CHAPTER III

RESULTS

A. Tea Seed Cake Extraction and Purification

The brown power 'TK" was obtained from tea seed cake extraction by the method of Laohapaibul and Tosukhovong(1981). The yield at this stage was 10±2% (10±2 gm of TK from 100 gm of crude tea seed cake). Column chromatography of 'TK' gave the light pale yellow precipitate (P) with the yield of 6.67% (0.6 gm from 9 gm of TK).

The thin layer chromatographic (TLC) patterns of TK and the light precipitate (P) obtained after partial purification of TK were as follows :-

a. Solvent system 1 (50% Ethanol in Chloroform) :

The light precipitate (P) gave at least 4 spots at the Rf values of 0, 0.214, 0.357 and 0.422 while TK gave at least 5 spots at the Rf values of 0, 0.110, 0.214, 0.357 and 0.422.

b. Solvent system 2 (Butanol : Acetic Acid : H_2O = 4 : 4 : 1) :

The light precipitate (P) gave at least 4 spots at the Rf values of 0.24, 0.387, 0.500 and 0.627 while TK gave at least 5 spots at the Rf values of 0.24, 0.273, 0.387, 0.500 and 0.627.

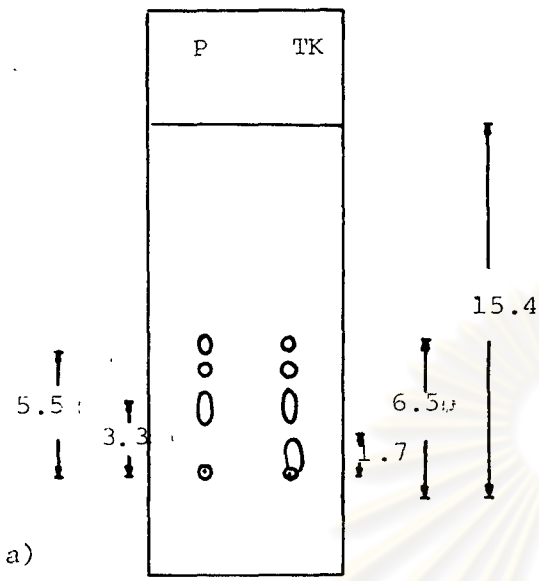
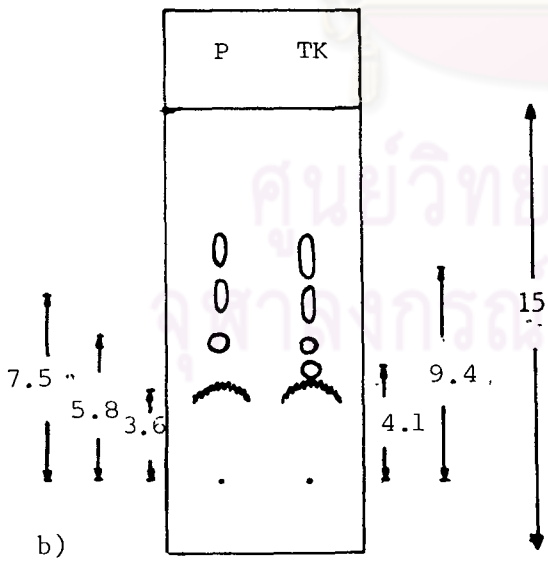


Figure 3.1 Thin layer chromatographic patterns of P and TK in 2 solvent systems

a) 50% ethanol in chloroform

b) butanol : acetic : H_2O =4:4:1

(distance in cm.)



B. Determination of Minimal Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFC)

1. Broth Dilution Method

The MIC and MFC of the light precipitate (P) and TK in 3 different media, YNB (pH 4.7), buffered YNB (pH 7.0) and SDB (pH 5.7) were obtained. The results were reported as MIC/MFC. Miconazole nitrate was used as the control drug. The MIC/MFC of the light precipitate (P) to *Candida albicans* in YNB, buffered YNB and SDB were 156/625, 2,500/5,000 and 500/1,000 µg/ml respectively and that to *Arthroderma benhamiae* in SDB was 5,000/10,000 µg/ml. The MIC/MFC of TK to *C. albicans* in YNB, buffered YNB and SDB were 156/312.5, 156/156 and 312.5/312.5 µg/ml respectively and that to *A. benhamiae* in SDB was 156/156 µg/ml. The MIC/MFC of miconazole nitrate to *C. albicans* in the same media were 12.5/50, 12.5/50 and 3.12/12.5 µg/ml respectively while that to *A. benhamiae* in SDB was 6.25/6.25 µg/ml.

2. Agar Dilution Method

The MFC of the light precipitate (P), TK, and miconazole nitrate were obtained. The MIC of the light precipitate (P) to *C. albicans* and *A. benhamiae* were 10,000 and 5,000 µg/ml while that of TK to the same organisms were 312.5 and 156 µg/ml respectively. The MIC of miconazole nitrate to *C. albicans* and *A. benhamiae* were 6.25 and 6.25 µg/ml respectively.

Table 3.1 MIC/MFC (µg/ml) of light precipitate (P), TK and miconazole to *Candida albicans* and *Arthroderma benhamiae* ; Broth dilution method

Organisms	*Candida albicans* ATCC 10231			*Arthroderma benhamiae* JCM 01886
Drugs \ media	YNB	buffered YNB	SDB	SDB
Light precipitate (P)	156/625	2,500/5,000	500/1,000	5,000/10,000
TK	156/312.5	156/156	312.5/312.5	156/156
Miconazole	12.5/50	12.5/50	3.12/12.5	6.25/6.25

Note: inoculum size used = 0.05ml of $1x10^6$ cells/ml stock cultures

Table 3.2 MIC (µg/ml) of light precipitate (P), TK and miconazole to *Candida albicans* and *Arthroderma benhamiae* : Agar dilution method

Organisms / Drugs	*Candida albicans* ATCC10231	*Arthroderma benhamiae* JCM 01886
Light precipitate (P)	10,000.00	5,000.00
TK	312.50	156.00
Miconazole	6.25	6.25

Note: inoculum size used = 0.01 ml of $1x10^6$ cells/ml stock cultures

C. Kinetics of Inhibition of Growth and Killing of Organisms

1. *Candida albicans*

The effect of TK on the viability of *C. albicans* is illustrated in figure 3.2. Measurement of the viability of the exponential phase yeast exposed to 200 and 1,000 µg/ml TK demonstrated that both 200 and 1,000 µg/ml TK appeared to be fungicidal to *C. albicans* with the onset of action at the 2-hour incubation. The 200 µg/ml TK reduced number of viable cells to 57.14%, 22.14%, 2.7% and 0.17% of the control cultures at the 2-, 4-, 6- and 8-hour incubations respectively. No viable cell was observed in this treatment at the 24-hour incubation. The 1,000 µg/ml TK seemed to have higher potency than

the 200 µg/ml TK as the number of viable cells in the 1,000 µg/ml treatment were 37.76% and 8.97% of the control cultures at the 2- and 4-hour incubations respectively and no viable count was observed since the 6-hour incubation. The optical density of the control and treated cultures (figure 3.3) got along with the viable counts data. In the 200 µg/ml treatment, the OD was reduced from 0.136 to 0.133, 0.100, 0.088, 0.081, and 0.076 at the 2-, 4-, 6-, 8-,and 24-hour incubations respectively. In the 1,000 µg/ml culture, the OD was markedly reduced from 0.127 to 0.116, 0.091, 0.085, 0.080 and 0.078 at the 2-, 4-,6-, 8- and 24-hour incubations respectively.

2. *Arthroderma benhamiae*

The effect of TK to *A. benhamiae* is demonstrated in figure 3.4. TK reduced the mycelial dry weight of the organism since the 1-day incubation while that of control were markedly increased from 3.10 mg to 9,27 , 11.07, 19.47 and 29.03 mg at the 1-, 3-, 5- and 7-day incubations respectively. The dry mycelial weight of 200 µg/ml TK treatment were reduced to 46.39%, 35% and 31.69% of the control culture at the 1-, 5-,and 7-day incubations respectively while that of 1,000 µg/ml TK were 33.44%, 13.82%, 8.73% and 2.65% of the control at the same incubation period.

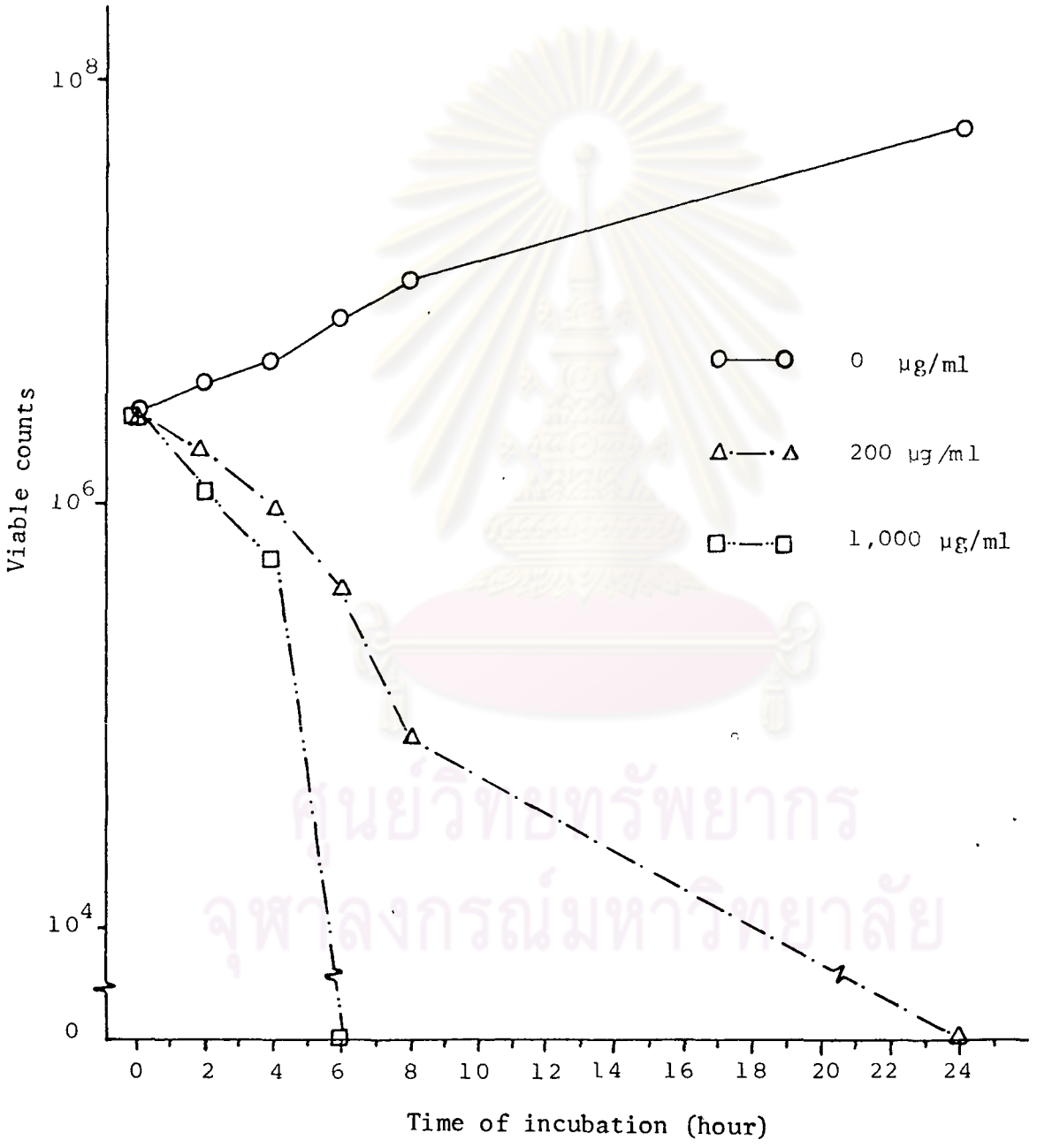


Figure 3.2 Kinetics of inhibition of growth and killing of *Candida albicans* treated with TK (viable cell counting), inoculum size = 2×10^6 cells/ml

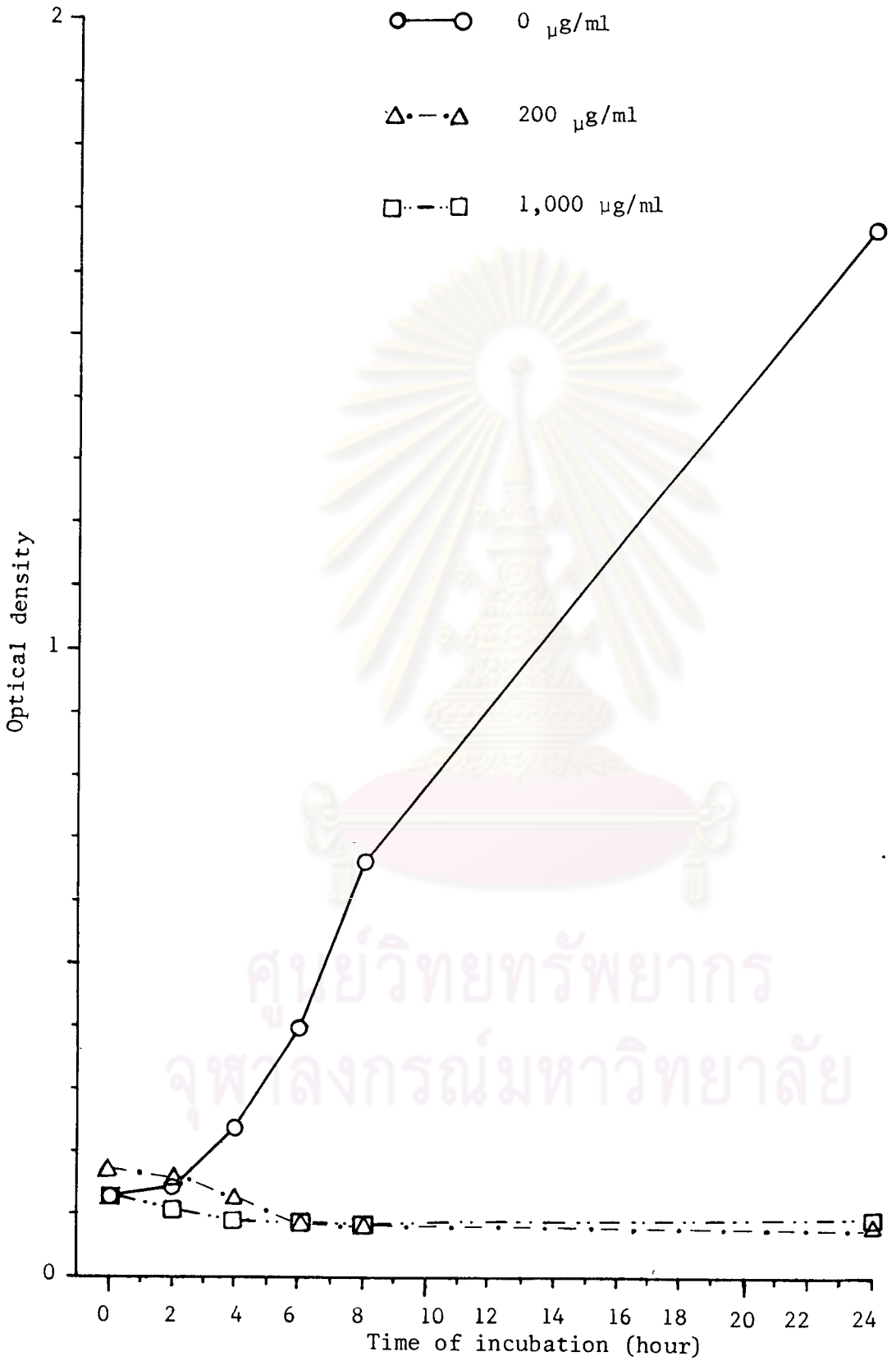


Figure 3.3 Kinetics of inhibition of growth and killing of *Candida albicans* treated with TK (Optical density measurement at 530 nm), inoculum size = $2x10^6$ cells/ml

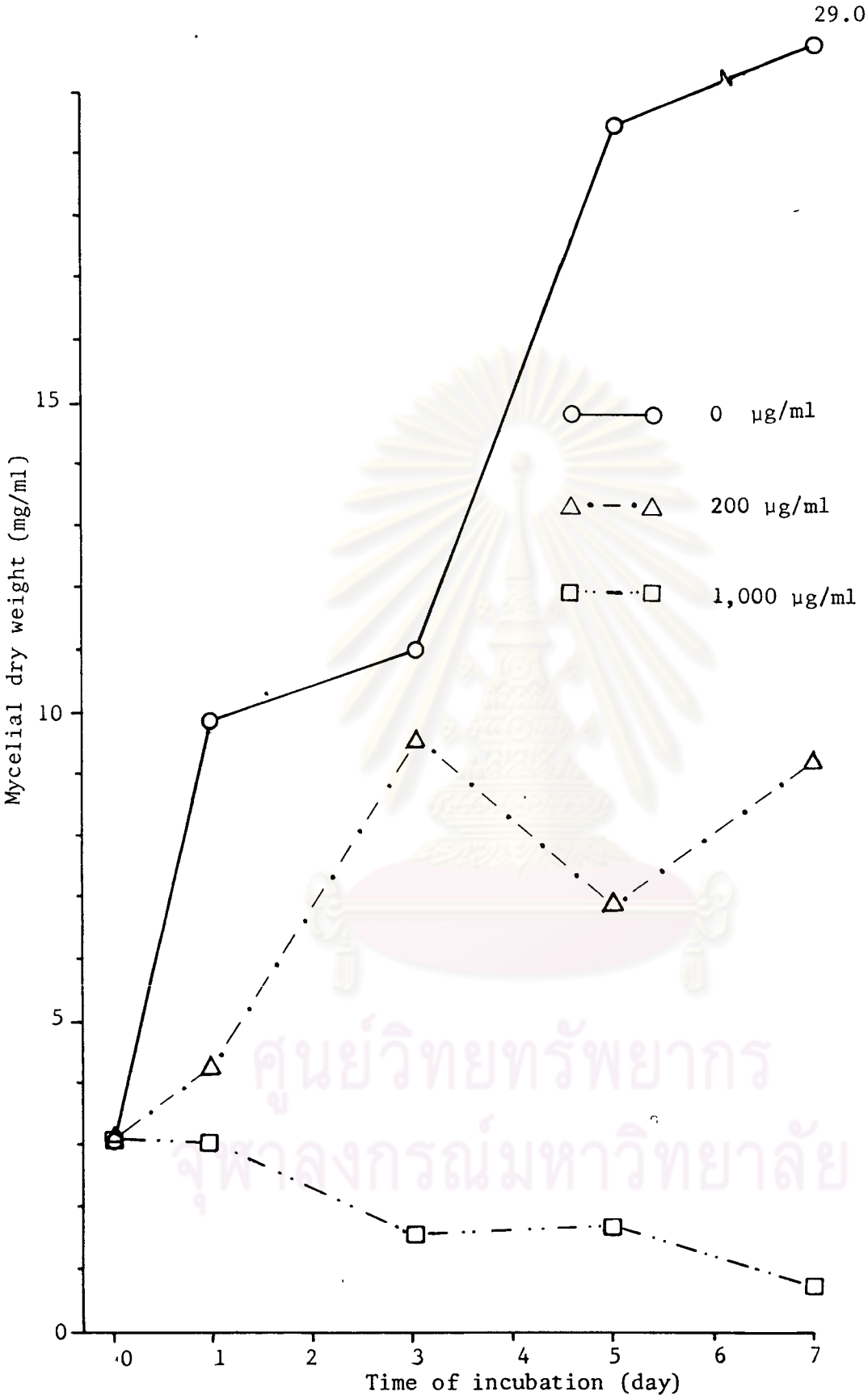


Figure 3.4 Kinetic of inhibition of growth and killing of *Arthroderma benhamiae* treated with TK ,
inoculumm size = 3 mg dry weight/ml

D. Light Microscopic Study

1. *Candida albicans*

TK killed the cells since the 2-hour incubation. Approximately 30%, 50% and 70% of the cell were killed by 200 µg/ml TK at the 2-, 4-, and 8-hour incubations respectively and no viable cell was observed at the 24-hour incubation. The 1,000 µg/ml TK killed about 50%, 80% and 100% of the cultures at the 2-, 4- and 8-hour incubations respectively. The control cultures grew exponentially with pseudomycelial formation and were not stained by Loeffer's methylene blue. The blastoconidia were spherical to oval with multiple budding and smooth surface (Plate 1, 2, 5, 8 and 11).

The TK treated necrotic cells were stained by Loeffer's methylene blue. Some had morphological changes (Plate 3,4), some seemed to have cell content leakage (Plate 6, 7) , some broke and lost their structures (Plate 10, 12, 13).

The alteration of cellular structure was dose-dependent since 1,000 µg/ml TK caused cell damage faster and more than 200 µg/ml TK. Increasing of the incubation time caused more alterations of cellular structure.

2. *Arthroderma benhamiae*

Control : Hyphal structure was regular with parallel wall and complete septum. Young mycelium was thin and long while the old one was thick and short as shown in plate 14, 15, 18 and 21. The culture from the 7-day incubation showed numerous microconidia and few macroconidia (Plate 21).

200 µg/ml TK Treated Culture : The 200 µg/ml TK treated cultures showed structural alterations since the 1-day incubation. The bulging mycelium (swelling of the mycelium) was the important feature in the 1-day treated culture and there were few necrotic hypha too (Plate 16). At the 3-day incubation, the mycelium was bulged and short with loose cellular content. At the 7-day incubation, there were a lot of necrotic hypha together with the short bulging hypha with loose cellular content.

1,000 µg/ml TK Treated Culture : The 1,000 µg/ml TK Treated cultures also showed structural alterations since the first day of incubation. There were short bulging mycelium and necrotic hypha. The necrotic hypha were found more in this treatment than in 200 µg/ml treatment at the same time of incubation (Plate 16, 17). At the third day of incubation, there were a lot of necrotic hypha and hyphal fragments. Short bulging mycelium were also present with very loose cellular content (Plate 20). At the seventh day of incubation, there were a lot of necrotic hyphal fragments and few amorphous mycelium (Plate 23).

The mycelial alteration of *A. benhamiae* treated with TK was dose-dependent too.

E. Electron Microscopic Study

1. Scanning Electron Microscopic Study (SEM)

1.1 *Candida albicans*

Control Cultures : Scanning electron micrographs of control culture at the 0-hour incubation showed numerous spherical to oval budding blastoconidia with almost smooth surface (Plate 24).

The 24-hour incubation culture showed numerous regular blastoconidia and pseudomycelial formation (Plate 31).

200 µg/ml TK Treated Cultures : Scanning electron micrographs of the 4-hour treatment showed wrinkled and shrink blastoconidia with cellular content liberation (Plate 25). The 8-hour incubation culture showed collapsed blastoconidia and collapsed pseudomycelium (Plate 27). At the twenty-forth hour of incubation, severe alteration of blastoconidia and pseudomycelium were observed with drastic cellular content leakage (Plate 29).

1,000 µg/ml TK Treated Cultures : Scanning electron micrographs of the 4-hour treatment showed severe alterations of the cellular structure. The necrotic blastoconidia were shrink, collapsed, broken and cellular content was released (Plate 26). The 8-hour treatment led to abnormal structures of blastoconidia. The spiny surface pseudomycelium, and very rough surface cell were shown with drastic cellular content liberation (Plate 28). At the twenty-forth hour of incubation, there were only abnormal blastoconidia. They were shrink, collapsed and broken with drastic cellular content leakage (Plate 30).

1.2 *Arthroderma benhamiae*

Control Cultures : Scanning electron micrographs of control cultures at the 0- and 7-day incubations showed normal hyphal structure with smooth surface and almost parallel wall (Plate 32.a and Plate 38).

200 µg/ml TK Treated Cultures : The 4-hour treatment showed severe alterations of hyphal structure. The mycelium had rough surface and it was shrink and bulge (Plate 32.b,c,d) The 3-day incubation culture showed the shrink bulging mycelium and irregular rough surface hypha (Plate 34) . At the seventh-day of incubation, severe damaged and amorphous mycelium were observed (Plate 36).

1,000 µg/ml TK Treated Cultures : The 1-day treatment led to severe alterations of hyphal structures. The shrink bulging mycelium was found among the amorphous mycelium (Plate 33). The severity of hyphal damage was greater at the 3-day incubation than that at the 1-day incubation. The shrink mycelium and hyphal fragments were shown (Plate 35). At the 7-day of incubation, there were only necrotic hypha with amorphous structure (Plate 37).

2. Transmission Electron Microscopic Study (TEM)

2.1 *Candida albicans*

Control Cultures : Transmission electron micrographs showed a consistent cytology over the 24-hour incubation. The blastoconidia were 2-6 µm x 3-9 µm , spherical to oval with thick cell wall. The plasmalemma exhibited typical double unit membrane with few convolutions and invaginations and it was closely adherent to the cell wall. The nucleus was eukaryotic with double unit nuclear membrane and nucleus. The intracytoplasmic organelles such as ribosome, endoplasmic reticulum, golgi apparatus, mesosome and vacuole were identified (Plate 39, 48).

200 µg/ml TK Treated Cultures : The 4-hour treatment showed various degrees of plasmolysis in cells with lipid bodies, dilated membrane fragments and deformed cell wall (Plate 40a). The intracytoplasmic organelles and cell membrane cannot be identified (Plate 40). At the 8-hour treatment, two irregular shaped blastoconidia with loose cytoplasmic content were present, one without cell membrane (Plate 42a) and the other with wrinkled cell membrane (Plate 42b). The 24-hour treatment led to severe alterations of cellular structure. The irregular shaped necrotic blastoconidia with absolute plasmolysis and lipid bodies were observed. The deformed cell wall was various in thickness and had dark bands inside. In addition, some cellular content was observed outside of the cells. (Plate 44).

1,000 µg/ml TK Treated Cultures : The 4-hour treatment showed severe alterations of blastoconidial structure. The deformed cell wall was various in thickness with dark bands inside. The cell membrane was absolutely absent, the nucleus was observed in one cell (Plate 41b) but other organelles cannot be identified. There are various degrees of plasmolysis in cells. Lipid body was also observed (Plate 41). The 8-hour treatment showed the distorted necrotic blastoconidia with dark bands in the deformed cell wall. The cells showed complete plasmolysis (Plate 43). A breaking blastoconidia with various stages of cell wall damage was observed in Plate 43c. At the 24-hour incubation, the distorted blastoconidia showed absolute plasmolysis with membrane fragments and lipid bodies inside. The deformed cell walls were various in thickness with the dark bands inside. There were broken cells with

cellular content liberation observed (Plate 45, 46 and 47).

2.2 *Arthroderma benhamiae*

Control Cultures : Transmission electron micrographs also showed a consistent cytology over the 7-day incubation. The hypha structure had long parallel wall with complete septum and the cell wall was electron translucent. The cell membrane exhibited double unit membrane with few convolutions and invaginations and it was closely adherent to the cell wall. The nucleus was surrounded by double unit membrane. The mitochondria were numerous in number. The intracytoplasmic organelles found were ribosomes, vacuole, membrane complexes and endoplasmic reticulum. Lipid bodies were observed too (Plate 49, 56).

200 μg/ml TK Treated Cultures : Transmission electron micrographs of the 1-day treatment showed the deformation of cell membrane with deep invaginations. The intracytoplasmic organelles could no longer be identified. Lipid bodies were found more than in the control culture (Plate 50). The 3-day treatment showed various degrees of mycelial damage, some got absolute plasmolysis with nearly absent cytoplasmic content, some got slightly damage with dilated membrane fragments and some seemed to be unchanged. The cell wall was rougher than control and cellular content was observed outside of the hypha (Plate 52). At the 7-day incubation, the hypha appeared severe structural alterations with absolute plasmolysis. The necrotic hypha was short and distorted, the cytoplasmic content was nearly absent from necrotic hypha and the cell wall was deformed and various in thickness. Lipid bodies were

found in the necrotic hypha while cytoplasmic content was found outside (Plate 54).

1,000 µg/ml TK Treated Cultures : Transmission electron micrographs of the 1-day treatment showed two degrees of damaged hypha, one with loose cytoplasmic membrane, dilated membrane fragments and lipid bodies but the cell membrane seemed to be unchanged; and the other with absolute plasmolysis where only membrane fragments were observed. The cytoplasmic content was found outside of the hypha (Plate 51). At the 3-day incubation, absolute necrotic hypha were shown with nearly absent of cytoplasmic content. Large lipid bodies were observed. The 7-day treatment led to severe damage of hyphal structure. The hyphal fragments were distorted with absolute plasmolysis. Cell wall fragments, deformed septum and lipid bodies were shown in Plate 55c. The cytoplasmic membrane fragments were observed outside of the hyphal fragments (Plate 55).

F. Determination of Macromolecular Biosynthesis

1. DNA Biosynthesis

1.1 *Candida albicans*

In contrast to control cultures, inhibition of ^{3}H-thymidine monophosphate into DNA in *C. albicans* treated with TK was observed both in perchloric acid fraction and precipitate since the second day of incubation. This inhibition features were dose-dependent since the 1,000 µg/ml showed greater inhibition than 200 µg/ml TK at the same time (Table A.7, A.8 and Figure 3.5).

1.2 *Arthroderma benhamiae*

In comparison to the control cultures, TK treated cultures showed the inhibition of ^{3}H-thymidine monophosphate incorporation into DNA in *A. benhamiae* both in perchloric acid soluble fraction and precipitate,(P) since the 1-day incubation (Table A.9,A.10 and Figure 3.6,3.7).

2. Carbohydrate Biosynthesis

2.1 *Candida albicans*

TK inhibited ^{14}C-glucose incorporation into carbohydrate in *C. albicans* in both perchloric acid soluble fraction and precipitate when compared to the control cultures since the second hour of incubation. This effect was dose-dependent since the 1,000 µg/ml TK showed greater inhibition than 200 µg/ml TK at the same time (Table A.11,A.12 and Figure 3.8,3.9).

2.2 *Arthroderma benhamiae*

In comparison to the control cultures, TK treated cultures showed the inhibition of ^{14}C-glucose uptake in *A. benhamiae* in both perchloric acid fraction and precipitate (P) since 1-day incubation. This effect was also dose-dependent since the 1,000 µg/ml TK showed greater inhibition than 200 µg/ml TK at the same time (Table A.13,A.14 and Figure 3.10,3.11).

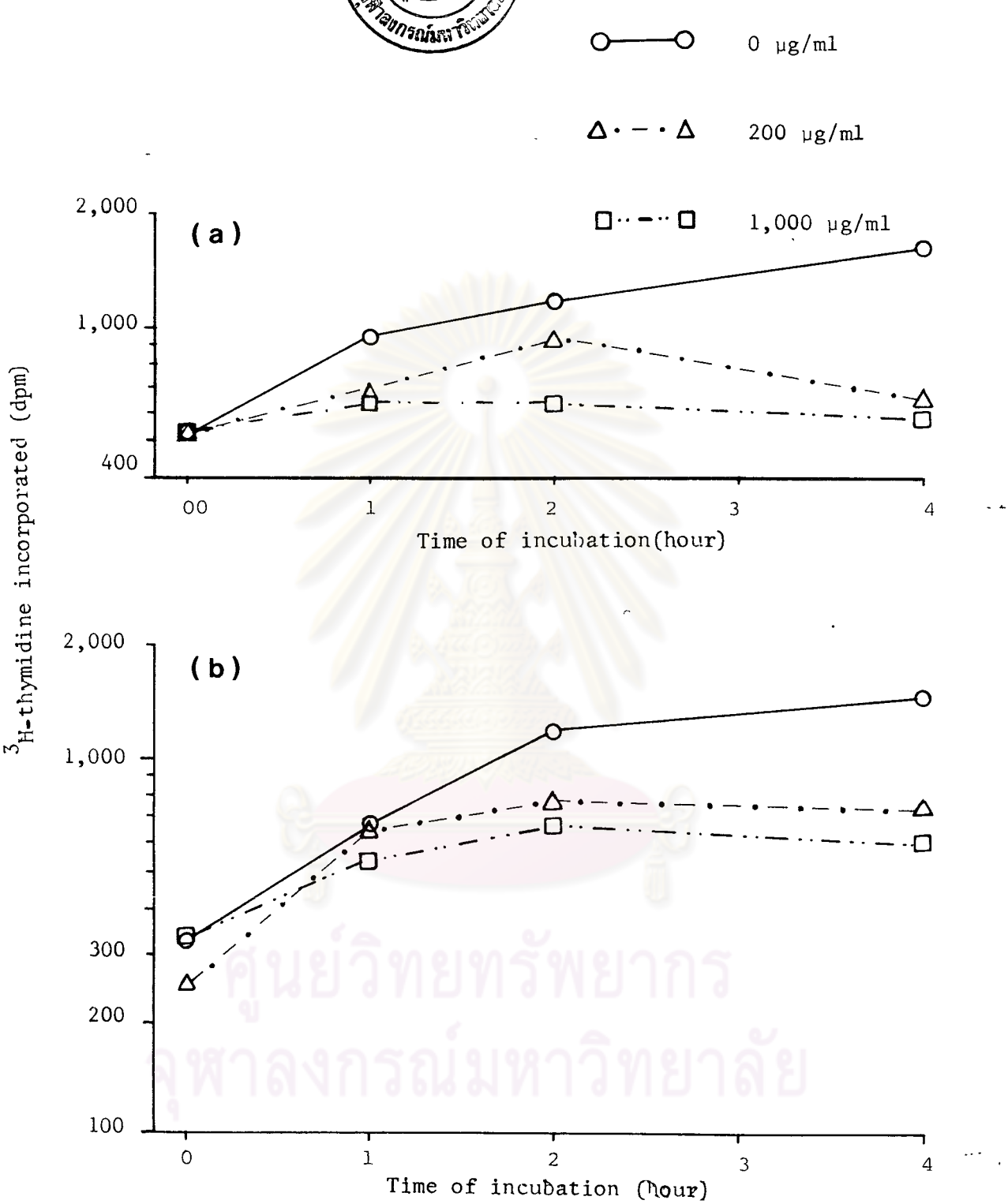


Figure 3.5 DNA biosynthesis in *Candida albicans* after treated with TK , inoculum size = $1x10^{6}$ cells/ml

a) perchloric acid soluble fraction

b) precipitate

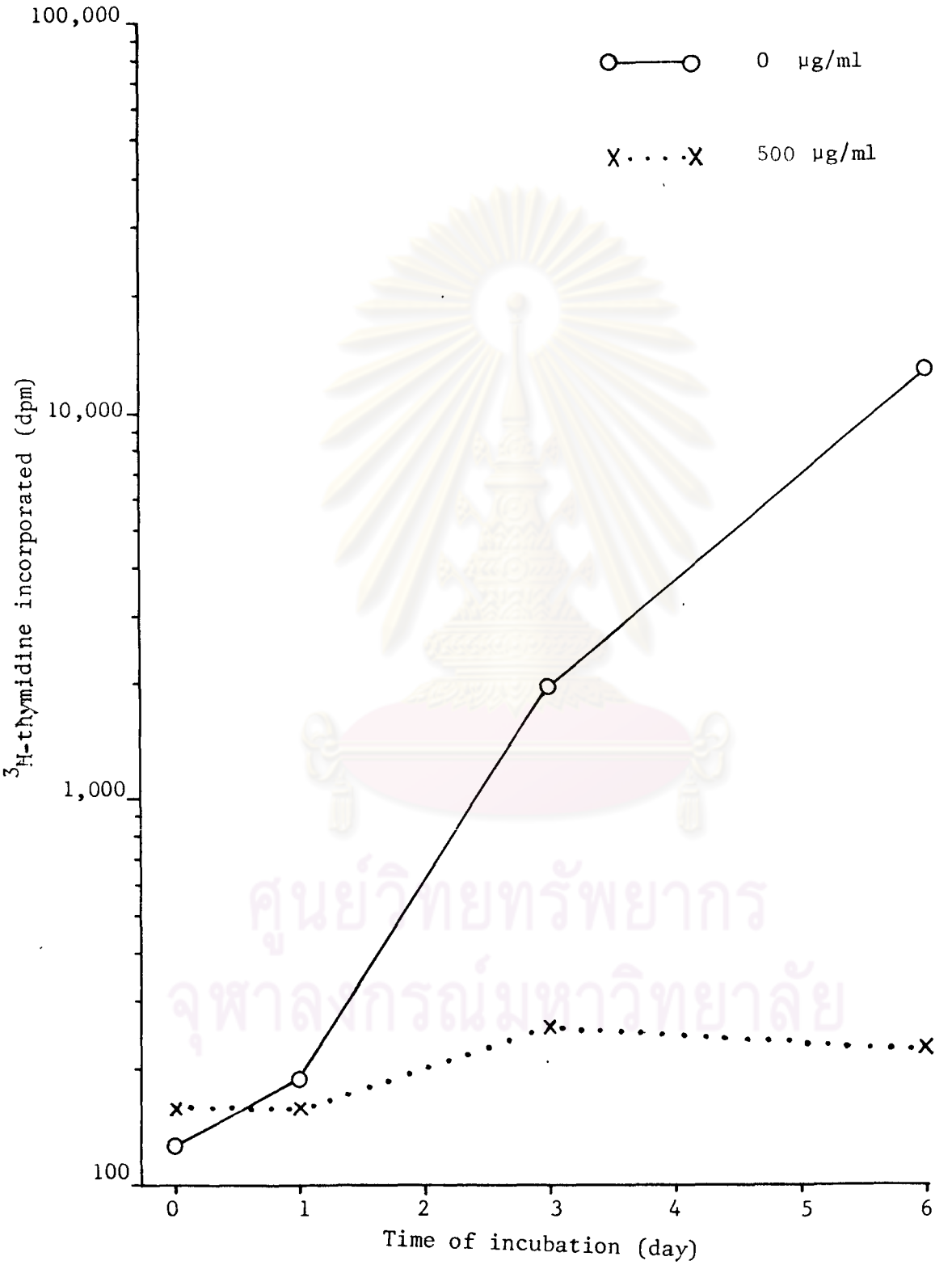


Figure 3.6 DNA biosynthesis in *Arthroderma benhamiae* after treated with TK (precipitate), inoculum size = $1x10^{6}$ cells/ml

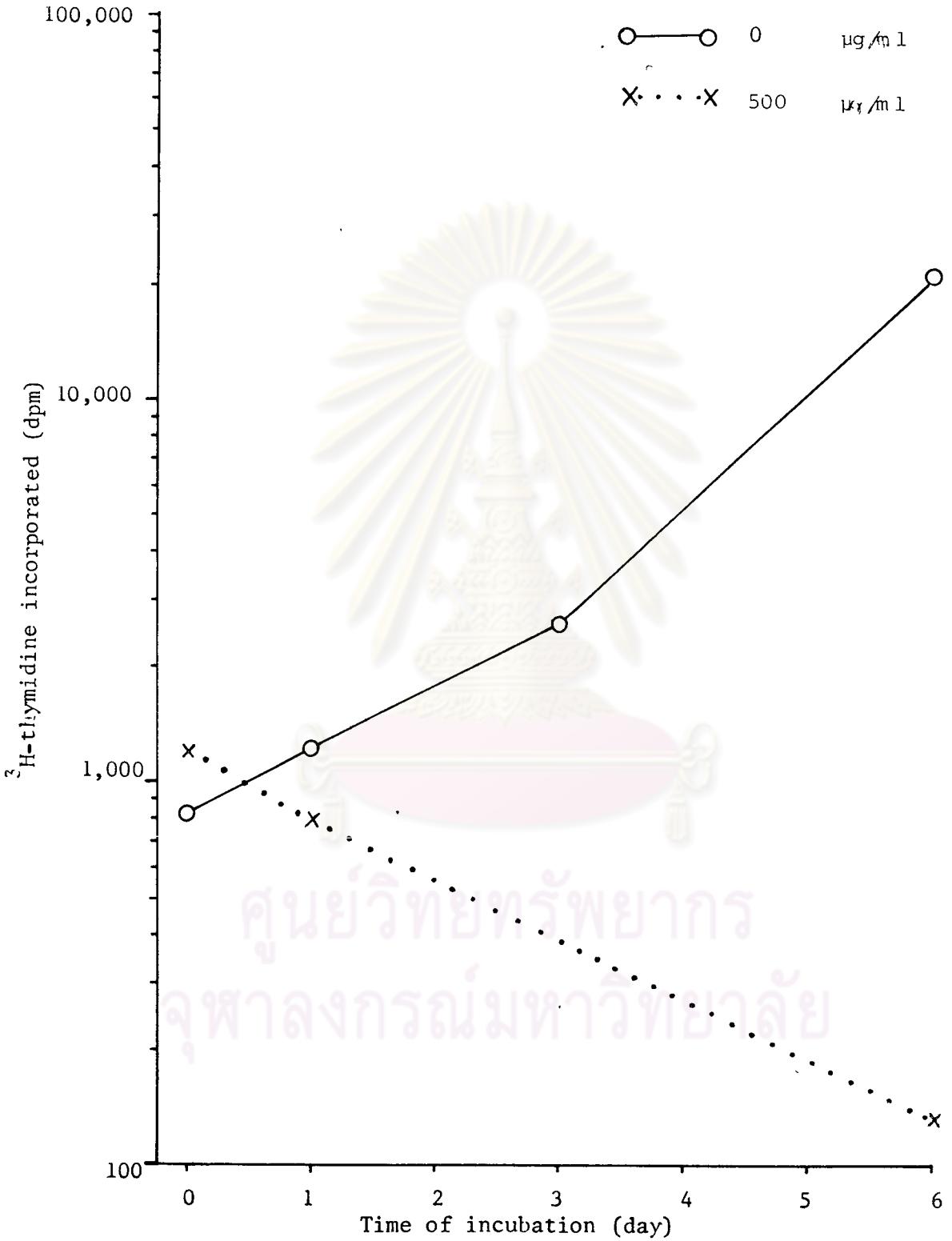


Figure 3.7 DNA biosynthesis in *Arthroderma benhamiae* after treated with TK (perchloric acid soluble fraction), inoculum size = $1x10^6$ cells/ml

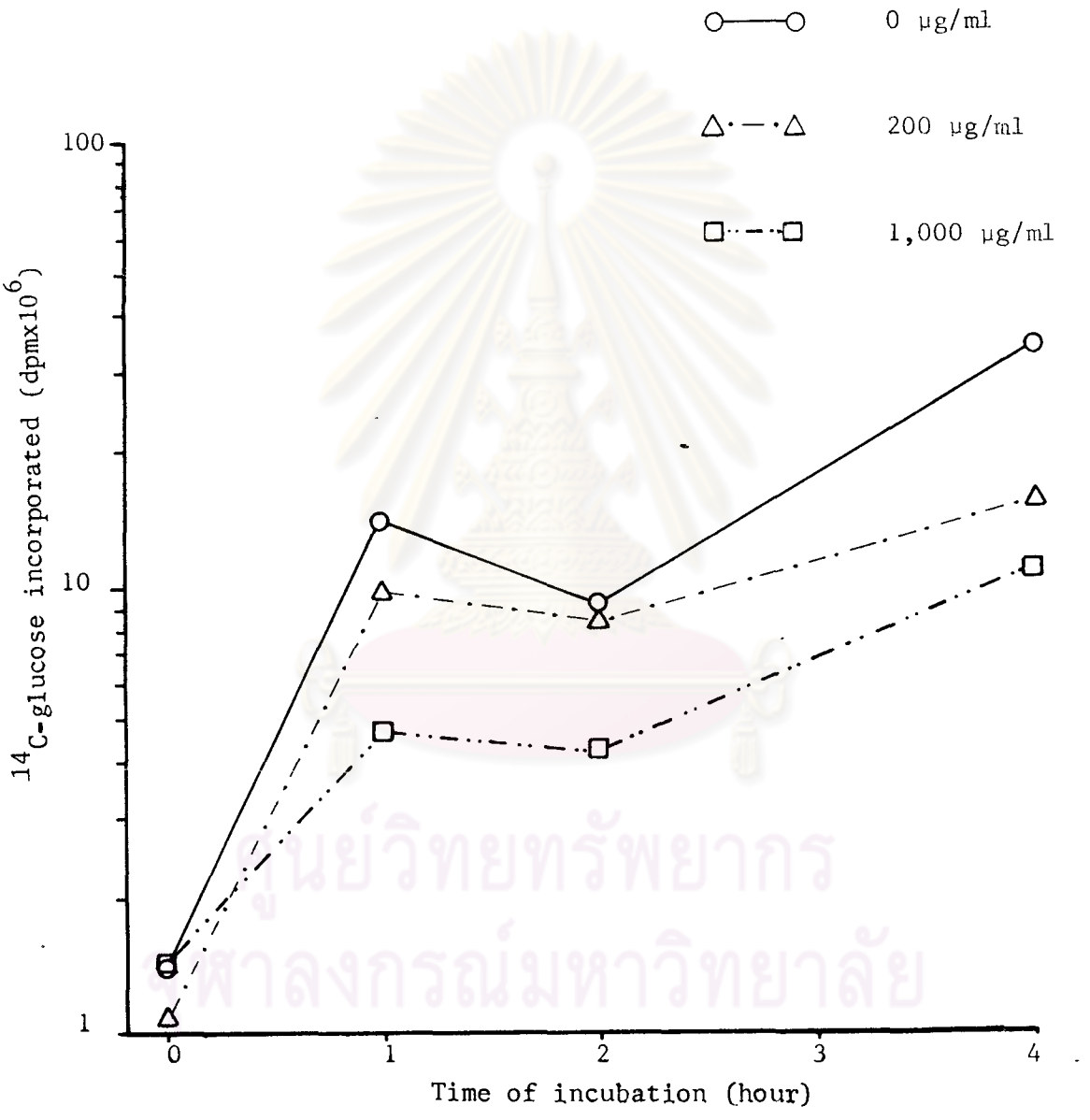


Figure 3.8 Carbohydrate biosynthesis in *Candida albicans* after treated with TK (precipitate), inoculum size = 1x10^6 cells/ml

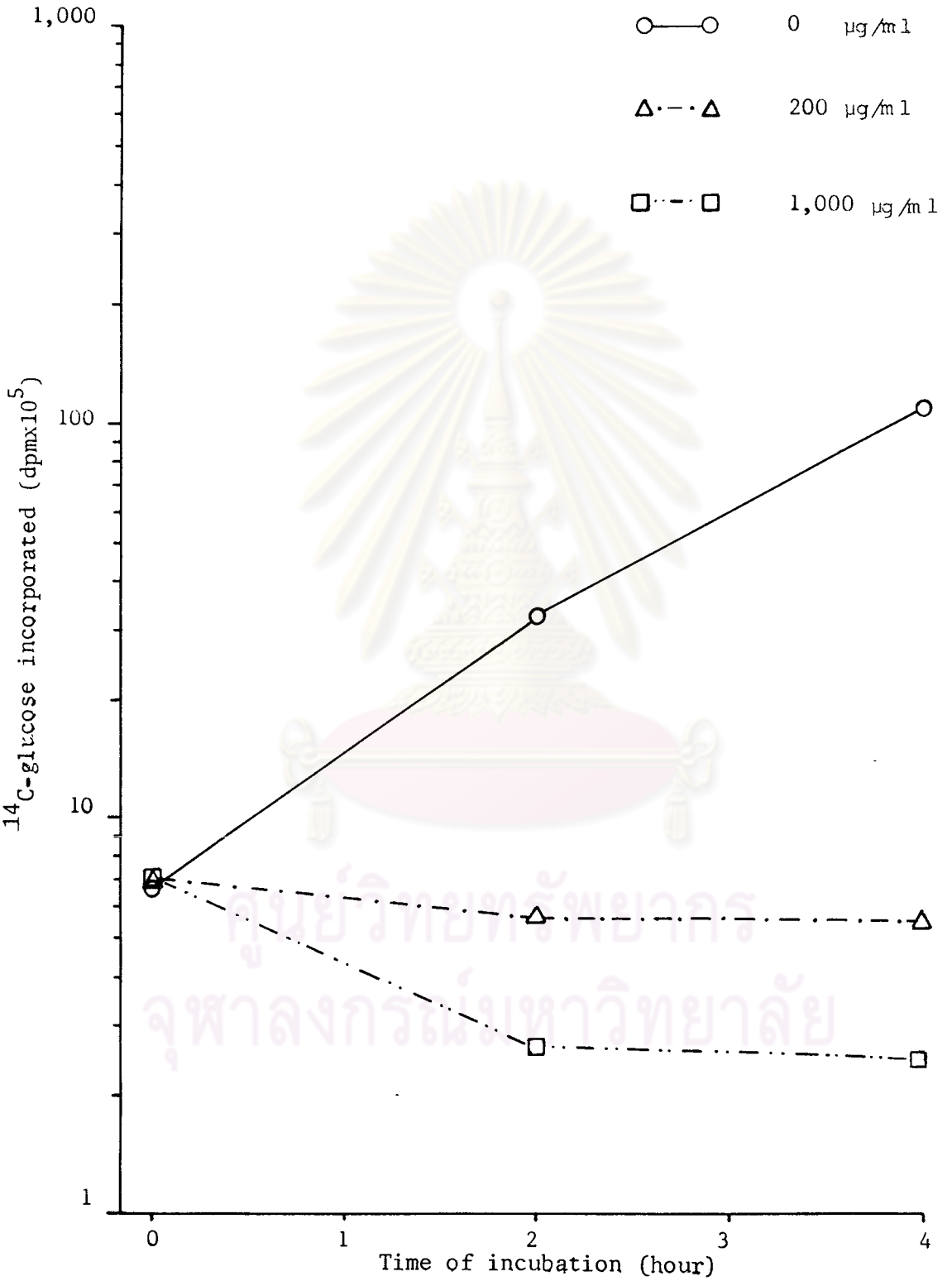


Figure 3.9 Carbohydrate biosynthesis in *Candida albicans* after treated with TK (perchloric acid soluble fraction) inoculum size = 1x10^6 cells/ml

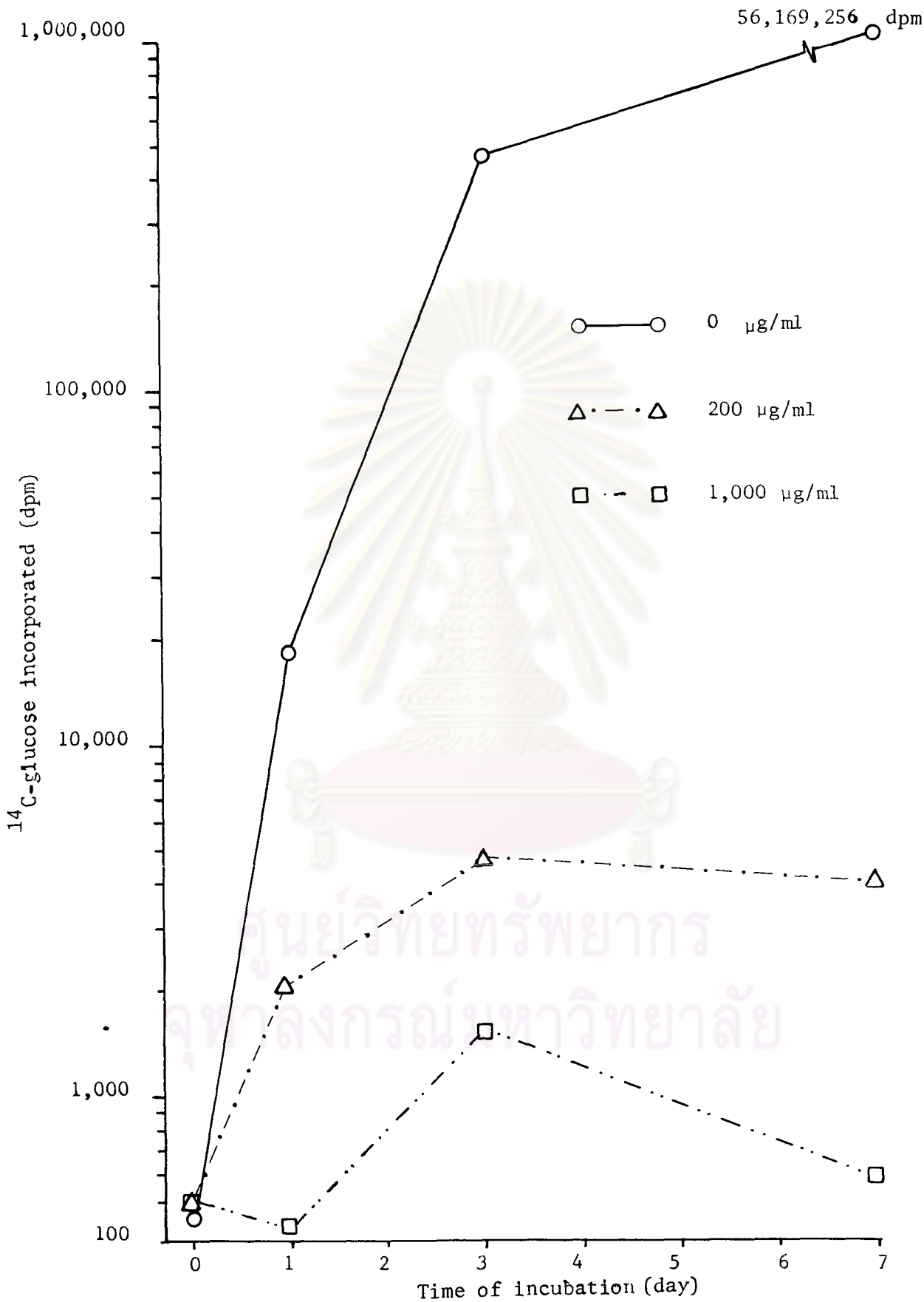


Figure 3.10 Carbohydrate biosynthesis in *Arthroderma benhamiae* after treated with TK (precipitate), inoculum size = $1x10^6$ cells/ml

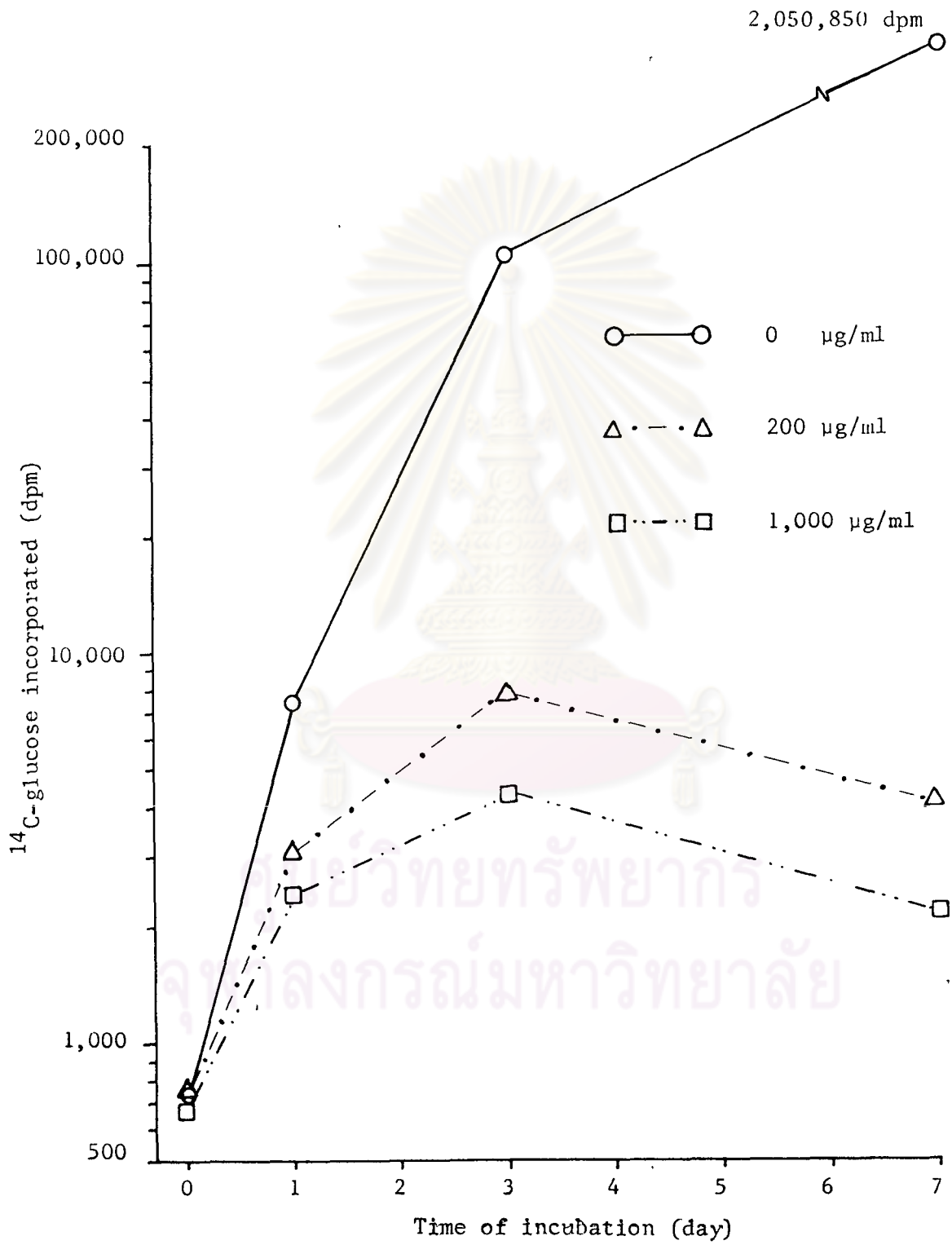


Figure 3.11 Carbohydrate biosynthesis in *Arthroderma benhamiae* after treated with TK (perchloric acid soluble fraction), inoculum size = $1x10^6$ cells/ml