CHAPTER 3
RESEARCH METHODOLOGY

3.1 Instruments and Chemicals

3.1.1 Instruments

1. Analytical balance: Satorius analytic model BP 221S, Satorius GmBH, Germany
2. Autoclave: Hirayama HVE-50, Unitated States; SS-320, Tomy, Seiko, Japan
3. Centrifuge: Firlabo SW 9 Model, UK; Hettich zentrifugen, ROTOFIX 32, Germany
4. Erlynmeyer flask: PYREX, USA
5. 2-L Fermentor: BioFlo 110, New Brunswick Scientific EDISON, N.J., USA
6. Dissolved oxygen probe: METTLER TOLEDO, Switzland
7. Oven: Venticell Medcenter Einrichtungen GmbH, Munche; Memmert 854, Memmert, Schwawach, Germany
8. Incubator shaker: Innava™ 4330 Refrigerated Incubator Shaker, New Brunswick Scientific Edison, New Jersey., USA
9. Laminar flow: HoltenLaminAir, Allerod, Denmark
10. Micropipette: Model P100, P200, P1000, P5000, Gilson, France
11. pH meter: UB-10, Denver Instrument, New York
12. Spectrophotometer: GENESYS 20, Spectronic Unicam, USA
13. Vortex mixer: vortex-2 Genie G-560E, Scientific Industries Inc., New York, US.
14. Water bath: model BS 01, Applied Medic, Bangkok, Thailand
15. Microscope: Olympus BX51 series microscope and Olympus Automatic Exposure Photomicrographic System Model PM-10 AK, Olympus Optical Co Ltd., Tokyo Japan
16. HPLC column: 300 mm × 6.5 mm (i.d.) Sugar-Pak™ I, Waters, USA
17. High Performance Liquid Chromatography model: Shimadzu, Japan.
18. Kjeldahl distillation: Buchi model 321 distillation, Ontario, Canada

3.1.2 Chemicals

- 3,5-Dinitrosalicylic acid, 1-Kestose, Dimethyl sulfoxide (DMSO), Sodium pyruvate: Sigma Aldrich, Switzerland.
- Ethyl alcohol absolute (C_2H_5OH): Carlo Erba Reagents, Italy.
- Ethyl acetate ($C_4H_8O_2$), Hexane (C_6H_{14}), Hydrochloric acid (HCl), Sulfuric acid (H_2SO_4): Mallinckrodt Chemicals, USA
- Fructose ($C_6H_{12}O_6$), Sulfuric acid (H_2SO_4), Yeast Malt extract medium: Merck, Germany.
- Sodium hydroxide (NaOH): Asia Pacific Specialty Chemicals, Australia.
- Ammonium Sulphat $(NH_4)_2SO_4$, Potassium Nitrate (KNO_3), Sucrose ($C_{12}H_{22}O_{11}$), Glucose ($C_6H_{12}O_6$), Zinc Chloride($ZnCl_2$), Tween 20: Ajak Finechem, New Zealand
- Ca EDTA ($C_{10}H_{12}CaNa_2Na_2O_8$): Fluka Bichemika, Switzerland
- *n*-hexadecane ($C_{16}H_{34}$): Fluka Analytical, Germany

3.2 Microbial Strain

The red yeast, *Xanthophyllomyces dendrorhous* TISTR 5730, was purchased from Thailand Institute of Scientific and Technology Research (TISTR) as freeze-dried cultures. The yeast was activated by culturing in YM medium at 22 °C, 200 rpm for 24 hours. Stock was prepared by suspending in YM medium supplemented with 20% glycerol and stored at -80 °C.

3.2.1 Starter Preparation

The yeast strain was quickly thawed at room temperature, and then inoculated into a 250-mL Erlenmeyer flask containing 50 mL of YM medium containing 10 g/L glucose, 5 g/L peptone, 3 g/L yeast extract, and 3 g/L malt extract. The flasks were maintained on an orbital shaker under 200 rpm agitation at 22 °C for a period of 48 hours. The seed culture was then streaked onto YM agar plates, and maintained on incubator at 22 °C for 72 hours. To prepare inoculum for all subsequent experiments, 2 loops full of yeast was activated in 250-mL Erlenmeyer flask with 50 mL YM medium and maintained on an orbital shaker at 200 rpm under 22 °C for 24 hours.

3.3 Pineapple Juice Preparation

Pineapple juice concentrate was obtained from canned pineapple factory. It is by-product obtained from pineapple waste, i.e., peel, core, and low quality pineapple fruit by the process of pressing and, then, evaporation resulting in pineapple juice concentrate. Currently, pineapple juice concentrate was employed as animal feed supplement. In this study, in order to prepare stock of pineapple juice concentrate, pineapple juice concentrate was diluted with DI water to appropriate final total sugar concentration and then filtered through a double-fold-cotton cloth. Particulate matter was further removed by centrifugation at 10,000 rpm for 10 minutes at 4°C. Stock solution containing sucrose, glucose, and fructose at the concentrations of 12.99, 32.54, and 42.88 g/L, respectively, whereas TKN and protein present were 0.042 and 0.26 % (w/w), respectively. Pineapple juice concentrate stock was then stored at -20°C until use.

3.4 Measurement of Biomass

Yeast cells from all experiments were separated from the liquid medium by centrifugation (4°C, 4000 rpm, 5 minutes) and washed twice with double distilled water. Cell pellet was resuspended in distilled water and cell concentration was determined turbidometrically at 660 nm (Kongtragool, 2006). The biomass concentration was directly calculated using standard curve constructed previously (Appendix B1).

3.5 Astaxanthin Extraction

Yeast cells were separated from the liquid medium by centrifugation (4°C, 4000 rpm, and 5 minutes) and washed twice with double distilled water. The extraction and analysis of carotenoid content in the yeast cells was conducted according to the method proposed by Sedmak *et al.* (1900). Briefly, the yeast cell suspension were subjected to centrifugation at 4°C, 4000 rpm for 5 minutes. Yeast cells (0.3 mL) was disrupted by treating with 1 mL Dimethyl Sulfoxide (DMSO) with glass bead and immersed in 55°C water bath for 5 minutes followed by vortexing for 10 minutes. Then, the mixture was extracted with hexane:ethyl acetate at 50:50 (v/v) and vortexing for 10 minutes. Cell and cell debris were then removed by centrifugation at 4°C, 4500 rpm for 5 minutes. Total carotenoid content was calculated from the absorbance measured at 480 nm using the correlation provided below:

$$\text{Astaxanthin } (\mu g/g \text{ yeast}) = \frac{\text{Volume of solvent} \times OD_{480}}{2100 \times \text{Cell dry weight}}$$

(Equation 3.1)

3.6 Determination of Sugar Concentration using HPLC

Sugars concentrations were analyzed by HPLC equipped with a Sugar-pak™ column (Falcão-Rodrigues *et al.*, 2007). Deionized water containing 5 ppm CaEDTA at flow rate of 0.5 mL/min served as mobile phase whereas the column temperature was maintained at 80°C. The injection volume of 20 µL sample was used and the peaks were detected with UV together with RID. Additionally, standard for each sugar was chromatographed individually and provided in Appendix B.

3.7 Cultivation of *X. dendrorhous* on YM Medium and Pineapple Juice Medium

250 mL Erlenmeyer flask containing 50 mL of YM medium and Pineapple juice diluted to 10 g/L total sugar were individually prepared and subjected to autoclaving at 121°C for 30 minutes. 10% inoculum prepared previously was then introduced to each flask. Flasks were then incubated at 22°C under 200 rpm agitation. Small sample was taken periodically and immediately subjected to centrifugation at 4°C, 4500 rpm for 5 minutes. Biomass production and astaxanthin accumulation were determined as described previously.

3.8 Screening of Factors Affecting Growth by Fractional Factorial Design (FFD)

Objective of this study was to screen the important nutrients and culture conditions influencing biomass production and, at the same time, astaxanthin production. Therefore, a 2^{6-2} two-level fractional factorial design of resolution IV was adopted to investigate effects of six variables namely, sucrose, glucose, $(NH_4)_2SO_4$, KNO_3, *n*-hexadecane, and pH, whose concentration ranges were provided in Table 3.1. The design mandates 2^4 runs while each variable was studied at two levels, high (1) and low (-1). Therefore, the total of nineteen runs were necessary including three center points

Table 3.1 The selected variables together with their corresponding concentrations employed in FFD

Variables	Low (-1)	Center (0)	High (+1)
A : Sucrose (g/L)	20.0	30.0	40.0
B : Glucose (g/L)	10.0	15.0	20.0
C : $(NH_4)_2SO_4$ (g/L)	1.0	2.0	3.0
D : KNO_3 (g/L)	1.0	2.0	3.0
E : *n*-hexadecane (% (v/v))	0	6.0	12.0
F : pH	4.5	5.5	6.5

Table 3.2 Experimental design of 2_{IV}^{6-2} fractional factorial design employed

Run No.	A	B	C	D	E	F
1	-1	-1	-1	-1	-1	-1
2	1	-1	-1	-1	1	-1
3	-1	1	-1	-1	1	1
4	1	1	-1	-1	-1	1
5	-1	-1	1	-1	1	1
6	1	-1	1	-1	-1	1
7	-1	1	1	-1	-1	-1
8	1	1	1	-1	1	-1
9	-1	-1	-1	1	-1	1
10	1	-1	-1	1	1	1
11	-1	1	-1	1	1	-1
12	1	1	-1	1	-1	-1
13	-1	-1	1	1	1	-1
14	1	-1	1	1	-1	-1
15	-1	1	1	1	-1	1
16	1	1	1	1	1	1
17	0	0	0	0	0	0
18	0	0	0	0	0	0
19	0	0	0	0	0	0

(Table 3.2). The design generators used in this screening experiment were E = ABC and F = BCD given that the defining relation of the form I = ABCE = BCDF = ADEF were adopted. Statistical analysis was performed using Minitab 14 software.

Individual experimental run was prepared according to experimental design (Table 3.2) in 250 Erlenmeyer flask containing the final volume of growth medium of 50 mL providing that pineapple juice concentrate diluted to contain 10 g/L final glucose concentration was employed as base growth medium. After autoclaving, 10% by volume of 24-hour-old inoculum was inoculated simultaneously with an introduction of *n*-hexadecane separately sterile by membrane filtration. All flasks were then incubated in an orbital shaker at 22 °C under 200 rpm agitation for 10 days. Samples were taken periodically at every 24 hour interval for the measurement of biomass and astaxanthin concentration.

3.9 Optimization of Growth using Doehlert Design

To optimize biomass production, a response surface methodology (RSM), particularly, Doehlert design, was employed for determining the optimal conditions of significant variables identified using the screening experiment (Box *et al.*, 1978). The experimental results were fitted to a predictive quadratic equation representing the correlation between the response variable and the independent variables. The analysis of variance (ANOVA) of the experimental data and the model coefficient were computed using Minitab 14. In addition, two-dimentional contour plot and three-dimentional surface plot were constructed for visual observation of the interactive effects of the significant variables on the responses using STATISTICA 7 (StatSoft, Inc. USA).

Sucrose, KNO_3, and *n*-hexadecane (obtained from screening design on growth) were adopted to optimize biomass production of *X.* dendrorhous, due to their strong effects according to FFD. The Doehlert design with triangular face plane projection was chosen (Figure 3.1). Therefore, KNO_3 and sucrose (the two more significant) were investigated at five levels in order to obtain the most information of the system whereas *n*-hexadecane (the less significant) was studied at only three levels (Table 3.3) given that three replicates at center point were as well included (Table 3.4). Table 3.4 shows the ranges and levels of the three chosen variables in triangular face Doehlert design.

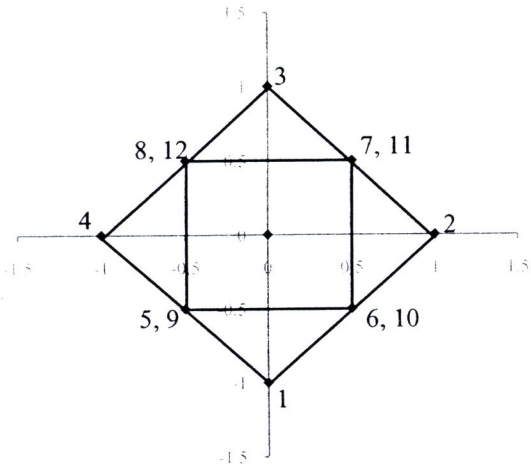

Figure 3.1 A triangular face plane projection of the three-variable Doehlert design

Table 3.3 Coded and actual values of the variables tested in Doehlert design for biomass production

Coded Level	X: Sucrose (g/L)	Y: KNO_3 (g/L)	Z: *n*-hexadecane (%v/v)
-1	25	0.25	-
-0.707	-	-	10.0
-0.5	30	0.63	-
0	35	1.0	8.0
0.5	40	1.38	-
0.707	-	-	6.0
1	45	1.75	-

The individual test medium was prepared according to experimental design (Table 3.4) given that pineapple juice diluted to the final glucose concentration of 10 g/L was employed as base medium. pH of the culture medium was then adjusted to 5.5. Subsequently, 10% by volume of 24-hour old inoculum was introduced and then incubated in an orbital shaker at 22 °C under 200 rpm agitation for 10 days. *n*-hexadecane was separately introduced into medium after autoclaving. Samples were taken from the culture flask every 24 hours for the measurement of biomass and astaxanthin concentration. All experiments were conducted using 250-mL Erlenmeyer flasks containing 50 mL growth medium (final volume).

Table 3.4 Three variable Doehlert experimental design for biomass production

Run No.	X	Y	Z
1	0	-1	0
2	1	0	0
3	0	1	0
4	-1	0	0
5	-0.5	-0.5	0.707
6	0.5	-0.5	0.707
7	0.5	0.5	0.707
8	-0.5	0.5	0.707
9	-0.5	-0.5	-0.707
10	0.5	-0.5	-0.707
11	0.5	0.5	-0.707
12	-0.5	0.5	-0.707
13	0	0	0
14	0	0	0
15	0	0	0

3.10 Screening of Factors Affecting Astaxanthin Production by Fractional Factorial Design (FFD)

With the completion of optimization of biomass production, screening of parameters influencing astaxanthin accumulation was undertaken. To screen factors significantly influencing astaxanthin production of *X. dendrorhous*, a 2^{4-1} two-level resolution IV half-fractional factorial design was again chosen to investigate effects of four variables, i.e., pyruvate, $ZnCl_2$, Tween20, and ethanol, whose concentration ranges were provided in Table 3.6. Therefore, the total of eleven runs were mandatory including three center points (Table 3.6). The design generators used in screening factors influencing astaxanthin production was D = ABC with no defining relation because this experiment contained only one design generator. Statistical analysis was performed by Minitab 14 software.

Table 3.5 The selected variables and their corresponding concentrations chosen for FFD range

Variables	Low (-1)	Center (0)	High (+1)
A : Pyruvate (mM)	10	25	40
B : $ZnCl_2$ (mM)	1	3	5
C : Tween20 (% v/v)	0.1	0.55	1
D : Ethanol (mM)	15	25	35

Table 3.6 Experimental design of 2^{4-1}_{IV} fractional factorial design

Run No.	A	B	C	D
1	-1	-1	-1	-1
2	1	-1	-1	1
3	-1	1	-1	1
4	1	1	-1	-1
5	-1	-1	1	1
6	1	-1	1	-1
7	-1	1	1	-1
8	1	1	1	1
9	0	0	0	0
10	0	0	0	0
11	0	0	0	0

The individual test medium was prepared according to experimental design provided in Table 3.6 and then incubated in an orbital shaker at 22°C under 200 rpm agitation for 10 days. The medium was prepared using 250-mL Erlenmeyer flasks containing the final volume of medium of 50 mL. The base medium containing 40.2 g/L sucrose, 1.23 g/L KNO_3 and 8.2 % v/v *n*-hexadecane together with pineapple juice diluted in such a way that the final to glucose concentration was at 10 g/L whereas the pH was adjusted to 5.5. *n*-hexadecane was separately introduced into medium after autoclaving. 24-hour old culture at 10% by volume was employed as inoculum. Samples were taken from the culture flask at every 24 hour interval for the measurement of biomass and astaxanthin concentration.

3.11 Optimization of Astaxanthin Production using Doehlert Design

Optimization is employed to investigate effect of a few identified variables on response and, at the same time, identify optimum conditions. However, to conduct optimization on a process with which prior knowledge is rarely available, steepest ascent may be employed to expedite the optimization of such system.

3.11.1 Path of Steepest Ascent

After performing a screening experiment and obtaining a linear model of the response, rapidly moving towards response maximization is preferable. Such task could be readily accomplished using the method of steepest ascent. To construct the path of steepest ascent requires the first-order model obtained from the screening experiment of the form

$$y = 835.9 + 41.0A - 124.8C$$

(Equation 3.2)

where y is astaxanthin content and A and C are the concentrations of pyruvate and Tween20, respectively. According to Equation 3.2, the path of steepest ascent would result in A moving in positive direction and C in a negative direction at a predetermined-stepwise manner, as can be seen in Table 3.9. Starting point of the path of steepest ascent was centered at A = 25 mM and C = 0.55%, which were the center point of FFD employed in screening experiment.

The individual test medium was prepared according to experimental design (Table 3.7) providing that the base medium always contained 40.2 g/L sucrose, 1.23 g/L KNO_3, 8.2 % v/v *n*-hexadecane and pineapple juice diluted to the final glucose concentration of 10 g/L. pH of the media was adjusted to 5.5. Additionally, both $ZnCl_2$ and ethanol were also included at the final concentrations of 1 and 35 mM, respectively. All experimental trials were inoculated with 24-hour old culture at 10% by volume. *n*-hexadecane and ethanol was introduced separately into cultivation medium after autoclaving. The medium was prepared using 250-mL Erlenmeyer flasks containing 50 mL of medium (final volume) and incubated in an orbital shaker at 22°C under 200 rpm agitation for 10 days. Samples were taken at every 24 hour interval for the measurement of biomass and astaxanthin concentration.

Table 3.7 Schematic representation of how to construct the path of steepest ascent.

Condition		Coded Unit		Uncoded Unit	
		A	C	A'	C'
1. Base levels (zero levels in the design)		0	0	25	0.55
2. Unit change		-	-	5	0.025
3. β_x from coded regression model*		41.0	-124.8	-	-
4. Uncoding of slope to original x scale (2× 3)		-	-	205	-3.12
5. ΔX (proportion of 4)		-	-	5	-0.076
6. Series of possible test runs along the path of steepest ascent	0	A_0	C_0	25	0.55
	1	$A_0+\Delta A$	$C_0+\Delta C$	30	0.47
	2	$A_0+2\Delta A$	$C_0+2\Delta C$	35	0.40
	3	$A_0+3\Delta A$	$C_0+3\Delta C$	40	0.32
	4	$A_0+4\Delta A$	$C_0+4\Delta C$	45	0.25
	5	$A_0+5\Delta A$	$C_0+5\Delta C$	50	0.17
	6	$A_0+6\Delta A$	$C_0+6\Delta C$	55	0.09
	7	$A_0+7\Delta A$	$C_0+7\Delta C$	60	0.02

* β_x represents coefficient of each variable according to the first-order model abtained from FFD

3.11.2 Optimization of Astaxanthin Production using Doehlert Design

After steepest ascent experiment for astaxanthin production was completed, Doehlert design, once more, was chosen for identifying the optimal levels of both pyruvate and Tween20 whose chosen ranges were 25-60 g/L and 0.02-0.55% (v/v), respectively. Pyruvate (the more significant) was investigated at five levels whereas Tween20 (the less significant), was studied at only three levels (Table 3.8) together with three replicates at center point as shown in Table 3.9.

Table 3.8 Coded and actual values of the variables employed in Doehlert design for astaxanthin production

Coded	X: Pyruvate (mM)	Y: Tween20(% v/v)
-1	20	-
-0.866	-	0.023
-0.5	35	-
0	50	0.17
0.5	65	-
0.866	-	0.317
1	80	-

Table 3.9 Two-variable Doehlert experimental designs for astaxanthin production

Run No.	X	Y
1	0.5	0.866
2	0.5	-0.866
3	-0.5	0.866
4	-0.5	-0.866
5	1	0
6	-1	0
7	0	0
8	0	0
9	0	0

The individual test medium was prepared according to experimental design given in Table 3.9 providing that the base medium always contained 40.2 g/L sucrose, 1.23 g/L KNO_3, 8.2 % v/v *n*-hexadecane and pineapple juice diluted to achieve the final glucose concentration of 10 g/L. Ethanol and *n*-hexadecane were introduced into medium in subsequent to autoclaving. pH of the growth medium was adjusted to 5.5. 24-hour old inoculum at 10% by volume was subsequently introduced. The medium were prepared using 250-mL Erlenmeyer flasks containing 50 mL of medium (final volume) and then incubated in an orbital shaker at 22°C under 200 rpm agitation for 10 days. Samples were taken at every 24 hour interval for determination of biomass and astaxanthin accumulation.

3.12 Validation Study in 2 Liter Fermentor

According to screening and optimization accomplished, respectively, with FFD and Doehlert design, the optimum condition leading to the highest biomass and astaxanthin production were identified. Therefore, it is of significant interest to validate the predictive efficiency of the obtained model using shake flask and 2 liter fermentor. The validation study was conducted at the previously established optimal conditions which were pineapple juice, the base medium containing 10 g/L glucose, supplemented with 40.2 g/L sucrose, 1.23 g/L KNO_3, 8.2 % (v/v) *n*-hexadecane, 0.15% Tween20, 49.8 mM pyruvate, 1 mM $Zncl_2$, and 35 mM ethanol. Ethanol and *n*-hexadecane were introduced separately into medium after autoclaving whereas pH of the medium was adjusted to 5.5. 24-hour old inoculum at 10% by volume was inoculated. For shake flask, experiments were performed using 250-mL Erlenmeyer flasks containing 50 mL of medium and incubated in an orbital shaker at 22°C under 200 rpm agitation for 10 days. Samples were taken on day 10 post inoculation for the measurement of biomass and astaxanthin concentration. For the 2-Liter fermentor, experiment was conducted with 1,500 mL medium. In addition, aeration and agitation were maintained at 1 vvm and 200 rpm, respectively. In batch cultivation using 2 liter fermentor, samples were taken periodically and frequently during the first 48 hour and from then on, at every 24 hour interval for determining biomass and astaxanthin production.