IV Results

IV.1 Sequential morphogenesis of *P. marneffei* conidia on Brain Heart Infusion agar (BHIA) at 37 °C

Observations on the morphogenesis of conidium to yeast-like phase had been carried out using light microscopy (Fig.4 - 7). Transformation began with germ tube production which occurred within 24 hours, elongation of hyphae and frequently septate formation to form intraseptal cells or arthroconidia - like cells . After detaching from hyphae, the arthroconidia then developed to become yeast-like cells which further divided by fission with one or two septa.

IV.2 Sequential morphogenesis of *P. marneffei* conidia in human blood

At 2 hours after inoculation, conidia of *P. marneffei* were phagocytosed by phagocytic cells. Transformation began with germination and elongation of hyphae. Germination occurred within 24 hours after inoculation. The hyphae then self-transformed by septum formation to form arthroconidia-like cells, which then gave rise to become yeast-like cells. The results are shown in Fig. 8 - 11.

IV.3 Effects of nitrogen sources on transformation of *P. marneffei*

At 25°C, *P. marneffei* grew as mycelial form and produced conidia on basal medium containing each nitrogen source. Red pigment production was reduced in all isolates when $(NH_4)_2 SO_4$ or L- arginine or L- cysteine was used as the nitrogen source. Microscopic morphology of *P. marneffei* on various nitrogen sources at 37°C are shown in Table 1 and Fig. 12-16. All isolates transformed into yeast-like cells on BHIA, numbers of yeast-like cells were scored as +3 to +4. No growth was found in all isolates when $NaNO_2$ was used as the nitrogen source. Few growth of only one isolate (isolate no.444) was observed on basal medium containing L-cystine with

slight amount of conversion. Transformation of *P. marneffei* from conidia to yeast-like cells was enhanced by the temperature of 37°C and some nitrogen compounds. Of all nitrogen compounds employed , L- glutamine stimulated conversion best (numbers of yeast-like cells of all isolates of *P. marneffei* were scored as +3 or +4). Other nitrogen compounds that were less effective to induce yeast-like development were L-asparagine, L-arginine and $(NH_4)_2SO_4$, respectively. Sequential transformation of conidia on basal medium containing nitrogen compounds occurred in the same manner as transformation on BHIA.

IV.4 Growth of *P. marneffei* on glutamine gradient plates (at 37°C)

At the lowest concentration of L-glutamine, all isolates of *P. marneffei* grew as yeast-like colonies with red pigment production. Creamy yeast-like colonies consisting entirely of fission yeasts were found at the highest concentration.The results are shown in Fig. 17 and 18.

IV.5 Effects of glutamine concentrations on transformation of *P. marneffei*

The results are shown in Table 2 and Fig.19 - 22. Production of yeast-like cells depended on concentrations of L-glutamine. Good conversion of all isolates occurred at the concentrations of 0.1 and 0.2 g. L-glutamine per 100 ml basal medium, the numbers of yeast-like cells were scored as +3 or +4.

IV.6 Assimilation reactions of yeast-like forms of *P. marneffei*

Yeast-like forms of *P. marneffei* assimilated glucose ,maltose and trehalose. Assimilation of glucose and maltose occurred within 24 hours which was observed by yellow zones of acid production and zones of growth around the discs. Assimilation of trehalose occurred more slowly than glucose and maltose. Hence, the effect of carbohydrate concentrations on transformation of *P. marneffei* would be tested with glucose and maltose.Assimilation reactions are shown in Table 3.

IV.7 Effects of carbon source concentrations on transformation of *P. marneffei*

The results are shown in Table 4 and Table 5. At 37°C, when L-glutamine (0.2 g. per 100 ml. medium) was used as the nitrogen source in the basal medium, the optimal concentrations of glucose or maltose for transformation of *P. marneffei* were 1 - 2 g% w/v or a C : N ratio of 10 : 1 to 20 : 1 (formation of yeast-like cells were scored as +3 to +4). Further increase of the concentration of each carbon source (3% or above) inhibited yeast-like production in all isolates.

IV. 8 The effect of temperature on transformation of *P. marneffei*

All seven isolates of *P. marneffei* were influenced by the temperature in the same pattern. No differences were observed among isolates obtained from patients, from soil and from the bamboo rat. The results are shown in Table 6. At 20°C, conidia developed slowly as mycelia without sporulation. Few yeast-like cells were observed at 33°C (the number of yeast-like cells was scored as +1 in all isolates). The optimal temperature for transformation of *P. marneffei* ranged between 35-37°C.At 39°C, transformation to yeast-like cells occurred but there was marked decrease of growth observed in all isolates. No growth was observed at 41°C.

IV.9 The effect of pH on transformation of *P. marneffei*

At 37°C, on BHIA slants, transformation to yeast-like cells of most isolates were enhanced at a pH range of 6.5 to 7.5. However, transformation of the isolate LUB 0601 occurred in a broader pH range (between 3.5 - 7.5). No growth was observed in the basic pH range of 8.5 to 9.0. The results are shown in Table 7.

IV.10 The effect of CO_2 on transformation of *P. marneffei* at 37°C

At 37°C, on BHIA or SDA slants, 5 - 10 % CO_2 has no effect on transformation of *P. marneffei* from conidia to become yeast-like cells. The results are shown in Table 8.

IV.11 Effects of sex steroid hormones on transformation of *P. marneffei*

There was no difference between transformation of *P. marneffei* on BHIA alone and on BHIA with ethanol (0.6 % v/v). Transformation of six of seven isolates were affected by the addition of testosterone only at the highest concentration (10^{-6} M). Formation of yeast-like cells was graded as +1. No such effect was found in other concentrations. Transformation of conidia of the isolate no. 233 was not inhibited by testosterone. 17-beta-estradiol had no effect on transformation of all isolates of *P. marneffei* at any concentration used in this study.The results are shown in Table 9 and Fig. 23-26.

IV.12 Chitin localization in *P. marneffei* using lectin labelling technique and Transmission Electron Microscope (TEM).

Wheat germ agglutinin conjugated to colloidal gold was reacted with chitin in whole cell sections of *P. marneffei*. Gold particles located in the regions of cell wall and in the septum of *P. marneffei*. Both yeast-like cells (cultured on BHIA or on basal salts medium supplemented with L-glutamine 0.2 g %) and hyphal cells (mycelial form) showed localization of chitin in the same regions. Gold particles were not found in control sections which were pretreated with chitinase enzyme. The results are shown in Fig. 27-34.

The numbers of mitochondria of cells in both mycelial phase and yeast-like phase varied between 2-7 mitochondria per cell. Hyphal cells of mycelial phase showed two types of mitochondria (Fig. 35), the small type which constricted in size

(0.15-0.2μm x 0.15-0.25 μm) and the large type (0.2-0.25 μm x 0.25-0.45 μm). The large typed mitochondria showed prominent cristae suggesting a high level of metabolic activity. In contrast, only the small type (0.1-0.2 μm x 0.15-0.25 μm) was found in yeast-like cells (Fig. 36).

Table 1. Effects of nitrogen sources on transformation of *P. marneffei*

Nitrogen sources	*P. marneffei* Isolates						
	500	518	LUB 0601	SB 0501	233	496	444
BHIA	+3	+4	+3	+3	+3	+3	+3
$NaNO_2$	NG	NG	NG	NG	NG	NG	NG
$NaNO_3$	+1	+1	+2	+1	+1	+1	+1
$(NH_4)_2SO_4$	+2	+3	+2	+3	+3	+1	+2
glycine	+1	+1	+2	+1	+1	+2	+1
glutamine	+3	+3	+3	+3	+4	+3	+3
arginine	+2	+3	+3	+2	+3	+2	+2
asparagine	+2	+2	+3	+3	+3	+3	+2
tyrosine	NG	+2*	NG	NG	+2*	+1*	+1*
cysteine	+1	+1	+2	+1	+1	+1	+1
cystine	NG	NG	NG	NG	NG	NG	+1*
phenylalanine	+1	+1	+1	+1	+1	+1	+1

Growth was scored as : +4 = heavy yeast-like growth (mycelium virtually absent)

+3 = heavy yeast-like growth (small amount of mycelium)

+2 = moderate yeast-like growth (moderate mycelium)

+1 = predominantly mycelium (slight amount of conversion)

0 = no conversion

Abbreviations : BHIA = Brain Heart Infusion Agar ; NG = no growth.

* = rare growth

Table 2 Effects of L-glutamine concentrations on transformation of *P. marneffei* at 37°C

P. marneffei isolates	Concentrations of L - glutamine (grams / 100 ml. basal medium)					
	.001	.005	.01	.05	.1	.2
500	+1	+1	+1	+2	+3	+3
518	+1	+1	+1	+2	+3	+3
LUB 0601	+1	+1	+2	+3	+3	+3
SB 0501	+1	+1	+1	+2	+3	+3
233	+1	+1	+2	+3	+4	+4
496	+1	+1	+2	+2	+3	+3
444	+1	+1	+2	+3	+3	+3

Growth was scored as : +4 = heavy yeast-like growth (mycelium virtually absent)

+3 = heavy yeast-like growth (small amount of mycelium)

+2 = moderate yeast-like growth (moderate mycelium)

+1 = predominantly mycelium (slight amount of conversion)

0 = no conversion

Table 3 Assimilation reactions of yeast-like forms of *P. marneffei*

P. marneffei	Sugars				
isolates	Glucose	Maltose	Sucrose	Lactose	Trehalose
500	+	+	-	-	+*
518	+	+	-	-	+*
LUB 0601	+	+	-	-	+*
SB 0501	+	+	-	-	+*
233	+	+	-	-	+*
496	+	+	-	-	+*
444	+	+	-	-	+*

* = Zones of acid production occurred more slowly.

Table 4 Effects of glucose concentrations on transformation of *P. marneffei* at 37°C

P. marneffei	% Glucose concentrations (w/v)				
isolates	0.5	1.0	2.0	3.0	4.0
500	+2	+3	+3	+2	+2
LUB 0601	+2	+3	+3	+2	+2
518	+2	+3	+4	+2	+2
233	+3	+4	+4	+3	+2
SB 0501	+2	+3	+3	+3	+2
444	+2	+3	+3	+2	+2
496	+2	+3	+3	+2	+2

Growth was scored as : +4 = heavy yeast-like growth (mycelium virtually absent)

+3 = heavy yeast-like growth (small amount of mycelium)

+2 = moderate yeast-like growth (moderate mycelium)

+1 = predominantly mycelium (slight amount of conversion)

0 = no conversion

Table 5 Effects of maltose concentrations on transformation of *P. marneffei* at 37°C

P. marneffei isolates	% Maltose concentrations (w/v)				
	0.5	1.0	2.0	3.0	4.0
500	+2	+3	+3	+2	+2
LUB 0601	+2	+3	+3	+3	+2
518	+2	+3	+3	+2	+2
233	+2	+3	+3	+3	+1
SB 0501	+2	+3	+3	+2	+2
444	+2	+3	+3	+2	+2
496	+2	+3	+3	+2	+2

Growth was scored as : +4 = heavy yeast-like growth (mycelium virtually absent)

+3 = heavy yeast-like growth (small amount of mycelium)

+2 = moderate yeast-like growth (moderate mycelium)

+1 = predominantly mycelium (slight amount of conversion)

0 = no conversion

Table 6 The effect of temperature on transformation of *P. marneffei*

P. marneffei	Temperature (°C)									
Isolates	20	22.5	25	27.5	30	33	35	37	39	41
500	0	0	0	0	0	+1	+3	+3	+3*	NG
LUB 0601	0	0	0	0	0	+1	+3	+3	+3*	NG
518	0	0	0	0	0	+1	+3	+3	+3*	NG
233	0	0	0	0	0	+1	+3	+3	+3*	NG
SB 0501	0	0	0	0	0	+1	+3	+3	+3*	NG
444	0	0	0	0	0	+1	+3	+3	+3*	NG
496	0	0	0	0	0	+1	+3	+3	+3*	NG

* = Marked decrease in the growth.

NG = No growth.

+4 = heavy yeast-like growth (mycelium virtually absent).

+3 = heavy yeast-like growth (small amount of mycelium).

+2 = moderate yeast-like growth (moderate mycelium).

+1 = predominantly mycelium (slight amount of conversion).

0 = no conversion.

Table 7 The effect of pH on transformation of *P. marneffei* at 37°C on BHIA

P. marneffei	pH						
isolates	3.5	4.5	5.5	6.5	7.5	8.5	9.5
444	+2	+2	+3	+3	+3	NG	NG
496	+2	+2	+3	+3	+3	NG	NG
518	+2	+2	+2	+3	+3	NG	NG
500	+2	+2	+2	+3	+3	NG	NG
233	+2	+2	+2	+3	+3	NG	NG
LUB 0601	+3	+3	+4	+4	+4	NG	NG
SB 0501	+2	+2	+2	+3	+3	NG	NG

Growth was scored as : +4 = heavy yeast-like growth (mycelium virtually absent)

+3 = heavy yeast-like growth (small amount of mycelium)

+2 = moderate yeast-like growth (moderate mycelium)

+1 = predominantly mycelium (slight amount of conversion)

0 = no conversion

Abbreviations : BHIA = Brain Heart Infusion Agar ; NG = no growth.

Table 8 The effect of CO_2 on transformation of *P. marneffei*

P. marneffei	BHIA		SDA	
isolates	5-10 % CO_2	without CO_2	5-10 % CO_2	without CO_2
500	+3	+3	+2	+2
LUB 0601	+3	+3	+2	+2
518	+4	+4	+2	+2
233	+3	+3	+2	+2
SB 0501	+3	+3	+2	+2
444	+3	+3	+2	+2
496	+3	+3	+2	+2

Growth was scored as : +4 = heavy yeast-like growth (mycelium virtually absent)

+3 = heavy yeast-like growth (small amount of mycelium)

+2 = moderate yeast-like growth (moderate mycelium)

+1 = predominantly mycelium (slight amount of conversion)

0 = no conversion

Abbreviations : BHIA = Brain Heart Infusion Agar

SDA = Sabouraud Dextrose Agar

Table 9 Effects of sex steroid hormones on transformation of *P. marneffei*.

P. marneffei	Control		10^{-6} M		10^{-8} M		10^{-10} M		10^{-12} M	
Isolates	BHIA	BHIA (EtOH 0.6%)	E	T	E	T	E	T	E	T
SB 0501	+3	+3	+3	+1	+3	+3	+3	+3	+3	+3
518	+3	+3	+3	+1	+3	+3	+3	+3	+3	+3
LUB 0601	+4	+4	+4	+1	+3	+4	+3	+3	+3	+3
496	+3	+3	+3	+1	+3	+3	+3	+3	+3	+3
233	+4	+4	+4	+3	+4	+3	+4	+4	+4	+4
444	+3	+3	+3	+1	+3	+3	+3	+3	+3	+3
500	+3	+3	+3	+1	+3	+3	+3	+3	+3	+3

Abbreviations : BHIA = Brain Heart Infusion Agar alone

BHIA (EtOH 0.6%) = Brain Heart Infusion Agar with ethanol

E = 17-beta-estradiol

T = Testosterone

+4 = heavy yeast-like growth (mycelium virtually absent)

+3 = heavy yeast-like growth (small amount of mycelium)

+2 = moderate yeast-like growth (moderate mycelium)

+1 = predominantly mycelium (slight amount of conversion)

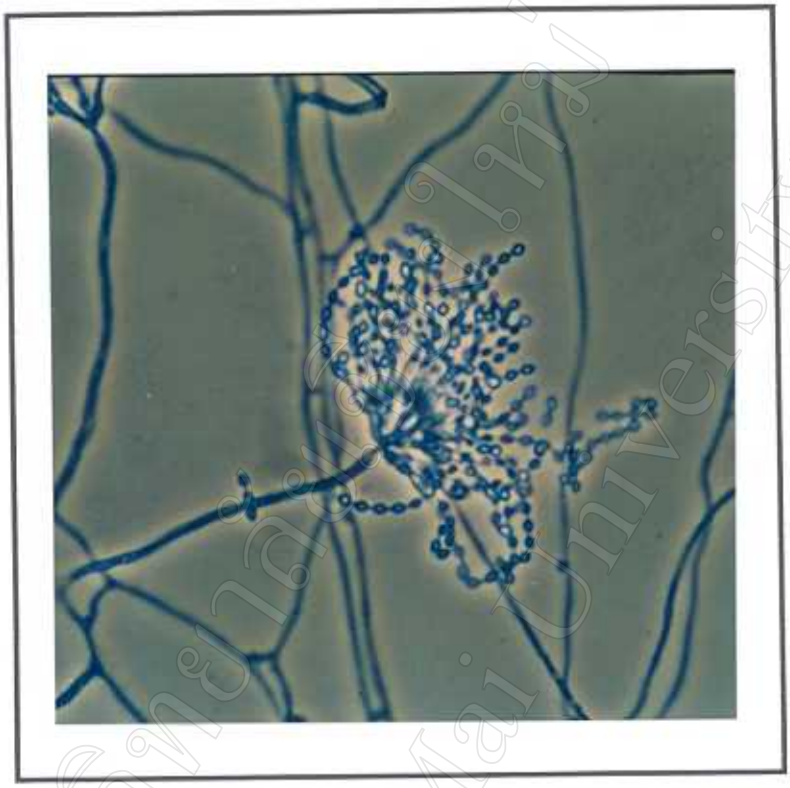

Figure 1. Microscopic morphology of *P. marneffei* cultured on SDA at 25°C (mycelial form) ; phase contrast, x600.

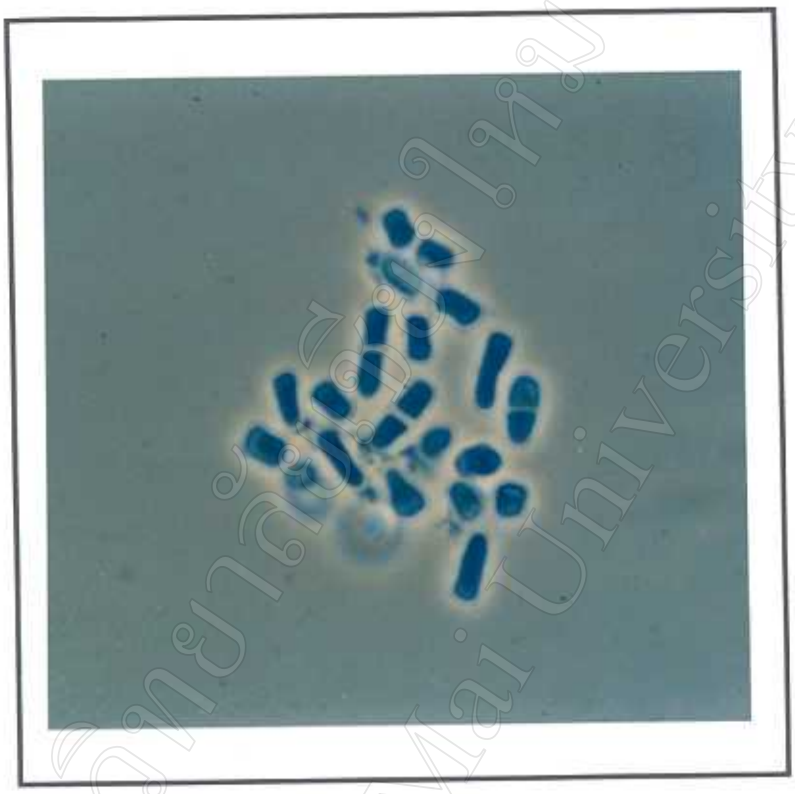

Figure 2. Microscopic morphology of *P. marneffei* cultured on BHIA at 37°C (yeast-like form) ; phase contrast, x1500.

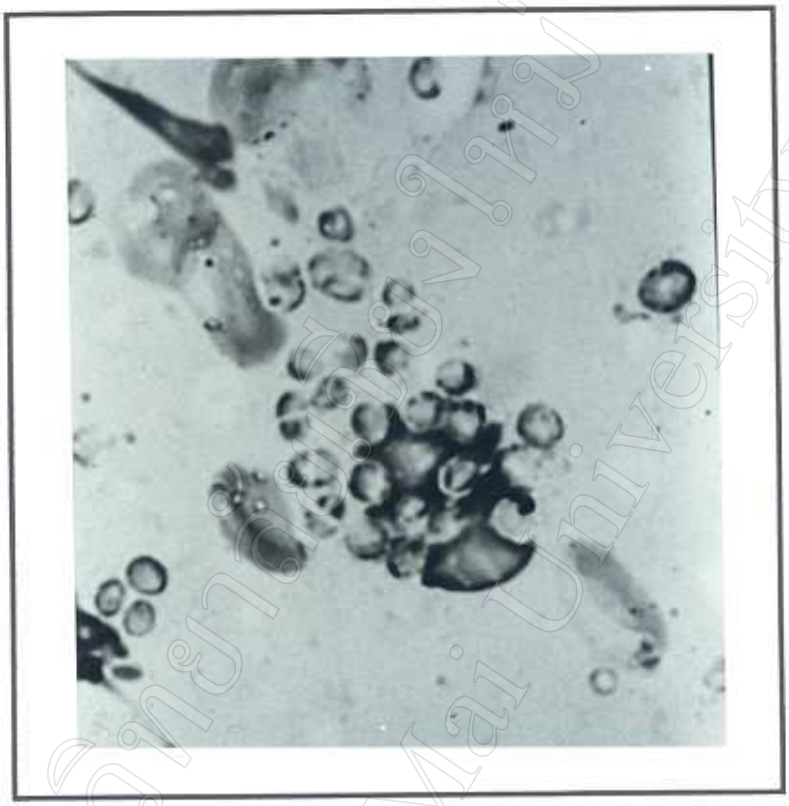

Figure 3. Yeast-like cells of *P. marneffei* in lesion of penicilliosis patient ; phase contrast, x1500.

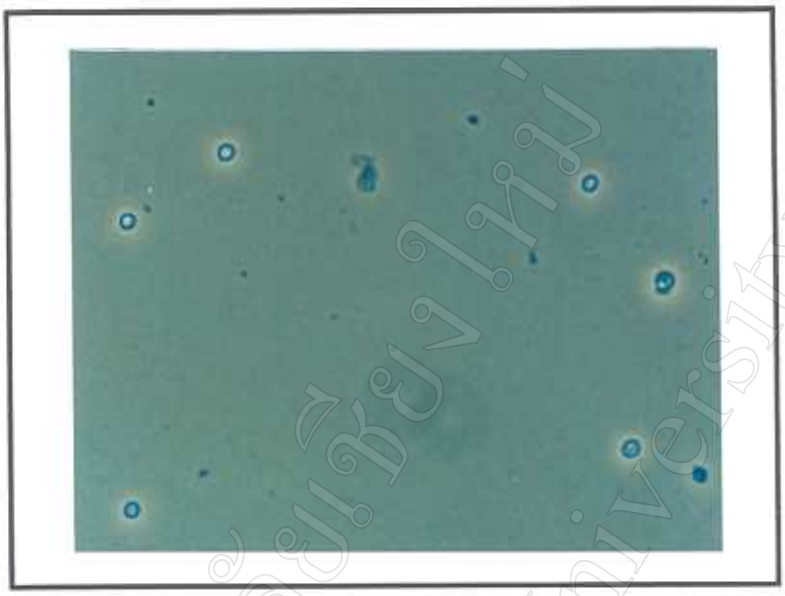

Figure 4. Transformation of *P. marneffei* on BHIA at 37°C showing conidia after inoculation ; phase contrast, x600.

Figure 5. Transformation of *P. marneffei* on BHIA at 37°C. Germination of conidia occurred at 24 hours after inoculation ; phase contrast, x600.

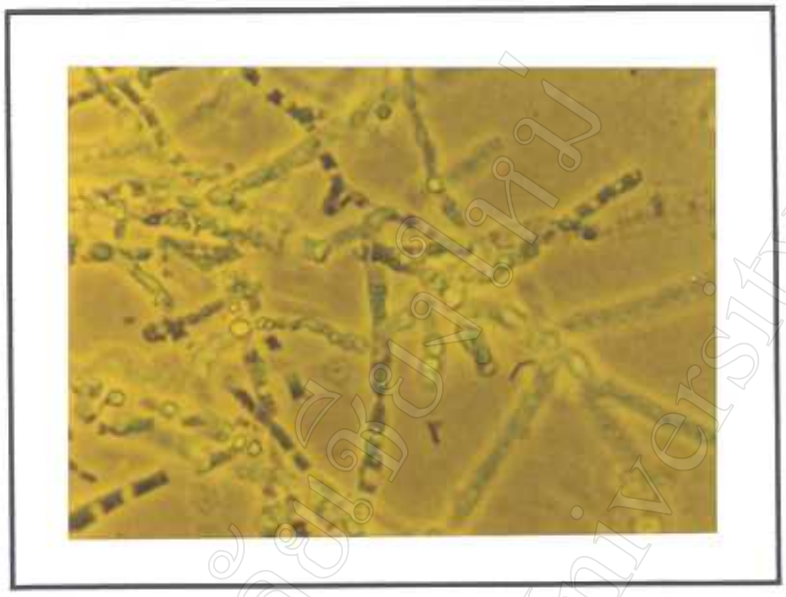

Figure 6 Transformation of *P. marneffei* on BHIA at 37°C. Elongation of hyphae and intraseptum formation, 5 days after inoculation ; phase contrast, x600.

Figure 7 Transformation of *P. marneffei* on BHIA at 37°C. Formation of intraseptal cells or arthroconidia-like cells, 10 days after inoculation; phase contrast, x600.

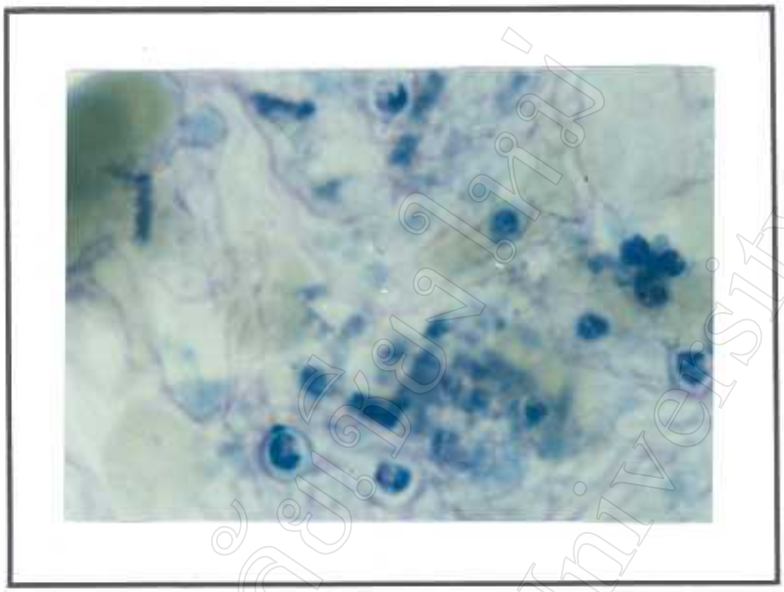

Figure 8 Transformation of P. *marneffei* in human blood at 37°C. Conidia were phagocytosed by phagocytic cells after inoculation ; phase contrast, x1500.

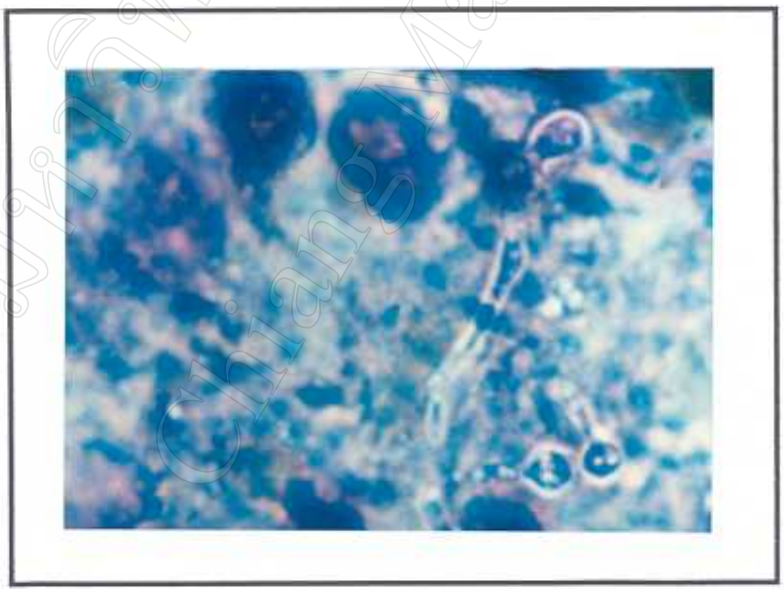

Figure 9 Transformation of *P. marneffei* in human blood. Germination of conidia occurred at 24 hours after inoculation ; phase contrast, x1500.

Figure 10 Transformation of *P. marneffei* in human blood. Elongation of hyphae and septum formation , 2 days after inoculation ; phase contrast, x1500.

Figure 11 Transformation of *P. marneffei* in human blood. Septum formation caused chains of arthroconidia-like cells, 4 days after inoculation ; phase contrast, x1500.

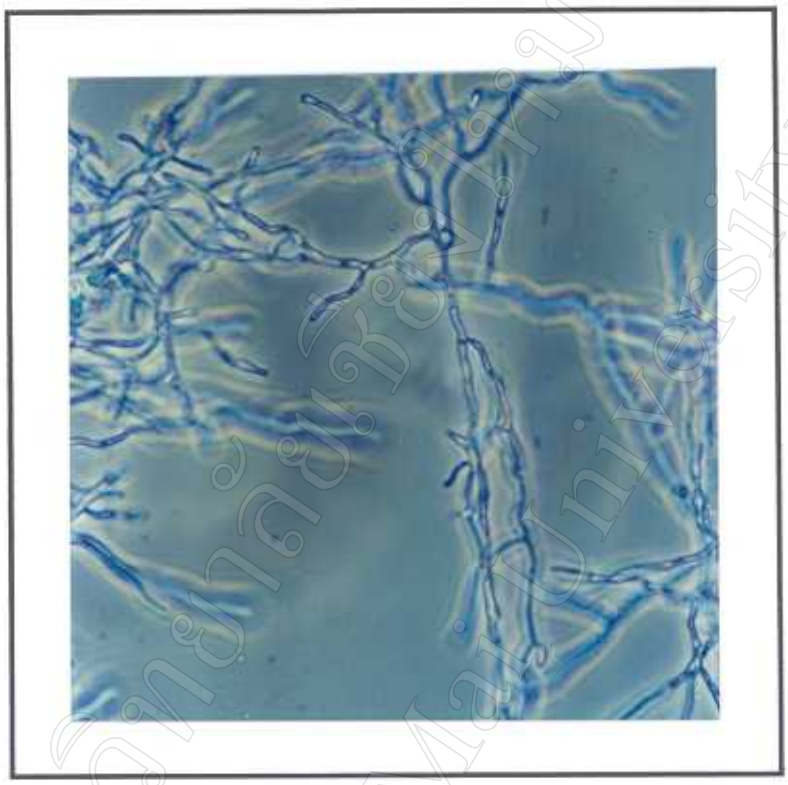

Figure 12 Microscopic morphology of *P. marneffei* cultured on basal medium supplemented with L-cysteine (0.1g%w/v) at 37°C, scored as +1 ; phase contrast, x600.

Figure 13 Microscopic morphology of *P. marneffei* cultured on basal medium supplemented with L-asparagine (0.1g% w/v) at 37°C, scored as +3 ; phase contrast x600.

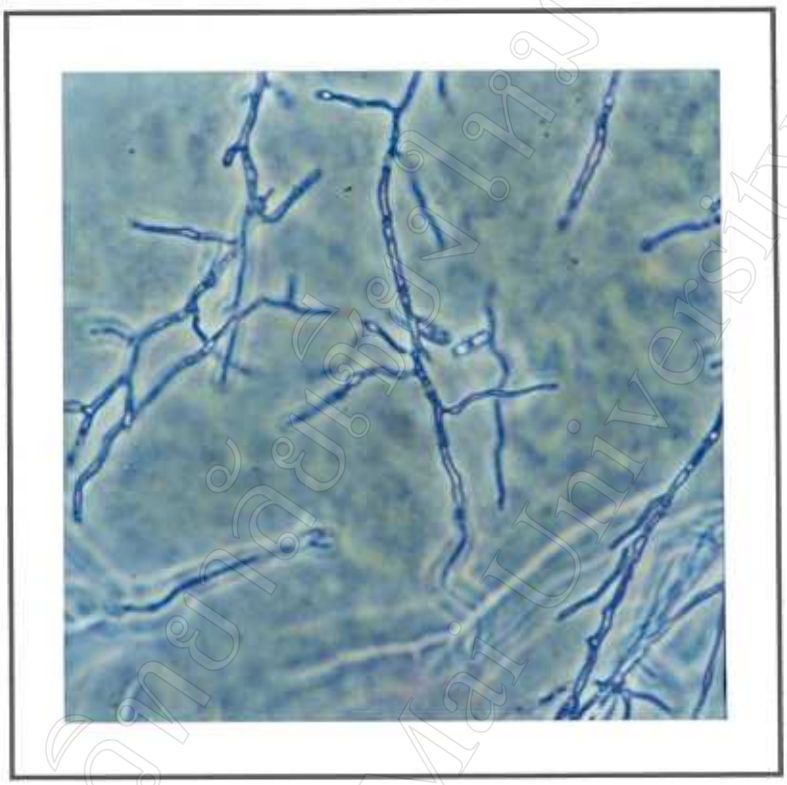

Figure 14 Microscopic morphology of *P. marneffei* cultured on basal medium supplemented with $NaNO_3$ (0.1g% w/v) at 37°C, scored as +1 ; phase contrast x 600.

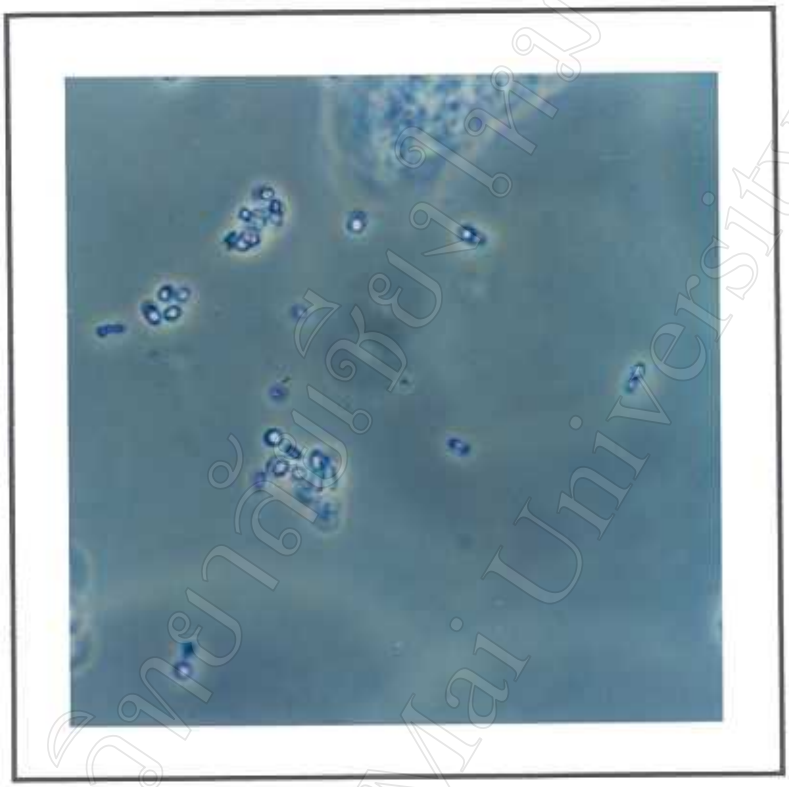

Figure 15 Microscopic morphology of *P. marneffei* cultured on basal medium supplemented with L-glutamine (0.1g% w/v) at 37°C, scored as +3 ; phase contrast x600.

Figure 16 Microscopic morphology of *P. marneffei* cultured on BHIA at 37°C, scored as +3 ; phase contrast x600.

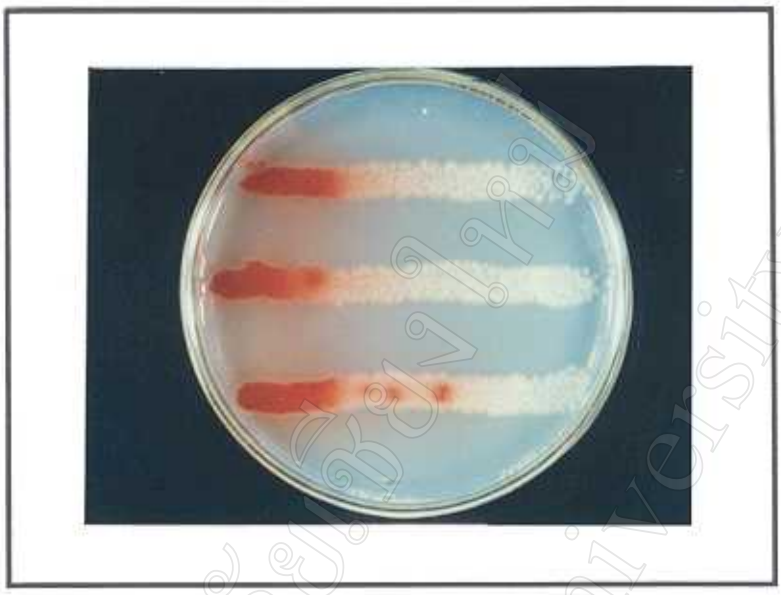

Figure 17 Growth on glutamine gradient plate of *P. marneffei* (isolate 518) at 37°C.

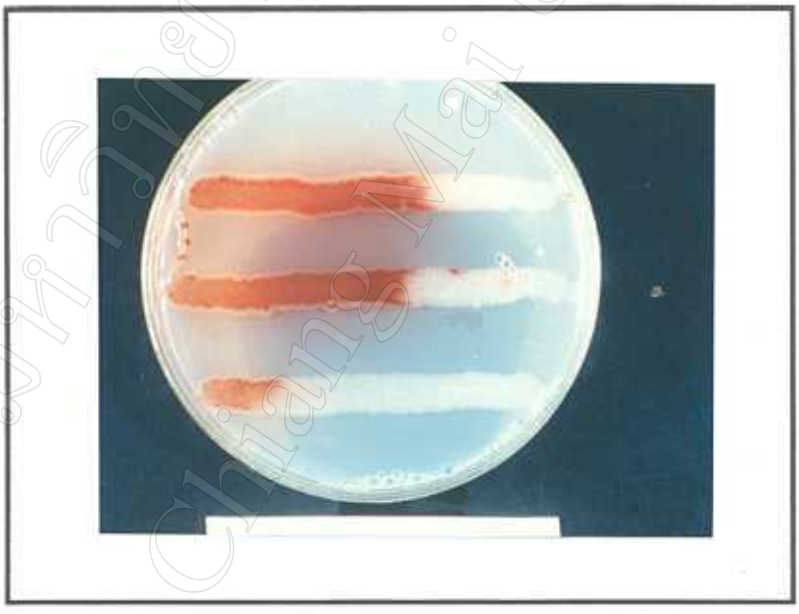

Figure 18 Growth on glutamine gradient plate of *P. marneffei* (isolate SB 0501) at 37°C.

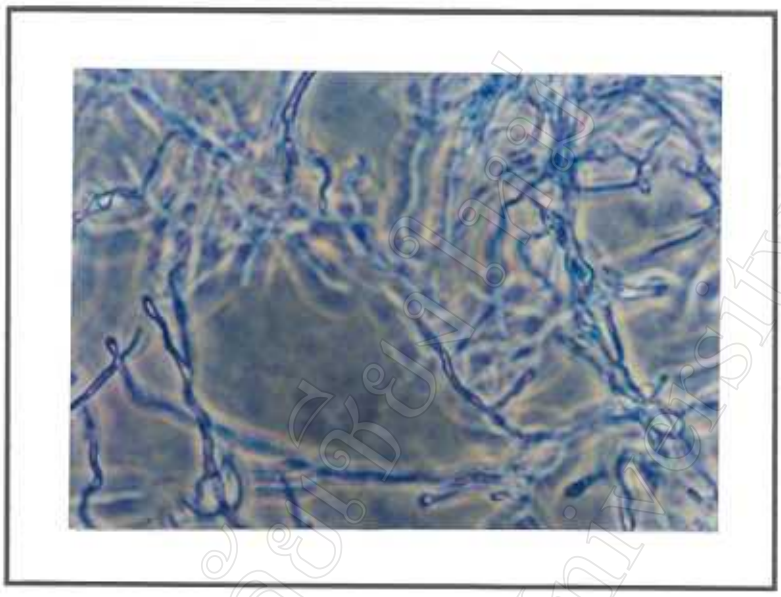

Figure 19 Microscopic morphology of *P. marneffei* cultured on basal medium supplemented with L-glutamine (0.01g% w/v) at 37°C, scored as +1 ; phase contrast x600.

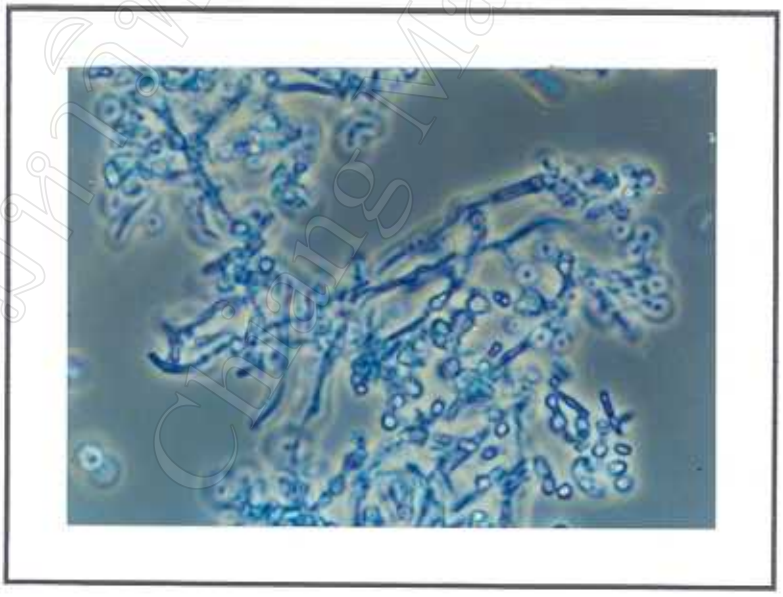

Figure 20 Microscopic morphology of *P. marneffei* cultured on basal medium supplemented with L-glutamine (0.05g% w/v) at 37°C, scored as +2 ; phase contrast x600.

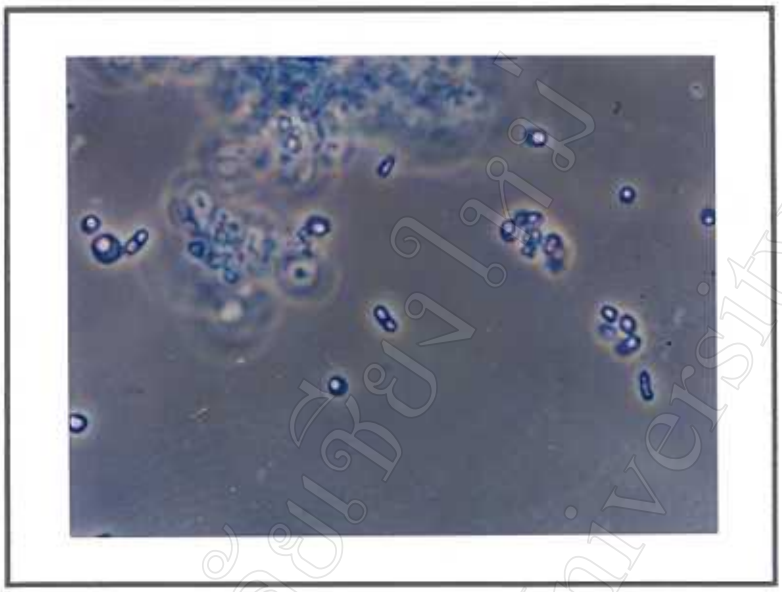

Figure 21 Microscopic morphology of *P. marneffei* cultured on basal medium supplemented with L-glutamine (0.1g% w/v) at 37°C, scored as +3 ; phase contrast x600.

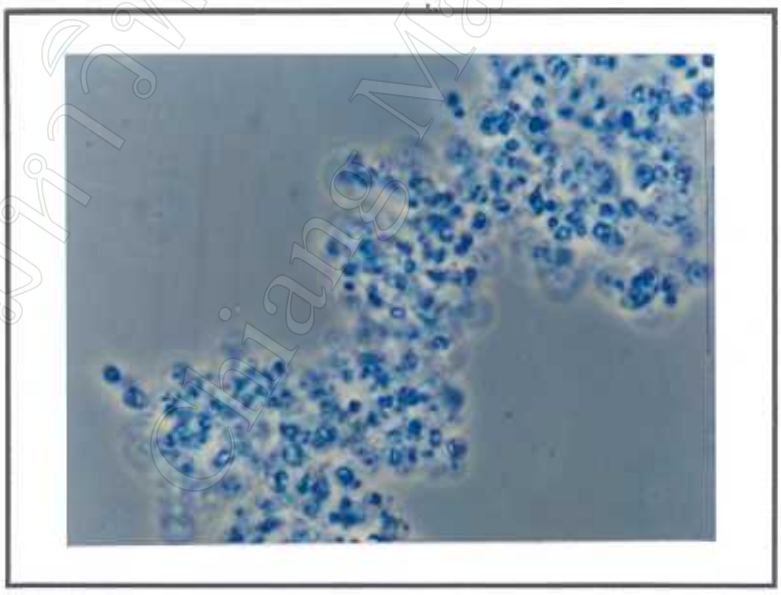

Figure 22 Microscopic morphology of *P. marneffei* cultured on basal medium supplemented with L-glutamine (0.2g% w/v) at 37°C, scored as +4 ; phase contrast x600.

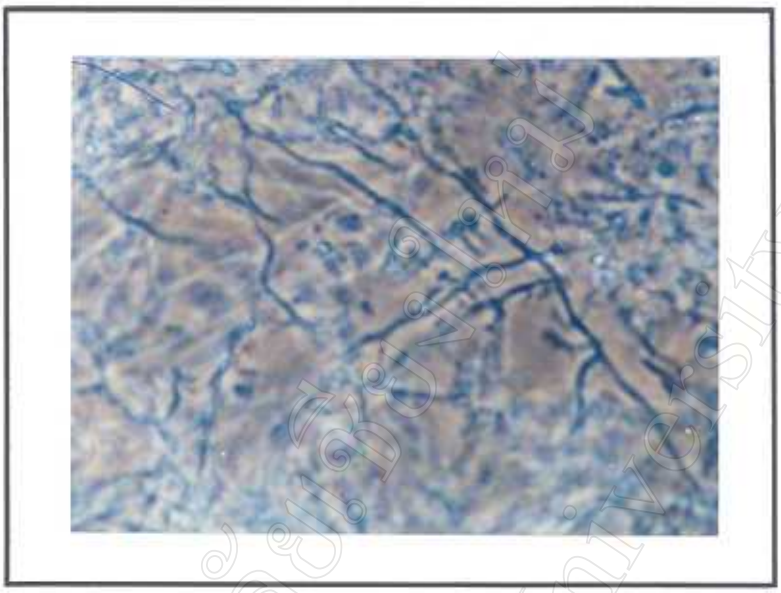

Figure 23 Microscopic morphology of *P. marneffei* isolate 496 on BHIA supplemented with testosterone (10^{-6} M) at 37°C, scored as +1 ; phase contrast x600.

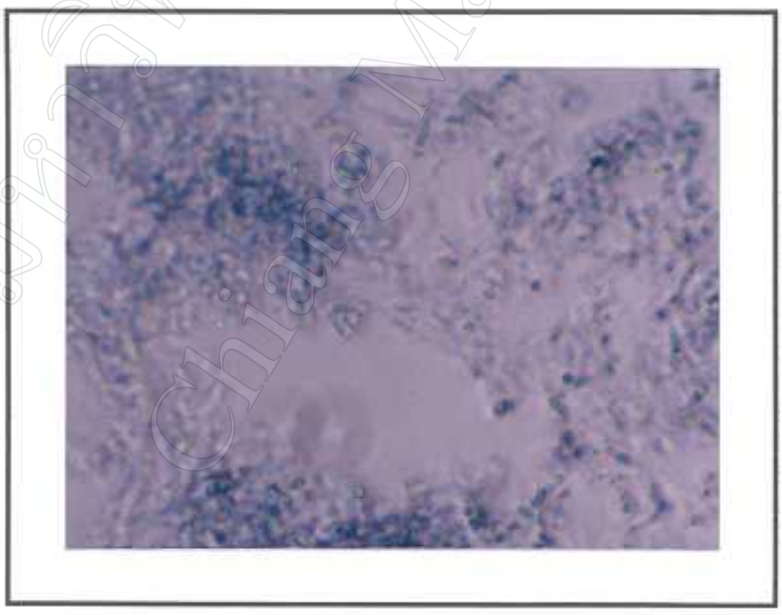

Figure 24 Microscopic morphology of *P. marneffei* isolate 496 on BHIA (with 0.6% ethanol) at 37°C, scored as +3 ; phase contrast x600.

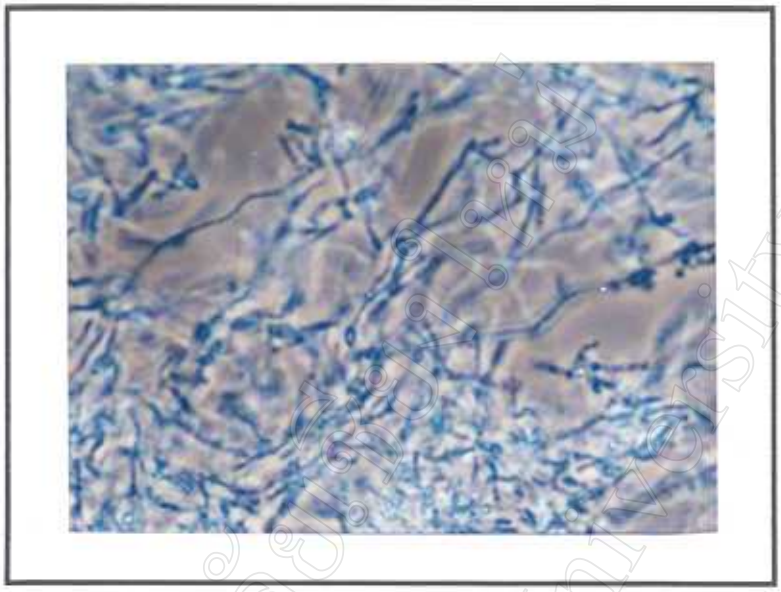

Figure 25 Microscopic morphology of *P. marneffei* isolate 518 on BHIA supplemented with testosterone (10^{-6} M)at 37°C, scored as +1 ; phase contrast x600.

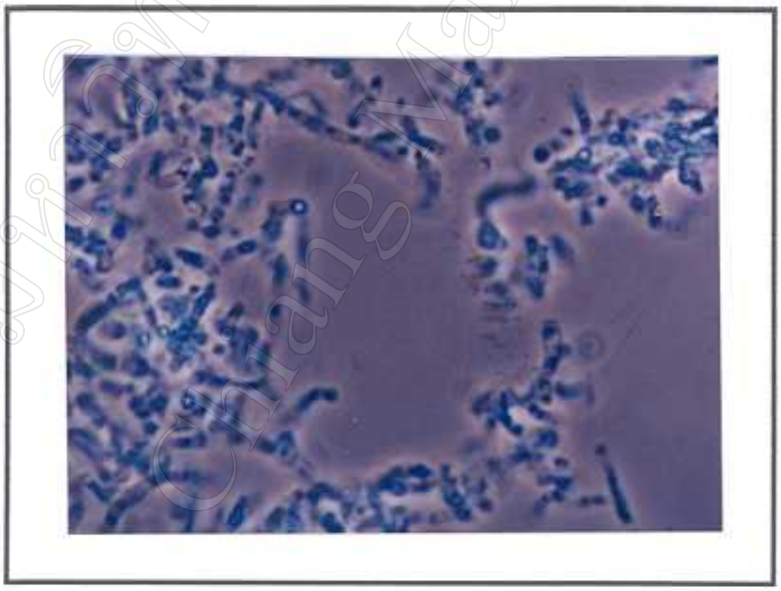

Figure 26 Microscopic morphology of *P. marneffei* isolate 518 on BHIA (with 0.6% ethanol) at 37°C, scored as +3 ; phase contrast x600.

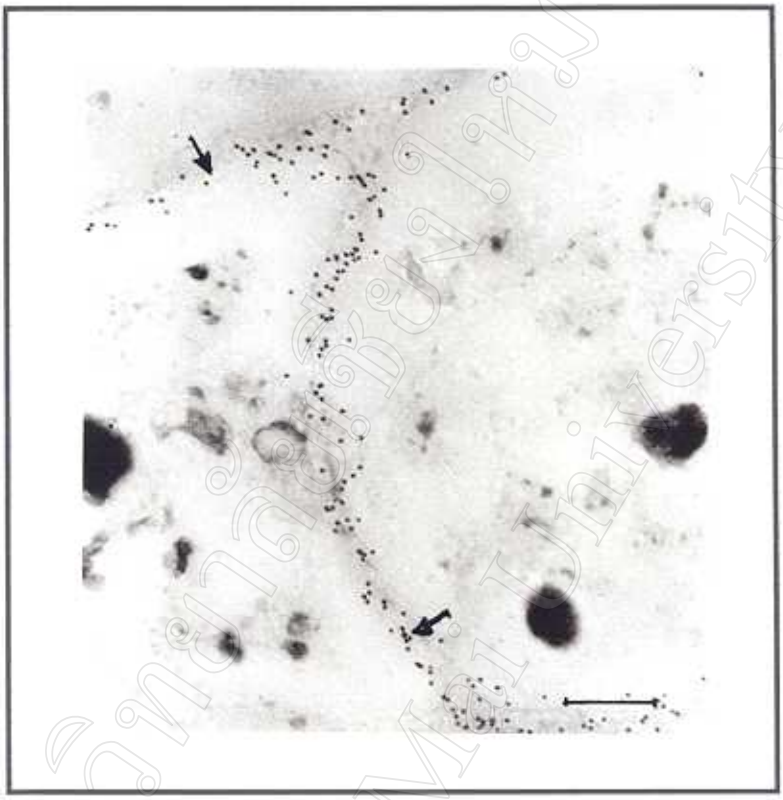

Figure 27 Chitin localization in yeast-like cells of *P. marneffei* (cultured on BHIA) showing gold particles located in the regions of cell wall and in the septum (arrows), bar represents 0.2 μm.

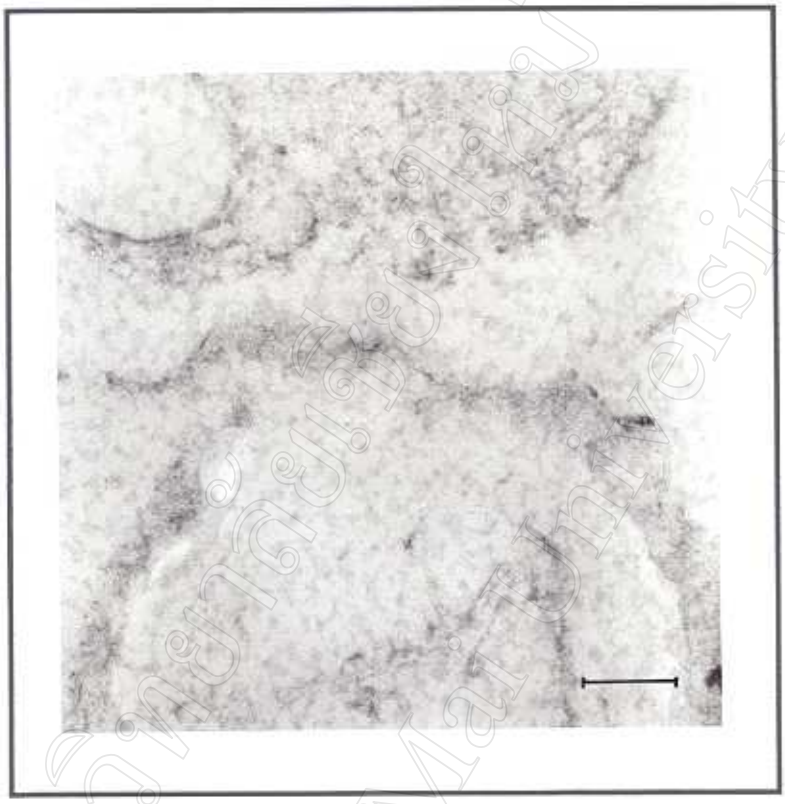

Figure 28 Control section of chitin localization in yeast-like cells of *P. marneffei* (cultured on BHIA). Gold particles were not seen. Bar represents 0.2 μm.

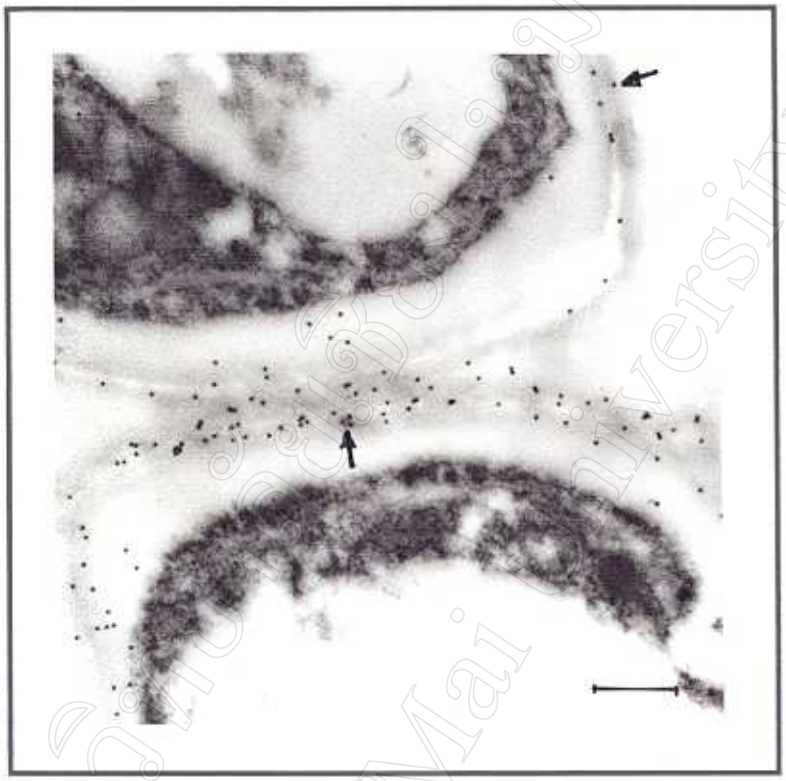

Figure 29 Chitin localization in yeast-like cells of *P. marneffei* (cultured on basal medium supplemented with L-glutamine 0.2g% w/v) showing gold particles located in the regions of cell wall and in the septum (arrows), bar represents 0.2 μm.

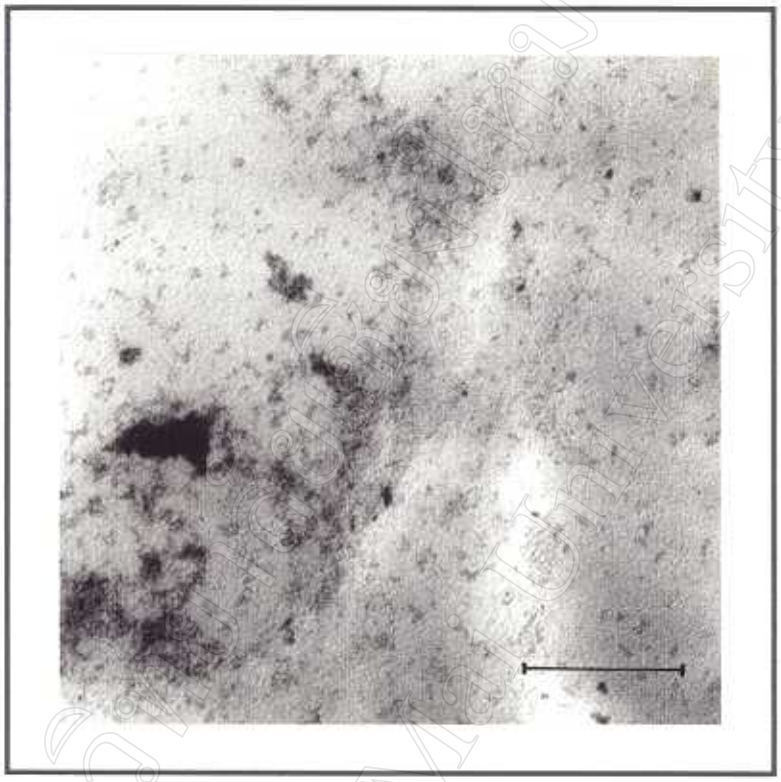

Figure 30 Control section of chitin localization in yeast-like cells of *P. marneffei* (cultured on basal medium supplemented with L-glutamine 0.2 g% w/v). Gold particles were not seen. Bar represents 0.2 μm.

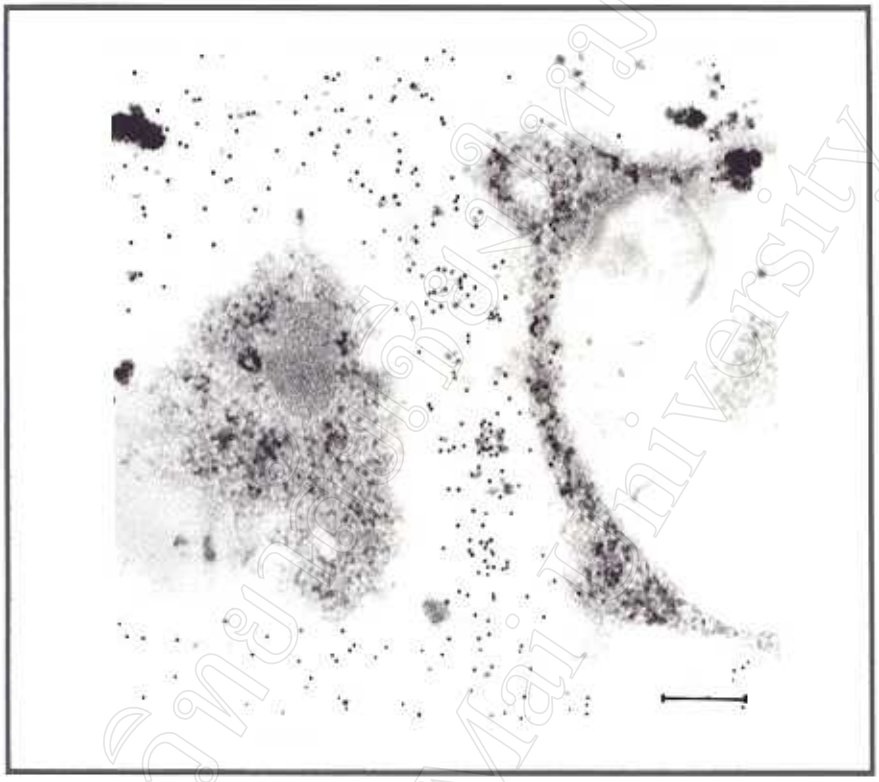

Figure 31 Chitin localization in yeast-like cells of *P. marneffei*, showing gold particles located in the septum.Bar represents 0.2 μm.

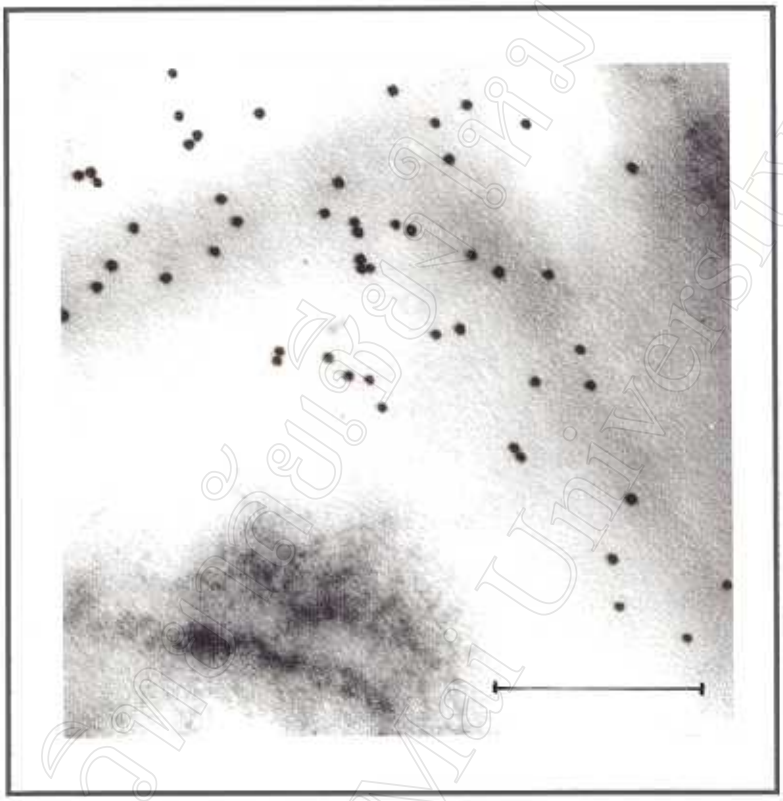

Figure 32 High magnification of gold particles located in the septum of *P. marneffei* yeast-like cells. Bar represents 0.2 μm.

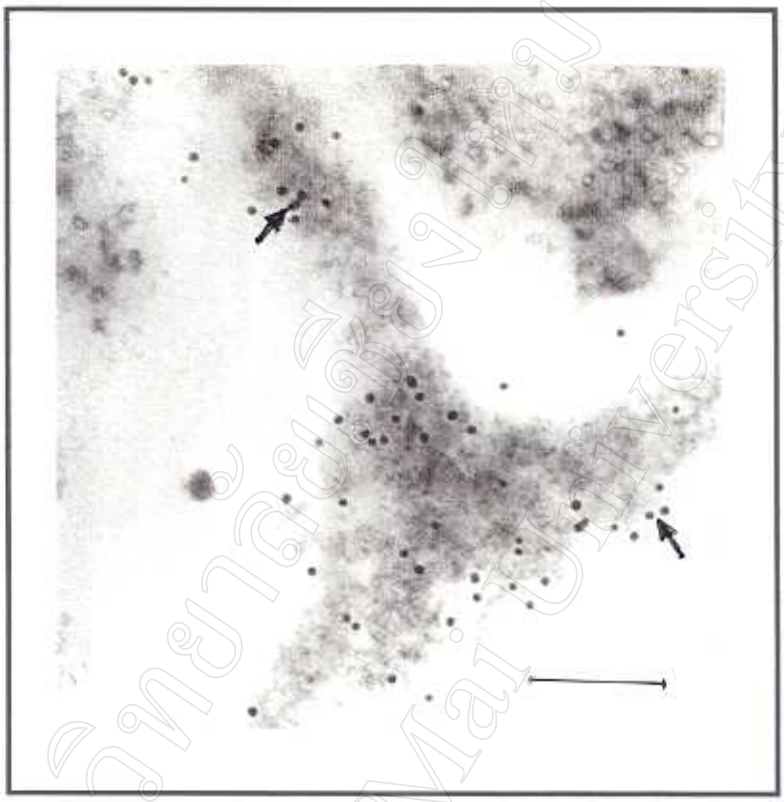

Figure 33 Chitin localization in hyphal cells of *P. marneffei* showing gold particles located in the regions of cell wall and in the septum (arrows), bar represents 0.2 μm.

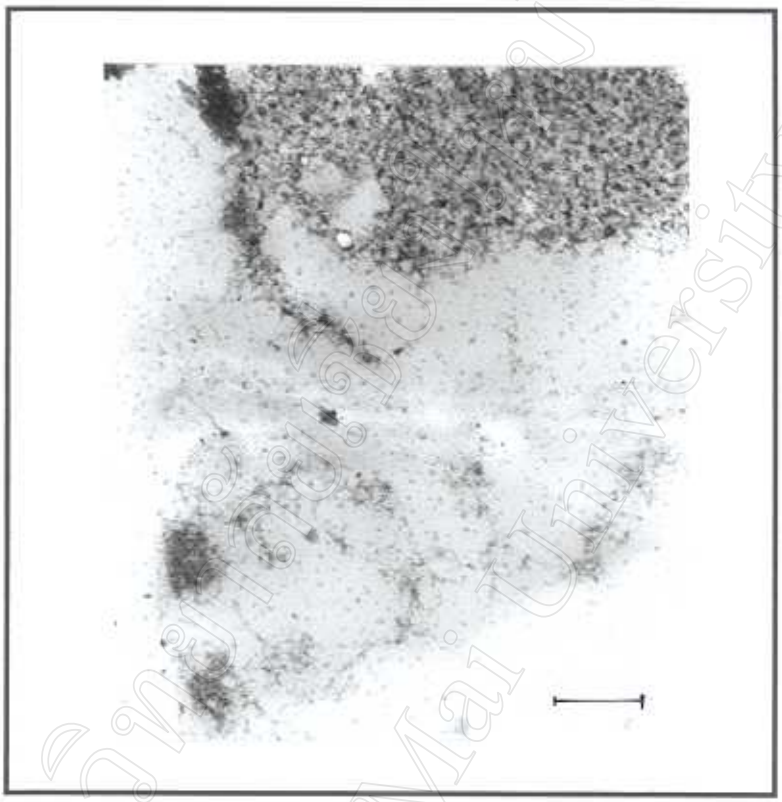

Figure 34 Control section of chitin localization in hyphal cells of *P. marneffei*. Gold particles were not seen. Bar represents 0.2 μm.

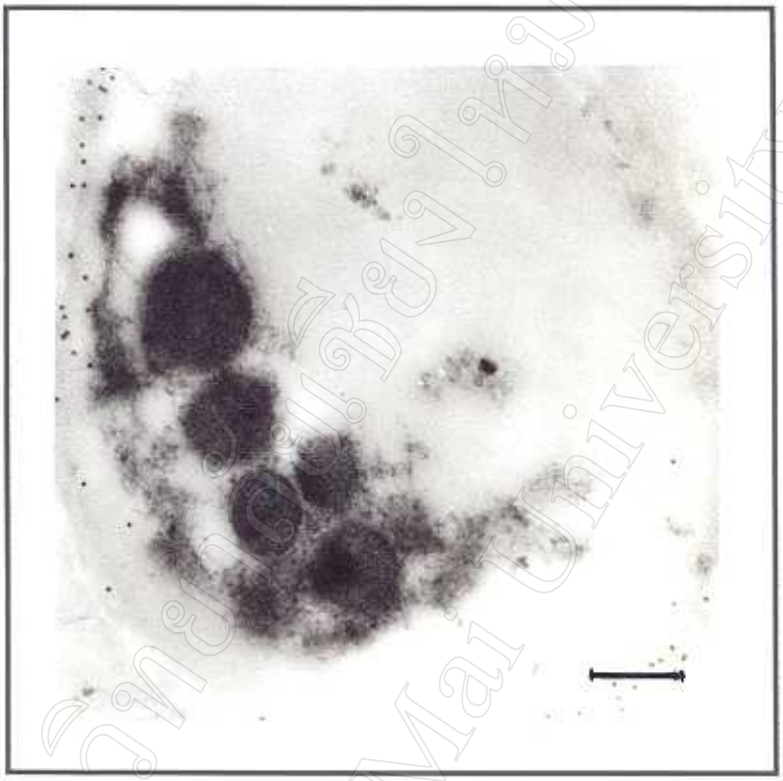

Figure 35 Yeast-like cell of *P. marneffei* showing the small typed mitochondria (S), bar represents 0.2 μm.

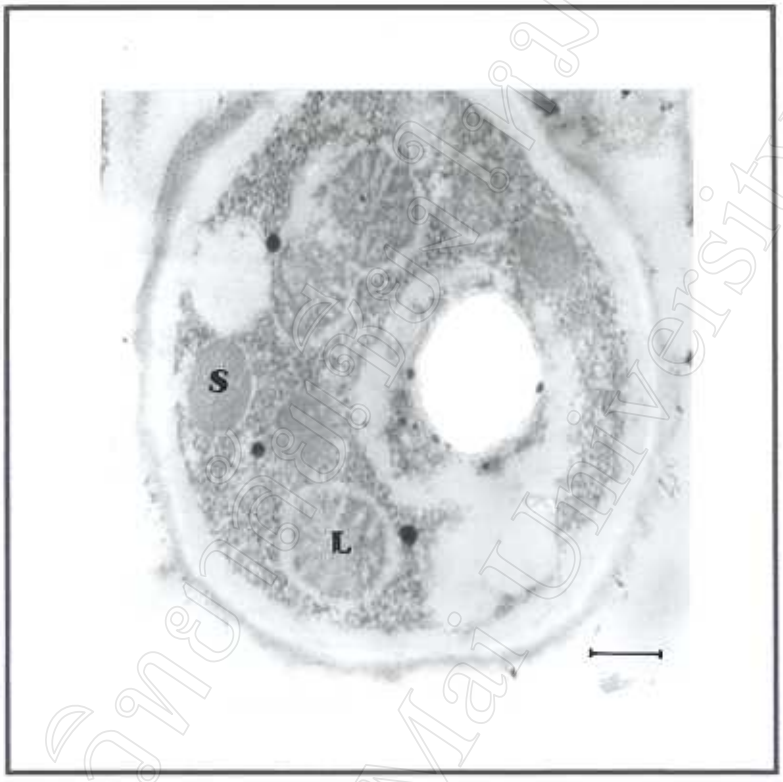

Figure 36 Hyphal cell (mycelial phase) of *P. marneffei* showing two types of mitochondria ; the small type (S) and the large type (L). The large typed mitochondria showing prominent cristae, bar represents 0.2 μm.