Chapter 3

Materials and Methods

The research work was conducted at the Institute of Biological Sciences, Faculty of Science, University of Malaya. The materials and analytical methods employed during this study are given below.

3.1. Microorganism

A white-rot fungus, *Pycnoporus sanguineus* (Murrill strain CY788) was used in this study. The fungal cultures were stored at 29 ± 1°C incubation.

3.2. Media preparation

P. sanguineus was used to produce cellulase in a submerged culture. The fungus was maintained on Potato Dextro Agar (PDA) plates and stored at 4^{o}C with periodic (30 days) sub-culturing. Potato dextrose agar (PDA) used for fungal growth were prepared according to the methods recommended by Harrigan (1998). The pH of media was adjusted by using 0.1 N NaOH and 0.1 N HCl.

Table 3.1. Composition of potato dextrose broth according to Harrigan (1998).

Ingredients	Quantity
Potatoes peeled and diced into small pieces	200 g
Glucose	20 g
Distilled water	1000 mL

The potato dextrose agar (PDA) was prepared by adding 1.5% agar to potato dextrose broth and then the medium was sterilized by autoclaving for 15 minutes at 121°C.

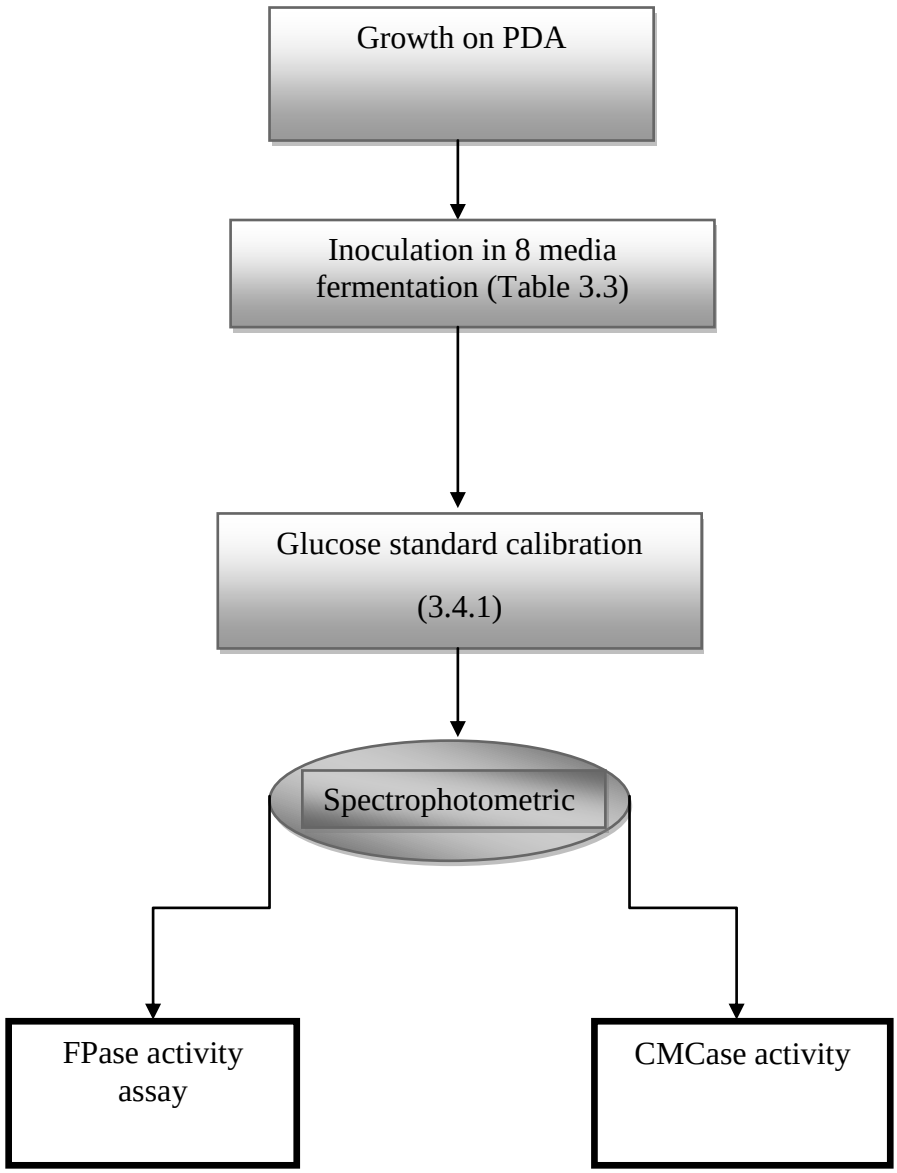

Figure 3.1. Schematic diagram for enzyme activity measurement.

3.3. Inoculation and incubation

The samples were first inoculated onto the PDA and incubated at 30°C for 144 to 168 hours and growth pattern was studied according to the suggestions and methods of Harrigan (1998). One (1) plug of mysellium (1 cm diameter) was used as inoculum for the following basal media for production of fungal biomass (Table 3.2).

Table 3.2. Basal medium composition.

Ingredients	Quantity (g L^{-1})
KH_4PO_4	0.5
K_2HPO_4	0.5
$(NH_4)SO_4$	1.0
$MgSO_4.7H_2O$	0.2
$CaCl_2$	0.1

The pH of media was adjusted by using 0.1 N NaOH and 0.1 N HCl. Then the medium was sterilized by autoclaving for 15 minutes at 121°C.

3.4. Enzyme production

Fungal cellulase production was carried out on the different media composition to investigate the effects of cellulose powder, carboxymethylcellulose (CMC), Tween 80 and yeast extract. All media were prepared at 1 L using special formulation (Table 3.3) according to Emtiazi *et al.*, (2007). All media were tested in 250 ml conical flasks, pH= 5.5, 25±1°C, 160 rpm.

Table 3.3. Media compositions for fungal cellulase production in shake flasks.

No. of media	Components			
	Cellulose ($g\ L^{-1}$)	CMC ($g\ L^{-1}$)	Tween 80 ($ml\ L^{-1}$)	Yeast extract ($g\ L^{-1}$)
1	20	_	_	_
2	20	_	2	_
3	10	_	_	10
4	10	_	2	10
5	_	20	_	_
6	_	20	2	_
7	_	10	_	10
8	_	10	2	10

Carboxymethylcellulose is an amorphous cellulose derivative. Therefore, endoglucanase (CMCase) could be used in hydrolyzing it. The enzyme locates randomly on the amorphous site along the cellulose polysaccharide chain and insert a water molecule in the intramolecular β-1,4-glucosidic bonds, producing a new reducing or non-reducing oligosaccharides of variable size and consequently new chain ends. On the other hand, filter paper is a crystalline cellulose structure in which exoglucanases cut cellulose polysaccharide chain at the terminal end to release cellobiose (Barr *et al*., 1996).

3.4.1. Glucose standard calibration

For the determination of reducing sugars, the glucose standards were prepared. The standard calibration was prepared using dinitrosalicylic acid reagent developed by Miller (1959) where 1 ml of enzyme supernatant filtered by fibre glass filter was mixed with 1 ml of sodium citrate buffer. 0.05 g CMC was added to the mixture in a test tube. The tubes were incubated at 50°C for 1 h in the water bath. The reaction was stopped with the addition of 2.0 ml dinitrosalicylic acid reagent and subsequent boiling in a water bath for 15 minutes. After that 1.0 ml Rochelle salt (40% sodium potassium tartarate) was added to the test tube and the absorbance was measured at 575 nm against blank i.e. distilled water.

For glucose calibration different concentrations of D-glucose *viz*. 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 g L^{-1} were prepared (Fig. 3.1).

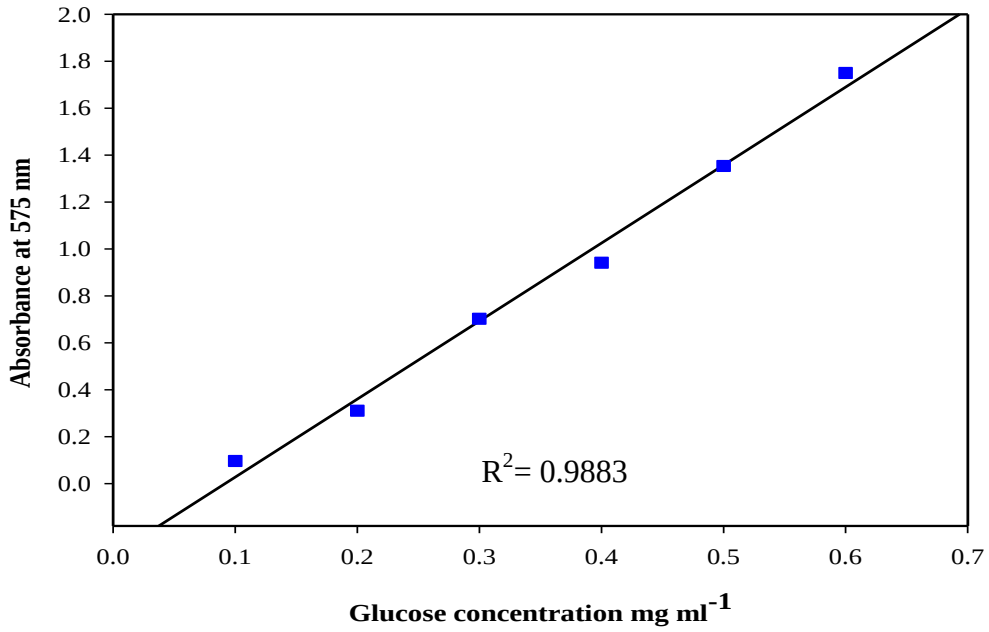

Figure 3.2. Glucose standard calibration.

The reducing sugars absorbance was measured by a spectrophotometer, Jasco V-630, Japan at 575 nm (Fig. 3.2). The spectrophotometer allows reducing sugars determination at constant temperature (25± 0.1°C) *via* thermostated cell compartment. The absorbance was converted to concentration using a calibration curve that fitted the following equation:

$$C_{reducing\ sugars} = 0.791 A_{575} \qquad \text{Eq. (3.1)}$$

where $C_{reducing\ sugars}$ is the concentration of the sugars in mg mL^{-1} and A_{575} is the absorbance of the solution at 575 nm. Equation (3.1) had a regression coefficient of 0.9883 and applied over a concentration range from 0 to 0.6 mg mL^{-1}.

Figure 3.3. Spectrophotometric system (V-630 Jusco).

3.4.2. CMCase activity using spectrophotometer

1.0 mL of culture filtrate was added to 0.05 g CMC, 1.0 mL 0.05 M citrate buffer pH 4.8 in a test tube and incubated at 50°C for 1 h (Mandel and Weber 1969). Then 2.0 mL DNS were added to the test tube and incubated at 100°C for 15 minutes. After that 1.0 mL sodium potassium tartarate was added to the test tube and then the released reducing sugars amount was determined *via* its optical density at 575 nm (Bush and Stutzenbrger 1997). The unit conversion was calculated as follows (Eq. 3.2):

$$\frac{mg\ glc}{ml} \times \frac{1\ \mu mol\ glc}{0.18016\ mg\ glc} \times \frac{1}{60\ min} = \mu mol\ ml^{-1}\ min^{-1} \qquad \text{Eq. (3.2)}$$

3.4.3. FPase activity using spectrometer

1.0 mL of culture filtrate was added to a test tube containing 0.05 g Whatman No.1 filter paper strips (10 x 1 mm) and 1.0 ml 0.05 M citrate buffer pH 4.8 and incubated at 50°C for 1 h (Mandel and Weber 1969). After that 2.0 ml DNS were added to the test tube and then incubated at 100°C for 15 minutes. Then 1.0 ml sodium potassium tartarate was added (Bush and Stutzenbrger 1997). Released reducing sugars amount was determined *via* its optical density at 575 nm. The unit conversion was calculated as in equation (3.2).

3.5. Enzyme activity using commercial cellulase from *Aspergilleus niger*

In order to validate the assays used to determine FPase and CMCase activities in fermentation mixture, a commercial cellulase enzyme was used (from *Aspergillus niger*, 1.4 U mg^{-1} solid, SIGMA). One unit of this enzyme will liberate 1.0 µmole of glucose from cellulose in 1 hour at pH 5.0 at 37 °C.

The amount of reducing sugar production was measured using carboxymethylcellulose and filter paper as substrate. For this purpose, cellulase was measured at 0.7 U, which was then dissolved in 3.0 mL of sodium citrate buffer (50 mM, pH 4.8). Subsequently, 0.05 g mL^{-1} filter paper (for FPase activity assay) and CMC (for CMCase activity assay) was added to the test tubes. The reaction was carried out at 50°C for 60 minutes and reducing sugar production was measured in 20 minutes interval using DNS method (Miller 1959).

3.6. Viscometric method for cellulase assay

The following flowchart displays the whole methodologies for this part of the present work.

Result 1 (No): it means that the mathematical models are different for each CMC concentration tested. The different concentrations of CMC affected the model form, and not only on the function coefficient;

Result 2(Yes): it means that the mathematical models are the same for both media (GYMP and PDA inocula) but differ only in coefficients, so the initial inoculum medium has no effect on enzyme production;

Result 3 (No): it means that the mathematical models for both media are different i. e, the initial inoculum medium affected enzyme production.

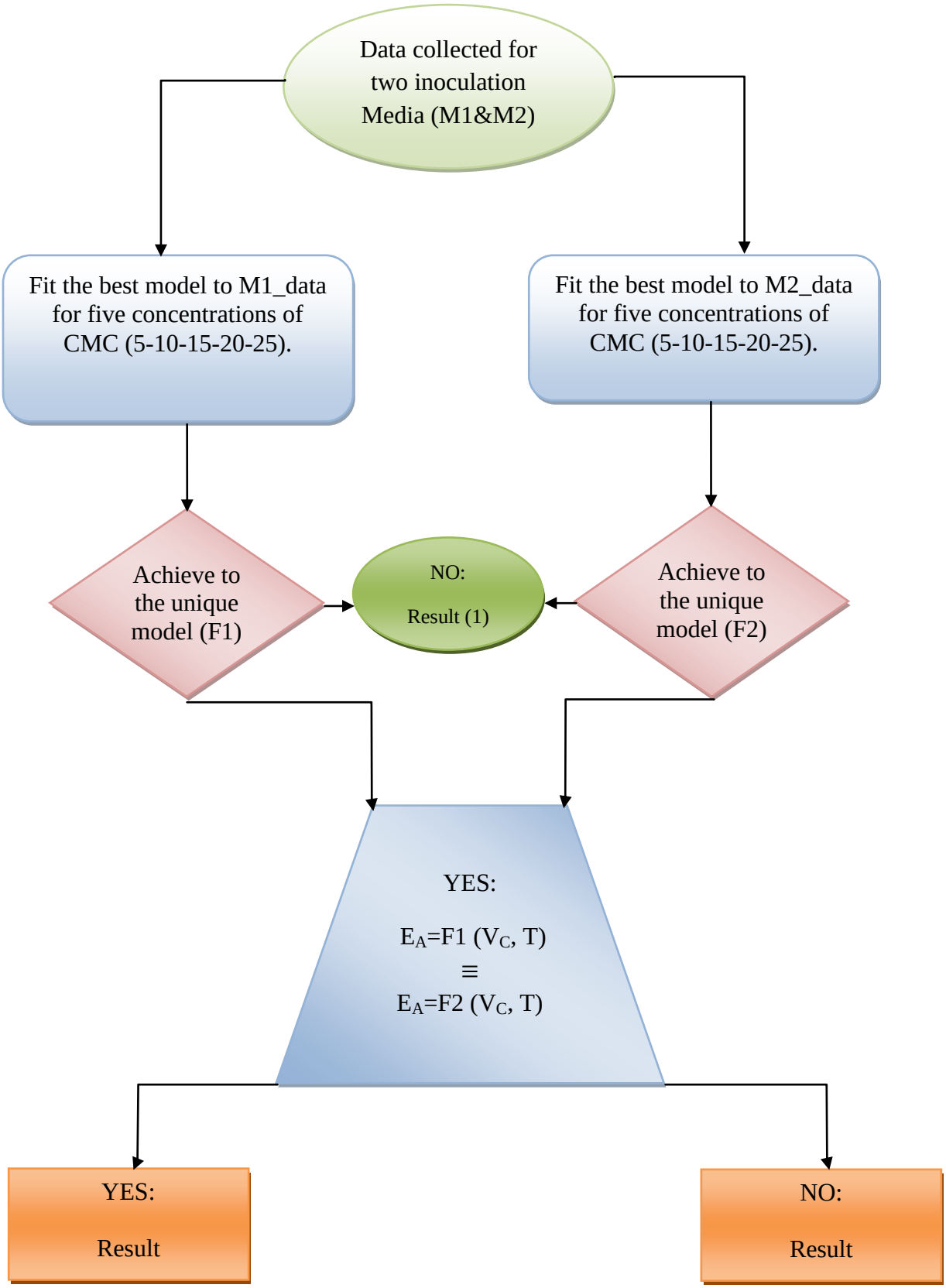

Figure 3.4. Schematic diagram for mathematical modeling methodology.

3.6.1. Media composition

The white-rot fungus, *P. sanguineus* (Murrill strain CY788) was used to produce cellulase in a submerged culture. The fungus was maintained on two different agar plates: first one (M1) was Potato Dextrose Agar, PDA (Table 3.1) and the second (M2) was Glucose Yeast Peptone Malt (GYMP) Agar, (Table 3.4). The plates were stored at 4^{o}C with periodic (30 days) sub-culturing. The growing fungus from these two different agar media was inoculated into the basal medium (Table 3.2), at different concentrations of carboxymethyl cellulose (CMC) sodium salt (low viscosity) (Sigma-Aldrich Netherlands) in 250 ml shake flasks, pH 5.5. One (1) plug of mycelium (1 cm diameter) was used as inoculum for each flask which was shaken at 160 rpm and 25^{o}C. The CMC concentrations used were 5, 10, 15, 20 and 25 g L^{-1}.

Table 3.4. Composition of GYMP Agar.

Ingredients	**Quantity (g L^{-1})**
Glucose	20
Yeast	2.0
Malt	2.0
Peptone	2.0
KH_2PO_4	1.0
K_2HPO_4	1.0
$MgSO_4.7H_2O$	0.5
Agar	18.0

Viscosity and cellulase production were measured every 12 hours in triplicate until 192 hours. Forty eight (48) flasks were prepared for each concentration of CMC. Since there were two different inoculum of media (PDA and GYMP) and five concentrations of CMC in basal medium, the number of the flasks were 240 for each different agar type; and 480 flasks in total.

3.6.2. CMCase activity measurement

Endo-1,4-β-glucanase activity was determined from the culture supernatant using the protocol described by Miller (1959). 1 ml of enzyme supernatant filtered by fibre glass filter was mixed with 1 ml of sodium citrate buffer. 0.05 g CMC was added to the mixture in a test tube. The tubes were incubated at 50°C for 1 h in the water bath. The reaction was stopped with the addition of 2.0 ml dinitrosalicylic acid reagent and subsequent boiling in a water bath for 15 minutes. After that 1.0 ml Rochelle salt (40% sodium potassium tartarate) was added to the test tube and The absorbance was measured spectrophotometrically at 575 nm against a blank, which was distilled water. All assays were performed in triplicate. The unit conversion was calculated as in equation (3.2).

3.6.3. Viscosity measurement

Viscosity of each sample was measured using Sine-wave Vibro Viscometer (SV series) in Clean Room, Department of Physics, University of Malaya. 15 ml of sample was filtered using glass fibre, thickness 0.55 mm, 13400-25J, Sartorius Stedim, France (Fig. 3.4).

Figure 3.5. Glass fibre for sample filtration.

The culture filtrate was collected into a sample cup (polycarbonate, volume 35 to 45 ml). The data of viscosity is transmitted during measurement to a PC using the standard accessory software “WinCT-Viscosity” (Fig. 3.6. b, a).

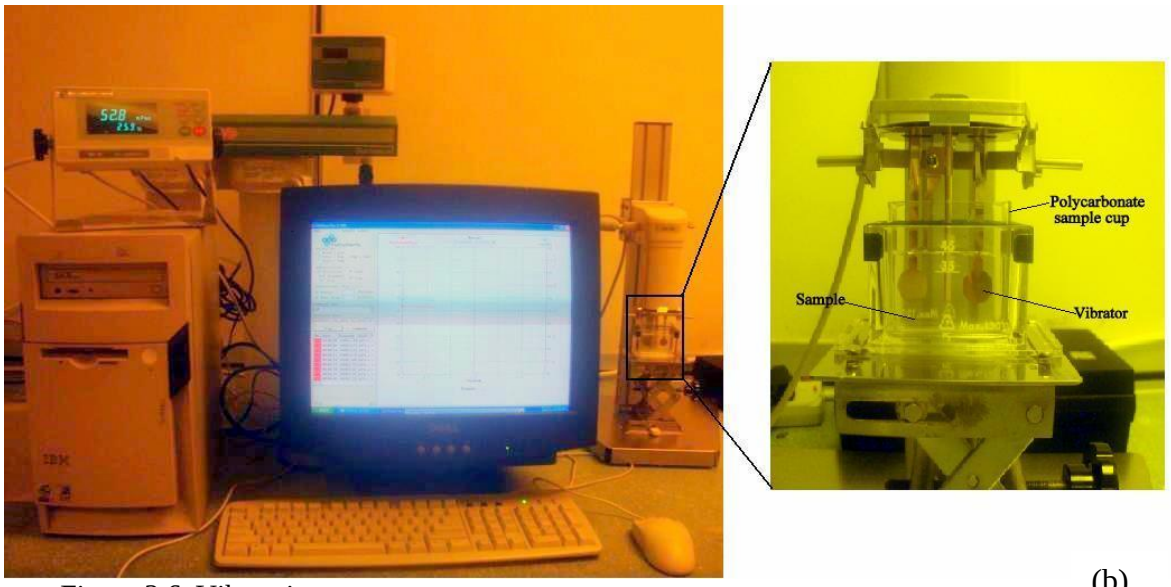

Figure 3.6. Vibro viscometer system.

(a) (b)

3.6.4. Vibro viscometer system

As shown in Fig. 3.6 (b) the thin sensor plates were immersed into the sample. When the spring plates are vibrated a uniform frequency, the amplitude varies in response to the quantity of the frictional force produced by the viscidity between the

sensor plates and the sample. The vibro viscometer controls the driving electric current to vibrate the spring plates in order to make uniform amplitude.

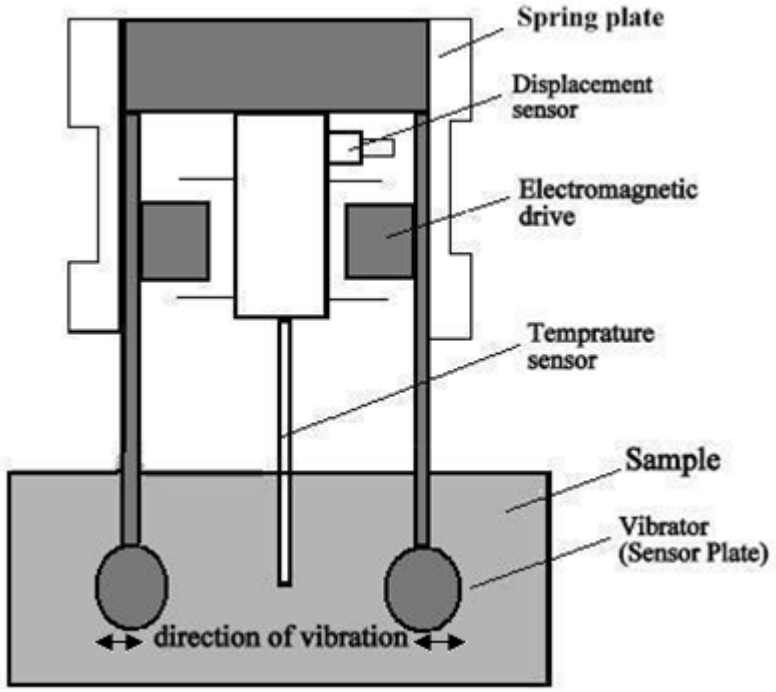

Figure 3. 7. Vibro viscometer (detection system).

Since the frictional force of viscosity is directly proportional to the viscosity, the driving electric current (driving power) for vibrating the spring plates with a constant frequency to make uniform amplitude is also directly proportional to the viscosity of each sample.

The vibro viscometer measures the driving current to vibrate the sensor plates with a uniform frequency and amplitude, and the viscosity is given by the positive correlation between the driving electric current and the viscosity.

Sine-wave Vibro Viscometer (SV) series materialize the dynamic ranges of viscosity measurement as wide as from 0.3 mPa.s to 10,000 mPa.s with SV-10 and from 1 Pa.s to 100 Pa.s with SV-100, and is capable of continuously measuring in the measurement ranges with excellent repeatability (accuracy) and stability. This wide dynamic range enable the measurement of viscosity changes in the processing of thixotropy liquid (gel from sol colloidal solution), or in the processing of curing of resin, adhesive or paint, which cannot be continuously measured with a conventional rotational viscometer.