II. Literature Reviews

Dimorphic fungi can generally be divided into three groups depending on the types of environmental factors triggering interconversion (Romano, 1966). The first group is temperature dependence dimorphism. In which temperture alone is sufficient to cause transformation, no nutritional or supplementary additions to growth media are necessary for yeast-mycelium-yeast morphogenesis. The example of dimorphic fungus in this group is *Paracoccidioides brasiliensis.* This species grows as yeasts at 37 °C and as mycelium at 20-25 °C. Major polysaccharide in yeast is α-1, 3-glucan whereas in mycelia is β-1, 3-glucan. Chitin and protein are also present. When the incubation temperature is shifted from 37 °C to 20 °C, the synthesis of α-glucan decreases and the synthesis of β-glucan increases. In addition, the activity of the enzyme β-1, 3-glucanase is higher in the mycelial form than in the yeast form and increases during the 37 °C to 20 °C shift. On the other hand, the activity of α-1, 3-glucanase is very low in both forms (Kanetsuna and Carbonell, 1970; Kanetsuna *et.al.*, 1972; Flores-Carreon *et.al.*, 1979). The yeast-phase cell contains α-glucan in an outer layer and chitin in an inner layer. β-glucan is presumed to be located at discrete budding sites in the wall, as in true yeasts such as *Saccharomyces cerevisiae.* At 37 °C, β-glucanase activity weakens the β-glucan at a budding site, and the weakened area balloons out as a bud. The relatively low activities of α- and β-glucanase result in a uniform weakening of the new wall, and spherical growth occurs. At 20 °C the increased activity of β-glucanase causes continued expansion at the budding site and the onset of tip growth. Apical growth is maintained by wall lysis

due to enhanced β-glucanase activity and by wall synthesis due to increased synthesis of β-glucans. The resulting wall is largely β-glucan with protein and chitin interwoven in an unlayered manner (Kanetsuna and Carbonall, 1970).

The second group is temperature and nutrient dependent dimorphism. The organisms included in this group are *Histoplasma capsulatum* and *Sporothrix schenckii. Histoplasma. capsulatum* also grows as a yeast at 37 °C, and mycelial growth results when the temperature is decreased to 25 °C. Campbell (1947) first described a blood agar medium containing cysteine which converted these fungi to yeast form many more isolates and more rapidly than blood agar alone. The inclusion of a sulfur containing component initiated a continuing series of investigations concerning the effect of sulfur, sulfides, mercaptans and Eh on the growth of the organism. Excellent conversion has been obtained on an egg-potato flour medium (Kurung and Yegian, 1954). The egg provided the sulfur compounds, and the potato flour probably detoxifided inhibitory fatty acid. The comparative nutrition of the two phases of *H. capsulatum* have been studied by many investigators. Some of them found the yeast phase required a minute amount of the sulfur containing vitamin biotin, whereas the hyphal stage did not have this requirement (Salvin, 1949). For maintenance of yeast-phase growth, some sulfur compound was necessary. This could be supplied by an organic sulfide or mercaptan. Sulfur-containing amino acids supported the best growth. In a medium containing 16 nonsulfur amino acids incubated at 37 °C the yeast converted to mycelium. However, if cystine or cysteine were incorporated in a salts glucose medium, yeast-phase

growth was maintained. Cystine and cysteine were the best organic sulfur compounds to support yeast growth. The tripeptide glutathione was less effective than cysteine. Among the several sulfur-containing organic molecules, lanthione has been found equal effectiveness to cysteine (Salvin, 1949). Pine formulated a synthetic medium containing salts and cysteine for the conversion of M to Y growth of *H.capsulatum*. Free -SH (sulfhydryl group) was found essential for initiating conversion, as SH-blocking agent (PCMS or parachloromercuriphenyl sulfonic acid) inhibited morphogenesis. He also noted that after 6 hours no free sulfhydryl could be detected in the media, but by that time conversion to yeast phase had already begun (Pine, 1954). The mycelium to yeast conversion occurs above 30 °C, with an optimum at 37 °C (Scheer, 1957). It has been suggested that the optimum temperature for mycelium to yeast transition in a given strain is directed dependent on genetic determinants that are strictly correlated with temperature (Medoff, 1986). It is clear that SH-containing compounds must be present in the culture medium to initiate mycelium to yeast transition. Scheer (1957) speculated that at least two factors would influence the mycelium to yeast phase transition (i) SH-containing compounds might represent a nutritional requirement in the yeast phase to compensate for an inability of the yeast phase to reduce sulfur-containing compounds at 37 °C; or (ii) -SH groups might regulate in some manner the redox potential of the growth medium necessary for yeast development (Scheer, 1957). The role of -SH group has been investigated the most thoroughly. Maresca *et.al.* (1978) reported that yeast and mycelial phases each take up ^{35}S-cystine at comparable rates at both 37 °C and 25 °C. while a yeast phase

mutant which was unable to take up cystine could still form mycelia. Thus cystine uptake was not required for the transition to the yeast phase nor for its maintenance. The enzyme cystine reductase that catalyzed the reduction of cystine to cysteine was localized at the plasmamembrane. This enzyme appeared during the transition from 25 °C to 37 °C and was present thereafter in yeast-cells. The enzyme seemed to be necessary for the maintenance of the yeast phase. Cells treated with an inhibitor of cystine reductase remain mycelia even at 37 °C. Thus, this enzyme might cause an increase in the level of cellular -SH groups, which were required for the transition of mycelium to yeast. Studies of cellular respiration rates during the transition from mycelium to yeast, Maresca *et.al.* (1978) demonstrated that there were three distinct metabolic changes. Following the temperature increase there was a rapid decrease in cell respiration and in the intracellular levels of cysteine and other amino acids. This might be due to a temperature induced increase in cellular redox potential. When the second stage was reached, respiration had completely stopped. It was at this stage that exogenous cysteine is required. Studies with isolated mitochondria showed that mitochondrial respiration was activated by cysteine. During the third stage the respiration rate rised, intracellular concentrations of cysteine and other amino acids increased, then the morphological transition was completed. It is thought that the requirement for cysteine to activate respiration and to maintain the yeast phase ensures that random temperature increases will not cause the morphological conversion and that the yeast phase will occur only in an appropriate host where cysteine is available (Maresca *et.al.*, 1981).

The third group of dimorphic fungi is nutrient-dependent dimorphism. Dimorphism in species such as *Candida albicans* and *Mucor rouxii* are regulated by environmental conditions other than temperature. *C. albicans* grows as yeast form at either 37 °C or 25 °C when incubated in culture media with high level of glucose, whereas the mycelial form develops in the presence of less readily utilizable carbon sources like glycogen. This shift from yeast to mycelium could be inhibited by the addition of cysteine, indicating the involvement of sulfhydryl groups (Romano, 1966). Micromolar concentrations of zinc inhibited mycelium formation at 25 °C but not at 37 °C (Bedell and Soll, 1979). *Mucor* spp. also exhibited nutrient-dependent dimorphism, but the key environmental factors appeared to be the level of CO_2 and hexose concentrations in the environment. *Mucor rouxii* grew as mycelial form when incubated either aerobically or anaerobically in the absence of CO_2. But when the CO_2 level reached 0.3 atm under anaerobic conditions or 0.9 atm under aerobic conditions, it grew as yeastlike form (Bartnicki-Garcia and Nickerson, 1962). Another important factor is the hexose concentration. Under anaerobic conditions and 0.3 atm CO_2, culture incubated on a medium containing a low glucose concentration developed as mycelium, whereas on a high glucose concentration yeast-like cells formed. In fact, CO_2 was not required at all for yeast development if the glucose level was high enough. Under aerobic conditions only mycelia were formed, regardless of the hexoselevels. The possible role of hexose was to inhibit hyphal development by inhibiting the initiation of apical growth in some way (Bartnicki-Garcia, 1968).

There are about 150 known species of *Penicillium*. Among them, *Penicillium marneffei* is the only species of *Penicillium* which is dimorphic (Pitt, 1979). This fungus is of interest to the medical mycologist because it is known to have pathogenic potential for both man (Disalvo *et.al.*, 1973; Segretain, 1962; Drouhet, 1993; Supparatpinyo *et.al.*, 1994) and animal (Capponi *et.al.*, 1956; Segretain, 1959). Segretain (1959) showed that *P. marneffei* needed an organic source of nitrogen for mycelial growth in a well defined synthetic chemical medium. Sodium nitrate and ammonium phosphate were not utilized. Optimal growth obtained when glucose was used as carbon source in the medium. The yeastlike phase of *P. marneffei in vivo* is the same in infected rodents and in infected human or *in vitro* when cultured at 37 °C on wort agar or Brain Heart Infusion agar. The pathological examination of biopsies shows fungal elements in the form of globose, ovoid and elongated yeast-like cells, are seen free and within macrophages. The multiplying cells have a central septum, but occasionally there is two septa, dividing the cell to two or three chambers. The cross-wall formation is somewhat similar to that observed in *Schizosaccharomyces* sp. No budding is visible. Aspects of the culturally induced mycelial-to-tissue phase transformation of *P. marneffei* were studies by electron microscopy of thin sections (Garrison and Boyd, 1973). The hyphal cell was observed to contain multiple, large lipid bodies scattered throughout the cytoplasm. The fine structure of the yeast-like phase of *P. marneffei* differed in certain aspects from that reported for yeast-like forms of *Blastomyces dermatitidis* (Edwards and Edwards, 1960), *Paracoccidioides brasiliensis* (Carbonell and Polack, 1963), *Histoplasma capsulatum* (Edwards

et.al., 1959), and *Sporothrix schenckii* (Lane *et.al.*, 1969). The tissue-phase cells of *P. marneffei* were usually elongated with one or more septa seen as the result of fission. The cell wall of *P. marneffei* tissue-phase appeared somewhat thinner than that of yeast-like cells of other dimorphic fungi and seemd to be composed of a single rather than a double layer (Garrison and Boyd, 1973). Morphological variation in pathogenic strains of *P. marneffei* isolated from patients in Thailand was studied by Pracharktam (1992). Variation in mycelium pigment was observed ranging from yellowish-green to orange with water soluble red pigment produced in every strain. Microscopic morphology of mycelial form was compared with that of the reference strain PLM 689. PLM 689 strain had only biverticillate penicilli, but all five strains from Thailand had both monoverticillate and biverticillate penicilli. By transmission electron microscopy, the yeast phase cells were rich in cytoplasmic structure. The nucleus was centrally located. Cytoplasmic organelles and storage bodies were arranged bipolarly. Mitochondria were scattered throughout the cytoplasm. The septum formation was initiated from the cell membrane as an early stage of cell division and underwent complete septum formation which subsequently gave rise to two similar daughter cells. Molecular epidemiology of *P.marneffei* was studied by restriction endonuclease analysis. Forty-six isolates of *P.marneffei* were differentiated into two DNA types on the basis of their restriction fragment length polymorphism. Of the 22 human isolates of *P.marneffei*, 16 (72.7%) were type I and 6 (27.3%) were type II. Of the 23 bamboo rat isolates, 20 from *Rhizomys sumatrensis* were type I and 3 from *Cannomys badius* were type II. The soil isolate was type II (Vanittanakom *et.al.*, 1996).

Histopathological and electron microscopical observations on experimental *Penicillium marneffei* infection were studied in mice. The conidia of *P.marneffei* were inoculated through the tail veins of BALB/c mice and BALB/c-nu/nu-SIc mice. In BALB/c mice, the conidia were phagocytosed by Kupffer cells soon after inoculation, and proliferated by fission in the cytoplasm. Marked proliferation of yeast cells was observed 7 and 14 days after inoculation. With proliferation of the fungus, the number of lysosomes in Kupffer cells increased, and numerous granulomas were formed in the liver. These granulomas consisted mainly of macrophages with yeast cells, together with a few polymorphonuclear leukocytes, lymphocytes and giant cells. From 28 days on yeast cells were gradually cleared from the granulomas, and 56 days after inoculation almost all the granulomas disappeared. In BALB/c-nu/nu-SIc mice, at an early stage of infection, similar pathological changes to those seen in BALB/c mice were observed. However, as the infection progressed, the number of granulomas continued to increase and yeast cells continued to proliferate although lymphocytes did not infiltrate these granulomas. With proliferation of yeast cells the liver tissue was replaced with both yeast cells engulfed by macrophages and extracellular yeasts and dissemination occurred (Cui, *et.al.*, 1997). The effect of nitric oxide (NO) and reactive nitrogen intermediates on the *in vitro* growth of *Penicillium marneffei* both in a cell-free system and in a novel macrophage culture system were investigated. In the cell-free system, NO that was chemically generated from $NaNO_2$ in acid media (pH 4 and 5) markly inhibited the growth of *P. marneffei*. On the contrary, inhibition of growth did not occurred in neutral medium (pH 7.4) in which NO was not produced. *P. marneffei* conidia were

phagocytized by nonstimulated murine J774 macrophages after 2 hrs. of incubation. During the following 24 hrs., *P. marneffei* grew as yeast-like cells replicating by fission in the J774 macrophages. The intracellular growth of *P. marneffei* damaged nonstimulated J774 macrophages, as confirmed by electron microscopy. When J774 cells were stimulated by gamma interferon and lipopolysaccharides, which led to enhanced production of reactive nitrogen intermediates, the percentage of yeast-like cells was significantly reduced and *P. marneffei* conidia were damaged in the J774 macrophages. The inhibition of NO synthesis by N-monomethyl-L-arginine restored the intracellular growth of *P. marneffei*. The inverse correlation between intramacrophage growth and the amount of nitrite detected in culture supernatants supported the hypothesis that the L-arginine-dependent NO pathway plays an important role in the murine macrophage immune-response against *P. marneffei* (Cogliati, *et.al.*,1997).