CHAPTER 1

INTRODUCTION

1.1 Statement and significance of problem

Fungi are ubiquitous plant pathogens and major spoilage agents of foods and feedstuffs. The infection of plants by various fungi results in reducing crop yield and quality, leading to significant economic loss. Moreover, the contamination of grains with fungal poisonous secondary metabolites called mycotoxins, causes acute liver damage, liver cirrhosis, induction of tumors and teratogenic effects because mycotoxins are both acutely and chronically toxic to men and animals (Onilude *et al*., 2005). One family of mycotoxins, the aflatoxins, is a group of structurally related toxic metabolites produced by *Aspergillus flavus* and *Aspergillus parasticus*. Among the major aflatoxins of concern, aflatoxin B_1 (AFB_1) is the most frequently found metabolite in contaminated samples and is classified as a human carcinogen. Aflatoxin B_2 (AFB_2), G_1 (AFG_1), G_2 (AFG_2) are generally not reported in the absence of AFB_1 and are classified as possible carcinogens to humans (Chun *et al*., 2007). The toxins have been reported in many countries, especially in tropical and subtropical regions where conditions of temperature and humidity are favorable for the growth of the molds and the production of the toxins. Unfortunately, aflatoxins are not eliminated completely in food chain. Furthermore the aflatoxins are heat-stable, therefore they are rarely degraded during cooking and processing, making it more difficult to control or eliminate aflatoxins in foods (Kim, 2007).

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Bird chili (*Capsicum frutescens* Linn.) is one of various favorable chilies for Thai people. Dried chili powder is highly contaminated with aflatoxigenic fungi. Because of potential health hazards to humans, regulatory levels have recently been documented. The range of worldwide regulations for AFB_1 was from 0 to 30 µg/kg with a total aflatoxins from 0 to 50 µg/kg (Creppy, 2002 ; FAO, 1997). In the European Union, the total aflatoxins and AFB_1 level in human commodities are regulated with maximum residue level (MRLs) that cannot be greater than 4 and 2 µg/kg, respectively (EEC, 1998). Recently, the Codex Alimentarius Commission, Joint FAO/WHO Food Standard program adopted a limit of 15 µg/kg for total aflatoxins (Codex, 2001). In Thailand, a residue limit of 20 µg/kg total aflatoxins for food stuffs has been established since 1986 (Minister of Health, 1986).

Aflatoxins are resistant to degradation under normal food processing conditions. The degradation methods that will effectively decompose aflatoxins, while retaining the nutritive quality of the treat food are needed. Many microorganisms including bacteria, yeasts, moulds, actinomycetes and algae are able to remove or degrade aflatoxins in foods and feeds (Line and Brackett, 1995). The productive effect of microorganisms against food mutagens such as aflatoxins has been studied by Lillehoj *et al*. (1967) who reported the ability of *Flavobacterium aurantiacum* NRRL B-184 to irreversibly remove AFB_1 from solutions. In addition, Smiley and Draughon (2000) demonstrated that the crude protein extracts from *F. aurantiacum* degraded AFB_1.

Several *Bacillus* species produce various antibiotics, some of which have antifungal activity. These antifungal antibiotics play a major role in biological control

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of plant pathogens and post-harvest spoilage fungi (Munimbazi and Bullerman, 1997). Kimura and Hirano (1988) isolated a *B. subtilis* strain NK-330 that could inhibit the growth and aflatoxin production of *A. flavus*. Moyne *et al*. (2001) reported that bacillomycin D, a lipopeptide from *B. subtilis* AU 195 inhibited the growth of *A. flavus*. Zhang *et al*. (2008) screened bacteria for antagonistic activity against *A. flavus* and identified a *B. subtilis* B-FS06 with high antifungal activity.

The purpose of this study is to isolate the aflatoxigenic fungi from dried bird chili powder and inhibit the growth of such fungi by the cell-free culture filtrate (CCF) of *Bacillus subtilis*. Furthermore, the hydrolytic enzymes secreted as the antifungal compounds will be studied.

1.2 Chili

The chili belongs to the family Solanaceae (also known as the nightshade family) and genus Capsicum (genus of flowering plants). In addition to grown worldwide as a food vegetable and a spice, Capsicum has also found use in medicines. In Thailand pungent chili is an economically important crop grown for local consumption, for domestic and international food industry market (Kraikruan *et al.*, 2008). Chilies are high in vitamin A and C, but low in calories and sodium and contain potassium, magnesium and folic acid. But capsaicinoids (vanillylamides of monocarboxyl acids) which are responsible for the pungency or bite are considered as active compounds in chilies. Capsaicin accounts for about 50% to 70% of the total capsaicinoids which ranges from 600 to 13,000 ppm in the fruits. It gives bite, but has no odor. Other bite-contributing components are: 20 to 25% dihydrocapsaicin, which

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together with capsaicin provides the fiery notes from the mid-palate to the throat; 7% nordihydrocapsaicin, which is fruity and sweet and has the least-burning sensation; and 1% homocapsaicin and 1% homodihydrocapsaicin, which give a numbing and prolonged burn. Bird chili (*C. frutescens* Linn.) is one of two chili types widely used in Thailand, known in Thai as "Prik Khee Nu" which is a small type (tiny cone) and only 0.75 to 1.5 inch in length, but it is very hot (50,000-100,000 scoville units) and commonly found in Cambodia, Laos, Vietnam, Thailand, Malaysia, Indonesia, the Philippines and Singapore. (Wangcharoen and Morasuk, 2009). Bird chili is a short-lived evergreen shrub usually 1 to 1.5 m in height and 1 to 3 cm in basal stem diameter. The shrub is supported by a short to long taproot (depending on soil conditions), many spreading lateral roots, and moderately abundant fibrous roots. The stem and larger branches of mature plants are woody but moderately soft and weak. Bark of stems and older branches is light gray. The form is upright, the abundant branching is often dichotomous, and the branches and twigs are slender. The ovate to ovatelanceolate leaves vary in size. The larger of them are 4 to 12 cm long and 1 to 4.5 cm broad. Greenish-white to yellowish-white flowers with blue, violet, or yellow anthers occur in groups of two or more at the nodes. The berries are red or red-orange at maturity, elongated with a pointed or rounded tip, 1.5 to 3.5 cm long and 0.5 to 1.2 cm thick. The fruits are somewhat dry and contain few to many (depending on fruit size) cream to yellow lenticular seeds about 3 mm in diameter. The fruits, especially the seeds and placenta, have a biting, pungent taste. The species has 2n = 24 chromosomes (Bosland and Votava, 2000).

Red pepper is used and loved worldwide as a condiment, added to food fresh, dried, refined, and ground (for Cayenne pepper and curry), and as the principal or

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incidental ingredient in sauces. The fruits are excellent sources of vitamins A and C. Products that may contain chili peppers include meat dishes, salad dressing, mayonnaise, dairy products, beverages, candies, baked goods, salsa, hot sauces, pharmaceuticals, and cosmetics (Bosland and Votava, 2000). Processing methods include canning, pickling, fermentation, dehydration, and freezing. In Thailand, chili is one of spices strongly important for Thai people's daily life because mostly Thai people like its spiciness and prefer using the chili as an ingredient in their daily cooking and they are popular for Thai people in both forms: fresh and dry. (Ooraikul *et al*., 2011) Both fresh and dried chilies are used as food ingredients or seasonings. The chili products of Thailand including chili sauce, chili paste, ground chili, and chili powder. Dried chili powder is highly contaminated with aflatoxigenic fungi, especially in tropical and subtropical regions where conditions of temperature and humidity are favorable for the growth of the molds and production of the toxin. (Kim, 2007)

Chili powder is the name given to any spice blend that is made mostly from chilies. Some chili powders contain only pure powdered chilies, but more often, they are mixtures of ground chilies and other spices that complement the heat and add depth. The mature chilies having deep red fruits with length about 2 centimeters were harvested. Natural sun drying is one of the most common ways to produce dried chili. The dried chili is produced mostly by spreading on the ground to be dried by sun and wind; this process may result in poor quality products due to contamination (Hirunlabh *et al*., 2004). But more often, the chili was spreaded in a single layer on each rack into a constructed polythene-covered stand, this process neither dries chili on the ground nor directly in the sun. After 4 to 7 days of sun drying, the fruits should

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be shriveled yet not brittle (moisture content of 7.5 to 8 percent) and then dried chili should be stored in a covered basket or a gunny sack and kept in dry place of the ground. (Agribusiness Development Centre, 2001) The chili pepper powder was produced by mill grinding machine and packed into the plastic bag.

Chilies are subject to various pest and disease constraints for optimal production (Paterson, 2007) because (1) there is a lack of cleaning process for freshly harvested chili pods, (2) the use of traditional sun drying in the open air, and (3) dried chilies are stored for a long time with moisture contents of approximately 10-12%, leading to contamination and development of mycotoxins (Duman, 2010).

1.3 Mycotoxins

Mycotoxin is reserved for the toxic chemical products formed by a few fungal species that readily colonize crops in the field or after harvest and thus pose a potential threat to human and animal health through the ingestion of food products prepared from these commodities (Scudamore, 2005). Mycotoxins are those secondary metabolites of fungi that have the capacity to impair food and feed quality and safety. Mycotoxin contamination of foods and feed materials frequently occurs in the field following infection of plants with particular pathogenic fungi or with symbiotic endophytes. Contamination may also occur during processing and storage of harvested products and feed whenever environmental conditions are appropriate for spoilage fungi (Mello, 2000). Mycotoxin can enter the human food chain directly by cereals, nuts, seeds, dried fruit, coffee, cocoa, spices, fruits (particularly apples), dried peas, beans, beverages, other plant materials, indirectly and directly by food products

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obtained from animals given contaminated feeds through residues in milk, meat, eggs and their derivates (Galvano *et al*., 2005; Scudamore, 2005). A list of the principal mycotoxins occurring in feeds and forages is given in **Table 1.1**, which also indicates the fungal species associated with the production of these contaminants. The toxigenic fungi involved in the human and animal food chains belong mainly to five genera: *Aspergillus*, *Fusarium*, *Claviceps*, *Stachybotrys* and *Penicillium* (Anonymous, 2003; Sweeney and Dobson, 1998; Santin, 2005). Environmental conditions are extremely important in pre-harvest mycotoxin contamination of grain and oilseed crops (Anonymous, 2003). Some fungal strains are capable of producing more than one mycotoxin and also a single mycotoxin is produced by more than one fungus. In some cases one species produces multiple mycotoxins (Devegowda and Murty, 2005; Santin, 2005). In stored grain, toxigenic fungal contamination, and mycotoxin production result from a complex interaction between moisture, temperature, substrate aeration, oxygen (O_2) and carbon dioxide (CO_2) concentration, inoculum concentrations, microbial interactions, mechanical damage, fungal abundance and insect infestation (Ominski et al. 1994; Anonymous, 2003; Santin, 2005). Moisture content and ambient temperature are key determinants of fungal colonization and mycotoxin production. It is conventional to subdivide toxigenic fungi into “field” (or plant-pathogenic) and “storage” (saprophytic/spoilage) organisms (Mello, 2000; Santin, 2005). Field fungi are those that invade the seeds while the crop is still in the field and require high moisture conditions (20-21 %). These include species of *Claviceps, Neoitphodium, Fusarium, Alternaria, Cladosporium, Diplodia, Gibberella* and *Helminthosporium.* Storage fungi (also called storage moulds) are those that invade grains or seeds during storage. These need less moisture than field fungi (13-

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18 %) and usually do not present any serious problem before harvest and they were those that could grow at moisture contents in equilibrium with relative humidity of 70 to 90% where no free water was present. Storage fungi include species *Aspergillus* and *Penicillium* (Anonymous, 2003; Mello, 2000; Santin, 2005). Fungi grow at temperatures between 20 °C and 30 °C. It is important to note that if the grain is at high temperature at harvest, it can maintain high temperature for several days or week after harvest unless the storage facility has cooling equipment (Santin, 2005).

Table 1.1 Origin of principal mycotoxins occurring in common feeds and forages (Mello, 2000)

Mycotoxins	**Fungal species**
Aflatoxins	*Aspergillus flavus; A. parasiticus*
Cyclopiazonic acid	*A. flavus*
Ochratoxin A	*A. ochraceus; Penicillium viridicatum; P. cyclopium*
Citrinin	*P. citrinum; P. expansum*
Patulin	*P. expansum*
Citreoviridin	*P. citreo-viride*
Deoxynivalenol	*Fusarium culmorum; F. graminearum*
T-2 toxin	*F. sporotrichioides; F. poae*
Diacetoxyscirpenol	*F. sporotrichioides; F. graminearum; F. poae*

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Mycotoxins	Fungal species
Zearalenone	*F. culmorum; F. graminearum; F. sporotrichioides*
Fumonisins; moniliformin; fusaric acid	*F. moniliforme*
Tenuazonic acid; alternariol; alternariol methyl ether; altenuene	*Alternaria alternata*
Ergopeptine alkaloids	*Neotyphodium coenophialum*
Lolitrem alkaloids	*N. lolii*
Ergot alkaloids	*Claviceps purpurea*
Phomopsins	*Phomopsis leptostromiformis*
Sporidesmin A	*Pithomyces chartarum*

1.3.1 Aflatoxins

This group includes aflatoxin B_1, B_2, G_1 and G_2 (AFB_1, AFB_2, AFG_1 and AFG_2, respectively). In addition, aflatoxin M_1 (AFM_1) has been identified in the milk of dairy cows consuming AFB_1-contaminated feeds. The aflatoxigenic *Aspergilli* are generally regarded as storage fungi, proliferating under conditions of relatively high moisture/humidity and temperature. Aflatoxin contamination is, therefore, almost exclusively confined to tropical feeds such as oilseed by-products derived from groundnuts, cotton seed and palm kernel. Aflatoxin contamination of maize is also an important problem in warm humid regions where *A. flavus* may infect the crop prior to harvest and remain viable during storage.

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1.3.2 Ochratoxins

The *Aspergillus* genus includes a species (*A. ochraceus*) that produces ochratoxins, a property it shares with at least two *Penicillium* species. Ochratoxin A (OA) and ochratoxin B are two forms that occur naturally as contaminants, with OA being more ubiquitous, occurring predominantly in cereal grains and in the tissues of animals reared on contaminated feed.

1.3.3 *Fusarium* mycotoxins

Extensive data now exist to indicate the global scale of contamination of cereal grains and animal feed with *Fusarium* mycotoxins. Of particular importance are the trichothecenes, zearalenone (ZEN) and the fumonisins. The trichothecenes are subdivided into four basic groups, with types A and B being the most important. Type A trichothecenes include T-2 toxin, HT-2 toxin, neosolaniol and diacetoxyscirpenol (DAS). Type B trichothecenes include deoxynivalenol (DON, also known as vomitoxin), nivalenol and fusarenon-X. The production of the two types of trichothecenes is characteristic for a particular *Fusarium* species. However, a common feature of the secondary metabolism of these fungi is their ability to synthesize ZEN which, consequently, occurs as a co-contaminant with certain trichothecenes. The fumonisins are synthesized by another distinct group of *Fusarium* species. Three members of this group (fumonisins B1, B2 and B3) often occur together in maize.

1.3.4 Endophyte alkaloids

The endophytic fungus *Neotyphodium coenophialum* occurs in close association with perennial tall fescue, while another related fungus, *N. lolii,* may be

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present in perennial ryegrass. Ergopeptine alkaloids, mainly ergovaline, occur in *N. coenophialum*-infected tall fescue, while the indole isoprenoid lolitrem alkaloids, particularly lolitrem B, are found in *N. lolii*-infected perennial ryegrass. The ergopeptine alkaloids reduce growth, reproductive performance and milk production in cattle, while the lolitrem compounds induce neurological effects in ruminants.

1.3.5 Phomopsins

In Australia, lupin stubble is valued as fodder for sheep, but infection with the fungus *Phomopsis leptostromiformis* is a major limiting factor because of toxicity arising from the production of phomopsins by the fungus. Mature or senescing parts of the plant, including stems, pods and seeds, are particularly prone to infection. Phomopsin A is considered to be the primary toxin, causing effects such as ill-thrift, liver damage, photosensitization and reduced reproductive performance in sheep.

1.3.6 Sporidesmin

Pithomyces chartarum is a ubiquitous saprophyte of pastures and has the capacity to synthesize sporidesmin A, a compound causing facial eczema and liver damage in sheep.

1.4 Aflatoxins

Aflatoxins, a group of mycotoxins, are secondary metabolites of various *Aspergillus* spp., commonly contaminated a wide variety of tropical and subtropical food/feed stuffs. These mycotoxins are known to have strong hepatotoxic and carcinogenic effects and are regulated by feed/food law in at least 100 countries

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(Egmond and Jonker, 2004). Chemically, aflatoxins are difuranocoumarin compounds and include B_1, B_2, G_1, G_2, M_1, and M_2 (Bilgrami and Choudhary, 1998) (**Figure 1.1**). These mycotoxins contaminate a wide variety of agricultural commodities including oilseed meals, dried fruits, spices, and cereals. Aflatoxins M_1 and M_2 however, mainly occur in milk (AFM_1 in small quantities also reported in eggs) as metabolites of the B_1 and B_2. Among the various types of aflatoxins, aflatoxin B_1 (AFB_1) is most commonly encountered and it is also considered to have higher toxicity than other aflatoxins (Galvano *et al*., 2005).

Aflatoxins are a group of approximately 20 related fungal metabolites produced primarily by the fungi *Aspergillus flavus* and *A. parasiticus*. The four major naturally produced aflatoxins are known as B_1, B_2, G_1, and G_2. “B” and “G” refer to the blue and green fluorescent colors produced under UV light on thin layer chromatography plates, while the subscript numbers 1 and 2 indicate major and minor compounds, respectively. Aflatoxin B_1, the most toxic of the aflatoxins, is the most potent naturally occurring chemical liver carcinogen known. Specific P450 enzymes in the liver metabolize aflatoxins into a reactive oxygen species (aflatoxin-8, 9-epoxide), which may then bind to proteins and cause acute toxicity (aflatoxicosis) or to DNA and induce liver cancer (Wild and Gong, 2010; Wu and Khlangwiset , 2010)

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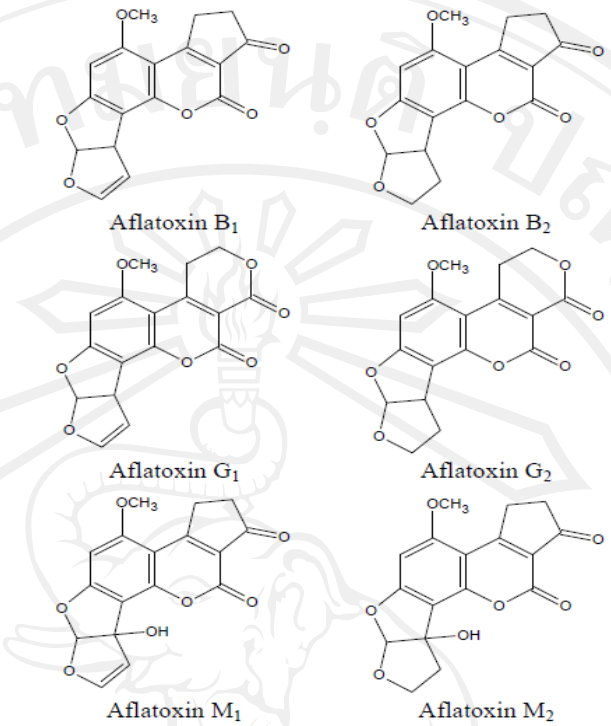

Figure 1.1 Structure of aflatoxins

Aflatoxins are a group of toxins having similar molecular structures. The toxin was first discovered in 1960 when there were mass deaths from liver disease of turkeys in England followed by deaths of other farm animals. More than 100,000 turkeys died within a few months. Scientists first called the new disease "Turkey X Disease" because they did not know its cause. It was finally established that all bird affected had been fed with feed prepared with contaminated groundnut meal. Examination of the incriminated groundnut meal revealed the presence of mould. The main microbial contaminant of the groundnut meal was identified to be *Aspergillus flavus* and the toxin was named aflatoxin. Since then, aflatoxin is considered as one of the most harmful mycotoxins in the world. (Liu *et al*., 2012)

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1.4.1 Aflatoxins and their health consequences

Aspergillus flavus and *A. parasiticus* colonize a wide variety of food commodities including maize, oilseeds, spices, groundnuts, tree nuts, milk, and dried fruit (Strosnider et al. 2006). Whether these fungi produce aflatoxin depends on drought stress and rainfall, suitability of crop genotype for its climate, insect damage, and agricultural practices (Wu and Khlangwiset 2010). These fungi can also produce aflatoxin in "postharvest" conditions: storage, transportation, and food processing. Aflatoxin contamination is a particular problem in maize, oilseeds, spices, peanuts, tree nuts (almonds, pistachios, hazelnuts, pecans, Brazil nuts, and walnuts), milk (in the form of aflatoxin B_1's metabolite aflatoxin M_1), and dried fruit (Shephard, 2008). Maize and peanuts are the main sources of human exposure to aflatoxin because they are so highly consumed worldwide and unfortunately are also the most susceptible crops to aflatoxin contamination (Wu and Khlangwiset 2010). **Figure 1.2** (Wu 2010) depicts the pathway by which aflatoxin accumulates in food crops and contributes to various adverse human health effects.

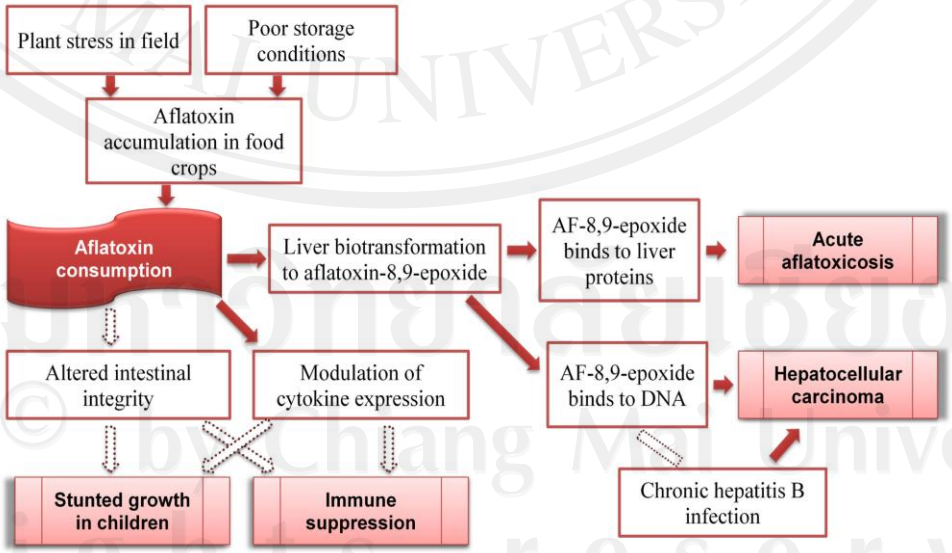

Figure 1.2 Aflatoxin and disease pathways in humans. Source: Wu (2010).

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The darker arrows in **Figure 1.2** denote linkages that have been well-established in agricultural and toxicological research, while the white arrows denote linkages that have been relatively less well-established (Wu 2010).

As the top portion of **Figure 1.2** shows, the main predisposing factor in pre-harvest aflatoxin contamination is stress of the host plant (such as maize or peanuts). Stress can be caused by multiple factors, including use of a hybrid type that is unsuitable for the local geography, drought stress, high temperatures, and/or insect damage. All these factors increase the risk of the crop plant being infected by *A. flavus* or *A. parasiticus*. The main predisposing factor in postharvest aflatoxin accumulation in food is poor storage conditions; namely, excessive heat and moisture, pest-related crop damage, and extensive periods of time spent in storage (exceeding several months).

When aflatoxin is consumed, it can exert toxicity in several ways. It may alter intestinal integrity (Gong *et al*., 2008) or modulate the expression of cytokines, proteins that "signal" to each other and to immune system components. Both of these effects may result in stunted growth in children and/or immune suppression (Wu 2010).

In the liver, aflatoxin may be transformed by certain P450 enzymes (CYP1A2, 3A4, 3A5, 3A7) to its DNA-reactive form aflatoxin-8,9-epoxide. This molecule may bind to liver proteins and lead to their failure, potentially resulting in acute aflatoxicosis. Alternatively, it may bind to DNA, a step that is a precursor for aflatoxin-induced hepatocellular carcinoma (liver cancer). As mentioned earlier, there may be a synergistic effect between aflatoxin and chronic infection with hepatitis B virus (HBV) that results in significantly higher liver cancer risk (Wu 2010).

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1.4.2 Acute exposure to aflatoxins

Acute aflatoxicosis, associated with extremely high doses of aflatoxin, is characterized by hemorrhage, acute liver damage, edema, and death in humans. Conditions increasing the likelihood of acute aflatoxicosis in humans include limited availability of food, environmental conditions that favor fungal development in crops and commodities, and lack of regulatory systems for aflatoxin monitoring and control. There have been several reported cases of acute aflatoxicosis in Africa associated with consumption of contaminated home-grown maize, including the outbreaks in Kenya in 1982, in which 12 people died, and in 2004, in which 317 people became ill and 125 people died in the central provinces (Azziz-Baumgartner et al., 2005; Probst *et al*., 2007; Lewis *et al*., 2005; Stosnider *et al*., 2006).

Acute aflatoxicosis can also occur in animals. In 1960, more than 100,000 turkeys died on in the United Kingdom over the course of a few months, prompting the name "Turkey X disease" (Asao, 1963). Later investigation revealed that the source of the disease was toxic peanut meal. In 1981, several hundred calves that had been fed on peanut hay died in Australia (McKenize *et al*., 1981), and in 2007, several hundred animal deaths occurred on a chinchilla farm in Argentina; both these occurrences were linked to aflatoxin (González Pereyra *et al*., 2008).

1.4.3 Chronic exposure to aflatoxins

Hepatocellular carcinoma (HCC) as a result of chronic aflatoxin exposure has been well documented, presenting most often in persons with chronic hepatitis B virus (HBV) infection (Qian *et al*., 1994, Groopman *et al*., 2008). For individuals chronically infected with HBV, aflatoxin consumption raises the risk of liver cancer

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up to thirty-fold, compared with either exposure alone (Groopman *et al*., 2008). Unfortunately, these two risk factors aflatoxin and HBV are especially prevalent in poor nations worldwide (Liu and Wu 2010).

In developing countries, many people subsist largely on cereal diets. Nutritional deficiencies are very prevalent in populations consuming high levels of cereals (Bankole and Adebanjp, 2003), particularly children. Additionally, many children in the developing world are also exposed to high levels of mycotoxins in their diets.

Aflatoxin and immunosuppression in humans has been relatively less well-characterized, but could in fact have enormous significance from a global health perspective. Several recent human studies have shown evidence of immunomodulation (Turner *et al*., 2003, Jiang *et al*., 2005), though the actual outcomes of such immunomodulation have yet to be characterized in humans. Indeed, aflatoxin's immunotoxicity may be one explanation for the stunted growth in children that appears to follow a dose-response relationship with aflatoxin exposure (Turner *et al*., 2003). The mechanism by which aflatoxin may result in growth impairment is not yet known; however, one possible explanation may be altered intestinal integrity through cell toxicity or immunomodulation (Gong *et al*., 2008).

Similarly, decades of animal studies have demonstrated that chronic exposure to aflatoxins in animals can also cause growth inhibition and immune suppression. Nursing animals may be affected, and aflatoxin M_1 may be excreted in the milk of dairy cattle and other dairy animals. This in turn poses potential health risks to both animals and humans that consume that milk. Chronic aflatoxin exposure in animals can result in impaired reproductive efficiency, reduced feed conversion efficiency,

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increased mortality rates, reduced weight gain, anemia, and jaundice. In the case of laying hens, aflatoxicosis causes an enlarged fatty liver and lowered egg production (Khlangwiset *et al*., 2011).

1.4.4 Aflatoxins screening test

Two types of screening tests are often used: blacklight tests and commercial test kits.

(i) The blacklight (also called ultraviolet light) test is a quick preliminary test that is a visual inspection for the presence of a greenish gold fluorescence under light at a wavelength of 365 nm. The greenish gold fluorescence looks like a firefly glow, and indicates the presence of an acid that is produced by actively growing *A. flavus* in the kernel. However, this test is an initial screening for the presence of the fungus, not the toxin, and the results must be verified by laboratory analysis. If there are less than eight glowing particles per 5 lb sample, this does not guarantee that the sample is free of aflatoxins, nor is it certain that the same count will work in future outbreaks. Furthermore, kernels with glowing particles are not necessarily the ones contaminated with aflatoxin. Screening indicates which samples could need further testing, and is potentially an initial rapid sorting for high throughput grain handling.

(ii) Commercial test kits with immunoassay or ELISA techniques are available for on-site tests for aflatoxin. Immunoassay analysis is based on the detection of specific proteins found in aflatoxins using antibodies to identify these proteins. The tests are very specific for aflatoxin, but they require operator training and practice to be accurate. Some tests determine only the presence or absence of aflatoxin; others can quantify, within a range, the amount of aflatoxin present. If a lot of corn is

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rejected based on the results of an immunoassay test kit, the results also should be confirmed by laboratory analysis. It is very important that the entire 5- to 10-lb sample is ground before removing the small subsample for the test kit. This reduces sampling error, although one can assume a 25 to 40 percent overall error on any test result for aflatoxin. Subdivision of 5- to 10-lb samples prior to grinding is a major source of error. Detected levels of aflatoxin in subsamples taken prior to grinding the 5- to 10-lb sample results most often in low levels of aflatoxin being detected with occasional very high levels.

Analytical laboratories use one of several procedures such as thin-layer chromatography, mini columns, gas chromatography, or mass spectroscopy to determine aflatoxin levels. These procedures are highly accurate and quantitative. The laboratory should grind the entire 5- to 10-lb sample of corn together before taking subsamples for analysis (Kress, 2012).

1.5 Aflatoxigenics fungi

Aflatoxins (AFs) are derived secondary metabolites of a polyketide family produced by several species of the *Aspergillus* spp. They are considered as potent hepatotoxins and carcinogens causing mortality and/or reducing the productivity of farm animals. Contaminated foodstuffs by these mycotoxins have also been associated with a high incidence of liver cancer in human (Stark 1980; Berry 1988; Ventura *et al*., 2004). The principal filamentous fungi involved in the AF production are *A. flavus* and *A. parasiticus.* Taxonomically, these 2 species belong to the section *flavi* of the *Aspergillus* genus that are phylogenetically related. However, other

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species belonging to the section *flavi* have also been reported as AF producers namely *A. nominus*, *A. tamarii* and *A. pseudotamarii* (Ito *et al*., 2001).

1.5.1 Morphological Studies of *A. flavus*, *A. parasiticus* and *A. nomius*

An important group of foodborne fungi are the aflatoxin producers: *A. flavus*, *A. parasiticus* and more recently *A. nomius*. A detailed morphological study of ex-type and other isolates of *A. flavus* and *A. parasiticus* was undertaken (Kozakiewicz, 1982). *Aspergillus parasiticus* Speare originally isolated from material on sugar cane in Hawaii (Speare, 1912) was subsequently subcultured around the world. Light and scanning electron microscopy studies of all extant isolates disclosed conidia of two distinct ornamentations or morphs, but never mixtures of both. When these morphs were assigned to their respective cultures a sharp dichotomy was revealed. One form occurs in the isolate derived from the type of *A. parasiticus* (**Figure 1.3a**) and the other has been established as that of *A. flavus* (**Figure 1.3b**). Consequently cultures for the type of *A. parasiticus* held in three major world collections are in fact *A. flavus*. More worryingly, since the publication of Kozakiewicz (1982) no other than the collection at CABI, Egham, UK (formerly IMI) which has re-disbursed the particular isolates, has confirmed that these mistakes have been rectified; implying that wrongly named material continues to be sold and distributed. Furthermore, in routine examinations of the IMI collection, ten additional isolates have been re-identified to date (Kozakiewicz, 1994).

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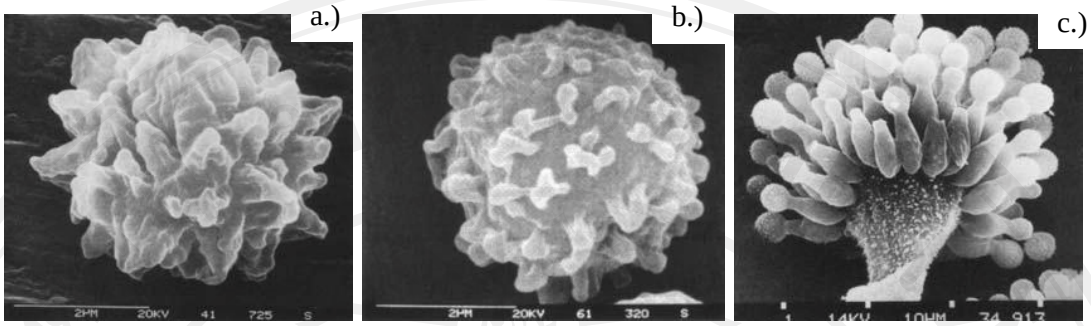

Figure. 1.3 Scanning Electron Micoroscopy pictures of (a) *A. parasiticus* and (b) *A. flavus* spores, where spore ornamentation differences are clearly seen; and of (c) *A. parasiticus* conidial head.

The situation is further complicated by the species *Aspergillus nomius.* Morphologically, it resembles *A. flavus* but differs by the production of small bullet-shaped sclerotia; those in *A. flavus* being more globose. However, it is unclear whether fresh isolates of *A. nomius* always produce these distinctive sclerotia. In their absence only isoenzyme patterns and mycotoxin production provide reliable identification techniques. That is, for *A. nomius* the detection of aflatoxins B_1, B_2, G_1, G_2 (as does *A. parasiticus*), but without the detection of the secondary metabolite cyclopiazonic acid. *A. flavus* produces detectable aflatoxin B_1 and B_2 and cyclopiazonic acid only (Rodrigues *et al*., 2007).

1.5.2 Morphological separation of *A. flavus* and *A. parasiticus*

The primary separation is the presence of metulae and phialides (biseriate conidial head) for *A. flavus* and phialides only (uniseriate conidial head) for *A. parasiticus* (**Figure 1.3**c). Examination of a large number of *A. parasiticus* isolates (Kozakiewicz, 1995) has shown that up to 10% of conidial heads in an *A. parasiticus* colony can have metulae and phialides (biseriate). Furthermore, not all *A. flavus*

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isolates consistently produce metulae. Conidial wall ornamentation is now regarded as the primary diagnostic character for separation of these two species. Conidia of *A. flavus* have relatively thin walls which are finely to moderately roughened. Their shape can vary from spherical to elliptical. Conidia of *A. parasiticus* are more spherical and noticeably echinulate or spinulose. Scanning Electron Micoroscopy (SEM) micrographs clearly show these ornamentation differences (**Figure 1.3a and 1.3b**). Furthermore, once SEM micrographs have been studied and compared, then with practice these differences become apparent using light microscopy (Rodrigues *et al*., 2007).

Additionally, there are a few selected media, which may be employed to help less trained mycologist: (i) *Aspergillus* differentiation agar (AFPA); (ii) coconut cream agar (CCA) and (iii) Czapek Dox agar (CZ). AFPA is a selective identification medium for the detection of *A. flavus* group strains (Pitt et al., 1983). With this method it is possible to distinguish these species from other *Aspergillus* based on the development of orange colour on the reverse of the plates (**Figure 1.4a**). The CCA is used to detect aflatoxin producer strains (**Figure 1.4b**). The production of aflatoxin is detected by a blue fluorescence when exposed to a UV-light (Davis *et al*., 1987).

When grown on CZ, colonies taxonomically between the two species can also be separated. Those of *A. flavus* being yellow-green and those of *A. parasiticus* a distinctly darker green, referred to as near Ivy green (**Figure 1.5**). **Table 1.2** summarises the morphological differences between the two species.

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Table 1.2 Morphological separation of *A. flavus* and *A. parasiticus*

	Colony color	**Seriation**[1]	**Conidia**	
			shape[2]	**texture**[3]
A. flavus	Yellow/green	b/u	gl/el	sm/fr
A. parasiticus	Ivy green	u/b	Gl	r

1- u = uniseriate; b = biseriate

2- gl = globose; el = elliptical

3- sm = smooth; fr = finely roughened; r = rough

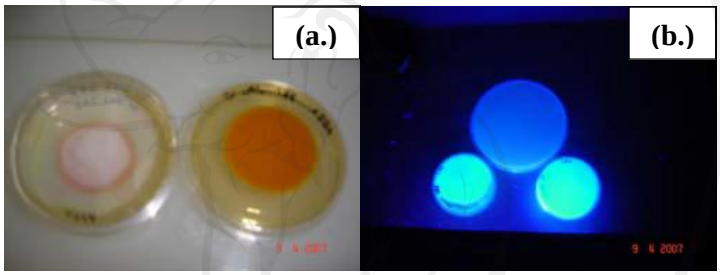

Figure. 1.4 **(a.)** *A. flavus* in AFPA, after 7 days incubation at 25ºC, with the characteristic orange colour on the reverse side of the plate; **(b.)** aflatoxigenic *A. flavus* grown on small plates of CCA under long-wave UV light, after 7 days incubation (large plate = uninoculated CCA plate).

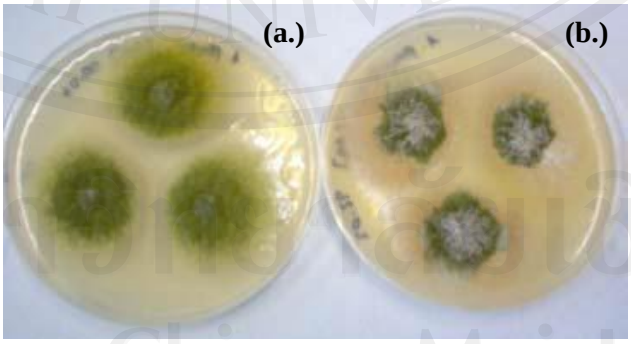

Figure 1.5 *A. flavus* **(a.)** and *A. parasiticus* **(b.)** strains growing on CZ.

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1.5.3 Molecular methods for *Aspergillus* Section *Flavi* species differentiation

The most widely used DNA target regions for discriminating *Aspergillus* species are the ones in the rDNA complex, mainly the internal transcribed spacer regions 1 and 2 (ITS1 and ITS2) and the variable regions at the 5' end of the 28S rRNA gene (D1-D2 region).

Single-copy conserved genes can also be used as targets for taxonomic studies within the *A. flavus* group, when multi-copy segments from the rDNA complex lack variability. Universal β-tubulin, calmodulin and topoisomerase II genes have been used in fungal species identification but only within distantly related species, since variability is generally low (Kanbe *et al*., 2002). Genes involved in secondary metabolism are considered to be more variable within closely related species. Several genes involved in aflatoxin biosynthesis have been identified, cloned and studied. They include a regulatory gene locus *aflR* from *A. flavus* and *A. parasiticus*, and several structural genes, *e.g. pks*A, *nor*-1, *ver*-1, *uvm*8 and *omt*A (Yu *et al*., 1995).

However, *A. flavus* group species are difficult to differentiate even genetically. *A. flavus*, *A. parasiticus*, *A. oryzae* and *A. sojae* have shown to possess high degrees of DNA relatedness and similar genome size. Furthermore, (a) *A. flavus* and *A. oryzae*, and (b) *A. parasiticus* and *A. sojae*, were considered virtually impossible to discriminate, since their DNA relatedness was found to be of 100% and 91%, respectively (Kutzman *et al*., 1986). But, a Random Amplified Polymorphic DNA (RAPD) analysis was able to distinguish *A. parasiticus* and *A. sojae* (Yuan *et al*., 1995)

Also mitochondrial DNA showed contrasting levels of variability. Moody and Tyler (1990) could not discriminate between similar species based on mtDNA studies,

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although Wang *et al*. (2001) reported a reliable tool for discriminating species based on cytochrome b sequence variability.

Genetic relatedness between *A. flavus* and *A. nomius* has also been studied, and showed contrasting results (Moody and Tyler, 1990), which means that larger numbers of *A. nomius* isolates have to be examined in order to determine relatedness with other *Aspergillus* species from the *A. flavus* group.

For studies within *A. flavus*, or for comparing *A. flavus* with other *Aspergillus* species, and even for differentiating aflatoxin producers from non-producers, several rDNA complex regions and structural aflatoxin genes have been tested for use as molecular markers, with different levels of success. Some of these studies are based on Polymerase Chain Reaction (PCR) amplification followed by sequencing for variability analysis (Shapira *et al*., 1996). But PCR amplification of known DNA target regions or genes followed by Restriction Fragment Length Polymorphisms (RFLP) (Kumeda and Asao, 1996; Somachekar *et al*., 2004), Single-Strand Conformation Polymorphisms (SSCP) (Kumeda and Asao, 1996) or Heteroduplex Mobility Assay (HMA) (Kumeda and Asao, 2001) are easier to apply in most laboratories for the study of numerous test samples, and NCBI information can be used for generating primers and DNA probes. Kumeda and Asao (1996) successfully applied PCR-SSCP and PCR-RFLP to differentiate *A. flavus* from other species (including *A. parasiticus*), based on a 600 bp fragment corresponding to the amplification of the ITS1/5.8S/ITS2 region with the primer pair ITS1-ITS4. These authors considered that, by using a fragment as big as 600 bp, they could eliminate the major problem associated to PCR-SSCP analysis, which is intraspecific variability. However, these analyses failed on differentiating between those strains where

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aflatoxin was detected or not detected. Chang *et al*. (1995) found the *afl*R gene to be virtually identical in *A. flavus* and *A. parasiticus*, but Somashekar et al (2004) , using a limited number of strains, were able to differentiate *A. parasiticus* from *A. flavus* based on the RFLP resulting from digesting an *afl*R gene fragment with the restriction enzyme *Pvu*II. Multiplex PCR, using several primer pairs for different target regions, is an alternative approach for species differentiation, and has been successfuly applied using *aflR*, *ver-1*, *omt-1* and *nor-1* genes (Shapira *et al*., 1996; Criseo *et al*., 2001).

1.6 *Aspergillus flavus*

Aspergillus flavus have a world-wide distribution and normally occur as saprophytes in soil and many kind of decaying organic matter (Dvorockova, 1990). These fungi readily colonize several important crops such as corn, cottonseed, peanuts, and tree nuts. Most *A. flavus* and *A. parasiticus* can produce polypeptide-derived secondary metabolites called aflatoxins, which are highly toxic, mutagenic and carcinogenic to animals (Hesseltine, 1965). They may also involve to some degree in primary liver cancer in humans (Van Rensburg et al, 1985). Possible cases of aflatoxicoses in human have been reported in many countries in Southeast Asia and Africa (Rodricks and Roberts, 1977). Aflatoxins have been implicated in hepatocellular carcinoma, acute hepatitis, Reye's syndrome, cirrhosis in malnourished children, and kwashiorkor (Gourama and Bullerman, 1995; Jelinek et al, 1989). *A. flavus* belongs to the genus *Aspergillus*. The genus *Aspergillus* belongs to a filamentous fungal group with a wide dispersion in the environment and consists of approximately 200 species. *A. flavus* is second most common species after *A. fumigatus.* As with fungi in general, *Aspergillus* taxonomy is complex and ever

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evolving. The genus is easily identified by its characteristic conidiophore, but species identification and differentiation is complex, for it is traditionally based on a range of morphological features. Macromorphological features which are considered include conidial and mycelial colour, colony diameter, colony reverse colour, production of exudates and soluble pigments, presence of sclerotia and cleistothecia. Micromorphology characterization is mainly dependent on seriation, shape and size of vesicle, conidia and stipe morphology, presence of Hülle cells, and morphology of cleistothecia and ascospores. Furthermore, all these morphological features have to be determined under standardized laboratory conditions (Okuda *et al*., 2000) by trained mycologists, in order to obtain an accurate identification. Several *Aspergillus* taxonomic keys and guides are available (Raper and Fennell, 1965).

Aspergillus Subgenus *Circumdati* Section *Flavi*, also refered to as the *Aspergillus flavus* group, has attracted worldwide attention for its industrial use and toxigenic potential. Section *Flavi* is divided into two groups of species. One includes the aflatoxigenic species *A. flavus*, *A. parasiticus* and *A. nomius*, which cause serious problems worldwide in agricultural commodities, and the other includes the nonaflatoxigenic species *A. oryzae, A. sojae* and *A. tamarii*, traditionally used for production of fermented foods in Asia (Kumeda and Asao, 2001).

1.6.1 Classification and characterization of *Aspergillus* section *Flavi*

A. flavus, a member of the subgenera, section *Flavi*, is the most common species implicated in preharvest contamination of crops with AF (Cotty *et al*., 1994). *A. flavus* populations are genetically and phenotypically diverse (Geiser *et al*., 2000) with some isolates producing abundant conidiospores, large (L) sclerotia, and variable

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amounts of AF, while another type produces abundant, small (S) sclerotia, fewer conidiospores and high levels of AF (Cotty, 1989; Cotty *et al*., 1994). *Aspergillus* section *Flavi* historically includes species with conidial heads in shades from yellow-green to brown, and dark sclerotia. Several species of section *Flavi* produce aflatoxins; *A. flavus* and *A. parasiticus* are pathogenic on peanuts, corn and cotton (Smith and Moss, 1985), while isolates of other so-called domesticated species, such as *A. oryzae* and *A. sojae* as well as *A. tamarii*, are used in oriental food fermentation processes, and as hosts for heterologous gene expression (Campbell-Platt and Cook, 1989). ITS sequences of type strains or representative isolates of the species and subspecies currently assigned to this section were analysed. Phylogenetic analysis of sequence data indicated that species of *Aspergillus* section *Flavi* form distinct clades (**Figure 1.6**) (Rigó *et al*., 2002). The three main clades identified based on sequence data could also be distinguished based on colony colour and their ubiquinone systems. The *A. flavus* clade includes species characterised with Q-10(H2) as their main ubiquinone, conidial colours in shades of green and dark sclerotia. Studies on the genetic variability of *A. flavus* indicated that the name is currently applied to a paraphyletic group of isolates that may produce aflatoxins B or G, and have large or small sclerotia. It was suggested that isolates with small sclerotia, able to produce both aflatoxins B and G (group II), deserve recognition as a new species. The other group (group I) includes isolates producing only aflatoxin B and having large or small sclerotia (**Table 1.3**). This group also includes isolates of *A. oryzae*, which has previously been described as having a recombining population structure (Geiser *et al*., 2000). Although several lines of evidence suggest that *A. oryzae* and *A. sojae* are morphological variants of *A. flavus* and *A. parasiticus*, respectively, it was suggested

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that these taxa should be retained as separate species due to the regulatory confusion that conspecificity might generate in the food industry. The *A. tamari* clade involves species with ubiquinone system Q-10 (H2), and conidia in shades from olive to brown, while the *A. alliaceus* clade consists of species with Q-10 ubiquinone system, and conidia in shades of ochre. Two species of this clade, *P. alliaceus* and *P. albertensis*, produce high amounts of OA (50–300 mg/mL), and are considered to be responsible for OA contamination of figs (Bayman *et al*., 2002; Varga *et al*., 1996). The synnematous species *A. coremiiformis* was closely related to species in the *A. tamarii* clade. The recently described aflatoxin producing species *A. pseudotamarii* and *A. bombycis* are closely related to *A. caelatus* and *A. nomius*, respectively. Physiological properties and mycotoxin producing abilities of these taxa justify their treatment as separate species (**Table 1.3**) (Peterson *et al*., 2002; Ito *et al*., 2001). While no evidence of genetic recombination was found in *A. bombycis*, cryptic genetic recombination was observed in an *A. nomius* population (Peterson *et al*., 2002). Two other species, *A. avenaceus* and *A. leporis*, were found to form separate lineages not closely related to any of the main clades identified. It is suggested that *A. clavatoflavus* and *A. zonatus* be excluded from *Aspergillus* section *Flavi*, a suggestion previously made by Kozakiewicz, based on her scanning electron microscopic studies of the group (Kozakiewicz, 1989).

Aflatoxin producing species (*A. flavus*, *A. parasiticus*, *A. nomius*, *A. bombycis* and *A. pseudotamarii*) were scattered throughout the dendrogram indicating that aflatoxin producing ability was lost (or gained) several times during evolution.

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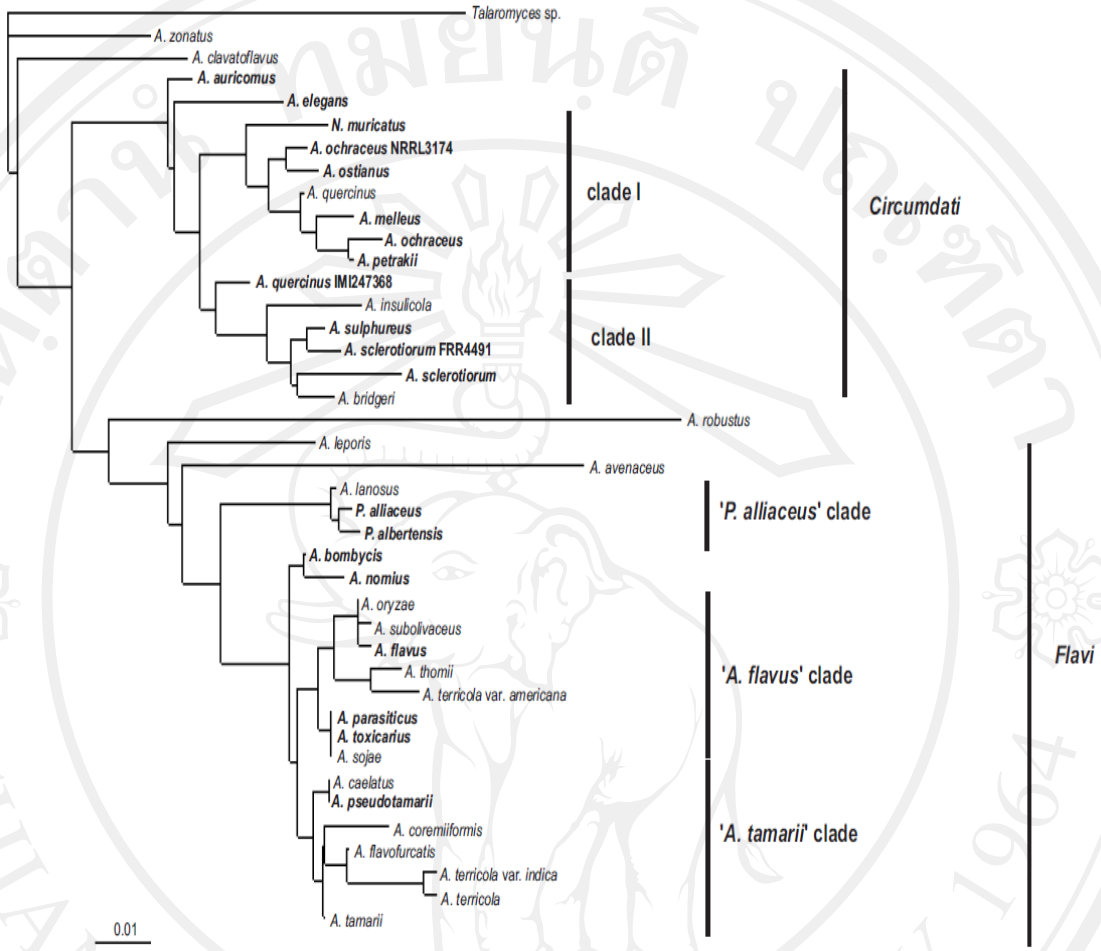

Figure 1.6 Neighbour-joining tree of ITS sequences of species assigned to *Aspergillus* sections *Circumdati* and *Flavi*. Ochratoxin producing isolates are set in bold type, while aflatoxin producing isolates are in bold small capitals. Species names alone indicate the positions of type strains.

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Table 1.3 Mycotoxin producing abilities of the economically important species of *Aspergillus* section *Flavi* (compiled from refs. Geiser *et al*., 2000; Peterson *et al*., 2002; Ito *et al*., 2001)

Species	Aflatoxins		CPA*	Kojic acid	Nominine
	B	G			
A. flavus group I	+	-	±	+	-
A. flavus group II	+	±	+	+	-
A. oryzae	-	-	±	+	-
A. parasiticus	+	+	-	+	-
A. sojae	-	-	-	+	-
A. nomius	+	+	-	+	+
A. bombycis	+	+	-	+	?
A. tamarii	-	-	+	+	-
A. caelatus	-	-	-	+	-
A. pseudotamarii	+	-	+	+	-

*CPA, cyclopiazonic acid

1.6.2 Morphological and biological characters of *A. flavus*

Colonies of *A. flavus* grow rapidly and the diameter will reach 6-7 cm in 10-14 days. The color of colony is initially yellow, and turns into yellow-green or olive green. The old colony appears dark green. The shape is smooth and some have radial wrinkles. The reverse is colorless or sandy beige. More details can be observed under scanning-electron microscope : Conidiospores are long (400-800 μ) and are often

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rough just beneath the globes vesicles (25-45 µ); phialides arise circumferentially and are biseriate (two layered) or sometimes uniseriate (single layered); the shape of conidial heads vary from columnar to radiate and globose; the arrangement of phialides on the vesicle dictates the shape of conidial head. Vesicles are elongated in shape and varies with the composition of the substrate. The diameter varies from 10 to 65 µm (Raper and Fennell, 1965; Pitt and Hocking, 1985). Conidia from *A. flavus* isolates are smooth to slightly roughen.

Structures of the conidiophores (spore forming structures) are very important for identification of *Aspergillus* spp as shown in **Figure 1.7**. The conidiophores originate from a basal cell located on the supporting hyphae and terminate in a vesicle. The morphology, colour and roughness of the conidiophores vary from species to species. Additionally, the position of the flask-shaped phialides (spore producing cells) on the vesicle is an important character. The phialides can cover the vesicle surface entirely ("radiate" head) or partially ("columnar" head) and the phialids can be attached to the vesicle directly (uniseriate) or attached via a supporting cell, called metula (biseriate). The phialides produce round conidia (2-5 µm in diameter) that form radial chains (**Figure 1.7**). The conidia of the different species can have different colours (**Table 1. 4**) (Jensen, 2013).

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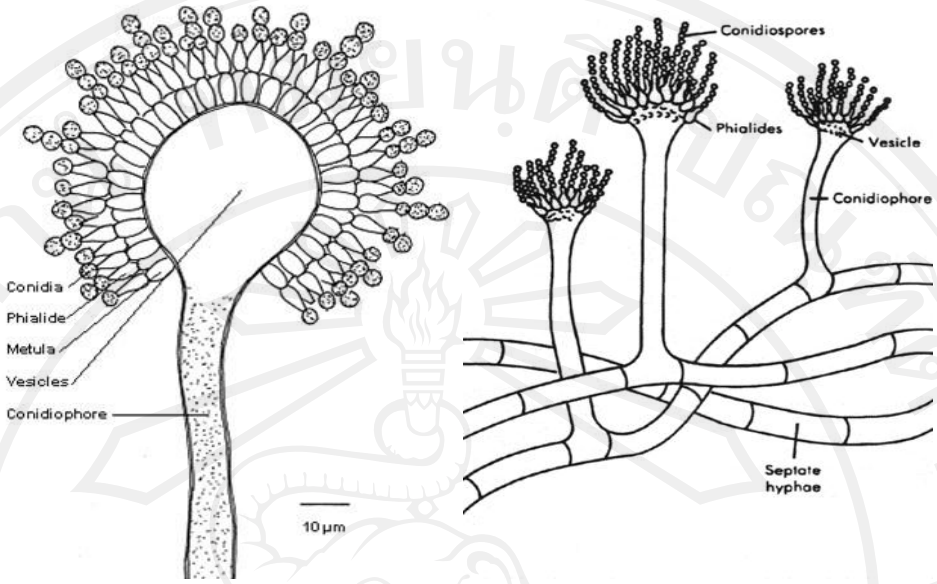

Figure 1.7 Structures of importance for identification of *Aspergillus* species (Jensen *et al*., 2013).

Table 1.4 Microscopic characters of three *Aspergillus* species (Jensen *et al*., 2013).

Species	Conidia colour	Conidiophores	Phialides	Vesicle
A. flavus	Yellow-green	Colourless. Rough	Uni/biseriate	Round, radiate head
A. fumigatus	Blue-green to grey	Short (<300µm), smooth, colourless-green	Uniseriate	Round, columnar head
A. niger	Black	Long, smooth, colourless or brown	Biseriate	Round, radiate head

1.6.3 Production of aflatoxin

The aflatoxin molecule contains a coumarin nucleus linked to a bifuran and either a pentanone, as in AFB_1 and the dihydro derivative AFB_2, or a six member lactone, as in AFG_1 and its corresponding derivative AFG_2 (Sanz *et al*., 1989). These four compounds are separated by the color of their fluorescence under long-wave

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ultraviolet illumination (B=blue; G= green) (Dutton and Heathcote, 1966). The subscripts relate to their relative chromatographic mobility. Of the four, B_1 is found in highest concentrations followed by G_1 and G_2. *A. flavus* only produces B_1 and B_2 and *A. parasiticus* produces these same metabolites along with G_1 and G_2. Dutton and Healthcote (1966) characterized the hemiacetal derivatives of these last toxins have been found in peanuts and maize. These derivatives, AFM_1 and AFM_2, were first isolated from the milk of cows fed on aflatoxin-contaminated rations. Various other minor aflatoxins are produced by *A. flavus* in culture, and in the liver and probably other organs.

Aflatoxin production is the consequence of the combination of species, substrate, and environment. The factors affecting aflatoxin production can be divided into three categories: physical, nutritional, and biological factors. Physical factors include temperature, pH, moisture, light, aeration and level of atmospheric gases. Aflatoxins are produced only between temperatures of 12 and 42 °C, and the optimal temperature is 25 °C to 35 °C (Diener and Davis, 1966). Aflatoxin production is particularly favored by very moist conditions. Maximum moisture content for aflatoxin production in corn kernels is 25% at 30 °C and the minimum relative humidity for aflatoxin production varies between 83% and 88%. Presence of CO_2 and O_2 influences mold growth and afltoxin. A 20% level of CO_2 in air depresses aflatoxin production and markedly depress mold growth. Decreasing the O_2 concentration of air to 10% depresses aflatoxin production, but only at O_2 levels of less than 1% are growth and aflatoxin production completely inhibited (Lenders *et al*., 1967). Many researches have reported that initial pH did not significantly affect aflatoxin production, while other investigators have shown that weak acid pH resulted

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in higher aflatoxin production and markedly depressed mold growth (Jarvis, 1971). Either natural or laboratory media exert a strong effect on aflatoxin production. Generally, the preferred carbon sources for aflatoxin production are glucose, sucrose or fructose. Zinc and manganese are essential for aflatoxin biosynthesis, but a mixture of cadmium and iron depressed mold growth and hence aflatoxin production.

In order to devise effective methods for preventing aflatoxin contamination of feed, elucidation of the aflatoxin biosynthetic mechanism in *A. flavus* species is very important (Since the first gene was isolated and described in 1992 (Chang *et al*., 1992; Skory *et al*., 1992), the last review on the molecular biology of aflatoxin biosynthesis, rapid and significant process followed the discovery that genes involved in the pathways of aflatoxin biosynthesis has been investigated). The aflatoxin biosynthetic pathway consists of at least 18 multienzymatic conversion reactions initiated by polypeptide synthesis from acetate, a process similar to fatty acid synthesis. The generally accepted pathway for aflatoxin B_1 formation is as follows (Prieto and Woloshuk, 1997):

norsolorinic acid (NOR) ⟶ averantin ⟶ averufanin ⟶ averufin(AVF) ⟶ hydroxyversicolorone ⟶ versiconal hemiacetal acetate (VHA) ⟶ versicolorin A ⟶ sterigmatocystin (ST) ⟶ ⟶ O-methylsterigmatocystin (OMST) ⟶ aflatoxin B_1.

In aflatoxin biosynthesis, norsolorinic acid (NOR) is the first stable intermediate. The conversions of ST to OMST and OMST to aflatoxin, which represent the final steps of the pathway, are unique to the aflatoxin-producting fungi *A. flavus* and *A. parasiticus* (Bennet and Papa, 1988; Yu *et al*., 1993; Yu *et al*., 1995). Some of the enzymes involved in aflatoxin biosynthesis have been characterized, and their

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respective genes have been cloned. These include the pksA, pksLl, faslA, nor-l, norA, avfl, vbs, verl, stcP, omtA, ordl, avnA and the aflR gene, which codes for a regulatory factor (AFLR) that activates the transcription of these pathway genes (Skory *et al*., 1992; Prieto and Woloshuk, 1997; Motomura *et al*., 1999).

Studies reveal that all of the identified genes related to aflatoxin biosynthesis are located within a 75-kb DNA region in both *A. flavus* and *A. parasiticus*, and their relative positions in the clusters of both fungal species are similar (Yu *et al*., 1995). On the other hand, several fungi (*A. nidulans*, *Bipolaris* spp., *Chaetomium* spp., *Farrowia* spp., *Monocillium* spp.) produce sterigmatocystin, a precursor of aflatoxins. Sterigmatocystin biosynthesis genes have been isolated and found to be located in a 60-kb cluster in the *A. nidulans* genome (Brown *et al*., 1996; Keller and Hohn, 1997).

1.6.4 Biocontrol of aflatoxin producing fungi and aflatoxin production

Aflatoxins are unique in being resistant to degradation under normal food processing conditions making it more difficult to control or eliminate aflatoxins in food (Ciegler and Vesonder, 1983). Various physical, chemical, and biological methods to reduce the aflatoxin level in food and feeds have been tried for many years. Preventing the contamination of food by the toxigenic fungi, *A. flavus* and *A. paraciticus* is the most rational and economic approach to avoid the potential hazards (Rania *et al*., 2005). Physical approaches to aflatoxins destruction generally involve treating with heat, UV, light or ionizing radiation (Aziz *et al*., 1990). Chemical degradation of aflatoxins is usually carried out by chlorination, oxidizing or hydrolytic agents. Ammoniation methods is the most widely accepted, however, effective ammoniation requires expensive equipment and may result in losses in

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nutritional quality of the treated feed (Samarajeewa *et al*., 1990). Moreover, chemical control methods are ineffective and non eco-friendly as they increase environmental and health hazards. Biological control by microorganisms including bacteria, yeasts, moulds, actinomycetes and algae are able to remove or degrade aflatoxins in foods and feeds (Line and Brackett, 1995). Numerous bacteria have been tested for biological control of *A. flavus*. Celestin and Lloyd (1998) used *B. pumilus* to inhibit aflatoxin production of *A. parasiticus* NRRL 2999. The inhibitory activity was due to extracellular metabolites that were produced in cell free supernatant fluids of cultured broth. Removal of AFB_1 by *Lactobacillus rhamnosus* strain Lb 50 isolated from traditional sourdough ferments, estimated to 45%, removed from the contaminated MRS broth (Abdellah *et al*., 2005). Zhang *et al*. (2008) investigated inhibition the growth of *A. flavus* by *B. subtilis* B-FS06. The antifungal compounds from *B. subtilis* was purified and identified to be bacillomycin compounds. Farzaneh *et al*. (2012) reported a destructive effect of *B. subtilis* onto aflatoxin structure, and its degradation activity was likely due to the extracellular nature of the produced enzyme in the media which could remove AFB_1. In the previous study, there were reports on antifungal activities of hydrolytic enzymes such as chitinase, β-1,3 glucanase, chitosanase and protease (Wang *et al*., 2002). El-Katany *et al*. (2001) showed that chitinase from *Streptomyces* sp. was able to lyse the fungal cell walls.

Aflatoxin contamination of crops can be minimized by early harvest, prevention of insect damage, and proper storage (Cotty, 1991). However, even under careful management, unacceptable aflatoxin levels may occur from unpreventable insect damage to the developing crop or from exposure of the mature crop to moisture either prior to harvest, or during storage in modules, handing, transportation, or even

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use (Cotty and Lee, 1989). For many diseases, traditional chemical control methods are not always economical nor effective, and fumigation as well as other chemical control methods may have unwanted health, safety, and environmental risks. The antifungal abilities of some beneficial microbes have been known since the 1930s, and there have been extensive efforts to use them for plant disease control since then. However, they are only now beginning to be used commercially. Aflatoxins cannot be readily removed from contaminated foods by detoxification. Therefore, there is interest in developing a biological control method that can increase crop safety by decreasing toxin content and that is based on the displacement of toxigenic isolates using atoxigenic isolates of the same species. It has been reported that aflatoxin production is inhibited by lactic acid bacteria, *Bacillus subtilis* and many molds. This inhibition may result from many factors including competition for space and nutrients in general, competition for nutrients required for aflatoxin production but not for growth, and production of antiaflatoxigenic metabolites by co-existing microorganisms (Liu *et al.*, 2012).

1.7 *Bacillus subtilis*

Bacillus subtilis is a ubiquitous naturally occurring saprophytic bacterium that is commonly recovered from soil, water, air and decomposing plant material. *B. subtilis* has been a model for Gram-positive and rod-shaped bacteria (0.3-2.2 x 1.2-7.0 µm), capable of forming endospores, growing in the mesophilic temperature range (optimal temperature is 25-35 °C), using their flagella for a swarming motility. *B. subtilis* were formerly considered strictly aerobic, meaning that they require

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oxygen to grow and they cannot undergo fermentation. However, more recent studies showed that they can indeed grow in anaerobic conditions making them facultative aerobes. *B. subtilis* was classified in procaryotea kingdom, Bacteria domain, Proteobacteria phylum, Bacilli class, Bacillales order, *Bacillus* genus, and *Bacillus subtilis* species.

Table 1.5 Morphology and characteristic of *B. subtilis*

Morphology	Characteristic
Form	Almost circular
Surface elevation	Flat
Surface of colony	Smooth
Edge/Margin	Entire or undulate
Opticity	Opaque
Chromogenesis	White and cream
Gram	Positive
Endospore forming	Positive (central oval)

Bacillus subtilis is used extensively in the industrial production of enzymes, biochemicals, antibiotics and insecticides (Harwood, 1992). *B. subtilis* shows antagonistic activities against several plant pathogens because they have a well-developed secretory system producing diverse secondary metabolites with a wide spectrum of antibiotic activity and hydrolytic enzymes. Therefore, they are widely used in biocontrol of plant diseases and become very valuable for medical and agricultural applications (Liu *et al*., 2007).

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B. subtilis is a promising candidate for controlling pathogenic species of the fungal genera *Fusarium* such as *F. oxysporum* and *F. graminearum.* Strains of *B. subtilis* are known to produce a spectrum of antifungal lipopeptides in both liquid and solid cultures, and these compounds, if present in a biological control product (BCP), could decrease the likelihood of developing pathogen resistance due to the multiplicity of antifungal factors (Kajimura *et al*., 1995; Magetdana and Peypoux, 1994; Mannanov and Sattarova, 2001; Nakayama *et al*., 1997; Schneider *et al*., 1999; Umezawa *et al*., 1986). In addition, *B. subtilis* forms endospores that can withstand harsh conditions either in storage or after application. BCP formulations can be manufactured that contain both an initial dose of antifungal lipopeptides produced during fermentation and a spore inoculum that will potentially allow the bacterial cells to colonize the plant root system and produce more active compounds *in situ*. The composition and relative concentrations of the lipopeptides produced are dependent on both the bacterial strain and the fermentation conditions in which it is cultivated (Akpa *et al*., 2001; Besson *et al*., 1987; Jacques *et al*., 1999; Ohno *et al*., 1992; Ozcengiz and Alaeddinoglu, 1991; Vanittanakom *et al*., 1986; Yamada *et al*., 1990). It is important to define the conditions in which the bacterial cells will produce metabolites with maximum antifungal activity, particularly if the composition of the biological control product can be shown to confer maximal activity against the target organisms. Besides the anti-fungal effects, some compounds produced by *B. subtilis* may also act as plant growth promoters (Compant *et al*., 2005).

B. subtilis is able to synthesize more than 60 different types of antibiotics, mainly in polypeptides, many of which possess antifungal effects and belong to the iturin family (Phae *et al*., 1991). Cyclic lipopeptide antibiotics of the iturin, surfactin

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and fengycin families are important metabolites produced by *Bacillus* species (Hassan et al., 2010; Nihorimbere et al., 2010; Yu *et al*., 2002). Strong antibiotic activity of iturin A and surfactin is well known (Asaka and Shoda, 1996; Hassan *et al*., 2010). Iturin A contains seven amino acid residues (L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser). Surfactin contains a hydroxy fatty acid with an ester peptide linkage to seven cyclic amino acid residues (L-Glu-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Leu) (Kowall *et al*., 1998; Yakimov *et al*., 1995).

The antifungal secondary metabolites of *B. subtilis* have been observed mainly during microbial growth in media. Many of these antifungal substances have been characterized and identified as peptide antibiotics (Katz and Demain, 1977). The antifungal peptides produced by *Bacillus* species, included mycobacillins (Majumdar and Bose, 1958; SenGupta *et al*., 1971), iturins (Delcambe and Devignat, 1957; Isogai *et al*., 1982), bacillomycins (Besson *et al*., 1977; Peypoux *et al*., 1981), surfactins (Kluge *et al*., 1988), mycosubtilins (Peypoux *et al*., 1976; Besson and Michel, 1990), fungistatins (Korzybski *et al*., 1978), and subsporins (Ebata *et al*., 1969). Most of these antibiotics are cyclic peptides composed entirely of amino acids, but some may contain other residues. However, a few peptide antibiotics are linear such as rhizocticins (Kluger *et al*., 1990). *Bacillus* spp. produce also a range of other metabolites including chitinases and other cell wall-degrading enzymes (Priest, 1977; Pelletier and Sygusch, 1990; Frändberg and Schnürer, 1994a, 1994b; Sadfi *et al*., 2001), volatiles (Fiddman and Rossal, 1993; Sadfi *et al*., 2001) and compounds which elicit plant resistance mechanisms (Kehlenbeck *et al*., 1994).

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1.8 Hydrolytic enzymes

The productions of several hydrolytic enzymes that degrade cell walls of pathogenic fungi were involved in parasitism of phytopathogenic fungi. Specially chitinases, glucanases and proteases which are considered key players in the lysis of cell walls of higher fungi and may be important factors in biological control. The potential of *Bacillus sp.* to synthesize a wide variety of antifungal and antimicrobial metabolites with industry interest is one determinant of their ability to control plant diseases when applied as a biological control agent (Leifert *et al*., 1995).

(i) Protease

Proteases refer to a group of enzymes whose catalytic function is to hydrolyze proteins or the peptide bonds forming the primary structure of protein. They are also called proteolytic enzymes or proteinases. Proteases are classified according to their structure or the properties of the active site. Through evolution, proteases have adapted to the wide range of conditions found in complex organisms (variations in pH, reductive environment and so on) and use different catalytic mechanisms for substrate hydrolysis; their mechanism of action classifies them as either serine, cysteine or threonine proteases (amino-terminal nucleophile hydrolases), or as aspartic, metallo and glutamic proteases (with glutamic proteases being the only subtype not found in mammals so far). Proteases specifically cleave protein substrates either from the N or C termini (aminopeptidases and carboxypeptidases, respectively) and/or in the middle of the molecule (endopeptidases) and their primary role was long considered to be protein degradation relevant to food digestion and intracellular protein turnover (Barrett *et al*., 2004).

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Proteases are one of the most important groups of industrial enzymes, and commercial proteases account for nearly 60% of the total industrial enzyme market. Microorganisms account for a two-third share of commercial protease production in the world (Kumar and Takagi, 1999). Microbial proteases are classified into various groups, dependent on whether they are active under acidic, neutral, or alkaline conditions and on the characteristics of the active site group of the enzyme, i.e. metallo- (EC.3.4.24), aspartic- (EC.3.4.23), cysteine- or sulphydryl- (EC.3.4.22), or serine-type (EC.3.4.21; Kalisz 1988; Rao *et al*., 1998). Alkaline proteases (EC.3.4.21–24, 99) are defined as those proteases which are active in a neutral to alkaline pH range. They either have a serine center (serine protease) or are of metallo-type (metalloprotease); and the alkaline serine proteases are the most important group of enzymes exploited commercially. Protease production is an inherent capacity of all microorganisms; and a large number of bacterial species are known to produce alkaline proteases of the serine-type, although very few are recognized as commercial producers. Only those microbes that produce substantial amounts of extracellular enzyme are of industrial importance. Several products based on bacterial alkaline proteases have been launched successfully in the market in past few years. *Bacillus subtilis* is one of the most widely used bacteria for the production of specific chemicals and industrial enzymes and also a major source of amylase and protease enzymes. Protease has a wide application in detergent, pharmaceutical, photography, leather, food and agricultural industries. It is also used in baking, brewing, meat tenderization, peptide synthesis, medical diagnosis, cheese making, certain medical treatments of inflammation and virulent wounds and in unhairing of sheepskins. It has also wide application in bioremediation process (Gupta *et al*., 2002). Alkaline

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proteases are produced by a wide range of microorganisms including bacteria, moulds and yeasts. In bacteria, this enzyme is produced mainly by many members belonging to genus *Bacillus* especially, *B. licheniformis*; *B. horikoshii, B. sphaericus, Bacillus furmis, Bacillus alcalophilus, Bacillus subtilis* (Ellaiah *et al*., 2002). Currently, a large proportion of commercially available proteases are derived from *Bacillus* strains (Mehrato *et al*., 1999).

The fungal cell walls consist of thick layers of chitin, (1–3)-β-d-glucan, (1, 6) β-glucans, lipids and peptides. Microbial proteases may play a significant role in cell wall lysis. Proteolytic enzymes or proteases catalyze the cleavage of peptide bonds in proteins. In the previous study, *B. subtilis* 21 showed activity against pathogenic fungi in plants infected with *Rhizoctonia solani* and *Fusarium verticillioides* and treated strawberry with *B. subtilis* 21 produced augment in the number of leaves per plant and an increment in the length of healthy leaves in comparison with untreated plants. Secreted proteins by *B. subtilis* 21 were studied, detecting the presence of proteases and bacteriocin-like inhibitor substances that could be implicated in its antagonistic activity. Chitinases and zwittermicin production could not be detected. Then, *B. subtilis* 21 could potentially be used to control phytopathogenic fungi that infect strawberry plants. (Basurto-Cadena *et al*., 2012)

(ii) Chitinase

Chitin $(C_8H_{13}O_5N)_n$, is a polymer of unbranched chains of the (1-4)-β-linked homopolymer of N-acetyl-D-glucosamine (GlcNAc; *N*-acetyl D-glucosamine; NAG). It is the most abundant biopolymer next to cellulose with an annual production of 10^{10}

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to 10^{11} tons per annum. Shrimp and crab shell, are waste products from canning industries. They are the best sources of chitin, and are a major structure component of most fungal cell walls. Cell walls of many microorganisms and plants are made of chitin cross-linked with other biopolymers. Chitin binds easily with other materials and can be cast into sheets or films. Chitin and most of its derivatives have a strong positive charge on them. This property has been exploited for many uses. Several reports are available regarding the presence of chitin in fungal cell walls, especially in filamentous fungi and basidiomycetes where it comprises up to 16% of the dry weight of the organism (Gooday, 1979). In yeasts the amount of chitin in cell wall is much lower but it is nevertheless important, particularly in the budding process. Bud scars have been shown to be largely composed of chitin (Cabib and Bowers, 1971). Since chitin is important in the growth of fungi, it is most likely that there exists a system whereby it can be remodeled by a concerted breakdown and buildup to allow a degree of plasticity in the wall, which would be important in budding and in the elongation of hyphae (Barrett-Bee and Hamilton, 1984). The x-ray diffraction studies revealed that chitin occurs in three polymorphic forms, that is, α-, β- and γ-chitins, which differ in the arrangement of molecular chains within the crystal cell. Among these, α-chitin is the most abundant form of chitin (Peberdy, 1985).

Fungal cell walls are mainly composed of polysaccharides (**Figure 1.8**). Two of them, β-1,3 glucan and chitin are common to all species in the fungal kingdom and constitute the skeleton of the cell wall. The three dimensional organisation of the fungal cell wall has been however studied in very few fungal species. A comparison of the chemical composition of cell walls from different fungi demonstrates that a common skeletal corestructure exists in almost all fungi. This core skeleton is similar

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for both yeasts and moulds. It is composed of a branched β-1,3- glucan to which chitin is linked through a β-1,4 linkage to a non-reducing end of the lateral β-1,3 glucan chains. This fibrillar core is further decorated with amorphous polysaccharides that are alkali-soluble. In contrast to the structural polysaccharides, the composition of these polysaccharides varies with the species studied and has some taxonomical foundations. Among the most important amorphous polysaccharides, we can cite B-1,6-glucans, α-1,3-glucans and mannans. Proteins of the cell wall do not play a role of linker in the structural organisation of the cell wall. Most cell wall proteins are in transit towards the external milieu. Some of the proteins that were originally anchored to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor play a role in remodeling cell wall polysaccharides whereas other GPI-proteins can become covalently bound to the cell wall polysaccharides (Latgé *et al*., 2007).

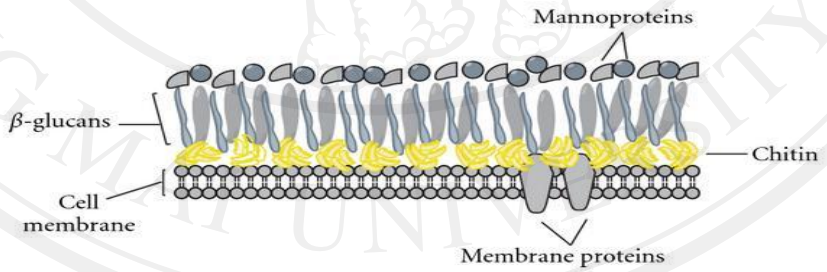

Figure 1.8 Fungal cell wall components. The fungal cell wall contains a cell membrane with various membrane proteins, a protective layer of chitin (yellow) as well as glucans (mostly beta), and mannoproteins on its surface. Different fungal cell walls contain different glucans. For example, the cell wall of A. fumigatus contains β-1, 3- and β-1,4-glucan, and α-1, 3-glucan while C. albicans contains β-1,3- and β-1,6-glucan. (Netea *et al*., 2008)

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Chitinase (EC 3.2.1.14) is an enzyme which can hydrolyze chitin to its oligomeric, dimeric and monomeric component. They are defined as enzymes cleaving a bond between the C1 and C4 of two consecutive *N*-acetylglucosamines of chitin. It occurs in several of organisms including viruses, bacteria, fungi, insects, higher plants, and animals. Many bacteria and fungi containing the chitinolitic enzyme convert chitin into carbon and nitrogen. (Flach *et al*., 1992; Park *et al*., 1997; Hunt *et al*., 2008; Adams, 2004; Graham and Sticklen, 1994). Chitinases are classified into two major groups. Endochitinases (EC 3.2.1.14) cleave chitin randomly at internal sites, generating low molecular mass mutimers of GlcNAc, such as chitotetraose, chitotriose, and diacetylchitobiose. Exochitinases can be divided into two subgroups: chitobiosidases (EC 3.2.1.29), which catalyze the progressive release of diacetylchitobiose starting at the nonreducing end of chitin microfibril, and β-1,4 *N*-acetyl glucosaminidases (EC 3.2.1.30), which cleave the oligomeric products of endochitinases and chitobiosidases, generating monomers of GlcNAc (Sahai and Manocha, 1993).

The production of chitinase by plants has been suggested to be a part of their defense mechanism against fungal pathogens (Harighi *et al*., 2007). Chitinases have received increased attention due to their wide range of biotechnological applications, especially in the biocontrol of fungal phytopathogens (Bhushan and Hoondal, 1998). Chitinase has been implicated in plant resistance against fungal pathogens because of its inducible nature and antifungal activities *in vitro*. Chitinase in fungi is thought to have autolytic, nutritional and morphogenetic roles. In viruses, chitinases are involved in pathogenesis (Dahiya *et al*., 2006). Due to multiple applications of chitinases in biocontrol, waste management, medicine and biotechnology they become interesting

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enzymes for study (Nawani and Kapadnis, 2005). Chitinase as a biocontrol agent against insect pest, *Monochamus alternatus*, a pine sawyer beetle which is one of the important factors causing pine tree destruction in Japan. Chitinase (50µ liter, 0.3 to 3µM) were individually administrated orally into the digestive tube of the pine sawyer beetle and most of the beetles died in 20 hrs. thus chitinase has potential as a pest-control agent (Koga, 2005). Other applications of chitinases are bioconversions of chitin waste to single cell proteins and ethanol and fertilizers. Industrial applications of chitinases have been governed mainly by key factors such as cost production, shelf-life stabilities and improvement in enzyme properties by immobilization (Bhushan, 2000). Chitinase producing marine bacteria play an important role in the degradation on chitin in the oceans (Park *et al*., 2000).

Biocontrol agents directly attack the plant pathogen by secreting lytic enzymes such as chitinase, β-1, 3-glucanase, cellulase and proteases. These enzymes hydrolyse the pathogen's cell wall components such as chitin, glucan, cellulose and proteins successfully limiting the growth of fungal pathogens (Lorito *et al*., 1994; Carsolio *et al*., 1999). As the skeleton of the fungal cell wall mainly contains chitin, glucan and proteins, mycoparasitism and enzymes that hydrolyze these components are one of the main mechanisms accounting for showing antagonistic activity against plant pathogenic fungi. Chitinase, β-1, 3-glucanase and cellulase are important in the hyper-parasitic mechanism.

Various species of *Bacillus* have been shown to secrete chitinase, including *B. circulans, B. licheniformis, B. laterosporus, B. amyloliquefaciens, B. magaterium, B. pabuli, B. stearothermophilus, B. subtilis, B. thuringiensis* (Shanmugaiah *et al*., 2008). In previous study, *B. subtilis* NPU 001 stain isolated from soil sample,

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excreted a chitinase when culture in a medium containing 2% (w/v) shrimp and crab shell powder as the major carbon source. The purified chitinase (2 mg ml^{-1}) inhibited hyphal extension of the fungus *Fusarium oxysporum* (Chang *et al*., 2010). Similarly, Karunya *et al*. (2011) suggested that chitinase produced by *B. subtilis* that was isolated from soil sample was a digestive enzyme that broke down glycosidic bonds in chitin and show antifungal activity against plant pathogens *viz, Aspergillus niger, A. flavus* and *Penicillium chysogenum*. The strain J9 of *B. subtilis* was able to reduce grey mold disease on strawberry under field conditions with percentage reduction ranging from 46.3 to 44.3% which was comparable to those of chemical fungicides respectively after treatments of 8 and 40 days (Badiaa *et al*., 2012). Then, the strain J9 was able to produce high antifungal enzymes inducible by the pathogen; chitinase (102 U/ml), protease (14.5 U/ml) and laminarinase (0.74U/ml).

(iii) Amylase

Starch is an important renewable biological resource and the most important industrial enzymes in use today include protease, carbohydrate-hydrolyzing enzymes, and ester cleavage fat hydrolyzing enzymes. It is one of the abundant carbon sources in nature (Syed *et al*., 2009; Sadhukham *et al*., 1992; Paquet *et al*., 1991). It is a major reserve carbohydrate of all higher plants. It is a heterogeneous polysaccharide composed of two high molecular weight entities called amylose and amylopectin. Amylose is composed of linear chains of α-1, 4 linked D-glucose residues. Hence it is extensively degraded by α-amylase. Amylopectin has a branched structure composed of chains of about 20-25 α-1, 4 linked D-glucose residues which is branched by α-1, 6 linkages and may contain 4 to 5% α-1, 6-D-glucosidic bonds (Aiyer, 2005).

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Amylases (endo-1, 4-α-D-glucanohydrolase) belong to hydrolase category of enzymes, which catalyze the hydrolysis of starch molecule to give diverse products including dextrin and progressively smaller polymers composed of glucose units. Amylases constitute a class of industrial enzymes having approximately 25% of the enzyme market (Hoque *et al*., 2006; Rao *et al*., 1998; Teresita *et al*., 1996). Amylases can be classified into α-amylase (EC 3.2.1.1), β-amylase (EC 3.2.1.2), and glucoamylase (EC 3.2.1.3). The α-amylase (endo 1, 4-α-D-glucan glucohydrolase) randomly cleaves internal α-1, 4 glycosidic linkages in starch molecules, giving oligosaccharides of varying chain lengths with an α-configuration and α-limited dextrins as products, which are further cleaved into glucose by exo-acting amylases and other amylolytic enzymes. The α-amylases have numerous biotechnological applications in the production of syrups containing oligosaccharides, maltose and glucose (Narayana and Vijayalakshmi, 2008). The β-amylase, distributed in higher plants and microorganisms, can attack the α-1, 4 linkages from the non-reducing ends of the starch molecules, giving maltose units. These enzymes are used for production of high maltose syrup from starch in combination with pullulanase, which can hydrolyze the α-1, 6-linkages in α-1, 6-branched α-1, 4-glucans. Maltose covers a wide range of application in the food and pharmaceutical industries since its properties are represented by mild sweetness, good thermal stability, low viscosity in solution, and lack of color formation. Glucoamylase cuts the 1, 6-α-D linkages in amylopectin in addition to the 1, 4-α-D linkages. Therefore, they can completely hydrolyze amylose, amylopectin, maltose, and malto-oligosaccharides and release glucose from the nonreducing ends of starch. They are widely used in the industrial conversion of starch into sugars. Besides, glucoamylase can hydrolyze isomaltose and

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nigerose but at slower rate than starch or starch degraded products. They are produced by fungi, bacteria, yeast and in certain animal tissue, such as liver, spleen, and intestine. Moreover, raw starch in plants and tubers is not attacked by glucoamylase (Najafi and Kembhavi, 2005; Shambe and Ejembi, 1986; Noda *et al*., 2001).

The most widely used enzyme in the industry for starch hydrolysis is α – amylase (EC.3.2.1.1). This enzyme accounts 65% of enzyme market in world and is extensively used in many industries including starch liquification, brewing, food, paper, textile and pharmaceuticals (Ohdan *et al*., 2000). Microorganisms like fungi and bacteria have been extensively screened for α-amylase production (Ivanova *et al*., 2001). *B. stearothermophilus*, *B. licheniformis*, *B. amyloliquefaciens*, and *B. subtilis* are known to be good producers of α-amylase, and they have been widely used for commercial production of the enzyme for various applications (Sivaramakrishnan *et al*., 2006).

(iv) Cellulase

Cellulose is a long chain of linked sugar molecules that is an important structural component of the primary cell wall of green plants, many forms of algae and the oomycetes. Cellulose is an organic compound with the formula $(C_6H_{10}O_5)_n$, a polysaccharide consisting of a linear chain of several hundred to over ten thousand β(1→4) linked D-glucose units. Cellulose is derived from D-glucose units, which condense through β(1→4)-glycosidic bonds. This linkage motif contrasts with that for α(1→4)-glycosidic bonds present in starch, glycogen, and other carbohydrates. The long chain is made by the linking of smaller molecules. The sugar units are linked when water is eliminated by combining the -OH group and H. The hydroxyl groups

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on the glucose from one chain form hydrogen bonds with oxygen atoms on the same or on a neighbor chain, and linking just two of these sugars produces a disaccharide called cellobiose. Cellulose is a polysaccharide produced by cellulose chain bristles with polar -OH groups. These groups form many hydrogen bonds with OH groups on adjacent chains, bundling the chains together. The chains also pack regularly in places to form hard, stable crystalline regions that give the bundled chains even more stability and strength (Kim *et al*., 2012; Yin *et al*., 2010).

The biological degradation of cellulose involves the synergistic action of cellulolytic enzymes. There are three classes of cellulolytic enzymes.

a.) Endo-1,4-β-glucanases randomly cleave internal bonds in the cellulose chain. These enzymes may be non-processive or processive (in processive enzymes, enzyme-substrate association is followed by several consecutive cuts in a single polysaccharide chain that is threaded through the active site).

b.) Exo-1, 4-β-glucanases attack the reducing or nonreducing end of the cellulose polymer. Processive exo-1,4-β-glucanases are referred to as cellobiohydrolases; they are among the most abundant components in natural and commercial cellulase mixtures and a subject of intense study.

c.) β-glucosidases convert cellobiose, the major product of the endo- and exo-glucanase mixture, to glucose (Horn *et al*., 2006; Horn *et al*., 2012; Payne *et al*., 2011).

These enzymes act synergistically because endo-acting enzymes generate new reducing and non-reducing chain ends for the exo-acting enzymes, which release cellobiose that is converted to glucose by β-glucosidases (Kostylev and Wilson, 2012; Wood and McCrae, 1979). The mechanism of cellulose degradation, cellulases are

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subdivided into either non-processive cellulases (endocellulases) or processive cellulases (including different exocellulases and some new processive endocellulases) (Barr *et al*., 1996; Reverbel-Leroy *et al*., 1997). Endocellulases can randomly cleave the cellulose at exposed positions and produce new reducing ends, however, the processive cellulases remain attached to the chain and release mainly cellobiose or cellotetraose units from one end of the chain (Sakon *et al*., 1997). These mechanisms are similar to those degradation of amylase (Robyt and French, 1967). During the degradation of cellulose, non-processive cellulases and processive cellulases have been found to work synergistically (Creuzet *et al*., 1983; Henrissat *et al*., 1985; Irwin *et al*., 1993). It has been generally accepted that effective biological hydrolysis of cellulose into glucose requires synergistic actions of three enzymes including endo-β-1,4-glucanase (EC 3.2.1.4, EG, randomly cleaving internal linkages), cellobiohydrolase (EC 3.2.1.91, CBH, specifically hydrolyzing cellobiosyl units from non-reducing ends), and β-*D*-glucosidase (EC 3.2.1.21, hydrolyzing glucosyl units from cellooligosaccharides) (Perez *et al*., 2002).

Currently, cellulase is commonly used in many industrial applications, especially in animal feed, textile, waste water, brewing and wine-making. Cellulases hold many potential industrial applications. In textile industries, they were used for the "Biopolishing" of fabrics for increasing its softness and brightness. They were also used in animal feeds for improving the nutritional quality and digestibility, in processing of fruit juices, and in baking, while de-inking of paper is yet another emerging application. It plays a major role in the conversion of renewable cellulosic biomass into commodity chemicals (Beguin and Aubert, 1994; Mandels, 1985)

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Cellulases are the inducible bioactive compounds produced by microorganisms during their growth on cellulosic matters. Increasing knowledge of mode of action of cellulase; they were used in enzymatic hydrolysis of cellulosic substances. Although a large number of microorganisms are capable of degrading cellulose, only a few of them produces significant quantities of cell-free bioactive compounds capable of completely hydrolyzing crystalline cellulose *in vitro.* Fungi are the main cellulase-producing microorganisms, though a few bacteria have also been reported to yield cellulase activity (Saraswati *et al*., 2012).

Most commercial cellulase is of fungal origin and produced by *Trichoderma* species and *Aspergillus* species. Recently some bacteria, *Clostridium thermocellum*, *C. cellulolyticum*, *C. cellulovorans*, *C. josui*, *Ruminococcus albus* and the actinomycetes, *Thermoactinomycetes* sp., *Thermomonospora cuvata*, *Streptomyced* sp., *B. subtilis*, *B. licheniformis* , *B. stearothermophilus* ,and *B. amyloliquefaciens* have also been reported as cellulase producers using different substrates e.g. cellulose, carboxymethylcellulose, starch and glucose as carbon source (Mohamed *et al*., 2010). Yin *et al*., (2010) reported that *B. subtilis* YJ1 could utilize natural wastes such as rice bran as substrate for growth and produce high levels of cellulase. According to substrate specificity, the purified cellulase has high specificity to CMC and was considered to be an endo-1,4-glucanase. The produced cellulase was considered to be endoglucanase and highly benefits to the industrial application. Saraswati *et al*., (2012) also found that the cow dung is served as the good isolation source for cellulase producing microorganism as it is rich in cellulose and *B. subtilis* strain (CELPTK1) was isolated from cow dung.

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The production of cellulolytic enzyme has been suggested to be antagonistic activity against the pathogenic fungus. Kanchanasin and Chaiyanan (2012) have been reported that *Streptomyces* isolate S22 could be a good biocontrol for *Pythium aphanidermatum*, a causative agent of seedling damping off and root rot diseases of plant due to their ability to synthesize antimicrobial substance and cellulolytic enzyme. Their extracellular enzymes and metabolic products can suppress the proliferation of *P. aphanidermatum*.

(V) Lipase

Lipids constitute a group of naturally occurring molecules that include fats, waxes, sterols, fat-soluble vitamins (such as vitamins A, D, E, and K), monoglycerides, diglycerides, triglycerides, phospholipids, and others. The main biological functions of lipids include energy storage, signaling, and acting as structural components of cell membranes. Lipids are a large and diverse group of naturally occurring organic compounds that are related by their solubility in nonpolar organic solvents (e.g. ether, chloroform, acetone & benzene) and general insolubility in water. Lipids constitute a large part of the earth's biomass, and lipolytic enzymes play an important role in the turnover of these water-insoluble compounds. Lipolytic enzymes are involved in the breakdown and thus in the mobilization of lipids within the cells of individual organisms as well as in the transfer of lipids from one organism to another (Beisson *et al*., 2000; Rohit *et al*., 2001).

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are hydrolases, which act under aqueous conditions on the carboxyl ester bonds present in triacylglycerols to liberate fatty acids and glycerol. The natural substrates of lipases are long-chain triacylglycerols, which have very low solubility in water; and the reaction is catalyzed

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at the lipid–water interface (Gupta *et al*., 2004). Lipases are ubiquitous enzymes of considerable physiological significance and industrial potential. Lipases catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids (Martinelle *et al*., 1995). The presence of lipases has been observed as early as in 1901 for *Bacillus prodigiosus, B. pyocyaneus* and *B. fluorescens* which represent today's best studied lipase producing bacteria now named *Serratia marcescens*, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*, respectively. *Bacillus* species have been found to produce a number of enzymes other than lipases that may be utilized industrially. In 1856, Claude Bernard first discovered a lipase in pancreatic juice as an enzyme that hydrolysed insoluble oil droplets and converted them to soluble products. Lipases have traditionally been obtained from animal pancreas and are used as a digestive aid for human consumption either in crude mixture with other hydrolases (pancreatin) or as a purified grade. Initial interest in microbial lipases was generated because of a shortage of pancreas and difficulties in collecting available material (Fariha *et al*., 2006).

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1.9 PURPOSE OF RESEARCH

The research on inhibitory activity of *Bacillus subtilis* BCC 6327 metabolites against growth of aflatoxigenic fungi isolated from bird chili powder were conducted with the following objectives:

1. To isolate aflatoxigenic fungi from bird chili powder

2. To inhibit the growth of isolated aflatoxigenic fungi by cell free culture of *B. subtilis*

3. To assay the selected hydrolytic enzyme activity from *B. subtilis*

4. To study effects of crude enzymes or hydrolytic enzymes from *B. subtilis* against aflatoxigenic fungi

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