CHAPTER 4 RESULTS AND DISCUSSION

4.1 Morphology of an endophytic fungus NHL-L 6/6

NHL-L 6/6 is an endophytic fungus isolated from *Stemona burkillii* leave which showed the highest activity against some fungal plant pathogens (Ratnaranthorn *et al*., 2009) (i.e. *Alternaria brassicicola*, *Penicillum* sp., *Fusarium solani* and *Fusarium oxysporum*) and bacterial plant pathogens (i.e. *Erwinia caratovora*, *Pseudomonas solanacearum* and *Xantromonas citrii*). The colony grew rapidly on Potato Dextrose Agar (PDA) at 26 °C. Its colony was white and velvety and pale yellow to brown on the reverse side. It cannot produce conidia on Potato Dextrose Agar (PDA) and other common mycological media including Malt Extract Agar (MEA), Corn Meal Agar (CMA) and M1D agar (Appendix B). Therefore, this endophytic fungus was firstly classified as "*Mycelia sterilia*". Colony of NHL-L 6/6 grew on PDA plate at 26 °C for 5 days was shown in Figure 4.1.

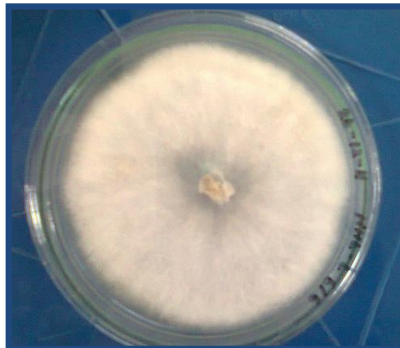

Figure 4.1 Colony morphology of fungal endophyte NHL-L 6/6 on PDA plate.

4.2 Identification of NHL-L 6/6 by using nucleotide sequence of the ITS regions of the ribosomal RNA gene

Classical identification of fungi is based on observable characteristics. Morphological species can be based on colony surface texture, hyphae pigment, margin shapes, growth rate and sporulation structure (Redlin and Carris, 1985). Unfortunately, this fungal isolate could not produce spores under tested condition. Therefore, molecular method of identification was performed. The filamentous fungus was identified by comparative

analysis of nucleotide sequence of the ITS regions (ITS1 and ITS2) of nuclear rRNA genes.

4.2.1 Preparation of genomic DNA and ITS regions

The quality of genomic DNA was examined using 0.8% (w/v) agarose gel electrophoresis. High quality of genomic DNA with approximate size of 21 Kb was shown in lane 1 in Figure 4.2 (a). The PCR amplification of internal transcribed spacer regions of rRNA genes using ITS1 and ITS4 (shown in Figure 2.2, Table 2.1) as primers was achieved and the product was analyzed according to the method provided in Appendix C. Figure 4.2 (b) shows the PCR product of approximate 500 bp of ITS regions of NHL-L 6/6. The PCR products were purified and submitted for sequencing to obtain the nucleotide sequence of the ITS regions of nuclear rRNA gene.

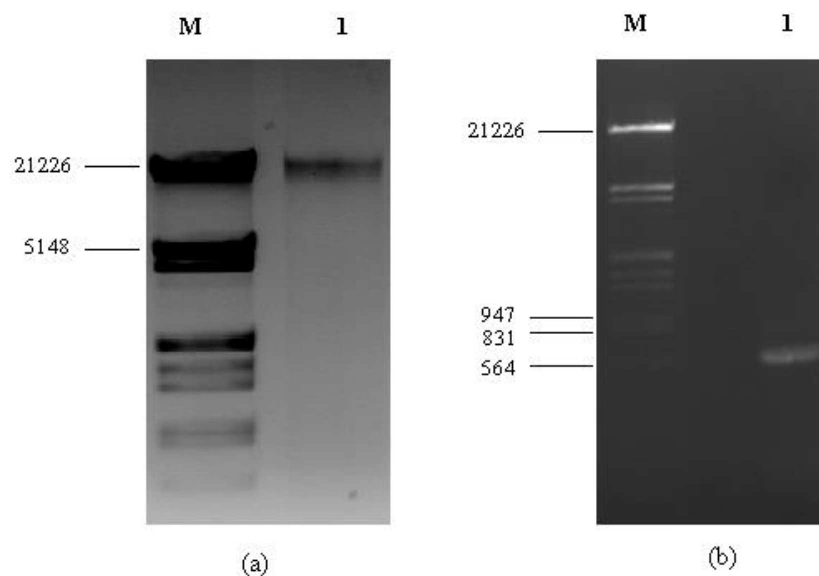

Figure 4.2 (a) The genomic DNA of NHL-L 6/6. (b) The PCR product of ITS region from NHL-L 6/6 DNA using ITS1 and ITS4 as primers. The genomic DNA of about 21 Kb (Lane 1 in Figure 4.2 (a)) and PCR products of about 564 bp (Lane 1 in Figure 4.2 (b)) were observed on 0.8% agarose gel. Lanes M (marker) contained Lambda DNA cut with EcoRI and Hind III restriction enzyme.

4.2.2 DNA sequence analysis

The filamentous fungus was identified by comparative analysis of nucleotide sequence of the ITS regions (ITS1 and ITS2) of nuclear rRNA gene. The search for similar ITS region sequence in GenBank by BLAST program showed that NHL-L 6/6 had the highest sequence similarlity of 99% with an endophytic *Nodulisporium* sp. CMU-UPE34 (accession no. JN558831.1, submitted on 04-AUG-2011), an endophytic *Nodulisporium* sp. JP3821 (accession no. AF280627.1, submitted on 21-JUN-2000), and a phytopathogenic *Hypoxylon* sp. WHCS-8 (accession no. JQ362418.1, submitted on 06-JAN-2012). According to the percentage identity performed using ClustalW 2.1, the result showed that NHL-L 6/6 had sequence similarlity of 99.79% with *Nodulisporium* sp. CMU-UPE34 and *Nodulisporium* sp. JP3821, and of 98.95% with *Hypoxylon* sp. WHCS-8 as shown in Table 4.1.

Table 4.1 Closest matches of fungal ITS sequences

Species	Accession No.	Length (bp)	% Similarlity
Nodulisporium sp. CMU-UPE 34	JN558831.1	484	99.79
Nodulisporium sp. JP3821	AF280627.1	477	99.79
Hypoxylon sp. WHCS-8	JQ362418.1	478	98.95

According to the result of sequence similarity, it is reasonable to identify the isolate NHL-L 6/6 as *Nodulisporium* sp. Many valuable bioactive metabolites with biological activities such as insecticidal (Polishook *et al*., 2001), antimicrobial (Narzir *et al*., 2012) and antitumor activities (Wu *et al*., 2010) have been successfully discovered from this endophytic fungus.

4.3 Cultivation of *Nodulisporium* sp. NHL-L 6/6 in Sucrose Yeast extract (SY) medium

According to Ratnarathorn *et al*. (2009), the endophytic fungus NHL-L 6/6 produced bioactive metabolites against some fungal and bacterial plant pathogens. Pairoj *et al*. (2011) investigated the effects of medium components on the antimicrobial activity of NHL-L 6/6 against *Erwinia caratovora* and *Penicillium* sp. by one-factor-at-a-time approach. Five different media were tested, i.e. M1D, PDB + 2% sucrose, SY (2%

sucrose and 0.5% yeast extract), Sucrose ammonium chloride (2% sucrose, 0.025% yeast extract, 2.9 g/L NH_4Cl) and Sucrose ammonium sulfate (2% sucrose, 0.025% yeast extract, 3.58 g/L $(NH_4)_2SO_4$). Sucrose yeast extract medium gave the best activities against the pathogen tested. After optimization of yeast extract concentration, it was found that sucrose yeast extract medium containing 2% sucrose and 0.3% yeast extract yielded maximum antimicrobial agent production by this fungus. Several studies reported that sucrose yeast extract medium was a very rich substrate for bioactive secondary metabolite production. Kosalec *et al*. (2005) found that gliotoxin, an antimicrobial compound, production was higher in the liquid medium (4.06 g/L), which containing 4% sucrose and 2% yeast extract, than in the synthetic Czapek-Dox liquid medium (1.07 g/L) after three days of fermentation. Ghanbary *et al*. (2012) reported that the maximum production of penicillin in *Penicillium chrysogenum* was found in the medium containing 2.1% sucrose and 0.3% yeast extract during 6-8 days after inoculation.

However, the kinetic profile of growth and secondary metabolite production of NHL-L 6/6 by using SY medium have not yet been carried out. In the present work, to gain more insight into growth and anti-phytopathogenic compound production, *Nodulisporium* sp. NHL-L 6/6 was grown on SY medium at 28 ºC, 150 rpm on a rotary shaker. The results (Figure 4.3) showed lag phase on the first day of cultivation, and then a rapid growth was observed during the period of 2-4 days, with the maximum cell dry weight of 4.85 g/L at day 4 of cultivation. Its growth was accompanied by acid production because the pH of the growth medium dropped from 5.5 to 4.5 during the exponential growth. The fungus entered the stationary phase after day 4 and then the death phase after day 6 of cultivation. The anti-phytopathogenic compound production by *Nodulisporium* sp. NHL-L 6/6 cultivated in SY medium increased rapidly and reached the maximum yield of 16.01±1.51 AU/mL at day 5 and decreased rapidly thereafter. It should be concerned that the harvest time of antimicrobial compounds is very important, early or late harvest may obtain the less yield. These extracellular metabolites were not produced during the growth phase but were produced during late exponential and the stationary phase. This indicated that anti-phytopathogenic compounds formation in *Nodulisporium* sp. NHL-L 6/6 was non-growth-associated. It should be concluded that the unknown antimicrobial compounds produced by this endophytic fungus in SY medium at 28 ºC, 150 rpm are secondary metabolites.

Furthermore, it was observed that 70% of total sugar in SY medium was quickly consumed within 3 days in exponential phase, indicating that sugar presented in the culture medium was favorable for rapid cell growth. Sugar in SY medium was continuously and slowly consumed until 7 days of cultivation because 30% of residual sugar remained in 7 day-old culture broth.

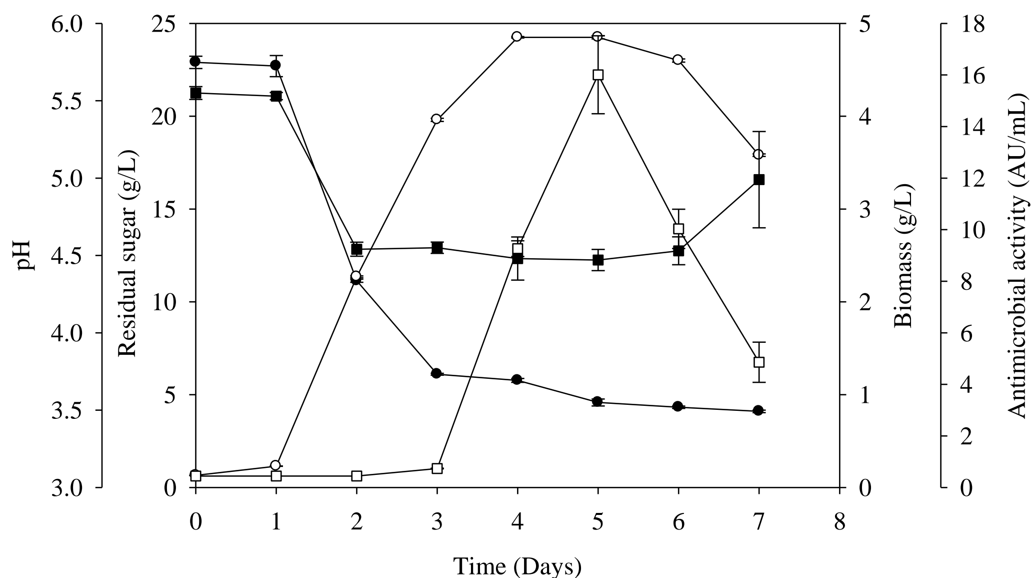

Figure 4.3 Cultivation of *Nodulisporium* sp. NHL-L 6/6 in Sucrose Yeast extract (SY) medium. The experimental data (symbol) for biomass (-○-), antimicrobial activity (-□-), residual sugar (-●-), and pH (-■-) were represented.

4.4 Effect of nitrogen and carbon sources on anti-phytopathogenic compound production

Fungal metabolite production depends upon adequate supply of energy or the quality of the culture medium. Hence, formulation of the medium is an essential part of successful fermentation experiments, as there is no common recommended medium for fermentation of secondary metabolites. Anti-phytopathogenic compounds are secondary metabolites and their production is regulated by culture condition and medium components such as carbon sources, nitrogen sources, phosphate, and trace elements (Betina, 1994). Therefore, the objective of this experiment was to screen for suitable sources of carbon and nitrogen for anti-phytopathogenic compound production from *Nodulisporium* sp. NHL-L 6/6 by one-factor-at-a-time. The test microorganism was

Erwinia caratovora, a plant pathogen which causes soft rot in a number of economically important crops such as potatoes, carrots, and beets.

4.4.1 Effect of nitrogen sources

Nitrogen source, usually supplied as organic or inorganic compounds, is a critical nutritional factor for the synthesis of enzymes involved in secondary metabolism, (Sanchez and Demain, 2002). To investigate the effects of nitrogen sources on antimicrobial compound production, sucrose was used as a carbon source.

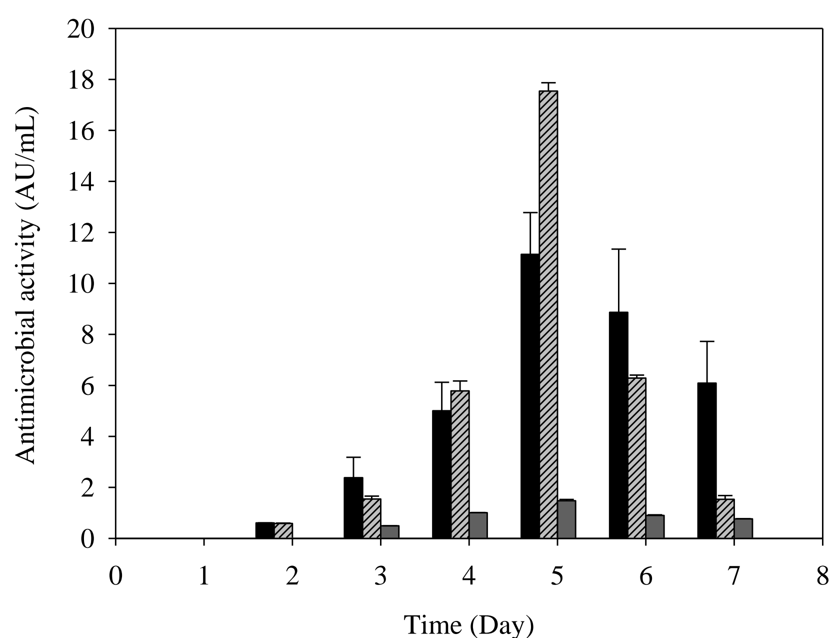

Figure 4.4 Effect of nitrogen sources on anti-phytopathogenic compound production. The experimental data (symbol) for whey ( ), yeast extract ( ), ammonium sulfate ( ), and urea ( ) were represented.

Of the four different nitrogen sources chosen (i.e. yeast extract, whey, urea and ammonium sulfate), yeast extract and whey were found to play significant role on the antimicrobial compound production. The results (Figure 4.4) indicated that among the various nitrogen sources studied, the maximum antimicrobial activity was achieved with yeast extract (17.54±0.32 AU/mL), followed by whey (11.14±1.64 AU/mL) at day 5. Considering the interesting titer of antimicrobial compounds produced by *Nodulisporium* sp. NHL-L 6/6, yeast extract and whey should be used as nitrogen

sources for subsequent experiment. Yeast extract provides various trace elements, vitamins and amino acids that encourage high production of secondary metabolites (Sanchez and Demain, 2002). Whey consists of lactose, nitrogen compounds, amino acids, trace elements and vitamins (Haast *et al*., 1986). Some amino acids present in yeast extract and whey may possibly function as inducers which turn on the production of fungal secondary metabolites. In a number of pathways, for example, methionine function as inducer of δ-(L-α-aminoadipyl)-L-cysteinyl-L-D-valine synthethase (ACVS), cyclase and expandase in the cephalosporin pathway of *Acremonium chrysogenum* (Zhang *et al*., 1988).

Low antimicrobial activity was observed with ammonium sulfate (1.47±0.06 AU/mL) while no antimicrobial activity was observed with urea. Among the test organic nitrogen sources, urea was a cheap nitrogen source and expected for its potential to replace more expensive commercial nitrogen source but it was not preferable for production of antimicrobial substances by *Nodulisporium* sp. NHL-L 6/6. This fungus grew slowly in ammonium sulfate and urea medium (data not shown). The initial concentration of ammonium sulfate or urea presence in the culture medium may be not suitable for fungal growth. This may be due to the negative effect of NH_4^+ which represses enzymes for fungal growth and/or biosynthesis of secondary metabolites (Sanchez and Demain, 2002). There are some studies reported about the negative effect of ammonium salt or urea on growth and/or secondary metabolite production in fungi. For instance, ammonium concentrations higher than 100 mM inhibited ß -lactam antibiotics production in the fungus *Cephalosporium acremonium* (Shen *et al*., 1984). The production of acetate-derived phenolic compounds such as trihydroxytoluene by *Aspergillus fumigatus* is under nitrogen source control (Ward and Packter, 1974) and addition of ammonia completely inhibits these polyketides production. The growth of *Cladosporium* sp. F14 grew and produced antibiotics slowly in ammonium salt containing media and not at all in urea containing media when glucose or xylose was used as carbon source (Xiong *et al*., 2009). Although some fungi grew well in ammonium and/or urea containing media, the secondary metabolites were not produced, for instance, lovastatin was not detected during cultivation of *Aspergillus terreus* in ammonium and urea containing media (Hajjaj *et al*., 2001).

It should be noted that pH in yeast extract and whey containing medium (pH = 4.0 - 5.5) promote antimicrobial compound production (Figure 4.5, Table A.2). It was observed that the increase in antimicrobial compound production was accompanied by the decrease of culture pH of these media, probably that some organic acids were formed in these growth media. The acidic pH may affect the expression of genes involved in regulation of secondary metabolites or on the genes directly involved in metabolite production in fungi, for example, the structural genes, *stcU* in *Aspergillus nidulans* and *ver-1* in *A. parasiticus*, were expressed at high levels in acidic media (pH 4.0 to 6.0) (Keller *et al*., 1997). Moreover, *Nodulisporium* sp. NHL-L 6/6 did not produce antimicrobial substances in the cultivation media containing urea. Since the high pH ranges were observed in media containing urea (pH = 5.4 - 7.8) during cultivations, the production of secondary metabolites was known to be affected by some culture condition (e.g. pH value). Vieira *et al.* (2008) reported that the production of antimicrobial substances at acidic pH values around 4.5 - 6.0 presented higher level than at higher pH values in *Polyporus tricholoma* Mont.

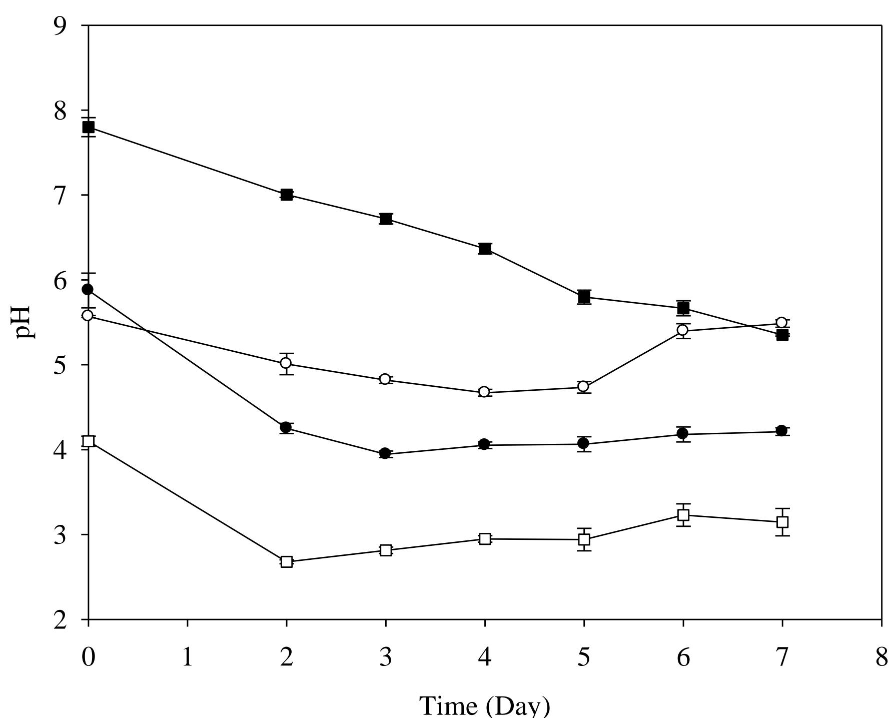

Figure 4.5 pH of culture media in preliminary screening of nitrogen sources. The experimental data (symbol) for whey (-●-), yeast extract (-○-), ammonium sulfate (-□-), and urea (-■-) were represented.

4.4.2 Effect of carbon sources

To investigate the effects of carbon sources (i.e. sucrose, glucose, cassava starch and molasses) on secondary metabolite production, yeast extract was adopted as a nitrogen source. The result showed that all tested carbon sources exhibited different effects on antimicrobial compound production. Among four different carbon sources, the highest antimicrobial activity was obtained with sucrose containing medium (17.53±0.34 AU/mL), followed by cassava starch (11.22±2.75 AU/mL) and glucose (10.04±1.18 AU/mL) (Figure 4.6). The result showed influences of sucrose, cassava starch and glucose on antimicrobial compound production, possibly that the endophytic fungus preferred to use these carbon sources for growth and product formation like *in planta*. Carbon sources within the endophytic niche in plant consist of several sugars such as sucrose, glucose, fructose, galactose and related carbohydrates derived from photosynthesis (Kuldau and Bacon, 2008). These sugars serve to regulate the biological activity of endophytic fungi and contribute to the diversity of secondary metabolites produced by specific genotypes of each endophyte species or strain. Among the carbon sources studied, sucrose was the most significant medium composition which influenced the production of antimicrobial compounds. According to Rodrı´guez-Ortiz *et al*. (2010), bikaverin production of *Fusarium fujikuroi* against some protozoa and phytopathogenic fungi was stimulated by sucrose, but strongly suppressed in the presence of equivalent carbon concentrations by glucose, galactose, fructose, maltose, xylose and glycerol. Sucrose is converted to glucose and fructose by invertase activity; however, neither fructose nor a mixture of glucose and fructose reproduced the sucrose stimulatory effect observed in *F. fujikuroi*, suggesting that the inducing signal is sucrose itself, and not a sucrose-derived metabolite.

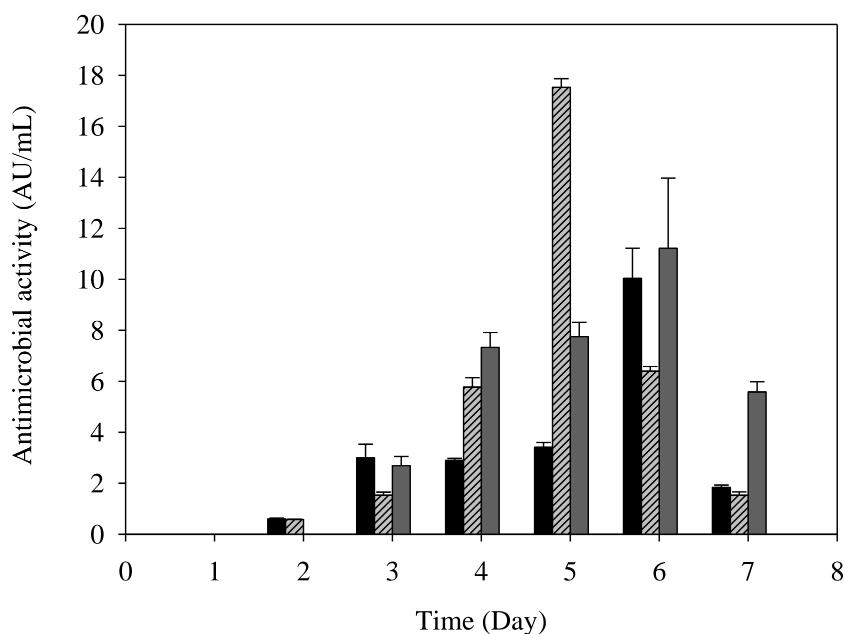

Figure 4.6 Effect of carbon sources on antimicrobial compound production.
The experimental data (symbol) for glucose ( ), sucrose ( ), cassava starch ( ), and molasses ( ) were represented.

Media containing glucose and cassava starch showed less antimicrobial activities and the cultivation time to obtain the highest antimicrobial compounds in these two media was longer than that in sucrose medium. The highest antimicrobial metabolite production by *Nodulisporium* sp. NHL-L 6/6 in cassava starch and glucose medium was reached at day 6 while it was reached at day 5 of cultivation in sucrose medium. The harvest time of glucose medium extended to 6 days may be due to it was more rapidly utilized by *Nodulisporium* sp. NHL-L 6/6 when compared to sucrose medium. The production of secondary metabolite usually occurs when rapidly utilized carbon sources in the media are limited. A possible explanation of this phenomenon is that rapidly utilizable carbon sources cause catabolite repression, in which the production of enzymes of secondary metabolite biosynthesis is inhibited. The delay in secondary metabolite formation is one of the major mechanisms that prevent suicide in antibiotic-producing microorganisms (Drew and Demain, 1977). Secondary metabolite production in fungi is often stimulated by slowly assimilated complex carbohydrates in the production media, and decreased when more rapidly utilized monosaccharides are present (Bertasso *et al.,* 2001).

The harvest time of antimicrobial compounds in cassava starch medium extended to 6 days when compared to sucrose medium. It was observed that pH of cassava starch medium decreased from 5.5 to 3.3 at day 3 (Figure 4.7, Table A.4), and increased to about 5.3 at the end of cultivation while pH of sucrose medium decreased from initial pH of 5.6 to 5.0 at day 2, which may due to the release of organic acids and slightly increased to 5.5 at the end of fermentation which may cause from cell autolysis. The pH of cassava starch medium rapidly decreased when compared to sucrose medium which may be due to the fact that cassava starch was more rapidly utilized and some organic acids were formed in the culture medium. Moreover, this fungus may produce enzymes (e.g. amylase) which hydrolyzed starch into glucose in the culture medium which possibly led to catabolite repression, which inhibit the production of enzymes of secondary metabolite biosynthesis.

Antimicrobial activity was not detectable in the medium containing molasses even though molasses is composed of sugars (e.g. sucrose, glucose and fructose), vitamins (e.g. biotin, pantothenic acid, riboflavin, and thiamine), organic compounds (e.g. protein, and nitrogen) and minerals (e.g. copper, iron, manganese and zinc) (Curtin, 1983), which may be beneficial for the fungal growth and secondary product formation. However, Demain (1992) reported that the rapidly utilized carbon sources which were favorable for fungal growth may not be beneficial to secondary metabolite production. Chang and Hua (2007) reported that adding molasses to culture media led to a nine-fold decrease in aflatoxin production by *Aspergillus flavus.* Moreover, molasses contains chelating agent which may reduce the availability of metal ions for aflatoxin production such as Zn^{2+} and Cu^{2+} (Ceuro and Ouellet, 2005).

In order to select the suitable nitrogen and carbon sources for further experiment, in addition to antimicrobial activity, the demand for low cost medium components should be considered. In this study, the antimicrobial compound production at the laboratory scale and raw material costs at the market price were compared. In case of nitrogen sources, the highest antimicrobial activity was detectable with yeast extract (17.54 AU/mL), followed by whey (11.14 AU/mL). Very low and no antimicrobial activity was found with ammonium sulfate (1.47 AU/mL) and urea. This result was implied that ammonium sulfate and urea were poor nitrogen sources for antimicrobial substance production. Yeast extract was expensive; however, it was the best nitrogen source for

antimicrobial compound production. Although whey showed a moderate antimicrobial activity, it was a cheap nitrogen source (1.8 Baht/L; Premier Dairy Foods Co., Ltd) expected for its potentials to reduce the quantity of an expensive nitrogen source. Therefore, yeast extract and whey were chosen for further experiment.

In case of carbon sources, sucrose showed the highest antimicrobial activity (17.53 AU/mL), followed by cassava starch (11.22 AU/mL) and glucose (10.04 AU/mL). The antimicrobial activity was not detectable in molasses medium; therefore, it was not chosen for antimicrobial compound production. The highest cost of carbon sources was cassava starch (60-90 Baht/Kg), followed by glucose (50 Baht/Kg) and sucrose (24 Baht/Kg). Cassava starch showed moderate antimicrobial activity, high cost and also needed to be gelatinized by heating prior autoclaving; therefore, it was not preferred for further experiment. Thus, the suitable carbon sources chosen for optimization experiment were sucrose and glucose.

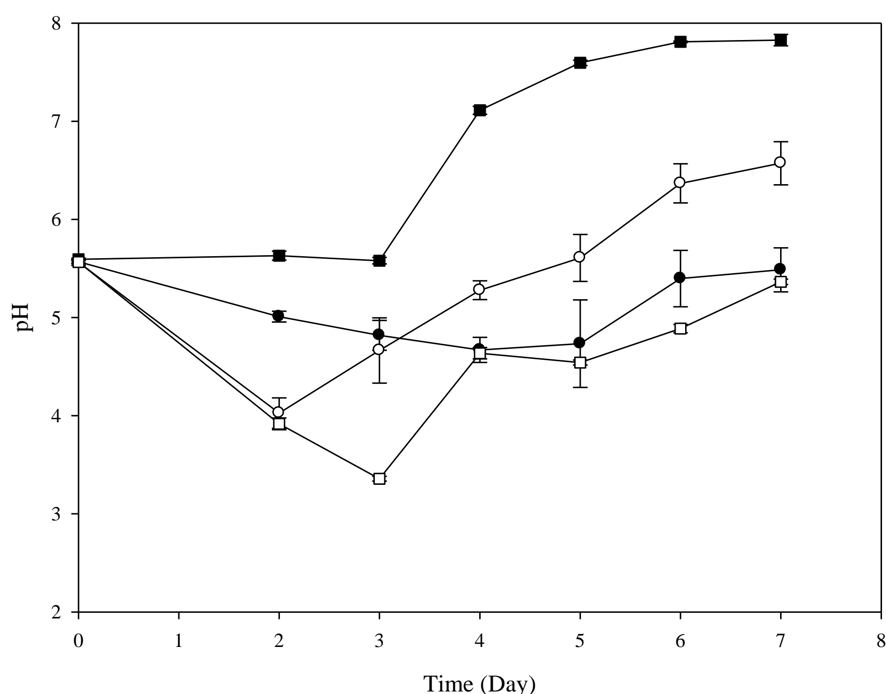

Figure 4.7 pH of culture media in preliminary screening of carbon sources. The experimental data (symbol) for sucrose (-●-), glucose (-○-), cassava starch (-□-), and molasses (-■-) were represented.

4.5 Experimental design and optimization by response surface methodology

4.5.1 Screening of medium components using fractional factorial design

A 2^{4-1} two level fractional factorial design (FFD) is a rapid method for screening significant variables. Fourteen runs were carried out to investigate the influence of four variables on anti-phytopathogenic compound and biomass production by endophytic fungus. The highest antimicrobial compound productions in section 4.4, accomplished by one-factor-at-a-time, were obtained at day 5 and day 6 of cultivation. In addition, the study of the production of antimicrobial substance by *Nodulisporium* sp. NHL-L 6/6 (Figure 4.4 and 4.6) revealed that cultivation time was crucial for maximum activity. All factors were combined according to FFD which may alter the optimal harvest time, therefore; samples were taken at day 4 to 8 of cultivation. The result showed that maximum antimicrobial activity was obtained at day 6 of cultivation as demonstrated in Table 4.2.

Prior statistical analysis, normality of data, antimicrobial activity, was tested using Anderson-Darling method. The normal probability plot (Figure A5, Appendix A) as an important diagnostic for the model roughly formed a straight line and dropped close to the fitted line, i.e. there were no signs of any problems in the data. Subsequently, statistical analysis was conducted using Minitab 15. The regressive analysis of FFD using antimicrobial activity obtained at day 6 as a response was carried out providing that coefficients, student's *t* distribution, and corresponding *p*-values along with parameters' estimated effects were shown in Table 4.3. The coefficient of determination (R^2) and adjusted R^2 obtained for antimicrobial activity were 97.65% and 93.88%, respectively, indicating great similarity between the observed and predicted value. Furthermore, analysis of variance (ANOVA) given in Table 4.4 showed that curvature was significant statistically (p<0.05) indicating that concentration of each variable investigated leading to maximum response was included within the chosen range. The regression equation correlating the level of antimicrobial compound production to the process variables: yeast extract, whey, sucrose and glucose were included in the Equation 1 regardless of their significance.

$$Y = 11.55 - 0.255x_1 - 1.975x_2 + 4.575x_3 + 4.310x_4 \qquad \text{(Equation 1)}$$

where Y is the response, that is, antimicrobial activity against *Erwinia caratovora* and x_1 ,x_2, x_3 and x_4 are the coded values of the test variables, yeast extract, whey, sucrose and glucose, respectively.

Regression showed that two carbon constituents, i.e. sucrose and glucose were considered statistically significant at a confidence level of 95% (p<0.05) and showed the largest influence on antimicrobial compound production. The effects of sucrose and glucose were positive, meaning that increasing their concentrations tended to increase antimicrobial compound production. While nitrogen sources, i.e. yeast extract and whey, were statistically insignificant at a confidence level of 95% (p>0.05) whose effects were negative, suggesting that increasing their concentrations would decrease antimicrobial compound production (Table 4.3). This could be due to catabolite repression of some components in yeast extract and whey such as some amino acids or trace elements. In general, the biosynthesis of fungal secondary metabolites is limited by nitrogen sources favoring cell growth. It may be due to the fact that some amino acids present in nitrogen sources inhibited the production of antimicrobial compound. For example, bikaverin formation by *Gibberella fujikuroi* is under nitrogen regulation in that when glycine is used as nitrogen source for the fermentation, bikaverin production begins only upon glycine exhaustion (Bu'Lock, 1974).

According to the regressive analysis of biomass (Table 4.5), the results showed that all constituents, i.e. yeast extract, whey, sucrose, and glucose were statistically significant at a confidence level of 95%. Yeast extract showed the largest influence on biomass production by NHL-L 6/6, followed by sucrose, glucose and whey, respectively. The effects of all constituents were positive, meaning that they tended to increase biomass production. The results of FFD indicated that nitrogen sources which are favorable for growth are not being beneficial to secondary metabolite production. Even though the objective of this experiment was to screen factors affecting antimicrobial compound production, it should be borne in mind that nitrogen is another key factor affecting cell growth which fungi used directly for building proteins or metabolites for other biosynthetic processes. It means that yeast extract and whey should be maintained as nitrogen sources for fungal cultivation. Therefore, all variables were chosen for further optimization study.

Table 4.2 The experimental design and results of 2^{4-1} FFD for biomass and antimicrobial activity at day 6

Run	Block	Yeast extract (g/L)	Whey (mL/L)	Sucrose (g/L)	Glucose (g/L)	Biomass (g/L)	Antimicrobial activity (AU/mL)
1	1	3 (1)	0 (-1)	10 (-1)	20 (1)	4.85	15.97
2	1	1 (-1)	20 (1)	10 (-1)	20 (1)	3.56	10.38
3	1	3 (1)	0 (-1)	30 (1)	0 (-1)	4.82	14.35
4	1	1 (-1)	20 (1)	30 (1)	0 (-1)	3.97	13.06
5	1	2 (0)	10 (0)	20 (0)	10 (0)	4.6	39.69
6	1	2 (0)	10 (0)	20 (0)	10 (0)	4.57	40.19
7	1	2 (0)	10 (0)	20 (0)	10 (0)	4.62	40.19
8	2	1 (-1)	0 (-1)	10 (-1)	0 (-1)	2.75	1.55
9	2	3 (1)	20 (1)	10 (-1)	0 (-1)	4.19	0.00
10	2	1 (-1)	0 (-1)	30 (1)	20 (1)	4.63	22.23
11	2	3 (1)	20 (1)	30 (1)	20 (1)	6.47	14.86
12	2	2 (0)	10 (0)	20 (0)	10 (0)	5.06	46.61
13	2	2 (0)	10 (0)	20 (0)	10 (0)	5.02	46.61
14	2	2 (0)	10 (0)	20 (0)	10 (0)	5.11	46.59

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

Table 4.3 Regressive analyses 2^{4-1} FFD using antimicrobial activity at day 6 as response.

Term	Effect	Coefficient	*t*	*p*
Constant	-	11.55	-	-
Block	-	-0.33	-0.29	0.784
yeast extract	-0.51	-0.26	-0.17	0.873
whey	-3.95	-1.97	-1.30	0.250
sucrose	9.15	4.58	3.02	0.030*
glucose	8.62	4.31	2.84	0.036*
yeast extract* sucrose	-2.530	-1.265	-0.83	0.443
yeast extract* glucose	-0.380	-0.190	-0.13	0.905

S = 4.29180 R^2 = 97.65% R^2 (adj) = 93.88%,

Note: * designates significance at $p<0.05$

Table 4.4 Analysis of Variance (ANOVA) for antimicrobial activity

Source	DF	Seq SS	Adj SS	Adj MS	*F-value*	*p-value*
Blocks	1	1.52	1.52	1.52	0.08	0.785
Main Effects	4	347.78	347.78	86.94	4.72	0.050
2-way Interactions	2	13.09	13.09	6.55	0.36	0.717
Curvature	1	3459.12	3459.12	3459.12	187.66	0.000*

Note: * designates significance at $p<0.05$

Table 4.5 Regressive analyses 2^{4-1} FFD using biomass at day 6 as response.

Term	Effect	Coefficient	*t*	*p*
Constant	-	4.41	-	-
Block	-	-0.16	-5.38	0.003*
yeast extract	1.36	0.68	17.24	0.000*
whey	0.29	0.14	3.63	0.015*
sucrose	1.14	0.57	14.44	0.000*
glucose	0.95	0.47	12.02	0.000*
yeast extract* sucrose	-0.0100	-0.0050	-0.13	0.904
yeast extract* glucose	0.2100	0.1050	2.67	0.044

S = 0.111176 R^2= 99.34% R^2 (adj) = 98.28%,

Note: * designates significance at $p<0.05$

Table 4.6 Analysis of Variance (ANOVA) for biomass

Source	DF	Seq SS	Adj SS	Adj MS	*F-value*	*p-value*
Blocks	1	0.3584	0.3584	0.3584	29	0.003*
Main Effects	4	8.197	8.197	2.04925	165.8	0.000*
2-way Interactions	2	0.0884	0.0884	0.0442	3.58	0.109
Curvature	1	0.61929	0.61929	0.61929	50.1	0.001*

Note: * designates significance at $p<0.05$

4.5.2 The steepest ascent experiment

According to FFD previously conducted, four medium components were selected for further optimization and the curvature was found to be significant statistically, indicating that the optimal region for the experimental condition lies in the present design range. 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 determine the proper direction of manipulating the variables, increasing or decreasing the concentration according to the sign of the main effects to improve anti-phytopathogenic compound production. The results of the steepest ascent method were shown in Table 4.7 and Figure 4.8 providing that the highest antimicrobial activity against *Erwinia caratovora* at the first step of day 7 (38.65±1.80 AU/mL) and the starting point of day 6 (37.61 ± 0.00 AU/mL) were of comparable level according to Duncan's New Multiple Range Test (IBM SPSS statistics version 19). Considering economic benefit, shorter cultivation time should be selected for production of microbial metabolites. The highest response was found at day 6 when the concentrations of yeast extract, sucrose, glucose and whey were 2, 20, 10 g/L and 10 mL/L, respectively, suggesting that this point was near the region of maximum production response. The antimicrobial activity decreased slightly thereafter, indicating that further manipulation of these variables would no longer result in an increase in antimicrobial compound production. Therefore, the concentrations at the beginning of the path of steepest ascent at day 6 were adopted and subsequently employed as the center point of the further optimization by Central Composite Design (CCD).

Table 4.7 Results of antimicrobial activity along the path of steepest ascent

Step	Antimicrobial activity (AU/mL)			
	Day 5	Day 6	Day 7	Day 8
0 (Starting Point)	17.58±0.00^{e}	37.61±0.00^{a}	30.90±0.00^{b}	16.62±1.67^{e}
1	12.32±0.00^{g}	27.30±3.12^{c}	38.65±1.80^{a}	21.13±0.00^{d}
2	6.93±0.50^{i}	12.32±0.00^{g}	14.35±0.58^{f}	7.96±0.00^{i}
3	6.77±0.00^{i}	10.38±0.00^{h}	10.75±0.65^{gh}	11.27±0.98^{gh}
4	4.68±0.00^{j}	6.36±0.00^{i}	10.38±0.00^{i}	12.32±0.00^{g}

Note: Within table, differences of means were tested by Duncan's New Multiple Range Test. Means ± S.D. followed by the different letters are significantly different (p<0.05).

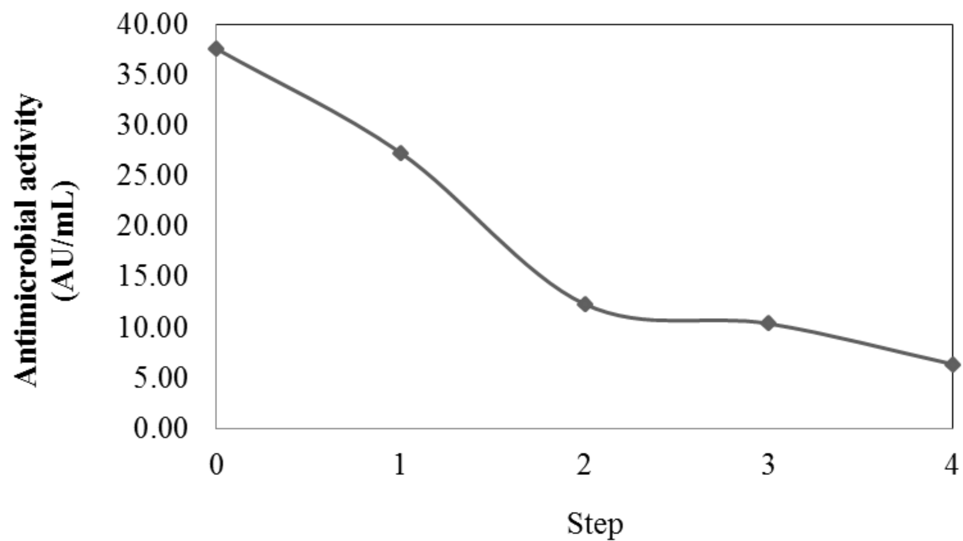

Figure 4.8 The path of steepest ascent and results for antimicrobial activity at day 6

4.5.3 Central composite design

A central composite design (CCD) was used to optimize the antimicrobial compound produced by *Nodulisporium* sp. NHL-L 6/6. Table 4.8 showed experimental design and antimicrobial compound production at day 6 of cultivation as the response. The antimicrobial activities ranged from 9.60 to 40.73 AU/mL. It is evident that the highest antimicrobial activity of 40.73 AU/mL was obtained in Run 23 and 25 where yeast extract, whey and sucrose were set at the middle level while glucose was set at the low to the middle level. The lowest antimicrobial activity of 9.60 AU/mL was obtained in Run 1 where all constituents were set at the low level.

As is evident from statistical analysis, the normal probability plot of the residuals (Figure A8, Appendix A) showed a linear pattern which verified normality in the error term (Wang and Liu, 2008), indicating that there were no signs of any problems in the data. The estimated regression coefficients for antimicrobial activity at day 6 were provided in Table 4.9. The significance of each coefficient in this study was determined by Student's *t*-test and *p*-value. The larger the magnitude of *t*-test and smaller the *p*-value, the more significant is the corresponding coefficient.

The linear term of x_1, x_3 and x_4 were significant at $p<0.05$. The positive coefficients for that yeast extract (x_1), sucrose (x_3) and glucose (x_4) indicated a linear effect to increase antimicrobial activity. The result suggested that yeast extract (x_1), sucrose (x_3) and glucose (x_4) played significant roles on antimicrobial compound production. Sucrose showed the largest influence on antimicrobial compound production, followed by glucose and yeast extract, respectively. This finding is similar to several studies which demonstrated that sucrose, glucose, and yeast extract have been widely used to enhance the synthesis of bioactive secondary metabolites in fungal culture. For example, in the culture medium of *Aspergillus* sp., the maximum antimicrobial compound production was noted when sucrose was used as a sole carbon source (Thakur *et al*., 2009). The highest bioactivity of the endophytic fungus *Aspergillus terreus* KC 582297 isolated from the *Codium decorticatum* seaweed was achieved in potato dextrose medium supplemented with sucrose as a carbon source and yeast extract as a nitrogen source (Mathan *et al*., 2013).

Table 4.8 The experimental design and results of CCD for antimicrobial activity at day 6.

Run	Yeast extract (g/L)	Whey (mL/L)	Sucrose (g/L)	Glucose (g/L)	Antimicrobial activity (AU/mL)
1	1.95 (-1)	8 (-1)	15 (-1)	5 (-1)	9.60
2	2.05 (+1)	8 (-1)	15 (-1)	5 (-1)	20.11
3	1.95 (-1)	12 (+1)	15 (-1)	5 (-1)	13.53
4	2.05 (+1)	12 (+1)	15 (-1)	5 (-1)	14.86
5	1.95 (-1)	8 (-1)	25 (+1)	5 (-1)	26.17
6	2.05 (+1)	8 (-1)	25 (+1)	5 (-1)	38.11
7	1.95 (-1)	12 (+1)	25 (+1)	5 (-1)	26.85
8	2.05 (+1)	12 (+1)	25 (+1)	5 (-1)	28.24
9	1.95 (-1)	8 (-1)	15 (-1)	15 (+1)	18.47
10	2.05 (+1)	8 (-1)	15 (-1)	15 (+1)	22.49
11	1.95 (-1)	12 (+1)	15 (-1)	15 (+1)	28.98
12	2.05 (+1)	12 (+1)	15 (-1)	15 (+1)	25.50
13	1.95 (-1)	8 (-1)	25 (+1)	15 (+1)	28.60
14	2.05 (+1)	8 (-1)	25 (+1)	15 (+1)	37.61
15	1.95 (-1)	12 (+1)	25 (+1)	15 (+1)	39.69
16	2.05 (+1)	12 (+1)	25 (+1)	15 (+1)	34.75
17	1.9 (-2)	10 (0)	20 (0)	10 (0)	10.38
18	2.1 (+2)	10 (0)	20 (0)	10 (0)	18.01
19	2 (0)	6 (-2)	20 (0)	10 (0)	15.97
20	2 (0)	14 (+2)	20 (0)	10 (0)	18.47
21	2 (0)	10 (0)	10 (-2)	10 (0)	10.99
22	2 (0)	10 (0)	30 (+2)	10 (0)	39.66
23	2 (0)	10 (0)	20 (0)	0 (-2)	40.73
24	2 (0)	10 (0)	20 (0)	20 (+2)	35.70
25	2 (0)	10 (0)	20 (0)	10 (0)	40.73
26	2 (0)	10 (0)	20 (0)	10 (0)	39.69
27	2 (0)	10 (0)	20 (0)	10 (0)	38.62
28	2 (0)	10 (0)	20 (0)	10 (0)	39.69
29	2 (0)	10 (0)	20 (0)	10 (0)	39.69
30	2 (0)	10 (0)	20 (0)	10 (0)	39.13
31	2 (0)	10 (0)	20 (0)	10 (0)	39.13

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.

Glucose was another carbon source influenced secondary metabolite production in *Nodulisporium* sp. NHL-L 6/6. Generally, culture media used for growth and production of antimicrobial agents usually contain glucose as carbon sources. Like other studies, glucose was the best carbon source for beauvericin, a mycotoxin which has insecticidal, antimicrobial, antiviral and cytotoxic activities produced by many fungi such as *Beaveria bassiana* and *Fusarium* spp. (Wang and Xu, 2012). In this study, the highest antimicrobial activity (40.73 AU/mL) was obtained in the medium with or without glucose as can be seen in Run 25 and 23, respectively. In the medium without glucose (Run 23), sucrose as a carbon source was utilized by the endophytic fungus. Generally, fungi utilize sucrose by secreting invertase into the medium (Carlson *et al*., 1981). However, some of this enzyme remains in the cell wall (Esmon *et al*., 1987). Invertase hydrolyzes sucrose into glucose and fructose, which are imported into the cell by a variety of hexose transporters. The presence of glucose in fungal cell enhanced the production of bioactive compounds could be explained by two possibilities: 1) the glycolysis of the glucose or the tricarboxylic acid cycle provided sufficient amounts of the intermediate products that are necessary for the biosynthesis of these bioactive compounds (Jeffries, 2006); 2) the metabolism of glucose generated a sufficient level of ATP necessary for the biosynthesis processes of these antimicrobial compounds.

Yeast extract was a nitrogen source influenced secondary metabolite production in *Nodulisporium* sp. NHL-L 6/6. In this study, yeast extract at the concentration about 2 g/L promoted antimicrobial compound production in the endophytic fungus *Nodulisporium* sp. NHL-L 6/6 after 6 days of cultivation. It should be noted that yeast extract provide peptides, free amino acids, vitamins, minerals, and carbohydrate, which are favorable for supporting fungal growth and secondary metabolite production. Zhao *et al*. (2011) reported that yeast extract at 10 g/L effectively enhanced the production of diepoxin (378.70 mg/L), a naphthoquinone derivative with notable antimicrobial, antifungal, antitumor, allelochemical and anti-leishmanial activities, in liquid culture of endophytic fungus Dzf12 from *Dioscorea zingiberensis* after 10 days culture. Zain *et al*. (2009) reported that the highest secondary metabolite production of *Aspergillus terreus*, *Penicillium janthinellum* and *Penicillium duclauxii* were significantly affected by yeast extract.

The quadratic term of yeast extract (x_1^2), whey (x_2^2) and sucrose (x_3^2) were significant at p<0.05. The negative coefficient for yeast extract (x_1^2), whey (x_2^2) and sucrose (x_3^2) showed negative effects on the antimicrobial compound production, indicating that the antimicrobial compound production increased as the level of these factors increased and decreased as the level of these parameters increased above certain values. The interactive terms of x_1x_2 and x_2x_4 were also significant at p<0.05. The coefficient of yeast extract*whey (x_1x_2) interaction was negative, indicating that their combination tended to decrease the antimicrobial compound production, even though the two individual variables increased the antimicrobial compound production. The coefficient of whey*glucose (x_2x_4) interaction was positive, indicating that their combination tended to increase the antimicrobial compound production.

Table 4.9 Regressive analysis of CCD using antimicrobial activity at day 6 as the response

Term	**Coefficient**	**SE**	***t***	***P***
Constant	39.5257	1.2074	32.735	0.000*
Yeast extract (X_1)	1.8767	0.6521	2.878	0.011*
Whey (X_2)	0.6767	0.6521	1.038	0.315
Sucrose (X_3)	6.8258	0.6521	10.468	0.000*
Glucose (X_4)	2.0233	0.6521	3.103	0.007*
Yeast extract*Yeast extract (X_1* X_1)	-5.9812	0.5974	-10.012	0.000*
Whey*Whey (X_2* X_2)	-5.2250	0.5974	-8.746	0.000*
Sucrose*Sucrose (X_3* X_3)	-3.1987	0.5974	-5.354	0.000*
Glucose*Glucose (X_4* X_4)	0.0238	0.5974	0.040	0.969
Yeast extract*Whey (X_1* X_2)	-2.5737	0.7987	-3.223	0.005*
Yeast extract*Sucrose (X_1* X_3)	0.3137	0.7987	0.393	0.700
Yeast extract*Glucose (X_1* X_4)	-1.2850	0.7987	-1.609	0.127
Whey*Sucrose (X_2* X_3)	-0.8225	0.7987	-1.030	0.318
Whey*Glucose (X_2* X_4)	2.0163	0.7987	2.525	0.023*
Sucrose*Glucose (X_3* X_4)	-1.0037	0.7987	-1.257	0.227

S = 3.195 R^2= 95.4% R^2 (adj) = 91.3 %

Note: * designates significance at p<0.05

The adequacy of the response surface quadratic model was determined using an analysis of variance (ANOVA) which was tested using Fisher's statistical analysis and the results were shown in Table 4.10. The Fisher's *F*-test revealed a *p*-value of zero (p = 0.000) which indicated that the model was highly significant. The goodness of fit of the model was examined by the determination coefficient R^2 which was calculated to be 0.954. Therefore, the model was able to explain 95.4% of the total variations. The R^2 also indicated that only 4.6% of the total variations were not explained by the model. Tests for the lack-of-fit of the model showed that the results were significant ($p<0.05$), this indicating that the polynomial model is not fitting the design points well. Despite the significance indicated by the lack-of-fit analysis, the adjusted R^2 value was very high, 0.913, which suggested a good correlation between the predicted values and the experimental results.

Table 4.10 Analysis of variance (ANOVA) for antimicrobial activity

Source	DF	Seq SS	Adj SS	Adj MS	*F-value*	*p-value*
Model	14	3350.07	3350.07	239.291	23.45	0.000
Residual Error	16	163.29	163.29	10.205	-	-
Lack of Fit	10	160.62	160.62	16.062	36.17	0.000
Pure Error	6	2.66	2.66	0.444	-	-
Total	30	3513.36	-	-	-	-

The production of antimicrobial compound could be described by

$$Y= -10393.6+9891.16x_1 +77.5669x_2+5.19712x_3+9.45239x_4-25.7375x_1 x_2+1.255x_1 x_3 -5.14x_1 x_4-0.08225x_2x_3+0.201625x_2x_4-0.04015x_3x_4-2392.49x_1^2-1.30624x_2^2 -0.127949x_3^2+0.00095119x_4^2 \quad \text{(Equation 2)}$$

where 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.

To analytically determine the optimal concentrations of yeast extract, whey, sucrose and glucose, equation 2 was individually differentiated respect to x_1 , x_2 , x_3 and x_4 and then equated to zero. The optimum concentration of yeast extract, whey, sucrose and glucose were 2.03 g/L, 9.86 mL/L, 25.08 g/L, and 12.99 g/L, respectively. The model

predicted that the production of antimicrobial compound could extend to 41.91 AU/mL by using the aforementioned optimized concentration of each variable.

The quadatic model could be further analyzed by Statistica 8.0 software (StatSoft Inc., Tulsa, USA) for contour and surface plot. The 3D response surface and their corresponding 2D contour plot of the significant interactions (i.e. Yeast extract*Whey (X_1* X_2), $p = 0.005$; Whey*Glucose (X_2* X_4), $p = 0.023$) for the antimicrobial compound production were presented in Figure 4.9 and 4.10. Such graphical tools provide useful information on the process conditions necessary to achieve the desired value of response. The optimum value of each variable was located based on the hump in the three dimensional plot, or from the central point of the corresponding contour plot. The shapes of the contour plots, circular or elliptical, indicate whether the mutual interactions between the variables are significant or not. A circular contour plot of response surfaces indicates that the interaction between the corresponding variables can be ignored, while an elliptical or saddle nature of the contour plot suggests that the interaction between the corresponding variables is significant (Muralidhar *et al*., 2001). The optimum level of each variable and the effect of their interactions on antimicrobial compound production as a function of two variables were studied by plotting contour and surface plots by keeping the other two factors constant at its middle level.

According to Figure 4.9, the elliptical nature of the contour plots stated clearly that the interaction between whey and yeast extract was significant. An increase in the yeast extract concentration with whey concentration up to the optimum point increased the antimicrobial compound production to a maximum level. The optimum for maximum antimicrobial compound production lies near the center point of the concentration of yeast extract and whey when the other two variables sucrose and glucose were kept constant at the middle level. The trend of further increase in the yeast extract concentration with whey concentration is reversed. According to Figure 4.10, the stationary point for interaction effect of glucose and whey was a saddle point, in which both a minimum and a maximum antimicrobial activity was obtained. Saddle point was observed at the middle level of whey and glucose when other two variables sucrose and yeast extract were kept constant at the middle level. The graphical result was in agreement with the analytical result using the quadratic model (Equation 2) which indicated that there were regions where the response could be maximized.

The results of this study indicated that the maximum antimicrobial activity obtained with unoptimized medium containing 3 g/L yeast extract and 20 g/L sucrose was 17.54 AU/mL whereas after optimization by RSM, the maximum antimicrobial compound production obtained with the optimum concentration of yeast extract, whey, sucrose and glucose at 2.03 g/L, 9.86 mL/L, 25.08 g/L, and 12.99 g/L, respectively, was 40.73 AU/mL. Confirmatory experiments of the optimal medium compositions produced maximum antimicrobial activity of 42.11 AU/mL in a six day of fermentation at 28 ^{o}C, 150 rpm. This result showed that the experimentally determined production value was in close agreement with the statistically predicted one which confirmed the model's authenticity. This represented an improvement in the optimal response predicted with 2.4 folds higher antimicrobial activity even though the cultivation time might be actually delayed from five to six days when compared to sucrose yeast extract (SY) medium using classical method. Generally, the production of fungal secondary metabolites is often stimulated by slowly assimilated complex medium components, and decreased when more rapidly utilized medium components are present (Bertasso *et al*., 2001). The delay in secondary metabolite formation is one of the major mechanisms that prevent suicide in antibiotic-producing microorganisms (Drew and Demain, 1997). This study revealed that the production of antimicrobial agents was promoted by the combination of medium compositions. High concentration of secondary metabolites was found when producing organisms grow in complex media containing more than one type of carbon source and nitrogen source (Martin and Demain, 1980). Experimental design can eliminate the limitation of single factor optimization and provide the optimum concentrations of medium ingredients to facilitate the maximum production of active substances (Zeng *et al*., 2006; Mao *et al*., 2007).

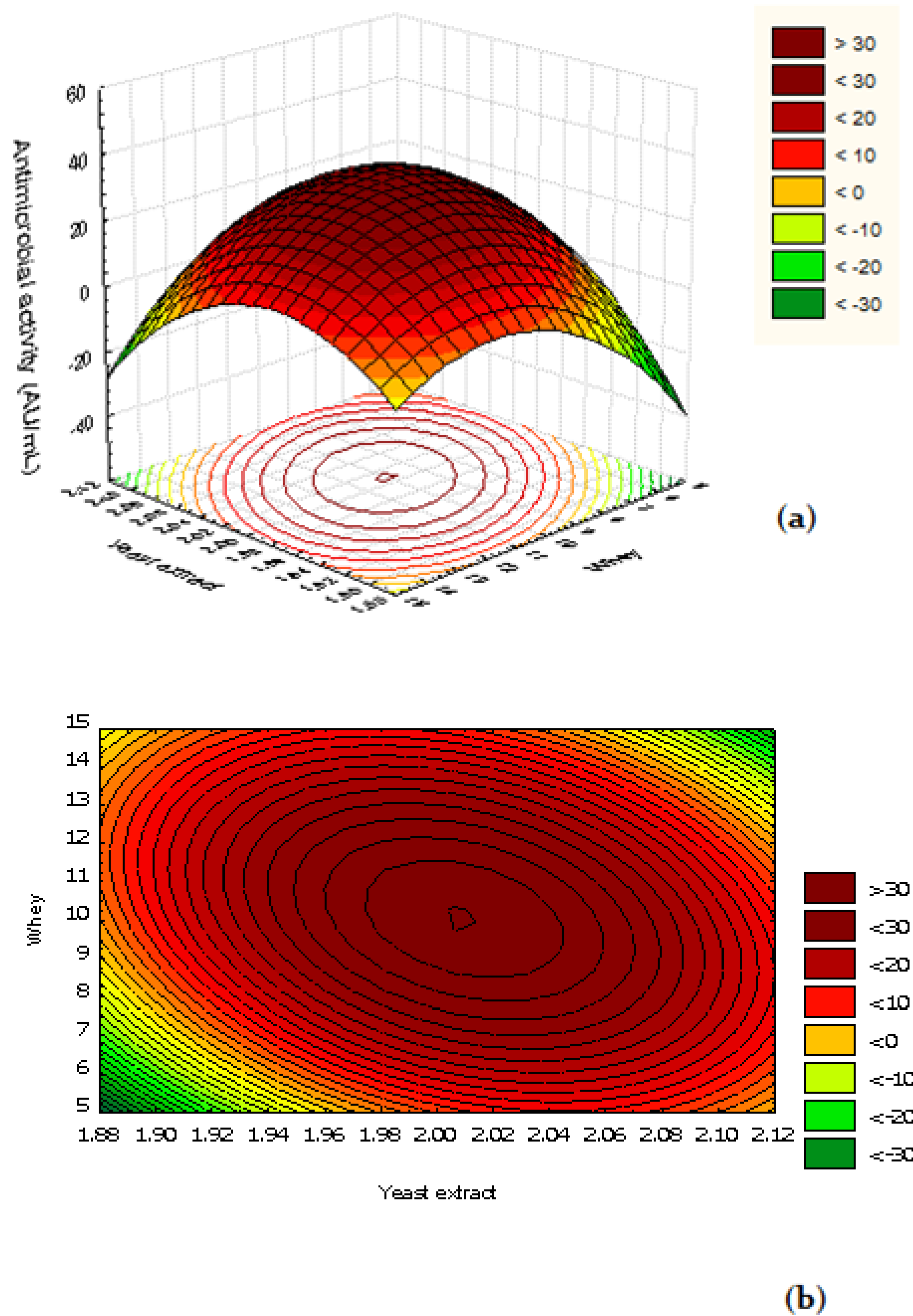

Figure 4.9 (a) Three-dimensional response surface and (b) contour plot of antimicrobial compound production at 28 °C under 150 rpm as the combined effect of yeast extract and whey

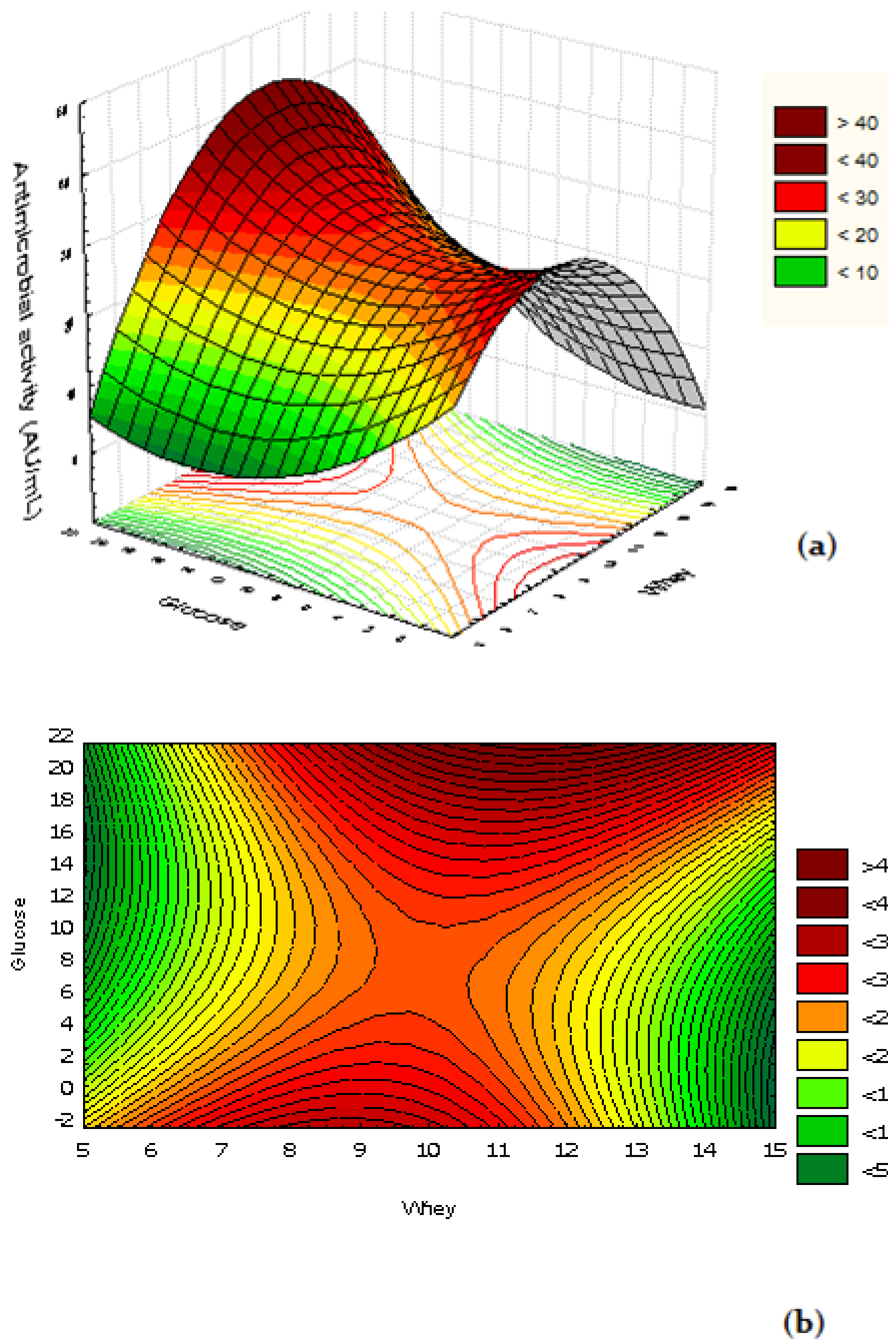

Figure 4.10 (a) Three-dimensional response surface and (b) contour plot of antimicrobial compound production at 28 °C under 150 rpm as the combined effect of whey and glucose

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

After obtaining the medium formula for production of antimicrobial compound, *Nodulisporium* sp. NHL-L 6/6 was cultured in optimized medium. The fermentation broth was harvested and filtered through a piece of nylon cloth. The filtrate was extracted with ethyl acetate twice. Ethyl acetate extract of *Nodulisporium* sp. was concentrated and resulted in a sticky brown color crude extract with the yield of 1.64 g/L of fermentation broth (Figure A8 in Appendix A). In the present investigation, crude extract was evaluated for antimicrobial activity against certain plant pathogenic microorganisms. Potency of crude extract was tested by dilution method. Minimum Inhibitory Concentration (MIC) is defined as the highest dilution or the lowest concentration of the crude extract that inhibits growth of plant pathogenic microorganisms. The concentration of antimicrobial agent that completely killed fungi and bacteria was taken as minimum fungicidal (MFC) and minimum bactericidal (MBC) concentrations, respectively (Sen *et al*., 2011). Several important plant pathogens were chosen, including bacteria, i.e. *Pseudomonas solanacearum*, *Xanthomonas citrii*, and *Erwinia caratovora*, and fungi, i.e. *Colletotrichum* sp., *Fusarium solani*, *F. oxysporum*, *Alternaria brassicicola*, *A. porri*, and *Penicillium* sp., in order to examine more closely the antimicrobial activity of this bioactive secondary metabolite. These plant pathogens cause severe diseases in agricultural crops and ornamental plants, resulting in significant production loss (Table 4.11).

Table 4.11 Plant pathogens and diseases

Plant Pathogens	Disease
Colletotrichum sp.	Anthracnose of chilli, pepper and mango
Fusarium solani	Fusarium crown and foot rot of squash and pumpkin, Fruit rot of cucurbit fruits
Alternaria brassicicola	Brassica dark leaf spot
Alternaria porri	Alternaria blights of the shoots of a wide host range (i.e. potato, tobacco, apple, pear, gooseberry, etc.)
Fusarium oxysporum	Fusarium wilt of a wide host range (i.e. cabbage, banana, linseed, and tomato etc.)
Penicillium sp.	Post-harvest rots of a wide host range (i.e. citrus fruits, apple, pear, strawberry, tomato, corn, and rice etc.)
Pseudomonas solanacearum	Brown rot of tomato, potato and tobacco etc.
Xanthomonas citrii	Citrus canker (or black spot)
Erwinia caratovora	Blackleg, aerial stem rot, and tuber soft rot of a wide host range (i.e. carrot, potato, tomato, leafy greens, squash and other cucurbits, onion, green peppers, etc.)

(Source: http://en.wikipedia.org/wiki/Category:Plant_pathogens_and_diseases)

The present work shows that crude extract exhibited strong inhibitory activity against various plant pathogens. According to the results given in the Table 4.12, the minimum inhibitory (MIC) concentrations of the extract that inhibited both bacterial and fungal pathogens were found in the range of 31.25 to 125 μg/mL. The minimum fungicidal (MFC) and minimum bactericidal (MBC) concentrations that completely killed fungi and bacteria were found in the range of 125-500 μg/mL.

Table 4.12 MIC, MBC and MFC of crude extract of *Nodulisporium* sp. NHL-L 6/6 culture broth against plant pathogens

Plant Pathogens	MIC (µg/mL)	MFC or MBC (µg/mL)
Fungi		
Colletotrichum sp.	62.5	125
Fusarium solani	62.5	125
Alternaria brassicicola	62.5	125
Alternaria porri	62.5	250
Fusarium oxysporum	62.5	250
Penicillium sp.	125	500
Bacteria		
Pseudomonas solanacearum	31.25	125
Xanthomonas citrii	31.25	125
Erwinia caratovora	125	250

Bacterial pathogens were found to be susceptible to antimicrobial agent more than fungal pathogens. In comparison between bacteria, *Pseudomonas solanacearum* and *Xanthomonas citrii* were found to be the most susceptible bacterial pathogens to the antimicrobial agent with the MIC value of 31.5 µg/mL, while *Erwinia caratovora* was the least susceptible bacterial pathogens with the MIC value of 125 µg/mL. The minimum bactericidal (MBC) concentrations of antimicrobial compound that completely killed *Pseudomonas solanacearum* and *Xanthomonas citrii* were found at 125 µg/mL, while *Erwinia caratovora* was completely inhibited at 250 µg/mL (Figure A9; Appendix A).

In consideration of fungi, *Penicillium* sp. was the least susceptible to antimicrobials with the MIC value of 125 µg/mL while other pathogenic fungi were more susceptible with the MIC value of 62.5 µg/mL. The minimum fungicidal (MFC) concentrations of antimicrobial compound that inhibited fungi completely depended on the strains. *Colletotrichum* sp., *Fusarium solani*, and *Alternaria brassicicola* were completely killed when exposed to antimicrobial agent at the concentration of 125 µg/mL. Additionally, *Alternaria porri* and *Fusarium oxysporum* were completely killed when exposed to 250 µg/mL, while *Penicillium* sp. was the least sensitive fungi which was

completely inhibited when exposed to 500 µg/mL of antimicrobial agent (Figure A10; Appendix A).

According to the results, it was found that the bioactive secondary metabolites showed the property of bacteriostatic or fungistatic agent at low concentration (≤ 125 µg/mL). Upon transfer of fungal culture plug and bacterial suspension to the new media without the antimicrobial agent, the fungi and bacteria usually start to grow again. There is not always a precise distinction between biostatics and biocides; high concentrations of some biostatic agents are also biocidal, whereas low concentrations of some biocidal agents are biostatic (Pangey, 2004). In this case, the antimicrobial agent showed the biocidal property that killed fungi and bacteria at high concentrations ranging from 125-500 µg/mL.

Table 4.13 Effect of crude extract of *Nodulisporium* sp. NHL-L 6/6 culture broth on fungal plant pathogen growth

Plant Pathogen	% Mycelial Growth Inhibition*			
	Concentration of crude extract (µg/mL)			
	15.625	31.25	62.5	125
A. porri	12.5±2.1	50.9±3.4	100.0±0.0	100.0±0.0
A. brassicicola	12.6±2.2	55.3±2.2	100.0±0.0	100.0±0.0
F. solani	11.1±2.8	41.9±2.8	100.0±0.0	100.0±0.0
F. oxysporum	22.1±2.4	52.6±4.0	100.0±0.0	100.0±0.0
Penicillium sp.	4.8±0.0	17.5±0.0	54.0±3.2	100.0±0.0
Colletotrichum sp.	34.6±3.8	48.9±2.5	100.0±0.0	100.0±0.0

* Mean percentages of mycelial growth inhibition ± S.D.

It was found that the concentrations of antimicrobial agent at the higher levels reduced the colonial growth of fungi. As shown in Table 4.13, the crude extract of *Nodulisporium* sp. NHL-L 6/6 at the concentration of 15.625 µg/mL showed weak inhibitory effects (less than 40% inhibition) on the growth of *Alternaria porri*, *A. brassicicola*, *Fusarium solani*, *F. oxysporum*, *Penicillium* sp. and *Colletotrichum* sp. The crude extract at the concentration of 31.25 µg/mL showed moderate inhibitory

effects (41-55%) on the growth of *A. porri*, *A. brassicicola*, *F. solani*, *F. oxysporum*, *Penicillium* sp. and *Colletotrichum* sp. The crude extract at the final concentration of 62.5 µg/mL showed moderate inhibitory effects on the growth of *Penicillium* sp. (54.0±3.2%). The mycelial growth of all plant pathogenic fungi was found to be inhibited with 100% efficacy at the concentrations of 62.5 to 125 µg/mL.

This study is a preliminary evaluation of bioactive compounds produced by *Nidulisporium* sp. NHL-L 6/6. The inhibitory effects of the culture extract of *Nidulisporium* sp. NHL-L 6/6 on both bacterial and fungal phytopathogens may be indicative of the presence of more than one broad-spectral antimicrobial compound or normally secondary metabolite. When compared the growth inhibitory effect on each group of phytopathogens (i.e. bacteria and fungi), MIC varied with different microbial species. This could be due to the different ecological niches of these pathogens since they were isolated from different infected plant species and tissues. Moreover, the protective mechanisms of pathogens also depend on pathogen strains and types of the antimicrobials.

The development of natural antimicrobial metabolite would help to decrease the negative effect of synthetic agents, such as residues, resistance and environmental pollution. In this respect, natural antimicrobials may be effective, biodegradable, and less toxic to the environment as well as agriculture industries. As the crude extract of bioactive metabolites from *Nodulisporium* sp. NHL-L 6/6 showed broad spectrum of antimicrobial activity against plant pathogens, thus, it can be concluded that the antimicrobial compounds from *Nidulisporium* sp. NHL-L 6/6 are promising novel types of natural pesticides to control several plant pathogens causing severe diseases in crops and vegetables.

CHAPTER 5 CONCLUSION AND RECOMMENDATION

5.1 Conclusion

5.1.1 Identification of NHL-L 6/6

The strain NHL-L 6/6 was identified by comparative analyse of nucleotide sequence of the internal transcribed spacer (ITS) region of the ribosomal RNA genes. The ITS region sequences were compared with the data in GenBank by BLAST program, and the percentage identity was performed by using ClustalW 2.1. The result showed that ITS of NHL-L 6/6 had sequence similarlity of 99.79% with that of *Nodulisporium* sp. CMU-UPE 34. Therefore, it is reasonable to conclude that NHL-L 6/6 is *Nodulisporium* sp.

5.1.2 Characteristics of growth and anti-phytopathogenic compound production

The characteristics of growth and anti-phytopathogenic compound production by *Nodulisporium* sp. NHL-L 6/6 was studied by cultivation in sucrose yeast extract medium. The maximum yield reached 16.01±1.51 AU/mL at day 5 of fermentation at 28 °C, 150 rpm. A rapid growth was exhibited during the period of 2-4 days, with the highest cell dry weight of 4.85 g/L at day 4 of cultivation. This indicated that anti-phytopathogenic compound formation in fungal endophyte NHL-L 6/6 was non growth-associated.

5.1.3 Effect of nitrogen and carbon sources on anti-phytopathogenic compound production

The suitable carbon and nitrogen sources were selected by one-factor-at-a-time. The result showed that yeast extract, whey, sucrose, glucose and cassava starch influence the anti-phytopathogenic compound production. Considering the price of raw materials and the antimicrobial activity produced, yeast extract and whey were used as nitrogen

sources while sucrose and glucose were used as carbon sources for subsequent experiment.

5.1.4 Experimental design and optimization by response surface methodology

The medium components including carbon and nitrogen sources were optimized for production of anti-phytopathognic compounds from this isolate using a sequential optimization strategy. The nitrogen and carbon sources that affected the antimicrobial activity significantly including whey, yeast extract, sucrose and glucose (except cassava starch due to its high cost) were selected to investigate the effects on antimicrobial activity by using fractional factorial design (FFD). Then, the steepest ascent experiment was used for moving towards response maximization. Finally, central composite design (CCD) was employed to optimize the concentrations of these components. 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. Maximum antimicrobial activity (42.11 AU/mL) was obtained under the optimized conditions, which gave 2.4 folds higher than that of unoptimized medium. The chosen methods were proved to be a powerful tool for the optimization of culture medium for antimicrobial compound production of *Nodulisporium* sp. NHL-L 6/6. Furthermore, the information obtained is considered fundamental and useful for the development of *Nodulisporium* sp. NHL-L 6/6 culture process for efficient production of antimicrobial compound on a large scale.

Table 5.1 Summary of experimental optimization in this study

Medium	Formula	Antimicrobial Activity (AU/mL)	Incubation Time (Days)
Unoptimized medium	3 g/L yeast extract, 20 g/L sucrose	17.54	5
Optimized medium by Response Surface Methodology (RSM)	2.03 g/L yeast extract, 9.86 mL/L whey, 25.08 g/L sucrose, 12.99 g/L glucose	42.11	6

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

This study was also carried out to evaluate the *in vitro* antimicrobial and antifungal efficacy of the crude extract against plant pathogens. The minimum inhibitory (MIC) concentrations of the extract that resulted in growth inhibition of both bacterial and fungal pathogens were found in the range of 31.25 to 125 µg/mL. The minimum fungicidal (MFC) and minimum bactericidal (MBC) concentrations that completely killed fungi and bacteria were found in the range of 125 to 500 µg/mL. The bacterial pathogen *Erwinia caratovora* and fungal pathogen *Penicillium* sp. were found to be least sensitive to antimicrobial agent.

5.2 Recommendation

In addition to medium components, medium conditions also play important role in secondary metabolite production. The parameter those effect secondary metabolite production (i.e. culture pH, medium volume, rotary speed, temperature, and inoculation volume) should be applied to improve the titer of antimicrobial compounds from *Nodulisporium* sp. NHL-L 6/6 by using statistical method.