CHAPTER II

Literature Reviews

2.1 General information of *Penicillium marneffei*

In 1956, *P. marneffei* was first isolated from the hepatic lesions of a Chinese bamboo rat (*Rhizomys sinensis*) maintained in captivity for experimental infections at the Pasteur Institute of Indochina at Dalat, South Vietnam and was described by Capponi *et al* (Vanittanakom *et al*., 2006; Viviani and Vanittanakom, 2010). This fungus was identified as a new species by G. Segretain and was named *P. marneffei* in honor of Hubert Marneffe who is Director of the Pasteur Institute of Indochina in 1959. In the same year, Segretain described the first case of penicilliosis marneffei in human. He accidentally punctured his finger with a needle filled with *P. marneffei* used to inoculate hamster. Nine days later, he developed a small nodule at the site of inoculation followed by lymphangitis and axillary adenopathy. The naturally occurring case of penicilliosis marneffei in human was reported in 1973 in an American minister with Hodgkin's disease who had visited Southeast Asia. The fungus was found in an isolated splenic abscess and also isolated from the spleen (Viviani and Vanittanakom, 2010). Subsequent reports in Asia were found in patients living in Thailand, Hong Kong and the Guangxi region of southern China. However, the human cases were very rare and usually misdiagnosed as histoplasmosis until the explosive epidemic of HIV-AIDs in Southeast Asia in 1980s. *P. marneffei* infection was observed in the patients and became the third most common HIV-related opportunistic infection following tuberculosis and cryptococcosis (Vanittanakom *et al*., 2006; Viviani and Vanittanakom, 2010). In northern Thailand reported cases of penicilliosis marneffei coincides with AIDs and is recognized as an AIDs-indicator disease (Cooper Jr and Vanittanakom, 2008). In addition, *P. marneffei* infection has also been found in other countries in Asia such as Vietnam, Taiwan, Manipur state of India, Guangxi region of China, Hong Kong and Malaysia.

The route of *P. marneffei* infection in human has not been clearly identified, however an infection is probably started by the inhalation of conidia into respiratory tract (Viviani and Vanittanakom, 2010; Cooper Jr and Vanittanakom, 2008). A protein presenting on surface of conidia recognizes host extracellular matrix proteins, laminin and fibronectin. This facilitates the attachment of conidia to the bronchoalveolar epithelium. Recently, the study of proteome profiling of *P. marneffei* extracellular proteins demonstrates that glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is upregulated during mycelial growth and acts as an adherence factor of conidia to host bronchioalveolar epithelium during the early stage of infection (Lau *et al*., 2013). After attachment, *P. marneffei* conidia are phagocytized by host alveolar macrophage where they are able to survive and multiply intracellularly. Within macrophages, *P. marneffei* can survive and cause the systemic disease in immunocompromised person (Cooper Jr and Vanittanakom, 2008). Penicilliosis marneffei is fatal, therefore the treatment of *P. marneffei* infection is required. *P. marneffei* are resistant to fluconazole and the most antifungal drugs used for treatment are amphotericin B, itraconazole, and ketoconazole (Viviani and Vanittanakom, 2010). In Thailand, the treatment includes a 2-week course of intravenous amphotericin B followed by itraconazole for 10 weeks.

2.2 Mycology

2.2.1 Morphology and physiology

P. marneffei is the only one species of genus *Penicillium* that is a thermally dimorphic fungus. At 25ºC, *P. marneffei* grows on Sabouraud's dextrose agar (SDA) as a mycelial fungus possessing yellowish green sporulating colonies with a pink or red center and dark green edges (Viviani and Vanittanakom, 2010). The fungus produces brick-red pigment and releases into the culture medium. At 25-30ºC, this pigment appears after 24-72 hours on PDA or SDA agar (Figure 2.1A). Under microscope (Figure 2.1B), mycelial form is identified by production of tortuous smooth-walled conidiophores (1.5-2 µm in width and 70-175 µm in length) grow away from aerial septate hyphae. Terminals of conidiophores are typically divaricate, verticils, either symmetrical or asymmetrical and rarely branching. Three to five metulae (2.5-3.5 x 7-11 µm) originate from the top of conidiophore. Each matulae produces 4-6 verticil-arranged phialides in an acropetal mode of division, such that the younger ones is over the older ones (Viviani and Vanittanakom, 2010; Cánovas and Andrianopoulos, 2007). These phialides have flask-like shape gradually tapering to slender collar with the same size as metulae and generate short disordered chains of globose (2-3 µm), smooth walled asexual spore (conidia) in a basipetal mode of division, in that the younger ones displace the older ones.

(A) **(B)**

Figure 2.1 Colony of *P. marneffei* on potato dextrose agar (PDA) incubated at 25ºC for 7 days (A) and morphology under bright field microscope (B). Scale bar represents 5 µm

At 35-37ºC, *P. marneffei* produces glabrous, off-white, and yeast-like colonies with little or no pigment on SDA (Figure 2.2A). Within liquid synthetic media containing amino acids and maltose or glucose such as SDB or BHI, *P. marneffei* grows as a yeast-like single-cell form called arthoconidia and divides by fission (Figure 2.2B). In rodent or human host, the globose, ovoid and elongated yeast-like cells (2-3 x 2-6.5 µm) are found outside or within macrophages with a single central septum in the multiplying cells. In addition, extracellular, elongated, and sausage-shaped cells 8-13 µm in length and more rarely short hyphae that no longer than 20 µm are also found (Viviani and Vanittanakom, 2010).

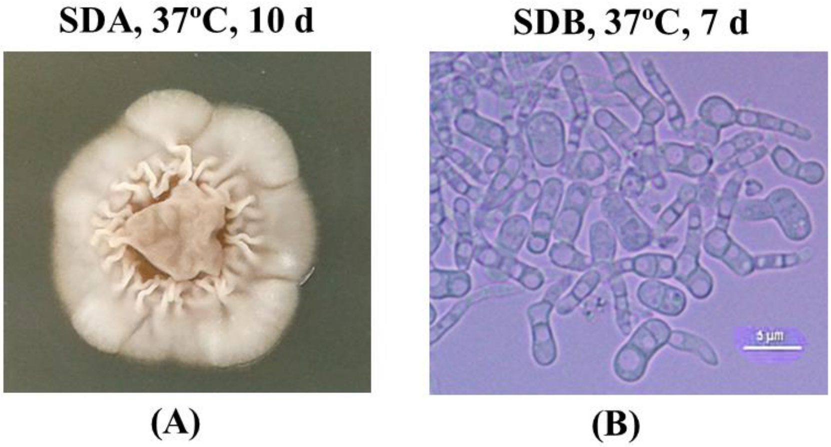

Figure 2.2 Colony of *P. marneffei* on Sabouraud dextrose agar (SDA) incubated at 37ºC for 10 days (A) and morphology of conidia inoculated in Sabouraud dextrose broth (SDB) and incubated at 37ºC for 7 days under bright field microscope (B). Scale bar represents 5 µm

Casein hydrolysate, peptone and asparagine but not $NaNO_3$ and $(NH_4)_2PO_4$ can be used as an organic nitrogen source for mycelial growth of *P. marneffei* (Viviani and Vanittanakom, 2010). For carbon sources, glucose (optimal growth), lactose, xylose, maltose, levulose, and mannitol are utilized. This fungus is sensitive to cycloheximide similar to most of saprophytic fungi. Enzymatic activities were analyzed in *P. marneffei* in both mycelial and yeast phases. The results demonstrated that alkaline phosphatase and naphthol-AS-BI-phosphohydrolase activities were expressed in both growth forms. In addition, several interesting enzymes including acid phosphatase, esterases and lipases are found in both mycelial and yeast form. These enzymes might be involved in virulence of this fungus (Viviani and Vanittanakom, 2010; Youngchim *et al*., 1999).

Cell wall of *P. marneffei* can be stained by periodic acid –Schiff (PAS) and Gomori's methenamine silver (GMS) stains. However, cells of this fungus are poorly stained by hematoxylin and eosin (H&E). In the past, *P. marneffei* yeast forms in tissue section are usually mistaken for yeast forms of *Histoplasma capsulatum* var. *capsulatum* because of their sizes. Nevertheless, the phenotypes of these two yeast forms are totally different, such that yeast forms of *P. marneffei* are oval, round and sausage-shaped with septa, whereas *H. capsulatum* yeast forms are uniform round or ovoid with budding (Viviani and Vanittanakom, 2010; Cooper Jr and Vanittanakom, 2008).

2.2.2 Identification

Based on Pitt's taxonomy, *P. marneffei* was classified in the subgenus *Biverticillium* because of the production of frequently biverticillate conidiophores and poor growth on 25 percent glycerol nitrate agar (Viviani and Vanittanakom, 2010). Recently, the relationship between the genus *Talaromyces* and *Penicillium* was analyzed and the phylogenetic analysis of species relationships using the inter transcriptional space (ITS) region demonstrated that *Talaromyces* species and most species of *Penicillium* subgenus *Biverticillium* sensu Pitt are in a monophyletic clade distant from other subgenera of *Penicillium*. This indicates that *Penicillium* subgenus Biverticillium including *P. marneffei* is different from other subgenera in Penicillium and *P. marneffei* is now combined in the genus *Talaromyces* (Samson *et al*., 2011).

The production of reddish pigment to the culture medium can be used for characterization and identification *P. marneffei*. Nevertheless, this reddish pigment is also produced by other *Penicillium* species in different manner, such that yellow-brown to reddish brown pigment for *P. citrinum*, orange-reddish to vinaceous-purple for *P. janthinellum*, red to purple-red for *P. purpurogenum* and *P. rubrum*, whereas *P. marneffei* possess brick-red pigment (Viviani and Vanittanakom, 2010).

An exoantigen test can be used in differentiation of *P. marneffei* from other *Penicillium* species. The exoantigen extracted from a slant culture of suspect fungus is tested by a reverse microimmunodiffusion method against rabbit anti- *P. marneffei* reference serum. The production of two precipitin lines of the same identity as the *P. marneffei* reference system confirms identification of the fungus. However, the significant standard to identify *P. marneffei* is the thermally dimorphism. At 37ºC, *P. marneffei* produces yeast-like colony and grows as yeast cell dividing by fission similar to *Schizosaccharomyces pombe*, while other *Penicillium* species are not.

2.2.3. Interaction with host cells

Most pathogenic fungi are ubiquitous in environment and usually incidentally infect the host by inhalation of spores or mycelial fragments. These fungal cells could then transform to parasitic phase inside the host body (Romani, 2011; Seider *et al*., 2010). For survival and disease establishment, pathogenic fungi require the adaptation to host environments and the evasion of host immune cells (Seider *et al*., 2010; Campos *et al*., 2005). *P. marneffei* infection is presumed to occur after inhalation of fungal conidia. These infectious conidia are small enough to reach the host alveoli (Cooper and Vanittanakom, 2008). The attachment of *P. marneffei* conidia to host extracellular matrix has been studied. This step is crucial for establishment of infection before the conidia are phagocytosed by host alveolar macrophages. It has been shown that sialic acid-containing receptor and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of *P. marneffei* conidia is required for binding of fungal conidia to host extracellular matrix proteins (fibronectin and laminin) and pneumocytes (Hamilton *et al*., 1998; Vanittanakom *et al*., 2006; Lao *et al*., 2013). In addition, conidia of *P. marneffei* can also bind to glycosaminoglycans including chondroitin sulfate B and heparin (Cooper and Vanittanakom, 2008).

After host establishment, fungal conidia have to encounter host immune cells especially alveolar macrophages. The interaction of *P. marneffei* conidia with host leukocytes and alveolar macrophages has been investigated. The conidia are able to bind to human monocyte-derived macrophage in the absence of opsonization. Unlike *Histoplasma capsulatum* and other unopsonized fungi, this binding is independent on divalent cation, the β chain of the CD18 complex, complement receptor 3 (CD11b/CD18), and LPS receptor (CD14). Nevertheless, the binding is inhibited in the presence of wheat germ agglutinin, a lectin that has affinity to N-acetyl-β-D-glucosaminyl residues and N-acetyl-β-D-gluconsamine oligomers suggesting that the major receptor(s) of *P. marneffei* conidia is a glycoprotein containing N-acetyl- β-D-glucosaminyl groups (Rongrungruang and Levitz, 1999). In immunocompetent host, after phagocytosis by macrophagees, *P. marneffei* are cleared via the L-arginine-dependent nitric oxide (NO) pathway stimulated with T-cell derived IFN-γ (Cooper and Vanittanakom, 2008). In addition, conidia of *P. marneffei* are able to stimulate respiratory burst of human peripheral blood mononuclear cell (PBMC) in the absence of opsonin and the secretion of tumor necrosis factor gamma (TNF-γ) in the presence of serum factors (Rongrungruang and Levitz, 1999). In immunocompromised patients, *P. marneffei* conidia can survive and transform into a yeast-like form inside macrophage with unknown mechanism. However, this might be the result of the expression of genes encoding acid phosphatase, SodA, CpeA and Hsp70 that can reduce host antifungal activity or improve survival of this fungus under oxidative stress inside macrophage (Cooper and Vanittanakom, 2008). The role of T lymphocytes of host immune against *P. marneffei* infection has also been studied in murine model. It has been shown that CD4$^+$ T cells play a crucial role in host defense against this fungus (Viviani and Vanittanakom, 2010). Hence, the systemic *P. marneffei* infection in AIDS patients may due to the decrease of CD4$^+$ T cell number.

2.3 Potential virulence factors of *Penicillium marneffei*

Enzymes or genes encoding proteins involved in survival inside host macrophages or inhibit antifungal activity of host phagocytes might act as potential virulence factors of *P. marneffei*. Superoxide dismutases (SODs) are enzymes that converts superoxide radicals which is a precursor for other reactive oxygen species (ROS) to hydrogen peroxide (H_2O_2) and oxygen (Cooper and Vanittanakom, 2008; Morano *et al*., 2012). These enzymes are ubiquitous antioxidants in the cells and are different in intracellular locations. SODs from different organisms require different metal cofactors and their activities depend on redox cycling of the bound metal cofactor. In budding yeast, *S. cerevisiae*, there are two types of SODs that play different roles during oxidative stress conditions including a cytoplasmic Cu, Zn-SOD (Sod1) and a mitochondrial matrix Mn-SOD (Sod2) (Morano *et al*., 2012). Catalase-peroxidase (Cpe) is a bifunctional enzyme that can either reduce H_2O_2 with peroxidase activity or convert H_2O_2 to O_2 and H_2O via catalase activity. Both SODs and Cpe are associated with intracellular survival and are concerned as the virulence factors of many pathogens such as *Mycobacterium tuberculosis, Candida albicans, Cryptococcus neoformans* and *Paracoccidioides braziliensis*. In *P. marneffei*, gene *sodA* and *cpeA* encoding putative Cu, Zn SOD and Cpe, respectively were identified (Thirach *et al*., 2007; Pongpom *et al*., 2005). The transcripts of these genes were found to upregulate during yeast growth and during macrophage infection suggesting the role of these genes in survival of this fungus inside macrophage phagsome. The other genes whose expressions are also increased during macrophage infection are *pakA* (*ste20* homologue) and *pakB* (*cla4* homologue) encoding for p21 activated kinases. PakA plays a crucial role at 37ºC which is a host temperature and involves in germinating of conidia during macrophage infection (Boyce and Andrianopoulos, 2011). The *pakB* gene expression is upregulated during macrophage infection and this gene is associated in formation of yeast cells inside macrophages.

Heat shock proteins (Hsp) are proteins that prevent damage of intracellular proteins upon temperature increase and play a crucial role in some pathogens during growth inside host cell. In *P. marneffei*, expression of *hsp70* gene encoding Hsp70 was upregulated during mycelial to yeast phase transition and during fungal cells encounter heat shock condition at 39ºC indicating its role in adaptation and heat stress response (Kummasook *et al*., 2007). In addition, transcript of gene coding for small heat shock protein, Hsp30 in yeast cells of *P. marneffei* was higher than in mycelial cell suggesting its role in adaptation inside host cells (Vanittanakom *et al*., 2009).

One of possible factors that might involve in virulence of some pathogenic fungi is pigment production. Fungal dark pigments, melanins, synthesized by either the dihydroxynapthalene or the 3, 4-dihydroxy-L-phenylalanine (L-DOPA) pathways are known as virulence factors in many pathogenic fungi. These melanins were found to participate in inhibition of phagocytic killing. It has been shown that L-DOPA melanin was produced by yeast cells of *P. marneffi in vivo* (Cooper and Vanittanakom, 2008). In addition, two polyketide synthase genes, *pks11* and *pks12*, encoding mitorubrinic acid and mitorubrinol yellow pigment biosynthesis of *P. marneffei* were identified. The results revealed that survival of *pks11* , *pks12* knockdown and *pks11pks12* double knockdown mutants in both J774 and THP1 macrophages were significantly decreased and survival of mice challenged with these mutants was significantly better than those were challenged with wild type strain indicating the role of yellow pigment biosynthesis in virulence factor of this fungus (Woo et al., 2012).

2.4 Molecular biology of *Penicillium marneffei* morphogenesis

2.4.1 Asexual development of *Penicillium marneffei*

The developmental program of conidiation or asexual development in *P. marneffei* is similar to the well-characterized *A. nidulans* in the regulated manner (Etxebeste *et al*., 2010). The program begins after expose of vegetative hyphae to the light and atmosphere at 25ºC (Cánovas and Andrianopoulos, 2007; Boyce and Andrianopoulos, 2013). The thickening of the wall of specific vegetative hyphae results in the foot cell. An aerial stalk cell wider than vegetative hyphae arises from the foot cell. This stalk is called conidiophore. In *Penicillium* spp., the conidophores often branch to produce second stalk cells (rama) (Cánovas and Andrianopoulos, 2007; Borneman *et al*., 2000). Inside these stalks, there are multiple nuclear divisions and the multiple budding of hypha-like cells or metulae. After entry of each nucleus into each metula, a septum is formed to separate metulae from the stalk cells. Three to five phialides apically bud from each metula by acropetal manner. These phialides produce chain of asexual spores or conidia in a basipetal mode of division (Cánovas and Andrianopoulos, 2007; Etxebeste *et al*., 2010).

Many genes involved in regulation of asexual development of *P. marneffei* have been characterized. The heterotrimeric Gα subunits GasA and GasC and protein downstream RasA are thought to regulate the onset of conidiation (Boyce and Andrianopoulos, 2013). These proteins activate a protein kinase A (PKA) signaling pathway that negatively controls the expression of gene *brlA* and *abaA* which are the primary regulator of asexual development and the downstream target regulator, respectively. The *brlA* gene encodes a C2H2-type transcription factor that regulates the expression of a number of conidiation specific genes. In *A. nidulans*, overexpression of BrlA in vegetative hyphae results in the production of conidia from the hypha tip, imitating the function of a phialide (Etxebeste *et al*., 2010). AbaA, a transcription factor, that controls phialide differentiation is the first known factor regulated by BrlA. This protein also activates expression of *wetA* gene desired for conidial pigmentation and integrity (Etxebeste *et al*., 2010). For *P. marneffei*, the *brlA* deletion mutant strain generates only conidiophore stalk, while the *abaA* deletion mutant fails to produce both phialide and conidia (Boyce and Andrianopoulos, 2013). Sensor hybrid histidine kinases (HHKs), DrkA and SlnA, are also involved in regulation of asexual development in *P. marneffei* via the *brlA* regulatory pathway. Deletion of these two genes reveals a delay in the onset of conidiation and a decrease in conidial density. In addition, proteins involved in actin polarization and septation also play a role in asexual development in *P. marneffei*. The Rho GTPase CflB is not only required for polarized growth of hyphae, but also involves in polarized growth of conidiophore structure. The p21-activated kinase protein (PAK), PakB, plays a crucial role in septation and cell separation of conidia from phialides (Boyce and Andrianopoulos, 2013).

2.4.2 Dimorphism and phase transition of *Penicillium marneffei*

All known species of genus *Penicillium* do not have dimorphism except *P. marneffei*. In *P. marneffei*, this process is controlled by temperature that influences the increase or decrease of particular gene expressions involved in cellular differentiation (Vanittanakom *et al*., 2006). Under proper conditions at 25ºC, uninucleated conidia of *P. marneffei* isotropically swell for 6 to 12 hours followed by polarized growth to produce germ tube. Germ tubes apically extend to form true hyphae and cross walls are produced subapically to generate individual cells. The older subapical cells are usually uninucleate, whereas the growing cellular compartments are multinucleate indicating that nuclear and cellular division of these active growing cells are uncoupled (Vanittanakom *et al*., 2006; Cánovas and Andrianopoulos, 2007). In addition, subapical cells can repolarize to form branches of filaments in apical extension manner to establish a mycelial network.

At 37ºC, *P. marneffei* conidia germinate to produce hyphae that are shorter and wider than those generated at 25ºC. These hyphae have more branching and cells and nuclear division become coupled within 48 hours (Vanittanakom *et al*., 2006). Hyphal cells are separated from each other by a double-layered septum and each cell contains single nucleus. In undisturbed condition, these cells called pre-arthroconidia remain attached to one another. Once these cells are slightly perturbed, cells can break apart along the midline of the double septum into separated cells called arthroconidia. At the same temperature, *P. marneffei* conidia start polarized growth for cell elongation. This is followed by nuclear division and cell dividing by fission to produce two uninucleate cells similar to cell division of *S. pombe* (Vanittanakom *et al*., 2006).

Arthroconidia formation and subsequent yeast cell production can occur in mold culture when the temperature shifts from 25ºC to 37ºC. This phase transition starts with couple division of nucleus and cell in apical cells of hyphal filament followed by arthroconidiogenesis and yeast cell formation, respectively. However, arthroconidia and yeast cell development are decreased when the temperature shifts from 37ºC to 25ºC. In this phase transition, nuclear and cellular divisions of apical growth are uncoupled similar to growth of mold phase (Vanittanakom *et al*., 2006). These indicate that phase transition in *P. marneffei* is temperature dependent and is reversible. However, no evidence has reported yeast-hyphal phase transition of *P. marneffei* in animal model and hyphae form has never been reported in patient infected with this fungus. It has been shown that after conidia are phagocytized by alveolar macrophages, conidia can survive as intracellular parasites and grow as yeast phase without first forming hyphae (Vanittanakom *et al*., 2006).

Molecular studies of *P. marneffei* phase transition have been reported. At 25ºC, both Rho GTPases orthologous to Cdc42 (CflA) and Rac (CflB) play a role in polarized growth of *P. marneffei* hyphae by recruiting actin and activating PAKs to control cellular division and proteins required for polarized growth (Boyce and Andrianopoulos, 2013). Another genes involved in hyphal formation are *pakB* and *tupA* encoding p21-activated kinase and asexual development transcriptional repressor, respectively. Binding of PakB to CflA or CflB and SfaD, the Gβ subunit of heterotrimeric G proteins, is required for hyphal morphogenesis signaling, whereas TupA is necessary for repressing transcription of genes expressed during yeast growth. When temperature increases to 37ºC or inside host cell, *P. marneffei* utilizes the sensor kinase protein to sense the environmental changing and activate the signaling pathways essential for the morphological transition to yeast growth (Boyce and Andrianopoulos, 2013). It has been shown that *slnA* and *drkA* genes encoding hybrid histidine kinases play a role in the generation of *P. marneffei* yeast cells *in vivo*. The *slnA* gene associates with the germination of conidia, whereas the *drkA* gene participates in dimorphic switching during macrophage infection (Boyce *et al*., 2011). The *myoB* and *pakB* genes also involve in yeast cell morphogenesis of *P. marneffei*. The *myoB* gene encodes the type II myosin in cytokinesis and is required for arthroconidiation. Procuct of *pakB* gene plays a role in yeast cell division during infection. PakB is found at septa and adjacent to the division site after cell wall deposition during yeast growth. However, in hyphal morphogenesis, this protein is localized at the tip of hyphal apex and might activate the polarisome (Boyce and Andrianopoulos, 2013).

2.5 Genome and mitochondrial genome of *Penicillium marneffei*

The numbers and sizes of *P. marneffei* chromosome isolated from arthoconidia of the 11 *P. marneffei* isolates have been determined using pulse-field gel electrophoresis (Yuen *et al*., 2003). The results demonstrate that *P. marneffei* genomes are composed of 6 to 12 telomeric fragments indicating three or more chromosomes. The genome size is about 17.8 to 26.2 Mb with 48.8 mol% G+C content. This genome size is relatively small comparing to other *Penicillium* species such as *P. chrysogenum*, *P. notatum* and *P. purpurogenum*. One explanation is that *P. marneffei* is the only one species that can cause the disease in susceptible host and the loss of some genes might occur during the adaptation to intracellular growth (Yuen *et al*., 2003). *P. marneffei* genome consists of genes encoding proteins involved in metabolism (33%), information transfer (59%) and compartmentalization (8%). Primary metabolic genes comprise of genes for carbohydrate metabolism (cytochrome P450 enzymes, glucokinase and enzymes in the glycolytic pathway), amino acid metabolism (aminotransferases and amino acid synthases), fatty acid metabolism (enzymes of fatty acid and phospholipid synthesis) and cell wall synthesis (endoglucanase and chitinase precursor). In addition, genes encoding for secondary metabolism (non-ribosomal peptide synthase and polyketide synthesis) are also found. Genes classified for information transfer include genes coding for ribosomal proteins, tRNA synthetase subunits and translation initiation and elongation factors. Another set of genes in this category are regulatory proteins associated in transcription, cell cycle control and differentiation as well as serine/threonine and tyrosine kinases, phosphatases and a class III adenylyl cyclase. For genes that encode compartmentalization proteins, a number of membrane transporters and permeases involved in transportation of carbohydrates, proteins, fatty acids and several multi-drug-resistance proteins similar to a fluconazole resistance protein are identified. Moreover, genes participated in mating type and mating pheromone similar to pheromone receptor gene *ste2* in *S. cerevisiae* are found even a teleomorph of *P. marneffei* has never been described. This suggests that *P. marneffei* might have a very short sexual stage in its life cycle. For ribosomal DNA (rDNA), the sequences of 18S and 28S RNAs are determined and the results demonstrate that the spacer regions of *P. marneffei* rDNA are highly similar to that of *Talaromyces* species.

Recently, the genome sequences and transcriptome profile during hyphal and yeast growth of *P. marneffei* PM1 were analyzed (Yang *et al*., 2013). The results reveal that genes involved in asexual development such as *brlA*, *gasC*, *stuA* and *abaA* which play a role in both asexual development and yeast growth are higher expressed at 25ºC than 37ºC. Genes participated in synthesis of melanin, a compound that plays a role in hindering the production of host cytokine production and apoptosis in macrophages in some pathogenic fungi are identified. There are two kinds of melanin, dihydroxynaphthalene-melanin (DHN-melanin) and pyomelanin. At 37ºC, genes involved in DHN-melanin biosynthesis are downregulated, whereas genes related to synthesis of pyomelanin are upregulated indicating the role of pyomelanin in pathogenicity of *P. marneffei*. Genome of *P. marneffei* also presents abundant of the tandem repeat sequences (TRSs) which are two or more adjacent copies of the same sequence of nucleotides. TRSs located in the coding region of genes result in repeated units of amino acids in the genes' protein products and expressions of 15 of 66 TRSs are found to be upregulated at 37ºC. The explanation is that these TRSs might play a crucial role in evading of fungal cells from host immune response. Surprisingly, very low amount of heat responsive genes (3.6%) and species-specific genes (13%) in *P. marneffei* are overexpressed at 37ºC.

P. marneffei mitochondrial DNA is a circular DNA of 3.5 kb with 25% G+C content (Woo *et al*., 2003). It contains genes encoding for 15 proteins including subunit of respiratory chain complexes (the cytochrome oxidase subunits I, II and III, apocytochrome *b* and the reduced nicotinamide adenine dinucleotide ubiquinone oxireductase (NAD) subunits), the ATPase subunits (ATP synthase subunits 6, 8, and 9), 28 tRNAs, the small and the large subunit rRNAs, and the ribosomal protein of the small ribosomal subunit. The cytochrome oxidase subunit I (*cox1*) gene, the NAD 1 (*nad1*) gene and the large subunit of ribosome (*rnl*) gene carry intron and each intron contains an open reading frame (ORF). The ORF in *rnl* gene encodes small ribosomal subunit (*rps*). The set of protein coding genes in *P. marneffei* mitochondrial genome is similar to that of *A. nidulans*. Phylogenetic tree analysis of predicted amino acid sequences from of protein coding genes from *P. marneffei* mitochondrial genome comparing with 24 other fungi was performed. The results demonstrate that closest relatives of *P. marneffei* are *A. nidulans* and other molds such as *Podospora anserina*, *Neurospora crassa*, and *Hypocrea jecorina* and *P. marneffei* is more distantly related to the yeasts including *Schizosaccharomyces* species, *Saccharomyces* species and *Candida* species. These indicate that the mitochondrial genome does not participate in the unique characterization of thermal dimorphism of *P. marneffei* and is more closely to those of molds than yeasts (Woo *et al*., 2003).

2.6 Oxidative stress in fungi

Molecular oxygen carries two unpaired electrons in its outer shell and is relatively unreactive and harmless in its ground state (Herrero *et al*., 2008; Morano *et al*., 2012). Nevertheless, when oxygen is reduced by one electron, the superoxide anion ($O_2^{\cdot -}$) is generated. The main source of $O_2^{\cdot -}$ in the cell is partial reduction oxygen in the respiration process (Gessler *et al*., 2007). Molecule of $O_2^{\cdot -}$ itself is not highly reactive, however, it is a precursor of most reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$) and a mediator in oxidative chain reactions. H_2O_2 is produced by the dismutation of $O_2^{\cdot -}$ and partial reduction of H_2O_2 in the presence of reduced transition metals generates the $^{\cdot}OH$ which is one of the strongest oxidants in nature. Natural sources of ROS are from environmental factors i.e. ionizing radiation (α-, β-, γ- and X-rays), UV radiation, visible light, temperature shifts and mechanical damage and from side reactions of normal aerobic metabolism such as mitochondrial respiration (Gessler *et al*., 2007; Morano *et al*., 2012). Endogenous ROS in yeast cells is also produced from several metabolic processes including peroxisomal fatty acid degradation in the β-oxidation pathway and oxidative deamination of amino acids by D-amino acid oxidases during using D-amino acids as a carbon source (Morano *et al*., 2012). Eukaryotes, utilize ROS to control proliferation, differentiation, extracellular signal transduction, ion transport and immune response. In immune cells, $O_2^{\cdot -}$ is produced by reduction of O_2 to NADPH by a conjugated enzyme complex NADPH-oxidase (NOX) including membrane-bound and cytosolic compounds. This results in respiratory burst that defends host cells against pathogenic invasion (Gessler *et al*., 2007).

After generation of ROS, the antioxidant enzymes and compounds are produced to detoxify and recover cellular damages caused by these toxic molecules. Nevertheless, when these cellular survival mechanisms of the cells are unable to handle the ROS, the oxidative stress occurs. Oxidative stress caused by ROS results in damage of cellular molecules composing of lipid peroxidation, protein oxidation, and DNA damage through DNA modification (Herrero *et al*., 2008; Morano *et al*., 2012). Therefore, the antioxidation systems are very important for organisms especially pathogens, because they have to encounter not only their own ROS but also those produced by host immune cells. Most fungi have defense mechanisms against ROS including both enzymatic and nonenzymatic antioxidants.

2.6.1 Enzymatic antioxidant systems

Fungi possess many protective enzymes to detoxify ROS produced by host or themselves including superoxide dismutases (SODs), catalases, peroxidases and peroxiredoxins.

1). Superoxide dismutases (SODs)

SODs catalyze the dismutation of superoxide anion to hydrogen peroxide (H_2O_2). These enzymes are thought to be the first line defense against oxidative stress in eukaryotes (Gessler *et al*., 2007). SODs are ubiquitous antioxidants, highly stable (withstand up to 100ºC for 1 min) and are resistant to wide range of pH. The activity of the enzymes depends on redox cycling of the bound metal cofactor. Fungi possess two SODs classified by their metal cofactor and intracellular location and they play different roles during oxidative stress conditions (Morano *et al*., 2012). A cytoplasmic Cu,Zn-SOD (Sod1) is found predominantly in cytoplasm and inner membrane space of mitochondria. Its function is to detoxify superoxide generated by respiration and externally-added oxidants such as paraquat or menadione (Herrero *et al*., 2008; Gessler *et al*., 2007). A mitochondrial matrix Mn-SOD (Sod2) plays a role in detoxification of superoxide generation from mitochondrial respiration during stationary phase growth. In *S. cerevisiae*, the expression of both Cu, Zn-SOD and Mn-SOD encoding genes was upregulated in the stationary growth phase to protect mitochondrial proteins from oxidative stress, whereas SOD activity of *Neurospora crassa* was increased during germination and transition to stationary phase (Gessler *et al*., 2007). In *P. marneffei*, the Cu, Zn-SOD encoding gene is characterized. The expression of this gene is found to accumulate in conidia and decreased in mycelia phase. Nevertheless, gene expression is upregulated during yeast phase and during macrophage infection suggesting the role of this gene in stress response of *P. marneffei* in adaptation to environment inside macrophage (Thirach *et al*., 2007).

2). Catalases

Catalases are the ubiquitous enzymes that decompose H_2O_2 generated by aerobic metabolism to H_2O and O_2. They are metallo-tatrameric enzymes containing a single heme per subunit (Gessler *et al*., 2007). There are two catalases in yeast including peroxisomal catalase A (Cta1) and cytosolic catalase T (Ctt1). Cta1 functions in detoxification of H_2O_2 produced by acyl-CoA oxidase during fatty acid β–oxidation at peroxisome, while Ctt1 is cytosolic and its expression is increased in response to various kinds of stresses i.e. heat, osmotic, nutrient starvation and H_2O_2 stresses (Herrero *et al*., 2008; Morano *et al*., 2012). *C. albicans* possesses a single catalase gene (*cat1*) whose expression is increased under oxidative stress *in vitro* and following phagocytosis by neutrophils *ex vivo*. However, deletion of this gene in both genome alleles does not reduce virulence of *C. albicans* in a murine model of candidiasis (Morano *et al*., 2012). There are at least two catalases presented in *A. nidulans*, CatA and CatB. The expression of CatA encoding gene is restricted to conidia, whereas CatB is produced in the mycelial phase. In other pathogenic fungi, it has been shown that catalase is able to inhibit the killing of

Aspergillus fumigatus, *Paracoccidioides brasiliensis* and *Blastomyces dermatitidis* by neutrophil and the killing of *Histoplasma capsulatum* by alveolar macrophages (Hamilton and Holdom, 1999).

3). Peroxidases

Peroxidases catalyze the reduction of organic and inorganic peroxides into the corresponding alcohols using cysteine thiols at their active sites. There are two types of eukaryotic peroxidases classified by their electron donors. Glutathione peroxidases (Gpxs) use the reducing power offered by GSH, whereas thioredoxin (Trx) peroxidases utilize Trxs as reductants (Herrero *et al*., 2008). Gpxs are divided into classical Gpxs that are multimeric, soluble and reduce both inorganic and organic hydroperoxides and phospholipid hydroperoxide Gpxs (PHGpxs) which are monomeric, membrane-associated and reduce both soluble hydroperoxides and lipid hydroperoxides in membranes. Unlike mammalian cells, yeast do not have classical Gpxs, but possesses three PHGpxs including Gpx1, Gpx2 and Gpx3 to protect membrane lipid from peroxidation (Gessler *et al*., 2007; Morano *et al*., 2012). In addition, yeast Gpxs does not contain selenium atoms in the composition of catalytic cysteine that are found in mammalian Gpxs. In *P. marneffei*, catalase-peroxidase (CpeA) encoding gene is isolated. Catalase-peroxidase is a bifunctional enzyme that is able to reduce H_2O_2 with either an external reductant (peroxidase function) or decompose it to H_2O and O_2 (catalase function). The expression of *P. marneffei cpeA* is upregulated at 37°C, in conidia and under H_2O_2 treatment suggesting the role of this gene in survival of this organism in host cell and oxidative stress response (Pongpom *et al*., 2005; Pongpom *et al*., 2013).

4). Peroxiredoxins (Prx)

Peroxiredoxins (Prx) are proteins that have multiple functions including stress protection, antioxidation, molecular chaperone and regulation of signal transduction (Morano *et al*., 2012). They reduce hydroperoxides to alcohols and also have peroxidase activity against H_2O_2, peroxynitrites, and many of organic hydroperoxides (Gessler *et al*., 2007). Based on the number of Cys residues in an active center, Prx are divided into 2 classes, the 1-Cys and 2-Cys Prx. However, their activity is similar including the reduction of their substrate, peroxide upon simultaneous oxidation of cysteine to sulfenic acid in their active center. Arginine is found in active center of all peroxiredoxins to stabilize the thiolate form of cysteine. The localizations of peroxiredoxins are cytosol and mitochondria and are also found to associated with nuclear and plasma membrane where they play a role in cell membrane protection against lipid peroxidation. There are three cytoplasmic 2-Cys Prx including Tsa1, Tsa2 and Ahp1. Tsa1 is a typical 2-Cys Prx that acts as an antioxidant in the hydroperoxide detoxification and also possesses a chaperone activity to promote heat and reductive stress resistances. In reduction of hydroperoxide, two Tsa1 are active as a dimer and a disulfide bond is formed between Cys of each subunit. Tsa2 plays similar roles as Tsa1, but the expression level is significantly lower. Unlike Tsa1 and Tsa2, Ahp1 does not play a major role in H_2O_2 antioxidant, but acts as an antioxidant against alkyl hydroperoxide (Morano *et al*., 2012). Unlike cytosol peroxiredoxins, activity of mitochondrial peroxiredoxins is pH-dependent and increases in response to acidic pH shift during transition to the stationary growth phase indicating their role in detoxification of ROS produced in the aerobic respiration process. In yeast, these proteins have a crucial role in H_2O_2 detoxification (Gessler *et al*., 2007).

2.6.2 Nonenzymatic antioxidants

Not only enzymatic systems, fungi produce several molecules that act as antioxidants to detoxify ROS.

1). Mannitol

Mannitol is used as an effective $^{\cdot}OH$ scavenger in laboratory. Fungi possess the acyclic hexitol mannitol and use it for osmotolerance (Gessler *et al*., 2007; Hamilton and Holdom, 1999). In *C. neoformans*, the mutant that produces low levels of mannitol is more susceptible to killing by polymorphonuclear cells than wild type and adding of extraneous mannitol to this mutant is able to shield it from $^{\cdot}OH$-generating model. This indicates the role of mannitol in protecting fungal cells from phagocyte killing via $^{\cdot}OH$ scavenging (Gessler *et al*., 2007; Hamilton and Holdom, 1999).

2). Vitamin E

Vitamin E is a molecule that has lipid-based radical chain breaking activity and contains scavenging capacity against free radicals including lipid peroxyl, alkoxyl and hydroxyl radicals (Herrero *et al*., 2008). Moreover, it has been shown that soluble form of vitamin E, Trolox, can decrease the levels of H_2O_2 and $O_2^{\cdot -}$ and regulate the activity of enzymatic antioxidants in yeast (Herrero *et al*., 2008).

3). Melanins

Melanins are dark brown or dark pigments with high molecular weight generated by the oxidative polymerization of phenolic compounds (Hamilton and Holdom, 1999). Melanins are located at the cell wall of mycelium and spore of fungi and protect fungal cells from ionizing radiation and host defense systems (Gessler *et al*., 2007). Melanin-deficient cells of *C. neoformans* are more susceptible to the antifungal effects of murine macrophages than the melanin producing cells (Hamilton and Holdom, 1999). It has been hypothesized that the antioxidant activity of melanins is due to their ability to bind metal ions that catalyze the free radical reaction (Gessler *et al*., 2007).

4). Glutathione (GSH)

Glutathione (GSH) is a thiol tripeptide involved in the metabolism of ROS, amino acid transport, nucleic acid synthesis and modulation of enzyme activity (Campos *et al*., 2005). This molecule protects the cells from oxidative stress by reacting with $^{\cdot}OH$ and hydroperoxides to produce water and corresponding alcohols, respectively. During reduction of hydroperoxides, GSH is oxidized to oxidized glutathione (GSSG) by glutathione peroxidase. GSSG is reduced back to GSH by glutathione reductase in the presence of NADPH (Costa and Moradas-Ferreira, 2001).

2.6.3 ROS compounds commonly used in laboratory

In yeast cells, there are two oxidative stress response systems. The first system functions in broad range of oxidative stress conditions, whereas the other one is required for oxidant-specific conditions. Therefore, only one oxidative compound cannot be used to study ROS response in fungi (Morano *et al*., 2012). There are many ROS compounds commonly used as models to investigate the oxidative stress response in these organisms.

1). Hydroperoxides

H_2O_2 is a byproduct of aerobic aspiration. This ubiquitous molecule functions as a signaling molecule required for regulation of many biological processes. Nevertheless, H_2O_2 is able to damage cells by promoting oxidative stress and have to be removed from cells to avoid the formation of highly reactive $^{\cdot}OH$ (Morano *et al*., 2012). H_2O_2 is detoxified to H_2O and O_2 by antioxidant enzyme, catalase (Gessler *et al*., 2007). Because of its water solubility and stability, H_2O_2 is commonly used as a model for oxidative stress condition.

To generate lipid peroxidation, organic hydroperoxides are used. These compounds includes cumene hydroperoxide ($C_9H_{12}O_2$), *tert*-butyl hydroperoxide [$(CH_3)_3COOH$] and linoleic acid hydroperoxide (LoaOOH). Cumene hydroperoxide is an aromatic lipid soluble hydroperoxide used as an intracellular source of ROS. This molecule is able to produce highly reactive free radicals that are high mutagenicity and toxicity (Morano *et al*., 2012). *tert*-butyl hydroperoxide is often used as a model for alkyl hydroperoxide condition, whereas linoleic acid hydroperoxide is used as lipid hydroperoxide model in yeast and is toxic to yeast cells at lower concentrations than H_2O_2 and other organic peroxide (Morano *et al*., 2012).

2). Superoxide anion

O_2^- is the major ROS product generated from electron leakage from the mitochondrial electron transport chain. This molecule is usually not highly reactive, but it is a precursor for H_2O_2 via dismutation and for $^{\cdot}OH$ via metal-catalyzed reactions (Morano *et al*., 2012). The redox-cycling drugs such as menodione and paraquat which can transfer electron to O_2 are frequently used as compounds for generating O_2^- in yeast cells (Morano *et al*., 2012).

3). Thiol-reactive compounds

Thiol-reactive compounds are compounds that indirectly bind to and deplete thiol groups as well as compounds that oxidize thiol groups. These activities result in generation of oxidative stress. The 1,-chloro-2,4-dinitrobenzene (CDNB) is a thiol-reactive compound that acts as a substrate for glutathione transferases and can deplete yeast cellular glutathione resulting in an accumulation of endogenous ROS (Morano *et al*., 2012). Diamide is a membrane-permeable compound and acts as a thiol-specific oxidant. This molecule is often used to induce oxidative stress in yeast by causing rapid oxidation of glutathione to the redox state (Morano *et al*., 2012).

4). Heavy metals

Heavy metals such as iron and cadmium are involved in generation of highly ROS in the cell. $^{\cdot}OH$ can be generated from H_2O_2 in the presence of reduced $Fe2^+$ via the Fenton reaction and from $O_2^{\cdot -}$ in the presence of oxidized $Fe3^+$ via the Haber-Weiss reaction. Cadmium is able to enter the cells and causes oxidative stress by depletion of glutathione and binding to sulhydryl groups (Morano *et al*., 2012).

2.7 Mitogen activated protein kinase (MAPK) pathways in fungi

To control the specific molecules inside the cells for cellular activities at a suitable place and time, a living cell requires the specific reaction between a particular molecule and its appropriate partners (Tanoue and Nishida, 2003). The mitogen-activated protein kinase (MAPK) pathways are the specific protein-protein interaction systems that transfer particular signals in the form of protein phosphorylation. Depending on their distinct signal transduction pathways, there are four subgroups of the MAPK cascades in mammalian cells, including ERK, ERK5, JNK/SAPK and p38. ERK is stimulated under mitogenic stimuli, whereas ERK5 is activated by growth factors, osmotic and oxidative stress. JNK/SAPK and p38 are mainly stimulated by stress stimuli or inflammatoty cytokines (Tanoue and Nishida, 2003). In addition, the MAPK pathways can be grouped by the presence of “X” in the regulatory TXY motif (threonine-any amino acid-tyrosine) such that glutamic acid (E) in ERKs, proline (P) in JNKs and glycine (G) in p38/Mpk2s. Dual phosphorylation of threonine and tyrosine at this motif is essential for the MAP kinase activation (Kumar *et al*., 1995).

The core structure of the MAPK pathways is quite conserved among eukaryotic cells. The structure is composed of three kinases, a MAPKKK or MEKK, a MAP kinase kinase (MAPKK) or MEK, and a MAP kinase (Figure 2.3). These proteins function by sequential phosphorylation (Román *et al*., 2007; Hohmann, 2002). The MAPKKK activates the MAPKK by phosphorylation on serine and threonine within a conserved N-terminal lobe of its kinase domain. The MAPKK subsequently phosphorylates and activates the MAP kinase on a threonine and tyrosine residues in the conserved TXY motif (Hohmann, 2002). Usually, phosphorylation of the MAP kinase activates its transfer from the cytosol to the nucleus. In the nucleus the MAP kinase stimulates the downstream effectors, transcription factors, by phosphorylation on serine/threonine followed by a proline resulting in the activation or repression of the corresponding target genes in response to environmental stimuli (Román *et al*., 2007;

Hohmann, 2002; Zhao *et al*., 2007). Nevertheless, a portion of activated MAP kinase also functions in the cytoplasm where it stimulates the function of other proteins such as protein kinases, cell cycle regulators and membrane proteins for activating the suitable cellular response (Smith *et al*., 2010).

The activation and repression of the MAPK cascades require the functions of the two-component signaling systems. These systems are firstly described in both pathogenic and non-pathogenic bacteria including a histidine kinase (HK) and a response regulator (RR). Usually, HK is a transmembrane protein that can autophosphorylate its own histidine molecule using ATP to response to environmental signal. The phosphate is subsequently transferred to aspartate residue on molecule of RR which normally acts as a transcription factor regulating gene expressions due to that environmental signal (Chauhan and Calderone, 2008). The phosphorelay systems in fungi have also been investigated in both yeast and filamentous mold (Hohmann, 2002; Hagiwara *et al*., 2008; Zhao *et al*., 2007). The major difference between prokaryotes and lower eukaryotes is the requirement of a histidine phosphotransfer protein (HPt) of the latter which functions in transferring phosphate from HK to RR (Chauhan and Calderone, 2008). In fungi, RR transfers the signal from the sensor kinase by activating the function of MAPK pathway (Figure 2.3). Fungal MAPK systems were firstly described in yeast *S. cerevisiae* (Zhao *et al*., 2007). It has been shown that *S. cerevisiae* possesses five MAPK pathways regulating mating, invasive growth, cell wall integrity, ascospore formation, and hyper-osmoregulation. In pathogenic fungi, the homologues of these pathways are also been investigated.

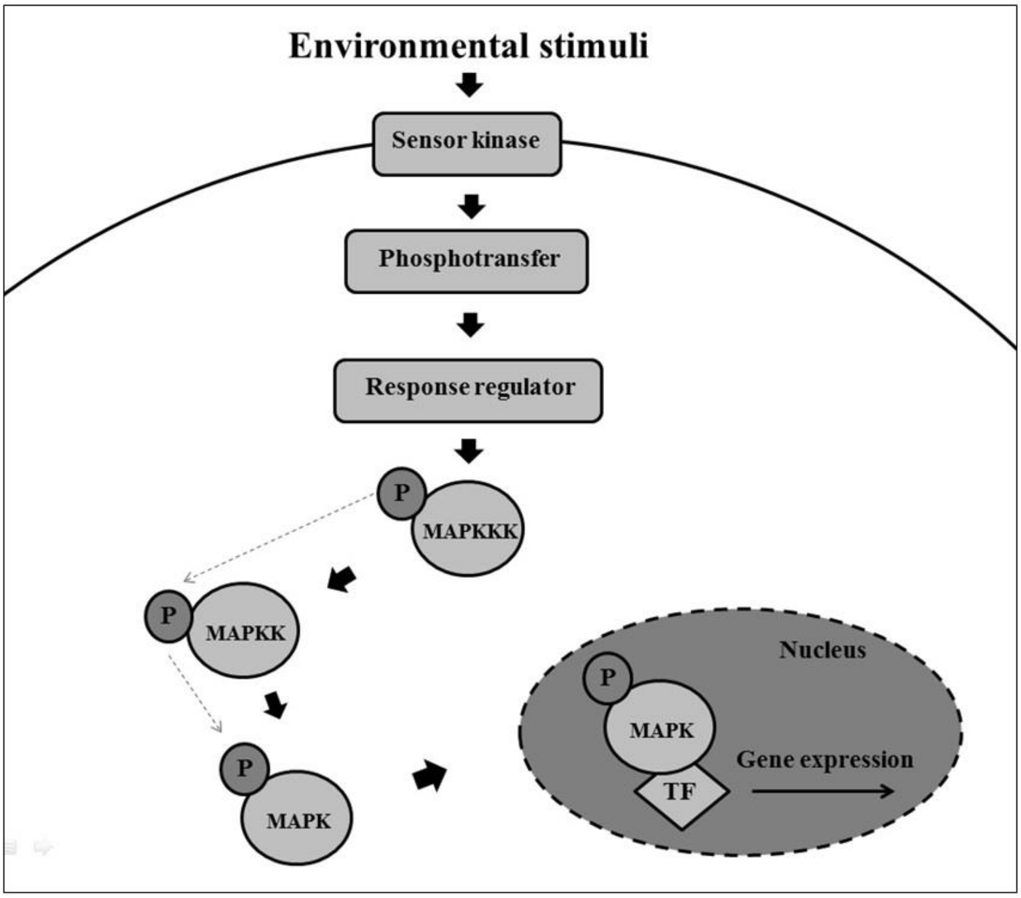

Figure 2.3 General diagram of phophorylay signaling pathway in fungi. The two-component system including histidine sensor kinase, phosphotransfer and response regulator senses the environmental signal and sends to the MAPK cascade. After activation, phosphorylated MAP kinase translocates from cytosol to the nucleus where it interacts and stimulates the downstream effector, transcription factor (TF). This activation results in upregulation or downregulation of specific gene expression in response to the stimuli (Adapted from Román *et al*., 2007).

2.7.1 The pheromone response and filamentation pathways

In *S. cerevisiae*, the pheromone response pathway or Ste (sterile) pathway is started by the binding of a peptide mating pheromone to a G-protein-coupled receptor (GPCR), Ste2 or Ste3 (Román *et al*., 2007; Xu, 2000; Zhao *et al.*, 2007). This binding results in the dissociation of an inhibitory Gα subunit (Gpa1) from stimulatory Gβγ subunits (Ste4, Ste18). The emancipated Gβ subsequently associates with a scaffold protein (Ste5) and a p21-activated protein (PAK) kinase, ste20. This protein complex is required for the activation of the Ste11-Ste7-Fus3/Kss1 MAPK pathway (Xu, 2000; Zhao *et al.*, 2007). The activated Fus3/Kss1 MAP kinase then translocates to nucleus where it activates the activity of the transcription factor, Ste12 and the cyclin-dependent kinase inhibitor, Far1 for controlling the mating process. There are several elements in the pheromone response cascade that play a role in the filamentation pathway including Ste20, Ste11, Ste7, Kss1, and Ste12. In *S. cerevisiae*, filamentous pseudohyphal development of a diploid cell occurs under nitrogen starvation, whereas invasive growth of a haploid cell is exhibited on rich media (Xu, 2000; Zhao *et al.*, 2007). In filamentation, the signal is sent from protein Ras2, Rho GTPase (Cdc42), 14-3-3 proteins (Bmh1, Bmh2), and Ste50 to activate the Kss1 MAPK cascade. After phosphorylation, Kss1 translocates to nucleus and stimulates the activities of transcription factors, Tec1 and Ste12 required for the transcription of genes participated in filament growth (Xu, 2000).

For filamentous and pathogenic fungi, homologues of the Fus3/Kss1 pathway have been investigated (Román *et al*., 2007; Zhao *et al*., 2007). It has been shown that several species of *Aspergillus* including *A. niger*, *A. nidulans* and *A. fumigatus*, possess Kss1 homologue, MpkB MAP kinase and Ste12 homolog, SteA. MpkB presents the TEY phosphorylation motif and its function has not been characterized, while SteA is essential for the development of ascogenous hyphae and cleistothecia (Zhao *et al.*, 2007). For *C. albicans*, the Kss1 homologue (Cek1) MAPK pathway has been identified. This MAPK cascade plays a role in virulence and yeast-hyphal transition (Román *et al*., 2007; Zhao *et al.*, 2007). *C. albicans cek1* mutants are attenuated both in the mouse systemic and in murine mastitis (superficial candidiasis) models and have a defect in transition from unicellular budding yeast growth to invasive hyphal growth on Spider or synthetic low-ammonium-dextrose (SLAD) medium. In addition, it has been shown that under different conditions that require active cell growth and cell wall remodeling of *C. albicans*, the transmembrane receptor, Sho1 plays a role in the Cek1 pathway activation. The *sho1* mutant has a defect in morphogenesis on media that induce hyphal growth and is more sensitive to oxidative and cell wall stress (Zhao *et al.*, 2007). The Fus3/Kss1 pathway homologue (Cpk1) has also been characterized in *C. neoformans*. The mating type-specific Ste11 and Ste12 homologues are found on *C. neoformans* mating type locus, however this mating type locus is not linked to the Cpk1 MAPK (Fus3/Kss1) and Ste7 homologues (Zhao *et al.*, 2007). The *C. neoformans cpk1* and *ste7* deletion mutants of both mating types *MATα* and *MATa* are impaired in mating and haploid fruiting but have virulence in disseminated mouse model similar to the wild-type strains indicating the role of Cpk1 MAPK pathway in mating and haploid fruiting but not in virulence. Overexpression of transcription factor, Ste12 can restore the defect of mating and haploid fruiting in the *cpk1* mutant. However, Ste12 is also found to play a role in virulence of *C. neoformans* in a serotype-specific manner. This suggests that Ste12 may function downstream from Cpk1 pathway in a non-strictly linear manner (Zhao *et al.*, 2007).

In *P. marneffei*, the Fus3/Kss1 pathway homologue has been identified. Comparing to *A. nidulans steA* and *C. neoformans ste12α* genes, *P. marneffei ste12* honologue, *stlA* possesses a distinct subclass of Ste12 homologues that have a C_2H_2 zinc-finger motif in addition to the homebox domain found in *ste12* genes. However, *P. marneffei* StlA is able to complement the defect in the sexual reproduction of the *A. nidulans steA* mutant, but is not required in asexual development and dimorphic switching (Zhao *et al.*, 2007; Borneman *et al*., 2001).

2.7.2 Cell wall integrity pathway

The Slt2 MAPK cascade of *S. cerevisiae* is involved in cell wall integrity and cell wall biosynthesis. In addition, this pathway also positively controls cell growth and differentiation and responds to environmental signals including high temperature, nutrient limitation, mating pheromones, alkaline pH and low osmolarity (Xu, 2000; Zhao *et al.*, 2007). The environmental signals are transmitted via surface sensors to a guanine exchange factor, Rom2 which transfers the signals to the GTP-binding protein, Rho1. Rho1 activates protein kinase C, Pkc1 which subsequently stimulates the Slt2 MAPK pathway including Bck1 MAPKKK, Mkk1 and Mkk2 MAPKKs, and Slt2 MAP kinase (Xu, 2000; Zhao *et al.*, 2007). Rlm1, Sbf and Swi6 are transcription factors that function downstream of MAPK pathway and regulate genes involved in cell wall synthesis and cell cycle (Zhao *et al.*, 2007).

In *A. nidulans*, the Slt2 MAPK pathway homologue, MpkA has been studied. *A. nidulans* contains *mpkA* gene that plays a crucial role in hyphal tip growth and conidial germination (Zhao *et al.*, 2007). For *C. albicans*, Slt2 homologue, Mkc1 participated in cell growth at high temperature (42ºC), regulation of cell wall integrity, morphological transition and pathogenesis (Román *et al.*, 2007; Zhao *et al.*, 2007). In addition, *C. albicans* Mkc1 is also activated by high concentration of calcium, low-temperature shock, antifungal drugs targeting to cell wall and membrane syntheses, various oxidant and certain osmotic stresses (Xu, 2000; Zhao *et al.*, 2007). In *C. neoformans*, several protein homologues in the Slt2 cascade have been identified including Rom2, Rho1, Mkk2, and Slt2 kinase (Mpk1). Similar to *C. albicans*, *C. neoformans* Mpk1 is involved in growth at elevate temperature (37ºC) and maintenance of cell wall integrity. Nevertheless, homologues of some yeast membrane-associated stress sensors have not been found in *C. neoformans* suggesting the different regulatory mechanism of cell integrity pathway in *C. neoformans* and other fungi (Zhao *et al.*, 2007).

In dimorphic fungus including *Blastomyces dermatitidis* and *Histoplasma capsulatum,* the histidine sensor kinase, Drk1 is involved in morphogenesis, cell wall intergrity and virulence (Román *et al*., 2007; Boyce *et al.*, 2011). In *P. marneffei,* sequence analysis demonstrates that *P. marneffei* genome contains Slt2 homologue, MpkA. In addition, deletions of two histidine sensor kinase encoding genes *slnA* and *drkA* result in defect of MpkA phosphorylation under cell wall stress, abnormal chitin deposition along the hyphae and frequent cell lysis. This indicates the role of *slnA* and *drkA* gene in cell wall integrity of *P. marneffei* (Boyce *et al.*, 2011).

2.7.3 The ascospore formation pathway

In *S. cerevisiae,* the ascospore formation or Smk1 pathway is essential for ascospore morphogenesis (Xu, 2000; Zhao *et al.*, 2007). It has been shown that the expression of Smk1 is activated by intracellular signals and occurs during the later stages of meiosis. This MAP kinase protein plays a role in regulation of ascospore assembly with unknown mechanism. The MAPKKK and MAPKK proteins upstream of Smk1 has not been identified, however the PAK kinase-like protein kinase, Sps1 seems to function upstream of Smk1 (Xu, 2000). Homologue of Smk1 in other fungi has not been found except for some ascomycetous fungi, such as filamentous pathogenic fungus *Ashbya gossypii* that causes stigmatomycosis in cotton and yeast *Kluyveromyces lactis* which has ability to assimilate and convert lactose to lactic acid (Zhao *et al.*, 2007).

2.7.4 Hyper-osmoregulation pathway

The high osmolarity glycerol (Hog1) MAPK pathway is the first stress-activated protein kinases (SAPKs) found in fungi. SAPKs are member of the MAPK family required for stress signaling in all eukaryotic cells (Ikner and Shiozaki, 2005; Smith *et al.*, 2010). In *S. cerevisiae,* this pathway plays a major role of growth under hyperosmotic conditions for maintaining the osmotic gradient of the plasma membrane by glycerol accumulation (Xu, 2000; Zhao *et al.*, 2007). In addition, it is also responsible for response to oxidative, heat, cold, citric acid, arsenite, methyl glyoxal, and weak acid stresses (Smith *et al.*, 2010). The Hog1 MAPK pathway is composed of the MAPKKK (suppressor of sensor kinase 2; Ssk2/Ssk22), the MAPKK (polymyxin B resistance protein 2, Pbs2), and the MAPK (Hog1). There are two branches of signaling systems required for activation of the Hog1 pathway, the Sln1 system and the Sho1 system. The Sln1 system is the two-component signaling system that is more sensitive and functions independently of the Sho1 pathway (Hohmann, 2002). In unstressed cells or under hypo-osmolarity condition, the membrane-bound histidine kinase, Sln1 is activated by autophosphorylation on a histidine residue at an H-box domain and the phosphate is transferred to an aspartate residue at a receiver domain of the same molecule or the different Sln1 molecule (Chauhan *et al.*, 2006; Hohmann, 2002). The phosphate is subsequently transferred to a histidine residue of the phospho transfer protein, Ypd1 and further transferred to an aspartate residue of the response regulator, Ssk1. Phosphorylated Ssk1 is unable to bind and activate Ssk2/Ssk22 of the Hog1 pathway (Chauhan *et al.*, 2006). Under stress conditions, the activation of the Sln1-Ypd1-Ssk1 pathway is inhibited. Binding of unphosphorylated Ssk1 activates the autophosphorylation of Ssk2/Ssk22 on a threonine residue resulting in the stimulation of the Hog1 MAPK pathway (Chauhan *et al.*, 2006; Hohmann, 2002). Signaling via Sho1 branch of the Hog1 pathway occurs as a protein complex at the cell surface. The Pbs2 protein of the Hog1 pathway binds to the SH3 domain of the transmembrane protein, Sho1 which is uncovered under stress conditions. This results in the recruitments of the Pbs2 and other proteins including a Rho-like G-protein (Cdc42), the p21-activated kinase (Ste20 or Cla4), and the MAPKKK (Ste11) to the cell surface (Hohmann, 2002). The forming of this signaling-component complex leads to the activation of Ste20 and the phosphorylation of Ste11 which subsequently phosphorylates and stimulates Pbs2. After the Hog1 pathway activation, phosphorylated Hog1 translocates to nucleus and regulates the transcription of genes responsible for cell adaptation (Chauhan *et al.*, 2006; Hohmann, 2002).

Fission yeast, *S. pombe*, possesses the Sty1/Spc1 MAPK pathway which is a homologue of the Hog1 pathway. The Sty1 pathway regulates the core environmental stress response (CESR) including hyperosmolarity, oxidative stress, heat shock, UV light, alkylating agents, and nutrient starvation (Hohmann, 2002; Ikner and Shiozaki, 2005; Smith *et al.*, 2010; Papadakis and Workman, 2014). A multistep phosphorylay system upstream of the Sty1 pathway includes the histidine kinases (Mak2 and Mak3) which do not have transmembrane domain found in Sln1, the phosphotransfer protein (Mpr or Spy1), and the aspartic-containing response regulator (Mcs4) (Hohmann, 2002; Papadakis and Workman, 2014). Under stress conditions, the Mak2/Mak3-Mpr-Mcs4 pathway transmits the signals to the Sty1 MAPK cascade. Mcs4, the Ssk1 homologue, directly binds and activates the MAPKKKs Wis4 and Win1 which subsequently stimulates the MAPKK Wis1 and the MAP kinase Sty1. After dual phosphorylation of a threonine-171 and a tyrosine-173 residue in a TXY motif, phosphorylated Sty1 translocates to the nucleus where it phosphorylates and activates the transcription factors in response to stress signals (Papadakis and Workman, 2014). There are three transcription factors involved in signaling thorough the Sty1 pathway including Atf1, Prr1, and Pap1 (Hohmann, 2002). Activating transcription factor, Atf1, is a bZip transcription factor closely relating to mammalian ATF2. Under environmental stresses such as oxidative or osmotic stress, Atf1 is activated by Sty1 and form heterodimer with another bZip transcription factor, Pcr1. This heterodimer binds to the CRE sequence (T[G/T]ACGT[C/A]A) of its target genes including catalase (*ctt1*), glycerol-3-phosphate dehydrogenase (*gpd1*), and trehalose-6-phosphate synthase (*tps1*) to regulate their transcriptions (Hohmann, 2002; Sakamoto *et al*., 2009; Wilkinson et al., 1996). Prr1 is a response regulator that has homology to Mcs4 and Skn7 which is a response regulator and a transcription factor in a Sln1-Ypd1-Skn7 pathway required for oxidative stress response and cell wall biosynthesis in *S. cerevisiae* (Vivancos *et al*., 2006; Chauhan *et al*., 2006; Ikner and Shiozaki, 2004). This protein is not directly involved in any upstream component of the Sty1 pathway, but it is found to control the expression of the Atf1 target gene, *ste11* in response to nitrogen starvation (Hohmann, 2002). Pap1 is a homologue of yeast activating protein 1 (Yap1) and mammalian AP-1 and is required for Sty1-dependent oxidative stress response. In stress-free condition, Pap1 is constitutively imported into the nucleus and exported to cytosol in a Crm1 (exporter)-dependent manner. Under oxidative stress, the export of Pap1 is inhibited by oxidant resulting in the localization of Pap1 inside the nucleus (Vivancos *et al*, 2006; Moye-Rowley, 2003). It has been shown that both Sty1 and Pap1 are essential for survival of *S. pombe* under oxidative stress. Pap1 is activated by moderate increase of H_2O_2 in Sty1-independent manner, whereas Sty1 responds to higher levels of the oxidant (Vivancos *et al.*, 2006; Papadakis and Workman, 2014). Nevertheless, Sty1 is found to have an effect on the nuclear localization of Pap1 with an unknown mechanism (Hohmann, 2002).

In filamentous fungi, *A. fumigatus*, *A. nidulans* and *A. oryzae*, the homologues of the Sty1 pathway including Ssk2/Ssk22, Pbs2 and SakA/HogA (Sty1 homologue) MAP kinase have been identified (Aguirre *et al.*, 2006; Kawasaki *et al.*, 2002; Du *et al.*, 2006). In *A. fumigatus, sakA* gene regulates the expressions of *dprA* gene associated with response to oxidative stress and resistance to phagocytic killing and *dprB* gene involved in osmotic and pH stress response. Moreover, *sakA* gene also controls morphogenesis of *A. fumigatus* such that it negatively regulates conidial germination under nitrogen starvation (Ma and Li, 2013). *A. nidulans sakA* is phosphorylated in asexual spore form and dephosphorylated during germination and is able to complement the function of sty1 under osmotic stress and cell elongation in *S. pombe spc1* (*sty1*) mutant (Kawasaki *et al.*, 2002). In addition, this gene also plays a role in repression of sexual development and is required for survival, oxidative and heat stress response of *A. nidulans* asexual spore (Kawasaki *et al.*, 2002). It has been shown that *A. nidulans* SakA translocates into the nucleus where it interacts with the bZip type transcription factor AtfA (Atf1 homologue) during conidiophore development and in response to oxidative stress of asexual spore and mycelia (Lara-Rojas *et al*., 2011). Both SakA and AtfA also regulate the expression of *catA* gene encoding spore-specific catalase during development and response to oxidative stress and nutrient starvation. Moreover, AtfA is found to involve in negative regulation of sexual development similar to SakA suggesting that these two proteins function in the same pathway (Lara-Rojas *et al*., 2011). In *A. oryzae*, *atfA* gene regulates trehalose content in conidia and glycerol content in germinating conidia (Sakamoto *et al*., 2009). Trehalose content is crucial for stress tolerance of conidia and is converted into glycerol during germination. In addition, *atfA* gene also involves in oxidative stress response, conidia germination and regulation of glutamate accumulation in conidia of *A. oryzae*.

In pathogenic fungus *C. albicans*, the two component signaling system homologues involved in stress response have been identified. There are three histidine kinases containing histidine kinase domain found in different related fungi (Smith *et al*., 2010). CaSln1 possesses histidine kinase homologous to Sln1 of *S. cerevisiae*, Chk1 is a homologue of the *S. pombe* Mak2 and Mak3, and Nik1 is homologous to the osmosensing Nik-1/Cos kinase in *N. crassa*. In addition, a single phosphotransfer protein (CaYpd1) and two response regulators (CaSsk1 and Skn7) have also been indicated. This two component signaling system is found to involve in regulation of the Hog1 MAPK pathway and virulence of *C. albicans* (Smith *et al*., 2010; Chauhan *et al*., 2006). *C. albicans* Hog1 MAPK pathway is composed of the MAPKKK CaSsk2, the MAPKK CaPbs2, and the MAP kinase CaHog1. It has been shown that the Hog1 pathway plays a crucial role in regulation of response to osmotic, oxidative and heavy metal stresses, morphogenesis, cell wall biosynthesis, chlamydospore formation and virulence in *C. albicans* (Alonso-Monge *et al*., 1999; Chauhan *et al*., 2006; Román *et al*., 2007; Enjalbert *et al*., 2006; Alonso-Monge *et al*, 2003). Moreover, the phosphorylation of Mkc1 MAP kinase involved in *C. albicans* cell wall integrity partially requires the CaHog1 pathway suggesting the relationship between two MAPK pathways (Zao *et al*., 2007). In pathogenic yeast *C.*

neoformans, the histidine kinase that activates the Hog1 MAPK pathway in response to stress has been investigated (Brown *et al*., 2007). The regulation of Pbs2-Hog1 MAPK cascade depends on the strain of *C. neoformans*. This MAPK pathway controls response to UV radiation and hyperosmotic stress in both a highly virulent serotype A H99 clinical isolate and less virulent laboratory-generated serotype D JEC21, but it regulates morphological differentiation, oxidative stress response and response to elevated temperature (40ºC) only in the virulence strain H99. Moreover, the Hog1 pathway also negatively regulates capsule and melanin synthesis and the mating process of H99 but not JEC21 strain (Zao *et al*., 2007; Bahn *et al*., 2005; Román *et al*., 2007). For downstream regulation, it has been shown that *C. neoforman atf1* gene is required for activation of thioredoxin (*trx*) gene under oxidative stress (Brown *et al*., 2007).

In pathogenic dimorphic fungi, a homologue of a histidine sensor kinase Sln1 (Drk1) has been identified in *B. dermatitidis* and *Histoplasma capsulatum*. However, the genome sequence analysis reveals that Drk1 of these fungi is a member of histidine kinase class that is different form the Sln1 histidine kinase found in *S. cerevisiae* (Boyce *et al*., 2011). *B. dermatitidis* Drk1 regulates the expression of the yeast-phase specific gene (*bad1*). In addition, Drk1 also play a role in transition at 37ºC and virulence of both *B. dermatitidis* and *H. capsulatum* (Boyce *et al*., 2011). In *P. marneffei*, it has been shown that the genome of this dimorphic fungus encodes two putative histidine sensor kinases that are homologous to both Drk1 (Drk1/Bos1/nikA homologue) found in *B. dermatitidis* and Sln1 (Sln1/tcsB homologue) found in *S. cerevisiae*. These two putative proteins are denoted DrkA and SlnA (Boyce *et al*., 2011). DrkA contains the HAMP domains that are specific to class III histidine kinases and lacks the transmembrane domains found in class VI histidine kinases. Both DrkA and SlnA are participated in osmoadaptation and SlnA also regulates the activation of the Hog1 pathway and phosphorylation of SakA under osmotic stress (Boyce *et al*., 2011). Moreover, *P. marneffei* genome also encodes other components involved in SAPK pathway including a putative phosphotransmitter (PMAA_040370), putative response regulators (Ssk1 and Skn7), putative MAPKKK (Ssk2 and Ssk22) and putative Hog1 (SakA) MAP kinase (Boyce *et al*., 2011; Lin *et al*, 2012). *P. marneffei* Skn7 is a response regulator and transcription factor required for oxidative stress response and the expressions of *ssk2* and hog1 (*sakA*) genes of SakA MAP kinase are increased during yeast phase suggesting the role of these genes inside host cell (Cao *et al*., 2009; Lin *et al*, 2012).

2.8 Genetic manipulation of Fungi

In fungi that act as industrial producer of primary and secondary metabolites, genetic manipulation tools have been developed to produce the improvement strains for biotechnical and pharmaceutical development (Kück and Hoff, 2010). For pathogenic fungi, these tools are used to characterize the functions of genes or proteins of interest in survival, virulence and pathogenicity. One of the methods for genetic manipulations used for functional genomic investigation in fungi is target gene deletion including homologous recombination methods and RNA interference (RNAi).

2.8.1 Homologous recombination methods

1). The split-marker system

Unlike DNA-mediated transformation in bacteria, self-replicating vectors in fungi are very rare and the transferred DNA of filamentous fungi is usually integrated ectopically into the fungal genomic DNA resulting in hundreds of transformants need to be screened (Kück and Hoff, 2010). Therefore, gene deletion experiment using homologous recombination method in filamentous fungi is time-consuming and laboring. The split-marker approach was invented to overcome these problems. This technique was first developed for yeast *S. cerevisiae* and was later applied in filamentous fungi. The concept of split-marker method is two PCR fragments containing 5’ and 3’ flanking regions of target gene and overlapping fragments of selectable marker gene generated by a two-step PCR reaction or by ligation-based fusion PCR are transformed into wild type strain. The first successful application was done in penicillin producer fungus, *Penicillium chrysogenum*. However, the efficiency is very low (0.14%). In 2003, Catlett and coworkers applied this method for more efficient result (Catlett *et al*., 2003). Their experiments were done in plant pathogens, *Cochliobolus heterostrophus* and *Gibberella zeae* and used hygromycin B resistance marker for selection. Two approaches including PCR fusion method and plasmid-based method were performed.

For PCR fusion method, only two rounds of PCR reactions are done and subcloning of the target sequences does not required (Catlett *et al*., 2003). In first round PCR, about 250-500 bp of 5’ and 3’ flanking regions of target gene and the overlapping fragments of selectable marker which is the hygromycin phosphotransferase cassette are separately amplified. PCR products from the first round PCR are used as templates for the second round PCR. The 5’ flanking region is mixed with one overlapping fragment and the 3’ flanking region is mixed with the other one. The 5’ extensions for primers used in amplifying the flanking regions contain nucleotide sequences that complementary to overlapping fragments of selectable marker. This allows the fusion between flanking region and overlapping fragments in the second round PCR using primers specific to flanking regions and selectable marker. One to two micrograms of two DNA fragments from the second round PCR are transformed into wild type strains using protoplast transformation. Three crossing-over incidents of two flanking regions and selectable marker gene result in substitution of target gene with the functional marker gene by homologous recombination (Kück and Hoff, 2010).

Plasmid-based method is used for longer flanking regions of target gene to avoid unknown PCR-induced mutations. Two plasmids are used and each plasmid comprises of partial fragment of selectable marker gene. The 5’ and 3’ flanking regions of target gene are separately amplified using specific primers containing recognition sequences of restriction enzymes for subcloning into each plasmid. The recombinant plasmids are digested to release the fragments containing flanking regions of target gene and partial sequence of selectable marker gene and transformed into the recipient strains (Kück and Hoff, 2010).

In *P. marneffei,* the split marker deletion strain was constructed to determine the functions of genes of interest such as *drkA* gene coding for the two-component histidine kinase involved in stress adaptation, asexual development, hyphal morphogenesis, cell wall integrity and dimorphic switching during macrophage infection (Boyce *et al.*, 2011).

2). Homologous recombination using a deficient non-homologous end joining strains

The limitations of split marker method are the decrease of transformation rates and the frequency of homologous recombination depends on the strains of recipients and/or genes of interest. One strategy used to increase the chance of homologous recombination is deletion of genes involved in non-homologous end joining (NHEJ) complex. The complex includes DNA-dependent protein kinase (DNA-PKcs), the DNA ligase IV-XRCC4 complex, the exonuclease Artemis, and the Ku70/80 heterodimer. The Ku protein is a heterodimeric protein complex and acts as a DNA end-binding factor (Krappmann, 2007). This complex binds to the ends of double-stranded DNA in a non-specific manner and is used as a platform for the subsequent recruitment of other protein complexes. The Ku70 and Ku80 components are the Ku subunits that are composed of a conserved core flanked by N-and C- terminal domains mediating the interactions with other proteins in the NHEJ pathway. It has been shown that disruption of either the *ku70* or *ku80* gene in *N. crassa* resulted in high degree of homologous recombination. The transformants exhibited integration at the target site were up to 100% when 2 kb of the 5’ and 3’ flanking regions were transformed to the recipient strain comparing to 10-30% found for wild type (Kück U and Hoff B, 2010). Nevertheless, the *ku* disruption strains are more susceptible to DNA-damaging agents including methyl methanesulfonate (MMS), ethyl methanesulfonate (EMS), and bleomycin than wild type strain. To improve this problem, disruptions of the other genes in NHEJ complex were investigated. Several studies demonstrated that elimination of gene encoding for DNA ligase IV (*lig4*), a homologue of the human lig4 and part of the DNA ligase IV-XRCC4 complex generated the strains that could increase the frequency of homologous recombination and had a weak susceptibility to DNA-damaging agents. This *lig4* deletion strain can be used as an alternative choice for the *ku* disruption strain. Therefore, the limitation of deficiency in NHEJ is the decrease function of fungal DNA repairing system.

2.8.2 RNA interference (RNAi) method

In eukaryotes, RNAi acts as a key of epigenetic mechanism to maintain genome stability and integrity and to protect cells form viruses (Billmyre *et al*., 2013). For genetic manipulation, RNAi method is used to downregulate genes of interest that are multi-copy or lethal. In the system, double stranded RNA (dsRNA) containing sequences that are complementary to genes of interest is generated by RNA dependent RNA polymerase (RdRp). This dsRNA is cleaved into small RNA interfering (siRNA) of 20-25 nt by double-strand RNA endonuclease, Dicer in cytoplasm. The small RNAs are loaded onto the RNA-induced silencing complex (RISC) or Argonaute and used to identify the complementary mRNA targets followed by the degradation of the target mRNAs (Billmyre *et al*., 2013; Kück U and Hoff B, 2010). The early application of RNAi in *C. neoformans* using sense and antisense RNA was reported. In the experiment, RNAi was used to downregulate expression of genes involved in biosynthesis of the polysaccharide capsule and downregulation of these genes resulted in avirulent strains of *C. neoformans* (Kück U and Hoff B, 2010). However, in this system, the downregulation of target gene expression is variable in individual transformants and strains used for studying gene functions have to be pre-selected. The vector systems were developed to solve these problems. The systems are composed of spacer sequences or intron between two target fragments that are inversely oriented. These results in double stranded RNA with hairpin structure giving stable and high silencing frequencies. In addition, vector systems are designed to downregulate a reporter gene and gene of interest, simultaneously allowing the transformants whose target gene is silenced are more easily to detect. The reporter genes applied to use with yeast and filamentous fungi including *ADE2*, *gfp* and *DsRed* genes (Kück U and Hoff B, 2010). The *ADE2* gene encodes phosphoribosylaminoimidazole carboxylase required for adenine biosynthesis and transformants with gene silencing can be determined by their pink colonies. The *gfp* and *DsRed* genes code for fluorescent proteins that the fluorescence of the transformants is decreased when genes are silenced. The limitation of these systems is the construction of vector containing stem-loop dsRNA is time-consuming and the vector can be propagated only in *Escherichia coli* stains with a *recA1* genotype. The alternative systems that are more easily constructed are dual-promoter plasmids in that a one target fragment is transcribed by divergently orientated fungal promoter sequences located at both sites. These result in two complementary single strand RNAs that can form a dsRNA. With these systems, RNAi gene library for testing the silence of a high number of target genes can be constructed. In vector systems, the transcriptions of target genes are not only controlled by the constitutive promoters but the inducible promoters can be used. The advantage of using inducible promoters is that the RNAi can be produced at specific time such as the *ku* gene silencing during transformation to generate homologous recombination transformants.

In addition, this system can be applied to explain developmental stages of the fungal life cycle in industrial processes (Kück U and Hoff B, 2010).

2.8.3 Selectable marker in filamentous fungi

1). Dominant selectable markers

The selectable markers used for fungal genetic manipulation have been developed in genus *Aspergillus*. It has been shown that some filamentous fungi are susceptible to several antibiotics produced by bacteria such as hygromycin and phleomycin/bleomycin (Brakhage and Langfelder, 2002). Hygromycin B is an aminoglycoside antibiotic that inhibits protein synthesis in both prokaryotes and eukaryotes and is synthesized by *Streptomyces hygroscopicus*. The *hph* gene encodes the hygromycin B phosphotransferase that phosphorylates hygromycin B molecule and inhibits its biological activity. The *hph* gene is frequently used as a selectable marker in *A. fumigatus* and other filamentous fungi. In the construct, the expression of this gene is usually under the control of the strong, constitutive promoter of *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) gene and the *trpC* terminator region. Bleomycin drug intercalates DNA strand of organisms resulting in DNA degradation. The *ble* gene codes for Ble protein that binds tightly to bleomycin preventing the DNA intercalating activity of this drug. This drug resistant gene is often used as the second selectable marker in strain that already carry the *hph* gene (Brakhage and Langfelder, 2002).

2). Auxotrophic markers

The auxotrophic markers can be used as the alternative selectable markers for transformation such as the *pyrG*, *sC* and *niaA* genes (Brakhage and Langfelder, 2002). The *pyrG* gene codes for the orotidine-5’-monophosphate decarboxylase enzyme required for the synthesis of uracil. The *pyrG* mutants are auxotrophic for uracil and are able to grow on media containing 5-fluoro-orotic acid (5’FOA). The *sC* gene encodes ATP sulfurylase. The *sC* mutant strains are unable to utilize sulfate as a sulfur source and resist to selenate which becomes toxic when is reduced inside the cell. The *niaA* gene codes for nitrate reductase. The mutant strains cannot use nitrate as the sole nitrogen source and resist to chlorate. The limitation of using auxotrophic markers is the mutants of these genes demonstrate significant reduction of virulence. Therefore, it is difficult to test the virulence in the deletion mutants.

3). Counter-selection systems

The counter-selection systems have been developed for the multiple gene deletions. One of these systems used in *Aspergillus* spp. and other fungi is the *ura*-blaster used to transform into a uracil-auxotrophic strain (Brakhage and Langfelder, 2002). In the construct, the *pyrG* gene is flanked by upstream and downstream flanking regions of target gene and in front of and behind the *pyrG* gene have to include direct repeats that can generate recombination. After transformation of this cassette, the selected transformants are uracil prototroph. The direct repeats of gene such as gene encoding neomycin phosphotransferase (*neo*) is used to split the *pyrG* gene from the transformant genome when the transformants are grown on medium supplemented with 5’FOA and uracil (the product of the *pyrG* gene converts 5’FOA to toxic compound). After losing the *pyrG* gene, the *ura*-blaster can be reused for the second round of transformation to delete other genes of interest.