CHAPTER 3

RESULTS AND DISCUSSIONS

3.1 Identification of fungal genera and frequency of occurrence in bird chili powder samples

The bird chili powder samples were collected from local market in Chiangmai, Thailand and preliminary screened for aflatoxin contamination. The contaminated fungi from three bird chili powder samples that aflatoxin contaminated were isolated by pour plate method (**2.4.1-2.4.2**). The culture plate appeared as two different fungal colonies, i.e. white (89.29%) and black (10.71%) fungal colonies, no fungal colony and white fungal colonies were found in chili powder sample 1, 2 and 3, respectively at 10^{-1} dilution (**Figure 3.1a, b and c**). The result indicated that fungal isolates from serial dilution were appeared mostly as white fungal colonies and some black colonies. Therefore 2 fungal genera identified were, most likely, *Aspergillus flavus* and *A. niger*, respectively. The frequency of occurrence of *A. flavus* (white colonies) in chili powder (94.62% of total 3 samples) was higher than black colonies or *A. niger* (5.38% of total 3 samples). After pure culture were isolated in fresh PDA plates, white colony turned to green colony by 4 days of incubation and morphological and reproductive characteristics after slide culture by microscope (40X) were similar to *A. flavus* (**Figure 3.2a, b**). While black fungal colony identified was similar to *A. niger* (**Figure 3.3a, b**). The identification was confirmed using a literature (Barnett and Hunter, 1987). Each *Aspergillus* species from different chili powder samples showed similar morphological and reproductive characteristics.

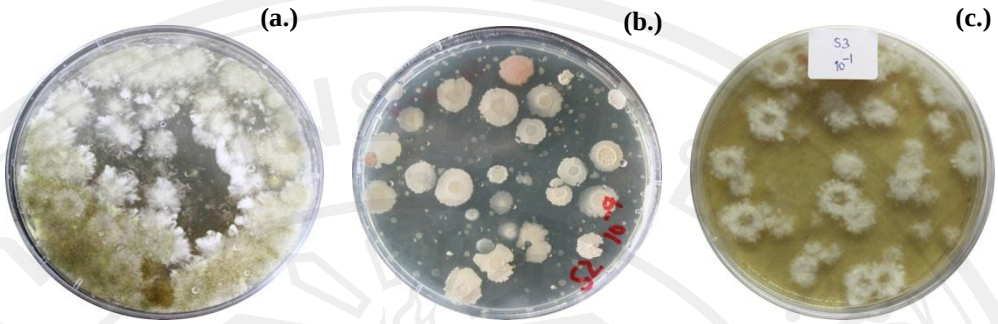

Figure 3.1 Pour plate cultures of bird chili powder samples on PDA (10^{-1}dilution)

(a.) sample 1 : white and black fungal colonies

(b.) sample 2 : no fungal colony but bacterial colonies

(c.) sample 3 : only white fungal colonies

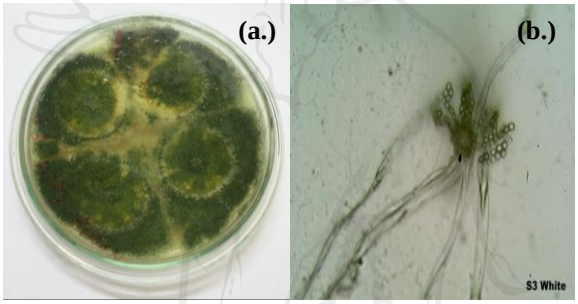

Figure 3.2 (a.) White fungal colonies after 4 days of incubation on PDA

(b.) Microscopic observation of white colony under 40X microscope *(A. flavus)*

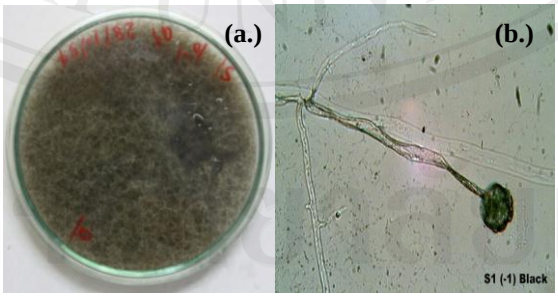

Figure 3.3 (a.) Black fungal colonies after 4 days of incubation on PDA

(b.) Microscopic observation of black colony under 40X microscope *(A. niger)*

3.2 Molecular identification of the fungal species

The mostly isolated fungi from bird chili powder that appeared in pour plate method (white fungal colonies) was identified for the fungal species by using 18S rRNA sequence (**2.4.3**). Analysis of 18S rRNA sequences is also currently the most powerful method for determining higher taxonomic relationships of *Aspergillus flavus*. The result showed that the aflatoxigenic fungi isolated from bird chili powder belonged to species *A. flavus*. The nucleotide sequence data reported in this study was submitted to the GenBank database with the accession number F1514/2556 and sample number RTP, as demonstrated in **Figure 3.4**. The obtained 610-bp DNA sequence was blasted with all DNA sequences in the GenBank database (05/03/2013). BLAST search result showed that 18S ribosomal DNA sequence had the 98.0% identity score that homology to *A. flavus* for 3 strains, *A. flavus* ATCC 11489, *A. flavus* ATCC 9643, and *A. flavus* ATCC 11497 with accession number: JQ 070072, HQ 026738 and GU 256760, respectively (shown in **Figure 3.6-3.7**). Alignments of 18S rRNA sequences followed by Clustal W 1.6 (Thompson *et al*., 1994) revealed that the sequence majority of the considered three strains of *A. flavus* had high similarity.

>RTP *Aspergillus flavus* (610 nucleotides)

CGGTTCGGGCTTTTTCGCGGGGGGGGGGACTCGGAAGGAACTACSGAGTGTAGGGTT
CCTAGCGAGCCCAACCTCCCACCCGTGTTTACTGTACCTTAGTTGCTTCGGCGGGCC
CGCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGGGCCCGCGCCCGCCGGAGACACC
ACGAACTCTGTCTGATCTAGTGAAGTCTGAGTTGATTGTATCGCAATCAGTTAAAAC
TTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATA
ACTAGTGTGAATTGCAGAATTCCGTGAATCATCGAGTCTTTGAACGCACATTGCGCC
CCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCATCAAGCACGG
CTTGTGTGTTGGGTCGTCGTCCCCTCTCCGGGGGGGACGGGCCCCAAAGGCAGCGGC
GGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGGCCCGG
CCGGCGCTTGCCGAACGCAAATCAATCTTTTYCCAGGTTGACCTCTATCAGGTAGGG
ATATACCCGACTTAAGCATACAAAAGAGGGGGGGAGAAAA

Figure 3.4 18S rRNA nucleotide sequence of the isolated *Aspergillus flavus*

Code : RTP (*Aspergillus flavus*)

> ***Aspergillus flavus* strain ATCC 11489** 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

Sequence ID: gb|JQ070072.1| Length: 587Number of Matches: 1

Related Information

Alignment statistics for match #1

Score Expect Identities Gaps Strand Frame 974 bits(527) 0.0() 552/564(98%) 5/564(0%) Plus/Plus

Features:

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Sbjct 560 GGATACCCGCTGAACTTAAGCATA 583
```

Figure 3.5 Alignment of 18S ribosomal RNA sequences from the isolated *Aspergillus flavus* and *A. flavus* ATCC 11489

>***Aspergillus flavus* strain ATCC 9643** 18S ribosomal RNA gene, partial sequence; internal

transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

Sequence ID: gb|HQ026738.1| Length: 576Number of Matches: 1

Related Information

Alignment statistics for match #1

Score Expect Identities Gaps Strand Frame 976 bits(528) 0.0() 555/568(98%) 6/568(1%) Plus/Plus

Features:

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Figure 3.6 Alignment of 18S rRNA sequences from the isolated *Aspergillus flavus* and *A. flavus* ATCC 9643

>***Aspergillus flavus* strain ATCC 11497** 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

Sequence ID: gb|GU256760.1| Length: 571Number of Matches: 1

Related Information

Alignment statistics for match #1

Score Expect Identities Gaps Strand Frame 976 bits(528) 0.0() 555/568(98%) 6/568(1%) Plus/Plus

Features:

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Sbjct 542 GGATACCCGCTGAACTTAAGCATATCAA 569
```

Figure 3.7 Alignment of 18S rRNA sequences from the isolated *Aspergillus flavus* and *A. flavus* 11497

3.3 Aflatoxin analysis in chili powder sample and *A. flavus* production

Aflatoxin B_1 of 3 extracts from bird chili powder were determined by MicroELISA assay. The amount of aflatoxin B_1 7.6, 4.4 and 31.0 µg/kg were found in chili powder sample 1, 2 and 3, respectively which correlated with the frequency of occurrence of white fungal colonies or *A. flavus* in chili powder samples. This suggested that *A. flavus* be possible to produce aflatoxin B_1. According to, Reddy *et al*. (2009) reported previously that *A. flavus* isolates from rice grains were shown to possess the ability to produce aflatoxin B_1. And, aflatoxin B_1 is one of several aflatoxins that can be isolated from the fermentation broth of the fungi *A. flavus*. Therefore, the white fungal colonies or the colonization of bird chili powder with *A. flavus* were used in the further experiment. The *A. flavus* culture flask with SMYK medium after 8 days of incubation was shown in **Figure 3.8**. The surface culture medium presents the white mycelia of *A. flavus*. The amount of aflatoxin B_1 in the fermentation broth of *A. flavus* was shown to be 27.6 µg/L.

Figure 3.8 Mycelia of *A. flavus* in SMYK medium for 8 days, 30 °C

3.4 Growth pattern of *B. subtilis* on nutrient broth medium

The *B. subtilis* was incubated in NB medium at 30°C with rotary shaker at 160 rpm while the growth rate was measured every 4 to 8 h for about 56 h by using a spectrophotometer with OD 610 nm. The growth curve was shown in **Figure 3.11** and revealed that during 4 to 28 h of incubation the culture was in exponential phase. And the culture reached stationary phase after 28 h of incubation. However the strain showed the maximum OD 610 nm at 48 h (1.656±0.001) after incubation. The growth of the strain was slightly decreased after 48 h of cultivation time. Thus, *B. subtilis* strain BCC6327 showed the maximum growth at 48 h. This result correlated with the report by Geetha and Manonmani (2008) that the exponential phase of *B. subtilis* subsp. *subtilis* was between 4 to 48 h and the stationary phase was seen after 48 h of inoculation.

Figure 3.9 *B. subtilis* colonies on NA

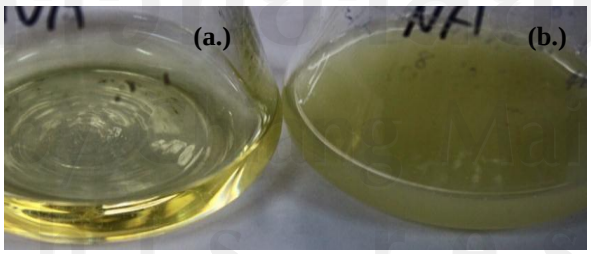

Figure 3.10 (a.) NB medium and (**b.)** Culture broth of *B. subtilis* in NB

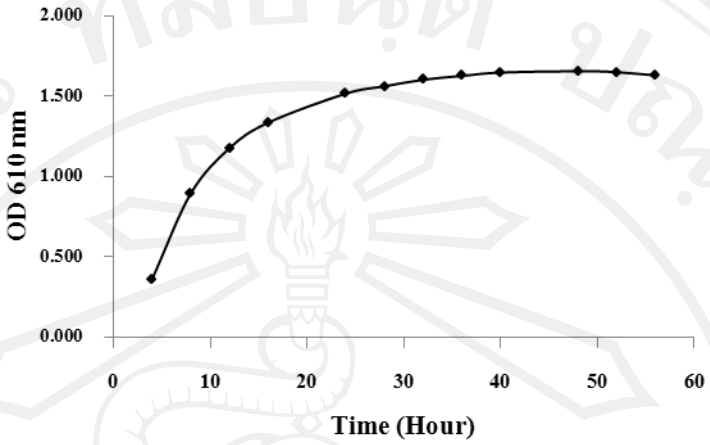

Figure 3.11 Growth curve of *B. subtilis* in NB medium for 56 h

3.5 Screening for the antagonistic activity of *B. subtilis* against *A. flavus*

The antagonist *B. subtilis* was screened for antagonism against isolated aflatoxigenic fungi or *A. flavus* by dual culture both spore suspension culture in broth medium and co-culture of antagonist (*B. subtilis*) and pathogen (*A. flavus*) on agar plate. The antagonistic activity on agar plate was screened in 2 features **i)** single streak of *B. subtilis* and **ii)** a plug of *A. flavus* placing onto the middle of test plate.

3.5.1 The antagonistic activity of *B. subtilis* against *A. flavus* in broth

Preliminary screening for the antagonistic activity of *B. subtilis* against isolated *A. flavus* was performed in broth medium (**2.7.1**). The dual culture test tubes were inoculated with both spore suspension of *A. flavus* and *B. subtilis*, and then incubated at 30 °C for 7 days (**Figure 3.12**). While, control test tubes were uninoculated with *B. subtilis*. The result in **Figure 3.13** revealed that dry weight of

mycelial mass which was used as measurement of the fungal growth. The results indicated that the control (15.7±2.3 mg) possessed a significantly higher dry weight of mycelial mass than treatment with *B. subtilis* (8.4±3.4 mg). Thus, test tubes incubated with *B. subtilis* strain had the reduced growth of isolated *A. flavus*. The percentage of growth inhibition of *A. flavus* was 46.4% at the 7 days of incubation. The results of this study were supported by Kimura and Hirano (1988). They reported that the isolated *B. subtilis* strain NK-330 could inhibit the growth and aflatoxin production of *A. flavus*.

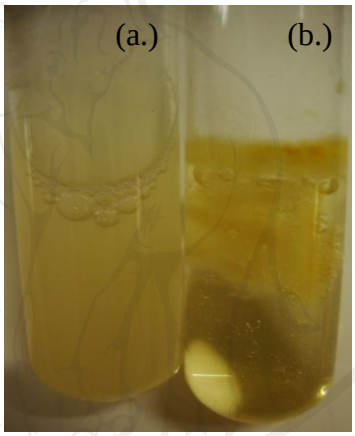

Figure 3.12 (**a.)** The dual culture of spore suspension of *B. subtilis* and *A. flavus* **(b.)** Control test tube without spore suspension of *B. subtilis*

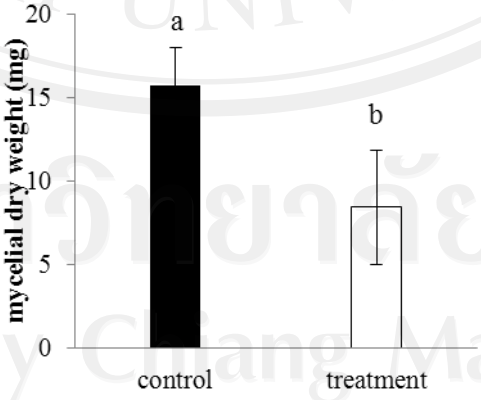

Figure 3.13 Dry weight of mycelia mass of *A. flavus* in control and dual culture with spore suspension of *B. subtilis* after 7 days incubation

3.5.2 The antagonistic activity of a single streak of *B. subtilis* against *A. flavus* on agar plate

The antagonistic activity of single streak of *B. subtilis* against *A. flavus* on PDA plate in three replicates (**2.7.2**). A single streak of *B. subtilis* in a length of 2 cm was applied onto the middle of test plate previously overlaid with *A. flavus*. After 7 days incubation, the inhibition zone around a streak of *B. subtilis* was observed. The results shown in **Figure 3.14** indicated that the strain *B. subtilis* effectively inhibited the mycelia growth of *A. flavus*. In dual culture, a clear zone of inhibition was observed exhibiting antagonism between pathogen (*A. flavus*) and antagonist (*B. subtilis*).

Figure 3.14 The dual culture plate of antagonistic of *B. subtilis* against the mycelia growth *A. flavus*.

3.5.3 The antagonistic activity of *B. subtilis* against a mycelial plug of *A. flavus* on plate

The antagonistic activity was determined by dual culture in NA plate against aflatoxigenic fungi (*A. flavus*). The spore suspension of antagonist *B. subtilis* was added onto NA as base layer of test plate and then place a plug of mycelial *A. flavus* onto the middle of plate (**2.7.3**). The dual culture on NA plate was determined by the fungal growth diameter that was compared between control (no inoculation of *B. subtilis* spore suspension) plates and dual culture plates. *A. flavus* was inhibited by *B. subtilis*. The result is as shown in **Table 3.1** and **Figure 3.15**, a mycelial fungus could not grow on the dual culture plates after 3 days inoculation and the fungal growth diameter was 0.60±0.00 cm that was the same as the original cut mycelial plug (0.60 cm). In control plates uninoculated with spore suspension of *B. subtilis*, the mycelial fungus grew on NA plates (2.98±0.36 cm) but not as good as growing in PDA (3.99±0.38 cm). Because PDA is a suitable media that is required for fungal growth, while NA media is mostly used for the culturing of bacteria (Nwagu *et al*., 2012). The percentage of inhibition of *A. flavus* growth was 79.9% when compared with NA control plate (2.98 cm) and 85.0% when compared with PDA control plate (3.99 cm). The result of antagonistic activity on dual culture NA plate was shown similar in PDB medium (**3.5.1**). *B. subtilis* able to inhibit the growth of *A. flavus* both dual culture on NA and PDB. The result indicated that *B. subtilis* culture able to retard the aflatoxigenic fungal growth due to *B. subtilis* inhibitory properties are considered the most efficient to antagonistic activity (Hernández-Suárez *et al*., 2011).

Table 3.1 Diameter of fungal growth in each treatment

Treatment	Diameter of fungal growth (cm)
PDA	3.99±0.38 [a]
NA	2.98±0.36 [b]
Dual culture in NA	0.60±0.00 [c]

Mean±SD (n=3)

[a-c] Means within a column with different superscripts are significantly different (P<0.05)

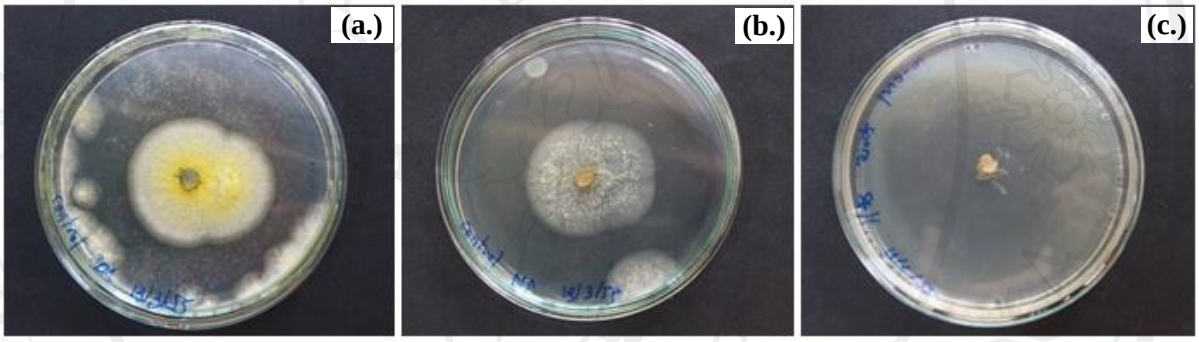

Figure 3.15 (a.), (b.), (c.) Growth of fungi on PDA, NA and dual culture plate in NA, respectively for 3 days after inoculation.

3.6 Inhibition of extracellular metabolites in cell free supernatant of *B. subtilis*

The mycelial fungal growth of *A. flavus* was shown to be inhibited by extracellular metabolites in cell free supernatant of *B. subtilis* both in broth medium and agar plate medium. And, the fungal morphology affected after inoculated with cell free supernatant of *B. subtilis* by slide culture, was observed under the microscope.

3.6.1 Inhibition of fungal mycelium by cell free supernatant of *B. subtilis* in broth medium

The inhibition of *A. flavus* mycelial growth was tested by cell free supernatant of *B. subtilis* that was collected at 12, 24, and 36 h of incubation in NB medium. The 25% (v/v) cell free supernatant of *B. subtilis* culture at 12, 24, and 36 h was individually added into PDB treatment flasks. The mycelial growth inhibition by cell free supernatant was determined after 5 days of incubation. Mycelia were filtered, dried and weighed (**2.8.1).** Compared with control flask without cell free supernatant, all treatment flasks with 25% v/v cell free supernatant resulted in a significant reduction in mycelial growth that exhibited on mycelium weight. As shown in **Table 3.2**, the highest dried mycelia of *A. flavus* were 0.3171±0.0271 g of control flask. It was significantly higher than treatment flasks with 25% (v/v) cell free supernatant at 12, 24, and 36 h which were 0.0250±0.0015, 0.0330±0.0015, and 0.0315±0.0028 g, respectively. The inhibition percentages of mycelium production were 92.1, 89.6 and 90.1% of cell free supernatants at 12, 24 and 36 h, respectively. **Figure 3.16 and 3.17** show the mycelial mass of fungi from control flask compared with 25% v/v cell free supernatant flask. All the cell free supernatants from 12, 24, and 36 h of incubation inhibited growth of aflatoxigenic fungi. Hai (2006) reported that the *B. subtilis* metabolites inhibited both spore germination and hyphal elongation, inducing the decrease of fungal development and consequent reduction of the aflatoxin production.

Table 3.2 Dried weight and percentage of the fungal mycelia inhibition by cell free supernatant of *B. subtilis* incubated at various time points

Supernatant collecting time (h)	**5 days dried weight (g)**	**%inhibition**
0	0.3171±0.0271 [a]	-
12	0.0250±0.0015 [b]	92.1
24	0.0330±0.0015 [b]	89.6
36	0.0315±0.0028 [b]	90.1

Mean±SD (n=3)

[a-b] Means within a column with different superscripts are significantly different (P<0.05)

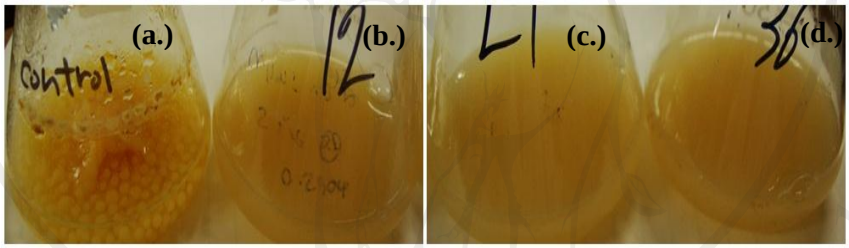

Figure 3.16 (**a.), (b.), (c.), (d.)** Mycelial growth of fungal on control, 12, 24 and 36 h collecting times of cell free supernatant flask, respectively

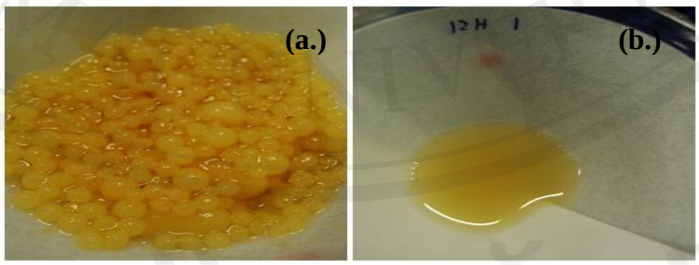

Figure 3.17 (a.), (b.) Mycelial mass of fungal in control and 12 h collecting time of cell free supernatant, respectively

3.6.2 Inhibition of fungal mycelium by cell free supernatant of *B. subtilis* in agar plate

The cell free supernatant collected during the stationary phase at 54 h, was used for growth inhibitory effect of *A. flavus* on PDA plate. The inhibition of fungal mycelium growth was observed on during 3-7 days of inoculation (**2.8.2**). The results were shown in **Table 3.3** and **Figure 3.18 a, b**, diameter of fungal growth was measured on during 3-7 days of inoculation. On control plates without cell free supernatant, the radial fungal growth was significantly increased and the maximum radius was reached 6 days after inoculation (6.90±0.46 cm). While, test plate with added cell free supernatant that collected for 54 h, the fungal diameter unchanged after 3 days of inoculation. Moreover, the result indicated that addition of cell free supernatant possessed a significantly less growth than control without addition. The diameter fungal growth in the presence of *B. subtilis* cell free supernatant from the stationary phase did not exceed 1.50±0.17 to 1.77±0.42 cm in diameter (3 to 7 days after incubation) in comparison with control (4.07±0.40 to 7.60±0.36 cm), and percentage of growth inhibition range was about 63-76%. The extracellular metabolites in cell free supernatant of *B. subtilis* were able to inhibit *A. flavus* growth when cultured on agar and broth medium.

Table 3.3 Diameter of fungal growth and percentage of the fungal mycelia inhibition by cell free supernatant (54h) of *B. subtilis*

Incubation time (days)	Diameter (cm)		% inhibition
	Control	54 h	
3	4.07 ± 0.040^{Ad}	1.50 ± 0.17^{B}	63.14
4	4.97 ± 0.35^{Acd}	1.60 ± 0.26^{B}	67.81
5	5.97 ± 0.45^{Abc}	1.63 ± 0.29^{B}	72.70
6	6.90 ± 0.46^{Aab}	1.73 ± 0.38^{B}	74.93
7	7.60 ± 0.36^{Aa}	1.77 ± 0.42^{B}	76.71

Mean±SD (n=3)
[a-d] Means within a column with different superscripts are significantly different (P<0.05)
[A-B] Means within a row with different superscripts are significantly different (P<0.05)

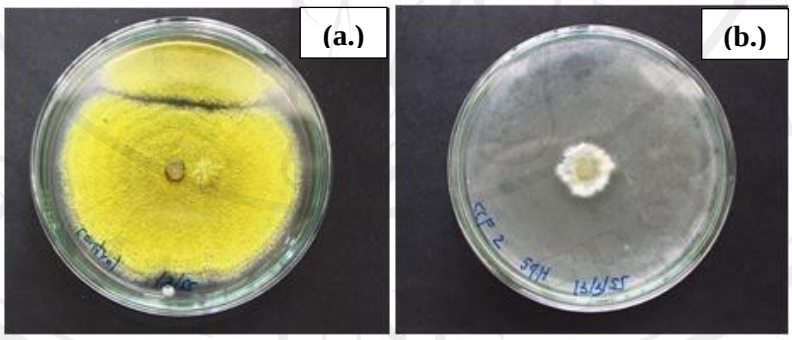

Figure 3.18 Growth of fungi on PDA without cell free supernatant (**a.**), and with 54 h bacterial cell free supernatant plate (**b.**) for 7 days after inoculation

3.6.3 Fungal hyphal morphology as affected by the crude extracellular metabolites

The slide culture of aflatoxigenic fungi on 10% v/v cell free supernatant culture of 9 h agar and control agar (without bacterial supernatant) were observed for their morphology under 10X and 40X microscope. **Figure 3.19 and 3.20** show fungal morphology had seen under 10X and 40X microscope, respectively in different culture agar. The control agar plate (**Figure 3.19 b.**) had more conidia and budding conidial spores of *A. flavus* than the cell free supernatant containing agar plate **(Figure 3.19 a.)** that had no conidial spores. Generally, the conidiophores (spore forming structures) are very important for identification of *Aspergillus* spp. The conidiophores originate from a basal cell located on the supporting hyphae and terminated in a vesicle and produced by *A. flavus* during reproduction (Jensen, 2013). The result was supported by the **Figure 3.21** showing slide culture of cell free supernatant and control agar. Fungal mycelium in agar plate with cell free supernatant was less denser than that in control plate. The result indicated that the growth of aflatoxigenic fungal was inhibited by cell free supernatant of *B. subtilis*.

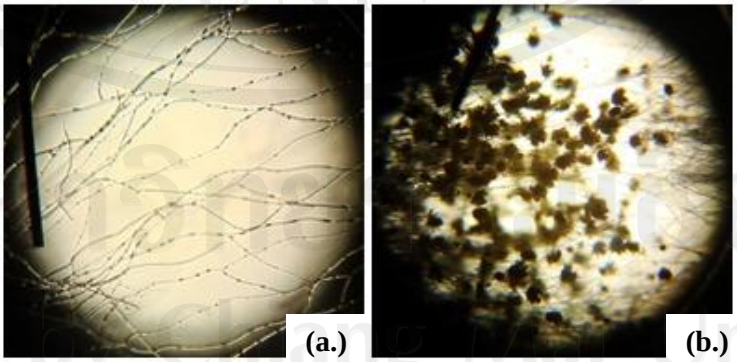

Figure 3.19 (**a.), (b.)** Morphology of fungi from PDA without and with bacterial cell free supernatant, respectively under 10X microscope

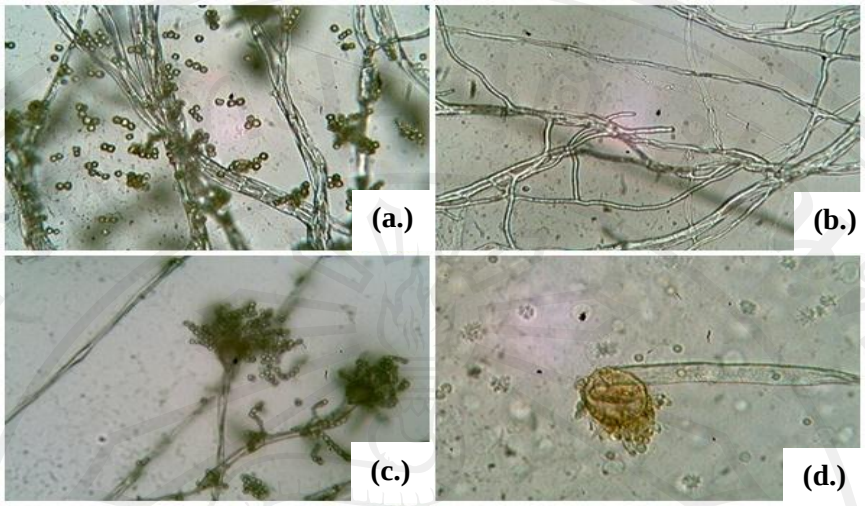

Figure 3.20 Morphology of fungi under 40X microscope on PDA without **(a.), (c.)** and with cell free supernatant **(b.), (d.)**

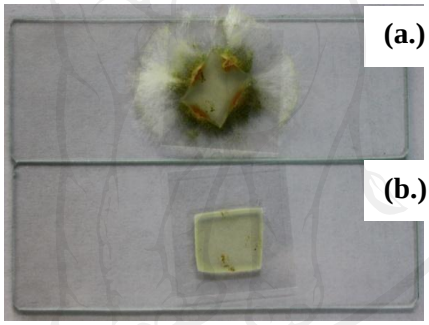

Figure 3.21 Slide culture on **(a.)** PDA and (**b.)** cell free supernatant

3.7 Plate screening of hydrolytic enzymes produced from *B. subtilis*

Preliminarily, *B. subtilis* was screened for its ability to produce hydrolytic enzymes by plate method (**2.9**). The *B. subtilis* was grown on plate agar with different substrates. The different substrates, *i.e.* soluble starch, colloidal chitin, casein, Na-carboxymethyl cellulose and Tween 20 were used to induce enzyme productions of amylase, chitinase, protease, cellulase and lipase, respectively. Three replicates for each enzyme treatment were incubated at 30 °C for 2 days. After incubation, the colony of *B. subtilis* which exhibited surrounding clear zone was considered as positive for enzyme production in chitinase and lipase production. In case of amylase, protease and cellulase, the plates were tested for positive by adding reagents 1% iodine in 2% potassium iodine, 25% TCA and 25% Congo red, respectively. The result shown in **Figure 3.22,** indicated that *B. subtilis* produced chitinase, protease and cellulase. The chitinase plate contained colloidal chitin as substrate. After 3 days of incubation time, clear zone surrounding the bacterial colony appeared (**Figure 3.22 b**). In the protease plates containing casein as a substrate and after 2-days incubation the plates were reacted with TCA. The clear zone surrounding the bacterial colony was revealed (**Figure 3.22 c**). The cellulase plate had Na-CMC as a substrate. After 2-day incubation, the plates were flooded with congo red and destained with 1 M NaCl. Clear zone surrounding the bacterial colony appeared in the CMC plates (**Figure 3.22 d**). *B. subtilis* has capacity to produce chitinase, protease and cellulase enzyme.

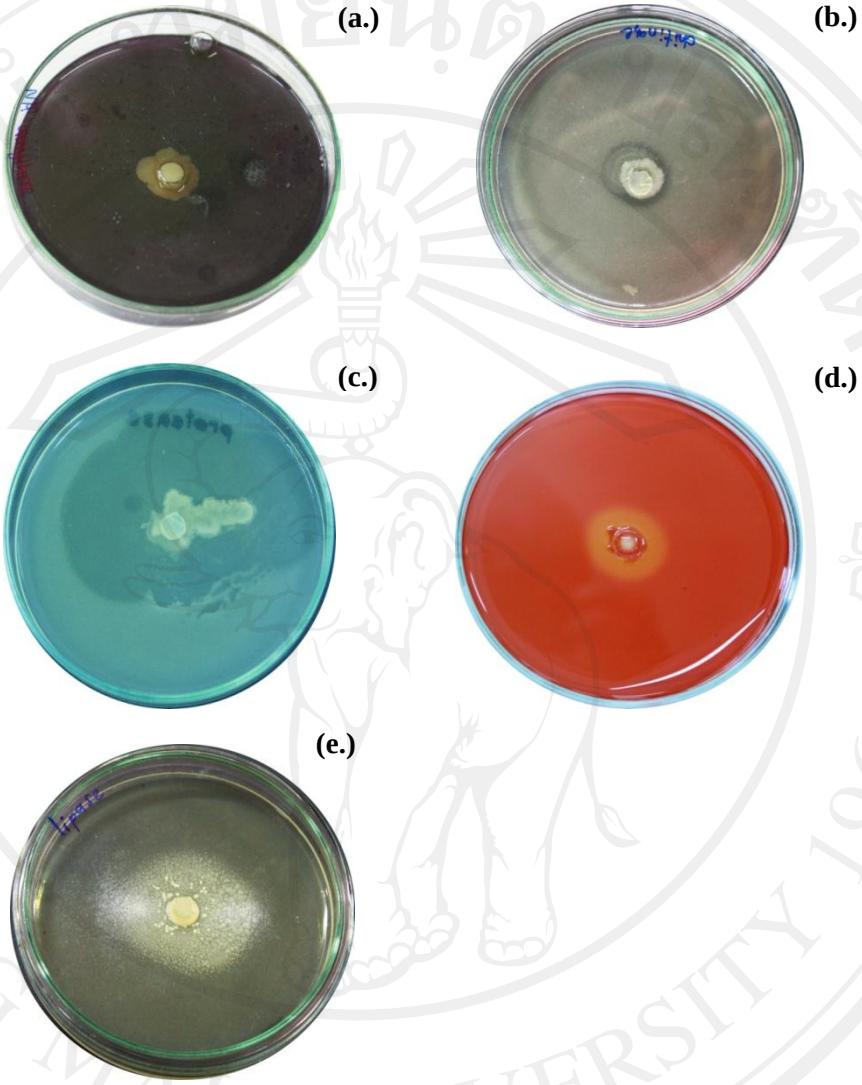

Figure 3.22 The hydrolytic enzyme productions on secretary plate of *B. subtilis*. Agar plate contained corresponding substrates for **(a.)** amylase, **(b.)** chitinase, **(c.)** protease, **(d.)** cellulase and **(e.)** lipase

3.8 Enzyme activity assay

3.8.1 Protease activity during growth phase of *B. subtilis*

B. subtilis was inoculated in liquid medium for protease production supplemented with 1% w/v peptone. Proteolytic activity was assayed by the colorimetric method along with the determination of cell growth. The result shown in **Figure 3.23**, indicated that the protease production of *B. subtilis* started in the early stationary phase (24 h) of growth and gradually increased during 24 to 32 h of growth. The maximum enzyme activity was achieved at 32 h of growth and rapidly decreased after 32 h of growth at stationary phase. The maximum protease activity of *B. subtilis* at 32 h of growth was 0.020+0.001 U/ml in the stationary phase. With the protein content, the specific activity of the crude protease from *B. subtilis* at 32 h of growth was calculated to be 1.449±0.045 U/mg protein. However, Hamid and Ikram (2008) reported that *B. subtilis* IH – 72 showed the enzyme production at 4 h up to 56 h of growth. It was found that the growth of *B. subtilis* and enzyme production was gradually increased as the fermentation process progressed and reached maximum after 40 h of incubation. The maximum growth and protease production was found to be 13.80+0.53 mg/ml and 7.0+0.36 U/ml, respectively. A rapid decline in the enzyme production was observed when the fermentation was extended beyond the optimum incubation period. There was very little production of the enzyme during lag and logarithm phase of the bacterial growth.

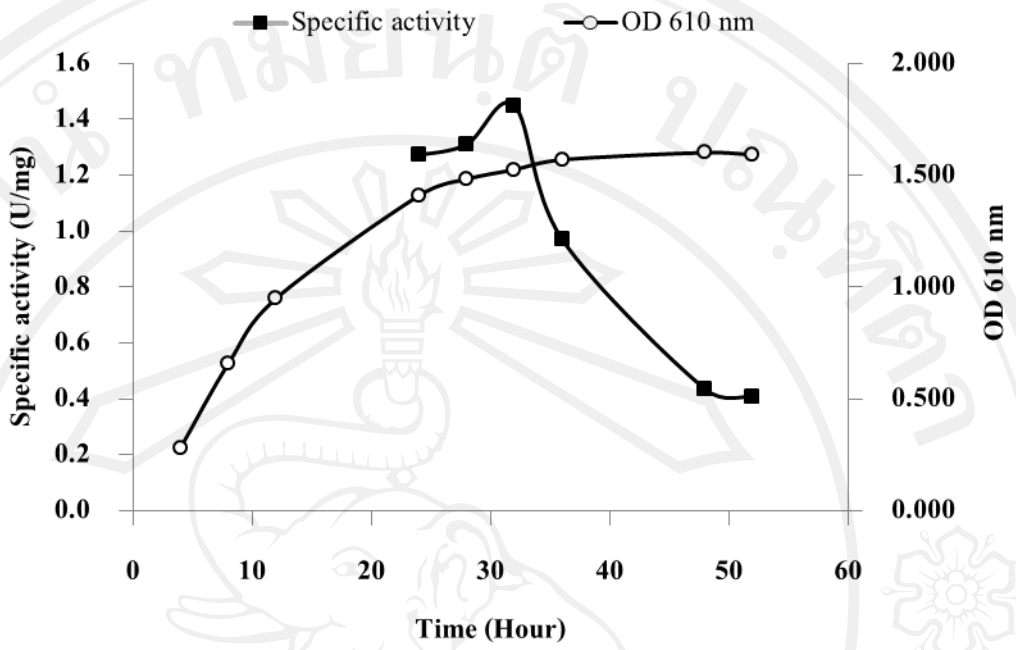

Figure 3.23 Specific activity of protease and OD 610 nm along growing time course of the *B. subtilis* in protease production liquid medium

3.8.2 Chitinase activity during growth phase of *B. subtilis*

Chitinase activity from *B. subtilis* was assayed along with cell growth observation represented by colony forming unit every 4 h for 56 h. The growth rate of B. subtilis on chitinase production medium unable to measure by optical density at 610 nm due to interfere of turbidity on culture broth, therefore the growth rate was measured by pour plate technique. The chitinase production was induced by 2% w/v colloidal chitin amended media and assayed by Somogyi's and Nelson's method. The result shown in **Figure 3.24** indicated that the growth and chitinase production patterns were closely associated. The chitinase production of *B. subtilis* started in the logarithm phase of growth (24 h) and rapidly increased at 24 h up to 36 h of growth in the logarithm phase and stationary phase. The maximum enzyme activity of

0.003046±0.000046 U/ml was achieved at 36 h of incubation of which the maximum growth was $4.7x10^7$ CFU/ml. And the specific activity of crude chitinase from *Bacillus* at 36 h of growth was calculated to be 0.1427 U/mg protein. A rapid decline in the enzyme production associated with the decreased cell number of growth after 36 h of incubation during death phase.

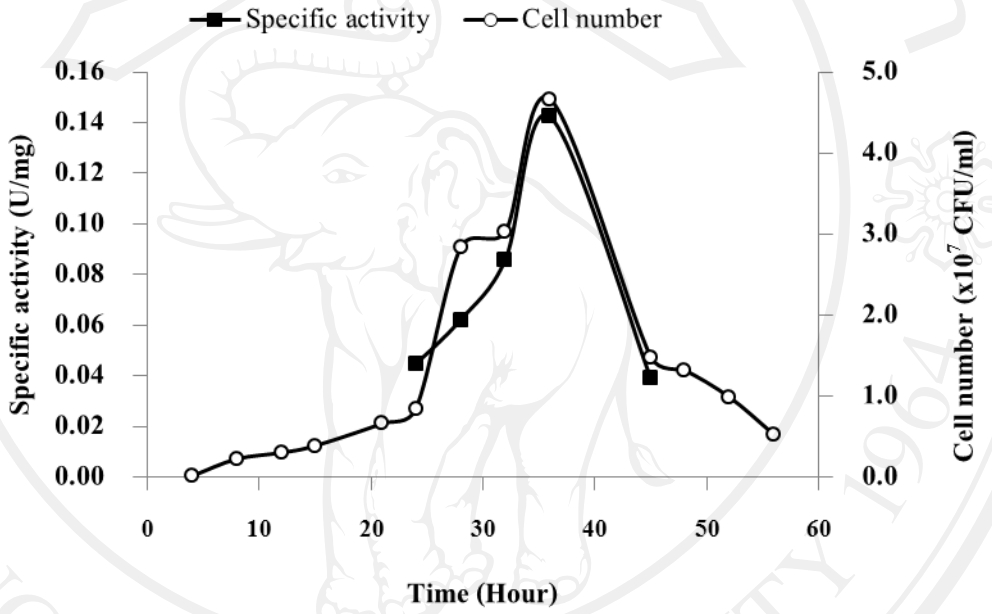

Figure 3.24 Specific activity of chitinase and cell number of bacteria of each growing time point of *B. subtilis* in colloidal chitin liquid medium

3.8.3 Cellulase activity during growth phase of *B. subtilis*

B. subtilis was inoculated in liquid medium for cellulase production supplemented with 0.2% w/v Na-carboxymethyl cellulose. Cellulase activity was assayed by DNS method along with the determination of cell growth. The result in

Appendix Table C.15 revealed that *B. subtilis* has no high activity of the cellulolytic enzyme or no enzyme activity in this condition.

3.9 Qualitative determination of lytic enzyme activity in the presence of fungal dried mycelia

Besides being a carbon source, the dried mycelia cell walls of *A. flavus* was tested as inducers of lytic enzyme synthesis from *B. subtilis*. A significant activities of protease, chitinase and β-1, 3- glucanase were produced by *B. subtilis* in culture media containing dried mycelium of *A. flavus* and NB without dried fungal mycelium (**Table 3.4**). However, dried mycelium supplemented in NB resulted in higher enzyme activities than NB without dried mycelium. Proteolytic specific activity of NB supplemented with dried mycelium (0.0907±0.0077 U/mg protein) was 1.4 times higher than NB without dried mycelium (0.0657±0.0024 U/mg protein). Hameed *et al*. (1994) reported that protease was an important enzyme in pathogenesis which attacks the plasma membrane after the degradation of cell wall by chitinase and glucanase.

The chitinase production of *B. subtilis* showed a high level when grown in NB supplemented with dried mycelium of *A. flavus* (0.0185±0.002 U/mg protein), about 2 times higher than NB media (0.0092±0.000 U/mg protein). The chitinase produced was active against fungi as measured by the release of sugars from their cell walls (Gupta *et al*., 1995).

The β-1,3- glucanase production in NB supplemented with dried mycelium present the specific activity of 2.2959±0.0383 U/mg protein was approximately 1.2

times higher than NB (1.9831±0.0318 U/mg protein). Pozo *et al.* (1999) reported that β-1,3- glucanases are able to partially degrade fungal cell walls by catalyzing the hydrolysis of β-1,3- glucosidic linkages in β-D-glucans, which are together with chitin, the major cell wall components of most fungi.

Production of extracellular β-(1,3)-glucanases, chitinases and protease increases significantly when *B. subtilis* are grown in media supplemented with dried mycelium cell wall of *A. flavus*. These observations, together with the fact that chitin, β-1,3- glucan and protein are the main structural components of most fungal cell walls (Peberdy, 1990) and are the basis for the suggestion that hydrolytic enzymes produced by *B. subtilis* play an important role in destruction of plant pathogens (Chet and Baker, 1981).

Incubation of dried mycelium of the aflatoxigenic fungi culture filtrates resulted in a high release of reducing sugars (**Table 3.5**). Aflatoxigenic dried mycelium was very sensitive to be hydrolyzed by *B. subtilis* crude enzyme. Higher released sugar from *B. subtilis* grown in media supplemented with dried mycelium, suggested that dried mycelium can act as an inducer for lytic enzyme synthesis. *B. subtilis* has the potential to produce cell wall degrading enzymes when chitin or isolated fungal cell wall material are present in the growth medium. The secreted hydrolytic enzymes such as protease, β-1, 3- glucanase and chitinase can penetrate and lyse the host cell wall of pathogenic fungi (Elad *et al*., 1983). This is one of the defensive mechanisms.

Table 3.4 Specific activity of protease, chitinase and β-1, 3-glucanase in NB with dried mycelium and NB

	Specific activity (U/mg)		
Media	**Protease**	**Chitinase**	**β-1, 3-glucanase**
NB with dried myceliam	0.0907 ± 0.0077 [a]	0.0185 ± 0.0002 [a]	2.2959 ± 0.0383 [a]
NB (control)	0.0657 ± 0.0024 [b]	0.0092 ± 0.0000 [b]	1.9831 ± 0.0318 [b]

Mean±SD (n=3)
[a-b] Means within a column with different superscripts are significantly different (P<0.05)

Table 3.5 The amount of sugar released in culture filtrate on NB with dried mycelium and NB media

Sugar released	**Media**	**Amount of sugar (µg/ml)**
Glc	NB with dried myceliam	2679.23 ± 27.01 [a]
	NB (control)	1228.20 ± 16.01 [b]
GlcNAc	NB with dried myceliam	869.84 ± 11.98 [a]
	NB (control)	538.10 ± 20.76 [b]
Total R.S.	NB and dried myceliam	3549.07 ± 32.88 [a]
	NB (control)	1766.30 ± 36.19 [b]

Mean±SD (n=3)
[a-b] Means within a column for each kind of releasing sugar with different superscripts are significantly different (P<0.05)
R.S. = Reducing sugar

3.10 Inhibition of fungal growth by cell free supernatant from each enzyme production of *B. subtilis*

The cell free supernatants of *B. subtilis* from NB, protease and chitinase production culture were used to inhibit the growth of *A. flavus* on potato dextrose agar plate. The result shown in **Table 3.6**, diameter of fungal growth was measured on 5 day of incubation and the against culture plate of NB, protease and chitinase production were collected during the exponential and stationary phase of NB, protease, and chitinase on culture media as shown previously in **Figure 3.11, 3.23, and 3.24**, respectively. The cell free supernatant of *B. subtilis* in NB medium were collected at 8 h of incubation for exponential phase and 24 and 48 h of incubation for stationary phase. The early stationary phase of protease production medium was started after 24 h of incubation. And, the cell free supernatant of *B. subtilis* in chitinase production medium were collected at 28, 36, and 45 h of incubation for used as exponential and stationary phase, respectively. The control plate without cell free supernatant showed maximum diameter (5.83±0.21 cm) of fungal colony and significantly higher than protease, chitinase production media and NB. The fungal diameter of each against culture plates on stationary phase were less than those of exponential phase. The minimum fungal diameter were 3.42±0.19, 2.03±0.15 and 3.03±0.15 cm on stationary phase of 24 h in NB, 32 h in protease production media and 36 h in chitinase production media, respectively. The result indicated that all "against" culture plates with cell free supernatant possessed a significantly less diameter of fungal growth than the control plate. The percentage of fungal growth inhibition from protease production media 32 h collecting time (on stationary phase) was shown highest antifungal activity (65.12%).

For the chitinase production media at 36 h collecting time showed antifungal activity with 47.97% fungal growth inhibition. Moreover, in stationary phase (24 h collecting time) of cell free supernatant from NB media was shown fungal inhibition (41.40%). The result indicated that *B. subtilis* able to produce hydrolytic enzyme or secondary metabolite on the culture medium. The production of several hydrolytic enzyme, especially protease and chitinase may degrade cell wall of aflatoxigenic fungi and inhibit the fungal growth. (Leifert *et al*., 1995). The result from all "against" culture plates indicated that the stationary culture filtrate possessed a significantly higher antifungal potential than the exponential culture filtrate.

The antifungal potential of exponential culture filtrate was probably related to the increased production of hydrolytic enzymes, both protease and chitinase (**Figure 3.23 and 3.24**). However, both exponential and stationary culture filtrates of *B. subtilis* inhibited the aflatoxigenic fungi. The percentage inhibition of fungal growth inhibitions were related to production of extracellular proteolytic and chitinolytic enzymes. The highest protease specific activity (1.449 U/mg proteins) were associated with the highest percentage of fungal growth inhibition (65.12%) at 32 h of incubation time and subsequently decreased both of protease specific activity (0.435 U/mg proteins) and percentage inhibition of fungal growth (25.65%) at 48 h of incubation time. The highest chitinase specific activity (0.143 U/mg proteins) were related to the highest percentage inhibition of fungal growth (47.97%) at 36 h of collecting time, while both of chitinase specific activity (0.039 U/mg proteins) and percentage inhibition of fungal growth (34.25%) were decreased at 45 h of collecting time. The production of protease and chitinase by *B. subtilis* were related to fungal growth inhibition due to the ability of *B. subtilis* to degrade fungal cell walls.

The production of several hydrolytic enzymes such as chitinase, glucanase and protease are considered key players in the lysis of cell walls of fungi and may be important factors in biological control, which was also reported by Essghaier *et al*. (2012). These findings imply that the antifungal potential of the stationary culture supernatant was probably related to increased production of hydrolytic enzymes and antibiotics. It has been reported that the glucanase enzyme in combination with the antibiotic showed a strong synergistic inhibitory effect on the hyphal growth of fungi (Leelasuphakul *et al*., 2006).

Table 3.6 Radii of fungal growth in each enzyme production treatment

Media	**Supernatant collecting time (h)**	**Specific activity (U/mg protein)**	**diameter of fungal colony (cm)**	**% inhibition**
PDA	-	-	5.83±0.21 ^{a}	-
NB	8	-	4.15±0.31 ^{bc}	28.82
	24	-	3.42±0.19 ^{def}	41.40
	48	-	3.40±0.10 ^{et}	41.68
Protease	24	1.273	2.37±0.15 ^{g}	59.41
	32	1.449	2.03±0.15 ^{g}	65.12
	48	0.435	4.33±0.15 ^{b}	25.67
Chitinase	28	0.062	3.87±0.42 ^{cd}	33.68
	36	0.143	3.03±0.15 ^{f}	47.97
	45	0.039	3.83±0.47 ^{cde}	34.25

Mean±SD (n=3)
$^{a-g}$ Means within a column with different superscripts are significantly different (P<0.05)

3.11 Antifungal activity of the cell free supernatant on bird chili powder

The *B. subtilis* cell free supernatant was used as *A. flavus* antifungal growth on dried bird chili powder sample. There are 4 treatment flasks shown in **Figure 3.25**, the control flask contained only dried bird chili powder, treatment 1 flask contained dried bird chili powder with cell free supernatant *B. subtilis*, treatment 2 flask contained dried bird chili powder with cell free supernatant of *B. subtilis* and spore suspension of *A. flavus* that used as fungal inhibition flask. Treatment 3 flask contained dried bird chili powder with spore suspension of *A. flavus*. The control flask and cell free supernatant *B. subtilis* contained flask had no mycelia of *A. flavus* after 7 days of incubation time when evaluated macroscopically and both flasks showed absence of *A. flavus* growth on the pour plate culture (**Figure 3.26**). In the flask containing cell free supernatant *B. subtilis* and spore suspension of *A. flavus* (fungal inhibition flask), evaluated macroscopically, there was no *A. flavus* growth after 7 days incubation but mycelia of *A. flavus* were found on the pour plate culture. However, the pour plate culture of fungal inhibition flask showed the growth of *A. flavus* less than the flask containing dried bird chili powder with spore suspension of *A. flavus*.

The result indicated that, the *B. subtilis* cell free supernatant possessed antagonistic activity against *A. flavus*, due to the growth of *A. flavus* was decreased when cell free supernatant of *B. subtilis* was supplemented to dried bird chili powder. From the result, the cell free supernatant of *B. subtilis* unable to inhibit the total growth of *A. flavus*, but able to decrease the growth of *A. flavus*. *A. flavus* was contaminated in chili powder sample because chili powder sample maybe un-

thoroughly coated with cell free supernatant of *B. subtilis*. *B. subtilis* was suggested to be a generally recognized as safe and able to be used for food, particularly *B. subtilis* stain BCC 6327 that was isolated from fermented soybean.

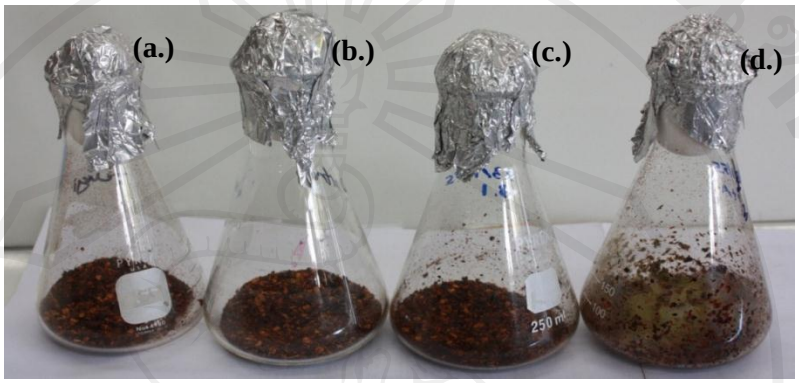

Figure 3.25 Treatment culture on bird chili powder flasks

(a.) Control: dried bird chili powder without *B. subtilis* cell free supernatant and spore suspension of *A. flavus* flask
(b.) Treatment 1 : dried bird chili powder supplemented with *B. subtilis* cell free supernatant flask
(c.) Treatment 2 : dried bird chili powder supplemented with *B. subtilis* cell free supernatant and spore suspension of *A. flavus* flask
(d.) Treatment 3 : dried bird chili powder supplemented with spore suspension of *A. flavus* flask

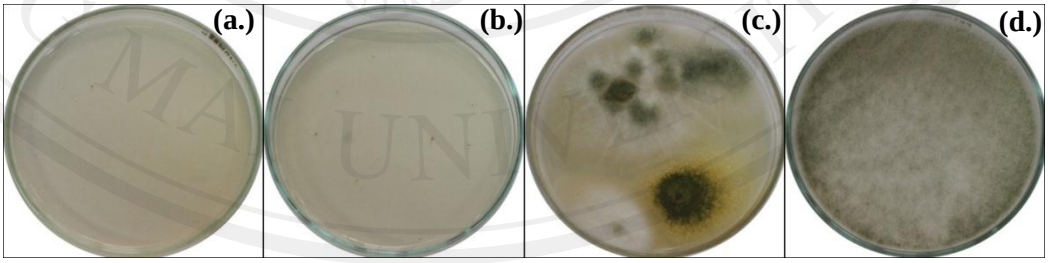

Figure 3.26 Pour plates (10^{-2} dilution) culture on PDA after 7 days of incubation

(a.) Control: Dried bird chili powder without *B. subtilis* cell free supernatant and spore suspension of *A. flavus* pour plate
(b.) Treatment 1: dried bird chili powder with *B. subtilis* cell free supernatant pour plate
(c.) Treatment 2: dried bird chili powder with *B. subtilis* cell free supernatant and spore suspension of *A. flavus* pour plate
(d.) Treatment 3: dried bird chili powder with spore suspension of *A. flavus* pour plate