provide as early protection as possible without being neutralized by the MDA (Fantay et al., 2015). The new IBD vaccine strain in Vietnam market is M.B., Israeli strain, isolated in 1989 by Abic scientists Drs Barbakov and Gutter. The name of M.B. was named by these scientists. It got the United States patent on Sep. 8, 1998 with the patent number 5804195. The M.B. strain belongs to genetic group 6 (Lazarus et al., 2008) and is able to break through MDA levels in broilers of 800 IDEXX ELISA while the normal intermediate IBD vaccines achieve this at a titer of 125 IDEXX ELISA and intermediate plus IBD vaccines at 500 IDEXX ELISA (De Wit, 2001).

The objective of this study was to evaluate the efficacy of the IBD M.B. vaccine strain and compare it with other commercial vaccines (LIBDV strain and 2512 strains) in commercial broiler chickens.

2. Materials and Methods

2.1. Experimental design

Six flocks (A1, A2, B1, B2, B3, and B4) (Figure 1) were kept in environmentally controlled broiler houses on 2 different commercially operated farms in Xuan Loc, Dong Nai. The flocks (A1, A2, B1, B2) were raised in farm 2 which was about 4 kilometers away from farm 1 (B3 and B4 flocks). There were 15,000 - 16,000 broiler chickens in each house. All chickens in farm 1 and 2 across the groups were subjected to the same management procedures and housing conditions.

A total of 95,000 day-old-chicks (Ross 308) used in this study were bought from the same breeding company, and hence they were assumed inherited the same MDA. This assumption was confirmed by testing the amount of MDA in chicks at 2 days old.

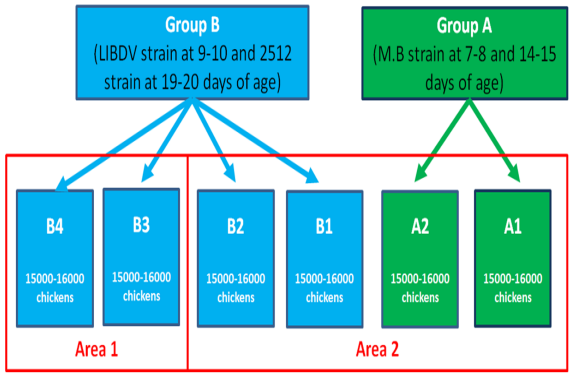

Figure 1. Design of experiments.

2.2. Vaccination schedule

There were two vaccination schedules which were different based on the Gumboro vaccination program (Table 1). All other vaccinations were exactly the same in both schedules. Schedule B was kept exactly as the vaccination program which was used in this farm for long time. It was considered as the standard vaccination program which was suitable for the condition and epidemiology of this farm. Schedule A was new set up based on the MDA levels at 2 days old. M.B. strain is able to break through MDA levels of 800 IDEXX ELISA, therefore, in schedule A, the 1st vaccination can be vaccinated as early as 7-8 days old. While LIBDV strain is able to break through MDA levels of less than 500 IDEXX ELISA, therefore, in schedule B, the 1st vaccination should be later (9-10 days old). The 2nd vaccination was 7-10 days after the 1st vaccination (Table 1).

2.3. Serology

Blood samples from twenty chickens per house were randomly collected immediately prior to the 1st IBD vaccination and subsequently at 21, 28 and 35 days of age for determination of IBD and ND antibodies. A commercial enzyme-linked immunosorbent assay kit (IDEXX, Maine, USA, Cat. number 99-09260) was used as described by the manufacturer for the detection of antibodies to IBD in chicken serum. The ND antibody titers were measured by the haemagglutination inhibition method according to Allan & Gough (1974)

Table 1. Vaccination schedule

Schedule A		Schedule B		Admission route
M.B. strain		LIBDV and 2512 trains		
Day old	Vaccine[1]	Day old	Vaccine[1]	
1	ND – IB	1	ND – IB	Spray
	ND kill		ND kill	SC
7 – 8	MB (1^{st})	9 – 10	LIBDV (1^{st})	DW
13	ND – IB	13 – 14	ND – IB	DW
14 – 15	MB (2^{nd})	19 – 20	2512 (2^{nd})	DW
27 – 28	ND – IB	27 – 28	ND – IB	DW

[1]SC: Subcutaneous; DW: Drinking water; ND: Newcastle disease; IB: Infectious bronchitis disease.

2.4. Bursa of Fabricius analysis

Five chickens per house unit were sacrificed at 28 and 35 days of age. Bursa index (BI) was calculated as bursa weight / body weight × 100 (Sellaoui et al., 2012). Bursa size vs. spleen size of the same chicken and lesions of BF were observed and compared among chickens of the same housing unit.

The BF samples were fixed in 10% formalin and stained with hematoxylin and eosin described by Fischer et al. (2008). Lesions were observed microscopically and were evaluated based on the scoring system from 0 to 5 described by Muskett et al. (1979).

2.5. Statistical analysis

Collected data were managed and simple calculation were performed in MS Excel 2010. Random effect models were used to detect any influence of factors: age, or vaccine on antibody levels or Bursa indices. These models were built in Stata 11 with farm factors as random variables.

3. Results

3.1. Maternally derived IBD antibodies

Right before the 1^{st} IBD vaccination was applied, 20 serum samples from birds that were randomly selected from each house were measured to determine their maternal ELISA antibody titers. The titers ranged from 100 to 2614 in group A1, from 271 to 2534 in group A2, from 175 to 2320 in group B1 and from 264 to 2673 in group B2. Although the day of the 1^{st} IBD vaccination was different among houses (Table 1), the maternal antibody levels at the day of the 1^{st} IBD vaccination in all houses in farm 2 were not significantly different ($P > 0.05$) (Table 2).

3.2. Induction of circulating IBD antibodies post-vaccination

At 28 days of age, circulating ELISA IBD antibodies ranged from 227 to 3190 in group A1 (mean titer: 1854, CV: 46%), from 140 to 4648 in group A2 (mean titer: 2431, CV: 47%), from 46 to 3182 in group B1 (mean titer 789, CV: 118%), from 0 to 4188 in group B2 (mean titer: 500, CV: 194%), from 0 to 2315 in group B3 (mean titer: 885, CV:106%) and from 972 to 2638 in group B4 (mean titer: 1787, CV: 34%). At 28 days of age, the induction of an active immune response in group A were significantly higher than in group B ($P < 0.001$) (Table 2). In addition, the antibody titers of the majority of samples in group A were above 1500. On the contrary, the antibody titers of the majority of samples in group B were below 1500 (Figure 2). The CV in group A was also much lower than that in group B (Table 2), which showed that group A was much more uniform than group B.

At 35 days of age, circulating ELISA IBD antibodies ranged from 682 to 4700 in group A1 (mean titer: 2741, CV: 36%), from 2014 to 3823 in group A2 (mean titer: 2851, CV: 20%), from 802 to 5890 in group B1 (mean titer: 2066, CV: 54%), from 33 to 5721 in group B2 (mean titer: 3127, CV: 51%), from 1346 to 4999 in group B3 (mean titer: 3174, CV: 32%) and from 1027 to 3666 in group B4 (mean titer: 2182, CV: 33%). The induction of an active immune response in group A was significantly higher than in group B ($P < 0.01$) (Table 2). Again, the CV in group A was still lower than that in group B (Table 2).

Table 2. Induction of circulating IBD antibodies

	Group A M.B. strain			Group B LIBDV + 2512 strains			
	Mean titer	CV (%)	N	Mean titer	CV (%)	N	*P*
IBD – before vac	1083.62	59.06	40	1158.2	49.37	40	0.583
IBD – 28 days old	2142.25	48.66	40	991.57	99.47	80	0.000
IBD – 35 days old	2795.77	28.51	40	2637.13	47.25	80	0.010

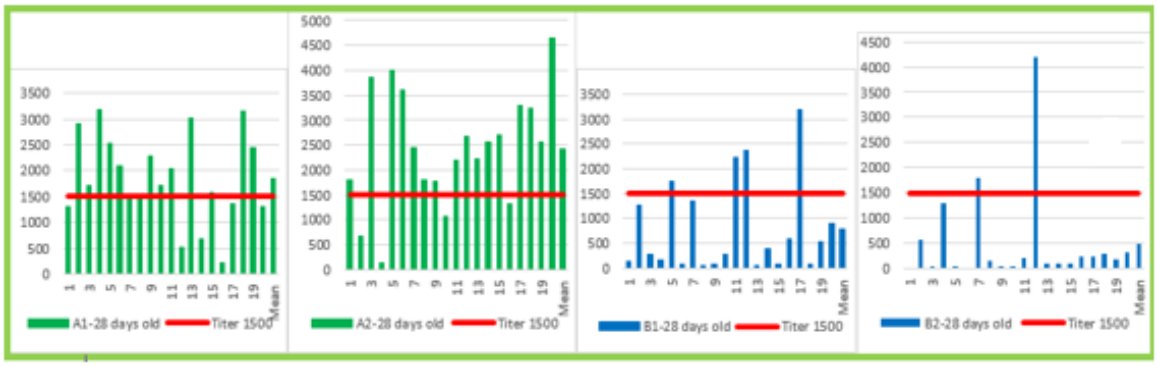

Figure 2. The serum IBD antibodies of chickens at 28 days old in farm 2.

Table 3. Induction of circulating ND antibodies

	Group A M.B. strain			Group B LIBDV + 2512 strains			
	Mean titer	CV (%)	N	Mean titer	CV (%)	N	*P*
ND – 21 days old	3.30	39.00	20	1.65	49.00	20	0.000
ND – 28 days old	3.35	49.00	40	3.45	57.39	80	0.777
ND – 35 days old	5.77	32.68	40	4.17	49.80	80	0.255

Table 4. Bursa index

	Group A			Group B			
	BI	CV (%)	N	BI	CV (%)	N	*P*
28	0.56	26.5	10	1.02	57.7	20	0.015
35	0.49	22.4	10	0.60	37.3	20	0.131

3.3. Induction of circulating ND antibodies post-vaccination

At 21 days of age, the induction of an active ND immune response in group A were significantly higher than that in group B ($P < 0.001$). However, at 28 and 35 days of age, the ND antibody titers of both groups were not significantly different ($P > 0.05$) (Table 3).

3.4. BI and the uniformity and lesions of BF

At 28 days of age, the BI of group A was significantly smaller than group B ($P < 0.05$). However, at 35 days of age, the BI of both groups were not significantly different ($P > 0.05$). Group A at both 28 and 35 days of age had fewer lesions (hemorrhages) than group B (Table 4). All data including CV (Table 4), comparing the size of bursa and spleen of the same chicken (Table 5) showed that the size of the bursa of group A was much more uniformity than group B's.

3.5. Histopathology studies

The score lesions of both groups at 28 and 35 days of age were not significantly different ($P > 0.05$) (Table 6).

Table 5. Detecting bursa lesions and comparing the size of bursa and spleen of the same chicken

	28 days old		35 days old	
	Group A (N=10)	Group B (N=20)	Group A (N=10)	Group B (N=20)
Hemorrhage inside (%)	10	20	0	15
Bigger than spleen (%)	0	15	0	5
Smaller or same size with spleen's = normal size (%)	100	75	100	95
Bursa atrophy* (%)	0	10	0	0

*if BI < 50% of BI average of that flock.

Table 6. Score lesions

Score lesions	Group A (N=10)	Group B (N=20)	*P*
28 days old	3.1	2.6	0.249
35 days old	1.8	2.2	0.205

4. Discussions

We compared two different IBD vaccination programs in maternal antibody positive broilers. Their efficacy by means of rapid and uniform IBD antibody immune response and interference to ND vaccination was investigated. Furthermore, the vaccines induced lesion development and effect on the size of the BF were compared.

Maternally derived IBD antibodies at the day of the 1st IBD vaccination in all housing units in farm 2 were checked and they were confirmed not significantly different. That mean both groups had the same starting point as the basis for comparing the increase of IBD antibodies later.

Young chickens are protected by maternal antibodies and then by active immunity which is induced by vaccination. There is a gap of immunity when maternal antibodies decrease to below protective levels and active immunity has not increased to the level of protection (Le Gros et al., 2009). To shorten this gap, a better IBD vaccine will be able to induce antibodies faster (Jackwood & Sommer, 1999; Van den Berg et al., 2000). Our study showed that the M.B. strain vaccine was able to induce faster and higher IBD antibody titers (Figure 2). If we choose the titer 1500 which is the titer of live IBD vaccines protecting against IBDV infection as the baseline (Bughio et al., 2017), at 28 days old, most of the chickens in group A had a titer above 1500 (72.5%), while most of the chickens in group B had a titer below 1500 (only 30% above 1500) (Figure 2 & 3). At 35 days of age, both groups had a good increase in titer and were protected (group A: 95%; group B: 85%).

Another vaccination strategy to control a disease is to use a vaccine to induce a uniform active immune response in all individual of the flock. In this way, the viruses in the field have no chance to attach, replicate and multiply at an extremely large number in any chickens. In addition, they have no chance to infect the other chickens in that flock. Therefore, a superior vaccine will produce better uniformity (CV is lower). The uniformity of both groups was improved from 28 days to 35 days of age. At both stages, the uniformity of group A was better than group B (group A: 48.66% decreased to 28.51%; group B: 99.47% decreased to 47.25%).

The IBD is characterized by immunosuppression and mortality in chickens of 3 to 6 weeks of age (Eterradossi & Saif, 2008; Sellaoui et al., 2012; Khenenou et al., 2017). Therefore, one of the common concerns for using live IBD vaccines is the cause of immunosuppression. At this young age, if chickens have immunosuppression, they will not be able to induce an immune response to other antigens such as ND (Allan et al., 1972; Van den Berg et al., 2000). In this study, both groups were using the same ND vaccine program. At 21days of age, group A had a statistically higher ND titers ($P < 0.001$) and more uniformity than group B (Table 3). Hence, the M.B. strain vaccine did not adversely affect immune response ability. It also may increase the health of the chickens, which enabled the chickens to have a better immune response to ND (Figure 4).

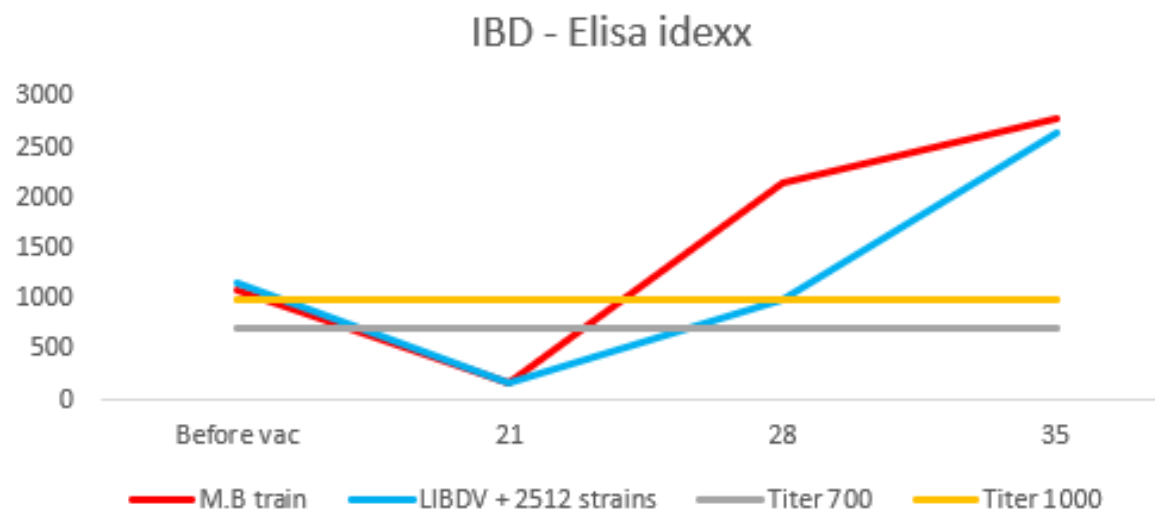

Figure 3. Induction of circulating IBD antibodies.

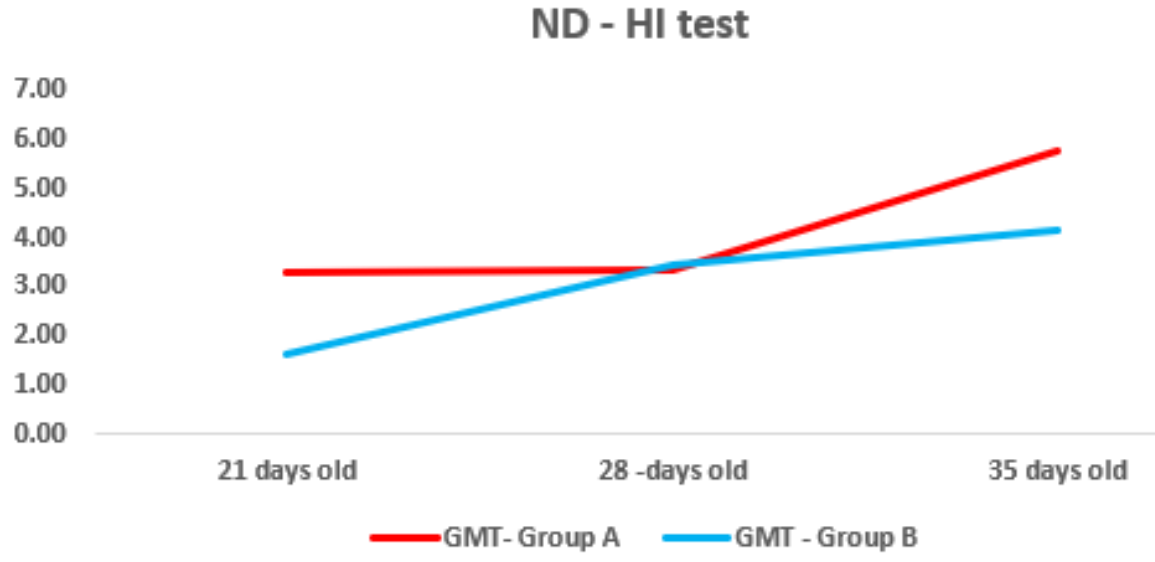

Figure 4. Induction of circulating ND antibodies.

The target organ of IBD viruses is the BF (Khenenou et al., 2017; Farhanah et al., 2018). IBD viruses need to travel through the chicken's body and only when they locate in the BF, will they be able to replicate and infect the chicken (Farhanah et al., 2018). During the gap of immunity, the chickens are at high risk for IBD infection due to the low level of both maternal antibody and humoral immunity (Lazarus et al., 2008; Le Gros et al., 2009). At this stage, a good IBD vaccine will protect chickens by rapidly locating the vaccine viruses in BF leaving no space left in the BF for field viruses to locate. Therefore, during the gap of immunity, the speed of location of vaccine viruses in BF is very important to protect chickens from IBD (Rautenshlein et al., 2005). In other words, a better IBD vaccine has a faster location of its vaccine viruses in the BF. During the development of the chicken, its BF is shrinking over time. It can also shrink due to the

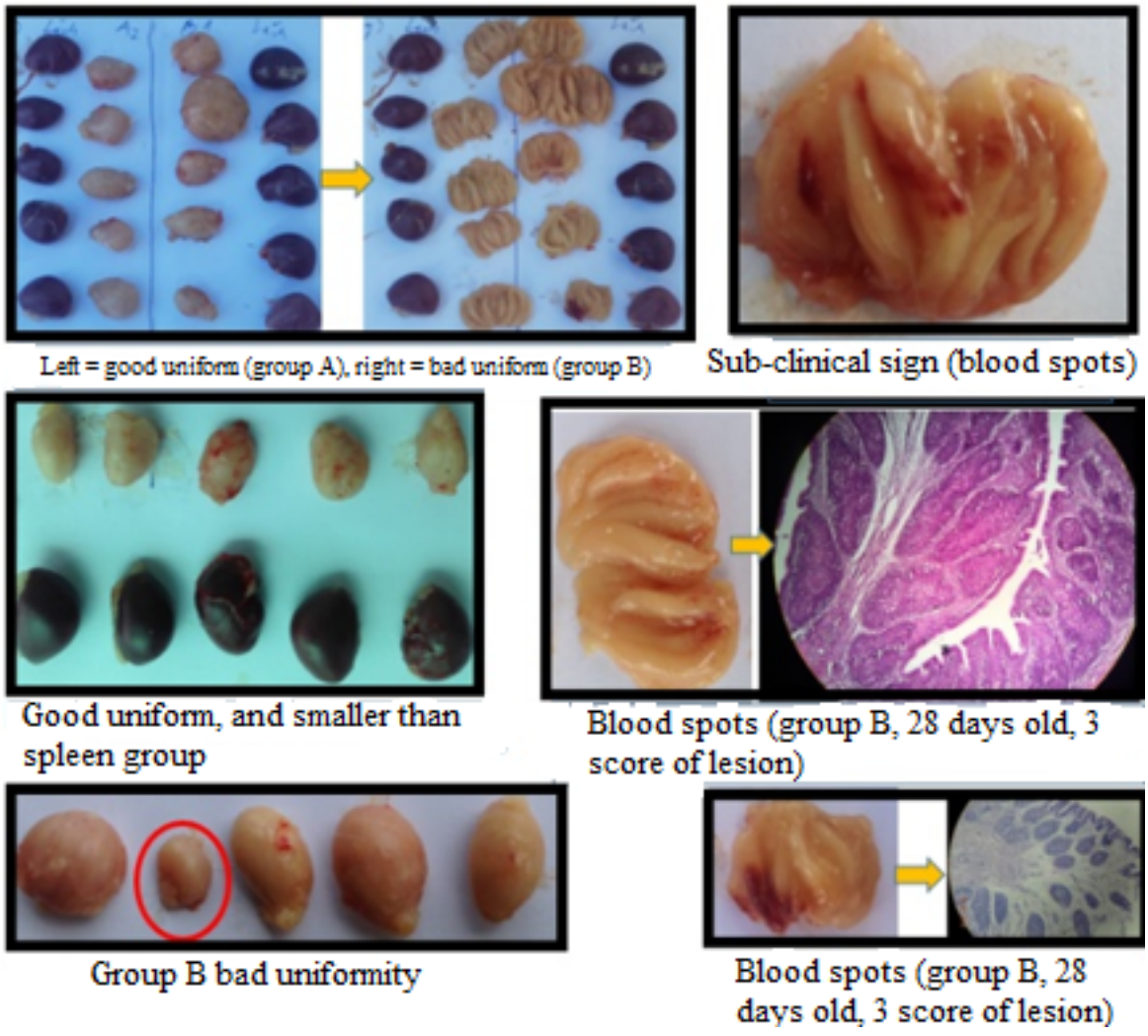

Figure 5. Sub clinical sign of BF and bursa size vs. spleen size.

location and replication of IBD viruses (Moraes et al., 2004). When the chickens are vaccinated with live IBD vaccine, these vaccine viruses are replicating in the bursa. As a result of this replication, the chicken's immune system is reacting with a humoral antibody response, which will protect the bird from the field strain. One of the signs of this replication is a change in the bursa size – the bursa is getting smaller (Moraes et al., 2004; Eterradossi & Saif, 2008). Therefore, the bursa size of a good IBD vaccine is smaller than normal and it has to be uniform, which means the vaccine provides good titer uniformity leading to uniformity in protection. When the bursa size is not uniform this means the protection is not uniform leading to poor protection and IBD can be subclinical or you may have a clinical outbreak. Our study found that at 28 days of age, the BI and CV of group A was smaller than group B, but at 35 days of age, the BI of both groups were similar. This indicated that the LIBDV and 2512 strains located and replicated in the BF later than the M.B. strain. They could only catch up with the M.B. strain at 35 days of age. However, in both stages (28 and 35 days), The M.B. strain was always more uniform than the other strains. Consistently, all data in Table 5 and Figure 5 indicated that the M.B. strain had better uniformity. One more time, it was confirmed that the IDB antibody titers of group A were much more uniform than group B (Figure 2).

5. Conclusions

Comparing different vaccine strains (M.B. strain vs. LIBDV and 2512 strain), the M.B. strain produced better protection for IBD in terms of shortening the immune gap, locating earlier in the BF, inducing higher and more uniform immune responses, and not causing immunosuppression.

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Detecting toxin genes of *Clostridium perfringens* isolated from diarrhea piglets using multiplex PCR

Dung H. M. Nguyen[1], Quynh T. X. Luong[1], Phuong T. Hoang[2], Duong T. T. Do[1], Thoai K. Tran[1], & Phat X. Dinh[1*]

[1]Department of Biotechnology, Nong Lam University, Ho Chi Minh City, Vietnam
[2]Vinh An Science and Technology Company Limited, Ho Chi Minh City, Vietnam

ARTICLE INFO

Research Paper

Received: September 15, 2018
Revised: October 29, 2018
Accepted: November 11, 2018

Keywords

Clostridium perfringens (*C. perfringens*)
Multiplex PCR (mPCR)
Piglet diarrhea
Piglets

***Corresponding author**

Dinh Xuan Phat
Email: dinhxuanphat@hcmuaf.edu.vn

ABSTRACT

Clostridium perfringens is currently classified into five types (A, B, C, D, E) based on the different toxins produced. Type A and C are known as the causative agent of enteritis and enterotoxemia in newborn and young piglets with severe intestinal lesions including edema, hemorrhage and necrosis. A multiplex PCR (mPCR) was developed in order to quickly and early determine the presence of genotypes of *C. perfringens* based on their genes of *cpa*, *cpb*, *cpb2* and *cpe* encoding alpha toxin, beta toxin, beta2 toxin and enterotoxin with predicted products of 324 bp, 196 bp, 107 bp and 257 bp respectively. The detection limit of the mPCR assay was 1×10^3 copies/reaction for each gene. Sequencing of mPCR products performed with clinical samples collected from *C. perfringens* suspected pigs showed that the mPCR test functioned specifically. In conclusion, the developed mPCR test successfully detected the presence of genes *cpa*, *cpb*, *cpb2* and *cpe* in the examined samples. Analysis of the bacteria isolated from field samples of diarrheal piglets collected in this study indicated that *C. perfringens* carrying gene *cpa* counted for 96.66% and 3.33% was identified as *C. perfringens* carrying genes *cpa* and *cpb* concurrently. Gene *cpe* was not found in this study, while gene *cpb2* was detected coincidently in 73.33% of the samples with *cpa* gene. The results indicate that the prevalence of these four toxin genes is *cpa*, *cpb2*, *cpb* and *cpe* in decending order.

Cited as: Nguyen, D. H. M., Luong, Q. T. X., Hoang, P. T., Do, D. T. T., Tran, T. K., & Dinh, P. X. (2018). Detecting toxin genes of *Clostridium perfringens* isolated from diarrhea piglets using multiplex PCR. *The Journal of Agriculture and Development* 17(6), 24-30.

1. Introduction

Diarrhea neonatal piglets is one of the most causes of economic losses in the swine industry. Among the common infectious agents, *Clostridium perfringens* (*C. perfringens*) plays a key role in enteric diseases not only in domestic animals but also in humans. *C. perfringens* is a Gram-positive, anaerobic, rod-shaped bacterium. It is known to produce various toxins including alpha (α), beta (β), epsilon (ε), and iota (ι). These toxins play important roles in the pathogenesis of the disease and are used to classify *C. perfringens* into five biotypes, designated A-E. These five types can be subdivided according to the production of two additional toxins: the enterotoxin (encoded by the *cpe* gene) and the β_2 toxin (encoded by *cpb2* gene) and described in Table 1. Type A and C strains cause diarrhea, dysentery and enterotoxaemia in pigs (Lebrun et al., 2010; Markey et al., 2013).

Conventional isolation on agar media usually

Table 1. *Clostridium perfringens* conventional toxinotypes (Leburn et al., 2010; Mcclane et al., 2006)

Genes	Toxin	Type A	Type B	Type C	Type D	Type E
cpa	α	X	X	X	X	X
cpb	β		X	X		
etx	ε		X		X	
iap/ibp	ι					X
cpe	Enterotoxin (X)					(X)
cpb2	β_2	(X)	(X)	(X)	(X)	(X)
Host		Pigs, humans, lambs, dogs, chickens	Lambs (under 3 weeks old), neonatal calves, foals	Piglets, lambs, calves, foals, adult sheep, chickens	Sheep (all ages, except neonates), (goats, calves)	Calves, rabbits

X Classic; (X) Potential.

takes longer time in routine diagnostic process. In this study, a multiplex PCR (mPCR) protocol was developed to determine the presence of toxin genes coding for alpha toxin (*cpa*), beta toxin (*cpb*), enterotoxin (*cpe*) and beta2 toxin (cpb2) of *C. perfringens* isolates.

2. Materials and Methods

2.1. Control and clinical samples

Positive control: DNA fragments of *cpb* gene (beta toxin) and *cpe* gene (enterotoxin) were synthesized by IDT (Integrated DNA Technologies - USA); and *C. perfringens* reference strains contained *cpa* gene (alpha toxin) and *cpb2* gene (β_2 toxin) were supplied by Sanphar Vietnam laboratory (belonging to Erber group, Austria). The presence of *cpa* and *cpb2* in this positive control sample was confirmed by sequencing. The resultant sequences of *cpa* and *cpb2* has 97-100% identity to the Genbank Id MH213493.1 and MG720638.1, respectively.

Negative control: viruses and bacteria potentially found in intestinal or fecal samples including *Salmonella* spp., *E. coli* (ATCC 25922), obtained from Sanphar's laboratory. *Salmonella* spp. was isolated from the field and identified by culture method as well as biochemical reaction; colonies of *Streptococcus suis* and a sample containing DNA of PCV2 virus confirmed by sequencing were obtained from the laboratory of Animal Molecular Pathogenesis and the Gene Technology laboratory respectively at the Department of Biotechnology, Nong Lam University, Ho Chi Minh City, Vietnam.

Clinical samples: Thirty isolates of *C. perfringens* were selected from different samples of anal swabs or feces taken from piglets (< 25 days of age) having the symptoms or lesions of: 1/ sudden death or dying shortly after bloody diarrhea; 2/ diarrhea; 3/ diarrhea with blood or necrotic patches of tissues;4/ Dead piglets usually have bulging stomach and/or intestines; 5/ Haemorrhagic and/or necrotic intestinal mucosa.

2.2. Isolation of total DNA

Clostridium perfringens isolates were collected from clinical samples (feces and swab samples from *C. perfringens* - suspected pigs with the symptoms described above) using blood agar medium (Cat#M975A, Himedia) in anaerobic condition and these colonies were determined as *C. perfringens* by morphology. After 24 to 48 hours of culture at 37^0C, these colonies appeared with round, smooth and glossy shapes, covered by a double hemolysis, complete hemolysis inner zone and partial hemolysis outer zone. Suspected colonies were further confirmed by biochemical reactions on gelatin medium to test sugar fermentation, nitrate to nitrite transfer and negative catalase test (Markey et al., 2013). Then, TPGY (Tryptone Peptone Glucose Yeast extract) (Cat#M969, Himedia) broth was used as an enrichment broth for obtaining a high rate of bacterial biomass. Thus, cells from 50 mL of overnight cultures of TPGY broth were harvested by cen-

trifugation at 13,000 rpm for 10 min at 4^0C. The cells were washed in 5 mL of 1X PBS pH 7.0 (Cat#10010023, Gibco), centrifuged and resuspended in 1 mL of the same buffer. Twenty microliters of the solution mixture with 300 μL TEN buffer (20mM Tris-HCl, 5mM EDTA, 140 mM NaCl, pH 8.0) and 30 μL lysozyme (10 ng/μL) (Cat#90082, Thermo Fisher Scientific). The solution was incubated at 37^0C for 15 min. After incubation of the mixture with 30 μL of SDS 20% solution at 37^0C for 15 min, the bacterial DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) solution (Cat#P1037, Sigma; Cat#25666, Merck). The tubes were kept inverted then still in 5 min and centrifugation at 13,000 rpm for 10 min. The upper aqueous layer was recovered for DNA precipitation with 900 μL ethanol 100% at -20^0C overnight. The DNA was pelleted, washed with 70% ethanol, allowed to dry and dissolved in 40 μL TE, pH 8.0. Extracted DNA was stored at -20^0C until being used. Two microliters were used in each mPCR reaction.

2.3. Primer design

Primer pairs CPA (encoding alpha toxin), and CPB (encoding beta toxin) were adopted from Meer and Songer (Meer et al., 1997). Besides, CPE (encoding enterotoxin) and CPB2 (encoding β_2 toxin) primers were designed by Primer3plus (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) using the sequence data of cpe gene and cpb2 *gene* obtained from NCBI (Table 2), and validated by NCBI BLAST, OligoAnalyzer 1.0.2. The annealing temperature and the size of the amplified product were adjusted to become appropriate to be combined with the two adopted primer pairs in a new mPCR. Primers were synthesized by IDT (Integrated DNA Technologies - USA).

2.4. Single PCR (sPCR) optimization

All primers were initially tested using gradient single PCRs according to the product specifications and protocols. The sPCR was performed in a 30 µl reaction mixture containing 1 μL DNA template, 0.33 μM each primer, 15 μL DreamTaq master mix 2X (Cat#K1081, Thermo Fisher Scientific), and nuclease-free water to adjust the final volume to 30 μL (Cat#R0582, Thermo Fisher Scientific). Nuclease-free water was also used as a negative control for all PCRs. The PCR was

Table 2. Primer sequences and estimated product sizes

Genes	Primers	Primer sequences (5' – 3')	Product size (bp)	Reference
cpa	CPA	F: GCTAATGTTACTGCCGTTGA R: CCTCTGATACATCGTGTAAG	324	Meer & Songer (1997)
cpb	CPB	F: GCGAATATGCTGAATCATCTA R: GCAGGAACATTAGTATATCTTC	196	Meer & Songer (1997)
cpe	CPE	F: ACAACTGCTGGTCCAAATGA R: GCAGCAGCTAAATCAAGGAT	257	Present study
cpb2	CPB2	F: TGCAACTTCAGGTTCAAGAGA R: CAGGGTTTTGACCATACACCA	107	Present study

carried out for pre-denaturation at 95^0C for 5 minutes, 35 cycles consisting of denaturation for 30 seconds at 95^0C, annealing at a temperature range for the gradient PCR: 53^0C, 55^0C, 57^0C, 59^0C, 61^0C for 30 seconds, extension for 70 seconds at 72^0C and a final extension of 72^0C for 10 minutes (model TC-512 GeneAmp PCR System; England). Ten microliters of amplified products were then analyzed by electrophoresis in a 2% (w/v) agarose gel in 1X Tris-acetate-EDTA (TAE) with Midori Green Advance DNA stain (Cat#AG10, Nippon) using 1 kb Plus DNA ladder (Cat#10787018, Invitrogen) as the molecular weight markers to indicate the sizes of the amplification products.

2.5. Multiplex PCR (mPCR)

After several rounds of optimization, four ratios of each primer were investigated. Finally, a primer mix including the four primer pairs was generated with a ratio of CPA:CPB:CPE:CPB2 to be 0.67 μM: 0.33 μM: 0.67 μM: 1.0 μM respectively. The annealing temperature of mPCR was 57^0C to detect equal signal for each PCR product. The final mPCR mix included 15 µl of DreamTaq 2X primer concentration is used as mentioned above; 4 μL DNA template mix; and nuclease-free water to adjust the final volume to 30 μL. The mPCR conditions were similar to those described for sPCRs. Gel electrophoresis was extended to 70 minutes at 60V for better separation of the amplicons. After that, DNA fragments were recovered from low melting agarose using phenol-chloroform method and sequenced by University of Medicine and Pharmacy, Ho Chi Minh city, Vietnam. The sequences of the products were aligned with the target genes.

2.6. Specificity and sensitivity of multiplex PCR

In order to confirm the specificity of the mPCR conditions, genomic DNA of *Salmonella* spp., *E. coli*, *Streptococcus suis*, and PCV2 were used as negative controls in the mPCR reactions as described above. Regarding the sensitivity, synthesized DNA fragments of *cpb* gene and *cpe* gene; and the purified PCR product of *cpa*, *cpb2* gene were used. These templates were diluted ten-fold serially in nuclease-free water and used for sensitivity test in the mPCR to estimate its limit of detection.

3. Results and Discussion

3.1. Multiplex PCR

In sPCRs, gel electrophoresis analysis confirmed the exact product size as predicted for each gene, including *cpa* - 324 bp, *cpb* - 196 bp, *cpe* - 257 bp, and *cpb2* - 107 bp. The results also indicated that 4 pairs of primers worked well in the annealing temperature range of 55^0C - 61^0C, and the 57^0C was chosen for mPCR. In addition, after the optimization of the mPCR, the products were clearly visible and easily distinguishable from each other, and sequencing of the four mPCR products showed that the mPCR functioned accurately (Figure 1).

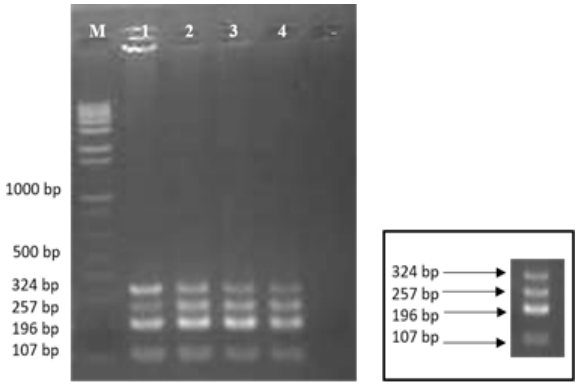

Figure 1. Results of the annealing temperature survey of multiplex PCR detecting four toxin genes of *C. perfringens*. *cpa* - 324 bp, *cpb* - 196 bp, *cpe* - 257 bp, and *cpb2* - 107 bp. M: 1 kb Plus ladder; (1) - (4): annealing temperature of 55^0C, 57^0C, 59^0C, 61^0C, respectively; (-) negative control with pure water.

Figure 2a is a result of the sensitivity testing of the optimized mPCR showing the four clear products. The mPCR could detect all four bands with equal signals when the template concentration present at 1 x 10^3 copies/reaction.

Specificity test of the mPCR was performed with unrelated DNA from virus and bacteria commonly found in the intestine and feces of pigs including *Salmonella* spp., *E. coli*, *Streptococcus suis*, and PCV2 as the four negative controls. Results showed that no amplified products were seen. It means that four primer pairs do not cross-react with DNA from the investigated organisms, avoiding false-positive results (Figure 2b).

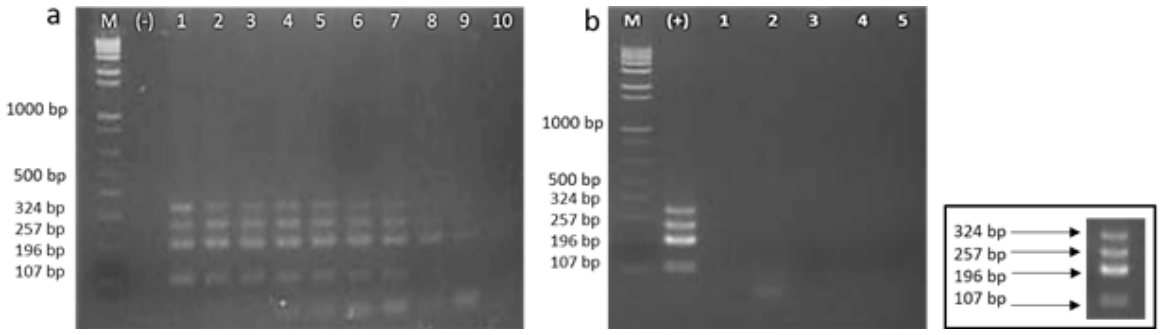

Figure 2. Multiplex PCR detecting four toxin genes of *C. perfringens*. *cpa* - 324 bp, *cpb* - 196 bp, *cpe* - 257 bp, and *cpb2* - 107 bp.
a. Sensitivity test. M: 1 kb Plus ladder; (-) negative control with pure water; (1) - (10): dilution starting from 1 x 10^9 to 1 x 10^0 DNA copies of each template.
b. Specificity test. (+): positive control; (1) - (4): negative controls (DNA of *Salmonella* spp., *E. coli*, *Streptococcus suis*, and PCV2 respectively); (5) negative control with pure water.

Figure 3. Multiplex PCR test using clinical samples.
M: 1 kb Plus ladder, (+) positive control, (-) negative control with pure water, (1) - (14) clinical samples.

3.2. Detecting the presence of toxin genes from clinical samples

The mPCR was evaluated using 30 colonies isolated from clinical samples of different farms suspected to be *C. perfringens* based on biochemical test following instruction by Markey et al. (2013). The results are summarized in Table 3 while Figure 3 showed the agarose gel analysis for mPCR products of 14 out of 30 isolates examined.

All 30 isolates were shown to carry the cpa gene (100%), further confirming these isolates are *C. perfringens* even though this is not surprising, as gene *cpa* has been reported to be the dominant genes of *C. perfringens* in swine. Only one out of 30 samples (3.33%), in the well number 10 showed positive for both alpha (*cpa*) and beta toxin (*cpb*) gene together (Figure 3). Recently, Yadav et al. (2017) also reported the presence of only 3% *C. perfringens* carrying the cpa and *cpb* gene from diarrheal cases in swine in India. Additionally, 22/30 isolates (73.33%) positive for the cpa and cpb2 gene (encoding β_2 toxin) in the present study was similar to the detection rate (70% - 90.3%) from previous reports (Van Asten et al., 2010; Chan et al., 2012; Yadav et al., 2017). It has been shown that β_2 toxin may play a key role in enteric diseases of pigs, even though the issue is still controversial. On the other hand, none of the isolates tested in this examination was *cpe*-positive, this is in accordance with a previous study carried out in America with 89 samples (Kanakaj et al., 1998). In the present communication, according to the toxinotypes of Leburn

Table 3. Results of mPCR detecting four toxin genes of thirty *C. perfringens* isolates from diarrheal piglets

Isolate	Genes (Toxin)			
	cpa (α)	*cpb* (β)	*cpe* (Entero-toxin)	*cpb2* (β_2)
1	(+)	(-)	(-)	(+)
2	(+)	(-)	(-)	(-)
3	(+)	(-)	(-)	(+)
4	(+)	(-)	(-)	(+)
5	(+)	(-)	(-)	(-)
6	(+)	(-)	(-)	(+)
7	(+)	(-)	(-)	(+)
8	(+)	(-)	(-)	(+)
9	(+)	(-)	(-)	(+)
10	(+)	(+)	(-)	(-)
11	(+)	(-)	(-)	(+)
12	(+)	(-)	(-)	(+)
13	(+)	(-)	(-)	(+)
14	(+)	(-)	(-)	(+)
15	(+)	(-)	(-)	(-)
16	(+)	(-)	(-)	(-)
17	(+)	(-)	(-)	(+)
18	(+)	(-)	(-)	(+)
19	(+)	(-)	(-)	(-)
20	(+)	(-)	(-)	(-)
21	(+)	(-)	(-)	(+)
22	(+)	(-)	(-)	(+)
23	(+)	(-)	(-)	(+)
24	(+)	(-)	(-)	(+)
25	(+)	(-)	(-)	(+)
26	(+)	(-)	(-)	(+)
27	(+)	(-)	(-)	(+)
28	(+)	(-)	(-)	(+)
29	(+)	(-)	(-)	(-)
30	(+)	(-)	(-)	(+)

(+):Positive;(-):Negative.

et al. (2010) and Mcclane et al. (2006) (Table 1), 96.66% of the isolates showing positive for *cpa* can be considered as *C. perfringens* type A; 3.33% isolates positive for both *cpa* and *cpb* can be considered as C. perfringens type C; 73.33% isolates showing positive for *cpa* and *cpb2* gene are *C. perfringens* type A carrying additional minor *cpb2* gene.

4. Conclusions

To summarize, the mPCR developed in this study enables the simultaneous detection of two major toxin genes (*cpa*, *cpb*) and two minor toxin genes (*cpe*, *cpb2*) of *C. perfringens*. The optimal annealing temperature was 57^0C/30 s. The ratio of primers CPA:CPB:CPE: CPB2 were 0.67 μM: 0.33 μM: 0.67 μM: 1.0 μM respectively. The mPCR was specific and the sensitivity was at 1 x 10^3 copies/template per reaction. Thirty colonies isolated from clinical samples were tested to determine the presence of these toxin genes. Results showed that in this set of samples, the detection rate of *cpa*, *cpb*, *cpb2* and *cpe* was 100%, 3.33%, 73.33% and 0% respectively. The results indicate that the prevalence of these four toxin genes is *cpa*, *cpb2*, *cpb* and *cpe* in decending order.

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Species composition and catch of sharks, rays and skates in Ba Ria - Vung Tau and Binh Thuan provinces of Vietnam

Manh Q. Bui[1*], Toan X. Nguyen[1], Anh H. T. Le[2], & Ali Ahmad[3]

[1]Research Institute for Marine and Fisheries, Vung Tau, Vietnam
[2]Directorate of Fisheries, Ha Noi, Vietnam
[3]Marine Fishery Resources Development and Management Department, Terengganu, Malaysia

ARTICLE INFO

Research Paper

Received: September 05, 2018
Revised: December 02, 2018
Accepted: December 26, 2018

Keywords

Ba Ria -Vung Tau province
Binh Thuan province
Ray
Shark
Skate

***Corresponding author**

Bui Quang Manh
Email: bqmanh79@gmail.com

ABSTRACT

Research on 2,626 individuals of sharks, rays and skates in total of 123 fishing boats were sampled during 2015 to 2016 in Ba Ria - Vung Tau and Binh Thuan provinces. The results identified 77 species of sharks, rays and skates belong to 22 families and 10 orders in Ba Ria - Vung Tau and Binh Thuan provinces. Of these, 57 species were recorded in Ba Ria - Vung Tau and 48 species in Binh Thuan. The families were found in the highest number of species such as *Carcharhinidae* family with 9 species, *Dasyatidae* family with 19 species and *Rajidae* family with 5 species. The total catch of sharks, rays and skates was 23,599 tons in Ba Ria - Vung Tau and was 24,355 tons in Binh Thuan. Sharks, rays and skates ratio made up from 0.2% to 0.5% in total catch landing from landing sites. Total length of sharks ranges from 21.0 cm to 366.0 cm, disc length of rays fluctuates from 11.0 cm to 248.0 cm and skates have a range from 0.7 cm to 152.0 cm in disc length.

Cited as: Bui, M. Q., Nguyen, T. X., Nguyen, H. T., Le, A. H. T., & Ahmad, A. (2018). Species composition and catch of sharks, rays and skates in Ba Ria - Vung Tau and Binh Thuan provinces of Vietnam. *The Journal of Agriculture and Development* 17(6), 31-41.

1. Introduction

Vietnam's territorial waters are home of a rich diversity of sharks, rays, skates and chimaeras (Class Chondrichthyes), which accounts for about 17% of the total number of species recorded worldwide and predominates in the surrounding area (Nguyen et al., 1972). Sharks, rays and skates in the cartilaginous category are high nutritional levels, long life cycles, late reproduction and low fertile reproduction. Sharks, rays and skates are caught by various fishing gears such as trawl nets, gillnets and longlines. Research on sharks, rays and skates have not yet conducted fully in freshwater, estuarine and the exclusive economic zone of Vietnam. In the period of 2000 -2005, there were 38 species belonging to 16 shark families (Vu & Tran, 2009) and 40 ray species belonging to 19 genera in 9 families of 2 Orders were statistically recorded (Tran & Vu, 2011) in Vietnam sea. There were 12 species belong to 5 families of shark was identified in Quy Nhon and neighboring waters (Vo et al., 2013).

A pilot project of Southeast Asian Fisheries Development Center (SEAFDEC) on recording landing data of sharks, rays and skates up to species level was conducted in the Ba Ria - Vung Tau and Binh Thuan provinces of Vietnam from 2015 to 2016. Vung Tau city, La Gi and Phan Thiet towns were selected as the study sites as it were the main landing sites of sharks, rays and skates in the states. The landing data were col-

lected at 7 jetties, with five jetties in Ba Ria - Vung Tau and two jetties in Binh Thuan. This report is a part of the research results of the project.

2. Materials and Methods

2.1. Selection of study sites

Ba Ria - Vung Tau and Binh Thuan are two main fish landing provinces in the Southeastern region of Vietnam. Vung Tau city and Lagi town are two major sites were selected as study sites for sharks, rays and skates data collection. The landing data were collected at 07 jetties, such as Ben Da, Incomat, Cat Lo, Phuoc Tinh and Ward 5 jetties in Ba Ria - Vung Tau province and Lagi, Phan Thiet jetties in Binh Thuan province (Figure 1).

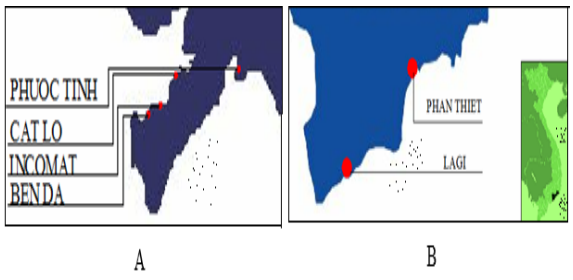

Figure 1. Location of study sites in Ba Ria - Vung Tau (A) and Binh Thuan (B) provinces.

2.2. Sampling methods

The sampling activity started in September 2015 to 31 August 2016. All enumerators were requested to record landing data and other related information via the standard. A standard SOP entitled 'SOP Sharks and Rays Data Collection in the Southeast Asian Waters' was produced. The content included Standard Operation Procedure and instructions to how enumerators to measure, weigh, record sharks, rays and skates species at sampling sites, name of enumerator, name of landing site, date of sampling, vessel registered number, vessel GRT, fishing area, price at landing sites, name of species (common name and scientific name), total catch of sharks, rays and skates commercial and low-value species from each sampling vessel.

2.3. Selection of fishing vessels and sampling activities

Between 1-4 fishing boats were selected for sampling each day for 5 days per month at each landing site. Measurement of total length (TL) was taken for all skates, sharks species and rays from the families *Rhynchobatidae, Rhinobatidae and Narcinidae*. While disc length (DL) was taken for all ray species having the tail is frequently absent or damaged (mainly from the families *Dasyatidae, Gymnuridae and Mobulidae*). All sharks, rays and skates were measured and weighed if the total number was less than 50 tails per vessel, otherwise, only 10-50% of them were sampled. The maturity stage for each individual was estimated according to Ahmad & Annie Lim (2012). The total catch of all sharks, rays and skates by species as well as the total catch of commercial and low-value species were also recorded for each sampling vessel.

2.4. Landing sample

In Ba Ria - Vung Tau province, 50 trawlers and 62 vessels were sampled. In Ba Binh Thuan province 103 trawlers, 09 vessels of gillnet and 11 ves-sels of longline fisheries were sampled.

2.5. Sample size

Ba Ria - Vung Tau province: A total of 1,037 tails belong to 239 rays, 398 sharks and 400 skates were sampled.

Binh Thuan province: A total of 1,589 tails belong to 409 rays, 199 sharks and 981 skates were sampled.

2.6. Classification

The classification (scientific names) used in this study follows that of Compagno (1999), Ahmad et al. (2013, 2014), and Ebert et al. (2013).

2.7. Data analysis

MS Excel software was used to analyse data.

Coefficient of species similarity analysis: Using formula of Magurran (1988).

3. Results and Discusions

3.1. Species diversity of sharks, rays and skates

The results of this study recorded 77 species of sharks, rays and skates that belong to 22 families and 10 orders (Table 1). In particular, 57 species were recored in Ba Ria - Vung Tau and 48 species in Binh Thuan. There were 29 species of sharks from six orders and 11 families, 43 species of rays from three orders and 10 families, and 5 species of skate from one order and one family were recorded (Table 1). The average species similarity index of sharks, rays and skates is 0.50 in the two sites. In particular, the coefficient of similarity among species groups ranged from 0.47 to 0.57.

For the shark, *Carcharhinidae* family was found 9 species, it was the highest number. Following, the family of *Scyliorhinidae* was identifed with 5 species and *Hemiscylliidae* with 4 species. The other shark families were numbered between 1 and 2 species (Figure 2). For the rays, the family of *Dasyatidae* was found the highest number of species with 19 species. Of these, the two families of *Myliobatidae* and *Narcinidae* found six species. The other ray families only found from 1 to 2 species. There was only one family of *Rajidae* of skate which has 5 species (Figure 3).

Vu & Tran (2009) recorded 38 species belong to 16 shark families in Vietnam from 2000 to 2005. Dao (2001) also recorded 20 species of 4 shark families in the Tonkin Gulf Seawaters. Vo et al. (2013) identified 12 species belong to 5 families of shark collections in Quy Nhon and neighboring waters. Meanwhile, Bui & Tran (2005) recorded 107 species belong to 15 shark families from different sources of Vietnam. A total of 40 rays species belonging to 19 genera in 9 families of 2 orders were observed in the period from 2000 to 2005. The species richness was observed in the South-eastern and central waters of Vietnam. Family of *Dasyatidae* got the highest abundance with 14 species (Tran & Vu, 2011). Previously, Nguyen et al. (1972) published 39 species of 12 ray families in Vietnam seawater. However, there are now many changes and rearrangement of shark and ray classification systems (Ahmad et al., 2017). Therefore, if it is corrected, the numbers of species and families of shark and rays will have many changes.

3.2. Fishing catch composition

The total catch of sharks, rays and skates was 23,599 tons in Ba Ria - Vung Tau and 24,355 tons in Binh thuan provinces.

In Ba Ria - Vung Tau, rays and skates were mainly sampled from trawl net and gillnet. The highest catch of rays and skates were 4,534.6 kg and 2,235.4 kg, respectively in October. Sharks were mainly sampled from both gillnet and trawl net in Ba Ria - Vung Tau in several months with 73% from gillnet and 27% from trawl net. Skates were collected only from trawl net fishery and reached 37% in total elasmobranch catch (Figure 4).

In Binh Thuan, rays and skates mainly were sampled from trawl net. The highest catch of rays was 1,046.9 kg in September and skates was 1,798 kg in April. Sharks mainly were sampled from longline reached 80% in May and June 2016, but sharks were sampled every months in gillnet and trawl net in light weight. Catch of skates and rays reached over 90% from trawl net (Figure 4).

3.3. Shark, ray and skate composition

A total of 3,602.57 tons of fish was landed in Ba Ria - Vung Tau from 112 landings during the study period. Sharks, rays and skates ratio made up 0.2%, 0.3% and 0.2% respectively in total catch landing, while landings of bony fish species were 99.34%. The elasmobranch catches gained small rate under 0.5% in total catch. The average landings per month for sharks, rays and skates were 504.8 kg, 754.2 kg and 721.3 kg respectively. The highest landing by month for sharks was 1,397.9kg in October, followed by 1,222.1kg in January. The highest landing of rays was 4,497.7 kg in October, followed by 1,046.9 kg in September. The highest landing of skates was 2,235.4 kg in October, followed by 1,793.0 kg in May (Figure 5).

At landing sites in Binh Thuan, total of 2,096.59 tons of fish was landed from 133 landings. Catch rate of sharks, rays and skates made up 0.4% and 0.3% and 0.5% respectively in the total landings. While landings of bony fish species was 98.81%. The average landings per month for sharks, rays and skates were 659.9 kg, 491.3 and 929.7 kg respectively. The highest landing by month for sharks was 3,894.9 kg in June, followed by 2,550 kg in May. The highest landing of rays

Table 1. Checklist of sharks, rays and skates species recorded during the study period

No.	Order/Families/species	Name	Ba Ria - Vung Tau	Binh Thuan
I	SHARKS		24	14
	CARCHARHINIFORMES			
	Carcharhinidae			
1	*Carcharhinus amblyrhynchos* (Bleeker, 1856)	Grey reef shark	+	
2	*Carcharhinus* cf. *falciformis* (Müller & Henle, 1839)	Silky shark	+	
3	*Carcharhinus dussumieri* (Müller & Henle, 1839)	Whitecheek shark	+	+
4	*Carcharhinus plumbeus* (Nardo, 1827)	Sandbar shark	+	+
5	*Carcharhinus limbatus* (Müller & Henle, 1839)	Common blacktip shark	+	+
6	*Carcharhinus sorrah* (Müller & Henle, 1839)	Spottail shark	+	+
7	*Carcharhinus* sp.			+
8	Galeocerdo cuvier (Person & Lesueur, 1822)	Tiger shark	+	
9	*Triaenodon obesus* (Rüppell, 1837)	Whitetip reef shark	+	
	Hemigaleidae			
10	*Hemigaleus microstoma* (Bleeker, 1852)	Sicklefin weasel shark	+	
	Scyliorhinidae			
11	*Atelomycterus marmoratus* (Anonymous [Bennett], 1830)	Coral catshark	+	+
12	*Atelomycterus baliensis* White, Last & Dharmadi, 2005	Bali catshark	+	+
13	*Cephaloscyllium sarawakensis* (Yano, Ahmad & Gambang, 2005)	Sarawak pygmy swell shark	+	
14	*Galeus* sp.			+
15	*Halaelurus buergeri* (Müller & Henle, 1838)	Blackspotted catshark	+	+
	Sphyrnidae			
16	*Sphyrna mokarran* (Rüppell, 1837)	Great hammerhead	+	
	Triakidae			
17	*Mustelus manazo* (Bleeker, 1855)	Starspotted smooth-hound	+	
	HEXANCHIFORMES			
	Hexanchidae			
18	*Heptranchias perlo* (Bonnaterre, 1788)	Sharpnose sevengill shark	+	
19	*Hexanchus griseus* (Bonnaterre, 1788)	Bluntnose sixgill shark	+	
	LAMNIFORMES			
	Alopiidae			
20	*Alopias pelagicus* (Nakamura, 1935)	Pelagic thresher	+	
21	*Alopias superciliosus* (Lowe, 1841)	Bigeye thresher		+

Table 1. Checklist of sharks, rays and skates species recorded during the study period (continue of page 34)

No.	Order/Families/species	Name	Ba Ria - Vung Tau	Binh Thuan
	ORECTOLOBIFORMES			
	Hemiscylliidae			
22	*Chiloscyllium plagiosum* (Anonymous [Bennett], 1830)	Whitespotted bambooshark	+	+
23	*Chiloscyllium punctatum* (Müller & Henle, 1838)	Brownbanded bambooshark	+	+
24	*Chiloscyllium* cf. *punctatum* (Müller & Henle, 1838)			+
25	*Chiloscyllium* sp.			+
	SQUALIFORMES			
	Centrophoridae			
26	*Centrophorus moluccensis* (Bleeker, 1860)	Smallfin gulper shark	+	
	Squalidae			
27	*Squalus megalops* (Macleay, 1881)	Shortnose spurdog	+	
	SQUATINIFORMES			
	Squatinidae			
28	*Squatina* sp.		+	
29	*Squatina tergocellatoides* (Chen, 1963)	Ocellated angelshark	+	
II	RAYS		29	31
	MYLIOBATIFORMES			
	Urolophidae			
30	*Urolophus aurantiacus* (Müller & Henle, 1841)	Sepia stingray	+	
	Dasyatidae			
31	*Dasyatis fluviorum* (Ogilby, 1908)	Estuary stingray		+
32	*Dasyatis parvonigra* (Last & White, 2008)	Dwarf black stingray		+
33	*Dasyatis sinensis* (Steindachner, 1892)	Chinese stingray	+	+
34	*Dasyatis* cf. *sinensis* (Steindachner, 1892)		+	+
35	*Dasyatis* sp.			+
36	*Dasyatis zugei* (Müller & Henle, 1841)	Pale-edged stingray	+	+
37	*Himantura imbricata* (Bloch & Schneider, 1801)	Bengal whipray	+	+
38	*Himantura jenkinsii* (Annandale, 1909)	Jenkins whipray	+	
39	*Himantura undulata* (Bleeker, 1852)	Leopard whipray	+	
40	*Himantura walga* (Müller & Henle, 1841)	Scaly whipray	+	+
41	*Himantura* cf. *javaensis* (Last & White, 2013)	Javan whipray		+
42	*Himantura* cf. *pastinacoides* (Bleeker, 1852)	Round whip ray	+	

Table 1. Checklist of sharks, rays and skates species recorded during the study period (continue of page 35)

No.	Order/Families/species	Name	Ba Ria - Vung Tau	Binh Thuan
43	*Maculabatis gerrardi* (Gray, 1851)	Sharpnose stingray	+	
44	*Neotrygon kuhlii* (Müller and Henle, 1841)	Blue-spotted stingray	+	+
45	*Neotrygon* sp.			+
46	*Pteroplatytrygon violacea* (Bonaparte, 1832)	Pelagic stingray	+	+
47	*Taeniurops meyeni* (Müller & Henle, 1841)	Round ribbontail ray	+	
48	Taeniura lymma (Forsskål, 1775)	Ribbontail stingray		+
49	*Urogymnus asperrimus* (Bloch & Schneider, 1801)	Porcupine whipray		+
	Gymnuridae			
50	*Gymnura japonica* (Temminck & Schlegel, 1850)	Japanese butterflyray		+
51	*Gymnura poecilura* (Shaw, 1804)	Long-tailed butterfly ray		+
	Myliobatidae		+	+
52	*Mobula thurstoni* (Lloyd, 1908)	Smoothtail mobula	+	
53	*Mobula japonica* (Müller & Henle, 1841)	Spinetail mobula	+	
54	*Mobula* sp.			+
55	*Aetobatus ocellatus* (Kuhl, 1823)	Ocellated eagle ray		+
56	*Aetomylaeus maculatus* (Gray, 1834)	Mottled eagle ray		+
57	*Myliobatis tobijei* Bleeker, 1854	Japanese eagle ray		+
	Pleisiobatidae			
58	*Plesiobatis daviesi* (Wallace, 1967)	Deep-water stingray	+	
	RHINOBATIFORMES Platyrhinidae			
59	*Platyrhina sinensis* (Bloch & Schneider, 1801)	Chinese fanray	+	+
60	*Platyrhina tangi* (Iwatsuki, Zhang & Nakaya, 2011)	Yellow-spotted fanray	+	+
	Rhinidae			
61	*Rhynchobatus australiae* (Whitley, 1939)	Bottlenose wedgefish	+	+
62	*Rhynchobatus palpebratus* (Compagno & Last, 2008)	Eyebrow wedgefish	+	
	Rhinobatidae			
63	*Rhinobatos formosensis* (Norman, 1926)	Taiwan guitarfish	+	+
64	*Rhinobatos* sp.		+	
	TORPEDIFORMES Narcinidae			
65	*Narcine brevilabiata* (Bessednov, 1966)	Shortlip electric ray	+	
66	*Narcine brunnea* (Annandale, 1909)	Brown numbfish	+	

Table 1. Checklist of sharks, rays and skates species recorded during the study period (continue of page 36)

No.	Order/Families/species	Name	Ba Ria - Vung Tau	Binh Thuan
67	*Narcine indica* (Bloch & Schneider, 1801)	Spotted numbfish	+	+
68	*Narcine* cf. *indica* (Bloch & Schneider, 1801)			+
69	*Narcine* sp.			+
70	*Narcine timlei* (Bloch & Schneider, 1801)	Spotted numbfish	+	+
	Narkidae			
71	*Narke dipterygia* (Bloch & Schneider, 1801)	Numbray		+
72	*Narke japonica* (Temminck & Schlegel, 1850)	Japanese sleeper ray	+	
III	SKATES RAJIFORMES		4	3
	Rajidae			
73	*Dipturus johannisdavisi* (Alcock, 1899)	Travancore skate	+	
74	*Dipturus kwangtungensis* (Chu, 1960)	Kwangtung skate	+	
75	*Okamejei cairae* (Last, Fahmi & Ishihara, 2010)	Borneo Sand Skate	+	+
76	*Okamejei hollandi* (Jordan & Richardson, 1909)	Yellow-spotted skate	+	+
77	*Okamejei* cf. *boesemani* (Ishihara, 1987)	Boeseman's skate		+
	TOTAL: 77 Species		57	48

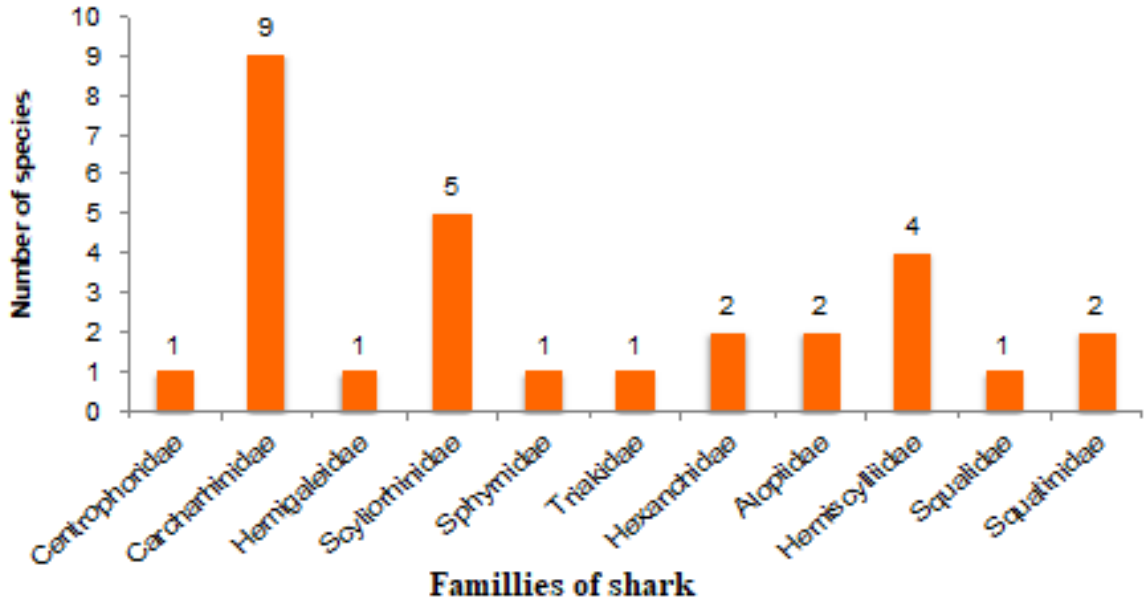

Figure 2. Number of species of shark families.

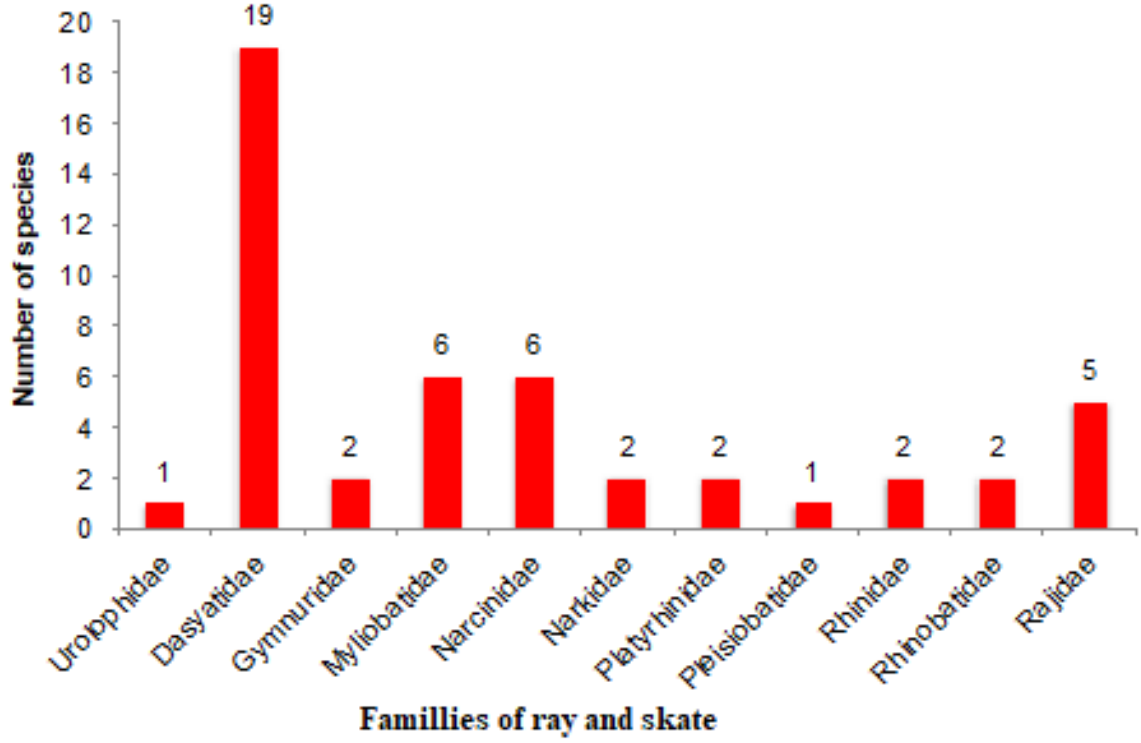

Figure 3. Number of species of shark families.

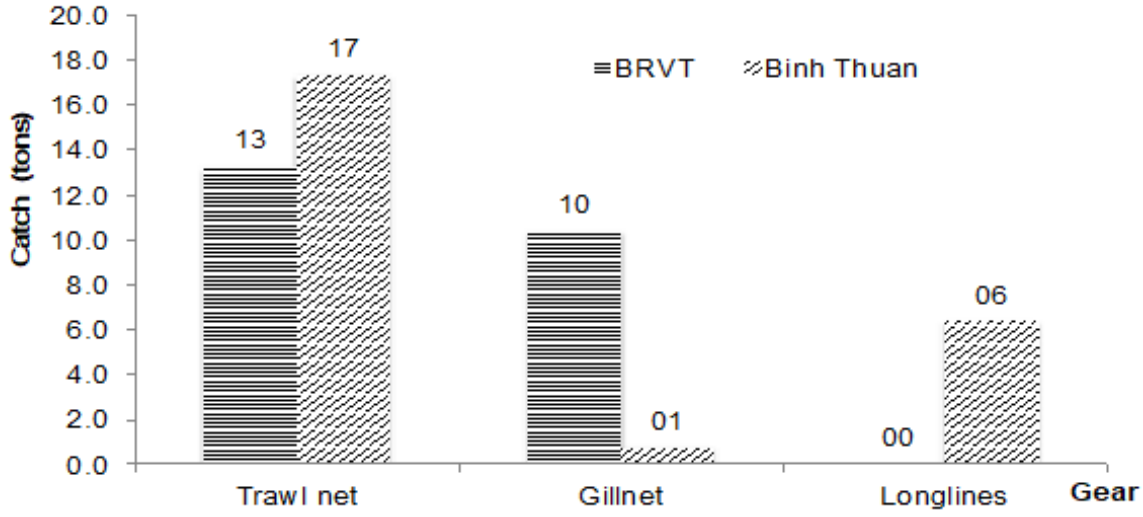

Figure 4. Sharks, rays and skates catch composition by gear type.

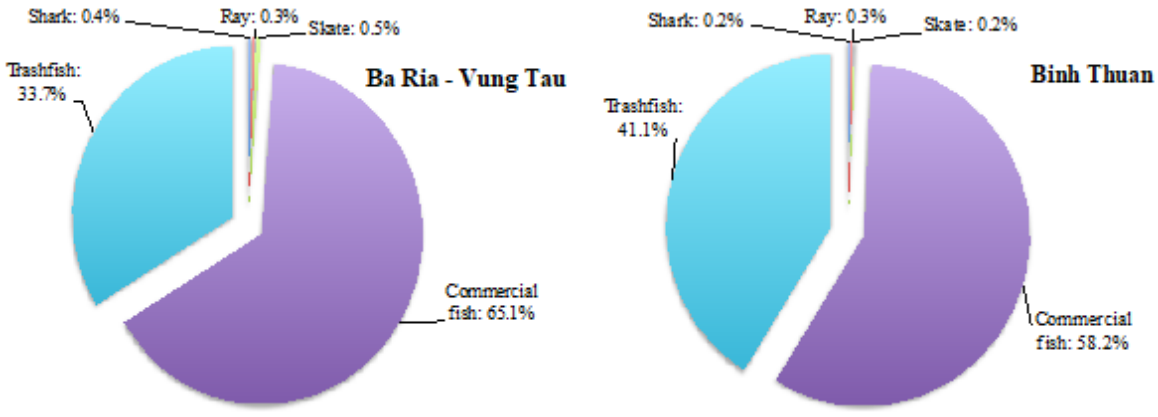

Figure 5. Sharks, rays and skates catch composition by gear type.

was 1,421.7 kg in October, followed by 1,046.9 kg in September, for skates was 1,798 kg in April, followed by 1,414 kg in January. The catch of sharks, rays and skates was under 1% in total catch of all fisheries in Binh Thuan province (Figure 5).

3.4. Weight of sharks, rays and skates by species

A total of 23,599.5 kg was landed in Ba Ria - Vung Tau province from 112 landings comprising 8,886.6 kg rays, 8,655.1 kg skates and 6,057.8 kg sharks. For rays, the highest landing by weight was *Mobula thurstoni*, followed by *Himantura jenkinsii*. For sharks, the highest landing was 10,810.73 kg for species of *Carcharhinus sorrah*, followed by 359 kg and 300 kg for *Carcharhinus limbatus* and *Galeus* sp. respectively. The highest landing weight of sharks by month was 3,871.2 kg of *Carcharhinus sorrah*, followed by *Chiloscyllium punctatum* was 779.2 kg. For skates, *Okamejei cairae* reached highest weight of 7,596.1 kg, the months of May, August, October and November was over 1,000 kg for the species.

In Binh Thuan province, total shark and ray species of 24,355.5 kg was landed from 133 landings, comprising 4,980.4 kg rays, 11,456.4 kg skates and 7,918.8 kg sharks. For rays, the

Table 2. Size range of sharks (Total length-TL), rays and skates (Disc length-DL) in Ba Ria - Vung Tau and Binh Thuan provinces from 2015 to 2016. All measurement in cm

Group	Ba Ria - Vung Tau province			Binh Thuan province		
	Max	Min	Average	Max	Min	Average
Shark (TL)	366.0	23.0	93.5 ± 62.1	366.0	21.0	96.8 ± 69.6
Ray (DL)	248.0	13.5	49.7 ± 43.8	127.0	11.0	48.0 ± 33.9
Skate (DL)	152.0	0.7 ± 34.0	26.4	58.0	11.0	30.7 ± 9.8

Table 3. Total operated days and total number of operations by gears sampled during the study period at Ba Ria - Vung Tau and Binh Thuan provinces in 2015 - 2016

Gears	Total operated days by gears in 2015 - 2016		Total number of operations by gears in 2015 - 2016	
	Ba Ria - Vung Tau	Binh Thuan	Ba Ria - Vung Tau	Binh Thuan
Gillnet	1,267	103	1,327	139
Trawl net	2,327	1,269	6,520	4,157
Longline	-	135	-	135
Total	3,594	1,507	7,847	4,431

highest landing by weight was *Himantura walga* amounted 1,586.5 kg, followed by 1,053.6 kg for *Himantura imbricata*. For sharks, the highest landing was 6,995.3 kg for species of *Carcharhinus sorrah*, followed by 329.5 kg and 300 kg for *Carcharhinus limbatus* and *Galeus* sp., respectively. For skates, *Okamejei cairae* reached highest weight of 9,904.8 kg, the months of from January to May and December was over 1,000 kg for the species.

3.5. Size range of sharks, rays and skates

In Ba Ria - Vung Tau province, the total length of sharks ranges from 23 cm to 366 cm (TL), reaching an average of 93.5 cm, rays have an average length of 49.7 cm in disc length (ranging from 13.5 cm to 248 cm) and skates have average length is 34 cm (DL). Also in Binh Thuan province, sharks have an average length of 96.8 cm (TL), rays are 48 cm (DL) and skate 30 cm (DL) (Table 2).

Most shark, ray and skate species landed in Ba Ria - Vung Tau province from January to May and from September to December were mature except to *Mobula thurstoni* (mature 198 cm). *Plesiobatis daviesi* (mature at 130 cm), *Atelomycterus marmoratus* (mature at 45 cm). *Carcharhinus limbatus* (mature at 120 cm), *Carcharhinus sorrah* matures at 103 - 128 cm (male) 110 - 118 cm (female). *Chilocyllium puctatum* matues at 68 - 76 cm. *Galeocerdo cuvier* matures at 300 - 305 cm for males and 250 - 350 cm for females (TL).

In Binh Thuan province, in general, all ray species sampled from January to May were mature. The most ray species landed from September to December were mature except for *Aetobatus ocellatus* (mature at 100 - 110 cm), *Gymnura poecilura* (mature at 45 cm). The most shark species landed from January, May and September to December were mature except for *Carcharhinus limbatus* (mature at 120 - 190 cm), *Carcharhinus sorrah* (mature at 103 cm), *Chiloscyllium plagiosum* (mature at 50 cm) and *Chilocyllium punctatum* (mature at 68 cm).

3.6. Fishing ground and fishing efforts

The main fishing grounds of shark, ray and skate vessels are in the Central and Southeast regions of Vietnam seawaters. In case of the gear of which annual effort excess 1000 days of operation or 1000 number of operations, CPUE (total of 12 months) was estimated by weight and number of individuals by species. Monthly fishing efforts (days at operation, total number of operation during the cruise) of the sampled vessels are summarized in Table 3.

4. Conclusion

A total of 77 species of sharks, rays and skates belong to 22 families and 10 orders are recorded in Ba Ria - Vung Tau and Binh Thuan provinces. Of these, 57 species were recored in BRVT and 48 species were found in Binh Thuan. The average species similarity index of sharks, rays and skates

is 0.50 in two study sites.

The *Carcharhinidae* family was found 9 species, *Dasyatidae* family identified 19 species and only one family of *Rajidae* of skate found 5 species. These families were recored the highest species number.

The total catch of sharks, rays and skates was 23,599 tons and 24,355 tons, respectively in Ba Ria - Vung Tau and Binh Thuan provinces.

The rate of sharks, rays and skates reached only from 0.2 to 0.5% in total catch landing from two sample locals.

The most abundant shark species in Ba Ria - Vung Tau are *Chiloscyllium punctatum*, *Carcharhinus sorrah* and *Atelomycterus marmoratus* while for rays are *Himantura walga*, *Himantura imbricata*, *Neotrygon kuhlii*, *Himantura jenkinsii* and *Dasyatis zugei*. The most common shark species are *Carcharhinus sorrah* while for rays *Himantura walga*, *Dasyatis zugei* and *Gymnura japonica*. The most abundant shark species in Binh Thuan are *Chiloscyllium punctatum*, *Carcharhinus sorrah* and for rays *Himantura imbricata*, *Dasyatis zugei* and *Himantura walga* and for skates, *Okamejei cairae*, *Okamejei hollandi*. Species of *Okamejei cairae* reached huge catch from trawl net from Lagi jetty of Binh Thuan province.

The total length of shark ranges from 21 to 366 cm, ray has disc length ranging from 11 to 248 cm and skate ranges from 0.7 to 152 cm in disc length.

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Concentrations of heavy metals in water from the Southern coast of Vietnam

Ha N. Nguyen[1], Tri N. Nguyen[2], Dong V. Nguyen[3], & Tu P. C. Nguyen[2*]

[1]Research Institute for Biotechnology and Environment, Nong Lam University, Ho Chi Minh City, Vietnam
[2]Faculty of Fisheries, Nong Lam University, Ho Chi Minh City, Vietnam
[3]Faculty of Chemistry, University of Science, Ho Chi Minh City, Vietnam

ARTICLE INFO

Research Paper

Received: September 10, 2018
Revised: November 12, 2018
Accepted: November 21, 2018

Keywords

Heavy metals
Southern coast
Suspended particulate matter
Vietnam
Water

***Corresponding author**

Nguyen Phuc Cam Tu
Email: npctu@hcmuaf.edu.vn

ABSTRACT

Concentrations of heavy metals (As, Cd, Pb and Hg) in water collected from seven coastal provinces between December 2012 and December 2015 were evaluated. The average total concentrations (μg/L) of As, Cd, Pb and Hg in water ranged from 2.90 to 6.38, $<$ 0.039 to 0.322, 4.26 to 10.5 and $<$ 0.01 to 0.118, respectively. The average concentrations (μg/L) of As, Pb and Hg in suspended particulate matters (SPM) ranged from 0.392 to 7.32, 0.365 to 18.7 and $<$ 0.01 to 0.038, respectively; whereas, Cd concentrations were not detected in most of SPM samples. There were positive linear relationships between concentrations of heavy metals in water and SPM, except for Cd. The results showed that the concentrations of metals analyzed in water remained below quality guidelines for the protection of aquatic life recommended by the international and Vietnamese organizations. However, As levels in 2/103 and 5/103 of water samples exceeded the QCVN 10:2015/BTNMT for maximum permitted level using for aquaculture and aquatic life protection (20 μg/L) and the Canadian water quality guidelines for the protection of aquatic life (12.5 μg/L), respectively.

Cited as: Nguyen, H. N., Nguyen, T. N., Nguyen, D. V., & Nguyen, T. P. C. (2018). Concentrations of heavy metals in water from the Southern coast of Vietnam. *The Journal of Agriculture and Development* 17(6), 42-49.

1. Introduction

Currently, nearly a half of Vietnam's population lives in the 28 coastal provinces, and there is an increasing migration into these regions where there are many large cities (e.g., Ho Chi Minh City, Da Nang, Hai Phong) and centralized industrial zones. These activities have created an increased pollution, most likely in hotspots such as the major estuaries and the coastline, which receive different kinds of wastes produced by inland industrial and population centers. The government findings estimate that 60% of the marine pollution originates from land-based sources, including nutrients, persistent organic pollutants and heavy metals (HMs) in water and sediments (MONRE, 2010).

The HMs, particularly As, Cd, Pb and Hg, are considered most toxic to biota and environment. HMs contamination can result in adverse effects including growth changes, metabolic process, and disease development (Morais et al., 2012). The fate and toxicity of HMs in the aquatic environment are significantly dependent on the distributing among the aqueous phase, suspended particulate matter (SPM) and sediments (Yang et al., 2016). Dissolved HMs existing in the pore waters are more bioavailable and toxic than particulate HMs (Chapman et al., 1998). While, SPM can play an important role in controlling the reactivity, transport, and biological impacts of HMs in the water (Yang et al., 2016). Understanding the main factors that influence the distribution of HMs in water would allow better prediction of

the changes in HMs toxicity to aquatic organisms (Atkinson et al., 2007). However, no study has comprehensively analyzed the multiphase partition of HMs for SPM-water in the Southern coast of Vietnam.

In the present study, the concentrations of four HMs (i.e. As, Cd, Pb and Hg) were analyzed in waters along the southern coast of Vietnam. Another objective of this study was to evaluate the relationships between the physico-chemical properties and HMs.

2. Materials and Methods

2.1. Sample collection and preparation

Water samples were collected from the extensive cockle culture along the Southern Vietnamese coast, including Ho Chi Minh City (HCM) and 6 provinces including Tien Giang (TGI), Ben Tre (BTR), Tra Vinh (TVI), Bac Lieu (BLI), Ca Mau (CMA), and Kien Giang (KGI) between December 2012 and December 2015 (Figure 1).

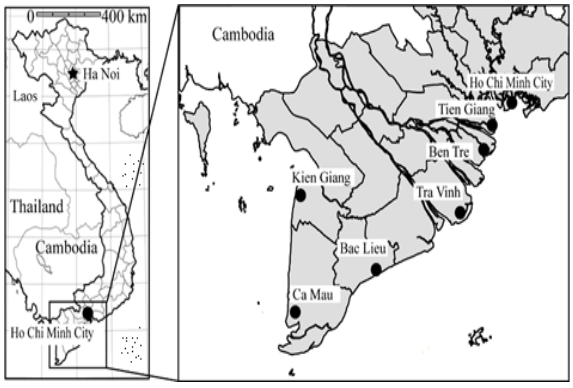

Figure 1. A map showing sampling locations in Southern coast of Vietnam.

Samples of 2 L of water were taken manually at a depth of approximately 20 cm below surface using acid-washed polyethylene bottles (VS, 1995a). Samples for total HMs determination were immediately acidified to pH 2 using 0.5 mL of concentrated HNO_3 (VS, 1995b). For analyzing HMs level in SPM fraction, approximately 500 mL of the water sample was filtered through 0.45-μm cellulose acetate membrane filters (Sartorius; cleaned with 5% v/v HNO_3 and washed in redistilled water) by vacuum pump. The membranes were kept in an ice box, transported to laboratory and stored in a deep freezer at – 20^0C before analysis.

At each sampling site, water quality parameters, including temperature, pH, electrical conductivity (EC and salinity), and dissolved oxygen (DO) were measured using a mercury thermometer, Denver pH meter UP-5, SevenGo pro^{TM} conductivity and MW600 standard portable DO meter, respectively.

2.2. Water sample analyses

Concentration of As in water was analyzed in accordance with the Vietnamese standard TCVN 6626-2000 (VS, 2000). For each sample, 50 mL of water was placed in a round-bottom flask with the addition of 5 mL of concentrated H_2SO_4 and 5 mL H_2O_2 30%, and then heated at 180^0C on a hot plate until white fume occurred. The solution was cooled, diluted to 25 mL and then transferred into a 40-mL glass screw-cap tube with a Teflon-faced silicone septum. Samples were prepared for hydride analysis by adding 0.5 mL of L-cysteine 5% and 4 mL of 3M HCl and heated in a water-bath at 90^0C for 45 min. Bring tube to 50 mL with 0.6 M HCl before analyzing.

Hg level in water was determined in accordance with TCVN 5991:1995 (VS, 1995c). Place a 50-mL portion of water in 50-mL glass screw-cap tube with a Teflon-faced silicone septum, add 0.4 mL of BrCl solution and kept in 3 hrs. To remove residue of BrCl, the solution was added some drops of 10% $NH_2OH.HCl$ before analyzing.

For analyzing Cd and Pb levels in water, 50 mL of samples were acidified by redistilled HNO3 and heated on hot plate at 90 – 95^0C until the volume was reduced to 15 – 20 mL. Samples were cooled, filtered and final volume was made up to 50 mL with double-distilled water.

Concentrations of Hg and As were determined by gold amalgamation cold vapour atomic absorption (CV-AAS) spectrometry and hydride generation atomic absorption spectrometry (HG-AAS), respectively. Levels of Cd and Pb were analyzed by an inductively coupled plasma mass spectrometry (ICP-MS).

2.3. SPM analyses

Whole membranes were placed in Teflon vials with the addition of 10 mL of concentrated

HNO_3, and then heated at 150^0C for 2 h. Digested solution was cooled, added 1 mL H_2O_2 and heated at 150^0C for 1 h. The whole procedure, including addition of H_2O_2 and heating was conducted in duplicate. The solution was then centrifuged, filtered, and then made up to 50 mL.

For As analysis, aliquot of 10 mL was placed in Erlenmeyer flask with addition of 0.5 mL of concentrated H_2SO_4 and heated on hot plate until white fume arose. And sample was treated and analyzed by the procedure used for total As analysis in water.

For Hg analysis, a portion of digested solution was added 1 ml of concentrated HCl and made up to 50 mL by double distilled water. Concentration of Hg was determined using a CV-AAS.

For Cd and Pb determination, the digested solution was directly analyzed by ICP-MS.

2.4. Quality assurance and quality control (QA/QC) procedure

Reagent grade chemicals were used in all analytical procedures. For each batch or 20 samples, at least one matrix spike, one matrix spike duplicate and two reagent blanks were used as the QA/QC procedure of the analysis. Recoveries of the HMs ranged from 80 to 120% of the spiked values. Detection limits for As, Cd, Pb and Hg were 0.13, 0.039, 0.14 and 0.01 μg/L, respectively. All data were expressed in μg/L.

2.5. Statistical analysis

One-half of the value of the respective limit of detection was substituted for those values below the limit of detection and used in statistical analysis (US EPA, 2000). All data were tested for goodness of fit to a normal distribution and homogeneity of variances with a Kolmogorov–Smirnov's one sample test and Levene test, respectively. Because most of the variables were not normally distributed, the data were logarithmically transformed and subjected to parametric statistics. Pearson correlation and simple linear regression were used to measure the relationships among concentrations of HMs in water, SPM and physicochemical parameters. For testing provincial differences, log transformed data were analyzed using one-way analysis of variance (ANOVA) with Duncan multiple range test as pairwise comparisons. A *P-value* < 0.05 was considered to indicate statistical significance. These statistical analyses were executed by the program SPSS (version 19, SPSS, Chicago, IL, USA).

3. Results and Discussion

3.1. Physicochemical parameters of water samples

Physicochemical parameters of water samples are shown in Table 1. Average temperature ranged from 27.8^0C in the BTR water to 32.1^0C in the HCM water. The lowest pH value (6.81) was found in water from HCM, and the highest value (7.93) occurred in KGI water, which was found to be within the prescribed limit of 6.5 – 8.5 (MONRE, 2015). pH values in water found in these regions were similar to those reported in the previous studies. Pham et al. (2007) reported that pH values in the coast of Can Gio, HCM City ranged from 7.8 to 8.1. During As accumulation study in surface water in some estuaries of the Mekong River Delta (MRD), Bui et al. (2011) observed that pH values remained same in sampling areas and were in range of 7.5 – 7.9. Similar results were reported by Nguyen (2007), the pH values ranged from 7.1 – 7.2 for the coastal regions of Ngoc Hien, Ca Mau. EC and salinity in water from the Southern coast found to be in the ranges of 20.9 – 35.9 mS/cm and 12.4 – 22.6‰, respectively. On comparison with other data by other works, it was found that the minimum and maximum values of EC and salinity were 0.29 – 43.1 mS/cm and 2.0 – 28.9‰, respectively (Bui et al., 2011), which are comparable to this study. From Table 1, it was clear that the range of the DO lies between 4.87 to 7.20 which follow the standards given by MONRE (2015).

3.2. Provincial differences in heavy metal concentrations

Concentrations of HMs in water (103 samples) and SPM (30 samples) collected from the Southern coast of Vietnam were exhibited in Table 2.

3.2.1. Arsenic

Mean As concentrations ranged from 2.90 μg/L in water from HCM to 6.38 μg/L in water from KGI (Table 2). No statistically significant difference was found among provinces (ANOVA, $P < 0.05$). Similar results were reported by Nguyen (2007), As concentrations in water ranged from

Table 1. Physicochemical parameters of water samples collected from the coasts of Vietnam[1]

Province	Temperature (^{0}C)	pH	EC (mS/cm)	Salinity (‰)	DO (mg/L)
KGI	31.1 ± 2.9	7.93 ± 0.42	21.1 ± 6.6	12.4 ± 4.3	7.20 ± 1.16
BLI	30.4 ± 3.3	7.84 ± 0.46	21.4 ± 9.6	12.9 ± 6.1	5.90 ± 1.66
CMA	30.0 ± 1.3	7.65 ± 0.26	32.0 ± 10.0	18.9 ± 5.9	6.08 ± 0.64
TRV	28.7 ± 1.7	7.40 ± 0.12	20.9 ± 7.6	12.6 ± 5.0	4.87 ± 0.47
BTR	27.8 ± 3.2	7.61 ± 0.93	24.0 ± 11.6	15.4 ± 8.1	6.40 ± 1.27
TGI	29.9 ± 2.1	7.10 ± 0.70	24.1 ± 14.9	14.6 ± 9.6	6.40 ± 1.32
HCM	32.1 ± 3.1	6.81 ± 0.65	35.9 ± 4.8	22.6 ± 3.4	6.67 ± 1.27

[1]Data are mean ± standard deviation.

Table 2. Heavy metal concentrations (μg/L) in water and SPM collected from the Southern coast of Vietnam[1]

Province	n	As	Cd	Pb	Hg
Water					
KGI	25	6.38 ± 5.44^{a}	< 0.039^{a}	10.2 ± 17.8^{a}	0.031 ± 0.023^{ab}
BLI	22	5.54 ± 3.99^{a}	0.167 ± 0.447^{a}	10.5 ± 16.4^{a}	0.064 ± 0.068^{bc}
CMA	26	3.41 ± 2.35^{a}	< 0.039^{a}	1.93 ± 2.29^{a}	0.118 ± 0.086^{c}
TRV	3	4.23 ± 2.24^{a}	< 0.039^{a}	8.81 ± 12.5^{a}	0.011 ± 0.010^{a}
BTR	15	5.09 ± 4.22^{a}	0.056 ± 0.082^{a}	5.87 ± 4.42^{a}	0.038 ± 0.064^{ab}
TGI	9	6.05 ± 7.40^{a}	0.322 ± 0.814^{a}	9.47 ± 12.0^{a}	0.049 ± 0.040^{bc}
HCM	3	2.90 ± 0.28^{a}	< 0.039^{a}	4.26 ± 5.11^{a}	0.022 ± 0.014^{ab}
SPM					
KGI	8	3.45 ± 3.39	ND	6.81 ± 7.05	0.019 ± 0.017
BLI	8	7.32 ± 5.63	ND	18.7 ± 19.8	0.038 ± 0.025
TRV	2	0.711 ± 0.419	ND	0.365 ± 0.132	< 0.01
BTR	6	2.61 ± 3.03	ND	4.32 ± 3.93	0.016 ± 0.012
TGI	5	6.76 ± 6.40	ND	9.32 ± 11.80	0.026 ± 0.028
HCM	1	0.392	ND	7.35	< 0.01

[1]Data are mean ± standard deviation. Values in the same column with different superscript letters are significantly different ($P < 0.05$, one-way ANOVA with Duncan test); ND: not detected.

0.40 – 23.3 μg/L for the coastal regions of Ngoc Hien, Ca Mau. In study of As accumulation in surface water in some estuaries of the Mekong River Delta (MRD), Bui et al. (2011) observed As levels in water between freshwater and brackish-water and saltwater were significantly different with mean levels of 1.48 ± 1.26 μg/L, 8.51 ± 7.79 μg/L and 49.47 ± 23.57 μg/L, respectively.

In this study, the general tendency in average As concentrations in SPM among different provinces were BLI > TGI > KGI > BTR > TVI > HCM. The positive relationship between As concentrations in water and SPM samples was relatively strong significant (Simple linear regression, $R^2 = 0.59$, $P < 0.001$) (Figure 2). This result was agreed with the work of Yang et al. (2016) at three large shallow lakes in China, e.g., Taihu, Chaohu, and Dianchi. These authors reported that total As in water had a significant positive relationship with the total As in SPM at all three lakes ($R^2 > 0.735$). As a result of fluctuations in physical, chemical, and biological factors occurred in natural water, As can be adsorbed onto SPM from aqueous dissolved phase, precipitated, and deposited to sediments. As reported by other works, the distribution of As between water and SPM can be influenced by concentrations and compositions of SPM (e.g., Fe and Mn oxides), salinity, pH, redox condition as well as biological effects (Balzer et al., 2013; Hong et al., 2016; Yang et al., 2016).

3.2.2. Cadmium

Average levels of Cd in our sample set ranged from < 0.039 μg/L to 0.322 ± 0.814 μg/L; the highest level was found in TGI, followed by BLI (0.167 ± 0.447 μg/L) and the lowest in KGI, CMA, TVI and HCM (< 0.039 μg/L). However, there was no statistically significant differences in

mean Cd concentration of water samples among provinces ($P > 0.05$). In contrast, concentrations of Cd were not detected in all SPM samples. This result was similar to that reported by Cenci and Martin (2004). In estuaries, Cd desorption should be expected due to chloride and sulfate complexation, and ionic strength effects. Mobilisation of Cd was recognized in many estuaries such as in the Amazon plume, Changjiang, Gironde, Mississippi, etc. (cited therein Cenci and Martin, 2004).

3.2.3. Lead

Lead concentrations in waters from these provinces varied widely, and extremely elevated levels were found in samples collected from BLI (Table 2). However, there were no statistically significant differences in mean Pb levels of samples among provinces ($P > 0.05$). While, Pb concentration in SPM samples ranged from 0.365 ± 0.132 μg/L in TRV to 18.7 ± 19.8 μg/L in BLI. The highly significant positive correlations found between total Pb concentrations in water and SPM samples ($R^2 = 0.71$, $P < 0.001$) (Figure 2).

3.2.4. Mercury

Average levels of Hg in water collected from the southern coast were highest in CMA (0.118 ± 0.086 μg/L), followed by BLI (0.064 ± 0.068 μg/L) and lowest in TVI (0.011 ± 0.010 μg/L). On the contrary, Hg concentrations were not detected in all SPM samples. There were statistically significant differences in mean Hg levels of water samples among provinces ($P < 0.05$) (Table 2). Log concentrations of Hg in water correlated positively with log concentrations of Hg in SPM ($R^2 = 0.84$, $P < 0.001$) (Figure 2).

Similar to As, in the estuarine waters, Pb and Hg distributed between the dissolved phase (water) and particulate (SPM). The dissolution of HMs between these phases depends on the physicochemical properties of the SPM as well as various ambient conditions such as salinity, pH, and the types and concentrations of dissolved organic matter (Wang et al., 2016). In this study, there were highly positive relationships between Pb and Hg concentrations in water and SPM samples. Yao et al. (2016) reported similar good linear regressions of total HMs concentrations between dissolved and particulate phase in estuarine waters.

3.3. Relationships between concentrations of total heavy metals in water and physicochemical properties

In the present study, relationships between physicochemical characteristics (temperature, pH, EC, salinity and DO) and HMs concentrations in water as well as among HMs were evaluated using Pearson correlations. The highly significant positive correlations discovered between EC and salinity ($r = 0.97$); therefore, just relationships between EC and other parameters were discussed. The weak positive correlations found between temperature and DO ($r = 0.31$), and concentrations of Cd and Pb ($r = 0.41$) in water; whereas the weak negative associations observed between EC and DO (r = -0.26), and concentrations of Pb and Hg (r = -0.29) in water. Moreover, EC and concentrations of Hg was weakly positively associated ($r = 0.25$). In addition, As levels were positively related with Cd ($r = 0.36$) and Pb ($r = 0.70$), but negatively related with Hg (r = -0.25). However, no correlations were found between pH and other parameters (Table 3).

3.4. Comparison with published data and guidelines

Analytical techniques have played an important role in assessing pollution of estuarine system and have been extensively used to determine various HMs levels in seawater, marine sediments and biota. However, the high salt content and a very low concentration for the HMs (ranging from < ng/L to μg/L) can be major obstacles to analyzing HMs in brackish and marine waters. Therefore, very little research has been done on concentrations of HMs in estuarine water.

Compared to other published works, average concentrations of As in water collected from the Vietnam southern coast were comparable with or lower. Levels of As in the coast water of Can Gio, HCM were 31 μg/L (Pham et al., 2007). While, accumulation levels of As in the coast water of Ngoc Hien, Ca Mau ranged 0.4 – 23,3 μg/L (Nguyen, 2007) (Table 4). Similarly, Bui et al. (2011) observed As levels in water between freshwater and brackish-water and saltwater were significantly different with mean levels of 1.48 ± 1.26 μg/L, 8.51 ± 7.79 μg/L and 49.47 ± 23.57 μg/L, respectively. Moreover, Phung and Huynh (2015) reported levels of As accumulated in water of Mekong River ranged 1.48 – 49.47

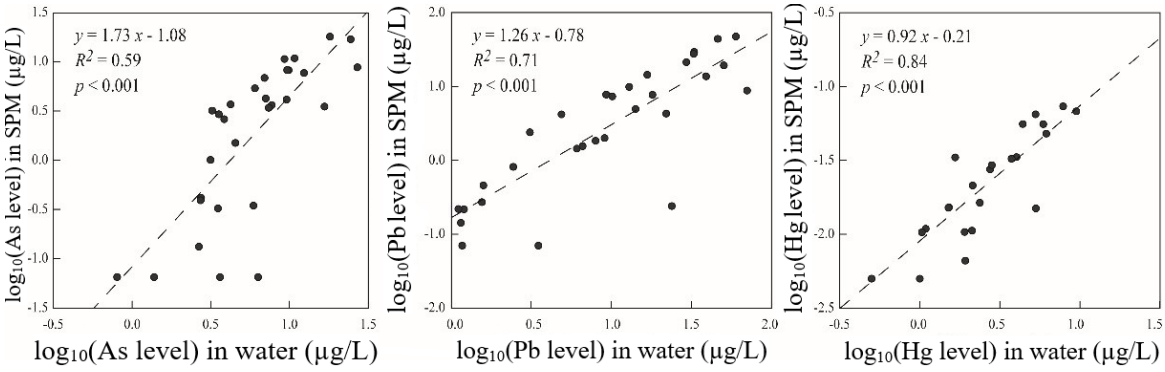

Figure 2. Relationships between heavy metal concentrations (μg/L, log-transformed) in water and SPM (n = 30) collected from the Southern coast of Vietnam.

Table 3. Correlation coefficients (r) for relationships between log-transformed concentrations (μg/L) of total heavy metals in water and physicochemical properties from the Southern coast of Vietnam[1]

	pH	EC	Salinity	DO	As	Cd	Pb	Hg
Temperature	-0.03	0.00	-0.10	**0.31**	-0.14	-0.17	-0.18	0.17
pH		0.00	-0.04	0.12	0.15	0.01	0.00	-0.17
EC			**0.97**	**-0.26**	0.14	-0.02	0.07	**0.25**
Salinity				**-0.26**	0.15	0.00	0.09	**0.21**
DO					-0.13	-0.20	-0.17	-0.12
As						**0.36**	**0.70**	**-0.25**
Cd							**0.41**	-0.19
Pb								**-0.29**

[1]Bold numbers in columns indicate significance at $P < 0.05$.

μg/L. However, concentrations of As in water in this study were lower than those collected in the Straits of Malacca, Malaysia (4.98 – 86.14 μg/L) (Looi et al., 2013). The concentrations of As analyzed in water remained below quality guidelines for the protection of aquatic life recommended by the Canadian Council of Ministers of the Environment (CCME, 2007) (12.5 μg/L) and the Vietnamese regulation (QCVN 10-MT : 2015/BTNMT, 20 μg/L) (CCME, 2007; MONRE, 2015) (Table 4). However, As levels in 2/103 and 5/103 of water samples exceeded the QCVN 10:2015/BTNMT and the CCME, respectively. The quality guidelines for As were developed from acute and chronic ecotoxicological data on marine species (CCME, 2007). Thus, the As contamination in the Southern coast of Vietnam did not exceed tolerable ecotoxicological risk levels.

Mean Cd levels in water collected from the estuary system were found to be similar to those reported from Mekong River (Cenci and Martin, 2004), but lower than those in water from the coast area of Can Gio, HCM (Pham et al., 2007), Ngoc Hien, Ca Mau (Nguyen, 2007) and the Malacca Straits (Looi et al., 2013). Concentrations of Cd detected in all water samples did not exceed the Vietnamese technical regulation on marine water quality (QCVN 10-MT: 2015/BTNMT, 5 μg/L), but, about 6% (6/103) of water samples had Cd levels exceeding the CCME guideline of 0.12 μg/L (Table 4).

Mean Pb concentrations in water collected from the estuary system were found to be higher than those reported from the coast area of Can Gio, HCM (Pham et al., 2007) and Ngoc Hien, Ca Mau (Nguyen, 2007), but comparable to samples from the Malacca Straits (Looi et al., 2013). Only 3/103 of water samples had Pb levels exceeding the Vietnamese technical regulation on marine water quality (QCVN 10-MT: 2015/BTNMT, 50 μg/L) (Table 4).

Generally, Hg is present in trace amounts in most of water samples. Compared with the

Vietnamese technical regulation on marine water quality (QCVN 10-MT : 2015/BTNMT, 1 μg/L) and quality guidelines for the protection of aquatic life recommended by the CCME (0.016 μg/L) (CCME, 2007; MONRE, 2015), Hg concentrations in all of samples were below the Vietnamese guideline, but 75/103 of samples had Hg levels exceeding the CCME guideline. According to the CCME (CCME, 2003), toxicity data on Hg for marine waters are much more limited. EC50s for inorganic Hg range from $<$ 5 - 55 μg/L for fish, from 1.2 - 20 μg/L for invertebrates and from 0.16 - 1002 μg/L for plants and algae. The LOAEL (lowest observed adverse effect level) of 0.16 μg/L was used to develop the guideline. The LOAEL was divided by a safety factor of 10 to give an interim Canadian water quality guideline of 0.016 μg/L or 16 ng/L.

4. Conclusions

Based on the results of this study, it may be concluded that the concentration of HMs in water collected from the Southern coast of Vietnam is quite uniform among provinces. The results indicated that the regions are in the range of 'unpolluted' water. These results could be useful for future relative studies of HMs contamination and monitoring programs to assess marine pollution originates from land-based sources. Further research on understanding the distribution and accumulation profiles of potential HMs in sediment and biota samples from these regions is clearly warranted.

Table 4. Comparison of concentrations of heavy metals (μg/L) in water with those from other sites and guidelines

Country	Region	As	Cd	Pb	Hg	Reference
Vietnam	South coast	5.07 ± 4.45	0.087 ± 0.320	7.28 ± 12.7	0.062 ± 0.070	This study
	Can Gio, HCM	31	1	3	< 0.5	Pham et al. (2007)
	Ngoc Hien, Ca Mau	0.4 - 23.3	0.18 - 2.63	0.06 - 8.96	Not detected	Nguyen (2007)
	Tien - Hau Rivers	1.48 - 49.47	-	-	-	Bui et al. (2011)
	Mekong river - dissolved	-	0.001 - 0.051	0.02 - 0.16	-	Cenci and Martin (2004)
	Mekong river - SPM	-	2 - 73	4 - 84	-	
Malaysia	Malacca Strait	4.98 - 86.14	Not detected - 5.66	Not detected - 28.6	-	Looi et al. (2013)
Vietnam guideline		20	5	50	1	MONRE (2015)
Canadian guideline		12.5	0.12	-	0.016	CCME (2007)

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Investigation of fermentation conditions for *Candida bombicola* ACTT22214 from molasses and soybean oil for sophorolipid production

Tho P. Le[1*], Huong T. T. Le[1], Hiep M. Dinh[2], & Hue B. T. Nguyen[1]

[1]Faculty of Biology and Biotechnology, University of Science, Ho Chi Minh City, Vietnam
[2]High-Tech Agriculture Park, Ho Chi Minh City, Vietnam

ARTICLE INFO

Research Paper

Received: October 18, 2018
Revised: December 02,2018
Accepted: December 14, 2018

Keywords

Antibacterial
Candida bombicola
Fermentation
Sophorolipid
Surfactant

***Corresponding author**

Le Phuoc Tho
Email: phuoctho022010@gmail.com

ABSTRACT

Sophorolipid (SL) is a biosurfactant belonging to the glycolipids group and was produced by harmless *Candida bombicola* ACTT22214 and has been widely used in many fields in our life. In order to search for appropriate condition for *C. bombicola* fermentation producing SL with high efficiency, this study focused on the investigation of the soy oil concentration, molasses concentration, fermentation time, pH and temperature. SL products were tested for antimicrobial activity, antioxidant, emulsifier, foaming ability. The highest content of SL was 43.27 ± 0.30 g/L under conditions of: soybean oil content 5%, molasses content 150 g/L, fermentation time 7 days, pH = 5, 28^0C fermentation temperature. The antibacterial activity of SL was good: the highest resistance to *Candida albicans* (16.33 ± 1.15 mm), good resistance to *Bacillus spizizenii* (13.67 ± 0.58 mm), resistance to *Staphylococcus aureus* (12.67 ± 1.15 mm), relatively weak resistance to *Pseudomonas aeruginosa* (11.33 ± 0.58 mm) and *Escherichia coli* (9.67 ± 0.58 mm). The antioxidant capacity of SL was quite high with an IC_{50} value of 6.024 mg/mL. The emulsifying capacity of SL was equivalent to the emulsification of the tween 20 at a concentration of 5 – 10 mg/mL. SL had the ability to foam evenly from concentrations of 5 to 20 mg/mL but not higher than the corresponding concentrations of tween 20, SL was smooth, even, stable longer than tween 20.

Cited as: Le, T. P., Le, H. T. T., Dinh, H. M., & Nguyen, H. B. T. (2018). Investigation of fermentation conditions for *Candida bombicola* ACTT22214 from molasses and soybean oil for sophorolipid production. *The Journal of Agriculture and Development* 17(6), 50-62.

1. Introduction

Surfactants are bipolar compounds reducing surface tension between liquids with liquids, solids or gases, therefore they are able to combine and dissolve in water or other liquids. Most used surfactants are originally from petroleum and chemically synthesized, these compounds are environmental hazards because of their low biodegradability and high toxicity when dissolved in water (Bogaert, 2008). Thus, searching for replaceable biological surfactants is a necessary strategy. Microbiologically synthesized surfactants, including sophorolipid produced by fermentation of *C. bombicola* are considered due to sophorolipid application and commercialization potential, and significantly because sophorolipid are produced by non-pathogenic and safe *C. bombicola* (Bogaert et al., 2011) which performs high productivity (Dubey et al., 2013).

Sophorolipid are glycolipid biological surfactants, they are bipolar molecules formed by one disaccharide sophorose group bonding hydroxyl group of the second-to-last carbon atom in the

C16 - C18 fatty acid chain. For the production of sophorolipid by the fermentation of *C. bombicola*, two main substrates are provided: hydrophiliccarbon source: glucose and hydrophobic carbon source: fatty acids, oil, fatty acid ester, alkan. There are two types of sophorolipid: free acid form and lactone ring (Bogaert et al., 2007). This difference results in distinction in sophorolipid physical and chemical characteristics, acidic sophorolipid show foaming ability and high solubility, otherwise, sophorolipid with lactone rings show antibacterial activity and reduce surface tension effectively.

Sophorolipid are used in food, medicine, cosmetics, detergents (Bogaert et al., 2007). Recent research provided some particular applications of SL. Sophorolipid are used in washing powder as a detergent (Gobbert et al., 1984). Sophorolipid emulsification is utilized in petrochemistry. They are used in recovery of secondary petroleum products, removing hydrocarbons in crude petroleum. Sophorolipid are used in treating hydrocarbon contaminated soil and water, absorbing heavy metals in sediment and improve the quality of flour in food industry (Gobbert et al., 1984; Mager et al., 1987; Daniel et al., 1998). Sophorolipid perform antibacterial activity in pimples , dandruff and body's smell treatment, protect skin and hair, stimulate metabolism of epithelial fibroblast cells and synthesis of collagen for skin (Gorin et al., 1961; Cooper & Paddock, 1984). Sophorolipid also inhibit free radicals and aging elastase enzyme, promoting skin healing and whitening (Isoda et al., 1997). Diacetyl lactone SL can kill carcinoma cell lines such as liver cancer cell line H7402, decrease mortality rate due to septic shock in lab rats (Kim et al., 2005; Daverey & Pakshirajan, 2009), inhibit the development of leukemia cells (Spencer et al., 1970).

For industrial production and commercialization, SL must be competitive with chemical surfactant in 3 main perspectives: cost, uses and yield. Therefore, searching for low-cost material and setting up procedure for producing SL effectively are important. This study investigates appropriate conditions for *C. bombicola* fermentation in producing SL from molasses and soybean oil, in addition to conducting test of physical, chemical and biological characteristics of obtained SL.

2. Material and Methods

2.1. Material

Lyophilized *C. bombicola* ATCC 22214 was provided by professor Kim Eun-Ki, Inha University, South Korea. Strain was grown in YM Broth (glucose 1%, yeast extract 0.6%, peptone 0.5%); 2,2-diphenyl-1-picrylhydrazyl (DPPH), 1',4"-sophorolactone 6',6"-diacetate were provided by Sigma (St. Louis, USA). Organic solvent: methanol, ethyl aceate, petroliumether were provided by Xilong company (China). Soy oil content 89.9% (Simply brand) was produced by Cai Lan vegetable oil company; molasses content 55% were provided by Kim Minh company; tested bacterial strains were provided by the Research Center of Bioactive Natural Products – University of Science, Ho Chi Minh City.

2.2. Methods

2.2.1. Propagation of *Candida bombicola* before fermentation

Lyophilized *C. bombicola* was propagated in YM media, after 48 hours, primary culture was sub cultured, producing secondary culture; *C. bombicola* propagation conditions include: temperature of 28^0C and shaking speed at 180 rpm in 48 hours. Secondary culture was used for fermentation and experiments.

2.2.2. Experiments for investigating appropriate conditions for *Candida bombicola* fermentation producing sophorolipid

Five one-factor experiments were randomly designed to investigate conditions including: molasses content, soybean oil content, temperature, pH and fermentation time (sophorolipid obtaining time) (Figure 1). Media used in these experiments contain: yeast extract 0.5%; KH_2PO_4 0.1%; $MgSO_4.7H_2O$ 0.05%, $CaCl_2.2H_2O$ 0.01%; NaCl 0.01%; peptone 0.07%. These experiments were conducted under conditions of shaking-speed at 180 rpm and fermentation medium was inoculated with 5% (v/v) seed medium. Erlenmeyer flasks (250 mL) with 50 mL of media were used in the above experiments. Crude sophorolipid yield was observed.

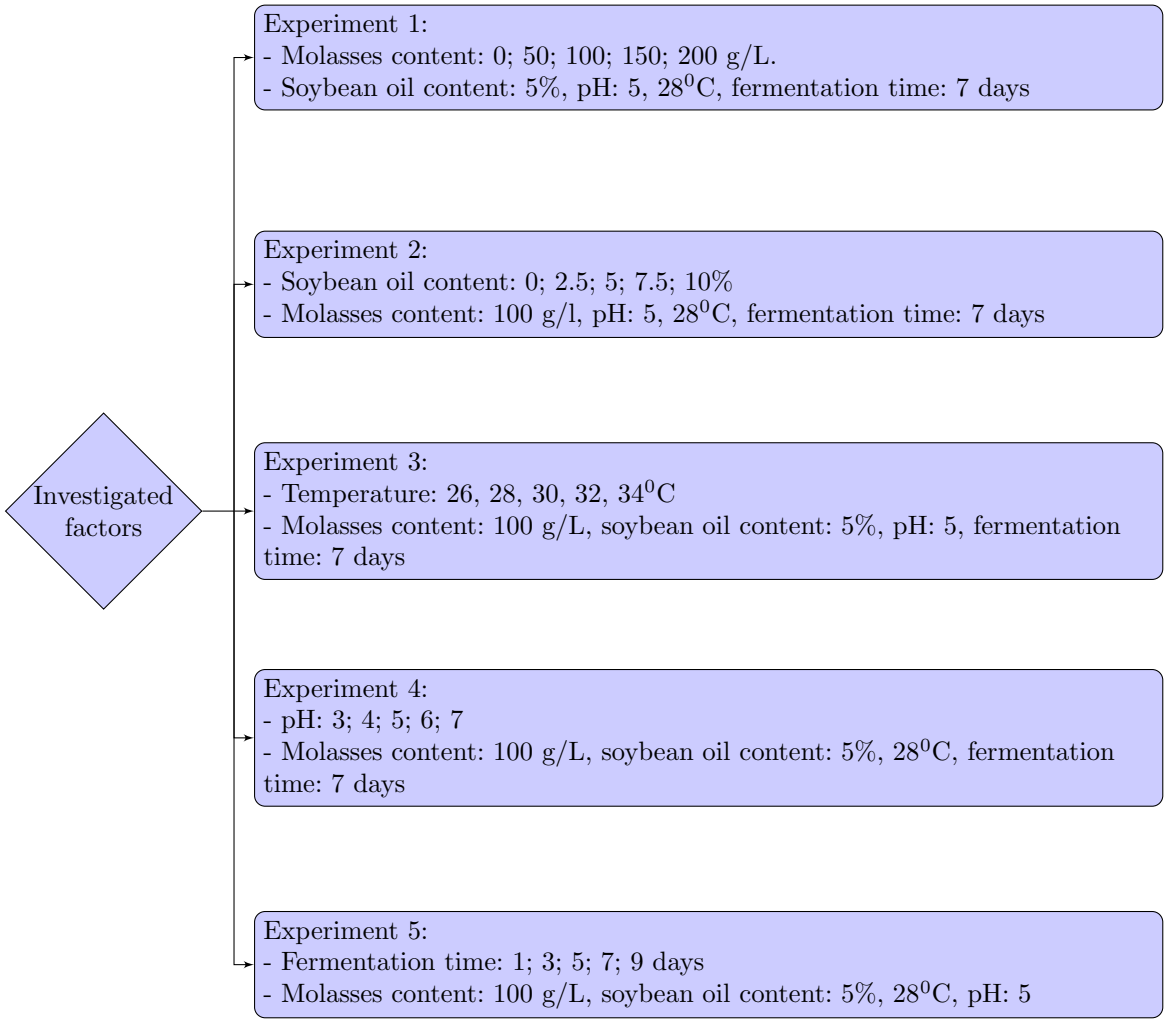

Figure 1. Experiments for investigating appropriate conditions for *C. bombicola* fermentation producing sophorolipid.

2.3. Sophorolipid separation from fermented broth and analysis of obtained SL by thin layer chromatogrphy TLC

2.3.1. Sophorolipid separation from fermented broth

Ethyl acetate (EtAc) was added into culture broth (1:1 v/v, twice), then centrifuged (6,000 rpm in 5 minutes), supernatant was collected and vacuum dried in rotary evaporatorat 40^0C to remove EtAc. After that, Petroliumether: Methanol (1:1 v/v, twice) was added and collect the lower layer (crude SL and methanol), then vacuum dried in rotary evaporator at 40^0C to remove methanol, crude SL was weighed (Figure 2).

2.3.2. Analysis of obtained SL by thin layer chromatogrphy TLC

Crude sophorolipid was spotted on chormatography plate then immersed in solvent system containedchloroform:methanol: H_2O (80:10:2 v/v/v) in 30 minutes. 1',4"-sophorolactone 6',6"-diacetate was used as the standard. After elution, the plate was sprayed with acid sulfuric 90% and dried at 100^0C, the spots were visualized.

2.4. Sophorolipid activity analysis

2.4.1. Emulsification test

Sophorolipid was diluted in DMSO 5% solution into different concentrations then add 5mL of each sample into test tubes (diameter: 1.6 cm, height: 16 cm). Add the same volume of diesel into these tubes then vortex in 2 minutes, leave for 10 minutes and observe emulsification after 10 minutes, 12 hours and 24 hours, measure the height of emulsified layer, emulsification index after 24 hours was calculated as the formula: E24 = (height of emulsified layer/total height) x 100.

2.4.2. Foaming ability of SL

Sophorolipid was diluted in DMSO 5% solution into different concentrations. After that, add the same volume of 10 mL of each sample into test tubes (diameter: 1.6 cm, height: 16 cm), tight the caps and shake the tubes vertically in 1 minute then leave them for a while. Foaming was observed, and height of foam layers was measured after shaking and after leaving for 5 minutes. Repeat the same procedure or the control (tween 20), the experiment was repeated 3 times.

2.4.3. Antibacterial activity

Testing of antibacterial activity was conducted using agar diffusion method. Bacterial strains: *Escherichia coli*, *Staphylococus aureus*, *Bacillus spizizenii*, *Pseudomonas aeruginos* were inoculated in LB agar plates and Candida albicansin SD agar plates with paper disks were placed on, 20 μL SL (100 mg/mL) was added to paper disks and incubated 37^0C in 1 – 2 days. Inhibition zones were observed.

2.4.4. Free radical scavenging activity

Free radical scavenging activity was determined using DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. Crude SL was dissolved in methanol into different concentrations, 100 μL of each sample was added into 96-well plate, 100 μL DPPH 300 μM was then added and mixed. The plate was incubated at 37^0C in 30 minutes, then OD was measured at 517 nm wavelength. The percentage of free radical scavenging was calculated as the formula: % antioxidant = (1 - OD sample/OD control) x 100.

3. Results and Discussion

3.1. Morphology of *C. bombicola*

C. bombicola was recovered and grown in YM media after 2-day inoculation at 28^0C. Culture was diluted into concentration of 10^{-9} and inoculated on YM agar plates, the colonies showed creamy color, smooth and glistening dome shape with entire margin, the diameter was from 0.4 - 0.7 cm. Observing under microscope using 100X objective lens showed the elongated oval shape of *C. bombicola* and its budding reproduction (Figure 3).

3.2. Testing of appropriate conditions for *C. bombicola* fermentation producing SL

Recovered *C. bombicola* was grown in YM media in 2 days at 28^0C, with 180 rpm of shaking speed to obtain the concentration of 109 CFU/mL. This culture was used as seed medium for below experiments.

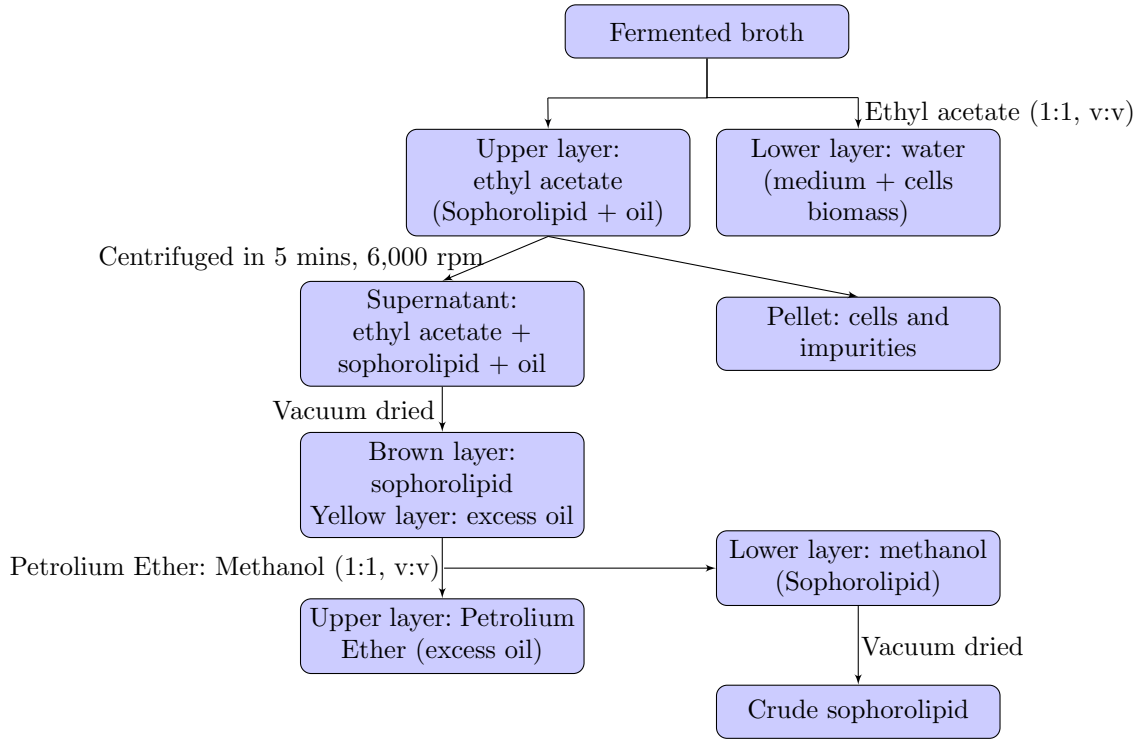

Figure 2. Procedure of separating sophorolipid from fermented broth.

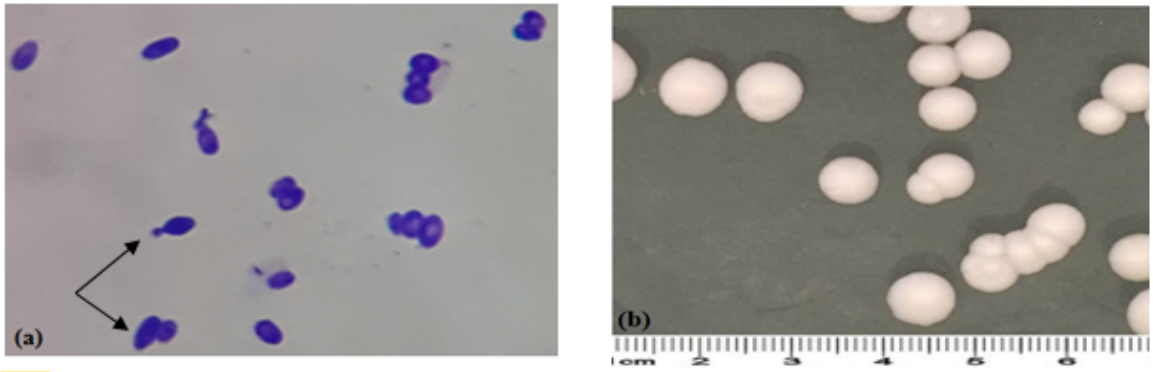

Figure 3. Morphology of yeast *C. bombicola*. (a) *C. bombicola* observed under microscope with 100X objective lens shows oval shape and budding reproduction, (b) *C. bombicola* colonies on YM agar plates after 48-hour incubation at 28^0C.

3.2.1. Investigation of molasses content affecting the fermentation of *C. bombicola* producing SL

C. bombicola was respectively inoculated into testing samples containing molasses contents of 0; 50; 100; 150, 200 g/L. The result showed that the highest SL production of C. bombicola was 43.27 ± 0.31 g/L when the fermentation media was composed of 150 g/L. The results were shown the difference from the media composed of 100 g/L was not significant. When increasing the molasses concentration to 200 g/L, SL production decreased. It was probably due to the high osmosis pressure caused by the high concentration. Therefore, the growth and development of *C. bombicola* were affected in the adapting stage (Table 1).

Table 1. Investigation of molasses content affecting the fermentation of *C. bombicola* producing SL

Treatments	Molasses content (g/L)					
	0	50	100	150	200	
Sophorolipid (g/L)	$0.33^{d} \pm 0.12$	$33.33^{c} \pm 0.50$	$41.87^{a} \pm 0.64$	$43.27^{a} \pm 0.31$	$38.33^{b} \pm 0.83$	

[a-d] Average values followed by letters were statistically significant difference by $LSD_{0.01}$ test.

3.2.2. Investigation of soybean content affecting the fermentation of *C. bombicola* producing SL

C. bombicola was respectively inoculated into testing samples containing soybean contents of 0; 2.5; 5.0; 7.5, 10%. The results showed the highest yield of SL was 42.27 ± 0.31 g/L at 5% of soybean content. However, there was no significant difference from the media containing the soy oil concentration of 7.5%. On the other hand, when increasing the amount of soy oil to 10%, SL production decreased significantly. This was probably due to the lower density of soy oil compared to water and its indissolubility in water. Therefore, the high concentration disrupted the diffusion of oxygen in the media (Table 2).

3.2.3. Investigation of temperature affecting the fermentation of *C. bombicola* producing SL

C. bombicola was respectively inoculated into testing samples for fermentation at the temperature 26; 28; 30; 32; 34^0C. The results showed the highest yield of SL was 41.87 ± 0.53 g/L at the temperature of 28^0C. At 34^0C, SL yield was nearly none because *C. bombicola* was almost unable to grow so the production of SL was stopped (Table 3).

3.2.4. Investigation of pH affecting the fermentation of *C. bombicola* producing SL

C. bombicola was respectively inoculated into testing samples for fermentation at pH conditions: 3; 4; 5; 6; 7. The results showed the highest yield of SL was 42.07 ± 0.53 g/L at pH = 5. We found that there was no significant difference from the result when pH = 6 (Table 4).

3.2.5. Investigation of product obtaining time affecting the fermentation of *C. bombicola* producing SL

C. bombicola was respectively inoculated into testing samples for fermentation with obtaining time 1; 3; 5; 7; 9 fermenting days. The result showed the highest SL concentration (41.67 ± 0,61 g/L) when the obtaining time was after 7 days. The SL production decreased significantly after 9 days of producing because when the substrate became exhausted, *C. bombicola* would use SL as a nutrient source. Therefore, the in-

Table 3. Investigation of temperature affecting the fermentation of *C. bombicola* producing SL

Treatments	Temperature (^{0}C)				
	26	28	30	32	34
Sophorolipid (g/L)	$38.40^{b} \pm 0.53$	$41.87^{a} \pm 0.31$	$41.00^{a} \pm 0.72$	$36.53^{c} \pm 0.61$	$0.20^{d} \pm 0.00$

[a-d]Average values followed by letters were statistically significant difference by $LSD_{0.01}$ test.

Table 2. Investigation of soy content affecting the fermentation of *C. bombicola* producing SL

Treatments	Soybean content (%)				
	0.0	2.5	5.0	7.5	10
Sophorolipid (g/L)	$13.40^{d} \pm 0.53$	$31.20^{c} \pm 0.72$	$42.27^{a} \pm 0.42$	$41.00^{a} \pm 0.20$	$38.53^{b} \pm 0.61$

[a-d]Average values followed by letters were statistically significant difference by $LSD_{0.01}$ test.

appropriate SL obtaining time decreased the SL amount significantly (Table 5).

Therefore, after the above experiments, the appropriate conditions for *C. bombicola* fermentation producing SL were shown: 150 g/L of molasses content, 5% of soybean oil content, 28^0C, pH = 5, obtaining time is after 7 days of fermentation. From the above data, fermentation of *C. bombicola* was conducted, the yield was 43.27 ± 0.30 g/L. SL obtained is brown,viscous liquid (Figure 4).

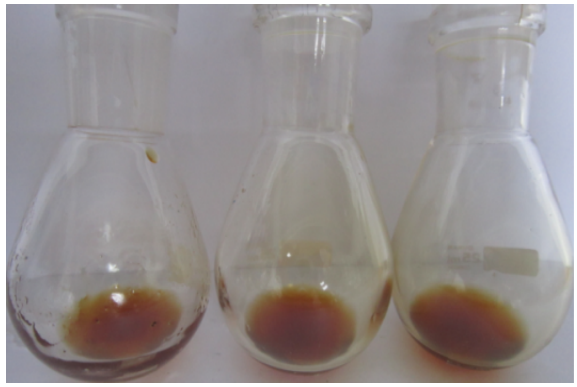

Figure 4. Obtained SL from the fermentaion of *C. bombicola.*

According to Table 6, SL yield was considered high (43.27 g/L), higher than results in the research of Asmer et al. (1988), Nguyen et al. (2017) and Le et al. (2016) which were 34; 21.8 and 14.6 g/L, respectively. However, SL yield was lower than in research of Cooper & Paddock (1984), Deshpande & Daniels (1995), and Zhou et al. (1992) which were 68; 97 and 138 g/L, respectively. The difference in SL yield can be due to different source of substrates, fermenting conditions as well as the time of obtaining SL. Therefore, it is important to have further research on optimal conditions as well as appropriate substrate contents for the fermentation.

3.3. Analysis of obtained SL by thin layer chromatogrphy TLC

Figure 5 shows the existance of 1',4"- sophorolactone 6',6"-diacetate in crude SL, proving the suitability of crude SL extraction in obtaining SL from fermented broth. In addition, visualized spots were at different positions showing different structures of obtained SL.

Table 4. Investigation of pH affecting the fermentation of *C. bombicola* producing SL

Treatments	pH					
	3	4	5	6	7
Sophorolipid (g/L)	$38.87^{c} \pm 0.31$	$40.13^{bc} \pm 0.42$	$42.07^{a} \pm 0.46$	$41.33^{ab} \pm 0.61$	$37.13^{d} \pm 0.76$

[a-d]Average values followed by letters were statistically significant difference by $LSD_{0.01}$ test.

Table 6. Comparison of SL production reported by others to our result

Media components (g/L)	Time (h)	Max level of SL (g/L)	References
Glucose: 100; oleic: 36; yeast extract: 10	120	34.00	Asmer et al. (1988)
Glucose: 100; sunflower oil: 95; yeast extract: 5	100	68.00	Cooper & Paddock (1984)
Glucose: 100; safflower oil:105	154	138.00	Zhou et al. (1992)
Glucose: 100; catfish fat: 100 yeast extract: 5	168	21.80	Nguyen et al. (2017)
Glucose: 100; coconut oil: 100; yeast extract: 5	168	14.60	Le et al. (2016)
Glucose: 100; fat: 100	60	97.00	Milind Deshpande & Lacy Daniels (1995)
Molasses: 150, soybean oil: 50; yeast extract: 5	168	43.27	This work

Table 5. Investigation of product obtaining time affecting the fermentation of *C. bombicola* producing SL

Treatments	Product obtaining time (days)				
	1	3	5	7	9
Sophorolipid (g/L)	$0.27^{d} \pm 0.12$	$5.40^{c} \pm 1.25$	$35.53^{b} \pm 1.10$	$41.67^{a} \pm 0.61$	$35.27^{b} \pm 0.90$

$^{a\text{-}d}$Average values followed by letters were statistically significant difference by $LSD_{0.01}$ test.

Figure 5. Chromatogram of SL analysis obtained SL; (C) standard substance (1',4"-sophorolactone 6',6"-diacetate), the Rf value is 0.55.

3.4. Testing of physical, chemical and biological of SL

3.4.1. Foaming ability of SL

To determine the foaming ability of SL, shake test tubes containing SL and 20 tween (control) dilutled in DMSO 5% solution into different concentrations were shaked vertically in 1 minute, observing and measure the height of foam layer twice: after shaking and after leaving for 5 minutes.

Based on Figure 6, the foaming ability of the two surfactants SL and tween 20 increased as their concentrations increased. The amount of foam produced by tween 20 was much higher than by SL. After 5 minutes, the amount of foam formed by the two surfactants was reduced but negligible, the foam volume was maintained quite well. At the same time, smooth, uniform, and stable foam produced by SL was formed, relatively durable compared to the tween 20.

3.4.2. Testing the emulsifying ability of SL

Determination of emulsifying ability of SL was carried out by uniformly vortexing SL and tween 20 solutions diluted in 5% DMSO solution into different concentrations in 2 minutes, leave them to stand still and observe the emulsion. After 10 minutes, 12 hours and after 24 hours, measure the height of the emulsified layer, determine the E24 index of solutions, with E24 being the emulsifying percentage after 24 hours.

Based on Figure 7, the emulsification of both SL and tween 20 increased as the concentration increased. However, at the concentration of 20 mg/mL, the emulsification of SL is very low, almost none; while at concentrations of 5 mg/mL and 10 mg/mL, the emulsification of SL is almost equivalent to tween 20. The decrease or loss of emulsification capacity when the concentration of SL increases is due to SL's tendency of aggregate into large masses, its contact and dispersion into the water phase and the oil phase decreases, sometimes the accumulation was too much, the SL mass becomes heavier, leading to deposition to the bottom and making the emulsification not good.

3.4.3. Examination of antibacterial activity of SL

In order to determine the antibacterial activity of SL, aspirate 20 μL of 100 mg/mL SL solution onto paper plates placed on the surface of agar containing the tested microorganisms. Positive antibiotics are gentamicin (100 μg/ml) or terbinafine (100 μg/mL) and negative methanol (90%).

Based on Figure 8 and Table 7, it shows that SL is most resistant to *C. albicans*, followed by *B. spizizenii*, then *S. aureus*, and finally weak resistance to *P. aeruginosa* and *E. coli*. Thus, it is possible to conclude that SL is more resistant to Gram (+) bacteria than Gram (-) bacteria. This finding is consistent with researches the research of Kim et al. (2005). The resistance to *C. albicans* which is a fungus parasitic on human body of SL proves that SL has the potential to be used as safe detergent and antiseptic.

Table 7. Results of the ring diameter of sophorolipid resistance for microorganisms

Microorganisms	Antibacterial ring diameter of SL (mm)
B. spizizenii	13.68 ± 0.58
C. albicans	16.33 ± 1.15
E. coli	09.67 ± 0.58
P. aeruginosa	11.33 ± 0.58
S. aureus	12.67 ± 1.15

3.4.4. Investigation of the antioxidant capacity of sophorolipid by DPPH (1,1-Diphenyl-2-picrylhydrazyl)

Add 100 μl of each SL solutions at different concentrations into 96 well microplates. Af-

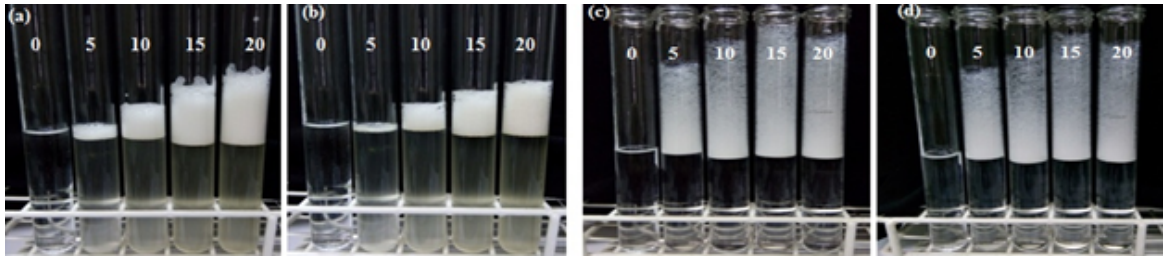

Figure 6. Results of the foaming test of SL and tween 20. (a) SL after shaking, (b) SL after leaving for 5 minutes, (c) Tween 20 after shaking, (d) Tween 20 after leaving for 5 minutes. SL/tween 20 concentrations were 0; 5; 10; 15; 20 (mg/mL).

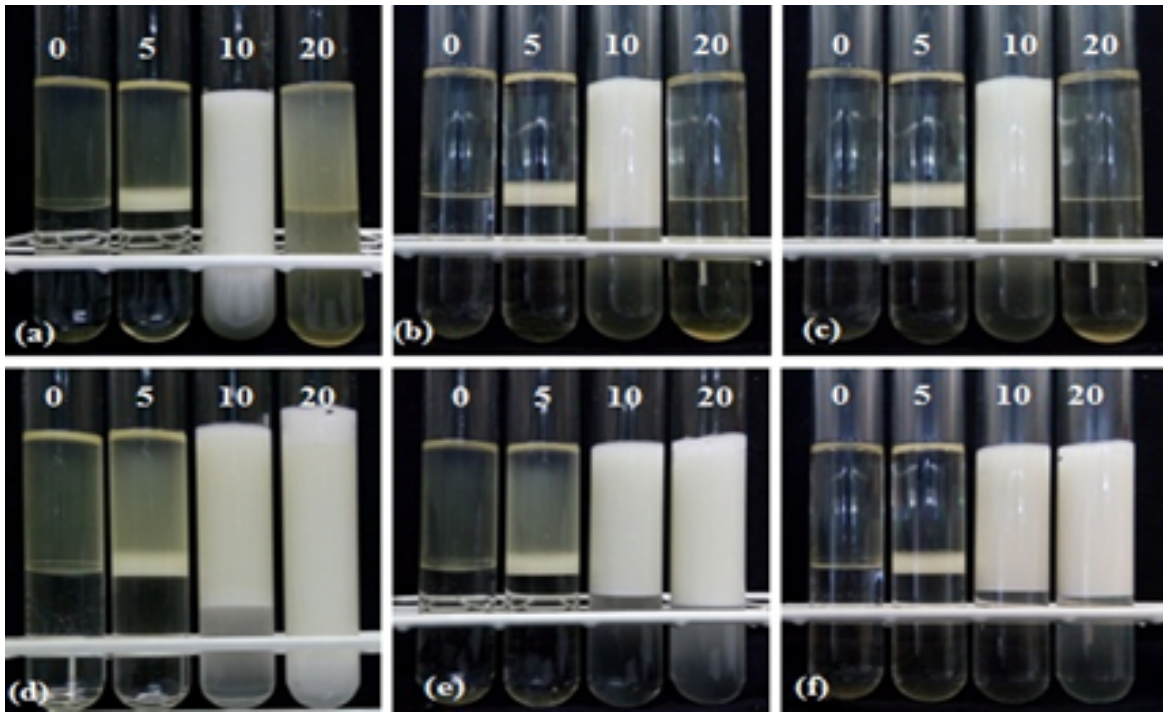

Figure 7. Results from emulsion investigations of SL and tween 20. (a), (b), (c) emulsification of SL after 20 minutes, 12 hours and 24 hours; (d), (e), (f) emulsification of tween 20 after 20 minutes, 12 hours and 24 hours. The investigated SL/tween 20 concentrations were 0; 5; 10; 20 (mg/mL).

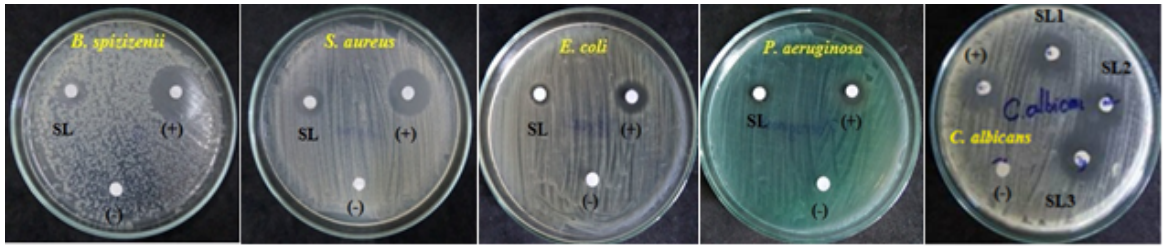

Figure 8. Antibacterial results of SL with paper disk method, (+): Positive test; (-): Negative test.

ter adding 100 μl DPPH, incubate at 37^0C for 30 minutes, then determine the color by ELISA reader and acknowledge the percentage of antioxidant corresponding to each concentration. Therefore, determination of the expressed curve shows the relationship between the substance concentration and the corresponding antioxidant percentage of SL.

From Figure 9, the IC_{50} value is 6.024 mg/mL. With IC_{50} value of 6.024 mg/mL, it can be seen that the antioxidant capacity of SL is quite good. This can be applied to produce useful antioxidants in cosmetic products such as skin lotion, aiming to care and protect the skin.

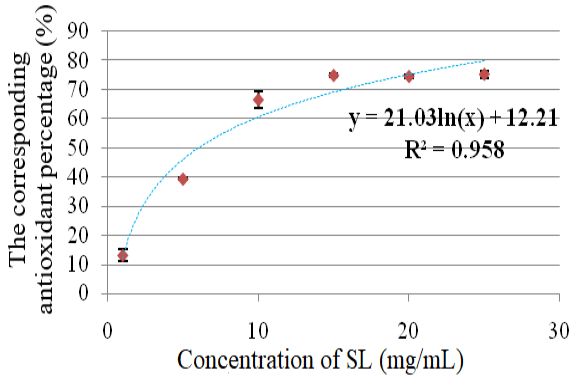

Figure 9. The graph shows the antioxidant percentages of SL by DPPH.

4. Conclusion

Initially, suitable conditions for fermentation of C. bombicola for SL production are found to be quite high at 43.27 g/L with the following parameters: 150 g/L of molasses; 5% of soybean oil, fermentation temperature is 28^0C, pH = 5, fermentation time is 7 days. SL product has good antimicrobial properties, antioxidant capacity with an IC_{50} of 6.024 mg/mL, the ability to foam, durable and stable emulsification equivalent to chemical surfactants. Therefore, it shows the high application potential of SL in this study in areas such as cosmetics and detergents.

Acknowledgements

The study was funded by the Ho Chi Minh City Department of Science and Technology.

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Effects of film coating materials on the quality of postharvest 'Sanh' orange fruits (*Citrus nobilis* var. Typica) during storage

Thach A. Nguyen*, & Ngan T. Nguyen
Department of Agriculture and Food Technology, Tien Giang University, My Tho, Vietnam

ARTICLE INFO

Research Paper

Received: September 28, 2018
Revised: November 16, 2018
Accepted: December 18, 2018

Keywords

Coating
PVC
'Sanh' orange
TSS
Weight loss

***Corresponding author**

Nguyen Ai Thach
Email: nguyenaithach2001@gmail.com

ABSTRACT

Postharvest orange fruit coating is an effective method to replace natural waxes which lost during washing and handling. The coatings can reduce water loss and impart gloss to the fruit. In this study, the oranges were stored at room temperature (30 ± 2^0C) with five coating materials: polyethylene (PE), and polypropylene (PP) bags, polyvinyl chloride (PVC) film, Citra Shine preservatives and 1% chitosan and the control were used. Some typical nutritional values and weight loss were determined during storage time. Research results show that PVC coated 'Sanh' oranges could be prolonged their shelf life up to 25 days with low damage ratios (7.10%, lower than other bags) which were acceptable in appearance with green peel color. Ascorbic acid content still maintained at a high level (12.32 mg/100 g), and weight loss relatively low (13.91%). Moreover, pH (3.77) and TSS (9.70) values did not significantly change during storage time.

Cited as: Nguyen, T. A., & Nguyen, N. T. (2018). Effects of film coating materials on the quality of postharvest 'Sanh' orange fruits (*Citrus nobilis* var. Typica) during storage. *The Journal of Agriculture and Development* 17(6), 63-71.

1. Introduction

'Sanh' orange (*Citrus nobilis* var. Typica) is the most popular grown fruit tree in the Mekong Delta. It is made up of soft texture, the fresh quality, juicy, light sweet flavor, and high nutrious value that are very important in human diet since it contains essential vitamins. 'Sanh' orange is a non-climateric fruit, and has a long shelf-life compared to other types of citrus, thus there is a potential for export markets. During the preservation of postharvest 'Sanh' orange, besides the effective of low temperature or cold storage in which is known as one of the most effective method of prolonging the postharvest life of fresh produce and slows down ripening, retards water loss, reduces decay and enhances visual quality, the utilization of synthetic films coating on fruits such as polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), chitosan,... (Trout et al., 1952; Bramlage, 1986; Drake & Nelson, 1990) was considered as one of several treatments developed to maintain the quality of products and reduce the post harvest decay. However, each fruit has own features (respiratory intensity, ethylene production, etc.), and this characteristics alter at different maturity period and in different environmental conditions (temperature and moisture). Porat et al. (2004) indicated that citrus was relatively hard to decay and can be stored for 6 - 8 days. Nevertheless, the varying degrees damage will be limited and lost due to commercial quality spoilage in the preservation of postharvest. Modifying the internal atmosphere in packaging and using coating films reduced effectively the injuried chilling when the types of different damage

to the left shell are not related to the cooling as hatching, fallow, wilting and ageing (Wilson & Wisniewski, 1989; Baldmin et al., 1995). Preserving 'Valencia' orange by using coatings of candelilla in the combination with PE packaging, was stored at 15 - 25^0C that retained its hamonious taste after 9 - 16 days during storage (Hagenmaier & Shaw, 2002). In addition, 'Sanh' orange trees usually flowers in the crop, therefore, the quantity of 'Sanh' orange is so large in the harvest that consumers can not be utilized in the short term. Thus, determining the appropriate preservation for 'Sanh' orange is too necessary in order to serve consumers in the long run as well as to supply raw materials for processing and export. The aim of this study is to characterize the effects of some coating applications on the quality changes, both physically and nutritionally on 'Sanh' orange at the room temperature storage.

2. Materials and Methods

2.1. Materials

Freshly 'Sanh' oranges were harvested in Tam Binh district, Vinh Long province at 7.5 months after flowering. The fruit was mature and ready for harvest at the orchard at 8 - 10 A.M, and 'Sanh' orange garden has been stopped spraying for 10 days before harvest. Fruits were selected for uniformity in maturity, shape, size, free of physical damage, and the average weight about 200 - 250 g.

2.2. Experiments

'Sanh' orange was washed by scrubbing gently the fruit surface in clean water to eliminate dirty on the shell, then dried with a soft towel in order to avoid the damage of its shell. Nextly, dip 'Sanh' oranges in a 0.04% chlorine solution for five minutes to demolish fungi on the surface. After five minutes, fruits were then dried naturally and ready to be used for the experiments. PE, PP and PVC packaging were purchased at local market in Viet Nam (produced by Tan Bach Dat Produce Trading Import Export Company Limited). Characteristics of some plastic packages used in this study: PE is flexible, durable and tear-resistant. A PP bag is non-toxic and high clarity, crystal clear bag. It provides a highly protective barrier against moisture and vapors. These poly bags delay evaporation and dehydration to preserve freshness and taste of packaged foods. PVC is non-toxic, light weight, good mechanical strength and toughness. It is resistant to vapors, chemical rotting, corrosion, shock and abrasion. Fruits were divided into six groups. Each group (sixteen fruits) was treated by different treatments including:

- Control: uncoated (CT).
- Fruits were wrapped with PE bag (one fruit/bag - size 15 cm x 20 cm, chisel five holes, hole diameter 1 mm) (PE).
- Fruits were wrapped with PP bag (one fruit/bag size 15 cm x 20 cm, unperforated) (PP).
- Fruits were wrapped a layer of PVC carefully (12.5 μm thick) pressed against the left shell (PVC).
- Fruits were coated into Citra Shine (ingredients: natural resins, fatty acid, polyethylene, casein, ammonium hydroxide 25%, pH 9-9.5, Cerexagri, Italy) 50% emulsion for 30 seconds and then take out (CITRA).
- Fruits were coated into 1% chitosan (powder made from shrimp shells, $\geq$ 75% (deacetylated), a bulk density of 0.15–0.3 g/cm^3, Merck) solution for 30 seconds and then take out (CHITO). The experiments were undertaken in four replications and each research unit was four fruits. All treated fruits were monitored at room temperature (30 $\pm$ 2^0C) and relative humidity (RH) 60 $\pm$ 5% for the quality characteristics.

2.3. Physico-chemical analyses

The chemico-physical analysis of the 'Sanh' orange was conducted in triplicates. Ascorbic acid content was analyzed by Association of Official Agricultural Chemists standard (AOAC, 2004). Accordingly, orange pulb samples (5 g) were extracted in 20 mL of 1% HCl using a chilled pestle and mortar, and then the homogenate was filtered. The juice pulp in 100 mL of distilled water was titrated with previously prepared 20 mL aliquot of standardized 0.001 N dichloroindophenol solution.

Grind about fifty gram orange pulp in a blender or pestle and mortar, transfer to a 100 mL beaker. Total soluble solids (TSS-oBrix) and pH value was determined by using a refractometer (Model Atago Digital DBX-5) and digital pH meter (Model PHS-2F), respectively.

The color of fruit peel is frequently determined at nine points randomly distributed on the equa-

torial region of a fruit by using Minolta colorimeter (Model CR-300, N.J.), with three fruits for each replicate and taking the average. This practice can lead to biased results because these points represent the equatorial region only and not the total area of the fruit peel.

Weight loss (%) was expressed as the percentage decrease in fruit weight, using the following formula: L (%) = $[(M_i–M_f)/M_i] \times 100$, with Mi being the initial weight (g) and Mf being the fruit weight after an indicated period of storage (g).

Incidence of fruit diseases damage was a determined as percentage of the number of decayed fruits from the initial number after an indicated period of storage.

The rate of stem loss = (total number of pods/total number of observations) × 100.

2.4. Statistical analysis

The data in this study was carried out using SPSS software (version 2011). Analysis of variance (ANOVA) evaluated differences between treatments. A value ($^*P < 0.05$, $^{**}P < 0.01$) was considered statistically significant for comparison by Duncan's method. The means and standard deviations were also caculated and plotted using Microsoft Excel software 2003 (Microsolft, USA).

3. Results and Discussion

3.1. Weight loss (%)

The results in Figure 1 showed that percentage weight loss of 'Sanh' orange increased with the storage period progressed. The percentage weight loss between samples had different significance ($P < 0.05$) during 30 days of storage. After 15 days of storage, the weight loss of control samples was 15.64%. At that time, the commercial value of 'Sanh' orange fruits had decreased due to the fruits started to get softer. Segments of the navel orange were difficult to separate which were bland in flavor. Orange peel turned into color yellow and wrinkled (data not shown in this research). Therefore, weight loss percentage was unacceptance (more than 15%) that has been recommended. At the end of the storage period, the percentage weight loss of wrapped PVC fruits was the lowest (15.10%) compared to those ones. The control sample (CT) was totally damaged (100%), coated chitosan fruits was the highest

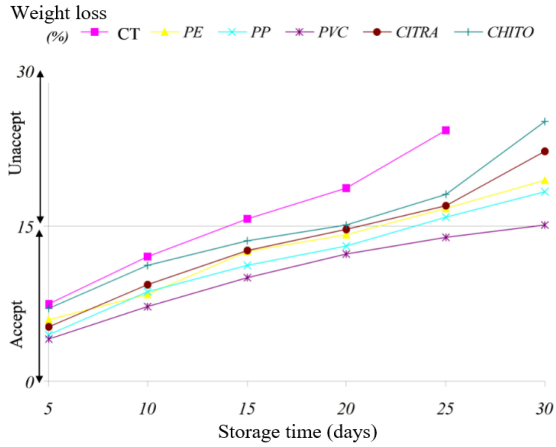

Figure 1. Effect of using films coating on the weight loss of 'Sanh' oranges during storage.

weight loss (25.10%), followed by coated Citra Shine (22.24%). Overall, the unacceptable percentage weight loss was observed in the wrapped PP samples (over 15%). The respiratory process enhances dehydration in fruits, increase water loss, the ripening process happens quickly, and thereby the decline in fruit quality. These findings are in agreement with other studies described a direct correlation between the internal gas modification of coated fruit and coating thickness, which depends on solid content and density of the coating formulation (Navarro-Tarazaga & Perez-Gago, 2006). According to Nguyen et al. (2005), they treated 'Sanh' orange with 4 - 6% potassium sorbate concentrations combined with 1% chitosan film only reduced the incidence of 4% weght loss after one month of storage at 4 - 6^0C. Simultaneously, the gel system of aging cells also has the tendency to lose water in fruits of water, so the evaporation rate increased significantly.

3.2. Decay incidence

After 15 days of storage, 'Sanh' orange was against the postharvest decay (Table 1) due to the fact that fruits were treated with chlorine before storage, reduced the incidence of fruit rot. Hence, the anti-fungal activities by using chlorine (0.04%) in 5 minutes killed the funguses on the surface of 'Sanh' orange, thus limiting harmful fungus. At the room temperature storage, 'Sanh' orange was damaged after the next 15 days of storage except for PVC, Citra Shine and chitosan samples that had lower decay percentage. How-

ever, there was a statistically significant difference at 1% in the values of incidence of fruit rot between the wrapped and unwrapped fruits, the highest decay incidence was the control sample (16.60%).

After 30 days of storage, the whole samples increased the decay incidence and had a statistically significant difference at $P < 0.01$, PP samples were the highest incidence of damage (55.80%), PVC samples (10.80%) and Citra Shine samples (12.90%) were the lowest. The high incidence of fruit rot does not satisfy conditions of preservation process due to several direct impacts on the quantity and the quality of fruit. Decay due to natural infection during the whole storage was relatively low as fruit were sanitized with a fungicide. The films and coatings can suspend decay by reducing senescence, which causes more susceptibility to pathogenic infection in produce due to damage of cellular or tissue integrity (Tanada-Palmu & Grosso, 2005). After 30 days of storage, the whole samples had the higher decay incidence (over 10%), the highest was PP samples (55.80%), the lowest was PVC samples (10.80%).

3.3. The rate of stem loss

The rate of stem loss did not occur afer the first 15 days of storage; however, in the next 15 storage days, this rate was noted in many samples and had a statistically significant difference at $P < 0.01$ (Table 2). After 20 days of storage, the shedding of fruit began to appear in the whole samples, the highest was still PP samples (24.60%), this rate was quite high in the control samples (16.10%), the lowest was PVC samples (3.70%). The rate of stem loss continued to increase over the storage process until the 30th day, all most samples appeared this problem, PP samples had the highest rate (44.80%), Citra Shine samples (16.10%) and the lowest was PVC samples (8.20%).

3.4. Color

Overall, the colourful alteration ($\triangle$E) of the shell increased with the stored period and after each time of observation, and had statistically significant difference between the samples varies. The results in Table 3 showed that $\triangle$E between treatments were statistically different at room temperature from the 5^{th} day to the 30^{th} day during the storage period. After 25 days of storage, the colourful alteration ($\triangle$E) of control samples (50.69) and PE samples (51.20) increased meaningfully and were statistically significant difference at 5%. The colourful change ($\triangle$E) of control samples and PE samples were larger than the other's. It may be due to dehydration in fruits, respiratory process, and ripening process occurs rapidly, and thereby chlorophyll content declines quickly. Additionally, appearance refers to color, glossiness, and absence of visual defects on food products. During their storage, food products are susceptible to oxidation reactions and enzymatic browning, which cause undesired color changes and moisture loss that lead to size shrinkage and gloss reduction. Skin blemishes may be caused by exposure to pests, microbial and mechanical damages. Applying films coating or packaging prior to the storage can inhibit this deleterious effects. Moreover, appropriate coating components can enhance the gloss and visual attractiveness of the food product. On the 30th day of storage, maximum colourful alteration value was observed in PE samples (52.77), whereas the minimum values were noted in Citra Shine samples (49.55), and followed by chitosan samples (49.6), and PVC samples (50.51). The lowest $\triangle$E values was alteration of three treatments (green to yellow) was slower than the other treatments.

3.5. pH value

Data in Table 4 shows the results of pH values of 'Sanh' orange that increased gradually as storage time progressed. However, during the 30 days of storage, pH values of the whole treatments was not statistically significant difference. So, using films coating to prolong the shelf-life 'Sanh' orange did not concern pH values. In contrast, according to Fereshteh et al. (2017), fruit juice pH increased during storage as was expected with the decline in fruit acidity. The different coatings evaluated in Fereshteh's study showed different effects on pH. These results are similar to found by Baldwin et al. (1999) who observed that pH value depends on the type of coating. Changes in pH might be due to the effect of treatment on the biochemical condition of the fruit. The metabolic activity particularly rate of respiration could be affected by the coating solution (Jitareerat et al., 2007).

Table 1. Effect of using the coating on the decay incidence of 'Sanh' oranges during storage

	Storage time (days)			
	15	20	25	30
CT[1]	$(16.60 \pm 1.32)^{a}$	$(21.60 \pm 1.09)^{b}$	$(42.80 \pm 2.53)^{a}$	Decayed
PE[1]	4.30 ± 0.21^{c}	6.90 ± 0.87^{d}	$(15.90 \pm 0.45)^{c}$	$(33.30 \pm 1.12)^{b}$
PP[1]	8.60 ± 0.35^{b}	$(25.80 \pm 1.21)^{a}$	$(43.10 \pm 3.81)^{a}$	$(55.80 \pm 4.21)^{a}$
PVC[1]	0.00 ± 0.00^{d}	4.00 ± 0.35^{e}	7.10 ± 0.70^{d}	$(10.80 \pm 0.67)^{d}$
CITRA[1]	0.00 ± 0.00^{d}	4.10 ± 0.29^{e}	8.60 ± 0.85^{d}	$(12.90 \pm 0.35)^{c}$
CHITO[1]	0.00 ± 0.00^{d}	8.50 ± 0.16^{c}	$(17.40 \pm 0.63)^{b}$	$(32.50 \pm 1.34)^{b}$
F	**	**	**	**
CV (%)	4.64	5.35	2.57	6.83

[a-e]Values on the same column with different letters are not significantly different from the Duncan test, **statistically significant difference at $P < 0.01$. Values in parentheses (): Analyze the rest values.
[1]CT: Control (uncoated), PE: Polyethylene, PP: Polypropylene, PVC: Polyvinyl chloride, CITRA: Citra shine, CHITO: Chitosan.

Table 2. Effect of using the coating on the rate of stem loss of 'Sanh' oranges during storage

	Storage time (days)			
	15	20	25	30
CT[1]	$(10.20 \pm 0.45)^{a}$	$(16.10 \pm 1.02)^{b}$	$(18.90 \pm 0.20)^{b}$	$(22.10 \pm 1.07)^{c}$
PE[1]	6.30 ± 0.32^{b}	11.50 ± 1.30^{c}	$(13.20 \pm 0.37)^{d}$	$(16.10 \pm 0.88)^{d}$
PP[1]	10.60 ± 0.68^{a}	$(24.60 \pm 1.12)^{a}$	$(32.70 \pm 2.08)^{a}$	$(44.80 \pm 3.25)^{a}$
PVC[1]	0.00 ± 0.00^{c}	3.70 ± 0.25^{e}	6.60 ± 0.51^{e}	$(8.20 \pm 0.27)^{e}$
CITRA[1]	6.40 ± 0.37^{b}	10.20 ± 0.85^{cd}	$(16.10 \pm 1.54)^{c}$	$(16.10 \pm 0.75)^{d}$
CHITO[1]	0.00 ± 0.00^{c}	9.60 ± 0.35^{d}	$(18.10 \pm 0.51)^{c}$	$(24.50 \pm 1.10)^{b}$
F	**	**	**	**
CV (%)	8.66	5.39	4.16	5.44

[a-e]Values on the same column with different letters are not significantly different from the Duncan test, **statistically significant difference at $P < 0.01$. Values in parentheses (): Analyze the rest values.
[1]CT: Control (uncoated), PE: Polyethylene, PP: Polypropylene, PVC: Polyvinyl chloride, CITRA: Citra shine, CHITO: Chitosan.

3.6. Total soluble solids

'Sanh' orange is a non-climacteric fruit, and they tend to decrease in total soluble solids in fruits, so °Brix value changes primarily due to dehydration in fruits. After 5 days storage, the whole values of total soluble solids (TSS) of 'Sanh' orange between the most treatments was not significantly different (Table 5). However, after 10 days of storage, ^{0}Brix values of the whole treatments were statistically significant difference at $P < 0.05$ and $P < 0.01$ on the 15^{th}, 20^{th}, 25^{th} and 30^{th} days. °Brix values of the control samples increased rapidly after 20 days of storage because respiration process was strong and the ripening process occurred quickly. Fruit TSS increased during storage due possibly to the cell wall disassembly (Cordenusi et al., 2005), and the enhancement in dry matter due to the reduction in the fruit water content during storage (Dhall, 2013). These findings are in agreement with results of researchers with other cultivars of citrus fruit such as 'Tacle' and 'Clara' mandarin (Rapisarda et al., 2008) as well as 'Ougan' and 'Hongju' mandarin (Ye et al., 2000). Moreover, the degradation of pectin, cellulose, and hemicellulose from cell walls within fruit segments might result in releasing soluble components which could have a direct effect on TSS (Iglesias & Echeverria, 2009). It has been shown that solubilization of the cell wall constituents under the effect of glucosidase and galactosidase present in citrus fruit, might have contributed to the increase in TSS levels (Iglesias & Echeverria, 2009). After 30 days of storage, °Brix value tends to decrease in the whole samples, may be because of the activity of respiratory process that consumed the most total soluble solids stored. ^{0}Brix values of PVC samples tends to increase slightly, because using PVC film wrapped 'Sanh' orange at room temperature that slows down the dehydration process and consumes more total soluble solids stored than other

Table 4. Effect of using the coating on the pH values of 'Sanh' oranges during storage

	Storage time (days)					
	5	10	15	20	25	30
CT[1]	3.62 ± 0.21	3.65 ± 0.22	3.80 ± 0.25	3.85 ± 0.24	3.84 ± 0.22	Decayed
PE[1]	3.62 ± 0.27	3.63 ± 0.27	3.67 ± 0.25	3.73 ± 0.30	3.82 ± 0.21	3.80 ± 0.26
PP[1]	3.54 ± 0.30	3.60 ± 0.29	3.63 ± 0.24	3.71 ± 0.28	3.78 ± 0.27	3.78 ± 0.31
PVC[1]	3.51 ± 0.25	3.61 ± 0.21	3.60 ± 0.30	3.74 ± 0.25	3.77 ± 0.28	3.69 ± 0.26
CITRA[1]	3.53 ± 0.22	3.62 ± 0.21	3.73 ± 0.31	3.74 ± 0.25	3.87 ± 0.27	3.85 ± 0.28
CHITO[1]	3.64 ± 0.27	3.68 ± 0.24	3.76 ± 0.26	3.84 ± 0.31	3.81 ± 0.30	3.85 ± 0.25

[1]CT: Control (uncoated), PE: Polyethylene, PP: Polypropylene, PVC: Polyvinyl chloride, CITRA: Citra shine, CHITO: Chitosan.

Table 3. Effect of using the coating on the colourful alteration at the shell of 'Sanh' oranges during storage

	Storage time (days)					
	5	10	15	20	25	30
CT[1]	42.98 ± 0.36^{b}	45.50 ± 0.29^{bc}	$(47.20 \pm 0.35)^{b}$	$(48.95 \pm 0.22)^{b}$	$(50.69 \pm 0.25)^{a}$	Decayed
PE[1]	44.28 ± 0.35^{a}	46.84 ± 0.23^{a}	48.80 ± 0.20^{a}	49.93 ± 0.12^{a}	$(51.20 \pm 0.35)^{a}$	$(52.77 \pm 0.30)^{a}$
PP[1]	44.35 ± 0.27^{a}	46.13 ± 0.35^{b}	47.62 ± 0.29^{b}	$(49.23 \pm 0.21)^{b}$	$(50.17 \pm 0.17)^{b}$	$(51.91 \pm 0.25)^{b}$
PVC[1]	42.96 ± 0.38^{b}	44.83 ± 0.40^{1c}	46.18 ± 0.33^{c}	47.62 ± 0.31^{c}	48.86 ± 0.34^{c}	$(50.51 \pm 0.45)^{c}$
CITRA[1]	41.21 ± 0.21^{c}	43.78 ± 0.31^{d}	46.44 ± 0.47^{c}	47.53 ± 0.33^{c}	$(48.50 \pm 0.370)^{c}$	$(49.55 \pm 0.58)^{c}$
CHITO[1]	40.94 ± 0.22^{c}	43.61 ± 0.28^{d}	46.30 ± 0.35^{c}	47.41 ± 0.35^{c}	$(48.45 \pm 0.35)^{c}$	$(49.61 \pm 0.55)^{c}$
F	*	*	*	*	*	*
CV (%)	10.24	10.44	8.67	8.30	9.14	7.88

[a-c]Values on the same column with different letters are not significantly different from the Duncan test, *statistically significant difference at $P < 0.05$. Values in parentheses (): Analyze the rest values.
[1]CT: Control (uncoated), PE: Polyethylene, PP: Polypropylene, PVC: Polyvinyl chloride, CITRA: Citra shine, CHITO: Chitosan.

Table 5. Effect of using the coating on ^{0}Brix values of 'Sanh' oranges during storage

	Storage time (days)					
	5	10	15	20	25	30
CT[1]	8.50 ± 0.33^{a}	8.90 ± 0.18^{ab}	(9.65 ± 0.25)^{a}	(10.05 ± 0.12)^{a}	(8.70 ± 0.53)^{b}	Decayed
PE[1]	8.60 ± 0.42^{a}	8.60 ± 0.22^{b}	8.95 ± 0.46^{ab}	9.10 ± 0.51^{b}	(10.00 ± 0.27^{a}	(9.10 ± 0.28)^{b}
PP[1]	8.40 ± 0.39^{a}	8.50 ± 0.34^{b}	8.75 ± 0.44^{b}	(9.20 ± 0.48)^{b}	(10.05 ± 0.25)^{a}	(9.05 ± 0.30)^{b}
PVC[1]	8.35 ± 0.45^{a}	8.55 ± 0.28^{b}	8.60 ± 0.59^{b}	9.05 ± 0.52^{b}	9.70 ± 0.21^{a}	(10.25 ± 0.34)^{a}
CITRA[1]	8.40 ± 0.28^{a}	8.60 ± 0.21^{b}	8.90 ± 0.51^{ab}	9.50 ± 0.44^{ab}	(9.00 ± 0.23)^{b}	(8.40 ± 0.22)^{c}
CHITO[1]	8.45 ± 0.44^{a}	9.05 ± 0.15^{a}	9.40 ± 0.22^{ab}	9.75 ± 0.19^{ab}	(8.90 ± 0.44)^{b}	(8.25 ± 0.20)^{c}
F	ns	*	**	**	**	**
CV(%)	2.69	6.40	6.74	5.72	9.90	4.66

[a-b]Values on the same column with different letters are not significantly different from the Duncan test, *statistically significant difference at $P < 0.05$; **statistically significant difference at $P < 0.01$. Values in parentheses (): Analyze the rest values; ns: not significant.
[1]CT: Control (uncoated), PE: Polyethylene, PP: Polypropylene, PVC: Polyvinyl chloride, CITRA: Citra shine, CHITO: Chitosan.

Table 6. Effect of using the coating on ascorbic acid content of 'Sanh' oranges during storage

	Storage time (days)					
	5	10	15	20	25	30
CT[1]	15.29 ± 0.35^{a}	12.54 ± 0.99^{b}	(10.58 ± 0.31)^{c}	(7.48 ± 0.88)^{d}	(6.82 ± 1.05)^{c}	Decayed
PE[1]	15.07 ± 0.28^{a}	14.41 ± 0.48^{a}	12.98 ± 0.35^{b}	11.00 ± 0.35^{c}	(9.9 ± 1.26)b	(8.91 ± 0.79)^{ab}
PP[1]	15.29 ± 0.37^{a}	14.85 ± 0.55^{a}	14.63 ± 0.28^{a}	(12.54 ± 0.58)^{ab}	(11.66 ± 0.51)^{ab}	(9.68 ± 0.23)^{a}
PVC[1]	15.29 ± 0.35^{a}	15.18 ± 0.67^{a}	14.41 ± 0.18^{ab}	13.20 ± 0.20^{a}	12.32 ± 0.30^{a}	9.79 ± 0.21^{a}
F	ns	*	*	*	**	**
CV (%)	4.45	6.31	6.88	8.16	9.48	6.29

[a-b]Values on the same column with different letters are not significantly different from the Duncan test, *statistically significant difference at $P < 0.05$; **statistically significant difference at $P < 0.01$. Values in parentheses (): Analyze the rest values; ns: not significant.
[1]CT: Control (uncoated), PE: Polyethylene, PP: Polypropylene, PVC: Polyvinyl chloride.

samples.

3.7. Ascorbic acid content

The main quality indexes of citrus fruit include TSS and ascorbic acid content. They were dynamically changed during postharvest storage (Ye et al., 2000). The ascorbic acid content in both wrapped and unwrapped fruits decreased gradually as storage period (Table 6). After 5 days of storage, the content of ascorbic acid of all samples was still quite high and there is no statistically significant difference. Ascorbic acid content gradually decreased in both treated and untreated fruit during storage. Khaliq et al. (2016) reported that mango fruit coated with gum arabic enriched with calcium chloride showed similar results. After 30 days of storage, ascorbic acid content in all samples had a statistically significant difference at 1%. At the end of the storage period, PP samples (9.68 mg/100 g), PE samples (8.91 mg/100 g) and PVC samples (9.79 mg/100 g) had a relatively high value of ascorbic acid compared to the control, chitosan, and Citra Shine samples. The results show that the utilization of synthetic films coating on fruits such as PP, PE, PVC is the most effective in preventing ascorbic acid losses. The differences reported might indicate that ascorbic acid loss during storage depends on the type of coating. Ascorbic acid plays an important role as an antioxidant and declines the oxidative stress (Fereshteh et al., 2017). The higher ascorbic acid content in treated fruit could be related to strengthening the defence system and maintaining the fruit quality without deterioration during storage condition (Khaliq et al., 2016).

4. Conclusions

Comparative analysis on the effects of several coating films and room storage conditions on the quality and the shelf-life of 'Sanh' orange, as observed in the present study, reveals that the utilization of wrapped PVC and storage at room temperature (30 ± 2^0C) was the best treatment for maintaining the quality and extending the shelf-life of 'Sanh' orange over other treatments or control, which was exhibited by the least weight loss percentages (13.91%), decay incidence, and the rate of stem loss, lower total soluble solids, higher ascorbic acid (12.32 mg/100 g), pH (3.77), maintained stable TSS values (9.7%) after 25 days of storage.

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Factors affecting betacyanin stability in juice of LD5 Red-fleshed dragon fruit (*Hylocereus polyrhizus*)

Anh H. T. Nguyen, Mai T. P. Phung, Trang T. T. Nguyen, Thuy T. P. Nguyen, Huan T. Phan, & Ngan N. T. Trinh*

Faculty of Food Science and Technology, Nong Lam University, Ho Chi Minh City, Vietnam

ARTICLE INFO

Research Paper

Received: September 20, 2018
Revised: November 11, 2018
Accepted: December 02, 2018

Keywords

Betacyanin
Binh Thuan dragon fruit
Colourant stability
LD5 variety
Fruit juice

***Corresponding author**

Trinh Ngoc Thao Ngan
Email: tntngan@hcmuaf.edu.vn

ABSTRACT

In Binh Thuan province (Vietnam), the red-fleshed dragon fruit (*Hylocereus polyrhizus*), concretely LD5 variety majorly grows and contains a large amount of betacyanin, a natural colourant that potentially applied to many products in the food industry. In this study, the processing factors possibly influencing the betacyanin stability in the red-fleshed dragon fruit juice were in turn investigated. The heating treatment included 2 factors: temperature (65, 75 and 85^0C) and heating time (10, 20 and 30 minutes); while the pH values ranged between 3.0 to 7.0 and the ascorbic acid addition varied in concentrations (0.1, 0.2, 0.3, 0.4 and 0.5% w/w). The processed fruit juice was stored in different packaging materials (plastic and glass) with and without light exposure for 5 weeks to monitor the retained betacyanin. The results showed that the betacyanin was remained with the highest proportion (0.84 ± 0.02) at 0.3% ascorbic acid addition, pH 4.0 and heat treatment at 65^0C for 10 minutes. In storage without light exposure, both plastic and glass packaging materials kept efficiently betacyanin in fruit juice. However, the glass material represented better efficiency in the betacyanin remaining than the plastic material did.

Cited as: Nguyen, A. H. T, Phung, M. T. P., Nguyen, T. T. T., Nguyen, T. T. P, Phan, H. T., & Trinh, N. N. T. (2018). Factors affecting betacyanin stability in juice of LD5 Red-fleshed dragon fruit (*Hylocereus polyrhizus*). *The Journal of Agriculture and Development* 17(6), 72-76.

1. Introduction

Dragon fruit belongs to the *Cactaceae* family that widely grown in several countries such as Taiwan, Vietnam and Malaysia (Nur' Aliaa et al., 2010). In Vietnam, it is one of the popular fruit-bearing trees with 2 available types: *Hylocereus undatus* (*H. undatus*) with pink skin and white-flesh; and *Hylocereus polyrhizus* (*H. polyrhizus*) with pink skin and red flesh. In recently, the red-fleshed dragon fruit has been extensively planted in many provinces such as Long An, Ninh Thuan, Tien Giang etc due to several benefits in terms of health, sensorial values, economic issues, etc. With more than 26,000 ha of growing dragon fruit, Binh Thuan Province has the largest area allocated to the fruit in the country, according to province's Agriculture and Rural Development Department. The dragon fruit planting area will predictably have increased to 28,000 ha with 750 000 tons of production by 2020 (Tran & Nguyen, 2017). The LD5 dragon fruit, a natural source of antioxidants and attractive colour betacyanin, mostly grows in Binh Thuan due to a high yield, good adaptation to the natural environment and (strong) resistance against diseases.

Betacyanin, made of water-soluble nitrogen-containing pigments (betalains), is a set compound contributing fundamentally to the colour of dragon fruit *H. polyrhizus* (Rebecca et al., 2010). It is unstable and easily degrades or breaks down into the degradation product such as cyclo-dopa 5-O-β-glucoside (colourless) and betalamic acid (bright yellow), leading to the discoloration

of the pigments (Herbach et al., 2004). Several factors including heat, oxygen, light, pH and moisture are reported to have significant effects on the betacyanin stability (Woo et al., 2011). In addition, the discoloration rate of red colour from garambullo tree (*Myrtillocactus geometrizans*), from red-fleshed dragon fruits (*H. polyrhizus*) was also diminished by the addition of some antioxidants such as ascorbic acid, citric acid (Reynoso et al., 1997; Wong & Siow, 2015).

The aim of this study was to investigate the effects of heat treatment, pH and ascorbic acid concentration on the betacyanin stability. The effects of storage conditions including light exposure and packaging material were also studied within 5 weeks at ambient temperature. These results are the preliminary findings for the further application into fruit juice process, that potentially contributes to value addition for red-fleshed dragon fruits in Binh Thuan Province.

2. Materials and Methods

2.1. Sample preparation

Red-fleshed dragon fruits were collected from farmer households in Binh Thuan province with the similarity in terms of maturity, weight and without any defects and/or crushes. The fresh fruits were stored at cool temperature (15-20^{0}C) before use. Red-fleshed dragon fruits were washed, peeled before collecting pulp. The pulp then crushed directly to collect the dragon fruit. The juice was centrifuged at 3000 g for 10 minutes (Z206A – Hermle – German) and filtered through a filter paper (Whatman) to remove the solid parts. The filtrate was then obtained to carry out the betacyanin analysis.

2.2. Experiments

Completely randomized design was serially applied to investigate the effects of heating condition, pH, ascorbic acid concentration and storage condition. The research was carried out at Food Engineering Laboratory, Faculty of Food Science and Technology, Nong Lam University Ho Chi Minh City.

Effects of heating conditions: The filtered dragon fruit juices (12 mL) at natural pH in test tube were treated at various heating conditions including 65, 75 and 85^{0}C for 10, 20 and 30 minutes in water bath. The time was counted since the center temperature rose up to the required temperature using the thermometer. The heated juices then cooled down in ice water and subsequently were subjected to betacyanin analysis.

Effects of pH: The filtered juice samples (12 mL) were adjusted to pH 3.0; 4.0; 5.0; 6.0 and 7.0 using 1M HCl and 1M NaOH. All these samples were subjected to heat treatment at 650C for 10 minutes and cooled immediately in iced water. The betacyanin analysis was then carried out Effects of ascorbic acid addition: Different concentrations of ascorbic acid including 0 (control); 0.1; 0.2; 0.3; 0.4 and 0.5% (w/w) were added into the juices. All the juice samples were then adjusted to pH 4.0 by 1M HCl and 1M NaOH; subjected to heat treatment at 65^{0}C for 10 minutes and cooled down in iced water before betacyanin analysis.

Effects of storage conditions: The dragon fruit juices treated with the optimal conditions from previous experiments were kept in 2 types of test tubes: the glass test tubes and the plastic test tubes. The glass ones were considered as imitation of glass packaging material, while the other represented for plastic packaging material. The tubes covered with aluminum-foil were the samples stored in light prevention condition; while the tubes without aluminum-foil were the samples stored in light exposure condition. All the samples were stored for 5 weeks at ambient temperature (30 $\pm$ 2^{0}C). The betacyanin analysis was determined after treating with heat and every week to compare the proportion of betacyanin retained during storage.

2.3. Betacyanin analysis and retained betacyanin proportion

The Mcllvaine buffer (pH 6.5) prepared from 0.1M citric acid (30 mL) and 0.2M dibasic sodium phosphate (70 mL) was used to dilute the dragon fruit juice. The juice sample, diluted into 40 times by adding 0.1 mL of juice sample to 3.9 mL of Mcllvaine buffer solution, was analyzed with UV-vis spectrophotometer (Shimadzu 1240, Japan). The wavelength was 540 nm which was tested preliminarily from 537 nm to 600 nm to attain the maximum absorbance. The Mcllvaine buffer solution with 4.0 mL (without sample) was a blank.

The concentration of betacyanin (Bc) is expressed as the following equation:

$$\mathrm{Bc}\left(\frac{\mathrm{mg}}{\mathrm{L}}\right) = \frac{\mathrm{A} \times \mathrm{F} \times \ \mathrm{MW} \times 1000}{\varepsilon \times \mathrm{l}}$$

Where:

A: Absorption value at $\lambda = 540$ nm

F: Dilution factor

MW: Molecular weight of betacyanin (550 g/mol)

ε: Molar extinction coefficient of betacyanin (60,000 L/mole × cm)

l: path length of the cuvette (1 cm)

The retained betacyanin proportion was calculated by the ratio between the betacyanin content in the untreated juice and the betacyanin content in the juice treated by factors of experiment, as following:

$$\text{Retained betacyanin proportion} = \frac{Bc_1}{Bc_0}$$

Where:

Bc_0: the initial betacyanin content (e.i betacyanin in the untreated/natural juice)

Bc_1: the betacyanin content in the juices treated by the factors of experiments)

2.4. Statistical analysis

All experiments were carried out in triplicate. Calculation, tabulating and graphing of data were carried out using Microsoft Excel 2007 (Microsolft, USA). Statistical analysis was performed by using JMP software version 10.0 (SAS Institute Inc, USA). The difference was considered significant at $P < 0.05$.

3. Results and Discussion

3.1. Effects of heating treatment

The betacyanin content remained in the juice after treating with heat is shown in Figure 1. Generally, heat treatment obviously caused decrease of betacyanin and the temperature has a significant effect on the retained betacyanin in the juice. The higher temperature applied, the less retained content in the juice was recorded. The betacyanin content decreased from initial content of 204.11 ± 2.40 (mg/L) to 175.54 ± 5.39; 140.85 ± 10.79 and 114.31 ± 4.09 (mg/L) as heating at 65^0C, 75^0C and 85^0C for 10 minutes, respectively ($P < 0.05$). The similar trend was also found when the fruit juices were subjected to heat for 20 and 30 minutes. Otherwise, the time fairly affected on the remained betacyanin content, particularly at the small variation in time. For examples, the juice heated at 65^0C contained 175.54 ± 5.39; 158.64 ± 13.03 and 145.62 ± 10.48 (mg/L) corresponding to 10, 20 and 30 minutes, respectively. Interestingly, when the temperature increased up to 85^0C, the retained betacyanin content decreased dramatically as compared to the results at the other temperatures and did not depend on the heating time. According to Herbach et al. (2004), the heating could accelerate the degradation of betanlains (the main structure of betacyanin) to cyclo-dopa 5-O-β-glucoside (colourless) and betalamic acid (bright yellow) by the bond cleavage, leading to the discoloration of the pigments. The results were supported by research of Reshmi et al. (2012): the betacyanin in basella alba fruit was maintained at 0, 10 and 20^0C and decreased when the temperature heated up from 40, 50 and 60^0C Similarly, Wong & Siow (2015) reported the proportion of betacyanin retained decreased when the temperatures increased 65, 75 and 85^0C in the long heating time.

As a result, the heating condition at 65^0C for 10 minutes was the optimal condition to remain efficiently the betacyanin content in the juice.

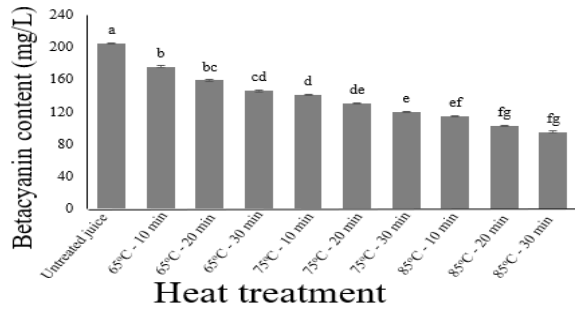

Figure 1. The betacyanin content in the different juice samples before and after heating.

3.2. Effects of pH

Figure 2 obviously represents the significant effects of pH on the betacyanin stability based on the results of betacyanin content in the juice samples and the retained betacyanin proportion. The betacyanin content of 204.03 ± 4.35 (mg/L) in the unheated juice decreased after heating to different amounts depending on the adjusted pH values. According to Reshmi et al. (2012), betacyanin was stable with red colour in neutral and slightly acidic media; on the contrary betacyanin was unstable and change colour from red into yellow in the alkaline medium at pH values upper 7.5. The acidic medium favored the connection between betalamic acid and cyclodopa-5-O-

β-glucoside to form betacyanin (Azeredo, 2009). The proportion of betacyanin retained was found to be the highest at pH 4.0 (with 0.87 ± 0.01 retained proportion of betacyanin) that was in consistent with the study of Herbach et al. (2007). But other researchers reported pH 5.0 was the optimal value to support the betacyanin stability (Tang & Norziah, 2010; Wong & Siow, 2015). The betacyanin content of pH 3.0 sample was the least at 118.98 ± 1.21 (mg/L) with 0.58 ± 0.01 retained proportion. It was explained by Azeredo (2009) that the CO_2 removal forms 17-decarboxybetanin with orange-red colour, or the dehydrogenation produces the neobetanin with yellow colour, leading to the pigment degradation. The pH 4.0 was the optimal for the betacyanin stability in the LD5 red-fleshed dragon juice.

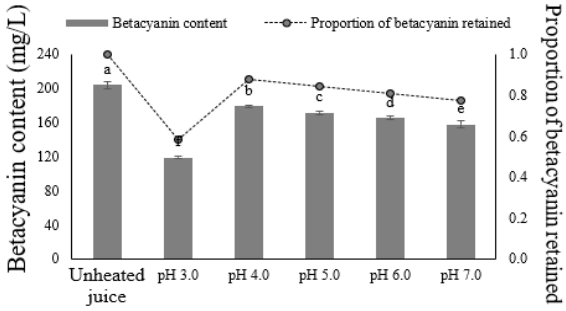

Figure 2. The betacyanin content and proportion of betacyanin retained in the juice samples after adjusting at different pH and heating.

3.3. Effects of ascorbic acid addition

Ascorbic acid has been well-known as an antioxidant due to its ability to combine with oxygen from surrounded environment – the main factor caused degradation of several sensitive compounds such as betacyanin (Attoe & von Elbe, 1982). Figure 3 shows the slightly positive effects of ascorbic acid on the betacyanin content remaining in the LD5 red-fleshed dragon fruit juice after processing. Generally, the betacyanin content in the unheated juice (203.79 ± 3.01 mg/L) reduced after heating process and in almost treatments the betacyanin proportion could be remained up to 75%. Without ascorbic acid and adding 0.1 and 0.2% ascorbic acid, the proportions of betacyanin remained were 0.80 ± 0.01; 0.79 ± 0.02 and 0.82 ± 0.01, respectively without any significant difference ($P > 0.05$). The highest retained betacyanin proportion was 0.84 ± 0.01 attained at 0.3% ascorbic acid addition with 171.18 ± 3.35 (mg/L) betacyanin content. Interestingly, the increment in concentration up to 0.4% ascorbic acid did not further increase the proportion of betacyanin retained (0.82 ± 0.01), while 0.5% ascorbic acid addition did not show any positive effect on the retained betacyanin proportion (0.78 ± 0.02). This result supported to the research of Wong & Siow (2015) that ascorbic acid efficiently remained the betacyanin at the concentrations less than 0.5% (w/w).

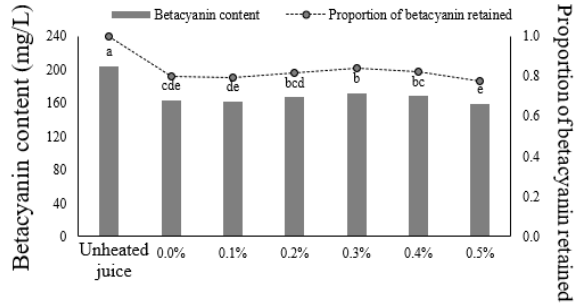

Figure 3. The betacyanin content and proportion of betacyanin retained in the juice samples after adding different ascorbic acid concentrations and heating.

3.4. Effects of storage conditions

Generally, the proportions of betacyanin retained decreased during the storage time for all the treatments with different rate (Figure 4). Obviously, during storage time, the juices in both plastic and glass test tubes in case of light exposure had the low retained betacyanin proportions and without any significant difference in results between these treatments in every week. The betacyanin content kept in the plastic tubes reduced down to almost half in the 1st week storage (0.57 ± 0.02) and continuously to 0.51 ± 0.03, 0.46 ± 0.03, 0.39 ± 0.03 and 0.33 ± 0.03 at the 2nd, 3rd, 4th and 5th week, respectively. Similar trend was found in the juice sample stored in the glass test tube in light exposure. Otherwise, the juice in case of without light exposure showed the better remained betacyanin proportion, especially as juice was kept in the glass packaging material. The juice in plastic tubes remained 0.63 ± 0.02 betacyanin proportion in the 1st week that slightly higher as compared to the formers; however, the proportion then in storage time was in the insignificant difference. The highest beta-

cyanin proportions during storage were observed at the juice in glass test tubes with 0.90 ± 0.01 in the 1st week, 0.76 ± 0.03 in the 2nd week, 0.74 ± 0.04 in the 3rd week, 0.64 ± 0.02 in the 4th week and 0.56 ± 0.02 in the last week. These findings were in agreement with the research conducted by Wong & Siow (2015) who also studied about betacyanin stability in red-fleshed dragon fruit juice. According to Jackman & Smith (1996), the pigment molecules absorb ultraviolet and visible light that excite π electrons into the higher state (π^*), resulted in the decrease of molecular stability.

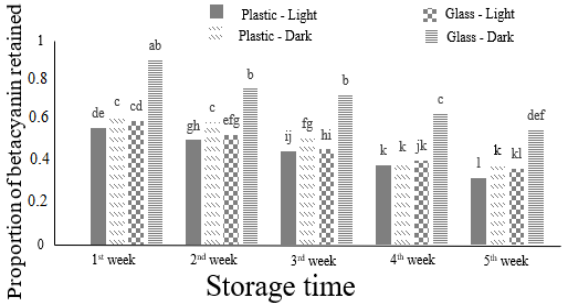

Figure 4. The retained proportion of betacyanin in the juice stored in different storage conditions during storage time.

4. Conclusions

The highest proportion of retained betacyanin was obtained from the dragon fruit juice that subjected to heat treatment at 65^0C for 10 minutes, pH 4.0 and 0.3% ascorbic acid addition. In storage, light exposure was an important factor that caused decrease of retained betacyanin in the fruit juice. In addition, the glass material represented better efficiency in the betacyanin remaining than the plastic material did.

Acknowledgement

This study was financially sponsored by Nong Lam University Ho Chi Minh City, Vietnam

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Total phenolic content and antioxidant activity of sesame cake aqueous extracts

Yen T. X. Nguyen[1], Costas Stathopoulos[2], Chockchaisawasdee Suwimol[2], Phuong L. Nguyen[3], & Tuyen C. Kha[1*]

[1]Faculty of Food Science and Technology, Nong Lam University, Ho Chi Minh City, Vietnam
[2]Faculty of Bioscience Engineering, Ghent University Global Campus, South Korea
[3]American International School, Ho Chi Minh City, Vietnam

ARTICLE INFO

Research Paper

Received: November 05, 2018
Revised: December 18, 2018
Accepted: December 26, 2018

Keywords

Antioxidant activity
Microwave pre-treatment
Sesame cake
Total phenolic content

***Corresponding author**

Kha Chan Tuyen
Email: khachantuyen@hcmuaf.edu.vn

ABSTRACT

The objective of this study was to investigate the efficiency of phenolic extraction and antioxidant activity from sesame cake using water extraction method and to evaluate the possibility of employing microwave irradiation to improve the extraction yield. The result showed that extraction temperature had major influence on total phenolic content and antioxidant activities of the extracts, whereas extraction time was found to be insignificant. The optimum extraction condition recommended were 90^0C for 30 min in this research. Furthermore, microwave pre-treatment at 120 s could have significantly positive influence on the overall extraction yield, especially the total phenolics and antioxidants based on FRAP assay. Therefore, the obtained results suggest that sesame aqueous extracts could be a source of antioxidants with more feasible applications in food as well as other industries.

Cited as: Nguyen, Y. T. X., Stathopoulos, C., Suwimol, C., Nguyen, P. L., & Kha, T. C. (2018). Total phenolic content and antioxidant activity of sesame cake aqueous extracts. *The Journal of Agriculture and Development* 17(6), 77-84.

1. Introduction

The potential applications of antioxidants, the consumer preferences of totally "natural" products, and the drawbacks of synthetic antioxidants, all has risen an emerging trend to look for a new source of antioxidants which can satisfy both the safety concerns and economic hindrance. Accordingly, the extraction of antioxidants from natural resources, especially from the utilization of residual products or waste, is recently of high considerations by many researchers and manufacturers. In several last decades, numerous studies and researches on such kinds of materials for their antioxidant composition, properties, and capacity, such as the studies of Guo et al. (2003), Pan et al. (2003), Wang et al. (2015) on the pulp, peel and seed parts of various kinds of fruits, those of Pinelo et al. (2005), Candrawinata et al. (2014), Saikia et al. (2015) on various sources of fruit pomace, and of Wanasundara et al. (1995), Suja et al. (2005), Terpinc et al. (2012) on different oilseed cake extracts, have been conducted with promising results.

Following up the current interests, sesame oil cake, which has been normally referred to as a by-product after sesame oil pressing and extraction process and is currently used as cattle feed, has been increasingly investigated and utilized as a potent source of bioactive compounds. The enormous amount of sesame cake produced annually by the oil-pressing process makes it interesting to evaluate what remains in the cake after the extraction process as well as to utilize the cake

as a source of value-added materials instead of a source of poultry feed. The antioxidant extraction and characteristics of sesame meal has already conducted by interested scientists and researchers. Yet, most of previous researches have not focused on the potential of aqueous phenolic, but the organic solvent extraction which employed organic solvents, hexane, methanol and ethanol were most frequently used, instead. In some cases, a mixture of solvents could be employed to optimize the extraction yield (Mohdaly et al., 2010; Reshma et al., 2013). The main drawbacks of organic solvents, however, are their high flammability and their potential hazards for both human health and environment. Moreover, solvent extraction also requires further steps for evaporation or removal of solvent to recover the extracts (Joana Gil-Chávez et al., 2013). Therefore, a search for an alternative which could minimize those merits of organic solvents has been continued for years.

Generally recognized as a green, safe, and cheap extraction solvent, water is recently referred to as a brilliant alternative for other organic solvents, which are usually toxic, volatile, and highly flammable. Another advantage of water-based extraction is that aqueous extracts do not require further concentration or separation steps as to the solvent extracts, which is sometimes very expensive and time-wasting process. The tendency of selecting water as the only solvent for extraction is evidently potential through the results of many recent (Çam & Aaby, 2010; Candrawinata et al., 2014). Moreover, in case of employing microwave as a pre-treatment method, the presence of water could facilitate the effectiveness of microwave irradiation in terms of phenolic recovery efficiency (Veggi et al., 2013). Microwave irradiation were selected as the pre-treatment method and compared with the effect of ultrasonic pre-treatment on sesame cake extraction in other unpublished papers. Microwave has been proposed by many researchers as a brilliant pretreatment candidate because of its effectiveness, simplicity and greatly lower time processing requirement (Azmir et al., 2013).

As a result, the objective of this present study was to investigate the efficiency of phenolic extraction as well as the antioxidant capacity from sesame cake through water extraction method. Furthermore, the work also focused on the effect and interactions of extraction temperature and time by the full factorial experiment design as well as the possibility of employing microwave irradiation as a pre-treatment method to improve the extraction yield.

2. Materials and Methods

2.1. Chemicals

All redox reagents, gallic acid and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were of analytical grade and were purchased from Sigma Aldrich Laboratory Chemicals.

2.2. Aqueous extraction of phenolic constituents from sesame cake

Sesame cake ($0.40 \pm 0.02\%$), after getting from commercial sesame oil producer, was stored at -18^0C until sampling. The cake was extracted in which water was the only solvent by preparing sample at the ration of 0.05 g sample/ mL distilled water, and then, placing and shaking gently in a water bath (model: JSSB-30T, South Korea) which was pre-set at an assigned temperature (40, 50, 60, 75, 90^0C) and time (30, 45, 60 min). The extracted mixture was cooled in water bath for 10 min so as to stabilize the phenolic extracted. The mixture was continuously centrifuged at 3500 rpm for 20 min and was filtered through Whatman filter paper to obtain the filtrate considered as the phenolic extract of sesame cake.

2.3. Pre-treatment with microwave on aqueous extraction of sesame cake

The previous experiment was determined the optimum condition for aqueous extraction of sesame cake. The further step was to investigate the effect of microwaves as pre-treatment method on the total phenolic yield and antioxidant capacity of aqueous sesame cake extract. The microwave model employed was LG MW233SK. The microwave power and frequency were set at 700 W and 2450 MHz respectively while pretreatment time was the variable parameter with 5 levels of interest (0, 30, 60, 90, 120 s). The levels were referenced from previous work that successfully applied microwave as pre-treatment method for extraction. Samples, after being pre-treated, continuously experimented under the optimum

extraction condition selected by the previous part of the research.

2.4. Analytical methods

2.4.1. Determination of moisture content

The moisture content of the sesam cake was determined by drying at a temperature of 105^0C until a constant weight was reached.

2.4.2. Determinations of the total phenolic content and the antioxidant activity

• Total phenolic content (TPC): TPC was determined by employing the Folin-Ciocalteu's colorimetric assay which was adapted from Thaipong et al. (2006) with some modifications. 150 μL of sesame cake extract was reacted with 150 μL of Folin Ciocalteu reagent (0.25) for 2 min, then 2400 μL of sodium carbonate (5% w/v) was added and mixed evenly using a Vortex. Absorbance was measured at 765 nm using spectrophotometer after the mixture was incubated for 1 h after mixing. The results were expressed in gallic acid equivalents (GAE, mg/g dry sample).

• Antioxidant activity measurement: The three assays, ABTS, DPPH, and FRAP, were optioned to measure antioxidant activity of the extracts.

◇ ABTS radical cation decoloration assay: The ABTS assay was employed with minor modifications from the method of Arnao et al. (2001) and Thaipong et al. (2006). The stock solution was $ABTS^+$ solution (7.4 mM) and potassium persulfate solution (2.6 mM). The working solution was prepared fresh every assay by mixing the two stock solutions at the volume ratio of 1:1 and was incubated in the dark at ambient temperature for 12-15 h before methanol was then added for dilution purpose at the ratio of 1:45 (v/v) of the working solution and methanol respectively so as to the mixture get an absorbance of 1.1 $\pm$ 0.02 units when measured at 734 nm by using the spectrophotometer. 150 μL of the sesame cake extract reacted with 2850 μL of ABTS solution added. The mixture was mixed well by using a Vortex and incubated for 2 h in dark room. Standard curve was prepared for the concentration of Trolox ranging from 100 to 600 μM (0.025 - 0.9 mg TE/ml water). The result was expressed as mg of Trolox equivalent (TE)/ml of water, which was then transferred to mg TE/g of dry sample.

◇ DPPH radical scavenging activity assay: The method to evaluate antioxidants using DPPH assay following the adapted method of Candrawinata et al. (2014), which was modified from method of Brand-Williams et al. (1995) with some modifications. Stock solution was prepared by dissolving 24 g DPPH reagent in 100 ml methanol. The solution was refrigerated at -20^0C before and after used. The working solution was prepared fresh every day of experiment by mixing stock solution and methanol at the ration 10:45 (v/v), respectively until the solution gained the absorbance of 1.1 $\pm$ 0.02 units. The absorbance was measured at 515 nM by spectrophotometer. 150 μL of the sesame cake extract reacted with 2850 μL of DPPH working solution added. The mixture was mixed well by using a Vortex and incubated for 30 min in dark room. Standard curve was prepared for the concentration of Trolox ranging from 100 to 800 μM (0.025 – 2.0 mg TE/ml water). The result was expressed as mg of Trolox equivalent (TE)/ml of water, which was then transferred to mg TE/g of dry sample.

◇ Ferric reducing/antioxidant power (FRAP) assay: The FRAP assay was adapted the method of Candrawinata et al. (2014) which modified from that of Thaipong et al. (2006) and Benzie and Strain (1996). Stock solutions was obtained by preparing three solution: acetate buffer (300 mM - pH = 3.6), TPTZ (10 mM) in HCl (40 mM) solution and $FeCl_3.H_2O$ (20 mM) solution. Water bath prepared at 50^0C was employed as preparing TPTZ 10 mM in HCl 40 mM solution to dissolve them well. The working solution was prepared fresh when used, at the ratio of 10 ml acetate buffer, 1 mL TPTZ and 1 mL $FeCl_3.H_2O$. Incubation time was set for 1 h before being available for reaction. After incubation, 150 μL of the sesame cake extract was added 2850 μL of repared FRAP working solution, mixed well and incubated for 30 min in dark room. Standard curve was prepared for the concentration of Trolox ranging from 100 to 800 μM (0.025 - 2.0 mg TE/ml water). The result was expressed as mg of Trolox equivalent (TE)/ml of water, which was then transferred to mg TE/g of dry sample.

2.5. Statistical analysis

Statistical analysis was performed by applying the JMP statistical software version 10 for Windows (SAS Institute INC., Cary, North California, USA). Data were subjected to analysis of

variance (ANOVA) ($P < 0.05$). All performances were conducted at least in triplicate and all were averaged. The confidence limits used in this study were based on 95% ($P < 0.05$).

3. Results and Discussion

3.1. Effect of extraction temperature and time on TPC and AAs

Statistical analysis expressed the effect of temperature and time on the phenolic extraction content (TPC) and the antioxidant activities (AAs). The results indicated that extraction time did not appear to significantly affect the TPC and the AA, whereas extraction temperature did have major effects in the TPC values and the AAs by the ABTS and FRAP assays. The AA of DPPH assays did not seem to be significantly affected by any individual or correlation effects of temperature and time.

Overall, as extraction temperature increased, the extraction efficiency, namely the phenolic extraction and antioxidant power by all assays, also increased (Figure 1). This experiment pattern was similar to that of other studies conducted by Spigno et al. (2007), Oancea et al. (2012), and Vergara-Salinas et al. (2012). Reportedly, an increased temperature during extraction could provide enough thermal energy to break down the interaction between solutes and the sample matrix. These internal forces could be the Van der Waals forces, hydrogen bonding, or the dipole attraction between the analyte molecules and the active sites in the matrix (Alupului et al., 2012). Furthermore, a high temperature could accelerate extraction yield by improving mass transfer rate of solutes out of the sample matrix towards the solution and reduce activation energy required for desorption process (Sparr Eskilsson & Björklund, 2000; Vergara-Salinas et al., 2012).

The results indicated that TPC values (Figure 1a) of aqueous sesame extracts ranged between 5.57 and 6.77 mg GAE/g of dry sesame cake which was highly comparable to that of other kinds of seed oil meal and by-products, and fruit peels in many studies. As the data comparison made sesame cake a potential source of natural antioxidants, the utilization of sesame meal could be practical in terms of phenolic extraction instead of a source of waste or fodder. Moreover, the higher TPC values of aqueous extracts of sesame cake than that of solvent extracts in many published studies (as discussed previously) also indicated that water could be used as an alternative solvent for other organic solvent in phenol extraction of sesame cake. This could potentially reduce the cost for extraction solvent as well as for evaporation or separation of solvent after the extract and make the sesame extracts more applicable and available especially in the food-based and health-related applications.

Antioxidant activities was measured from the sesame extract by using ABTS, DPPH and FRAP assays (Figure 4b, c and d, respectively). The average values of AA of each assay ranged from 16.57 to 19.20 mg TE/g of dry sesame cake, from 3.69 to 3.95 mg TE/g of dry sesame cake, and from 6.87 to 7.47 mg TE/g of dry sesame cake for ABTS, DPPH and FRAP assays, respectively.

The $ABTS^{\bullet+}$-scavenging data indicates that antioxidant components of the sesame extract had ability to scavenge the free radicals via a mechanism of electron/hydrogen donation. The $ABTS^{+}$ scavenging activities of the sesame cake extracts was greater than that of $DPPH^{+}$ radical. Mohdaly et al. (2010) explained the possible reasons due to the stereoselectivity of radicals and the different solubility of extracts at different assays. Some compounds possessing $ABTS^{+}$-scavenging activity in the extracts may not expose to show similar DPPH-scavenging activity. Floegel et al. (2011) noted that ABTS assays were more preferable to express the antioxidant contents than DPPH assays. ABTS assays could be employed in both hydrophilic and lipophilic solvent extraction while DPPH assays were only applicable in case of organic solvent which is most frequently under lipophilic conditions. In case of sesame cake extract, the oil-pressing process has effectively extracted. Hence, most of oil-soluble antioxidants was released out together with the sesame oil. Moreover, under the aqueous extraction, a vast majority of phenolics extracted were supposed to be more hydrophilic rather than lipophilic. Therefore, the aqueous conditions and the basic instinct of the two assays, ABTS assay was recorded for its better reflects the antioxidant contents than DPPH assay in case of sesame cake aqueous extracts.

$DPPH^{\bullet}$ is considered as a stable of hydrophobic free radical which was employed to measure the free radical scavenging capacities of the extracts. DPPH radical scavenging activity of the extracts may be attributed to their hydrogen do-

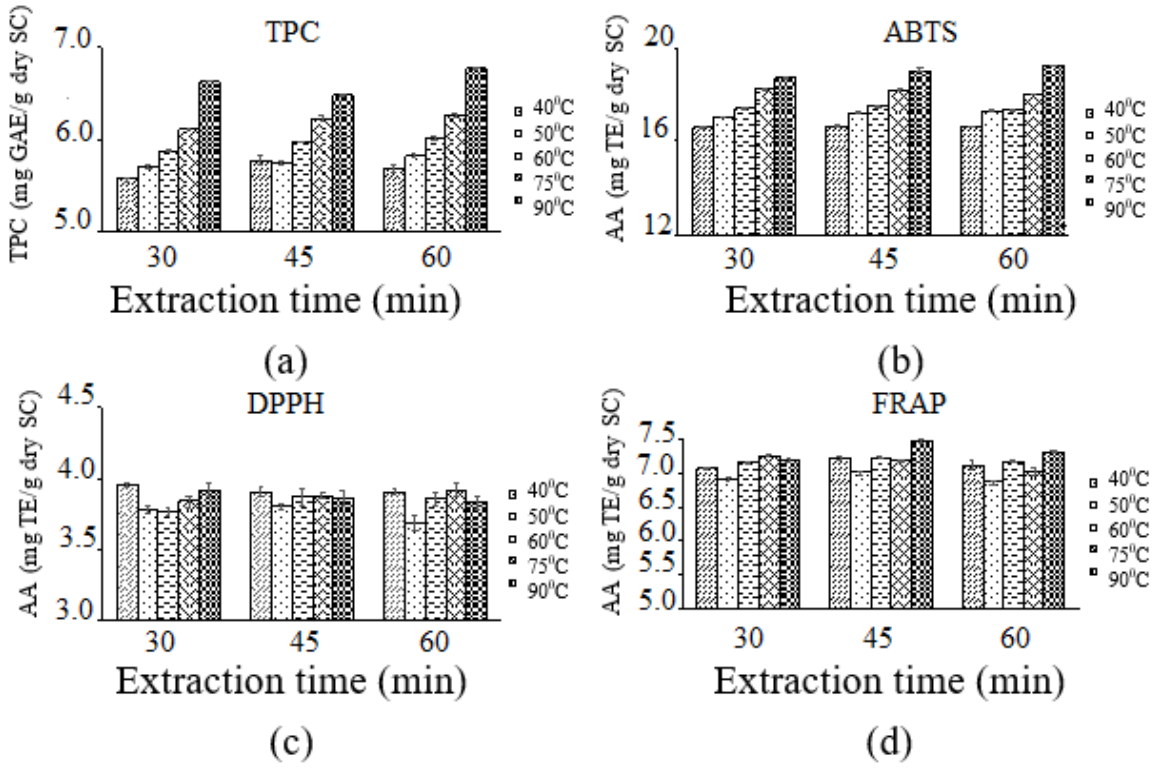

Figure 1. Effect of temperature and time on total phenolic content (TPC) and antioxidant activity (AA).

nation ability (Mohdaly et al., 2010; Reshma et al., 2013). As antioxidants in the extracts reacted with DPPH$^{\bullet}$ radicals, the number of free radical decreased, resulting in the discoloration visually from dark-purple to yellow. The scavenging activity of extracts against DPPH$^{\bullet}$ radical was concentration-dependent. Hence, higher results show greater antiradical capacity of the extracts.

In case of FRAP assay, under the presence of antioxidants acting as reductants, the ferric-tripyridyltriazine (Fe^{III} -TPTZ) complex is reduced to the ferrous (Fe^{II}) form with an intense blue color (Benzie & Strain, 1996). Results from antioxidant activities based on the FRAP assays showed that the sesame cake extracts did possess the ferric reducing capacity under the experiment conditions.

All responses obtained highest values at the experiments of 90^0C. Yet, only TPC values and AA values obtain from ABTS assays were significantly higher at 90^0C compared to lower levels of temperature. The possible reasons that antioxidants possessing the ability to scavenge DPPH$^{\bullet}$ radicals and their reducing power were not effectively involved to the increasing temperature and the net increase in their antioxidant values are not enough to be significant. Since time did not take into significant account to the overall effects during extraction, statistic results showed that the optimum extraction condition should be set at 90^0C for 30 min for the optimal extraction efficiency.

Correlations of TPC and AAs of the sesame extracts:

Correlations between the TPC and the antioxidant activities (AA) by all assays were analyzed by using the Pearson's correlation analysis (Table 1). Only the correlation of TPC values and AA based on ABTS assay was significantly high ($r = 0.85$, $P < 0.05$). The positively high correlation informed that the concentration of phenolic extracts possibly plays major roles in the antioxidant activity (Candrawinata et al., 2014).

Mohdaly et al. (2010) suggested it was better to use ABTS^{+} radical instead of DPPH$^{\bullet}$ radical to determine the potential free radical scavenging activity of the sample in case that the issues of

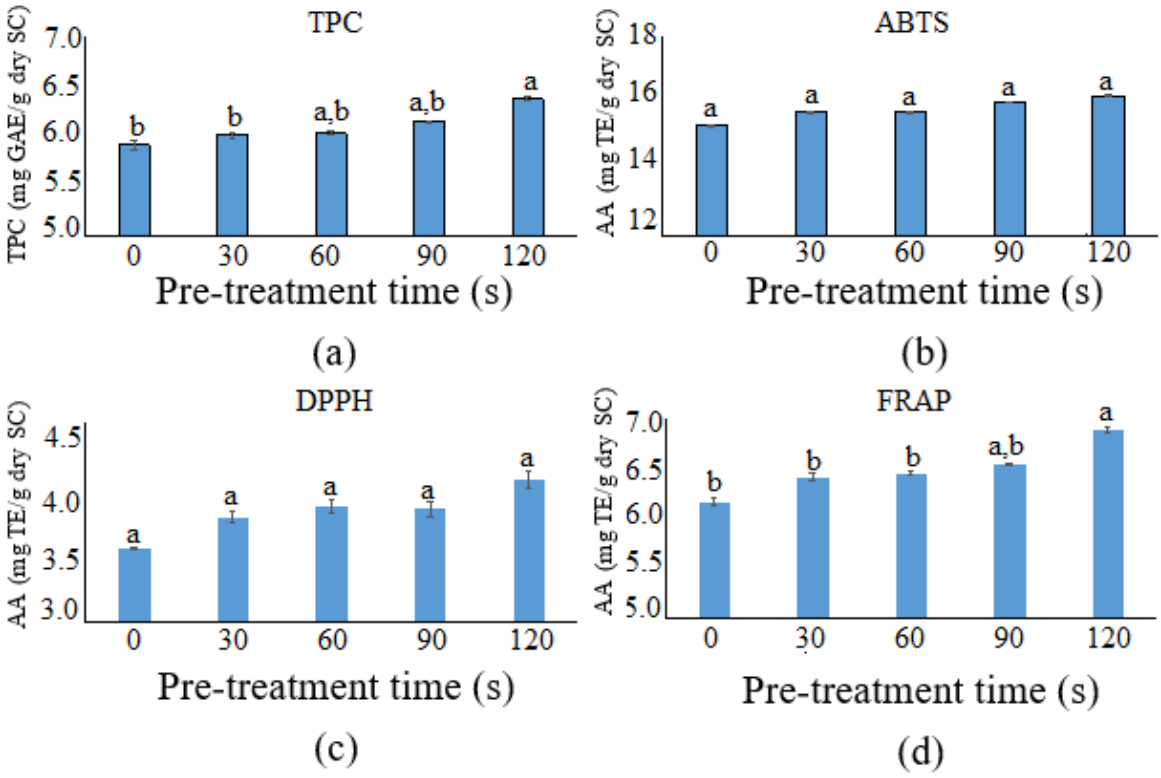

Figure 2. Effect of microwave pre-treatment time on TPC and AAs.
Values are means ± standard errors for different extraction times and those not sharing a superscript are significantly different ($P < 0.05$).

Table 1. Pearson's correlation coefficients of TPC and AAs of the sesame cake extracts

	TPC	ABTS	DPPH
ABTS	0.85*		
DPPH	0.11	-0.06	
FRAP	0.41	0.28	0.35

*: significant at $P < 0.05$.

sample solubility are a matter. The composition of antioxidants, different selectivity of radical and the solubility of extracts could influence the capacity of extract to react with other radicals in each assay (Mohdaly et al., 2010). As mentioned previously, the high hydrophobicity of DPPH$^{\bullet}$ radicals led the assay less acceptable than the more flexible nature of ABTS under the aqueous extraction condition. Therefore, the significantly higher r values between TPC x ABTS and TPC x DPPH and TPC x FRAP suggested that ABTS assays would be appropriate to determine quickly antioxidant activities from sesame cake aqueous extract. Nevertheless, none of the assay was recommended to be used alone to avoid interferences resulting in the underestimate or overestimate the overall antioxidant capacities of samples.

3.2. Effects of pre-treatment by microwave on TPC and AA

As the microwave power and frequency was preset at 700W and 2450 Hz respectively, the pre-treatment time was the only independent parameters. Data showed it witnessed an increase in TPC and AA as the pre-treatment time by microwave irradiation increased (Figure 2). Even though all responses became progressively greater, statistical analysis indicated that only the TPC (Figure 2a) and AA based on FRAP (Figure 2d) assays was considerably improved as the samples were pre-treated by microwave irradiation for 120s as compared with the non-pre-treated. The ABTS$^{\bullet+}$ (Figure 2b) and DPPH$^{\bullet}$

(Figure 2c) scavenging capacity was not greatly elevated by the pre-treatment of microwave irradiation.

Microwave pre-treatment has been reported to have more significant support effects than ultrasound pretreatment in the extraction efficiency of sesame cake extracts. In an unpublished research, data showed ultrasound seems to have no significant role in improving the efficiency of extraction. Wataniyakul et al. (2012) did conclude microwave was possibly applied to treat samples before water extraction. The presence of water with rather high dielectric constant results in a phenomenon called "superheating" in which microwave energy were well absorbed by water in the matrix but poor dissipated. The cells got heating very fast and became ruptured to release more solutes which is partially antioxidants, leading to a higher extraction recoveries (Veggi et al., 2013).

Although results showed a significant increase in TPC values for pre-treated samples relative to the non-pretreated, that recoveries was not related to an increase in AA values of DPPH and ABTS assays, indicating that the free radical scavenging abilities of the extracts were either treated for long time enough that made the two treatment insignificant or the newly-extracted phenolics did not retain the scavenging ability. In case on FRAP assays, a significantly greater AA values at 120s relative to other treatments showed the possible connection between microwave irradiation and reducing ability of antioxidant. Yet, there is little evidence to prove this case of phenomenon.

4. Conclusion

Polyphenol aqueous extraction from sesame cake proposed a promising replacement of organic solvents by water. It was the extraction temperature that had major influence on the TPC and antioxidant activities of the extracts while in the experiment range of present studies, extraction time was found to be insignificant. The optimum extraction condition recommended were 90^0C for 30 min in this research. Moreover, microwave pre-treatment at 120s could have significantly positive influence on the overall extraction yield, especially the total phenolics and antioxidants based on FRAP assays. All three assays showed the positive antioxidant activities of the extracts, ABTS assays, however, was suggested to be the most appropriate assays among the three assays. Moreover, the differences in antioxidant efficiencies are also implying that phenolic from the extracts are not the only contributor to the total antioxidant activities in the sesame cake. The obtained results, in the overall view, suggest that sesame aqueous extracts could be a source of antioxidants with more feasible applications in food as well as other industry. Further studies could focus on determining more appropriate antioxidant activities assays to obtain a more comprehensive understanding on antioxidant properties of the extracts.

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