CHAPTER IV

RESEARCH METHODOLOGY

This chapter presents selection of research method, experimental design, sample selection criteria, sample preparation procedure, experimental section which include chemical, enzymes experimental apparatus, experiments and experimental procedure, data collecting method, statistical analysis method and chapter summary.

4.1 Selection of Research Method

Due to the need to control the condition of the experiments and to monitor the relationships between different levels of independent variables and dependent variables of interest, a laboratory experiment was selected as the research method to ensure a high level of internal validity of this study.

The experiments were performed on small scale using 150-ml beakers as reactors. The veneers from commercially grown teak were cut into uniform square shape of size 3.2x3.2 mm.

Except for the experiments that performed at room temperature, the water bath was used as a mean to control the temperature.

The system selected was a closed system.

The data were collected using image processing technique as described in section 4.4 and 4.6. The results which represent lignin removal indirectly were reported in percentage change in average gray scale.

4.2 Experimental Design

The factorial experimental design with 4 independent variables is used in this study. The list of the dependent and independent variables and their levels is presented in Table 4.1

Table 4.1 Dependent and independent variables and their level

Variables	Number of level	Level detail
Dependent variable : % change in gray scale		
Independent variables :		
H_2O_2	2	2% 10%
Xylanase	3	0.25 u/ml 0.5 u/ml 1.0 u/ml
Laccase	3	0.05 u/ml 0.25 u/ml 1.0 u/ml
times	3	0.5 hour 1.0 hour 2.0 hour

4.2.1 Examination of each chemical component.

4.2.1.1 H_2O_2

As shown in Table 4.1, H_2O_2 has 2 levels of concentrations, 2% and 10%. Therefore, a 2x3 factorial with the replication of 3 is used. See Table 4.2 for detail representation of the experimental design.

Table 4.2 2x3 Factorial experimental design for H_2O_2 and time

Time (hour)	% change in average gray scale	
	2 H_2O_2 (%)	10 H_2O_2 (%)
0.5	RRR	RRR
1	RRR	RRR
2	RRR	RRR

Note: R represent 1 run of experiments

4.2.1.2 Xylanase

As shown in Table 4.1 xylanase has 3 levels of concentrations, 0.25, 0.50 and 1.00 u/ml. Therefore, a 3x3 factorial design with the replication of 3 is used. See Table 4.3 for detail representation of the experimental design.

Table 4.3 3x3 Factorial experimental design for xylanase and time

Time (hour)	% change in average gray scale		
	0.25 xylanase (u/ml)	0.5 xylanase (u/ml)	1.00 xylanase (u/ml)
0.5	RRR	RRR	RRR
1	RRR	RRR	RRR
2	RRR	RRR	RRR

Note: R represent 1 run of experiments

4.2.1.3 Laccase

As shown in Table 4.1 laccase has 3 levels of concentrations, 0.05, 0.25, and 1.00 u/ml. Therefore, a 3x3 factorial design with the replication of 3 is used. See Table 4.4 for detail representation of the experimental design.

Table 4.4 3x3 Factorial experimental design for laccase and time

Time (hour)	% change in average gray scale		
	0.05 laccase (u/ml)	0.25 laccase (u/ml)	1.00 laccase (u/ml)
0.5	RRR	RRR	RRR
1	RRR	RRR	RRR
2	RRR	RRR	RRR

Note: R represent 1 run of experiments

4.2.2 Examination of combination of chemical components

4.2.2.1 H_2O_2, xylanase and time

The 2x3x3 factorial experimental design with 3 replicates is used. See Table 4.5 for detail representation of the experimental design.

Table 4.5 2x3x3 Factorial experimental design for H_2O_2, xylanase and time

Time (hour)	H_2O_2 (%)	% change in average gray scale		
		0.25 xylanase (u/ml)	0.5 xylanase (u/ml)	1.00 xylanase (u/ml)
0.5	2	RRR	RRR	RRR
	10	RRR	RRR	RRR
1	2	RRR	RRR	RRR
	10	RRR	RRR	RRR
2	2	RRR	RRR	RRR
	10	RRR	RRR	RRR

Note: R represent 1 run of experiments

4.2.2.2 H_2O_2, laccase and time

The 2x3x3 factorial experimental design with 3 replicates is used. See Table 4.6 for detail representation of the experimental design.

Table 4.6 2x3x3 Factorial experimental design for H_2O_2, laccase and time

Time (hour)	H_2O_2 (%)	% change in average gray scale		
		0.05 laccase (u/ml)	0.25 laccase (u/ml)	1.00 laccase (u/ml)
0.5	2	RRR	RRR	RRR
	10	RRR	RRR	RRR
1	2	RRR	RRR	RRR
	10	RRR	RRR	RRR
2	2	RRR	RRR	RRR
	10	RRR	RRR	RRR

Note: R represent 1 run of experiments

4.2.2.3 Xylanase, laccase and time

The 3x3x3 factorial experimental design with 3 replicates is used. See Table 4.7 for detail representation of the experimental design.

Table 4.7 3x3x3 Factorial experimental design for xylanase, laccase and time

Time (hour)	xylanase (u/ml)	% change in average gray scale		
		0.05 laccase (u/ml)	0.25 laccase (u/ml)	1.00 laccase (u/ml)
0.5	0.25	RRR	RRR	RRR
	0.50	RRR	RRR	RRR
	1.00	RRR	RRR	RRR
1	0.25	RRR	RRR	RRR
	0.50	RRR	RRR	RRR
	1.00	RRR	RRR	RRR
2	0.25	RRR	RRR	RRR
	0.50	RRR	RRR	RRR
	1.00	RRR	RRR	RRR

Note: R represent 1 run of experiments

All experiment design established in section 4.2.1 and 4.2.2 and summarized in Table 4.8

Table 4.8 Summary of the established factorial experimental design

Experimental design	Number of experiments
1. single chemical component	
2 x 3 H_2O_2 and time	6
3 x 3 xylanase and time	9
3 x 3 laccase and time	9
2. combination of chemical component	
2 x 3 x 3 H_2O_2, xylanase and time	18
2 x 3 x 3 H_2O_2, laccase and time	18
3 x 3 x 3 xylanase, laccase and time	27

From experimental design summarized in Table 4.8, the total number of experiments will be 87 experiments. Therefore, with 3 replicates the sample size used in the experiment is 87 x 3 or 261 samples.

4.3 Sample Selection Criteria

This section presents criteria required in selecting teak veneer samples.

4.3.1 Selection of population

The population selected for experiments in this study was a commercially grown teak from the northern part of Thailand where most of commercially grown teak is populated. In this study veneers from teak in the area previously mention were obtained from veneer company in Nonthaburi to represent population of teak with poor color quality in veneer industry.

4.3.2 Selection of sample

The samples used in this experiment come from teak mention in section 4.3.1. The qualifications of samples used in this experiment are summarized in Table 4.9.

Table 4.9 Qualification requirement of samples

descriptions	Requirement
color	Yellow brown
thickness	0.5 mm.
Prebleaching sample	Not allow
Scar	Not allow
Reaction wood	Not allow

4.4 Sample Preparation Procedure

After samples were selected from criteria in Table 4.9, the samples must be prepared before they can be used in the experiments. The procedure in preparing veneer samples are as followed.

4.4.1 Each piece of veneer was cleaned with damp cloth to remove dust.

4.4.2 Veneers were then carefully cut into square pieces of 3.2x3.2 mm.

4.4.3 Veneers were then left to dry for 1 day in the room that temperature was controlled between 25 and 27 degree Celsius.

4.4.4 Each piece of veneer was labelled then scanned by Epson scanner model 2480 photo. The images acquired were stored in bitmap file. The detail procedure is in Appendix A

4.4.5 Each image was analyzed using computer program run on matlab. The results are in average gray scale. The computer program was written and provided by Mr. Chawalit Danulux. Image processing technique detail is in Appendix A.

4.4.6 Each sample is weighed by a 4-digit balance.

4.4.7 The samples are then stored in labeled zip lock plastic bag for experiments.

4.5 Experimental

This section presents chemicals, enzymes, and experimental apparatus used in this experiment, experiments and experimental procedure.

4.5.1 Chemicals

4.5.1.1 Hydrogen peroxide from Merck, Inc.

4.5.1.2 Distilled water.

4.5.1.3 Monobasic sodium phosphate monohydrate from Merck, Inc.

4.5.1.4 Dibasic sodium phosphate heptahydrate from Fluka, Inc.

4.5.1.5 Sodium acetate anhydrous from Merck, Inc.

4.5.1.6 Acetic acid from Merck, Inc.

4.5.2 Enzymes

4.5.2.1 Xylanase from Sigma, Inc.

4.5.2.2 Laccase from Sigma, Inc.

4.5.3 Experimental Apparatus

4.5.3.1 TAITEC xy-80 Water bath shaker from Bang Trading

4.5.3.2 4-digit balance

4.5.3.3 Epson scanner model 2480 photo

4.5.3.4 Magnetic stirrer

4.5.4 Experiments

Experiments were performed according to the established experimental design presented in section 4.2

The data collected from these experiments are reported in percentage change in average gray scale as an indirect measurement of lignin removed as stated in section 3.2

4.5.4.1 Single chemical component

These experiments were performed to examine the effect that each of the independent variables have on the removal of lignin from teak veneer.

For hydrogen peroxide, 6 experiments with 3 replicates were performed at 2 levels of hydrogen peroxide concentration (2 and 10%) at 3 levels of time (0.5, 1 and 2 hour). The total number of run was 18. All run were performed at 60 °C and pH 6.5. Phosphate buffer at 0.02 M was used to regulate pH of the solution.

For xylanase, 9 experiments with 3 replicates were performed at 3 levels of xylanase concentration (0.25 u/ml, 0.50 u/ml and 1.00 u/ml) at 3 levels of time (0.5, 1 and 2 hour). The total number of run was 27. All run were performed at room

temperature and pH 4.5. Acetate buffer at 0.02 M was used to regulate pH of the solution.

For laccase, 9 experiments with 3 replicates were performed at 3 levels of laccase concentration (0.05 u/ml, 0.25 u/ml and 1.00 u/ml) at 3 levels of time (0.5, 1 and 2 hour). The total number of run was 27. All runs were performed at room temperature and pH 6.5. Phosphate buffer at 0.02 M was used to regulate pH of the solution.

4.5.4.2 Combination of chemical component

These experiments were performed to examine the effect of the combination of different levels of hydrogen peroxide, xylanase, laccase and time on lignin removal from teak veneer.

1. H_2O_2, xylanase and time

In this experiment, 18 experiments with 3 replicates were performed using the combination presented in Table 4.5. These experiments were performed at 60°C and at pH 6.5 using 0.02 M phosphate buffer to regulate the pH.

2. H_2O_2, laccase and time

In this experiment, 18 experiments with 3 replicates were performed using the combination presented in Table 4.6. These experiments were performed at 60°C and at pH 6.5 using 0.02 M phosphate buffer to regulate the pH.

3. Xylanase, laccase and time

In this set of experiments, 27 experiments with 3 replicates were performed using the combination presented in Table 4.7. These experiments were performed at room temperature and at pH 4.5 using 0.02 M acetate buffer to regulate the pH.

4.5.5 Experimental procedure

This section presents the experimental procedure for the experiments outlined in section 4.5.4

1. Prepare 0.2M acetate buffer or 0.2M phosphate buffer according to detail in Appendix B.
2. Prepare solution of xylanase, laccase, hydrogen peroxide or their combination at specified concentration and pH as required by each experiment using distilled water and buffer solution in step 1.
3. Measure 20 ml of solution in step 2 and poured into 150-ml beakers.
4. Place the prepared wood samples into beakers and covered the beaker with plastic wrap to ensure closed system.
5. For experiments at 60°C, place the beakers into the water bath. For experiment at room temperature, placed the beakers on shelf and recorded room temperature.
6. Remove each sample from solution at scheduled of removal time.
7. Each veneer sample is washed with water, dried with cloth and left to dry for 1 day in the room that temperature was controlled between 25 and 27 degree

4.6 Data Collecting Method

After the samples were left to dry for 1 day in the room that temperature was controlled between 25 and 27 degree C, the samples were then processed as followed.

4.6.1 Each piece of sample was scanned and the acquired image stored in bitmap file.

4.6.2 Each bitmap image was analyzed using computer program run on Matlab. The results are in average gray scale.

4.6.3 The difference between the average gray scale before and after the experiment for each experiment was recorded, and the percentage change in gray scale was calculated.

4.6.4 Each sample was weighed by a 4-digit balance, and the different in weight was recorded.

4.6.5 All data from all experiments were collected, recorded and prepared for data analysis in the next chapter.

4.7 Chapter Summary

In this chapter, research method was selected and a small scale laboratory experiment with 150 ml beakers as reactors was chosen. The experimental design was established as laid out in Table 4.2 through 4.7. To ensure the consistency in veneer samples, the sample selection criteria was set and any veneer samples not meeting the requirement were rejected. The sample preparation procedure was set to establish the validity of the veneer samples. The experimental condition and procedure are presented.