CHAPTER 2

LITERATURE REVIEW

2.1 BIOTRANSFORMATION

Biotransformations are chemical reactions catalyzed by cells, organ or enzymes. These chemical reactions are possibly used to generate new or novel products or improve products more efficiently (Giri *et al*., 2001; Loughlin, 2000). Biotransformations may carry out specific conversions of complex substrates using plant, animal or microbial cells or purified enzymes as catalyst (Giri *et al*., 2001). These techniques are useful for producing medicinal and agricultural chemicals compounds (Prasad *et al*., 2008). They can be also applied to predict metabolic pathways in animals, mammals and plants (Asha and Vidyavathi, 2008; Giri *et al*., 2001; Zhan *et al*., 2002b).

Microorganisms are able to perform a variety of chemical reactions because they can produce a large number of enzymes (Leyenberger, 1990). The application of microorganisms and their enzymes in biotransformation of medicinal chemicals are well- known, for example, albendazole which is used as anti-helminthic drug was transformed by *Cunninghamella blakesleeana* into albendazole sulfoxide, albendazole sulfone and N-methylated analogue of albendazole sulfoxide, tolbutamide which is used in the treatment of first stage diabetes was transformed into 4′-hydroxytolbutamide by *C. blakesleeana* AS 3.910 and diosgenin which is a starting

material for the production of steroid hormones was transformed by *Syncephalastrum racemosum* into five metabolites which showed cytotoxic activity against the human myelogenous leukemia K562 cell lines (Huang *et al*., 2006; Nikolova and Ward, 1993; Prasad *et al*., 2008; Schulze and Wubbolts, 1999; Ward and Singh, 2000; Zhao *et al*., 2010). In addition, microorganisms have short doubling time and the methods for genetic manipulation are well established (Giri *et al*., 2001).

According to the International Union of Biochemistry, enzymes can be classified into six main groups, as shown in Table 2.1. The major of enzymes which are useful in biotransformations are in the hydrolase and oxidoreductase group.

Table 2.1 Classification of chemical reaction types catalyzed by enzymes

Class of enzymes		Chemical reaction types
EC1	Oxidoreductases	Oxidization-reduction: oxygenation of C-H, C-C and C=C bonds, removal of hydrogen atom equivalents
EC2	Transferases	Transfer of groups such as acyl, sugar, phosphoryl, aldehydic and ketonic
EC3	Hydrolases	Hydrolysis of glycoside, anhydrides, esters, amides, peptides and other C-N containing functions
EC4	Lyases	Reaction such as the addition or HX to double bonds as in C=C, C=N and C=O and the reverse process
EC5	Isomerases	Isomerizations such as C=C bond migration, *cis-trans*-isomerization and racemization
EC6	Ligases	Formation of C-O, C-S, C-N, C-C and phosphate ester bonds

2.1.1 Advantages and disadvantages of biotransformation (Alotaibi, 2009; Giri *et al*., 2001; Leyenberger, 1990; Loughlin, 2000)

Biotransformations are more efficient routes to react target compounds when compare to traditional synthetic chemistry. Biotransformations can carry out reactions which are difficult or not economically feasible by chemical synthesis due to the specificity and selectivity properties of biocatalysts or enzymes in whole cells or isolated enzymes (Alotaibi, 2009; Giri *et al*., 2001; Loughlin, 2000). For chemoselectivity, the enzymes in biotransformation allow the conversion of substrate by react with selected functional groups. For regioselectivity, one of several groups with similar chemical reactivity can be reacted with enzymes. For enantioselectivity, the enzymes in biotransformations display chiral catalytic activity for selective and asymmetric conversion (Loughlin, 2000; Sweers and Wong, 1986). Biotransformations are environmental friendly methodology with less by products and solvent waste and use mild reaction conditions (Prasad *et al*., 2008). In general chemical synthesis processes are performed under extreme conditions, for example, high temperature, low or high pH, or high pressure, which may be hazardous to health and the environment while the general operating conditions of biotransformation is between 20-40^{o}C, at pH values near neutrality and at normal pressure which can prevent undesired side-reaction (Andrus *et al*., 2003; Leyenberger, 1990; Loughlin, 2000). The whole organism cells are widely used in biotransformation because of the variety of enzymes productions which are capable of converting different substrates and can be applied with some organic solvents (Alotaibi, 2009). However, the organisms used in biotransformation can produce some inhibitors that may affect transformation ability. In addition, biotransformations proceed under improperly

temperature, pH or salt concentrations; these may lead to the deactivation of enzymatic activity in biotransformation (Loughlin, 2000). Moreover, the transformed products are low when compare to chemical synthesis (Alotaibi, 2009). Several mechanisms of reaction in biotransformations of whole cell organisms are not well-defined and the productions of desired product are sensitive to environmental conditions. These disadvantages cause the industrial inertia to replace chemical transformation with biotransformation (Alotaibi, 2009; Collins and Kennedy, 1999).

2.1.2 Methodology of biotransformation (Leyenberger, 1990)

The interaction of substrates and enzymes is required for biotransformation. The substrate should be able to pass through the cell membrane and should not show inhibitory effects against organisms or their enzymes. In addition, the optimal growth phase for supplemented substrate and its concentration should be considered. The typical biotransformation system can be categorized as the following systems.

Biotransformations with growing culture (Single-stage biotransformation): The substrate is added to the culture medium at that time of inoculation or during growth of organisms which the growth and the biotransformation take place simultaneously. This technique is suitable for screening the biotransformation ability of organisms in large scale. Moreover, this system requires short incubation time and the enzymes are active during the growth phase.

Biotransformations with the previous growth (Two-stage biotransformation): Cells are grown and then harvested. The harvested cells are resuspended in transformation medium which usually consists of a buffer solution containing the

soluble substrate. Several successful biotransformation researches also supplied the transformation medium with metabolizable nutrients in order to maintain viability of organisms and biocatalytic activity of the cells (Wang *et al*., 2007). The cell suspension is incubated until the highest yield of transformed product is obtained. The advantages of this technique are the elimination of growth-inhibitory effects by substrate or product, the cell concentration can be controlled and the product isolation is more easily than the biotransformations with growing culture. However, this technique increases the risk of contamination by undesired microorganisms during the biotransformation.

Biotransformations with the purified enzymes: This technique is commonly used in case of the substrate cannot permeable through the cell membrane or the unwanted reactions are generated due to the presence of the other enzymes in the cells. The supplement of specific cofactors such as NAD(H), NADP(H), FAD(H_2) or ADP/ATP are required for reactions by oxidoreductase and kinase. Therefore, the using of purified enzymes in biotransformation is expensive.

Biotransformations with immobilized cells or enzymes: Many techniques have been used for cells or enzymes immobilization, for example, entrapment, adsorption, ionic or covalent binding and crosslinking (Leyenberger, 1990). The immobilized enzymes or cells are easily to remove from the system and can be used for continuous biotransformation process.

Biotransformations with liquid two-phase systems: Cells and enzymes usually show their activities in aqueous media. Hence, the biotransformations of lipophilic substrates are limited due to the low solubility in medium. As results, detergents are

supplemented in order to improve the solubility. However, detergents may inhibit enzyme activities or cell growths. This problem can be overcome by addition a second water-immiscible phase, for example, hexane, dichloromethane, chloroform, nitrobenzene and chlorobenzene. Thus, the enzymes remain in aqueous phase while the lipophilic substrate and the transformed product are presence in the organic phase. The biotransformations are generated either in aqueous phase or interphase. An ideal organic solvent used for this technique should show high solubility for substrate and transformed product and should not inhibit enzyme activities. The advantages of this technique are simple separation and repeated use of biocatalysts.

2.2 ARTEMISININ (Li and Zhou, 2010)

The emergence of malaria strains that resist to almost traditional antimalarial drugs become the health problem. Forty years ago, a consortium of Chinese scientists supported by Chinese government attempted to combat malaria. This led to the discovery of artemisinin which is effective agent to kill malaria parasites. The World Health Organization (WHO) has recommended Artemisinin Combination Therapies (ACTs) as the first line treatment of multidrug-resistant *Plasmodium falciparum*. This parasites strain cannot be eliminated by traditional antimalarial drugs, quinoline and antifolate antibiotic (Zhan *et al*., 2002b). Artemisinin also shows inhibitory effects against other parasites such as Leishmania, Schistosoma and Toxoplasma (Jones-Brando *et al*., 2006; Sen *et al*., 2007; Utzinger *et al*., 2000; Utzinger *et al*., 2007). The additional biological activities of artemisinin are antiviral and anticancer which possible use in virus and cancer therapies (Li *et al*., 2008; Romero *et al*., 2006).

However, artemisinin has low solubility in both water and oil which lead to difficult of administration through intravenous injection. The other disadvantages of artemisinin are short half-life after administration and high recrudescence rate of infection with artemisinin monotherapy. Thus, the development of antimalarial drugs based on the artemisinin prototype has been investigated in order to improve its stability, efficacy and reduce toxicity.

2.2.1 The History of Artemisinin

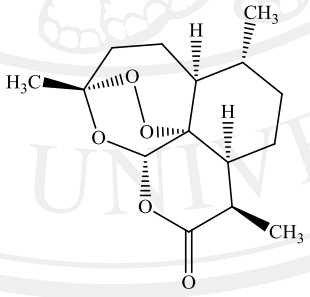
Artemisinin was first discovered in 1956. After the successful extraction and isolation of artemisinin from *Artemisia annua* or wormwood plant (Figure 2.1), artemisinin was introduced to the rest of the world in 1979. The chemical synthesis of artemisinin is interesting because of low production derived from nature. In 1983, the successful semi-synthesis method of artemisinin from artemisinic acid was first reported by Schmid and Hofheinz (Schmid and Hofheinz, 1983). Later, other methods were developed with different raw materials and routes. Medicinal chemistry studies based on endoperoxide of artemisinin lead to the development of peroxide, trioxane, tetraoxane and other derivatives of artemether and artesunate, which are C-10 modified derivatives of artemisinin.

Figure 2.1 *Artemisia annua*, L. (Sweet wormwood)

(From: http://www.naturaldrugs.blogspot.com, 27/3/2012)

2.2.2 General information

Artemisinin is a sesquiterpene lactone containing an endoperoxide with a molecular weight of 282.332 g/mol (Figure 2.2). Artemisinin has poor solubility in water and oil. The melting point is at about 156-157°C (Lin *et al*., 1985).



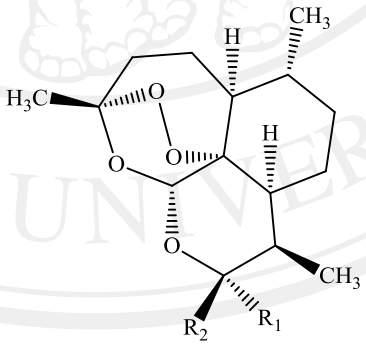
Formula: $C_{15}H_{22}O_5$, Molecular mass: 282.332 g/mol

Density: 1.24 ± 0.1 g/cm^3, Melting point: 156-157^{o}C

Figure 2.2 Chemical structure of artemisinin

Due to the poor solubility property of artemisinin, the development of pharmaceutical properties of artemisinin derivatives has been studies. The most

important derivatives are dihydroartemisinin, artemether, arteether, artelinic acid and artesunate (Figure 2.3). Arteether, oil soluble derivative, can be used in the treatment of acute *P. falciparum* (Pareek *et al*., 2006). Deoxyartemisinin, a sesquiterpene without endoperoxide (Figure 2.4), is prepared by hydrogenation with Pd/C as catalyst. This compound has nearly no inhibitory effect on malaria parasites (Li and Zhou, 2010). The lactone group of artemisinin is reduced with sodium borohydride, resulting in the formation of dihydroartemisinin which shows higher *in vitro* antimalarial activity than artemisinin (Figure 2.5). The other derivatives have been synthesized from dihydroartemisinin, for example, reduction of artemisinin by sodium borohydride at -5^{o}C and then methylation with methanol resulting in artemether (Figure 2.6) (Slade *et al*., 2009) which is oil soluble antimalarial compound and shows superior antimalarial activity to that of artemisinin and arteether, and artesunate which is now available in the antimalarial drug monograph of the U.S. pharmacopeia.



R_1 = H	R_2 = OH	Dihydroartemisinin
R_1 = H	R_2 = OCH_3	Artemether
R_1 = H	R_2 = OC_2H_5	Arteether
R_1 = H	R_2 = $OCH_2C_6H_5CO_2H$	Artelinic acid
R_1 = $O_2CCH_2CH_2CO_2H$	R_2 = H	Artesunic acid (Artesunate)

Figure 2.3 Chemical structures of some artemisinin derivatives

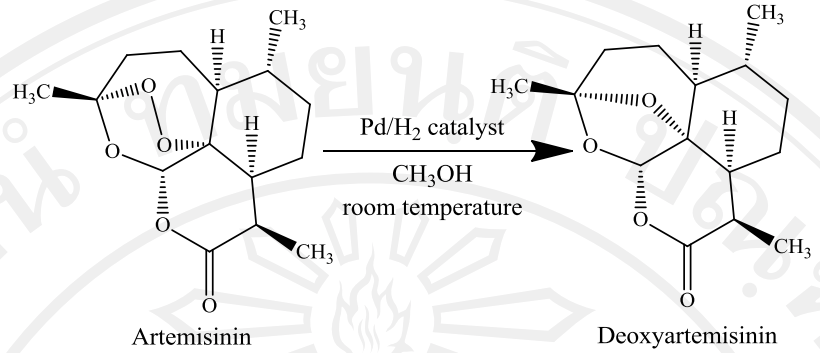


Figure 2.4 Hydrogenation with Pd/C as catalyst at room temperature resulting in deoxyartemisinin (Li and Zhou, 2010)

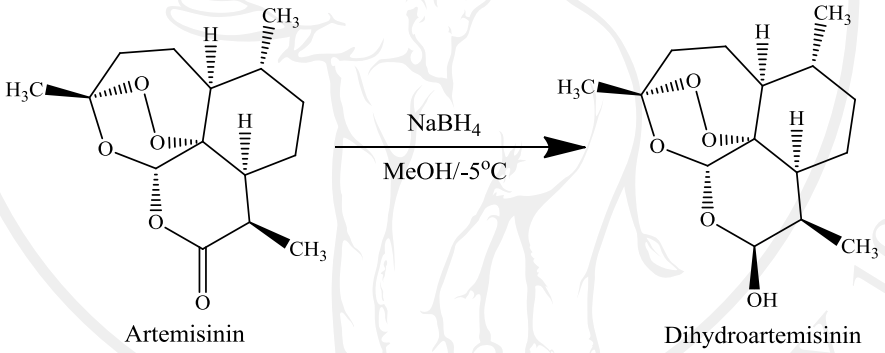


Figure 2.5 Reduction of artemisinin by sodium borohydride at -5°C resulting in dihydroartemisinin (Slade *et al*., 2009)

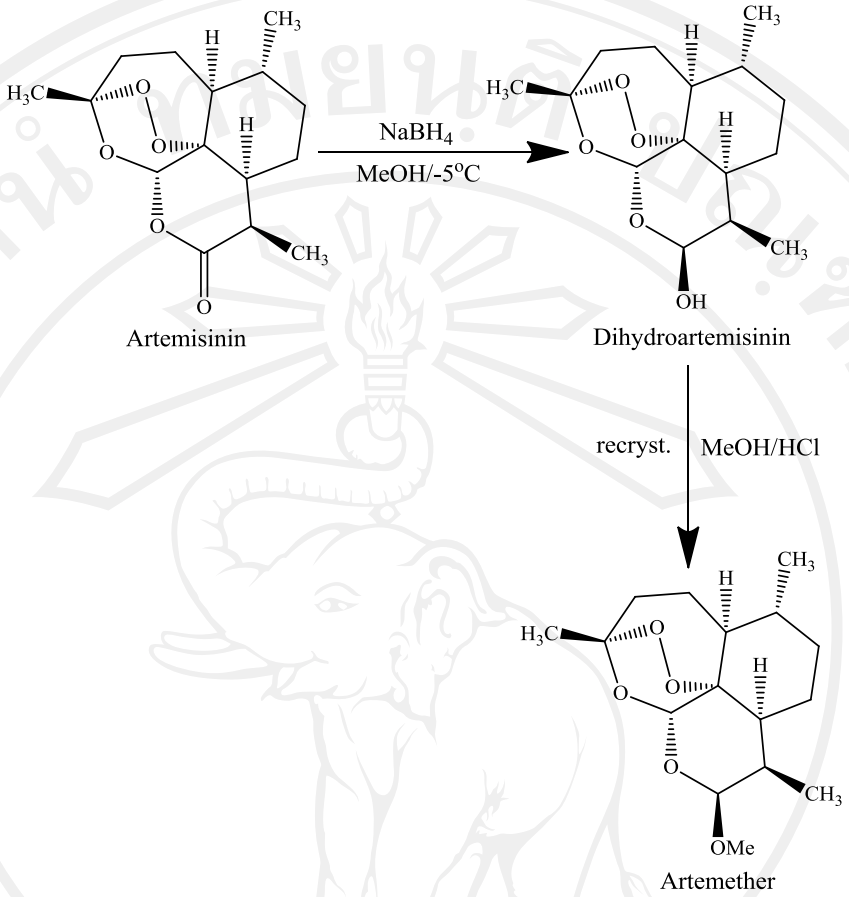


Figure 2.6 Reduction of artemisinin by sodium borohydride at -5°C and then methylation with methanol resulting in artemether (Slade *et al*., 2009)

2.2.3 Antimalarial activity of artemisinin (Li and Zhou, 2010)

Artemisinin is effective against multidrug-resistant strains of *P. falciparum* and has specific activity against the *Plasmodium* life cycle including activity throughout the asexual blood stages and the sexsual gametocyte stages which may reduce the spread of the disease (Kumar and Zheng, 1990; O'Neill *et al*., 2010).

2.2.3.1 Biological activity in parasites

The hemoglobin of parasite host is degraded by protease activities of malaria parasites to release peptides and amino acids which are important for growth and development of parasites. During the degradation of hemoglobin, hematin, a toxic compound, is formed by hydrogen bonding of heme monomers. This toxin is detoxified and biomineralized by malaria parasites to form insoluble non-toxic hemazoin, a heme crystal, as shown in Figure 2.7 (O'Neill *et al*., 2010; Timothy J, 2008).

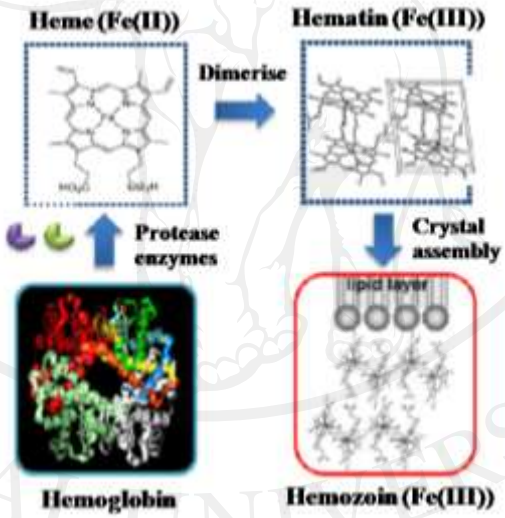


Figure 2.7 Detoxification of hematin (O'Neill *et al*., 2010)

Quinine, chloroquine, amodiaquine, mefloquine, halofantrine and lumefantrine are powerful antimalarial drugs which active during hemozoin producing stages of malaria parasites. These drugs inhibit the biomineralization of hematin into hemozoin and result in accumulation of toxic compound in malaria parasite. However, *P. falciparum* can resist to these drugs due to an increase in capacity for malaria parasite to evacuate drugs at a rate that does not allow these drug to reach levels required for

inhibition of hemazoin formation (Foley and Tilley, 1997). On the contrary, artemisinin and derivatives are effective not only against multidrug-resistant *P. falciparum* strains but have wide stage specificity against the life cycle of *Plasmodium* parasites including activity throughout the asexual blood and the sexual gametocyte stages. The antimalarial mechanism of artemisinin and its derivatives are not associated with detoxification of hematin. However, the accurate mechanism of artemisinin is still debated (O'Neill *et al*., 2010).

2.2.3.2 Proposed mechanisms of artemisinin on antimalarial activity

Many scientists have been proposed the mechanisms of artemisinin on malarial parasites as showed in Figure 2.8.

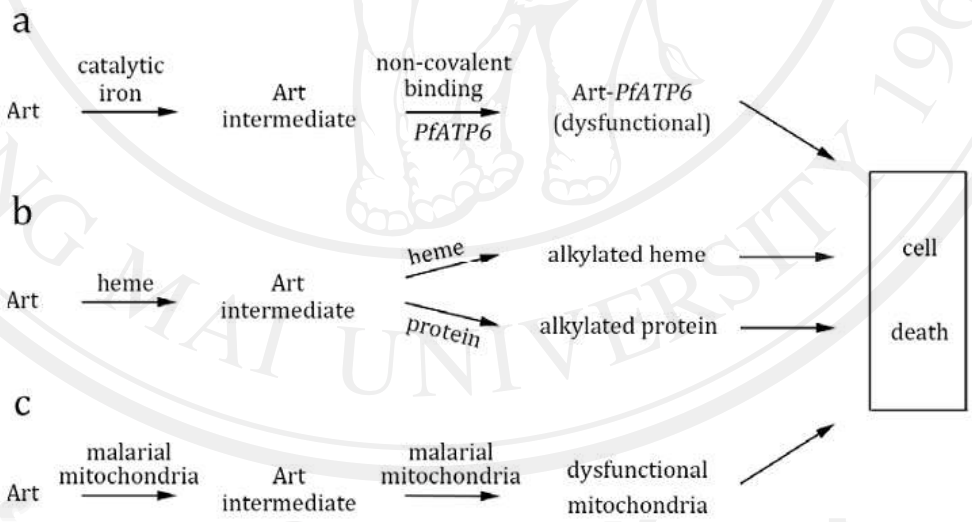


Figure 2.8 Proposed biological models for the action of artemisinin (Art). (a) The PfATP6 model (b) The heme model (c) The mitochondria model

a. PfATP6 as the target: Artemisinin possibly binds specifically to PfATP6 which is a critical calcium-pumping enzyme in *P. falciparum*. However, his proposed has not been supported by the findings on how the backbone and chirality of artemisinins can vary without diminishing their antimalarial activities (Figure 2.8A).

b. Heme as the activator and target: Artemisinin is activated by heme in parasite vacuoles to produce of reactive oxygen species (ROS), via a reductive scissor or an open peroxide model. The reductive scission model proposed that the carbon centred radicals are formed by the rearrangement of the oxygen centered radicals (Jefford *et al*., 1996; O'Neill *et al*., 2010; Posner and Oh, 1992; Posner *et al*., 1994). Artemisinin is bound with ferrous ion from heme or exogenous Fe^{2+}. After that the reductive scission of peroxide bridge is induced by electron transferring to generate oxygen centered radicals which give carbon centered radicals as shown in Figure 2.9A (O'Neill *et al*., 2010). The open peroxide model, Figure 2.9B, suggests that the protonation of peroxide or the complexation by Fe^{2+} open the artemisinin ring and generate the open hydroperoxide (Haynes *et al*., 1999; Haynes and Vonwiller, 1996). The oxygen atom stabilizes of positive charge and uses low energy for opening ring according to transition state theory. The capture of heterolytic cleavage of the endoperoxide bridge leads to an unsatured hydroperoxide formation which is able to modify malaria protein residues by oxidation. Then, a hydroxyl radical, which is a species that can oxidize parasite protein, is produced by Fenton degradation of the hydroperoxide (Haynes *et al*., 1999; O'Neill *et al*., 2010). This mechanism may associate with the antimalarial activity of artemisinin and its derivatives to generate reactive oxygen species. However, the other compounds without heme reactivity also

display potent antimalarial activity. Therefore, the inhibition of heme formation does not prevent the antimalarial activity of artemisinin (Figure 2.8B).

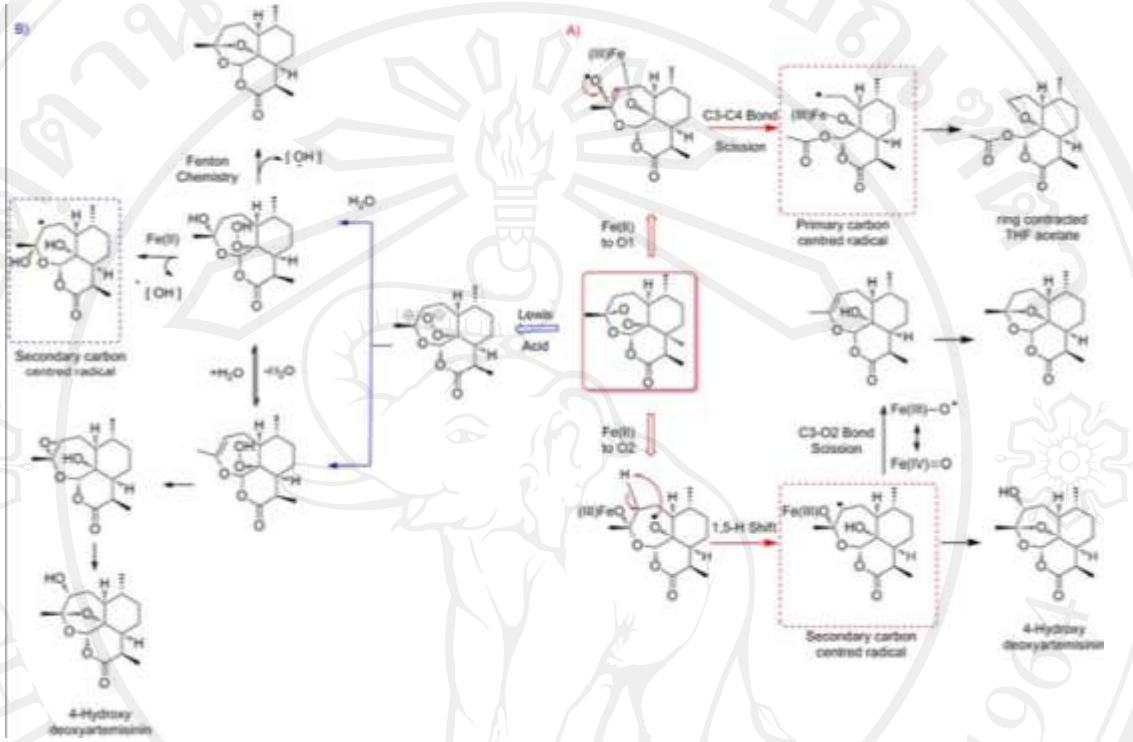


Figure 2.9 Biological activity of artemisinin; A) Reductive scission model; B) Open peroxide model (O'Neill *et al*., 2010)

C. Mitochondria as the target: Artemisinin is activated by malaria mitochondria and then the free radicals non-specifically damage surrounding molecules. This proposed model is demonstrated in yeast, but further supporting evidence is required in parasite (Figure 2.8C).

2.2.4. Cytotoxicity of artemisinin against tumor cells

Artemisinin have been investigated for their cytotoxicity against a variety of cancer cell lines, colon, breast, lung, pancreatic cancers and leukemia (Krishna *et al*., 2008). Artemisinin and derivatives such as dihydroartemisinin, artesunate, sodium

artesunate and deoxyartemisinin shows cytotoxic activity in micromolar range (Woerdenbag *et al*., 1993). Artemisinin may inhibit cancer cells by decreasing proliferation, increasing level of oxidative stress, inducing of apoptosis and inhibiting of angiogenesis (Krishna *et al*., 2008). Artemisinin also shows cytotoxicity against drug and radiation resistant cancer cell lines. These findings suggest that artemisinin associate with a different cytotoxic mechanism when compare to traditional anticancer therapies (Efferth *et al*., 2003). The current consensus suggests that the releasing of reactive oxygen species (ROS) or carbon centered radicals form the iron (II) mediated can induce DNA damage, mitrochondrial depolarization and apoptosis (Efferth, 2006; Efferth *et al*., 2004; Mercer *et al*., 2007). Furthermore, there are the other factors involving in cytotoxicity such as the ability of the tumor cell to transport and maintain ferrous iron. However, the cytotoxic mechanism of artemisinin and its derivatives in cancer cell is still unclear (O'Neill *et al*., 2010),

2.2.5 Pharmacokinetic data of artemisinin and derivatives

The pharmacokinetic data of artemisinin and its derivatives is shown in Table 2.2. Artemisinin and its derivatives can be absorbed rapidly, but incompletely when they are used as oral formulations. These compounds usually have short elimination half-life. The peak concentration of artemisinin and its derivatives in plasma is detected within 3 hours (Balint, 2001).

Table 2.2 The pharmacokinetic data of artemisinin and its derivatives (Balint, 2001)

Drug	Artemisinin	Artemether	Arteether	Artesunate	Dihydroartemisinin
Absorption	Rapid and incomplete	Rapid and incomplete		Rapid and incomplete	
Distribution volume (L/kg)		0.67	0.72	0.14	0.90
Oral plasma clearance (L/h)	400			2.33	1.10
Elimination Half-life (h)	2-5	3-11	> 20	< 1	3.1
Bioavailability (%)	8-10	54	34	82	85
Peak plasma concentration (h)	< 2	3			0.65
Protein-binding (%)	80				
First-pass effect	Extensive	Extensive		Extensive	
Usual oral (dose/kg)	20 mg	4 mg		4 mg	

2.2.6 Clinical data (Balint, 2001)

Many malaria-infected patients, particularly in China, Vietnam, and Thailand, have been treated with dihydroartemisinin, artesunate, arteether and artemether. The side effects of these artemisinin derivatives have not been discovered and the significant differences in efficacy or toxicity profiles are not found as well. These

antimalarial agents are effective against all human *Plasmodium* strains of malaria. Dihydroartemisinin, artesunate, arteether and artemether showed fast antimalarial treatment, good tolerability, and eliminate parasites from the blood within 2 days. Artemisinin and its derivatives should be administered in combination with schizonticide in order to reduce recrudescence and to prevent the development of resistance.

2.2.7 Resistance of artemisinin and derivatives (Meshnick, 2002)

There is currently no evidence for clinically relevant artemisinin resistance. However, the resistance of artemisinin and its derivatives possibly develop since these antimalarial agents are being widely used. There are several reasons to support the delay of the appearance of artemisinin and derivatives resistant parasites. The subtherapeutic concentrations do not affect to the malaria parasites because artemisinin and derivatives have a short half-life. Artemisinin and derivatives can reduce transmission of malaria parasites by inhibitory of the sexual gametocyte stages of malaria parasites. Thus, parasites in artemisinin derivatives treated patients have less possible to infect mosquitoes and other humans. In addition, artemisinin and derivatives are now widely used in combination with other antimalarials such as mefloquine or lumefantrine which are able to delay the onset of resistance.

2.2.8 Recrudescence of artemisinin and derivatives

The recrudescence rate refers to the high rate of treatment failure and the cure rate refers to the high rate of treatment success. Artemisinin and derivatives are very effective compounds against all human malaria parasites, particularly *P. falciparum* and *P vivax*. Clinical recovery of patients treated with these drugs is faster than with

other antimalarial drugs. In 3-day courses of oral, intramuscularly, intravenously, or intrarectally administrations with artemisinin, artesunate, artemether or dihydroartemisinin, the recrudescence rate vary from 44 to 54%. With 5-7 days of remedy, the cure rate increased above 90% (van Agtmael *et al*., 1999). The recrudescence after treatment with artemisinin derivatives is thought to be due to host metabolism (Meshnick, 2002).

2.2.9 Toxicity of artemisinin and derivatives

Adverse effects are limited in patients treated with artemisinin derivatives. In a study of over 3,500 patients in Thailand, the result demonstrated that no evidence for serious adverse effects. In animal studies, artemisinin derivatives at high doses have been shown to cause neurotoxicity. According to Brewer *et al*. (1998) study, the dogs administered with arteether at 20 mg/kg/day or 15 mg/kg/day has been found to develop neurological symptoms, neuropathological findings, and electrocardiographic abnormalities (Brewer *et al*., 1998). Artemether and arteether also show neurotoxicity against mice after oral administration at high doses (300 mg/kg/day) (Nontprasert *et al*., 2000). These findings are in agreement with the *in vitro* study in neuronal cells. (Meshnick, 2002).

2.2.10 The structural modifications of artemisinin and derivatives by chemical and biological methods

The biological activities of artemisinin and its derivatives are limited due to their short elimination half-time, toxicity, and solubility in oil and water (Krishna *et al*., 2004; Meshnick, 2002; Rydén and Kayser, 2007). The structural modifications of

artemisinin and its derivatives by chemical and biological methods are believed to solve these problems.

The chemical modifications of artemisinin and its derivatives have provided a variety of artemisinin derivatives which perform more desired pharmacological properties than artemisinin. One of the successful modifications of artemisinin derivative is revealed by Li and his co-workers. More than 30 artemisinin derivatives containing an amino group are synthesized. Almost of all derivatives are the β-isomers and these derivatives are combined with organic acids such as oxalic acid, maleic acid to yield the corresponding salts derivatives. The malenate derivatives provide better solubility in water than the oxalates. The aqueous solutions of these salts are stable at room temperature for several weeks without any decomposition. Furthermore, 1-dimethylamino-2-(10β-dihydroartemisinoxy) ethane oxalate and 1-morpholino-3-(10β-dihydroartemisinoxy) propane malenate shows higher antimalarial activity against *P.berghei* than artesunic acid in mice but poorer than that of artesunic acid against *P. knowlesi* in rhesus monkeys (Li *et al*., 2000). Ekthawachai *et al*. (2001) also reported the successful modification of artemisitene into C-16-derived artemisinin monomers, dimers, trimers, and tetramers. The majority of the C-16 artemisinin derivatives show high antimalarial activity comparable to that of artemisinin with low cytotoxicity against human epidermoid carcinoma (KB), human breast cancer (BC), and African green monkey kidney fibroblast (Vero cells). Several artemisinin dimers, trimers and tetramers also show higher antimalarial activity towards *P. falciparum* strain K1 than artemisinin and display high cytotoxicity against KB, BC, and Vero cell lines (Ekthawatchai *et al*., 2001). Galal *et al.* (2002) synthesized deoxyartemisinin derivatives from photooxygenation of

anhydrodeoxydihydroartemisinin. Among these derivatives, deoxyartemisitene (Figure 2.10) shows cytotoxicity against several human tumor cell lines for example leukemia HL-60 (TB), CCFR-CEM, K-562, RPMI-8226 and SR cell lines with IC_{50} values of 0.69, 0.92, 0.94, 1.68 and 1.89 µg/ml, respectively. It also shows the ovarian cancer IGROVI, the non-small cell lung cancer HOP-92 and the breast cancer MCF-7 and BT-549 cell lines with IC_{50} values of 0.89, 0.92, 1.46 and 1.97µg/ml, respectively (Galal *et al*., 2002).

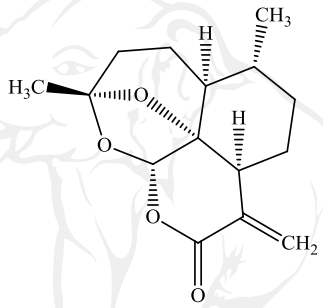


Figure 2.10 Chemical structure of deoxyartemisitene

Another approach for structural modification is the biological method which is called biotransformation or bioconversion. Biotransformations of artemisinin and its derivatives usually perform by bacteria, fungi and plant cells. For biotransformation by bacteria, artemisinin is transformed by *Streptomyces griseus* ATCC 13273 into artemisitone-9, 9α and 9β-hydroxy-artemisinin (Figure 2.11). The mechanism of generating these metabolites suggests that artemisinin is hydroxylated at C-9 position by a hydroxylase to generate the monohydroxylated compounds, 9α and 9β-hydroxyartemisinin. Upon the formation of these monohydroxylated compounds, an alcohol dehydrogenase in the organism catalyzes a dehydrogenation reaction, in the presence of NAD^+ or $NADP^+$ as a cofactor, to form artemisitone-9 (Liu *et al*., 2006b).

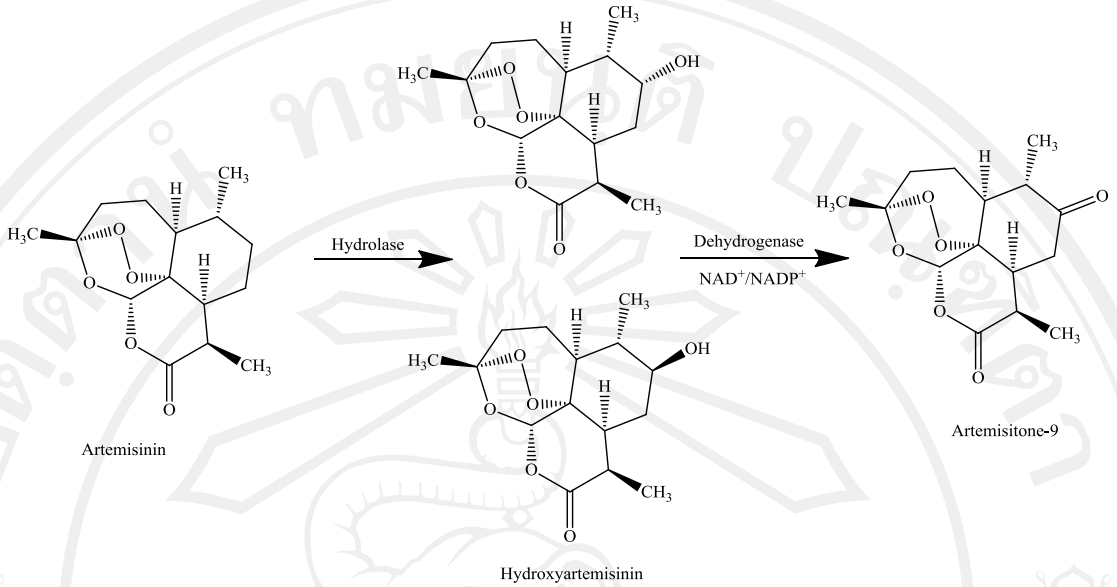


Figure 2.11 Biotransformation of artemisinin by *Streptomyces griseus* ATCC13273 into artemisitone-9, 9α and 9β-hydroxy-artemisinin.

There are several studies on biotransformations of artemisinin and its derivatives by fungi. *Nocardia corallina* ATCC 19070 and *Penicillium chrysogenum* ATCC 9480 are able to transform artemisinin into deoxyartemisinin and 3α-hydroxydeoxyartemisinin (Lee *et al*., 1989). The transformation of artemisinin by *Cunninghamella elegans* yields as 7β-hydroxyartemisinin, 7β-hydroxy-9α-artemisinin, 4α-hydroxy-1-deoxyartemisinin and 6β-hydroxyartemisinin at 78.6%, 6.0, 5.4 and 6.5%, respectively. These transformation products are purified and identified by high performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR) and mass spectrometry (MS). The hydroxygenated compound, 7β-hydroxyartemisinin, provides a better anti-malarial activity and water solubility when compared with artemisinin (Avery *et al*., 1999; Zhan *et al*., 2002b). Zhan *et al.* (2002) also reported that *Mucor polymorphosporus* is employed to convert artemisinin into 9β-hydroxyartemisinin, 3β-hydroxyartemisinin, deoxyartemisinin and 3β-

hydroxydeoxyartemisinin (Zhan *et al*., 2002a). Fiaux de Medeiros *et al*. (2002) revealed that 10-deoxoartemisinin is transformed to 7β-hydroxydeoxoartemisinin by using *Mucor ramannianus* (Fiaux de Medeiros *et al*., 2002). Transformation of artemisinin to 5β-hydroxyartemisinin and 7β-hydroxyartemisinin by the fungi *Eurotium amstelodami* is reported by Parshikov and his co-workers (Parshikov *et al*., 2006). Srivastava *et al.* (2009) shows the successful transformation of artemisinin by *Aspergillus flavus* as deoxyartemisinin with higher antibacterial activity against *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus mutans* than that of artemisinin (Srivastava *et al*., 2009). Goswami *et al.* (2010) found that artemisinin is transformed to 9β-acetoxy artemisinin and 9α-hydroxyartemisinin by *Penicillium simplissimum* (Figure 2.12). 9β-acetoxy artemisinin shows particularly inhibitory to colon HCT-15, lung A549 and neuroblastoma IMR-32 cell lines (Goswami *et al*., 2010). These transformations suggest that fungi are able to transform artemisinin by hydroxylation, deoxygenation and acetoxygenation.

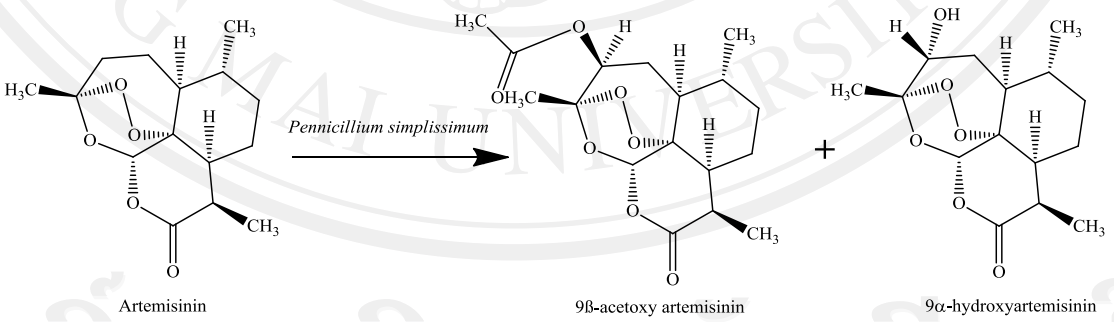


Figure 2.12 Biotransformation of artemisinin into 9β-acetoxy artemisinin and 9α-hydroxyartemisinin by *Penicillium simplissimum*

Artemisinin is also transformed by plant cells. C*atharanthus roseus* (L.) G. Don., *Lavandura officinalis* L. and *Withania somnifera* suspensions transform artemisinin into deoxyartemisinin, as showed in Figure 2.13 (Patel *et al*., 2010; Sabir *et al*., 2010).

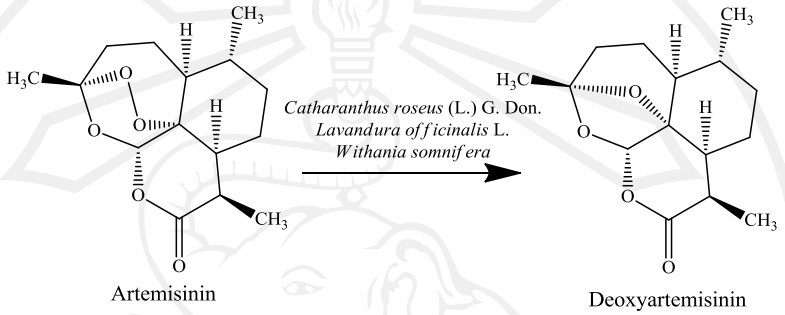


Figure 2.13 Biotransformation of artemisinin into deoxyartemisinin by plant cell suspension cultures of C*atharanthus roseus* (L.) G. Don., *Lavandura officinalis* L.and *Withania somnifera*

2.3 *ASPERGILLUS* SPECIES

The genus *Aspergillus* belongs to the family Trichocomaceae, class Euascomycetes and Phylum Ascomycota with seven subgenera. There are 184 species of *Aspergillus* sp. in each subgenus and more than 60 species of *Aspergillus* are plant, animal and human pathogens (Thom, 1926). However, several species of *Aspergillus* spp. have been used in medicinal and commercial industries.

2.3.1 Description and nature habitats

Aspergillus is a well-known filamentous fungus. It is commonly found in nature, soil, decaying vegetation and indoor air environment (Ozer *et al*., 2009). *Aspergillus* sp. is anamorphic fungus which is produce asexual spore. It produces conidia on uniseriate or biseriate phialides for reproduction. The colors of *Aspergillus* colonies are white, green, yellow, or black colonies (Murray, 2007). However, some of the *Aspergillus* sp. is teleomorphic fungi. Teleomorphic is a sexually reproducing stage, for example, *A. flavus*, *A. fumigatus*, *A. lentulus*, *A. calidoustus*, *A. versicolor* and *A. terreus* (Geiser, 2009).

2.3.2 Growth and distribution

Aspergillus sp. is highly aerobic fungus which can be found in almost all oxygen-rich environments. It commonly grows on the surface of a substrate which has the high oxygen tension. *Aspergillus* sp. can contaminate starchy foods, such as bread and potatoes, as well as grow on many plants. Many species of *Aspergillus* are oligotrophic fungi which can grow in very low levels or lack of nutrients in the environments.

2.3.3 Commercial importance

Aspergillus species are important in commercial fermented foods and drinks. *A. oryzae* is used to break down starches into sugars in the fermentation of Japanese sake, rice vinegars and soy sauce. Katsoubushi, an extraordinary fermented fish, is fermented by *A. ochraceus* and possibly *A. glancus*. Kaffir beer, an African drink made from grains such as sorghum and maize, is saccharified through the action of *A.*

flavus. In addition, *Aspergillus* species are capable to produce a large amount of commercial enzymes. *A. niger* and *A. oryzae* are used for commercial production of α-amylase and cellulase (Erb *et al*., 1948; Hurst *et al*., 1977). *A. saccharolyticus* can produce β-glucosidase which plays an important role for efficient and complete hydrolysis of lignocellulosic biomass for production of biofuels and bioproducts (Sorensen *et al*., 2011). The production of citric acid is one of the most commercial important of the *Aspergillus* fungi. Citric acid is widely used as a flavoring and preservative in food and beverages, especially soft drinks. *A. niger* and *A. awamori* are well-known as a citric acid producers. Citric acid produced by *A. niger* is also respected as Generally recognized as safe (GRAS) by the United States Food and Drug Administration.

2.3.4 *Aspergillus oryzae*

A. oryzae is a filamentous fungus which is an essential microorganism for oriental food production (Truong *et al*., 2004). The fungus is used for the production of soy sauce, miso and rice vinegars and also used in saccharification of rice, potatoes or other grains in order to produce alcoholic beverages such as huangjiu, sake and shochu (Haruta *et al*., 2006). *A. oryzae* is safe fungi because it does not produce harmful products such as aflatoxins or any other carcinogen metabolites (Barbesgaard *et al*., 1992). Due to its application in food industries, *A. oryzae* is listed as GRAS by the United State Food and Drug Administration. Machida *et al.* (2005) was reported that *A. oryzae* has the largest expansion of hydrolytic genes (approximately 135 protease genes) and suggested that *A. oryzae* is the most important protease producer. This fungus is also recognized as a wide range of enzymes producer that is able to

metabolize a variety of complex organic molecules (Truong *et al*., 2004). The abilities of *A. oryzae* in enzyme productions and transformations of complex organic molecules lead to expand the application of *A. oryzae* in biotechnology (Machida *et al*., 2005)

Figure 2.14 *Aspergillus oryzae* (Ozykat-1)

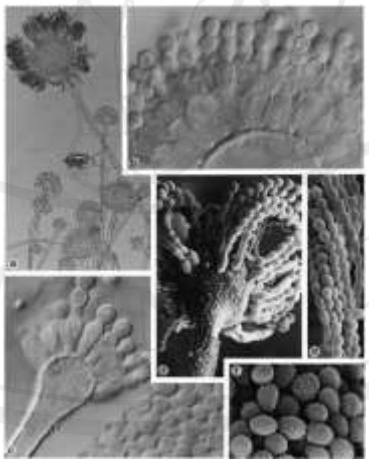


Figure 2.15 Morphology of *Aspergillus oryzae*

(From: http://www.mycobank.org, 27/3/2012)

2.3.5 *Aspergillus terricola*

A. terricola is a typical soil filamentous fungus which can produce soluble nutrient materials for soil flora. This fungus can grow rapidly when it is cultivated on

potato, beef gelatin and beef agar. The colonies of *A. terricola* are a yellow ochre color with a white border and appear a raw umber color when the colonies are a month-old. The conidia are a light amber color, round, thick walled and varicose. *A. terricola* can produce a variety of enzymes, for instance, inulase, diastase, invertase, maltase, alcoholoxydase, lipase, protease and amidase. In addition, this fungus is able to produce tannase when grown in media supplemented with a tannin solution *(*Scales, 1914). *A. terricola* is also used in production of xylanolytic enzymes for industrial applications.

Figure 2.16 *Aspergillus terricola* TISTR 3109

2.3.6 *Aspergillus niger*

A. niger is a filamentous human pathogenic fungus which is commonly found in the environment. This fungus can be found in nuts, graped, dried vine fruits, apples and tomatoes, soils and decomposing plant material (Perfect *et al*., 2001). It can produce ochatoxin A which is toxic to human due to the nephrotoxic, immunotoxic, teratogenic and carcinogenic effects. *A. niger* is the most famous fungus which is well-known as a citric acid producer. *A. niger* is served as a model fungal in citric acid production process because it can produce citric acid more than one million

metric tons annually (Lotfy *et al*., 2007). Moreover, *A. niger* can produce a variety of the extracellular enzymes which are important in biotechnology industries. Many *A. niger* enzymes such as lactase, protease and lipase and citric acid that are produced by *A. niger* are considered as GRAS by the United States Food and Drug Administration. *A. niger* is also used for waste treatment (Schuster *et al*., 2002). In addition, this fungus is important in the global carbon cycle with a production of hydrolytic and oxidative enzymes involved in the breakdown of plant lignocelluloses (Baker, 2006). *A. niger* can grow rapidly with woolly colonies on potato dextrose agar at 25^{o}C. The color of colony is initially white becoming black to deep brown as showed in Figure 2.17.

Figure 2.17 *Aspergillus niger* TISTR 3254

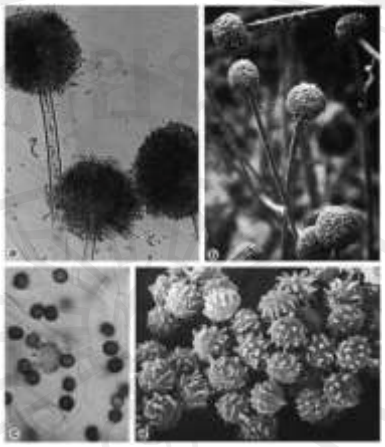


Figure 2.18 Morphology of *Aspergillus niger*

(From: http://www.mycobank.org, 27/3/2012)

2.3.7 *Aspergillus usamii*

A. usamii is a filamentous fungus which is closely related to *A. niger* in taxonomy and other properties (Zhou *et al*., 2008). This fungus can produce acidic xylanase, carboxypepidase and β-glucosidase (So *et al*., 2010; Wang *et al*., 2011). The xylanase gene (xynII) from *A. usamii* is successful cloned and obtained the recombinant protein using *E. coli* expression system. However, the expression level of this xylanase is too low for industrial application.

Figure 2.19 *Aspergillus usamii* TISTR 3258

2.3.8 *Aspergillus melleus*

A. melleus is plant pathogen which can cause a disease in *Psidium cattleianum, Passiflora mollisma* and *Clidemia hirta*. Colonies of *A. melleus* are raised at center with 2.5-3.0 cm. in diameter when *A. melleus* was cultured in Czapek's solution agar (CzA) at 24-26°C for 10 days. *A. melleus* are widespread in forest soils of tropical and subtropical regions. This fungus also grows on groundnut, soya beans, rice and corn, milled rice and dried salted food. *A. melleus* is also isolated from the cotton rhizosphere as an antagonistic fungus (Domsch, 1980). *A. melleus* is able to produce alkaline proteinase and aminoacylase (Bakker *et al*., 2001).

Figure 2.20 *Aspergillus melleus* (Ondeyka *et al*., 2003)