CHAPTER 3 METHODOLOGY

3.1 Microorganism

3.1.1 Endophytic fungal strain

An endophytic fungus, NHL-L 6/6, was isolated from *Stemona burkillii* leaves collected from Nahaew District, Loei Province, Thailand (Ratnarathorn *et al*., 2009). It showed high activity against many plant pathogenic fungi and bacteria suggesting that NHL-L 6/6 produced powerful antibiotics against phytopathogens. Therefore, it was adopted for production of anti-phytopathogenic compound in this study.

3.1.2 Plant pathogenic strains

The bacterial pathogenic strain used as indicator microorganisms in optimization experiment was *Erwinia caratovora.* The pathogenic strains selected for antimicrobial activity test included both bacterial pathogens (i.e. *Pseudomonas solanacearum* and *Xanthomonas citrii*), and fungal pathogens (i.e. *Alternaria brassicicola, A. porri, Penicillum* sp., *Fusarium solani, F. oxysporum* and *Colletotrichum* sp.).

3.2 Preservation of microorganism

For fungal culture preservation, fungi were grown on Potato Dextrose Agar (PDA) (Appendix B) and incubated at 26°C for 5 days, while bacteria were grown on Potato Sucrose Agar (PSA) (Appendix B) and incubated at 30°C overnight. The stock cultures were maintained at 4°C until used and were subcultured in the same medium once a month. For long-term storage, a few small pieces of fungal mycelia or active bacterial cells were kept in 15% glycerol and stored at -80°C.

3.3 Molecular technique based identification of an endophytic fungus

NHL-L 6/6 cannot produce spores in fungal media such as M1D Agar, Malt extract agar (MEA), Potato Dextrose Agar (PDA) and Corn Meal Agar (CMA) (Appendix B); however, NHL-L 6/6 could be identified using nucleotide sequence analysis of the ITS region of the ribosomal RNA genes.

3.3.1 Fungal DNA extraction

Fungal endophyte NHL-L 6/6 was grown on PDA at 26 °C for 5 days. The 12 corks (6 mm diameter) of NHL-L 6/6 were inoculated aseptically to 250 mL Erlenmeyer flask containing 100 mL modified M1D medium (Appendix B) and incubated in a rotary shaker (INNOVA 4330, New Brunswick Scientific, New Jersey, USA) at150 rpm, 28°C for 3 days. Mycelia were collected by filtration through a piece of nylon cloth and washed with sterile distilled water twice. Washed mycelia were kept at -30 °C until use. Genomic DNA was extracted from freshly frozen mycelia using modified DNA extraction method proposed by Raeder and Broda (1985).

1. Put a small amount (approximately 50-70 mg) of frozen mycelia in a chilled mortar, and then quickly pour liquid nitrogen into the mortar. The frozen mycelium was ground finely for approximately 30 seconds with a pestle.
2. The ground mycelium was transferred into a sterile eppendorf and resuspended in 500 µL of extraction buffer (Appendix B 2.5)
3. The slurry was mixed with 500 µL of phenol:chloroform:isoamyl alcohol (25:24:1 (v/v)) and the solution was mixed manually for 15 minutes.
4. The mixture was centrifuged at 13,000 rpm for 15 minutes at room temperature.
5. The upper aqueous phase was transfered to a new tube and 25 µL of RNAse A (10 µg/µL) was added, mixed gently and incubated for 30 minutes at room temperature.
6. The mixture was then extracted once with chloroform:isoamyl alcohol (24:1 (v/v)) and centrifuged at 13,000 rpm for 10 minutes at room temperature.

7. To precipitate the DNA, the upper aqueous phase was transferred to a new eppendorf; two volumes of cold absolute ethanol were added to one volume of the DNA sample, mixed well and incubated at -30 °C for an hour.
8. The pellet of DNA was collected by centrifugation at 13,000 rpm for 10 minutes at room temperature. The supernatant was carefully discarded.
9. The pellet of DNA was washed with 1 mL of cold 70% ethanol and then the mixture was centrifuged at 13,000 rpm for 3 minutes. The supernatant was carefully discarded by using micropipette. The pellet of DNA was allowed to stand for 1-2 hours at room temperature to dry, and then resuspened in 50 µL of deionized distilled water and was kept at -30 °C.
10. Size and quality of genomic DNA were determined by agarose gel electrophoresis (Appendix C)

3.3.2 Measurement of DNA concentration

Prior to measure the concentration, the DNA solution was diluted to a proper dilution with deionized distilled water. A measurement of DNA concentration was carried out at the wavelengths of 260 nm and 280 nm using a Biophotometer^{TM} (Eppendorf, Hamberg, Germany). The ratio between the reading at 260 nm and 280 nm (OD_{260}/OD_{280}) provided an estimated purity of the DNA sample. Pure preparation of DNA has OD_{260}/OD_{280} value of 1.65-2.00.

3.3.3 Polymerase chain reaction (PCR)

A primer pair, ITS1 (5’-TCCGTAGGTGAACCTGCGG-3’) and ITS4 (5’TCCTCCGCTTATTGATATGC-3’) (White *et al.*, 1990) was used to amplify the flanking of internal transcribed spacer region 1 (ITS1) and region 2 (ITS2) including 5.8S gene. The amplification was performed in a 50 µL reaction volume containing 50 ng of DNA template, 20 pmol of each primers (ITS1 and ITS4), 0.2 mM of dNTPs, 1X reaction buffer (Tris with 15 mM $MgCl_2$) and 2.5 units of *Taq* DNA polymerase. The thermal cycle consisted of 5 minutes of initial denaturation at 95 °C, followed by 30 cycles of 30 seconds denaturation at 94 °C, 30 seconds primer annealing at 50 °C, 1 minute extension at 72 °C, and a final 7 minutes extension at 72 °C. The size and purity of PCR products were estimated by agarose gel electrophoresis (Appendix C).

3.3.4 Purification of PCR products

The PCR products were purified using the HiYield™ Gel/PCR DNA Fragment Extraction Kit according to the manufacturer's instructions.

3.3.5 DNA sequencing

Purified PCR products were submitted to Bio Basic Canada Inc. for sequencing using ABI Prism 3730XL DNA sequencer. Primers ITS1 and ITS4 (White *et al*., 1990) were used in the sequencing reactions. The ITS sequences of NHL-L 6/6 were compared with ITS regions of rRNA gene sequences available in GenBank database by blasting (http://www.ncbi.nlm.nih.gov/BLAST). The pairwise alignment of ITS sequences of NHL-L6/6 and other organisms retrieved from GenBank database were done using ClustalW2.1 program available at http://www.ebi.ac.uk/Tools/msa/clustalw2/. The percentages of nucleotide sequence homology in comparison with other organisms were reported.

3.4 Inoculum preparation for liquid culture

For inoculum preparation, NHL-L6/6 was grown on Potato Dextrose Agar (PDA) plates and incubated at 26°C for 5 days. Four plates of 5-day-old fungal culture and 100 mL of sterile distilled water were blended at high speed for 1 minute using a sterile blender (model 34BL47, Waring, Torington, CT., USA).

3.5 Cultivation of NHL-L 6/6 in liquid medium

3.5.1 Cultivation of NHL-L 6/6 in Sucrose Yeast extract medium

One hundred milliliters of sucrose-yeast extract medium (2% sucrose and 0.3% yeast extract) in 250 mL Erlenmeyer flask were prepared. The initial pH of the medium was adjusted to 5.5 and then autoclaved at 121°C for 15 minutes. Five milliliters of prepared inoculum as described in 3.4 was then introduced into each flask. The flasks were then incubated at 28 °C, 150 rpm on a rotary shaker (INNOVA 4330, New Brunswick Scientific, New Jersey, USA). Samples were taken every day for 7 days. Biomass, final pH, residual sugar and antimicrobial activities against *E. caratovara* were determined (Appendix C). Experiments were conducted in triplicate.

3.5.2 Screening of the optimal nitrogen and carbon sources for anti-phytopathogenic compound production

To investigate the effects of nitrogen sources on antimicrobial compound production, four nitrogen sources (yeast extract, whey, urea and ammonium sulfate) at the final concentration of 3 g/L (3 g dried whey is approximately 25 mL liquid whey) were added to the trace elements solution containing $Ca(NO_3)_2$ (0.28 g/L), KNO_3 (0.8 g/L), KCl (0.6 g/L), $MgSO_4$ (3.6 g/L) and NaH_2PO_4 (0.2 g/L). The carbon source used in this experiment was sucrose at the final concentration of 20 g/L. To investigate the effects of carbon sources, four carbon sources (sucrose, glucose, cassava starch and molasses at the final concentration of 20 g/L) were added to the trace elements solution as described above. The nitrogen source used in this experiment was yeast extract at the final concentration of 3 g/L. Prior autoclaving, the initial pH of the medium was adjusted to 5.5. Inoculum was prepared as described in section 3.4. Fermentation medium in each flask was inoculated with 5% (v/v) inoculum and incubated at 28 °C, 150 rpm on a rotary shaker (INNOVA 4330, New Brunswick Scientific, New Jersey, USA). Each trial was performed in triplicate. Two milliliters of culture broth were collected at day 0, 2, 3, 4, 5, 6 and 7 and centrifuged at 13,000 rpm, 4 °C for 10 minutes. The supernatants were stored at -30 °C for further analysis. The antimicrobial activities were determined by agar well diffusion assay (Appendix C).

3.6 Experimental design

3.6.1 Selection of the significant medium components using a fractional factorial design (FFD)

The aim of this experimental step was to screen the medium components which show significant effect on anti-phytopathogenic compound production. Nitrogen sources (yeast extract and whey) and carbon source (sucrose and glucose) that promote secondary metabolite production were those identified in section 3.5.1. Therefore, a 2^{4-1} two level fractional factorial design of resolution III was adopted to investigate influence of these four variables, whose concentration ranges were provided in Table 3.1. Each variable was studied at two levels, high (+1) and low (-1). Since samples were taken for 5 days at day 4, 5, 6, 7 and 8 and the number of experimental runs was fourteen, the total number of culture flasks was seventy. Thus, they were divided into two homogeneous blocks. The totals of 14 runs including two blocks with three center points for each block were shown in Table 3.2. The first-order model to be fitted is of the form:

$$\hat{Y} = b_0 + \sum b_i x_i$$

where $\hat{Y}$ and b_0 are the predicted response and the constant coefficient, respectively, whilst b_i and x_i are the linear coefficient and the coded independent variable, respectively.

Individual run was performed according to the experimental design (Table 3.2) in 250 mL Erlenmeyer flasks at the working volume of 100 mL. After autoclaving, 5% (v/v) inoculums prepared as described in section 3.4 was inoculated individually into each medium. All flasks were then incubated at 28 °C, 150 rpm on a rotary shaker (INNOVA 4330, New Brunswick Scientific, New Jersey, USA). Samples were taken at day 4, 5, 6, 7 and 8 for determination of biomass and antimicrobial activity (Appendix C). The statistical software, Minitab 15, was used for the experimental design and analysis of the data.

Table 3.1 The selected variables and their corresponding concentrations employed in FFD.

Factor	Variables	Levels		
		Low (-1)	Center (0)	High (+1)
X_1	Yeast Extract (g/L)	1	2	3
X_2	Whey (mL/L)	0	10	20
X_3	Sucrose (g/L)	10	20	30
X_4	Glucose (g/L)	0	10	20

Table 3.2 Experimental design of 2^{4-1} fractional factorial design employed.

Run No.	Blocks	Yeast Extract (g/L)	Whey (mL/L)	Sucrose (g/L)	Glucose (g/L)
1	1	3 (1)	0 (-1)	10 (-1)	20 (1)
2	1	1 (-1)	20 (1)	10 (-1)	20 (1)
3	1	3 (1)	0 (-1)	30 (1)	0 (-1)
4	1	1 (-1)	20 (1)	30 (1)	0 (-1)
5	1	2 (0)	10 (0)	20 (0)	10 (0)
6	1	2 (0)	10 (0)	20 (0)	10 (0)
7	1	2 (0)	10 (0)	20 (0)	10 (0)
8	2	1 (-1)	0 (-1)	10 (-1)	0 (-1)
9	2	3 (1)	20 (1)	10 (-1)	0 (-1)
10	2	1 (-1)	0 (-1)	30 (1)	20 (1)
11	2	3 (1)	20 (1)	30 (1)	20 (1)
12	2	2 (0)	10 (0)	20 (0)	10 (0)
13	2	2 (0)	10 (0)	20 (0)	10 (0)
14	2	2 (0)	10 (0)	20 (0)	10 (0)

Note: The number in front of the parenthesis represent to actual value and the number in the parenthesis represent to coded value: -1 for the low level, 0 for the zero level and +1 for the high level.

3.6.2 Locating the region of optimum response by the steepest ascent design

After performing a screening experiment and obtaining a linear model of the response, rapid moving towards response maximization is preferable. The path of steepest ascent was endeavored to identify the proper direction of variable manipulation, increasing or decreasing the concentration according to the sign of the main effects to improve anti-phytopathogenic compound production. The first-order model obtained from the screening experiment using 2^{4-1} two-level fractional factorial design of the form $\hat{Y}$ = 11.55 - 0.255x_1 - 1.975x_2 + 4.575x_3 + 4.310x_4 is necessary to construct the path of steepest ascent, where $\hat{Y}$ is the response, that is, antimicrobial activity against *Erwinia caratovora* and x_1, x_2, x_3 and x_4 are yeast extract, whey, sucrose and glucose, respectively.

The path of steepest ascent was designed using Minitab 15 in such a manner that the concentrations of yeast extract (x_1), whey (x_2), sucrose (x_3) and glucose (x_4) were increasing and decreasing in a step wise manner, as shown in Table 3.3. Starting point of the path of steepest ascent was located at x_1 = 2 g/L, x_2 = 10 mL/L, x_3 = 20 g/L, x_4 = 10 g/L, which was the center point of FFD. Experiments were performed along the steepest ascent path until an increase in response is reached. Individual run was performed according to the experimental design (Table 3.3) in 250 mL Erlenmeyer flask with the working volume of 100 mL. After autoclaving, 5% (v/v) inoculum was inoculated individually into each flask. All flasks were then incubated at 28 °C, 150 rpm. Samples were taken at day 4, 5, 6, 7 and 8 for determination of antimicrobial activity (Appendix C).

Table 3.3 The concentrations of medium compositions along the path of steepest ascent

Condition	Code Unit				Uncode Unit			
	A	B	C	D	Yeast Extract (g/L)	Whey (mL/L)	Sucrose (g/L)	Glucose (g/L)
1. Base levels	0	0	0	0	2	10	20	10
2. Unit change	-	-	-	-	0.449	4.49	4.5	4.24
3. b_x from coded regression model (first-order model)	-0.255	-1.975	4.575	4.31	-	-	-	-
4. Uncoding of slope to original x scale	-	-	-	-	-0.11	-8.88	20.59	19.4
5. ΔX	-	-	-	-	-0.025	-1.94	4.5	4.24
6. Series of possible test runs along the path of steepest ascent	A_0	B_0	C_0	D_0	2	10	20	10
	$A_0+\Delta A$	$B_0+\Delta B$	$C_0+\Delta C$	$D_0+\Delta D$	1.98	8.06	24.5	14.24
	$A_0+\Delta 2A$	$B_0+\Delta 2B$	$C_0+\Delta 2C$	$D_0+\Delta 2D$	1.95	6.12	29	18.48
	$A_0+\Delta 3A$	$B_0+\Delta 3B$	$C_0+\Delta 3C$	$D_0+\Delta 3D$	1.93	4.17	33.5	22.72
	$A_0+\Delta 4A$	$B_0+\Delta 4B$	$C_0+\Delta 4C$	$D_0+\Delta 4D$	1.9	2.23	38	26.96

3.6.3 Optimization of the significant components concentrations using a Central Composite Design (CCD)

The Central Composite Design (CCD) was adopted to investigate the influence of four variables (i.e. yeast extract, whey, sucrose and glucose) on the anti-phytopathogenic compound production. As an α can be calculated using $\alpha = \pm 2^{n/4}$ where n = number of factor investigated; therefore, $\alpha = \pm 2^{4/4} = \pm 2$. The CCD for 4 factors requires 24 combinations including the replicates at the center point (16 cube points, 8 axial points and 7 center points to estimate the experimental error and to investigate the suitability of the proposed model). Levels of these variables used in CCD were provided in Table 3.4. The compositions of each experimental run necessary for CCD were given in Table 3.5. Once the experiments had been performed, the experimental results were fitted to a second-order polynomial function. The Student t-test permitted us to check the statistical significance of the regression coefficients, and the analysis of variances (ANOVA) was performed on the experimental data to evaluate the statistical significance of the model. The model for the response was expressed in terms of coded variables. Individual run was performed according to the experimental design (Table 3.5) in 250 mL Erlenmeyer flask with 100 mL working volume. After autoclaving, 5% (v/v) inoculum was inoculated individually into each medium. All flasks were then incubated in a shaker at 28 °C and 150 rpm. Samples were taken at day 5, 6, and 7 for determination of antimicrobial activity (Appendix C). Minitab 15 was used for the experimental design and analysis of the data.

Table 3.4 Levels of variables used in the CCD experiment

Factor	Variables	Levels				
		-2	-1	0	+1	+2
X_1	Yeast Extract (g/L)	1.9	1.95	2	2.05	2.1
X_2	Whey (mL/L)	6	8	10	12	14
X_3	Sucrose (g/L)	10	15	20	25	30
X_4	Glucose (g/L)	0	5	10	15	20

According to the optimization of anti-phytopathogenic compound production by CCD, the optimal conditions for the culture of *Nodulisporium* sp. NHL-L 6/6 were 2.03 g/L yeast extract, 9.86 mL/L whey, 25.08 g/L sucrose and 12.99 g/L glucose. The experiment was done in four replicates using 250 mL Erlenmeyer flask with 100 mL working volume to verify the modeling results. After autoclaving, 5% (v/v) inoculum prepared in section 3.4 was inoculated individually into each medium. All flasks were incubated at 28 °C, 150 rpm for 6 days. The antimicrobial activities of culture filtrates were determined by agar well diffusion assay (Appendix C).

Table 3.5 The concentrations of composition used in the CCD experiment

Run No.	Concentrations			
	Yeast Extract (g/L)	Whey (mL/L)	Sucrose (g/L)	Glucose (g/L)
1	1.95 (-1)	8 (-1)	15 (-1)	5 (-1)
2	2.05 (+1)	8 (-1)	15 (-1)	5 (-1)
3	1.95 (-1)	12 (+1)	15 (-1)	5 (-1)
4	2.05 (+1)	12 (+1)	15 (-1)	5 (-1)
5	1.95 (-1)	8 (-1)	25 (+1)	5 (-1)
6	2.05 (+1)	8 (-1)	25 (+1)	5 (-1)
7	1.95 (-1)	12 (+1)	25 (+1)	5 (-1)
8	2.05 (+1)	12 (+1)	25 (+1)	5 (-1)
9	1.95 (-1)	8 (-1)	15 (-1)	15 (+1)
10	2.05 (+1)	8 (-1)	15 (-1)	15 (+1)
11	1.95 (-1)	12 (+1)	15 (-1)	15 (+1)
12	2.05 (+1)	12 (+1)	15 (-1)	15 (+1)
13	1.95 (-1)	8 (-1)	25 (+1)	15 (+1)
14	2.05 (+1)	8 (-1)	25 (+1)	15 (+1)
15	1.95 (-1)	12 (+1)	25 (+1)	15 (+1)
16	2.05 (+1)	12 (+1)	25 (+1)	15 (+1)
17	1.9 (-2)	10 (0)	20 (0)	10 (0)
18	2.1 (+2)	10 (0)	20 (0)	10 (0)
19	2 (0)	6 (-2)	20 (0)	10 (0)
20	2 (0)	14 (+2)	20 (0)	10 (0)
21	2 (0)	10 (0)	10 (-2)	10 (0)
22	2 (0)	10 (0)	30 (+2)	10 (0)
23	2 (0)	10 (0)	20 (0)	0 (-2)
24	2 (0)	10 (0)	20 (0)	20 (+2)
25	2 (0)	10 (0)	20 (0)	10 (0)
26	2 (0)	10 (0)	20 (0)	10 (0)
27	2 (0)	10 (0)	20 (0)	10 (0)
28	2 (0)	10 (0)	20 (0)	10 (0)
29	2 (0)	10 (0)	20 (0)	10 (0)
30	2 (0)	10 (0)	20 (0)	10 (0)
31	2 (0)	10 (0)	20 (0)	10 (0)

Note: The number in front of the parenthesis denotes the actual value and the number in the parenthesis represents the coded value: -2 for the lower level, -1 for the low level, 0 for the zero level, +1 for the high level and +2 for the higher level.

3.7 Antimicrobial activity against bacterial and fungal pathogens of anti-phytopathogenic compounds

3.7.1 Anti-phytopathogenic compound preparation

The endophytic fungus *Nodulisporium* sp. NHL-L 6/6 was cultured in 250 mL Erlenmeyer flasks containing 100 mL optimized medium. Fermentation medium in each flask was inoculated with 5% (v/v) inoculum prepared in section 3.4 and incubated at 28 °C, 150 rpm for 6 days. The fermentation broth was harvested and filtered through nylon filter cloth to remove the fungal biomass. The filtrate was extracted with one volume of ethyl acetate twice at room temperature. The pooled extract was evaporated in a rotary vacuum evaporator (Buchi Model Rotavapor R-250) and weighed to constitute a crude extract. The crude extract was dissolved in 5% (v/v) solution of dimethyl sulfoxide (DMSO, Merck, Germany) to give a final concentration of 10 mg/mL as stock solution and kept at 4 °C in container wrapped with aluminum foil to protect the antimicrobial compound from light until use.

3.7.2 Determination of minimum inhibitory concentrations (MIC)

The antimicrobial activities of the bioactive metabolites were assayed against bacteria and fungi. The minimum inhibitory concentrations (MIC) against bacterial and fungal pathogens were determined by using broth dilution method and agar dilution method, respectively

3.7.2.1 Antibacterial activity assay

The two-fold serial dilution of crude extract in 5% (v/v) DMSO was performed in the PSB medium to give the final concentrations of 15.625, 31.25, 62.5, 125, 250 and 500 μg/mL. The solution of 5% (v/v) DMSO in Potato Sucrose Broth (PSB) was used as control. A single colony of bacterial pathogens (i.e., *E. caratovora*, *P. solanacearum* and *X. citrii*) grown on Potato Sucrose Agar (PSA) at 30 °C overnight was transferred into PSB and incubated overnight at 30 °C and 150 rpm. Bacterial liquid cultures were then added to PSB containing crude extract to obtain a final OD of 0.1 at 540 nm. A final volume of media used for each organism tested was 5.0 mL. All experiments were performed in duplicate. The samples were incubated at 30 °C for 24 hours. The MIC was defined as the lowest antimicrobial compound concentration at which bacterial growth was not observed when compared to that produced by control.

3.7.2.2 Antifungal activity assay

The stock of crude extract in 5% (v/v) DMSO was added to the melted PDA to make the final concentrations of 15.625, 31.25, 62.5, 125, 250 and 500 μg/mL. The agars and crude solutions were mixed thoroughly and poured into petri dishes. For control, the PDA with a final concentration of 5% DMSO was performed. The agars were allowed to solidify at room temperature. The plates were placed in laminar flow hood for approximately 30 minutes with their lids open to quickly dry agar surface. Fungal pathogens (i.e. *Alternaria brassicicola*, *A. porri*, *Penicillum* sp., *Fusarium solani, F. oxysporum* and *Colletotrichum* sp.) were grown on Potato Dextrose Agar (PDA) and incubated at 30°C for 7 days. The 7-day-old fungal pathogens were cut by using a 6 mm diameter cock borer, and then transferred on to PDA plates with or without antimicrobial solutions. All experiments were performed in duplicate. The culture plates were incubated at 30 °C for 5 days. The MIC was defined as the lowest antimicrobial compound concentration resulting in the absence of growth compared to that produced by the control plate. Sensitivity of each fungal species to antimicrobial agent was calculated as percentage of mycelial growth inhibition, according to the formula described by Pandey *et al*. (1982): (dc-dt)/dc x 100, where dc = average diameter of the fungal colony of the negative control and dt = average diameter of the fungal colony treated with the antimicrobial agent.

3.7.3 Determination of minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC)

To evaluate the MBC, 100 μL aliquots from the antimicrobial compound dilution tubes with no visible growth were spread onto PSA. These plates were incubated at 30 ºC for 24 hours. To evaluate the MFC, the agar plugs of fungal pathogens with no visible growth were placed onto the center of new PDA plates and incubated at 30ºC for 5 days. The complete absence of growth on the agar surface at the lowest concentration of sample was defined as MBC or MFC.