CHAPTER 3

MATERIALS AND METHODS

3.1 Microorganism and growth medium

P. sanguineus (Linn.: Fr.) Murrill was maintained by weekly transfer to potato dextose agar (PDA) which is incubated at 27°C for 7 days, after which it was stored at 4ºC until required.

Glucose yeast extract malt extract peptone (GYMP) medium will be used to grow the fungus in liquid suspensions. The composition of the medium (g L^{-1}): 0.5 $MgSO_4$, 1.0 K_2HPO_4, 0.46 KH_2PO_4, 20.0 glucose, 2.0 peptone (Becton-Dickinson), 2.0 yeast extract (Becton-Dickinson), and 2.0 malt extract (Becton-Dickinson). Glucose solution was prepared and autoclaved separately. The medium were autoclaved for 10 minutes at 121 °C, 15 psi and cooled at room temperature (25 ± 1 °C) before used.

3.2 Growth and preparation of fungal pellets

The fungal that have grown on PDA plates will be used to prepare the biomass pellet in the liquid medium identified above. Mycelium suspension prepared by pouring 20 ml of 1 % Tween-80 into the plate that contained the mycelia. The surface of the mycelia was scratch to release the mycelium and spores. A 2.0 ml aliquot of mycelia suspension was inoculated into GYMP medium in Erlenmeyer flasks (Figure 3.1) followed by incubation on a rotary shaker for four (4) days. A number of flasks were

started in parallel. Suspension of pellets produced was filtered using 1.5 mm^2 sieve to recover the spherical pellets.

Figure 3.1: Pellet of *P. sanguineus* in GYMP medium

3.3 Stock preparation of crystal violet

Crystal violet ($C_{25}H_{30}ClN_3$; molar mass of 407.98 g M^{-1}) was purchased from BDH Chemicals (United Kingdom). This commercial tryphenylmethane dye was chosen as the model compound for decolorization. The stock solution of crystal violet was prepared at the concentration 80 mg L^{-1} and stored in amber bottle to protect it from direct sunlight. Spectral analysis of the crystal violet dye was initially done following to dye decolorization analysis. This was to determine the light wavelength at which the crystal violet absorbs the strongest. Standard calibration curve of dye concentration and absorbance was constructed. Absorbance was measured at the wavelength 590 nm based on spectral analysis.

3.4 Decolorization and optimization process

Five (5) g of wet fungal pellet was added to the 100 ml dye solution. It was then agitated on rotary shaker in a 250 ml flask. The decolorization process was optimized by studying the effect of different factors on decolorization percentage. The experiments were carried out at varying initial dye concentration (5 to 40 ppm), speed (60 to 160 rpm) and decolorization time (1 to 3 days). Filtrate of the dye solution after decolorization was centrifuged at 6000 rpm for 15 minutes to separate the biomass debris in the dye solution.

The decolorization yield was expressed as the degree of decrease in the absorbance at 590 nm (Jasco V-630 UV-VIS spectrophotometer, Japan) against the initial absorbance at the same wavelength. The reduction of dye concentration of the test solutions was calculated by extrapolation from the standard curve calibration. All experiments were been performed at 25 ± 1 °C. Heat killed pellets inoculated into the dye solution was used as control. The amount of dye absorbed by the heat killed pellets was used in the material balance to calculate the actual amount of dye lost due to biological decolorization. See equation (*1*).

$$[\text{Crystal violet}]_{\text{decolorized}} = [\text{Crystal violet}]_{\text{initial}} - [\text{Crystal violet}]_{\text{residual}} - [\text{Crystal violet}]_{\text{absorbed}} \quad (1)$$

where $[\text{Crystal violet}]_{\text{decolorized}}$ is the dye concentration decolorized due to biological activity, $[\text{Crystal violet}]_{\text{initial}}$ is the initial concentration of dye, $[\text{Crystal violet}]_{\text{residual}}$ is the concentration of dye remaining in solution, and $[\text{Crystal violet}]_{\text{absorbed}}$ is the concentration adsorbed by the pellets.

The experimental design comprised of 45 variable combinations including a center points with each combination performed in triplicate (Table 3.1). The actual and coded values of this experimental design are shown in Table 3.2.

Table 3.1: The three factors and levels Box-Behnken design

Run	X1	X2	X3
1	-1	-1	0
2	0	0	0
3	0	0	0
4	1	0	1
5	0	1	1
6	1	1	0
7	-1	0	-1
8	0	0	0
9	0	0	0
10	1	0	-1
11	0	-1	-1
12	0	1	-1
13	1	1	0
14	-1	-1	0
15	1	0	1
16	1	1	0
17	-1	1	0
18	-1	0	1
19	0	-1	1
20	0	0	0
21	-1	0	1
22	0	1	1
23	0	-1	1
24	0	1	-1
25	0	-1	1
26	-1	1	0
27	1	-1	0
28	0	-1	-1
29	-1	-1	0
30	0	1	-1
31	1	-1	0
32	1	-1	0
33	0	0	0
34	-1	0	-1
35	0	0	0

36	1	0	-1
37	0	0	0
38	-1	0	1
39	0	0	0
40	1	0	-1
41	0	1	1
42	1	0	1
43	-1	0	-1
44	0	0	0
45	0	-1	-1

X1 = process time (day) *X2* = agitation speed (rpm) *X3* = initial dye concentration (ppm)

Table 3.2: Coded level for process time, agitation speed and initial dye concentration variable

Coded level	Process time (day)	Agitation speed (rpm)	Initial dye concentration (ppm)
1	1	60	5
0	2	110	22.5
-1	3	160	40

3.5 Laccase activity determination using syringaldazine

The activity of laccase was assayed spectrophotometrically by monitoring the absorbance increase from oxidation of syringaldazine at 530 nm (ε = 65 mM^{-1} cm^{-1}) with a Jasco V-630 UV-VIS spectrophotometer (Japan) at 25 ± 1 °C (light path 1 cm). The assay mixture inside the test cuvette consisted of 3 ml sodium citrate buffer, 0.2 ml of 0.1 mM syringaldazine (Sigma, Germany) in 50 % ethanol and 0.2 ml of filtrate from pellet cultivation medium. The assay mixture for the blank cuvette consisted of 3 ml sodium citrate buffer, 0.2 ml of 0.1 mM syringaldazine in 50 % ethanol and 0.2 ml of distilled water. The reaction was started by the addition of syringaldazine solution and immediate mixing by inversion (Pearce and Ride, 1980)

The absorbance of a sample is directly proportional to concentration (Beer's Law) and to sample thickness (Lambert's Law). When these two relationships are combined, it resulted in the Beer-Lambert equation *(2)*:

$$A = \varepsilon * c * l \qquad (2)$$

where ε = extinction coefficient of syringaldazine (65000 M^{-1} cm^{-1}), a characteristic constant for a given absorbing substance; c = concentration of substrate in mol L^{-1}; l = thickness of the sample in cm (1 cm). One unit of enzyme activity is defined as the amount of enzyme that oxidized 1 micromole of syringaldazine per minute. Thus, the laccase activity was calculated using:

$$\frac{dc}{dt} = \frac{dA}{dt} * \frac{1}{\varepsilon * l} \qquad (3)$$

where $\frac{dA}{dt}$ = the rate change in absorbance;

$\frac{dc}{dt}$ = the rate change for substrate

Hence $\frac{dc}{dt} = \frac{dA}{min^{-1}} * \frac{1}{65000\ M^{-1}\ cm^{-1} * 1\ cm}$ *(4)*

$= M\ min^{-1}$

$= 10^6\ \mu mol\ L^{-1}\ min^{-1}$

Figure 3.2 show the change of absorbance against the time during laccase activity assay of the crystal violet filtrate after decolorization. The slope is represent the rate change of absorbance per unit of time (dA/dt).

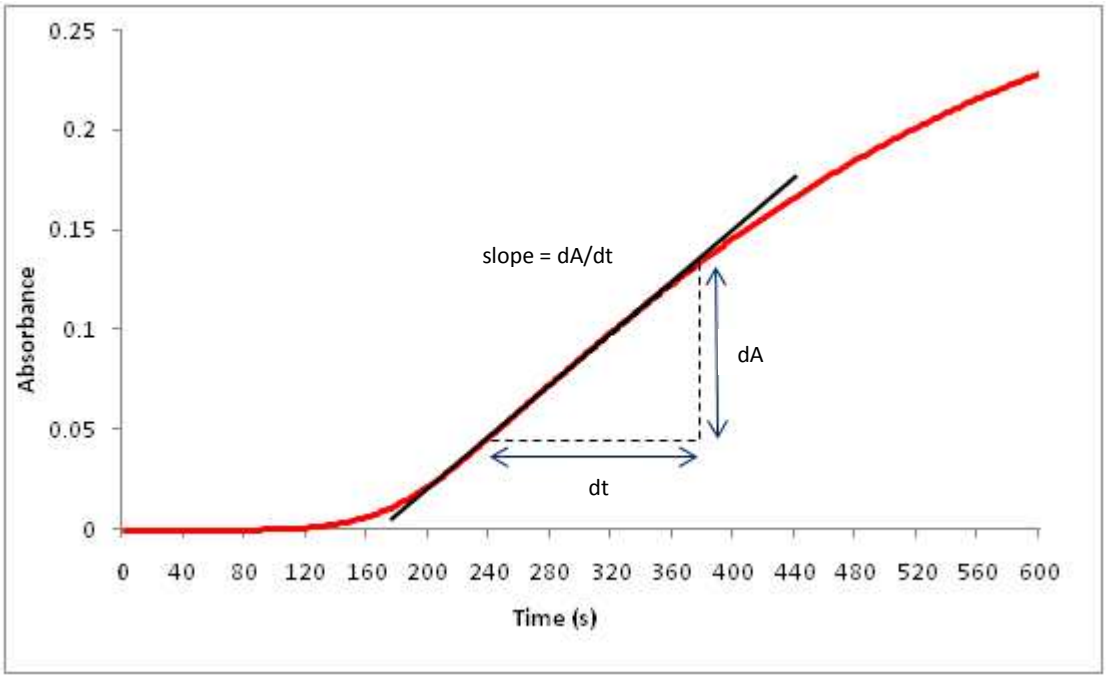


Figure 3.2: Laccase activity in the dye filtrate after decolorization

3.6 Statistical analysis and modeling

Data were subjected to the analysis of variance (ANOVA) and fitted according to a general polynomial model shown by equation (*5*).

$$Y = \beta_0 + \sum_{i=1}^{N} \beta_i X_i + \sum_{i=1}^{N} \beta_{ii} X_i + \sum_{i=1}^{N} \sum_{j=1}^{N} \beta_{ij} X_i X_j \qquad (5)$$

where Y is the predicted response variable, N is the number of design variables, β_o the intercept, β_i the first-order coefficient, β_{ij} the interaction coefficient and X_i and X_j are the coded forms of the input variables. To estimate the impact of each independent variable on the response, regardless of the presence of the other variables, main effects were calculated by equation (*6*):

$$Y = \beta_0 + \beta_i X_i \qquad (6)$$

Statistical examination of results and generation of response surfaces were performed using Minitab® 14 software.

3.7 Stirred tank reactor (STR)

Following the optimization process in the shake flasks, an attempt was made to perform the process in a stirred tank reactor. Decolorization process was carried out in a 1 L stirred tank reactor (Figure 3.2) filled with 0.8 L of dye solution. The tank reactors (Figure 3.2) were equipped with a single impeller stirrer and four baffle plates. Approximately 50 g L^{-1} of fungal pellets in 40 ppm crystal violet dye solution was used in the STR decolorization experiment.

The experiments were conducted at varying speeds *i.e.* 60 rpm to 120 rpm with different impeller geometry *viz.* angled blade 60° impeller (Figure 3.4) and curved blade impeller (Figure 3.5). Both of the impellers consisted of 6 blades and the diameter of each impeller is 5 cm. Figure 3.6 and 3.7 show the detail schematic of angled blade 60° impeller and curved blade.

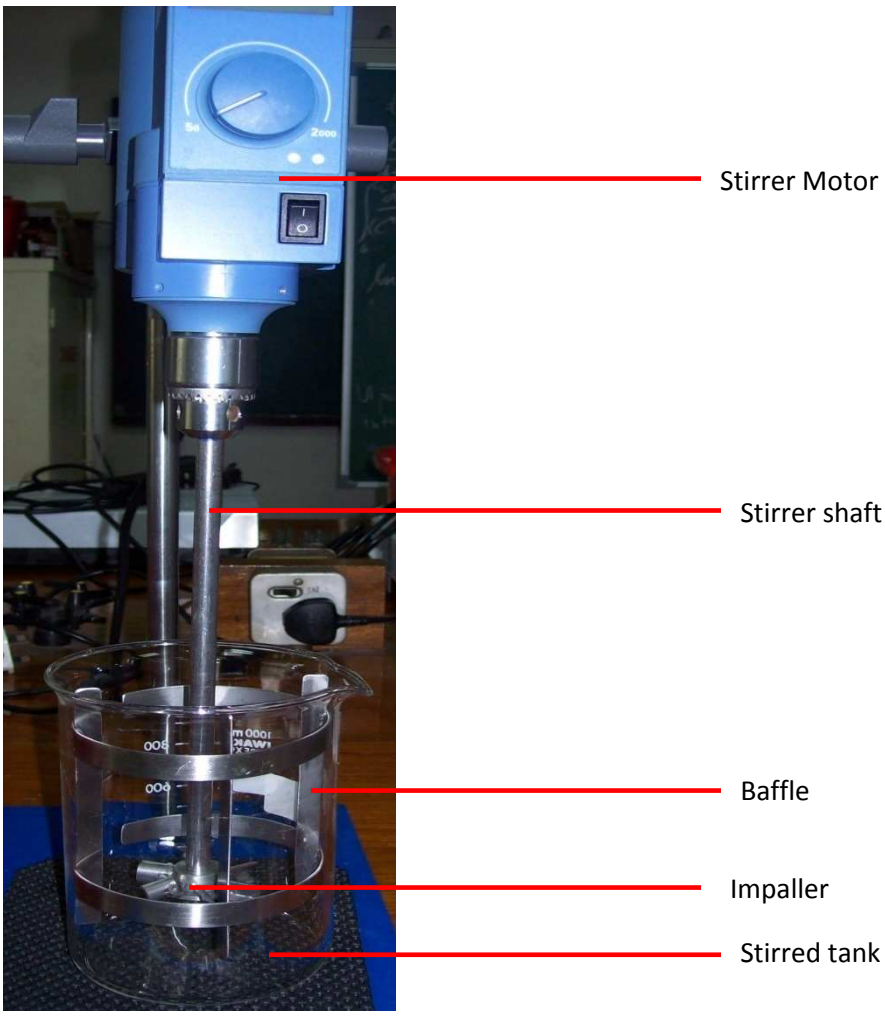


Figure 3.3: Stirred tank reactor

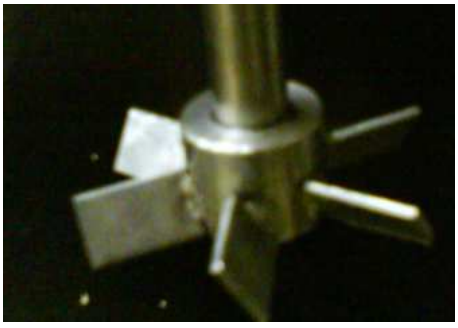
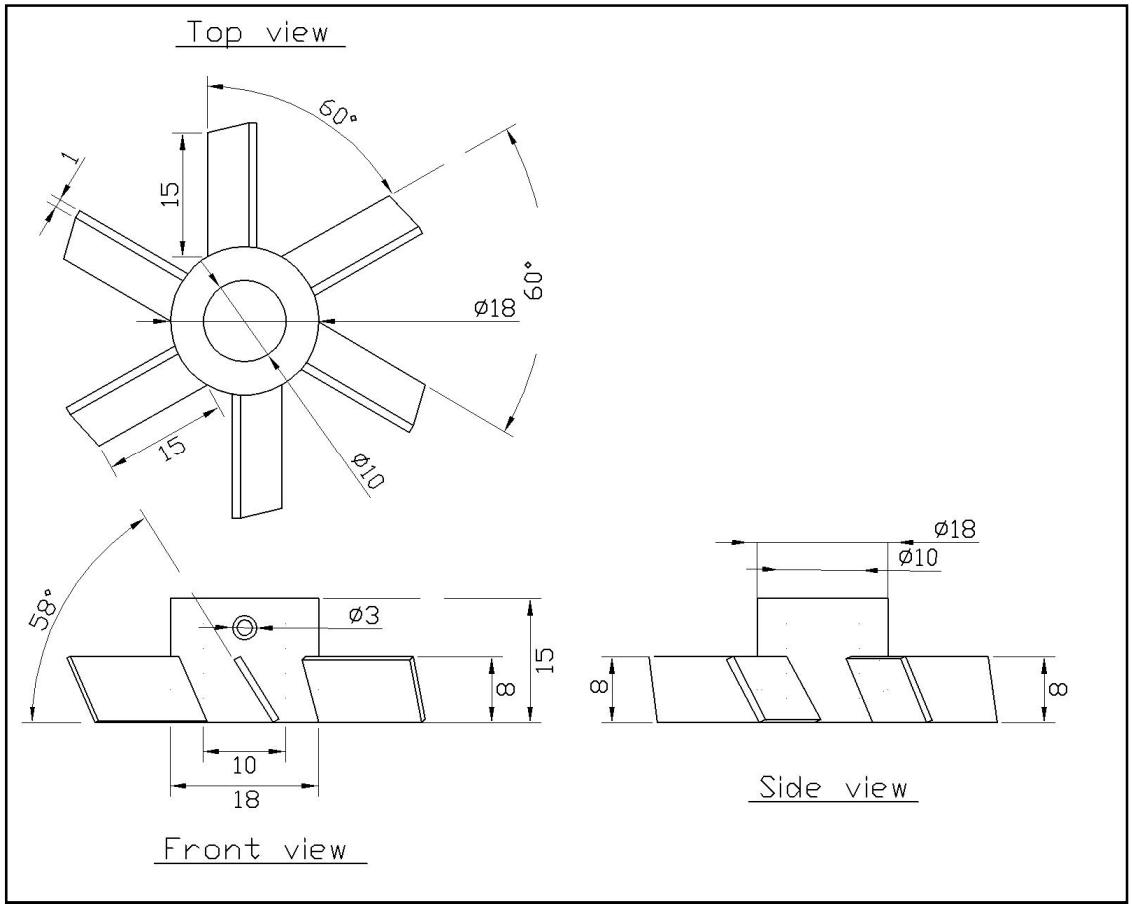


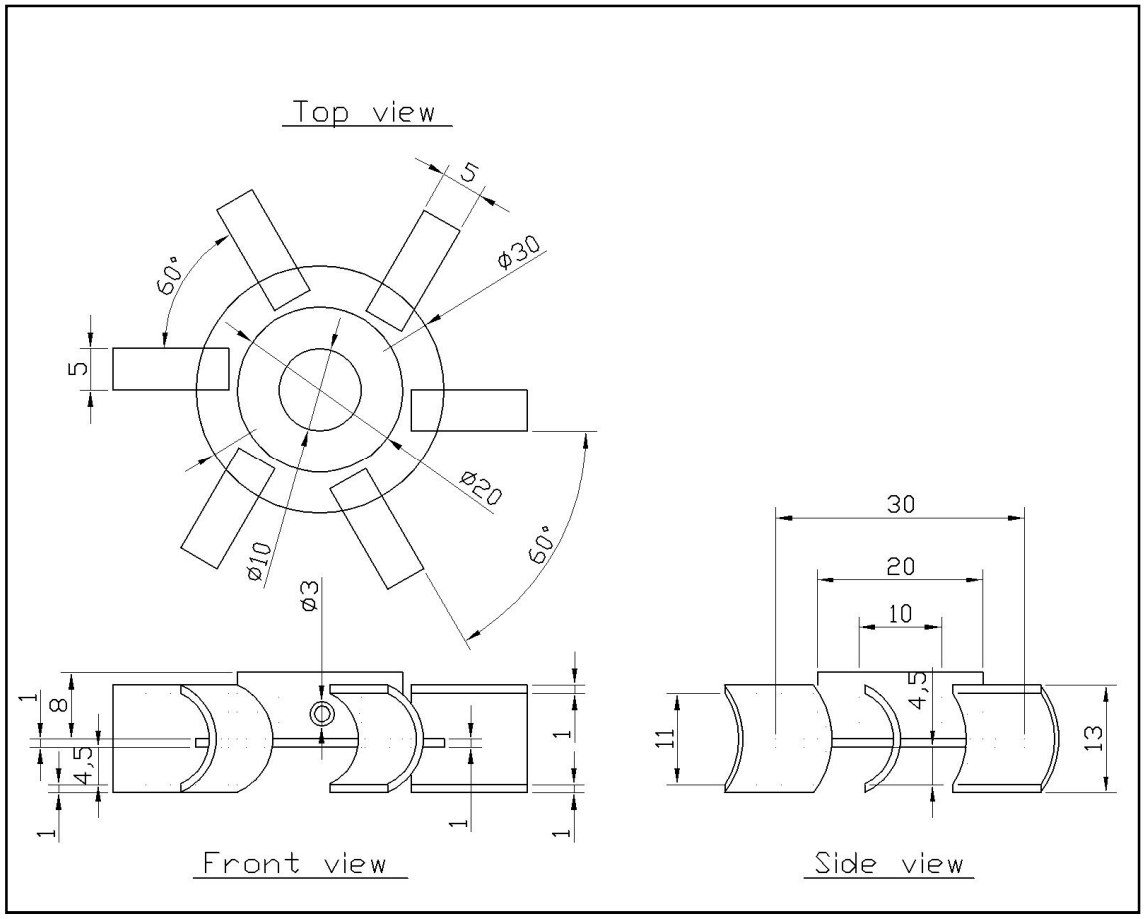
Figure 3.4: Angled blade 60° impeller

Figure 3.5: Curved blade impeller



*All measurements are in mm.

Figure 3.6: Schematic for six angled blade 60° impeller with no central disc



*All measurements are in mm.

Figure 3.7: Schematic for curved blade impeller with central disc

Sampling was done at regular interval for 72 hours to observe the pattern of dye decolorization. The dimensions of the stirred vessel and impellers are shown in Table 3.3 and Figure 3.8.

Table 3.3: Dimension of the stirred tank reactor and mixing equipment

Reactor Dimension	Angled blade 60° impeller	Curved blade impeller
Tank diameter, *T* (mm)	100	100
Liquid height, *H* (mm)	100	100
Liquid volume, *V* (ml)	800	800
Baffle width, *BW* (mm)	10	10
Baffle length, *BL* (mm)	125	125
Number of baffles, *nB*	4	4
Impeller diameter, *D* (mm)	50	50
Impeller clearance, *C* (mm)	50	50
Blade width, *B* (mm)	9	12
Number of blades, *nb*	6	6
Blade angle, α (°)	45	180

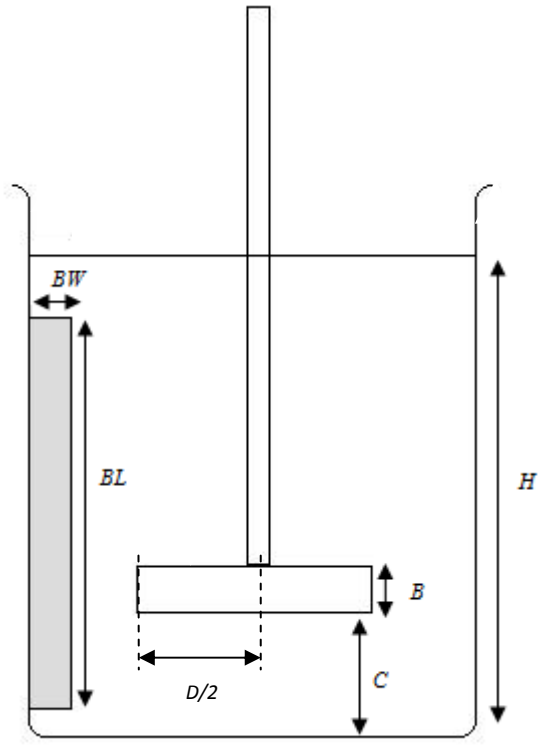


Figure 3.8: Dimension of stirred tank reactor and mixing equipment