PRODUCTION OF FLAVONOID, AN ANTI-INFLAMMATORY AGENT FROM *Trametes lactinea*

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By

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In the Name of Allah, the Most Gracious, the Most Merciful. *Alhamdulillah* (Praise to God) and *Selawat* (Prayer for peace and prosperity) to noble Prophet Muhammad SAW and upon his family and companions, the honorable followers.

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α	Product formation constant	
β	Product formation constant	
σ	Variance	
A	Input variable – initial pH	Day
B	Input variable – inoculum volume	mL
C	Input variable-Incubation temperature	(^{o}C)
D	Input variable-Incubation time	day
dX/dt	Growth rate	g/L.d
K_S	Substrate concentration at one – half the maximum specific growth rate	g/L
m_S	Maintenance coefficient	g/g.d
n	Average cell count per square	-
P_o	Product concentration at initial time	µg/mL
P_t	Product concentration at any time	µg/mL
S	Limited substrate concentration	g/L
S_o	Substrate concentration at initial time	g/L
S_t	Substrate concentration gas at any time	g/L
t	Time	hour
t_o	Initial time	hour
X	Biomass concentration	g/L
x_i	The level of independent variable	-
x_j	Coded independent variable	-
X_m	Maximum biomass	g/L

x_{max}	Maximum value of the natural variable	-
x_{min}	Minimum value of the natural variable	-
X_o	Initial biomass	g/L
$Y_{X/S}$	Biomass yield on the utilized substrate	g X/g S

ANN	Artificial neural network
ANOVA	Analysis of variance
BBD	Box-Behnken design
CCD	Central Composite design
DMSO	Dimetyl sulfoxide
DNS	Dintrosalicyclic acid
DO	Dissolved oxygen
DoE	Design of experiment
EPS	Exopolysaccharides
FRIM	Forest Research Institute Malaysia
GA	Genetic algorithm
HPLC	High performance liquid chromatograph
LLP	Logistic incorporated Luedeking-Piret
L-M	Levenberg-Marquardt
LMLP	Logistic incorporated Modified Luedeking-Piret
MEA	Malt extract agar
MLLP	Modified Logistic incorporated Luedeking-Piret
MLMLP	Modified Logistic incorporated Modified Luedeking-Piret
MMLP	Monod incorporated Modified Luedeking-Piret
MTT	3-(4,5-dimetylthylthiazolyl-2)-2,5-diphenyltetrazolium bromide
OFAT	One-factor-at-a-time
OTR	Oxygen transfer rate
OUR	Oxygen uptake rate

pO_2	Partial dissolved oxygen concentration
RMSD	Root mean square deviation
RSM	Response surface methodology
TLC	Thin layer chromatography
UV	Ultraviolet

PENGHASILAN FLAVONOID, IAITU AGEN ANTI-RADANG DARIPADA *Trametes lactinea*

ABSTRAK

Flavonoid adalah salah satu sebatioan bioaktif yang berharga dan mempunyai aktiviti farmakologi yang luas termasuk anti-radang. Pendekatan ke arah biologi yang mudah dan berkesan bagi menghasilkan flavonoid dari enam spesies tempatan yang telah dipencil dan diperolehi daripada pusat pengumpulan kultur telahpun dijalankan. *Trametes lactinea* telah didapati menghasilkan flavonoid tertinggi di dalam kaldu kulturnya, dan merencat aktiviti enzim anti-radang, iaitu hialuronidase dan lipoksigenase. Di dalam kultur kelalang goncang, kesan keadaan kultur ke atas biojisim dan penghasilan flavonoid oleh *T. lactinea* (komposisi medium, pH awalan, suhu dan masa eraman) telah dioptimum menggunakan kaedah satu-faktor-di-satu-masa (OFAT). Keputusan menunjukkan biojisim tertinggi diperolehi dalam medium 3 yang mempunyai glukosa, yis dan pepton. Bagaimanapun, medium ini tidak menggalakkan penghasilan flavonoid oleh kulat terpilih, tetapi lebih kepada medium yang mempunyai kandungan karbon dan mineral yang sedikit (Medium 2). Empat parameter terpilih yang diperolehi dari OFAT telah dioptimumkan lebih lanjut untuk penghasilan flavonoid dengan menggunakan kaedah sambutan permukaan (RSM) berdasarkan rekabentuk Box-Behnken. Empat parameter yang terlibat di dalam kajian ini ialah pH awalan, isipadu inokulum, suhu eraman dan masa eraman. Penghasilan flavonoid yang tinggi (20.02 μg/mL) telah diperolehi dalam medium penghasilan yang mempunyai pH awalan 6, suhu inkubasi 35^{o}C dan masa inkubasi 8.1 hari. Untuk penghasilan biojisim dan flavonoid yang tinggi, prestasi di dalam 2.5 L bioreaktor tangki teraduk telah dikaji dengan mengambil kira kesan kadar pengudaraan (0.5 to 1.5 vvm), kelajuan pengadukan (100 to 300 rpm) dan awalan

kepekatan glukosa (10-60 g/L). Biojisim dan penghasilan flavonoid tertinggi telah diperolehi pada kelajuan pengaduk 300 dan 100 psm, dan awalan kepekatan glukosa pada 40 dan 20 g/L. Lanjutan daripada itu, pekali pemindahan oksigen (k_La) oleh *T. lactinea* juga telah dikaji dengan mengambil kira kesan kadar pengudaraan dan kelajuan pengadukan. Keputusan menunjukkan nilai k_La meningkat dengan peningkatan kadar pengudaraan daripada 0.5 kepada 1.5 vvm, dan kelajuan pengadukan daripada 100 kepada 300 psm. Fermentasi secara suapan kelompok oleh *T. lactinea* di dalam bioreaktor tangki teraduk juga telah dijalankan, dan strategi glukosa suapan telah meningkatkan penghasilan flavonoid berbanding dengan di dalam mod kelompok dan kultur kelalang goncang. Kinetik untuk pertumbuhan, penghasilan produk, penggunaan and perencatan glukosa oleh kulat yang diuji telah juga diperhatikan. Untuk pertumbuhan kulat, model 'Logistic', model 'Modified Logistic' dan model 'Gompertz' adalah berpadanan dengan data eksperimen dengan nilai R^2 > 0.98 dan RMSD <0.050 pada kepekatan glukosa 40 g/L. Model 'modified Gompertz' pula lebih berpadanan dengan data eksperimen untuk penghasilan flavonoid dengan nilai R^2 > 0.90 dan nilai RMSD yang rendah pada kepekatan glukosa 20 g/L. Untuk penggunaan dan perencatan glukosa, model modified Gompertz and model Luong telah memberikan padanan yang baik dengan nilai R^2 yang tinggi dan RMSD yang rendah. Rutin telah didapati di dalam ektrak akues *T. lactinea* apabila ianya dianalisa dengan menggunakan pelbagai jenis alatan analitis (TLC, UV-vis spectrophotometer and HPLC). Ujian ketoksikan menunjukkan ektrak akues *T. lactinea* ini tidakpun memberikan kesan ke atas sel warisan paru-paru "Chinese hamster (V79-4)" yang telah didedahkan selama 24 jam.

PRODUCTION OF FLAVONOID, AN ANTI-INFLAMMATORY AGENT FROM *Trametes lactinea*

ABSTRACT

Flavonoid is one of the remarkable bioactive compound that possesses broad pharmacological activities including anti-inflammatory. A simple and effective bioroute approach for the production of flavonoid by six locally isolated strain obtained from culture collection center were looked at. *Trametes lactinea* was found to produce the highest flavonoid in its culture broth and inhibited the anti-inflammatory enzyme activities, hyaluronidase and lipoxygenase. In shake flask culture, the effect of culture conditions on biomass and flavonoid production by *T. lactinea* (media composition, initial pH, inoculum volume, temperature and incubation time) were optimized using one-factor-at-a-time (OFAT) method. Results showed that the highest biomass was obtained in the Medium 3, containing glucose, yeast and peptone. However, this production medium was not favourable for flavonoid production by the tested fungus but it preferred to condition of less carbon media with a small amount of minerals (Medium 2). Four selected parameters obtained in OFAT was further optimized for flavonoid production by *T. lactinea* using a Response Surface Methodology (RSM) coupled with Box Behnken design (BBD). Four parameters that involved in this study were initial pH, incubation temperature and incubation time. The highest flavonoid production (20.02 μg/mL) was obtained in the production media with initial pH 6, incubation temperature 35^{o}C and incubation time 8.1 days, respectively. To achieve higher biomass and flavonoid production, the performance of 2.5 L stirred tank bioreactor was investigated by considering effect of aeration rates (0.5 to 1.5 vvm), agitation speed (100 to 300 rpm)

and initial glucose concentration 10 to 60 g/L). The highest biomass and flavonoid production were obtained at agitation speed 300 and 100 rpm, and initial glucose concentration at 40 and 20 g/L, respectively. The volumetric oxygen transfer coefficient (k_La) of *T. lactinea* was also studied by considering the effect of aeration rate and agitation speed. Results showed that k_La values increased with increased of aeration rate from 0.5 to 1.5 vvm and agitation speed from 100 to 300 rpm, respectively. Fed batch fermentations of *T. lactinea* in a stirred tank bioreactor was also conducted and the results showed that a glucose feeding strategy was favourable process that enhanced the flavonoid production compared to batch mode and a shake flask culture. Kinetics of growth, product formation, glucose utilization and inhibition of the tested fungus were also looked at. For *T. lactinea* growth, the Logistic, Modified Logistic and Gompertz models fitted well with the experimental data with $R^2 > 0.98$ and RMSD <0.050 at initial glucose concentration at 40 g/L. The modified Gompertz model provided a more accurate description with R^2 values > 0.90 and smaller RMSD for the flavonoid production at initial glucose concentration at 20 g/L. For substrate utilization and inhibition, the modified Gompertz model and the Luong model described well the experimental data with high R^2 and low RMSD values. Rutin was found in the aqueous extract of *T. lactinea* when analyzed using various analytical tools (TLC, UV-vis spectrophotometer and HPLC). Cytotoxicity test revealed that the aqueous extract of *T. lactinea* possessed no toxic effect towards Chinese hamster lung (V79-4) cell lines within 24 hour exposure.

CHAPTER 1

INTRODUCTION

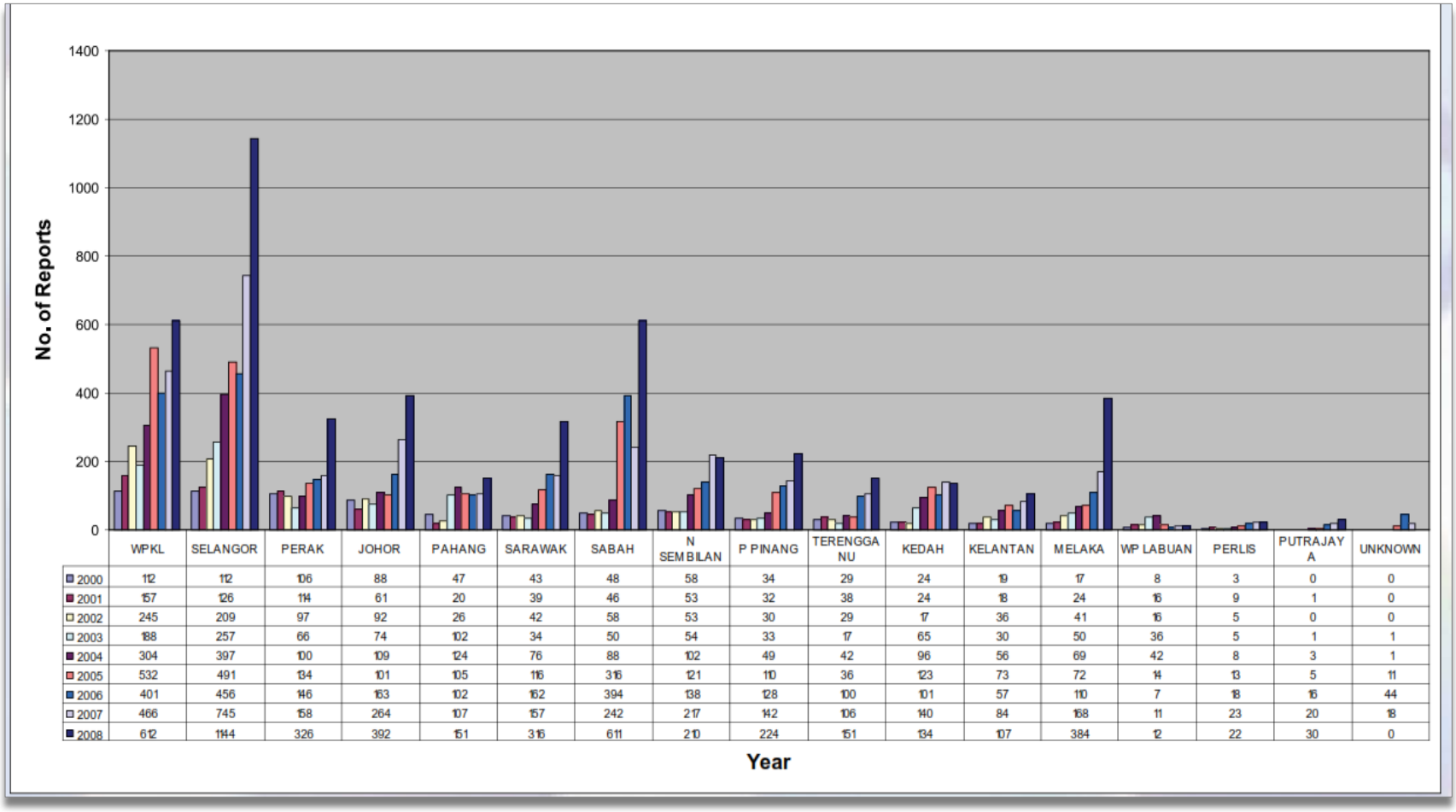
1.1 Research background

Inflammation is recognized as a major risk factor in human diseases (Pan *et al.*, 2010). Stoner and Wang (2013) reported that inflammation is caused by numerous factors such as oxidative stress, environmental pollutants, microbial agents, and physical damage to tissues. In classical features, inflammation was defined as redness, warmth, swelling, and pain (Gautam and Jachak, 2009). Basically, inflammation has its beneficial effect as it leads to removal of offending factors and restoration of tissue structure and physiological function (Ricciotti and FitzGerald, 2011). As the inflammation became uncontrolled and caused an extreme host cell damaged, the anti-inflammatory drugs such as glucocorticoids and aspirin were used to inhibit the enzymes (phospholipase A_2 and cyclooxygenase) that promoted the inflammatory pathways (Vane and Botting, 1987).

The history of anti-inflammatory agent started 3,500 years ago at a time when the Greek physician Hippocrates prescribed an extract from willow bark and leaves to treat fever and inflammation (Rao and Knaus, 2008). Later, the active ingredient of willow bark salicin was identified and improved by Felix Hoffman, who was working in Bayer Company which has become the most widely used medicines until today (Vane and Botting, 2003). The non-steroid anti-inflammatory drugs (NSAIDs) are heterogeneous group of compounds that possessed anti-inflammatory, analgesic, and antipyretic properties which including aspirin, ibuprofen, naproxen, indomethacin, celebrex and etc (Charlier and Michaux, 2003; Fendrick and Greenberg, 2009).

Nevertheless, utilization of these NSAIDs and other drugs in inflammation treatment was reported to produce unwanted side effects on human. In 2002, about 30,000 cases of acetaminophen ingestion were reported to the American Association of Poison Control Centers with 110 deaths were due to acetaminophen ingestion (Watson *et al.*, 2003; Bartlett, 2004). According to Dugowson and Gnanashanmugam (2006), the usage of non-selective NSAIDs can lead to the development of gastro duodenal ulcers four to eight times during therapy. In the United States, NSAIDs was reported to be used regularly at least by 13 million people with various arthritides. Out of that, 16,500 NSAID-related deaths occurred among patients with rheumatoid arthritis or osteoarthritis every year in the United States (Wolfe *et al.*, 1999). In fact, this number was greater than the number of deaths due to asthma, cancer and other diseases. On the other hand, there were also some of them, including Vioxx® and lumiracoxib (Prexige®) which were withdrawn from the market in 2004 and 2007 due to their side effects (Rao and Knaus, 2008).

As for Malaysia, the Malaysia Adverse Drug Reactions Newsletter on May 2009 stated that a total of 4826 local spontaneous reports of suspected adverse drug reactions (ADRs) were recorded in 2008. Number of reports has increased more than 90% since 1987. Figure 1.1 showed that Selangor was the leading state reported on the adverse drug reaction followed by Wilayah Persekutuan (612) and Sabah. The newsletter also reported that the most number of suspected ADRs were attributed to the pharmacological group cardiovascular, and the suspected drug that contributed to the highest number of ADR reports was perindopril followed by aspirin and diclofenac. Due to that, demand for an alternative and safe anti-inflammatory preventive drug has become an urgent need today.



	WPKL	SELANGOR	PERAK	JOHOR	PAHANG	SARAWAK	SABAH	N SEMBILAN	P PINANG	TERENGGANU	KEDAH	KELANTAN	MELAKA	WP LABUAN	PERLIS	PUTRAJAYA	UNKNOWN
2000	112	112	106	88	47	43	48	58	34	29	24	19	17	8	3	0	0
2001	157	126	114	61	20	39	46	53	32	38	24	18	24	16	9	1	0
2002	245	209	97	92	26	42	58	53	30	29	17	36	41	16	5	0	0
2003	188	257	66	74	102	34	50	54	33	17	65	30	50	36	5	1	1
2004	304	397	100	109	124	76	88	102	49	42	96	56	69	42	8	3	1
2005	532	491	134	101	105	116	316	121	110	36	123	73	72	14	13	5	11
2006	401	456	146	163	102	162	394	138	128	100	101	57	110	7	18	16	44
2007	466	745	158	264	107	157	242	217	142	106	140	84	168	11	23	20	18
2008	612	1144	326	392	151	316	611	210	224	151	134	107	384	12	22	30	0

Figure 1.1: Total number of ADR Reports Received Categorized by the states of Malaysia (Source: Malaysian Adverse Drug Reactions Newsletter, 2009).

Over the millennia, utilization of natural products from plants, herbs, fruits, animals and fungi have played a significant role in human health. Indeed, it also made an enormous contribution in drug development. Wang *et al.* (2007) in their research stated that about 60% of antitumor/anti-infectious drugs that were already in the market or under clinical investigations were of natural origin. In fact, natural products and its derived drugs were well represented in the top 35 worldwide selling ethical drugs in 2000, 2001 and 2002. Previously, Butler (2004) mentioned that the percentage of natural product-derived drugs was 40% in 2000 and remained approximately constant at 24% in 2001, 26% in 2002 and significantly contributed to the profitability of many companies.

These natural products were reported to have different chemical classes such as alkaloids, steroids, terpenoids, polyphenolics, flavonoids, phenylpropanoids, fatty acids and lipids, and various miscellaneous compounds which could inhibited the inflammatory cytokines and inflammatory mediators such as IL-1, IL-6, IL-10, TNF-a, NF-κB, NO, iNOS and COX-2 (Gautam and Jachak, 2009; Debnath *et al.*, 2013). One of the remarkable active compounds in natural product is flavonoids, which naturally distributed in the plant kingdom. In plant, its function is as flower colorant, and the distribution between plant taxa has made the first move for researchers to study on these compounds, thus leading to its first documented article in the late 1960s. According to Winkel-Shirley (2001), besides providing beautiful colorant in leaves, flower, fruits and seeds, flavonoids also played its roles in male fertility of some species, signaling between plants and microbes, antimicrobial agents and feeding deterrents and UV protection.

Flavonoids were known for their beneficial effects on health long before they were isolated as effective compounds (Nijveldt *et al.*, 2001). Pan *et al.* (2010) reported that

flavonoid was a potent antioxidant and exhibited broad pharmacological activities including anti-inflammatory, antimicrobial, anti-viral, anti-bacteria and anti-carcinogenic. In fact, the health benefit of products such as *Ginkgo biloba* containing natural-flavonoids (quercetin, kaempferol and isorhamnetin) has reached its annual sales more than USD 100 million worldwide in 1996 (Springen and Cowley, 1997). Nowadays, the usage of flavonoids compound has gone beyond nutritional supplements. The conjugated anthocyanins, such as cyanidin glucoside are used as natural colorants for flavor and fragrance industries. The unit price of cyaniding could reached up to USD 600 /kg (Springen and Cowley, 1997).

The commercialization of flavonoids in a large-scale production platform becomes more important either for health related research or commercial nutritional applications. Previously, flavonoids compound are produced with two methods; chemical synthesis and isolation from plant (Koopman *et al.*, 2012). Flavonoid produced from chemical synthesis need an extreme condition and used toxic chemicals such as 2', 4'-Dihydroxyacetophenone and 2'-Hydroxy-5' methoxyacetophenone (Lim *et al.*, 2001; Leonard *et al.*, 2008). This method was seen uneconomic as it added to cost production when the effluent wastes need to go through the wastewater treatment. In fact, toxic solid wastes have created another disposal problem to the environment. Hence, it geared the researchers direction to isolate the flavonoids compound from the plants (Koopman *et al.*, 2012).

Various extraction techniques were widely investigated to extract valuable compounds from plant. Traditionally, soxhlet, hydrostillation and maceration with an alcohol–water mixture or hot fat were used to extract these compounds for commercialization (Wang and Weller, 2006). These extraction methods took several

hours and many solvent for the extraction process to be completed. Due to that, green extraction methods using ultrasonic and microwave were developed to overcome such issues and become the biggest challenge for researchers is to maintain the availability of flavonoids production from the plant (Hemwimol *et al.*, 2006; Liazid *et al.*, 2010). Nevertheless, the commercialization of flavonoids from plant species is not successfully applied due to low growth rate of the plants (Wang *et al.*, 2011; Koopman *et al.*, 2012).

Again, it geared the researchers direction to approach metabolic engineering using microorganism such as *Saccharomyces cerevisiae* and *E. coli* to enhance higher value flavonoid products such as naringenin and pinocembrin (Chemler *et al.*, 2006; Leonard *et al.*, 2008). Four catalytic steps were involved for the conversion of the aromatic amino acid L-tyrinne to the main flavanone precursor, naringenin. In this process, the conversion of L-tyrosine to the phenylpropanoic acid p-coumaric acid go through the action of the enzyme tyrosine ammonia lyase (TAL). Once p-coumaric acid has been generated, 4-coumarate: CoA ligase (4CL) mediates the formation of its corresponding CoA ester, coumaroyl-CoA. This compound was subsequently condensed with three malonyl-CoA units by the sequential action of the type III polyketide synthase, chalcone synthase (CHS). The final step of this process resulting naringenin chalcone stereospecifically isomerized by chalcone isomerase (CHI) to form the (2S)-flavanone naringenin. Hence, discovery of this compound has become a starting point for the synthesis of varieties of other flavonoid molecules, which are created through the combined actions of functionalizing enzymes which hydroxylated, reduced, alkylated, oxidized, and glucosylated this phenylpropanoids core structure, accordingly (Kaneko *et al.*, 2003; Fowler and Koffas, 2009; Santos *et al.*, 2011).

However, Santos *et al.* (2011) stated that the approach of metabolic engineering using microorganism for the production of flavonoids has two major drawbacks: (1) the requirement for expensive phenylpropanoic (acid amino) precursors supplemented into the media, and (2) the need for two separate media formulations for biomass/protein generation and flavonoid production. Traditionally, the flavonoids fermentation protocol required two separation steps to achieve higher flavonoid titers. Strains were grown in rich media for biomass growth and adequate heterologous protein formation. After reaching a target density, cells were harvested and then transferred into minimal media supplemented with phenylpropanoic precursors for flavonoids production. These two separation steps of biomass seemed to be unrealistic in large scale of fermentation processes as it added to cost production. In fact, an expensive price of L-tyrosine, a flavonoid precursor (RM2500/kg-Sigma Aldrich) also attributed to this major drawback for commercialization. Then, medium formulation for flavonoids production was developed using two *E. coli* strains directly from glucose without phenylpropanoic precursors. They managed to produce 29 mg/l naringenin from glucose and up to 84 mg/l naringenin with the additional of fatty acid inhibitor, cerulenin. This showed that although metabolic engineering is perceived to have a solution for higher flavonoids production, the need for fatty acid inhibitor in the process made it possible to reach an efficient and cost efficient production. Due to that, according to Wang *et al.* (2011) only few flavonoid compounds are being produced by fermentation at an industrial scale.

1.2 Problem statement

Various physical and chemical methods have been used extensively on laboratory scale to produce flavonoids. The use of toxic chemicals and an extreme reaction conditions are of vital concern. In fact, commercialization of flavonoids from plant using established extraction techniques did not give much solution due to plant's slow growth. The need of expensive flavonoid precursor in the metabolic engineering has also limited its production in large scale.

Flavonoids were reported to be infrequent in fungi. However, Bird and Marshall (1969) had successfully isolated flavonoids compound, chlorflavonin (3'-chloro-5,2'-dihydroxy-3,7&trimethoxy- flavone) from cultures of *Aspergillus candidus*. Continuing research by Abou-Zaid *et al.* (1997) have isolated another two novel C-methylflavonols in the *Colletotrichum dematium* f. sp. *epilobii* cultures extract. Although there was no discussion on whether flavonoids might have originated from the nutrient medium, the results obtained from the research carried out indicated that flavonoids compound were biosynthesized *de vono* by the fungi (Bohm, 1998). According to Hyun *et al.* (2011), fungi as well as plant are able to synthesize phenylalanine via the shikimic acid pathway. This phenylalanine is used either for protein synthesis in plants or metabolized through the phenylpropanoid pathway. Phenylpropanoid metabolism involved the action of phenylalanine ammonia lyase (PAL) leads to the biosynthesis of a wide array of phenylpropanoid secondary products including flavonoids. In fact, the PAL activity was

detected in a few basidiomycetes, deuteromycetes and one ascomycete, *Nectria cinnabarina* (Bandoni *et al.*, 1968; Vance *et al.*, 1975; Hyun *et al.*, 2011).

Fungi are particularly useful producers of secondary metabolites from an industrial point of view, due to their high production level and extra cellular secretion, as well as the relative ease of cultivation (Jami *et al.*, 2010; Coleman *et al.*, 2011). In fact, fungi is known as the second secondary metabolites group producers after Actinobacteria with industrial application (Barreiro *et al.*, 2011). The estimated number of macrofungi on Earth is 140 000 however only 700 species of them were reported to possess significant pharmacological properties (Wasser, 2002). Unfortunately, Malaysia is still poorly represented in this research field compared to China and Japan. Occupied with the richest fungi biota, Malaysia could be a good base for more extensive research in exploration and biological evaluation of natural products from fungi.

Many papers have reported on the genera *Ganoderma, Schizophyllum, Inonotus, Phellinus*, *Lentinus* and *Trametes* for their medicinal properties. Wasser (2002) reported that macrofungi have been intensively investigated for medicinal effects in *in vivo* and *in vitro* model systems for almost 40 years. The medicinal effects including anti-inflammatory, antioxidant, antimicrobial and anticancer (Barros *et al.*, 2007a; Koyama *et al.*, 2008; Standish *et al.*, 2008; Abah and Abah, 2010; Lu *et al.*, 2010). However, comprehensive study on *Trametes lactinea*, a species belonging to the family of Polyporaceae which can possibly be used as a model organism for the production of flavonoids is still limited.

Fungi fermentation can be influenced by both physical and chemical parameters. These including media composition, pH of medium, inoculum volume, incubation time and etc (Fazenda *et al.*, 2008). However, the detailed study of these parameters on

flavonoids production by fungi is still scarce in literature. The techniques for experiments range from conventional one factor at a time (OFAT) to more appropriate statistical and mathematical tools in order to optimize the effect of selected parameters for fungi fermentation.

Fermentation systems with a scalable method using stirred tank bioreactor were a promising device for better production strategies. According to Scragg (1991), such bioreactor was design with dual advantages; low capital and low operating cost. In fact, an efficient mixing, excellent mass transfer and foam breaker provide several advantages as compared to other type of bioreactors.

In engineering point of view, kinetics and mathematical modelling makes possible applications of principles and practices in understanding the critical parameters that influences the fungi growth and product formation in batch systems. Meanwhile, the fed-batch mode strategies is anticipated to address the limitation and problems faced with the batch mode strategies of stirred tank bioreactor, thereby improving the fungus performance. Such conditions for flavonoid production has not been observed independently and a detailed answer let alone exploited. As such, greater focus is required in predicting flavonoid fermentation performance as a function of chemical and physical parameters and mathematical modeling related to it.

1.3 Research Objectives

The main objective of this research is to use a white rot fungus, *Trametes lactinea* as a model organism for flavonoid production using batch and fed batch fermentation. Prediction of kinetic rate behavior by a series of phases in the fermentation process was also carried out. The extract from this fungus was analyzed for its compound and tested on its toxicity. The specific objectives of the research are:

1. To screen six locally isolated macrofungi for their potential of producing anti-inflammatory agent via flavonoid and *in vitro* enzyme inhibition assays.
2. To optimize the culture conditions for growth and flavonoid production by *Trametes lactinea* (*T. lactinea*) using one-factor-at-one-time (OFAT) method and a statistical tool in shake flask cultures.
3. To study the growth and flavonoid production by *T. lactinea* in a 2.5 L stirred tank bioreactor operated as batch and fed batch modes.
4. To select and validate the kinetics models of *T. lactinea* growth, flavonoid production, substrate consumption and inhibition of batch process.
5. To determine the flavonoid compounds presence in *T. lactinea* extract, and tested for its toxicity against Chinese hamster lung cell lines.

1.4 Scope of Study

For preliminary studies, six locally isolated macrofungi (*Trametes pocas, Trametes feei, Trametes lactinea, Pycnoporus sanguineus*, *Schizophyllum commune* and *Lentinus sajor cajo*) were selected and screened for their capability of producing anti-inflammatory agent, via flavonoids content and *in vitro* enzyme inhibition assays. The selected enzymes were hyaluronidase, lipoxygenase and xanthine oxidase. These enzymes were chosen as they were known to be involved in the inflammation such as asthma, gout and allergic (Brash, 1999; Kong *et al.*, 2000; Samee *et al.*, 2009).

Optimization of process variables on growth and flavonoid production of the selected fungus were determined using one-factor-at- a-time (OFAT) method in shake flask cultivation. The selected independent parameters on the targeted responses were further optimized using a response surface methodology approach. In this work, Box Behnken design was used for the optimization for flavonoid production.

The optimum condition of flavonoid production that was determined previously, was then studied in a stirred tank bioreactor (STB) of batch mode. Effect of different culture conditions such as agitation, aeration and initial glucose concentration was investigated. Fed batch fermentation was also performed to identify the significance of the process over the conventional batch mode fermentation. To describe the kinetics of flavonoid production in stirred tank bioreactor, different kinetic models for growth, product formation, substrate utilization and inhibition were selected. These models were

then validated using coefficient of determination (R^2) and root mean square deviation (RMSD). The extracted compounds of flavonoid were determined and MTT (3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cytotoxicity test was carried out on its toxicity against Chinese hamster lung cells lines.

1.5 Organization of the thesis

There are five chapters in the thesis and each chapter describes the sequence of this research.

Chapter One gives the introductory of this research. It starts with a brief introduction and overview of inflammation, anti-inflammatory drugs and medicinal remedies of natural products. This chapter also presents the problem statement, research objectives, and scope of study.

Chapter Two provides literature review of this research. This includes history, sources, structures and biological activities of flavonoids. Optimization of culture conditions using statistical analysis, fermentation kinetic and models were also highlighted in detail.

Chapter 3 describes the materials and methods applied in this research. This includes screening for the presence of flavonoid and optimization of process parameters in shake flask and stirred tank bioreactor (STB) either of batch or fed batch mode. The kinetics of growth and flavonoid production of batch mode and analytical procedures were also described in this chapter.

Chapter 4 presents the results and discussion of experiments that were carried out, together with data analysis at various operating condition and process parameters. This chapter is organized into several main sections: determination of anti-inflammatory potential of the selected macrofungi via presence of flavonoids and enzyme inhibition

assays, fermentation of flavonoid in shake flask and stirred tank bioreactor either batch or fed batch mode. Optimization studies for the flavonoid was obtained using OFAT method and response surface methodology. The kinetics and modeling for the fermentation was also presented.

Chapter 5 concludes the research. Recommendations for future research were also given highlighted.

CHAPTER TWO

LITERATURE REVIEW

2.1 Flavonoid, as anti-inflammatory agent

Flavonoid have been consumed by humans for about 4 million years (Kumar and Pandey, 2013). They have wide biological properties that stimulate human health and reduce the risk of diseases.

2.1.1 History of flavonoid production

Szent-Gyorgyi first isolated a metabolite from orange in 1936 and known as vitamin P (Renaud and de Lorgeril, 1992; Tapas *et al.*, 2008). Later, this metabolite was claimed as flavonoid (Kumar and Pandey, 2013). According to Groot and Rauen (1998), over 4,000 varieties of flavonoids have been identified. In fact, many members of flavonoid family possessed attractive colours and responsible for the brilliant shades of blue, scarlet, orange, etc, in flowers, fruits and leaves.

As reported in the literature, flavonoids are ubiquitously distributed in plant kingdom, which in attached to sugars (glycosides), although occasionally they are found as aglycones (Ross and Kasum, 2002; Lin and Weng, 2006; Tapas *et al.*, 2008). According to Nijveldt *et al.* (2001), research on flavonoids have received great attention after discovery of the French paradox, ie, the low cardiovascular mortality rate observed in Mediterranean populations in association with red wine consumption and a high saturated fat intake.

According to Andersen and Markham (2010), in the early 1960s, flavonoids were only viewed as metabolic waste products that were stored in the plant vacuole. In fact, flavonoids were reported can be found in only plant kingdom. However, a few years

later, there were reports stated the presence of flavonoids in mosses and liverworts and even their occurrence in an alga. Meanwhile, the presence of flavonoid compounds were also reported in fungi species (Abou-Zaid *et al.*, 1997). Barros *et al.* (2007) stated that flavonoids were found as one of the major component in the methanolic extracts of Portuguese wild edible mushrooms (*Lactarius deliciosus*, *Sarcodon imbricatu*s). However, detailed study of flavonoids producing fungi were very scarce in literature compared to flavonoids producing plant. Most of the works that were carried out only reported the presence of flavonoids in the fungal extract without any detail discussion on the flavonoids subclasses or compounds that were successfully produced or isolated in the fungal extract.

Flavonoids are very important for human health. According to the US Department of Agriculture (USDA), flavonoids are consumed by human from foods. Flavonoids can be obtained in fruits, nuts and vegetables such as parsleys, tomatos, pears, strawberries, oranges, black beans and etc. The interest in the possible health benefits of flavonoids has increased owing to their potent antioxidant and free-radical scavenging activities observed *in vitro* (Ross and Kasum, 2002). In fact, flavonoids compound such as quercetin, kaempferol, morin, myricetin and rutin were reported acting as antioxidants and exhibited beneficial effects such as anti-inflammatory, anti-allergic, anti-viral, as well as anti-cancer activities (Tapas *et al.*, 2008). Previously, studies carried out by Knekt *et al.* (2002) revealed that higher intake of quercetin caused lower mortality from ischemic heart disease, lung cancer incidence and asthma incidence. While, risk cerebrovascular disease was lower due to higher intakes of kaempferol, naringenin and hesperetin. Hence, indicated that flavonoid do cause good to human being.

2.1.2 Structure of flavonoid

Based on the US Department of Agriculture (USDA), there are more than 5000 flavonoid compounds with dietary flavonoids consisted of five subclasses of monomeric flavonoids (flavonols, flavones, flavanones, flavans and anthocyanidins), polymeric proanthocyanidins and isoflavones. According to a few researchers, the basic structural feature of flavonoid compounds was the 2-phenyl-benzo[α]pyrane or flavane nucleus, which consisted of two benzene rings (A and B) linked through a heterocyclic pyrane ring (C) (Figure 2.1) (Brown, 1980; Cushnie and Lamb, 2005) Flavonoids can be divided into various classes on the basis of their molecular structure (Nijveldt *et al.*, 2001). Six major flavonoids subclasses are summarized in Table 2.1. As mentioned by Middleton *et al.* (2000), this subclasses was primarily based on the presence (or absence) of a double bond on position 4 of the C (middle) ring, the presence (or absence) of a double bond between carbon atoms 2 and 3 of the C ring, and the presence of hydroxyl groups in the B ring. Example, most of the flavonoid structure have a phenyl group substituted at the 2nd -position of the pyrone ring. As for isoflavonoids, the substitution occurred at the 3rd -position (Table 2.1). Uniquely, flavonoids also shared the common structure with tocopherols (vitamin E), the chromane ring (Middleton *et al.*, 2000).

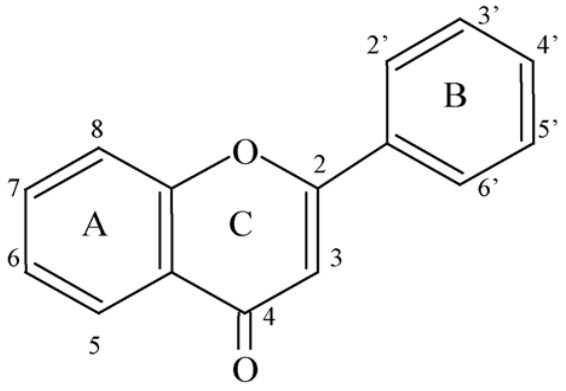
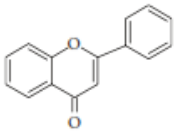
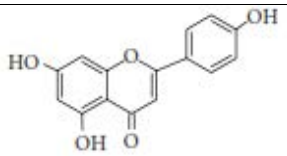
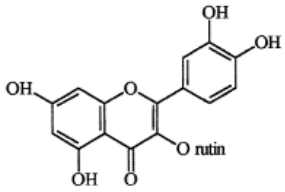
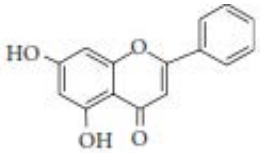
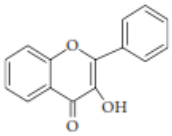
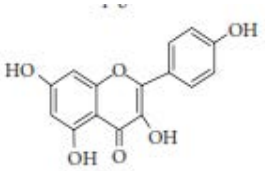
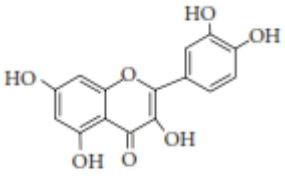
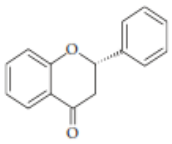
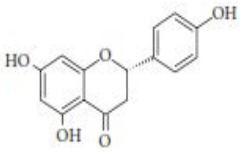
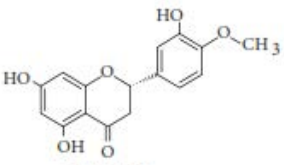
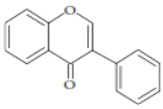
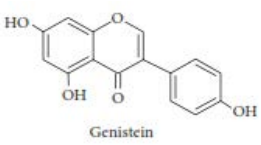
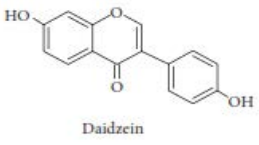
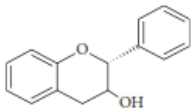
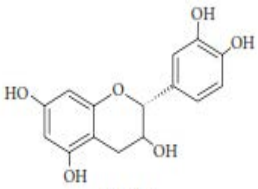
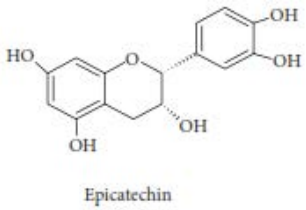
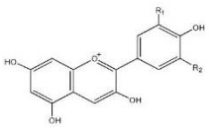
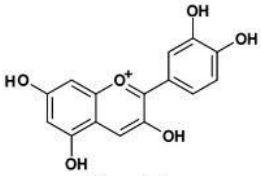
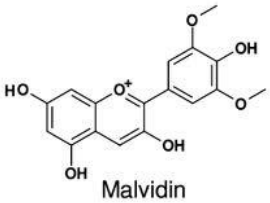


Figure 2.1: Basic structure of flavonoid compounds (Cushnie and Lamb, 2005).

Table 2.1: Subclasses, structure and sources of flavonoids (Nijveldt *et al.*, 2001; Agrawal, 2011; Kumar and Pandey, 2013).

Group of flavonoids and its structure backbone	Examples	Sources
Flavones 	 Apigenin  rutin  Chrysin	Apple skins, berries, broccoli, celery, olives, onions, grapes, parsley
Flavonols 	 Kaempferol  Quercetin	Apple, berries, broccoli, onions
Flavanones 	 Naringenin  Hesperetin	Citrus fruits Citrus peel
Isoflavones 	 Genistein  Daidzein	Soybeans, soy foods, legumes

Continue….

Group of flavonoids and its structure backbone	Examples	Sources
Flavan-3-ols 	 Catechin  Epicatechin	Red wine, Tea
Anthocyanidins 	 Cyanidin  Malvidin	Tea, strawberries, cherries, grapes

2.1.3 Chemical and Physical Properties of flavonoid

Most flavones and flavonols exhibited two major absorption bands: Band I (320–385 nm) represented the B ring absorption, while Band II (250–285 nm) corresponded to the A ring absorption (Yao *et al.*, 2004; Kumar and Pandey, 2013;). On the other hand, the functional groups attached to the flavonoid skeleton might caused a shift in absorption such as from 367 nm in Kaempferol (3,5,7,4'-hydroxyl groups) to 371 nm in quercetin (3,5,7,3', 4'-hydroxyl groups) and to 374 nm in myricetin (3,5,7,3', 4', 5'-hydroxyl groups). The absence of a 3-hydroxyl group in flavones distinguished them from flavonols. Hence, Band I always absorbed at a shorter wavelength by 20–30 nm, such as the 337 nm required for apigenin (Rice-evans *et al.*, 1995; Rice-Evans *et al.*, 1996; Rice-Evans *et al.*, 1997; Yao *et al.*, 2004). As for flavanones, it have a saturated

heterocyclic C ring, with no conjugation between the A and B rings, as determined by their UV spectral characteristics (Rice-Evans *et al.*, 1996). Meanwhile, flavanones exhibit a very strong Band II absorption and maximum between 270 and 295 nm, namely, naringenin-288nm and taxifolin-285 nm, and only a shoulder for Band I at 326 and 327 nm. According to Yao *et al.* (2004), Band II appeared as one peak (270 nm) in compounds with a monosubstituted B ring, but as two peaks or one peak (258 nm) with a shoulder (272 nm) when a di-, tri-, or o-substituted B ring was present. As for anthocyanins, the compounds show distinctive Band I peak in the 450–560 nm region due to hydroxyl cinnamoyl system of the B ring, and Band II peaks in the 240–280 nm region due to the benzoyl system of the A ring. In fact, the colour of the anthocyanins varied with the number and position of the hydroxyl groups (Wollenweber and Dietz, 1981; Yao *et al.*, 2004). Yao *et al.* (2004) in their research revealed that the chemical structure and relative orientation of various moieties in the flavonoids molecule greatly influenced their biochemical and metabolites properties.

2.1.4 Separation and quantification of flavonoid

Flavonoids especially glycosides can be degraded by enzyme action when the material is fresh (wet). Due to that Andersen and Markham (2010) suggested that the material should be dried and in powdered form before proceeding to the extraction process. In flavonoids extraction, the polarity of solvent used is very important. Table 2.2 listed choice of solvent used for flavonoids extraction.

Table 2.2 Choice of solvent for flavonoid extraction (Andersen and Markham, 2010)

Flavonoids	Solvent

Less polar	
Isoflavones, flavanones, methylated flavones, flavonols	Chloroform, dichloromethane, diethyl ether, or ethyl acetate
Polar	
Flavonoids glycosides, more polar aglycones	Alcohols or alcohol-water mixtures

Traditionally, soxhlet extraction and maceration were used for many years in flavonoids extraction. In this extraction, hexane was firstly used to remove lipids followed by ethyl acetate or ethanol to obtain phenolics compound (Andersen and Markham, 2010). However, these techniques need longer time and consumed a lot of solvent such as hexane that caused harmful effects on human and environment (Bimakr *et al.*, 2013). Due to that green extraction technology using ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), supercritical fluid extraction and accelerated solvent extraction (ASE) were developed to overcome such issue (Wang and Weller, 2006).

In another study by Veličković *et al.* (2007), flavonoids were extracted from *Salvia officinalis* L and *Salvia glutinosa* L using ultrasonic method within 20 min compared to maceration technique that completed after 6 hours. The flavonoids composition depend on the tested species, the polarity of the extracting solvents and the extraction techniques applied. On the other hand, the smaller solvent volume, 0.5 mL

and extraction time 5 min for extraction of fungal metabolites using ultrasonic was recorded by Smedsgaard (1997). In fact, the concentration of these extracts was sufficient to determine all the secondary metabolites presence using a HPLC.

As for supercritical fluid extraction (SFE), the technique relied on the solubilizing properties of supercritical fluids and ideal for the extraction of diffusion controlled matrices, such as plant tissues (Andersen and Markham, 2010). In another study by Dean *et al.* (1998), methanol was added as a modifiers for flavonoids extraction.

2.1.5 Anti-inflammatory assays of flavonoid

The significant contribution of enzyme inhibition assays is a basis of drug action which cannot be overestimated as this is demonstrated by the therapeutic benefits of classical examples of enzyme-inhibitory drugs such as penicillin, sulfonarnides, physostigmine, digitalis, methotrexate, and aspirin (Kalman, 1981). Flavonoids are known to inhibit a number of enzymes such as lipoxygenase, hyaluronidase, nitric oxide and cyclooxygenase-2, xanthine oxidase, lipases and etc (Havsteen, 1983; Wang *et al.*, 1994; Raso *et al.*, 2001; Kim *et al.*, 2005). Maeda *et al.* (1990) claimed that inhibition of hyaluronidase activity can also be used to evaluate the anti-allergic reaction. A study carried out by Wang *et al.* (1994) revealed the capability of flavonoids to inhibit human preadipocyte aromatase which caused breast cancer. Meanwhile, the ability of flavonoids to inhibit acid arachidonic acid metabolite, leukotrienes C_4 and D_4 was reported by Ban *et al.* (1989). These metabolites is initiated by the reaction of arachidonic acid with 5-lipoxygenase enzyme and caused constriction in blood vessel and enhanced vascular permeability. In fact, Pidgeon *et al.* (2007) suggested that lipoxygenase inhibitors might lead to the design of biologically and pharmacologically

targeted therapeutic strategies inhibiting lipoxygenase isoforms and/or their biologically active metabolites, thus might be useful in cancer treatment.

On the other hand, Zhu *et al.* (2004) have reported on the inhibition of xanthine oxidase by *Biota orientalis* extract. This species is rich in flavonoids compound such as quercetin and rutin. They observed that no hypouricemic effect in the mouse liver which could be due to inhibition of xanthine oxidase activities by these flavonoids compound. Meanwhile, apigenin and quercetin (0.5–50 M) were found to be the most potent inhibitors of nitric oxide (NO) production and markedly decreased prostaglandin E_2 (PGE_2) release and cyclooxygenase-2 (COX-2) expression in a concentration-dependent manner (Raso *et al.*, 2001).

2.2 White rot fungi

2.2.1 Morphology of white rot fungi and their applications in food and pharmaceutical industries, and environmental.

White rot fungi is defined as a group of fungi that degrade the brown colored lignin, leaving the white cellulose and giving the wood a bleached or pale appearance and transforming it into a fibrous mass that crumbles with a blow (Maheswari, 2012). According to Hickman *et al.* (2011), many wood rot fungi can be identified by the distinctive shape, color, and texture of the fruiting bodies that form on trees. These structures, called conks or brackets, often are located around wounds in bark, at branch scars, or around the root crown. Some decay fungi such as *Armillaria mellea* produce typical, fleshy, mushroom shaped fruiting bodies at the base of infected trees after a rain in fall or winter. Some fruiting bodies such as *Armillaria* mushrooms are annual (i.e.,

they appear soon after the beginning of seasonal rains), but many are perennial and grow by adding a new layer each year.

It is widely distributed in nature and grown rapidly under the warm and moist conditions of tropic climates, made Malaysia an ideal place for many fungi to flourish. They can be classified into categories of edible, medicinal and poisonous species. According to Salmiah (1997), white rot fungi is a mesophilic and thermophilic microorganisms that produced cellulase, laccase and lignase enzymes. These enzymes are used to digest the component of wood cell walls. It broke down lignin and cellulose and commonly caused rotted wood to feel moist, soft, spongy, or stringy and appear white or yellow (Hickman *et al.*, 2011). Most of common white rot fungi *are Ganoderma applanatum, Pycnoporus sanguineus, Schizophyllum commune, Lentinus sajor-caju*, *Trametes feei* and *Trametes versicolor* and etc. In fact, these fungi has significantly revealed their enormous potential in human health as a source of nutritive food, pharmaceutical industries and also playing a role in controlling environmental (Salmiah, 1997; Miles and Chang, 2004).

Rich in proteins and other individual nutrition such as fat, phosphorus, iron, and vitamins including thiamine, riboflavin, ascorbic acid, ergosterol, and niacin made edible white rot fungi such as *Schizophyllum commune, Lentinus sajor-caju* and *P. ostreatus* as a good source of nutritious food (Garcha *et al.*, 1993; Salmiah, 1997; Bonatti *et al.*, 2004; Miles and Chang, 2004). This can be explained by the dramatic elevation of total worldwide cultivated mushroom production which is 0.90 million tons in 1975; to 6.16 million tons in 1997 (Miles and Chang, 2004).

Extensive research carried out by several researchers revealed that white rot fungi is a powerful lignin degrader (Wu *et al.*, 2005). Lignin and its derivatives are one of the