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Optimization of the enzyme-assisted vacuum-distillation of essential oil from the leaves of *Citrus limonia* Osbeck by response surface method

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Abstract
The process of enzyme-assisted vacuum-distillation for extraction of leaves essential oil from *Citrus limonia* Osbeck was studied and optimized using response surface methodology (RSM). The factors considered were material to water ratio (A), incubation time (B) and concentration of Viscozyme-L (C). These parameters were varied at two levels. The material to water ratio of 0.26 g/ml, incubation time 71.25 min and Viscozyme -L concentration of 8.46 U/ml were predicted as the optimum conditions. These factors gave an optimum oil yield of 0.28%. Analysis of variance (ANOVA) indicates that the model was significant as evidenced from R^2 of 0.9018 and the model F-value of 6.12. The experimental value (0.27 % oil yield) was close to the predicted value (0.28%). Therefore, the model can be used for prediction of oil yield in essential oil extraction from *Citrus limonia* Osbeck leaves via enzyme-assisted vacuum-distillation method.

Keywords: optimization, response surface methodology, enzyme-assisted vacuum-distillation, essential oil, *Citrus limonia* Osbeck

Introduction

Citrus is a common term and genus of flowering plants that belongs to the *rue* family, *Rutaceae*, originating and growing extensively in tropical and subtropical southern regions of Asia (Calabrese, 2002) [3]. *Citrus* is the most abundant fruit crop around the world with an annual production of around 115 million tons in 2011 (FAO, 2014) [4].

In citrus, EOs are mainly found from peels, leaves and flowers. Citrus leaf essential oils are mainly consisted of limonene, β- pinene, neral and geranial (Bhuiyan, 2009; Majnooni, 2012; Paoli, 2016; Yuanzheng, 2000) [2, 8, 10, 14] With antimicrobial and antioxidant activities (Hamdan, 2013; Mohammad, 2017; Reddy, 2012) [5, 9, 12], *citrus* leaf essential oils can replace synthetic agents to lengthen the food shelf life and avoid health-related problems, off odors, unpleasant tastes or changes in color. It is necessary to enhance and improve the recovery processes and integrate methods that allow the maximal use of raw materials. Currently, the extraction of citrus oil is still done using conventional methods, which is hydro-distillation, that generally have high yield (Mohammad, 2017; Reddy, 2012) [9, 12]. However, the hydrolysable compounds such as ester, as well as thermally labile components, may be decomposed during the distillation process (Houghton, 1998) [6]. In addition, the hydro-distillation method take relatively long time. Therefore, consideration should be given to using a new technique in the extraction of essential oils to improve the yield without alteration of components of the product extracted with minimum time usage. Until now it has developed new methods to extract essential oils, one of which is using the enzyme (enzyme-assisted extraction).

Previous research has shown that the extraction using the enzyme is an alternative that could be developed than the conventional methods, because of the high levels of product purity, no altering compounds and short processing time. Some extraction with enzyme application that has been reported as an excellent alternative providing a more efficient recovery of compounds of product extracted compared with conventional methods (Bhat, 2000; Sowbhagya, 2011) [1, 13]. The use of enzyme for thyme and rosemary essential oil extraction were reported. In thyme essential oil extraction, application enzymes induced 109% increase in the essential oil yields. In case of rosemary essential oil, enzymatic treatment resulted in enhanced oil yields by 20% (Karim, 2013) [7].

This study proposes an alternative for essential oil production using enzymatic pre-treatment of vacuum-distillation. Vacuum distillation used in the study is modified from hydro-distillation method to reduce the boiling point and avoid altering volatile compounds while application of enzyme is to hydrolyze partial or complete of the cell walls and increase of oil yield. In the extraction by enzyme-assisted vacuum distillation, the extraction parameters need to be optimized. Response Surface Methodology (RSM) is a collection of mathematical and statistical techniques useful for modeling and analyzing of problems in which a response of interest is influenced by some quantitative variables with the objective of optimizing the response. The aim of this study is optimizing the conditions of enzyme-assisted vacuum distillation of *citrus* leaf essential oil (*Citrus limonia* Osbeck) using Response Surface methodology.

Materials and Methods

Enzyme-assisted vacuum distillation method

Leaves of *Citrus limonia* Osbeck were obtained from Da Lat province, Vietnam. The essential oils of *citrus* leaves were extracted using a modified Clevenger-type apparatus. Essential oil was extracted from plant materials by vacuum-distillation. The maintenance pressure of the system was controlled at 575mm Hg. Before extraction, *citrus* leaves were subjected to a preliminary treatment by soaking in water, then put into a 2 L glass beaker equipped with a hot plate and a stirrer. After adjusting the medium to a pH value of 4.5, Viscozyme-L enzyme was added. Incubation was then performed under stirring at 45ºC for 60-120 min. Then, the material was vacuum distilled in a Clevenger-type apparatus for specified times (3 hours). The obtained essential oil was dried over anhydrous sodium sulfate and stored in a sealed vial at 4 °C in the dark prior to analysis. Oil yield is calculated based on the mass of essential oil obtained and the mass of the initial material

Yield (%) =

$$\frac{\text{Weight of extracts recovered}}{\text{Weight of fresh leaf}} \times 100 \quad \text{(Equation 1)}$$

Design of experiments

Response surface methodology, in particular the Box-Behnken design (Rajasimman, 2009) [11] was employed to estimate the effect of 3 reaction parameters namely material to water ratio (A), incubation time (B) and Viscozyme-L concentration (C) on yield of essential oil. These variables each at two levels, low and high: A (0.2-0.3 g/ml), B (60-120 min) and C (6-10 U/ml) are presented in Table 1. These levels were chosen based on the capacity of the experimental set up for variables A and B, while C was selected based on experimental run time. The experimental design is shown in Table 2.

Table 1: Design summary

Factor	Name	Units	Low actual	High actual	Low code	High code
A	Material to water ratio	g/ml	0.2	0.3	-1	1
B	Incubation time	Min	60	120	-1	1
C	Concentration of Viscozyme L	U/ml	6	10	-1	1

The regression analysis was performed to estimate the response function as a second order polynomiala

$$Y = \beta o + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} {X_i}^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j \quad \text{(Equation 2)}$$

where Y is the predicted response, Xi is the uncoded value of the i-th test variable, β0, βi and βij are coefficients estimated from the regression according to Rajasimman (Rajasimman, 2009) [11]. A statistical program package Design-Expert version 11 (State-Ease Inc., Minneapolis, MN, USA) was used for regression analysis of the data and for estimating the coefficient of the regression equation. The equations were validated by the analysis of variance (ANOVA) test. Model and regression coefficients were considered significant when the p-value was lower than 0.05.

Table 2: Box-behnken matrix

Run	A	B	C	Yield %
1	0.3	60	8	0.25
2	0.2	60	8	0.2
3	0.3	120	8	0.24
4	0.2	120	8	0.15
5	0.25	60	6	0.17
6	0.25	120	6	0.18
7	0.25	60	10	0.24
8	0.25	120	10	0.15
9	0.3	90	6	0.14
10	0.2	90	6	0.15
11	0.3	90	10	0.18
12	0.2	90	10	0.18
13	0.25	90	8	0.28
14	0.25	90	8	0.28
15	0.25	90	8	0.25
16	0.25	90	8	0.26

Results and Discussion

Table 2 presents the Box-Behnken Design matrix and the *citrus* peel oil yield obtained for each experimental run. Analysis of variance for the response surface model is presented in Table 3. The analysis indicates that the model F-value of 6.12 implies the model is significant. There is only a 1.95% chance that an F-value this large could occur due to noise, the model also has a satisfactory level of adequacy (R^2). In this study, A^2 and C^2 are significant model terms ($p < 0.05$). The lack of Fit F-value of 4.41 implies the lack of Fit is not significant relative to the pure error. There is a 12.73 % chance that a lack of Fit F-value this large could occur due to noise.

Table 3: Analysis of variance (ANOVA) for response surface quadratic model to identify significant factors affecting the *citrus* oil yield

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	0.0335	9	0.0037	6.12	0.0195
A-Material to water ratio	0.0021	1	0.0021	3.47	0.1117
B-Incubation time	0.0024	1	0.0024	4.03	0.0916
C-Concentration of Viscozyme-L	0.0015	1	0.0015	2.49	0.1659
AB	0.0004	1	0.0004	0.6575	0.4484
AC	0.0000	1	0.0000	0.0411	0.8461
BC	0.0025	1	0.0025	4.11	0.0890
A^2	0.0064	1	0.0064	10.52	0.0176
B^2	0.0012	1	0.0012	2.01	0.2057
C^2	0.0169	1	0.0169	27.78	0.0019
Residual	0.0036	6	0.0006		
Lack of Fit	0.0030	3	0.0010	4.41	0.1273
Pure Error	0.0007	3	0.0002		
Cor Total	0.0372	15			

Standard deviation: 0.0247; R^2: 0.9018; Adj R^2: 0.7545; Pred R^2:-0.3127; Adeq precision: 6.7952 *Significant variable

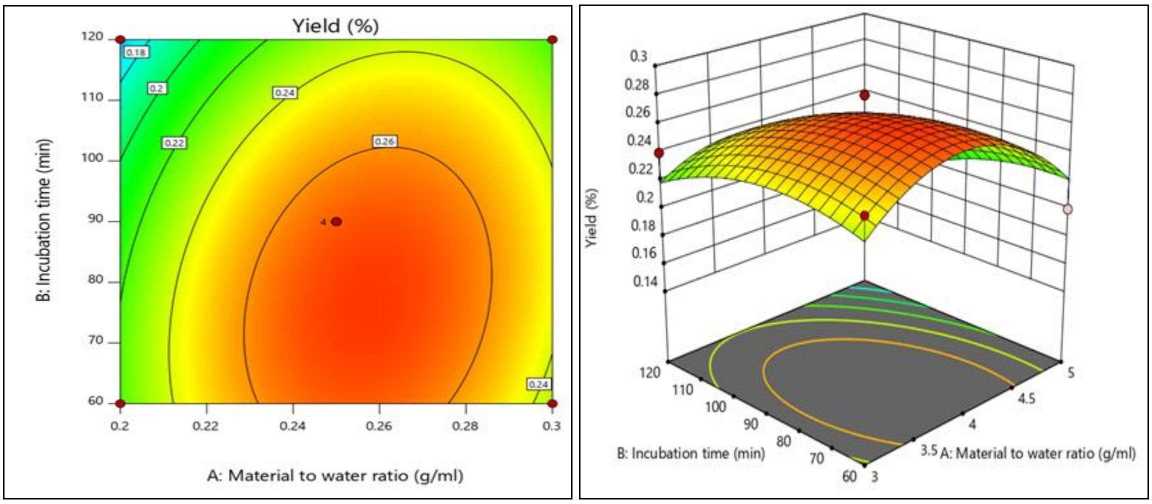
The Pred R^2 of -0.3127 implies that the overall mean is a better predictor of the response than the current model. Adeq Precision measures the signal to noise ratio; the ratio of 6.7952 obtained indicates an adequate signal (a ratio greater than 4 is desirable). Therefore the model can be used to navigate the design space.

The effects of interactions between material to water, incubation time and Viscozyme-L concentration on the essential oil yield could be intuitively reflected by the curved surface drawing and contour map. Figure 2a shows the effects of the interaction of A (material to water ratio) and B (incubation time) on the essential oil yield at 8 U/ml Viscozyme-L concentration. When the material to water ratio was constant, essential oil yield increased quickly at the beginning first, then began to decline slowly with the increasing of incubation time. When the incubation time remained constant, the citrus essential oil yield increased slowly to a maximum level, then began to decline with the rising of the material to water ratio. And the essential oil yield increased or declined more quickly with the increase of the concentration of incubation time at less than 100 minutes of incubation than at other incubation time

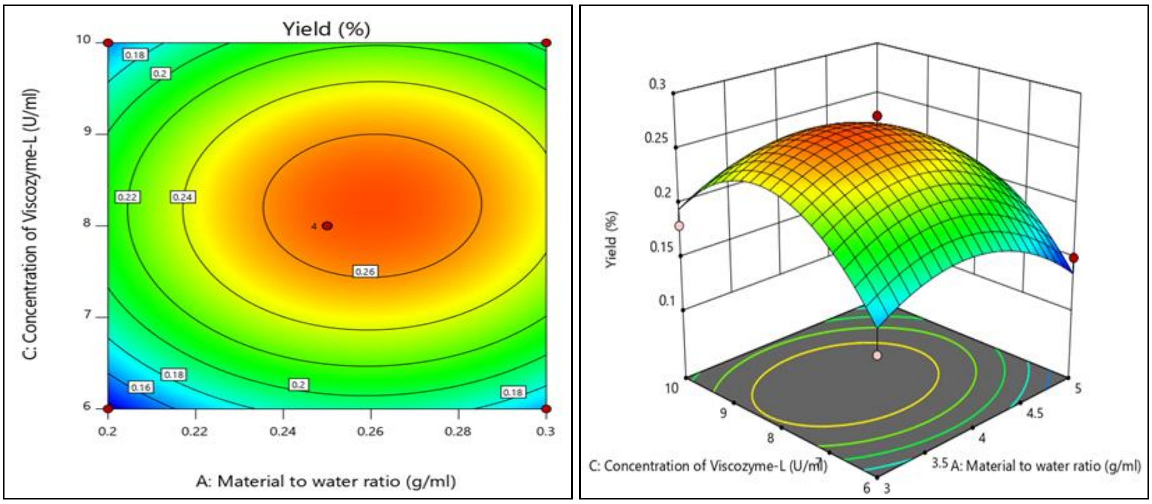
Figure 2b shows the effects of the interaction of A (material to water ratio) and C (concentration of Viscozyme-L) on the essential oil yield at 90 minutes of incubation. At a constant material to water ratio, the essential oil yield increased with the increase of Viscozyme-L concentration within a certain range; when the essential oil yield had reached a certain level, it began to decline with the increase of Viscozyme-L concentration value. If the Viscozyme-L concentration value remained unchanged, the essential oil yield would increase with the increase of material to water ratio within a certain scope; but beyond this range, the essential oil yield began to decline.

Figure 2c shows the effects of the interaction of B (incubation time) and C (concentration of Viscozyme-L concentration) on the citrus essential oil yield at 0.25 g/ml material to water ratio. When the concentration time value was constant, essential oil yield increased first, and then declined with the increase of incubation time. When the incubation time was constant, the essential oil yield increased to a maximum level and then declined slowly with the increase of Viscozyme –L concentration value. And the essential oil yield increased or declined more quickly with the increase of incubation time at the Viscozyme-L concentration less than 9 U/ml.

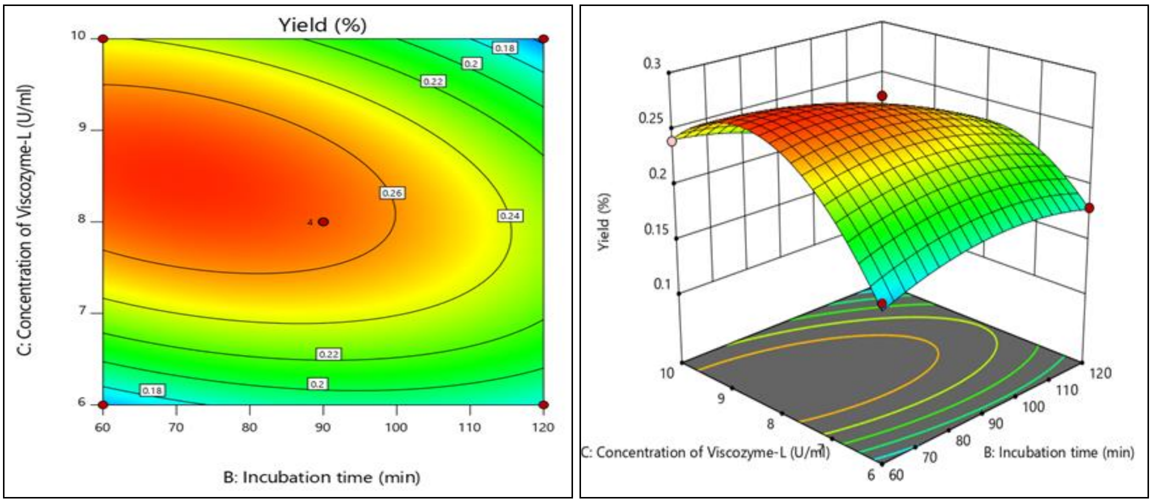
The response surface curve are plotted to understand the interaction of variables and the optimum level of each variables for maximum response (Rajasimman, 2009) [11]. The interaction effects between the variables are significant, as evidenced from the elliptical nature of the contours.



(a)



(b)



(c)

Fig 2: Response surface showing effect of (a) material to water ratio and incubation time on the yield at constant Viscozyme-L concentration (8 U/ml); (b) material to water ratio and Viscozyme-L concentration on the yield at constant incubation time (90 mins); (c) incubation time and Viscozyme-L concentration on the yield at material to water ratio (0.25 g/ml)

Final equation in terms of actual variables is given by Equation 3.

Yield = -2.11375 + 7.525 A + 0.004583 B + 0.298125 C + 0.006667 AB + 0.025 AC – 0.000417 BC - 16 A^2 – 0.000019 B^2 – 0.01625 C^2 (Equation 3)

Where A is material to water ratio, B is incubation time and C is Viscozyme-L concentration.

Experimental data generated in Table 2 in terms of actual values were substituted into Equation 3 and the predicted *citrus* peel oil yield was obtained. Actual *citrus* oil yield and the predicted yield are presented in Figure 3. Figure 3 indicates that the predicted values and actual values are in close agreement. This suggests good reliability of the model as also evidenced from the statistical parameters of the model such as standard deviation of 0.0247, R^2 of 0.9018 and F-value of 6.12. This shows fitness of the data for the model.

The optimum conditions for the response were predicted by numerical optimization method via Design-Expert version 11 (State-Ease Inc., Minneapolis, MN, USA). The material to water ratio of 0.26 g/ml, incubation time 71.25 min and Viscozyme-L concentration of 8.46 U/ml were the optimum conditions for the yield. This condition gives a yield of 0.28%.

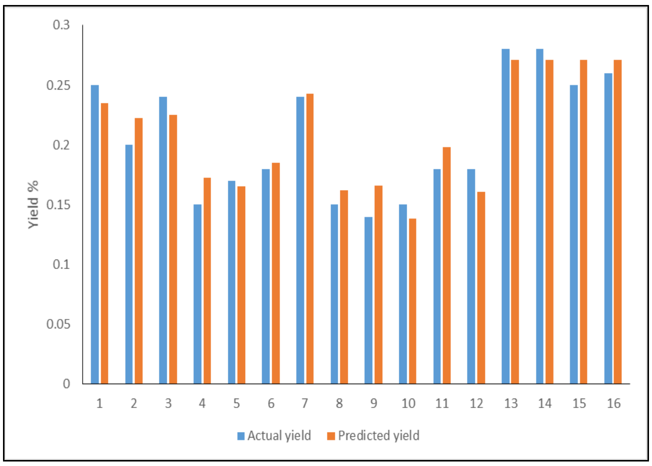


Fig 3: Predicted and actual oil yield from the model

Conclusion

The response surface methodology technique is applied for optimization model to extract citrus peel essential oil by enzyme-assisted vacuum-distillation. The model fits experimental data. Material to water ratio, incubation time and concentration of Viscozyme-L were the major process parameters found to significantly influence the oil yield. The optimum conditions predicted were 0.26 g/ml material to water ratio, 71.25 min incubation time and 8.46 U/ml Viscozyme-L concentration, which gives 0.28 % oil yield.

Acknowledgments

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