CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to Fungi

The organisms of the fungal lineage include mushrooms, rusts, smuts, puffballs, truffles, morels, molds, and yeasts, as well as many less well-known organisms (Alexopoulos *et al*., 1996). Griffin (1981) stated that all organism recognize as fungi have the basic characteristics of eucaryotic cells. More than 70,000 species of fungi have been described, however, some estimates of total numbers suggest that 1.5 million species may exist (Hawksworth *et al*., 1995; Hawksworth, 1991). Fungi are important as agent of decay in the cycling of carbon, nitrogen and other nutrients and in the deterioration of useful materials and product due to their ability to synthesize many strange and wonderful compound including enzyme, important materials for food, drugs and chemical industries (Griffin, 1981).

The degradation capacities of fungi, in particular white-rot strains, were studied for more than twenty years (Alaoui *et al*., 2008). A lot of data were obtained concerning treatment of pulp and paper, (Kondo *et al*. 1994) dyes and textile (*Lucas et al*. 2008), oxidation of polycyclic aromatic hydrocarbons (Zheng and Obbard, 2002) and olive oil mill wastewaters (Sayadi and Ellouz, 1995). Most of fungi can carry out fermentation process in the presence of oxygen (Griffin, 1981). Naturally, lignin degradation occur as the fungal decay of woods and utilization of the carbohydrates for growth (Kirk *et al.*, 1978). Kirk *et al.* (1978) also listed three aspects of lignin decomposition by fungus which set this process apart from other biodegradation process:

i. Fungi are the only organism that have been clearly demonstrated to be able to degrade lignin (able to modify natural lignin);
ii. Lignin degradation does not occur by the predominantly hydrolytic attack and that yields monomeric units in solution, as thus the digestion of polysaccharides, proteins and nucleic acids;
iii. Lignin degradation does not provide a primary source of carbon and energy for fungal growth, but is probably a necessary step in utilization of polysaccharide content of plant wastes.

2.2 Fungal Growth

Fungi can be grown in submerged cultures in several different morphological forms: suspended mycelia, clumps, or pellets (Metz and Kossen, 1977). Many studies have discussed the advantages and disadvantages of growth morphologies in terms of different products (Liao *et al.,* 2007). It has been concluded that fungal growth in pellet form is a favourable alternative which benefits most of the fungal fermentations since it not only makes repeated-batch fungal fermentation possible but also significantly improves the culture rheology which results in better mass and oxygen transfer into the biomass and lower energy consumption for aeration and agitation (Sjijdam *et al.,* 1980).

Fungal morphology is a key feature in industrial metabolite production in suspended cultures (Tamerler & Keshavarz 1999). El-Enshary *et al.,* (1999) stated that the pellets obtained in shake-flask cultures showed distinct layers of mycelial density with only the thin outer layer consisting of a dense mycelial network. It was shown that the process of pellet formation occurred in two steps:

i. Aggregation of free spores to spore clusters with subsequent germination and formation of small aggregates surrounded by a loose hyphal network;
ii. Aggregation of the primary aggregates to the final full-size pellets.

Liao *et al.* (2007) reported that a change in fungal morphology is influenced by medium composition, inoculum, pH, medium shear, additives (polymers, surfactants, and chelators), culture temperature, and medium viscosity. However, for individual strains, each factor has different importance to the growth morphologies thus much of the study on fungal pellet formation is limited to the level of the individual strain (Metz and Kossen, 1977).

Peptone was demonstrated to have a positive effect on pellet formation which is represented by uniform pellets (Liao *et al*., 2007). Metal ions are a very important factor in the metabolism of fungi. Foster and Waksman (1939) stated that the organism utilized the energy three times more efficiently when metal ions were added to the medium, which made for a relatively fast and abundant fungal growth. However, Liao *et al*. (2007) demonstrated that the metal ions caused the fungus grew so fast and caused the filamentous to tangle with each other and ultimately forming a clumpy morphology.

Carbon source and pH were not main factors on pellet formation (Liao *et al*., 2007). However, both of them had significant influences on fungal pellet size. Pellets from cultures grown with calcium carbonate and Potato Dextrose Broth (PDB) had bigger size than those grown without calcium carbonate, while pellets cultured on both PDB and glucose with calcium carbonate were much smoother than those without calcium carbonate. Calcium carbonate works as a neutralizer, preventing pH from

dropping into the low pH range of 2 to 3 which is not favourable for the biomass accumulation (Znidarsic *et al*., 2000).

Vasdev *et al*. (1995) reported that rapid growth of *C. bulleri* could have resulted in fast depletion of nutrients (C source) from the medium, and this in turn might have caused the start of the idiophase in this fungus, as reported for *Phanerochaete chrysosporium* (Faison and Kirk, 1985; Jeffries and Kirk, 1981). The nitrogen concentration in the culture medium also influenced the growth of *Trametes villosa* and *P. sanguineus*, with a greater biomass being obtained in the condition of nitrogen sufficiency (Machado *et al*., 2006). Fang *et al.* (2002) observed the effect of inoculum size on *Ganoderma lucidum* growth. They stated that a small inoculum size led to a low final cell density while the large inoculation density resulted in relatively small pellet size. However, Foster (1949) suggested that too high the inoculum size with more interaction with the hyphae, the more possibility the clump will be formed.

2.3 Decolorization process

Shaken condition during decolorization process generally results in higher dye decolorization than obtained with static cultures due to an increase in mass and oxygen transfer between cells and the medium (Katia *et al*., 2006). Katia *et al.* (2006) also found that the initial concentration of the dye influenced the decolorization capacity of *T. villosa*.

Katia *et al*. (2006) stated the higher glucose consumption by the *T. villosa* in the presence of synthetic effluent might be evidence of increased metabolism due to the detoxification mechanism. The presence of the effluent stimulated the growth of *P.*

sanguineus, with an approximately 0.5 g higher biomass as compared to control. The higher biomass observed might indicate the utilization of the dyes as a carbon source for growth.

2.4 *Pycnoporus sanguineus* (L.) Murril 1904

Pycnoporus sanguineus is one of the white-rot fungi that can grow on solid wood as a substrate since it is able to produce ligninolytic enzymes which are naturally produced for wood degradation (Heerden *et al*., 2008). This ligninolytic fungus, also produces extracellular cellulases for the utilization of carbohydrates (Dey *et al*.,1994). Pointing *et al.* (2000) reported that *P. sanguineus* decolorized azo and triphenylmethane dyes while producing laccase as a sole ligninolytic modifying enzyme.

2.5 Ligninolytic Modifying Enzymes (LME) produced by White-rot fungi

According to Pointing (2001), the ligninolytic enzymes of white-rot fungi has a broad specificity and have been implicated in transformation and mineralization of organo pollutant with structural similarities to lignin include synthetic dye**.** They secrete one or more of three extracellular enzymes essential for lignin degradation which are collectively referred to as lignin modifying enzyme (LME) *i.e.* lignin peroxidase (LiP), manganase dependent peroxidase (MnP) (Orth and Tien, 1995) and Laccase (Lac) (Thurston, 1994).

According to Buswell *et al.* (1984), LME production occurs during secondary metabolism and is induces by limited nutrient levels, particularly nitrogen. Pointing (2001) stated that nutrient, nitrogen levels, mediator compound and required metal

concentration affected the transcription level of LME. Laccases are involved in the biodegradation of lignin, which constitute the main non-carbohydrate component in wood and are among the most abundant groups of biopolymers in the biosphere. A great number of white-rot fungi have been reported to produce the lignin-degrading enzymes laccase (Lac), lignin peroxidases (LiP) , and manganese peroxidases (MnP), or at least one of these enzymes (Eggert *et al*., 1996; Dey *et al*., 1994).

2.6 Laccase

Laccase is a polyphenol oxidase containing copper atoms, can oxidize an array of organic and inorganic substrates, including mono-, di-, and polyphenols, aminophenols, methoxyphenols, as well as metal complexes: ferrocene, ferrocyanide or iodide, by concomitant four-electron reduction of oxygen to water (Edens *et al.* 1999).

Thurston (1994) stated that laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) have very broad substrate specificity with respect to the electron donor. They catalyze the removal of a hydrogen atom from the hydroxyl group of *ortho*- and *para*-substituted mono and polyphenolic substrates and from aromatic amines by one-electron abstraction to form free radicals capable of undergoing further depolymerization, repolymerization, demethylation, or quinone formation. Oxidation of methoxyhydroquinones during lignin degradation followed by autooxidation of the resulting methoxysemiquinones results in the formation of superoxide anion radicals, which can undergo further reactions (Guillén, 2000).

Elias *et al.* (2000) stated, the rather broad substrate specificity of laccase may be additionally expanded by addition of redox mediators, such as ABTS [2,29-azino bis[3

ethylbenzthiazolinesulfonic acid], 1-hydroxybenzotriazole, or compounds secreted by lignolytic fungi .The yields of laccase from *Pycnoporus sanguineus* can be increased 50 times using 20 mM xylidine (Pointing *et al.* 2000) while syringaldazine can induce the constitutive form of laccase from *Coriolus hirsutus* to a 10-fold level (Koroljova-Skorobogat'ko *et al.,* 1998). According to Galhaup *et al.* (2002), concentrations and types of carbon and nitrogen sources and metal ions such as copper in the media also influence laccase synthesis activity.

2.7 Synthetic dyes and their treatment

There are more than 100,000 commercially available dyes with over 7×10^5 ton of dyestuff produced (Meyer, 1981) particularly for textile, leather, pharmaceutical, food, pulp and paper industries (Seyis and Subasioglu, 2008). According to Gregory (1993), synthetic dye can be divided to azo, triphenylmethane, heterocyclic and polymeric structure. It is usually not readily biodegradable and therefore persistent in the environment and also toxic (Micheals and Lewis, 1985). Synthetic dyes are designed to be resistant to light, water and oxidizing agents, so it is difficult to remove them once they are released into the environment (Wesenberg *et al*., 2003).

The textile and dyestuff industrial wastes are generally treated by physicochemical methods (*viz.* adsorption, chemical precipitation and flocculation, oxidation by chlorine, H_2O_2 and ozone electrolysis, electrochemical treatment and ion pair extraction). These treatment methods are significantly different in color removal, volume capability, operating speeds and capital costs (Zollinger, 1987). Dye-containing effluents are only slightly decolorized by conventional biological wastewater

treatments. Due to high cost using physico-chemical treatment, unreliability and inefficiency of decolorization using conventional biological treatment, several works have been done and revealed the ability of white-rot fungi to decolorize synthetic dye (Pointing *et al*., 2000; Pasti-Grigsby *et al*., 1992).

2.8 Triphenylmethane dyes

Triphenylmethane dyes are aromatic xenobiotic compounds, and are used extensively in textile industries for dying nylon, wool, silk and cotton (Gregory, 1993). Some triphenylmethane dyes have been shown to be mutagens, mitotic poisons or clastogens (Au *et al.*, 1979, 1978). It was found that 15% of triphenylmethane dyes are lost to waste water as a result of inefficiency in the chemical and physical treatment processes (Zollinger, 1987).

2.9 Crystal violet

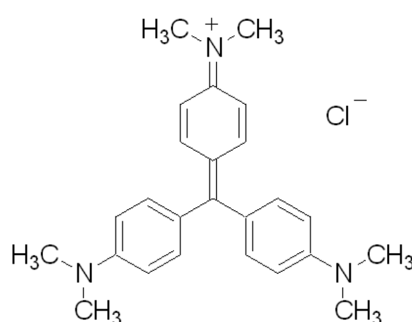

Figure 2.1: Crystal violet structure

Figure 2.1 showed the chemical structure of crystal violet. Crystal violet is a triphenylmethane dye which is aromatic xenobiotic compounds. It is used extensively in

textile industries for dying nylon, wool, silk and cotton (Gregory, 1993), for human and veterinary medicine as biological stain (Kean and Haskins, 1978; Kingsland and Anderson, 1976) and shown to inhibit glutathione S-transferases from rat liver (Debnam *et al*., 1993). Recently, it exhibited pronounced phototoxicity toward L1210 leukemia cells but comparatively small toxic effects toward normal hematopoietic cells (Indig *et al*., 2000). It was used as oral medication for treatment of pinworms and other tropical disease because of it great effect in controlling fungal growth under varying condition (Zolinger, 1987). Beside of antifungal properties, Ryan, (1992) reported that crystal violet was also used to treat skin infections by *Staphylococcus aureus* in humans and animals. Crystal violet decolorization was also found independent of pH of the medium when equal amounts of dye were added to the same amounts of buffer (pH 2-10). No significant changes in the absorbance of the dye were observed (Vasdev *et al*., 1995).

However, effluent of crystal violet may contaminate aqueous habitats (Micheals and Lewis, 1985). It has been suggested to be responsible for promotion of tumor growth in some species of fish (Nelson and Hites, 1980), a mitotic poisoning agent and regarded as biohazard substance (Au *et al*., 1978). It also reduces the penetration of light into the water and may effected aquatic flora.

Due to these problems, several studies on the decolorization of crystal violet decolorization by white rot fungi were carried out. Crystal violet was degraded by ligninolytic culture of *Phanerochaete chrysosporium*, and its initial oxidation proceeds via *N*-demethylation catalyzed by lignin peroxidase (Bumpus and Brock, 1988). Decolorization of crystal violet was found to be carried out by laccase in extracellular fluid from *Cyathus bulleri* (Vasdev et al., 1995), and by peroxidase from *Pleurotus*

ostreatus (Shin and Kim, 1998). Kumar and Uttam (1999) reviewed several studies on crystal violet decolorization by different species of fungus and bacteria (Table 2.1).

Table 2.1: Studied on crystal violet decolorization (Kumar & Uttam, 1999)

Organism	Crystal violet concentration (ppm)	Growth time (h)	DT (h)	D (%)	Refferences
Pseudomonas pseudomallei 13NA	20.4	20	120	96	Yatome *et al.* (1981)
Rhodotorulae rubra and Rhodotorulae sp.	10	24	96	99	Kwasniewska (1985)
P. chrysosporium BKM-F-1767	5	144	6	65	Bumpus and Brock (1988)
Bacillus subtilis IFO 13719	0.852	24	24	100	Yatome *et al.* (1991)
Nocardia globerula	0.852	24	24	96	Yatome *et al.* (1991)
Nocardia corallina	0.933	18	1.5	80	Yatome *et al.* (1993)
P. chrysosporium ME446	5	144	72	62	Yesilada (1995)
Coriolus versicolor	5	144	72	92	Yesilada (1995)
Funalia trogii	5	144	72	82	Yesilada (1995)
Laetiporus sulphureus	5	144	72	86	Yesilada (1995)
Cyathus bulleri	29.38	192	96	96	Vasdev *et al.* (1995)
Cyathus stercoreus	29.38	192	96	84	Vasdev *et al.* (1995)
Cyathus striatus	29.38	192	96	75	Vasdev *et al.* (1995)
P. chrysosporium NCIM 1197	20	144	216	92	Knapp *et al.* (1995)
P. chrysosporium MTCC no. 787	5	120	70	90	Sani *et al.* (1998)

Notes. DT, decolorization time; D, decolorization percentage

2.10 Laccase activity assay

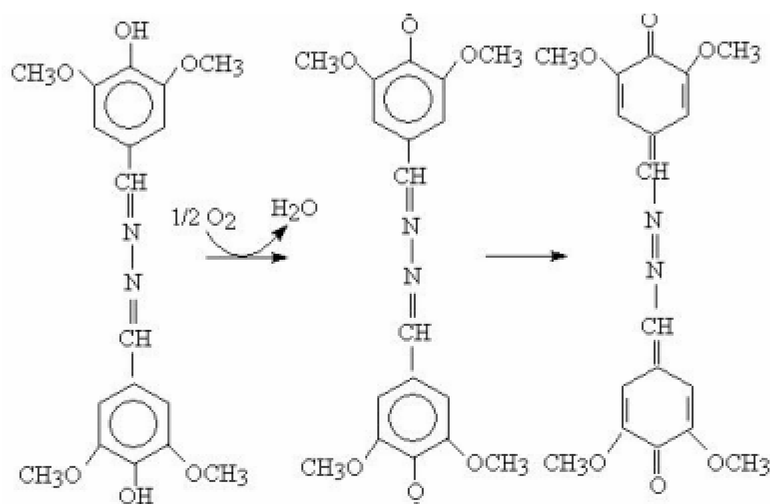

Figure 2.2: Oxidation of syringaldazine catalyzed by laccase to the corresponding quinone (Sanchez-Amat and Solano, 1997)

Laccase is regarded as the simplest enzyme that can be used to define the structure-function relations of copper containing proteins. Only laccase presents the possibility to oxidize activated methoxyphenols like syringaldazine (Thurston, 1994). Figure 2.2 demonstrated the oxidation of 4,4’- [azinobis(methanylylidene)] bis (2,6-dimethoxyphenol) (syringaldazine) to the corresponding quinone, 4,4’-[azinobis (methanylylidene)] bis (2,6-dimethoxycyclohexa-2,5-diene-1-one) (Manole *et al.*, 2008).

Table 2.2: Substrate used for laccase activity assay

Substrate	Extinction Coefficient, ε (M^{-1} cm^{-1})	Buffer	Abs (nm)	Author
Syringaldazine	65000	Britton (pH 5)	525	Manole *et al.*, 2008
		Sodium citrate (pH 4.8)	525	Manole *et al.*, 2008
		Mcllvaine	525	Sklarz *et al*., 1989
		Potassium phosphate (pH 6.5)	530	Sigma Aldrich Manual
ABTS	29300	Acetate (pH 5)	436	Srinivasan *et al*., 1995
		Na_2HPO_4/citric acid (pH 3)	415	Madzak *et a*l., 2006
		glycine-HCl (pH 3)	436	Niku-Paavola *et al*., 1988
6-hydroxydopamine	2300	Sodium acetate (pH 5.5)	490	Padglia *et al.,* 1994

Table 2.2 showed the substrates that are usually used for laccase activity assay *viz*. syringaldazine (Manole *et al*., 2008; Szklarz *et al*., 1989), ABTS (Madzak *et al*., 2006; Srinivasan *et al*., 1995; Niku-Paavola *et al*., 1988) and 6-hydroxydopamine (Padglia *et al.,* 1994). However, other studies also showed that guaiacol (Harkin and Obst, 1973), N,N-dimethylphenylenediamine (Reinhainmar, 1970), gallic acid, 3,4-dihydroxybenzaldehyde, guaiacol and pyrogallol (Wang *et al.,* 2000) can be used as substrate for laccase activity assay.

Terron *et al.* (2004) stated that tannic acid (TA) is able to carry out the chemical reduction of ABTS. This results in an overestimation of laccase activity values determined by this method in biological samples containing TA or related aromatic

compounds. Manole *et al.* (2008) was studying laccase substrate specificity for four different phenolic compounds (syringaldazine, hydroquinone, vannilic acid and tannic acid), and it can be observed that the enzyme is catalyzing the oxidation of all this compounds. However, it is observed that the activity of laccase in the oxidation of syringaldazine is higher than that of the other phenolics.

2.11 Stirred tank reactor

According to Doran (1995), impellers are broadly classified into axial flow or radial flow types. Axial flow impellers have blades which make angle of less than 90° to the plane of rotation and promote axial top-to-bottom motion. The fluid leaving the impeller is driven downwards until it is deflected from the floor of the vessel. It then spreads out over the floor and flows up against the tank wall before returning back to the impeller. Radial-flow impellers have blades which are parallel to the axis of rotation of the stirrer shaft and tank base. When radial-flow is set up by high speed rotation, the liquid is driven radials from the impeller against the walls of the tank where it divides into two streams, one flowing up to the top of the tank wall and the other flowing down to the bottom.

2.12 Experimental design

Methods of experimental design are used to evaluate the effects of several different treatments on a response variable. It is a specific procedure that stipulates exactly how each factor is to be varied to obtain the most information from the experimental data (Montgomery, 2001). Response surface methodology (RSM) term is referred to the methods that are directed towards investigating on effect of treatments

and relationship among them, using tools from experimental design and regression analysis (Hinkelmann and Kempthone, 1994). Regression analysis was used to detect the treatment effects of the experiment and simple relation among them. All of this information is important to find the treatment combination which gives the optimal response.

Box and Behnken (1960) have proposed Box-Behnken designs (BBD) *viz*. some three-level designs for fitting response surface. These designs are formed by combining 2^k factorials with incomplete block designs. The resulting designs are usually very efficient in terms of the number of required runs, and they are either rotatable or nearly rotatable (Montgomery, 2001). For three factors its graphical representation can be seen in two forms (Ferreira *et al*., 2007):

i. A cube that consists of the central point and the middle points of the edges, as can observed in Figure 2.3a.
ii. A figure of three interlocking 2^2 factorial designs and a central point, as shown in Figure 2.3b.

(a)

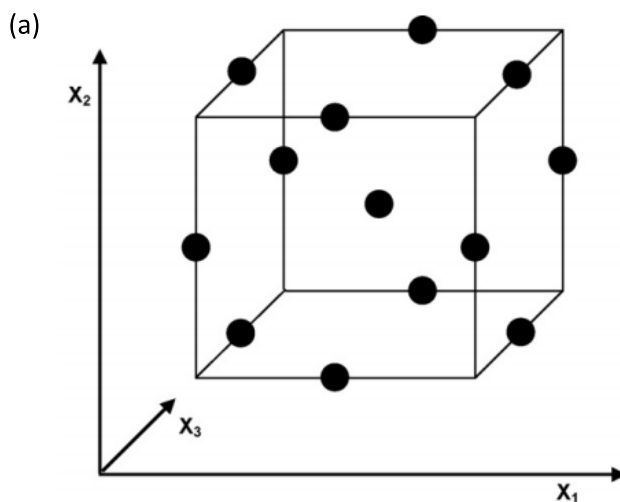

Figure 2.3: (a) the cube for BBD

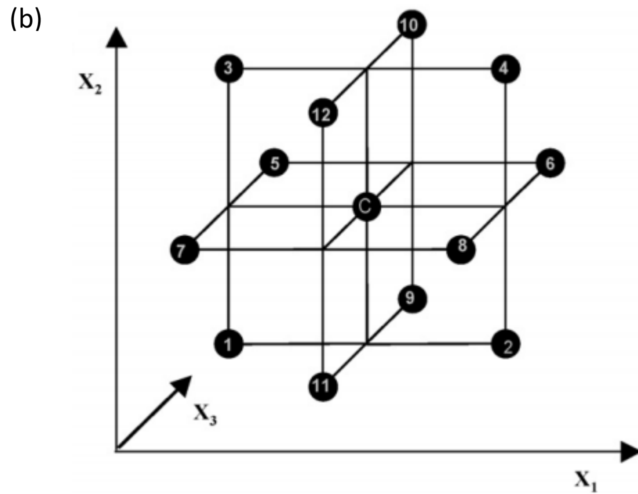

Figure 2.3: (b) three interlocking 2^2 factorial design

The number of experiments (N) required for the development of BBD is defined as $N = 2k(k\text{-}1) + C_0$ (where k is number of factors and C_0 is the number of central points). Table 2.3 contained the coded values of the three level factors of BBD (Ferreira *et al.*, 2007):

Table 2.3: Coded factor levels for a Box-Behnken design of a three-variable system

Experiment	*X1*	*X2*	*X3*
1	-1	-1	0
2	1	-1	0
3	-1	1	0
4	1	1	0
5	-1	0	-1
6	1	0	-1
7	-1	0	1
8	1	0	1
9	0	-1	-1
10	0	1	-1
11	0	-1	1
12	0	1	1
C	0	0	0
C	0	0	0
C	0	0	0
	0	0	0

Ferreira *et al.* (2007) also stated BBD and Doehlert matrix are more efficient than other RSM (central composite and three-level full factorial designs). The efficiency of one experimental design is defined as the number of coefficients in the estimated model divided by number of experiments. Beside the efficiency of BBD, it also does not contain combinations for which all factors are simultaneously at their highest or lowest levels. So, these designs are useful in avoiding experiments performed under extreme condition for which unsatisfactory results might occur. Conversely, they are not indicated for situations in which we would like to know the responses at the extremes, that is, at the vertices of the cube (Ferreira *et al.*, 2007).

2.13 Statistical Analysis

In the analysis of variance, it is usually more effective to check the normality with residuals. A check of the normality assumption could be made by plotting histogram residuals. However with small sample, considerable fluctuation often occurs and further analysis is required. A useful procedure is to construct a normal probability plot of residuals. If the underlying error distribution is normal, this plot will resemble a straight line (Montgomery, 2001). Montgomery (2001) also stated that plotting the residuals in time order of data collection is helpful in detecting correlation between the residuals. A tendency to have runs of positive and negative residuals indicates positive correlation. This would imply that the independence assumption on the errors has been violated.