CHAPTER 5

DISCUSSION

5.1 SCREENING OF ABILITY OF FUNGI IN BIOTRANSFORMATION

Artemisinin is an antimalarial agent which is effective against chloroquine-resistant parasites. However, the usefulness of artemisinin is limited because of water insolubility (Parshikov *et al*., 2004). Hence, several researches attempted to produce the synthetic or semi-synthetic artemisinin derivatives to offer more desirable bioactive compounds for pharmacological applications. In this research, five strains of *Aspergillus* sp., *A. oryzae* (Ozykat-1), *A. niger* TISTR 3254, *A. usamii* TISTR 3258, *A. terricola* TISTR 3109 and *A. melleus* TISTR 3128, were examined for their abilities to transform artemisinin. Among these strains, *A. oryzae* (Ozykat-1) and *A. terricola* TISTR 3109 showed their abilities to transform artemisinin to its derivative. *A. oryzae* has a wide range of enzymes and is capable to metabolize complexes of organic compounds (Truong *et al*., 2004). This fungus belongs to the section *Flavi* of the subgenus *Circumdati* of *Asperigillus* as same as *A. flavus* which is able to transform artemisinin into deoxyartemisinin (Machida *et al*., 2005; Srivastava *et al*., 2009). Unlike *A. flavus*, *A. oryzae* is safe because it produces no harmful products and it is commonly fungi used in food industries (Machida *et al*., 2005; Truong *et al*., 2004). *A. terricola* is a filamentous fungus that produces a wide range of enzymes such as inulase, diastase, invertase, maltase, alcoholoxydase, lipase, protease, amidase

and xylanase (Michelin *et al*., 2011; Scales, 1914). In addition, *A.terricola* was reported as an important bioconverter in transformation of bicyclo[3.2.0]hept-2-en-6-one to (-)-(1S,5R)-lactone which is used for the synthesis of prostaglandins (Borges *et al*., 2009). Therefore, *A. oryzae* (Ozykat-1) and *A.terricola* (TISTR 3109) were selected for further study.

5.2 OPTIMIZATION CONDITIONS

Biotransformation of artemisinin was conducted to determine the effect of temperature, transformed medium, artemisinin concentration, fungal pre-incubated period and incubation time on the growth of *A. oryzae* (Ozykat-1) and *A. terricola* TISTR 3109 and the production of the transformed product

5.2.1 Effect of temperature on the growth of fungi and the production of the transformed product

The effect of temperature on the growth of *A. oryzae* (Ozykat-1) and *A. terricola* TISTR 3109 were observed at room temperature (26^{o}C), 30^{o}C and 37^{o}C. The temperature of 37^{o}C is found to be an optimal temperature for the growth of *A. oryzae* (Ozykat-1) and *A. terricola* TISTR 3109. Nasseri and his co-worker (2002) revealed that the optimal temperature for the growth and enzymatic activity of *A. oryzae* is between 24-40^{o}C. Any temperature below 24^{o}C or more than 40^{o}C can slow down in *A. oryzae*'s growth and enzymatic activity (Nasseri, 2002). The study by Michelin *et al*. (2011) showed that *A. terricola* reached a maximum growth at 25^{o}C when it was cultured in Segato Rizzatti medium (Michelin *et al*., 2011). These indicated that the growth of *Aspergillus* sp. is depended on the concentration of carbon, nitrogen sources and salt solution in the medium. Different nutrients may affect the functional

and structural development of fungi (Carvalho *et al*., 2010; Pattron, 2006; Srinubabu, 2007).

The effect of temperature on transformation of artemisinin by *A. oryzae* (Ozykat-1) and *A. terricola* TISTR 3109 were studied at 30°C and 37°C. A temperature of 37°C is the most favorable for transformation when the extent of transformation was assessed based on the production of the transformed product. The production of the transformed product by both fungi was lower at 37°C. At lower temperatures, some enzymes may not be in active state (Prasad *et al*., 2008). Wright and Honek (1991) also reported that temperature fluctuations affected a number of cellular factors, including transport, protein synthesis, and membrane fluidity (Wright and Honek, 1991).

5.2.2 Effect of the transformed medium on the production of the transformed product

Transformations of artemisinin were carried out using transformed medium I (PDB) and II (SDB) for single-stage transformation and transformed medium III (0.05M Phosphate Buffer pH 7.0) for two-stage transformation. Transformed medium I and II are usually used as the medium in the transformation of artemisinin by fungi (Goswami *et al*., 2010; Srivastava *et al*., 2009; Zhan *et al*., 2002a; Zhan *et al*., 2002b). In this research, transformed medium II and III appeared to be the better media for the transformation of artemisinin by *A. oryzae* (Ozykat-1) and *A. terricola* TISTR 3109. Fantin *et al*. (2006) reported that transformed medium II was the best medium for *A. terricola* and *Aspergillus amazonicus* in the transformation of bicyclo[3.2.0]hept-2-en-6-one to (-)-(1S,5R)-lactone when compared to a synthetic medium and in the

modified synthetic medium by substituting glucose with mannitol (Fantin *et al*., 2006). However, the two-stage biotransformation required long time in conversion of artemisinin into the transformed product and the yield of the transformed product were lower than that of the single-stage biotransformation. In addition, the transformed product was not reproducible in each batch of biotransformation. In this study, the TLC chromatogram of the obtained crude extract from transformed medium I containing only artemisinin showed a spot which has similar R_f as the transformed product of *A. oryzae* (Ozykat-1) and *A. terricola* TISTR 3109. This result indicated that the components in transformed medium I could react with artemisinin and give deoxyartemisinin. This is supported by the finding by Zhan and colleagues (2002a) who revealed that artemisinin can be reacted to catalysis by Fe^{2+} in a potato medium (Zhan *et al*., 2002a). Therefore, the single-stage transformation of artemisinin in transformed medium II (SDB) was used a preferable medium in this research.

5.2.3 Effect of artemisinin concentration on the growth of fungi and the production of the transformed product

The addition of artemisinin to the *A. oryzae* (Okykat-1) and *A. terricola* TISTR 3109 cultures resulted in a noticeable decrease in the growth of fungi. The fungal culture containing artemisinin showed a reduction in growth when comparing to the fungal control. It has been speculated by Petal and his co-workers (2010) that the growth reduction is accounted for the concentration of artemisinin (Patel *et al*., 2010). In addition, Dhingra and his co-worker (2000) reported that artemisinin shows antifungal activity against *Aspergillus* sp., *A. flavus*, *A. niger* (Dhingra *et al*., 2000). The pH profile of *A.oryzae* (Ozykat-1) culture added with artemisinin was slightly

lower than non-treated control. On the other hand, the pH profiles of *A. terricola* TISTR 3109 after adding artemisinin was found to slightly higher than non-treated control but lower than the initial pH. These results indicated that *A.oryzae* (Ozykat-1) and *A. terricola* TISTR 3109 may produce acidic metabolites such as kojic acid and malic acid before leach out to the medium (Kumar and Zheng, 1990; van Agtmael *et al*., 1999). The addition of artemisinin to the medium causes an unusual fungal pellet formation of *A. oryzae* (Ozykat-1) and *A. terricola* TISTR 3109. The fungal pellets become loose and formed a hairy structure in the outer layer. This type of fungal structure was found in the growth of *Aspergillus awamori* in the medium containing wheat bran and *A. oryzae* IFO 30113 which was used for treating of cassava starch processing wastewater (Cui *et al*., 1998; Truong *et al*., 2004). *A.oryzae* (Ozykat-1) and *A. terricola* TISTR 3109 is able to transform artemisinin at a concentration less than 1 mg/ml. However, the highest conversion of artemisinin was 29 and 20% when introducing artemisinin at the concentration of 0.5 and 0.75 mg/ml into *A.oryzae* (Ozykat-1) and *A. terricola* TISTR 3109, respectively. However, adding 0.5 mg/ml of artemisinin into the *A. terricola* TISTR 3109 culture gave 19% yield of the transformed product, which was not significantly different from that of 0.5 mg/ml of artemisinin addition. In addition, increasing of the concentration of artemisinin caused the decreasing in growth of fungi. The inappropriate concentration of artemisinin causes unusual pellet formations and increased the viscosity of the medium that limited the induction of the oxygen and substrate (Truong *et al*., 2004). Therefore, 0.5 mg/ml of artemisinin were selected in favor of artemisinin in *A. oryzae* (Ozykat-1) and *A. terricola* TISTR 3109 systems.

5.2.4 Effect of pre-incubation period on the production of the transformed product

Artemisinin was introduced to the fungal cultures at lag, log and stationary phases and the cultures were harvested every other day. The highest yields of the transformed product were observed when *A. oryzae* (Ozykat-1) and *A. terricola* TISTR 3109 were pre-incubated at log phase prior to the introduction of artemisinin and further incubated for 6 days. The results, as showed in Figure 5.1, showed that both fungi could transform artemisinin into the transformed product after 4 days of artemisinin addition to the lag phase. Both fungi were able to transform artemisinin 2 days after the addition of artemisinin to the log and stationary phase of growth. These might suggest that during early lag phase, the fungi slightly grow and they produce enzymes which are essential for growth but are not associated with artemisinin transformation whereas the fungal cells may alter the medium through uptake of substrate artemisinin and then excrete metabolic or transformed product during log or stationary phase.

Adding artemisinin at the different pre-incubation time of growth did not affect on the production of the transformed product. *A. oryzae* (Ozykat-1) and *A. terricola* TISTR 3109 may produce the active enzymes for biotransformation of artemisinin at all growth states. In addition, both fungi were found to be able to convert artemisinin into the transformed product in the highest yield after the addition of artemisinin for 6 days at all growth states and the highest yield of the transformed product did not significantly differ in each growth states. These results may be due to the fact that the enzymes associated to artemisinin transformation have low activity or

limited because the number of initial spore or cells inoculation were controlled to 2×10^6 spore/ml as the method reported by Goswami and co-workers (Goswami *et al*., 2010). The yield of the transformed product may increase when the fungi were inoculated more than 2×10^6 spore/ml. However, there are the other factors also concerned with the transformation of artemisinin such as the substrate concentration and the culture conditions. Moreover, the result obtained from the experiments using biotransformation of artemisinin in free-cell culture demonstrated that the enzymes performing the biotransformation of artemisinin are intracellular enzymes which are present in the cells. Artemisinin might be deoxygenated in the cell and released the transformed product into the medium because the free-cell cultures extracts of both fungi did not have the ability to transform artemisinin.

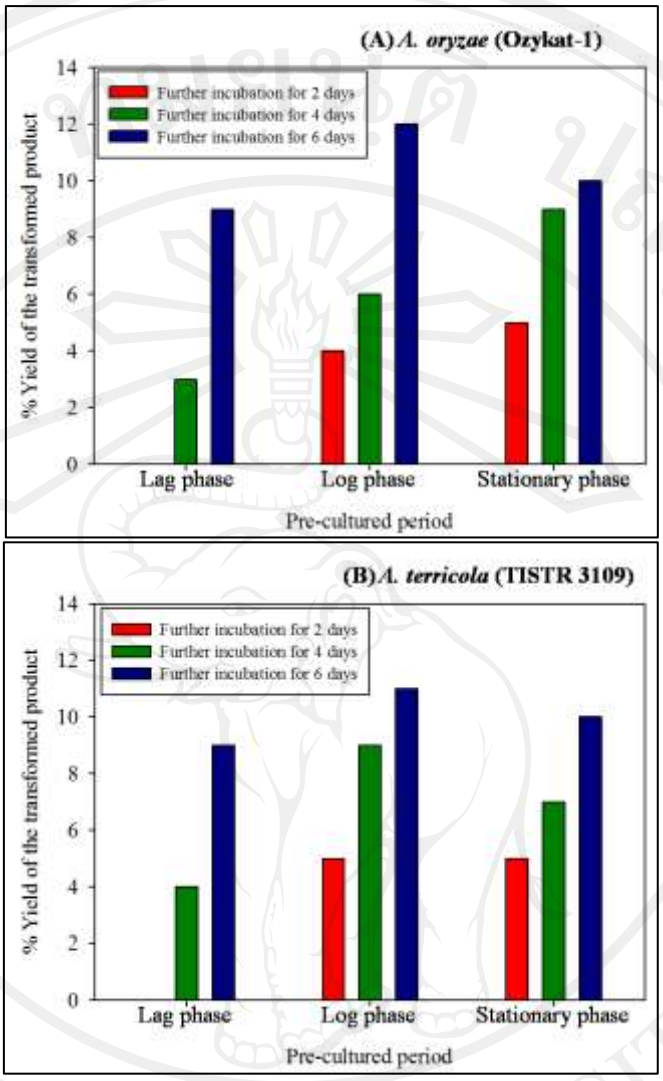
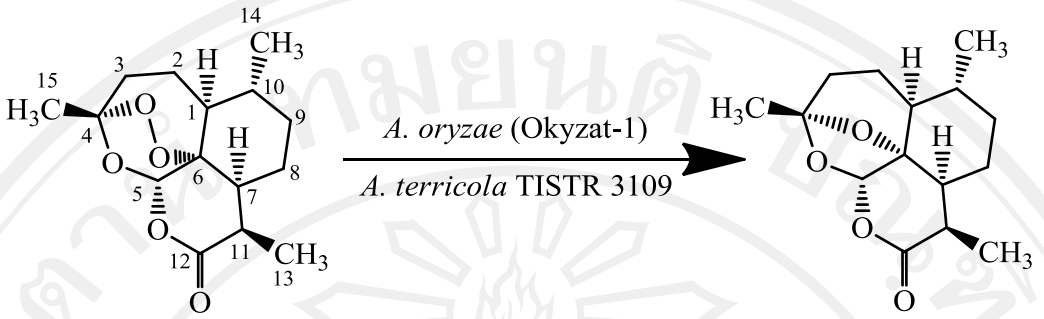


Figure 5.1 The yield of the transformed product when artemisinin was added to the lag, log and stationary phase of *A. oryzae* (Ozykat-1) (A) and *A. terricola* TISTR 3109 (B)

5.3 CHEMICAL CHARACTERIZATION OF THE TRANSFORMED PRODUCT

The high-resolution positive ESIMS data of the purified transformed product showed a molecular weight of 266.1538 (calculation for the molecular formular $C_{15}H_{22}O_4$, 266.3328 *m/z*), suggesting that an oxygen atom had been removed from artemisinin molecule. The FT-IR spectrum revealed the existence of lactone skeleton. The ^{1}H- and ^{13}C-NMR spectrum of the transformed product showed three methyl, four methylene and five methine groups. The 5-methine proton had shifted downfiled from chemical shift at 5.85 to 5.71 ppm as compared with artemisinin. From the above result, a single oxygen atom was removed from the endoperoxide position. The structure of the transformed product was confirmed by further analysis of 2D data (COSY, NOESY and HMQC spectra) and by comparison of the spectroscopic data with those of the literature values (Lee *et al*., 1989). The melting point of this non-endoperoxide derivative was between 110 to 111^{o}C. On the whole, this transformed product was identified as deoxyartemisinin (Figure 5.2) which was reported to be the transformed products of artemisinin by *Nocardia coralline* ATCC 19070, *Penicillium chrysogenum* ATCC 9480, *Mucor polymorphosporus*, *A. niger* and *A. flavas* (Lee *et al*., 1989; Srivastava *et al*., 2009; Zhan *et al*., 2002a).



Artemisinin Deoxyartemisinin

Figure 5.2 Biotransformation of artemisinin to deoxyartemisinin by *A. oryzae* (Ozykat-1) and *A.terricola* TISTR 3109

5.4 BIOLOGICAL ACTIVITY OF DEOXYARTEMISININ

The biological evaluation of deoxyartemisinin was based on antimalarial activity, antimicrobial activity and cytotoxicity assays in order to obtain the important information of this transformed product.

5.4.1 Antimalarial activity of deoxyartemisinin

Deoxyartemisinin showed less antimalarial activity than artemisinin, dihydroartemisinin and mefloquine. This result was in good conformed to the previous reports that deoxyartemisinin is an inactive antimalarial compound because it lacks of endoperoxide structure which is an essential structure for antimalarial activity (Abourashed and Hufford, 1996; Liu *et al*., 2006a). Although the mechanism of the action of artemisinin and its endoperoxide derivatives have not been fully elucidated (Slade *et al*., 2009). There are several hypotheses that have been proposed to explain their actions (Li and Zhou, 2010). The first hypothesis is that artemisinin binds specifically to a target in malaria parasites, such as PfATP6 which is a critical

calcium-pumping enzyme in *P. falciparum*. Artemisinin undergoes activation by reacting with catalytic iron, and after interaction with PfATP6, the free radicals are formed and then exert irreversible damages to this target protein. The second hypothesis speculated that artemisinin is uniquely activated by heme in malaria vacuoles. Artemisinin is activated by heme to produce reactive oxygen species (ROS), via a reductive scissor or an open peroxide model (O'Neill *et al*., 2010) follow by alkylation of heme and/or other malarial proteins. A reductive scissor model suggested that low valent transition ions (ferrous heme or non-heme exogenous Fe^{2+}) are bound to artemisinin and induced reductive scission of the peroxide bridge to produce oxygen centered radicals which rearrange to give carbon centered radicals. The open peroxide model suggested that ring opening is driven by protonation of peroxide or by complexation by Fe^{2+}. Another hypothesis was the mitochondria model which suggested that artemisinin is activated by malaria mitochondria and generates the free radicals non-specifically to damage surrounding molecules. This model has been demonstrated in yeast model (Li and Zhou, 2010a). All hypotheses suggest that the endoperoxide in the backbone of artemisinin is the essential pharmacophore. In this study, artemisinin which is endoperoxide compound showed antimalarial activity against *P. falciparum* K1 at the 50% inhibition of cell growth (IC_{50}) value of 0.0110 ± 0.001 µM, whereas a non-endoperoxide compound deoxyartemisinin showed 100-fold less antimalarial activity than artemisinin with IC_{50} value of 1.158 ± 0.081 µM. The previous studies suggested that deoxyartemisinin had no effect on membrane potential in mitochondria, PfATP6 inhibition and protein alkylation (O'Neill *et al*., 2010; Wang *et al*., 2010). The antimalarial activity of deoxyartemisinin in this study is unclear. However, it should be noted that the

antimalarial of deoxyartemisinin possibly concern with the ROS production. Further study should investigate the mechanism which may concern with the activity of deoxyartemisinin.

5.4.2 Antimicrobial activity of deoxyartemisinin

According to antimicrobial results, it was found that deoxyartemisinin did not show any antimicrobial activity. It is interesting that these findings were different from the prior published study which suggested that deoxyartemisinin exhibited *in vitro* antibacterial activity against *S. aureus, S. epidermidis* and *S. mutans* at a minimum inhibitory concentration (MIC) of 1.0 mg/ml that was lower than artemisinin (Srivastava *et al*., 2009). Artemisinin has also been reported to have antifungal activity against *A. flavus, A. niger*, *A. fumigates*, *C. albicans* and *C. neoformans* (Dhingra *et al*., 2000; Gautam *et al*., 2011). These different findings can be explained by variations in species or subspecies of microorganisms. Deoxyartemisinin could inhibit targets or processes only presented in some species or subspecies of microorganisms (Galal *et al*., 2005; Klayman, 1985). In addition, the MIC method which performed on planktonic (free floating) bacterial cells is more sensitivity than the agar disc and well diffusion methods.

5.4.3 Cytotoxicity of deoxyartemisinin

The cytotoxic assessment activity of deoxyartemisinin and artemisinin were showed in Table 5.1. Deoxyartemisinin showed the inhibitory effects of growth against all tested cell lines at the lethal dose (LD_{50}) value of 1.621 ± 0.095 mg/ml for normal mouse fibroblast L929 (ATCC-CCL-1) and at the 50% inhibition of cell

growth (IC_{50}) values of 0.585 ± 0.058, 0.394 ± 0.049, 1.292 ± 0.213 and 0.940 ± 0.087 mg/ml for mouse melanoma B10F16 (ATCC-CRL-6475), human lung carcinoma A549 (ATCC-CCL-185), human colorectal adenocarcinoma HT-29 and human colorectal adenocarcinoma Caco-2 cell lines, respectively. Nevertheless, artemisinin exhibited inhibited growth at the LD_{50} value of 1.108 ± 0.130 mg/ml for L929 cells and the IC_{50} values of 0.871 ± 0.070, 0.589 ± 0.102 and 0.080 ± 0.002 mg/ml for B16F10, A549 and HT-29 cells, respectively. These results indicated that deoxyartemisinin could inhibibit growth of Caco-2 cells at the lower concentration than that of artemisinin. The inhibitory effects of artemisinin and doxorubicin against Caco-2 were not determined because the highest concentration of artemisinin (2.5 mg/ml) and doxorubicin (0.01 mg/ml) could not inhibit the growth of Caco-2 cells. These finding could be explained by the fact that Caco-2 cells are the most resistant to doxorubicin when compared to other human colorectal adenocarcinoma cell lines, HT-29, LoVo and SW620 (Beaumont *et al*., 1998). The cytotoxicity of artemisinin and its derivatives to cancer cells was first reported by Woerdenbag *et al*. (1993) which revealed that derivatives dihydroartemisinin, artemether, arteether, sodium artesunate, artelinic acid, and sodium anelinate showed more potent cytotoxicity than artemisinin (Woerdenbag *et al*., 1993). Artemisinin inhibits cancer cells by decreasing proliferation, increasing level of oxidative stress, inducing of apoptosis and inhibiting of angiogenesis (Krishna *et al*., 2008). Endoperoxide has been proposed as a key group that might be responsible for the anticancer activity by inducing free radicals formation from iron content in cancer cells which lead to oxidative damage and iron depletion in the cells. However, the precise cytotoxic mechanism of artemisinin and its derivatives in cancer cell is still unclear (O'Neill *et al*., 2010). Reports on

cytotoxicity of the deoxyartemisinin and derivatives are limited; however, some studies have reported cytotoxic activity of these compounds toward tumor cells (Galal *et al*., 2002). Beekman *et al*. (1997) reported that artemisinin was 100-fold more cytotoxic than deoxyartemisinin, which lacks the endoperoxide bridge, against murine EN2 cells, a cloned Ehrlich ascites tumor cell line. However, the dimer of dihydrodeoxyartemisinin lacking the endoperoxide bridges was also effective in the inhibition of EN2 cells based on MTT assay, although it was 4-fold less cytotoxic than the dimer of dihydroartemisinin. These results suggested that the endoperoxide bridge appeared not to be certainly essential for cytotoxicity of the tumor cells (Beekman *et al*., 1997). Deoxyartemisinin and artemisinin also show cytotoxicity against P388 mouse leukemia cells (Charoenteeraboon, 2006). These suggested that the cytotoxicity against cancer cells may be exerting by other factors.

In this research, the maximum concentration of deoxyartemisinin and artemisinin were 2.5 mg/ml due to the poor solubility in culture medium. Deoxyartemisinin which is non-endoperoxide compound was significantly more effective than artemisinin in the inhibition of growth toward B16F10 cell line but not significantly more effective than that of the endoperoxide compound against A549 cell line at 95% significance level. In addition, the ratio of the lethal dose (LD_{50}) to the inhibition concentration (IC_{50}) of all cancer cell lines suggested that deoxyartemisinin showed the most effective in the inhibition of A549 cells growth with the ratio value of 4.120 ± 0.523 (Table 5.1). The high value of LD_{50} to IC_{50} ratio indicates that the compound show high cytotoxicity toward the cancer cells but low cytotoxicity toward the normal cells. However, the U. S. National Cancer Institute (NCI) defined that the pure compound which showed anticancer activity against

cancer cells must inhibit 50% of the cancer cells growth at the concentration lower than 4 µg/ml (Geran *et al.*, 1972). Thus, deoxyartemisinin and artemisinin did not show cytotoxicity against all tested cell lines in this study.

Table 5.1 The ratio of the lethal dose (LD_{50}) to the inhibition concentration (IC_{50}) of mouse melanoma B10F16, human lung carcinoma A549, human colorectal adenocarcinoma HT-29 and human colorectal adenocarcinoma Caco-2 cell lines treated with deoxyartemisinin

Cells	**The ratio of the lethal dose (LD_{50}) to the inhibition concentration (IC_{50})**
B16F10	2.763 ± 0.302
A549	4.120 ± 0.523
HT-29	1.271 ± 0.134
Caco-2	1.718 ± 0.161

*The data presented are the mean ± SD of triplicate samples