# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Chemical Reagents

| Chemical Reagents | Production Company |
|---|---|
| Ammonium Dihydrogen Phosphate | MERCK |
| Ammonium Sulfate | MERCK |
| Dinitrosalicylic Acid | SIGMA |
| Glucose | FLUKA |
| Malt Extract | MERCK |
| Peptone | HIMEDA |
| Phenol | J.K. BAKER |
| Potassium Sodium Tartrate | CARLO |
| Sodium Hydroxide | J.K. BAKER |
| Yeast Extract | MERCK |

**2.2 Equipments**

| **Name of Equipments** | **Production Company** |
|---|---|
| Air Lock | - |
| Analytical Balance | PRESICA |
| Autoclave model ACV-3167 | IWAKI |
| Beaker (50, 100, 500, and 1000 ml) | PYREX |
| Centrifuge | EPPENDORF |
| Erlenmeyer Flask (250, 500 and 1000 mL) | PYREX |
| Graduated Cylinder (50, 100 and 500 mL) | PYREX |
| Glass Distilllator | - |
| Glass Pipette | PYREX |
| Hand Refractometer (0-32 $^{o}$Brix) | N.O.W. TOKYO |
| Hot Air Oven | MEMMERT |
| Hypodermic Syringe | HP |
| Laminar Air Flow | LABCONCO |
| Micropipette | EPPENDORF |
| pH Meter | CYBERSCAN |
| Shaker | KUHNER |
| UV/Vis Spectrophotometer | JASCO |
| Volumetric Flask (50 and 100 mL) | PYREX |
| Vortex Mixer | VORTEX-2-GENIE |

## 2.3 Methods

### 2.3.1 Relationship Between Alcohol and Carbon Dioxide Production during Wine Fermentation by Using Grape Juice

2.3.1.1 Starter preparation

Grape (malaga white grape) was crushed and filtered through cheese cloth. The total soluble solid of grape juice was adjusted to 5 $^{o}$Brix with deionized water. Then, 300 mL of grape juice was poured into a 500 mL flask. Two flasks were prepared. The flasks were boiled for 2-3 min, then plugged with cotton wool. Then, they were cool down to ambient temperature. After that, 110 mg/L of dried yeast cells (*saccharomyces cerevisiae* V116) were aseptically transferred into the flasks. They were shaken at 100 rpm for 24 h at ambient temperature before use.

2.3.1.2 Must preparation

Grape was crushed and filtered through cheese cloth. The total soluble solid of grape juice was adjusted to 18 $^{o}$Brix with glucose. KMS of 100 ppm was added into the grape juice. After that, 90 mL of the grape juice was aseptically filled into a 250 mL flask. Sixty four flasks were prepared. All the flasks were plugged with cotton wool. Then, they were kept at 20 $^{o}$C for overnight.



2.3.1.3 Wine fermentation

Ten mL of starter (10 per cent by volume) was aseptically added into 90 mL of must in a 250 mL flask. All the flasks were inoculated, plugged with air locks and weighed before keeping in the controlled room at 20 $^{o}$C for wine fermentation. Sampling procedure was as follows; during the first 12 h, the interval sampling time was every 3 h. From 12 to 24 h of fermentation, the interval sampling time was every hour. From 24 to 30 h of fermentation, the interval sampling

time was 2 h. From 30 to 48 h of fermentation, the interval sampling time was 3 h. From 48 to 84 h of fermentation, the interval sampling time was 6 h. Two flasks were taken as the sample at a time.

### 2.3.2 The Suitable Time for the Addition of Ammonium Salt in the Must as Nitrogen Sources for *Saccaromyces cerevisiae*

2.3.2.1 Starter preparation

It was similar to 2.3.1.1

2.3.2.2 Must preparation

It was similar to 2.3.1.2, but 450 mL of grape juice was aseptically filled into a 1000 ml flask. Nine flasks were prepared.

2.3.2.3 Wine fermentation

Fifty mL of starter (10 per cent by volume) was aseptically added into 450 ml of must in a 1000 ml flask. All flasks were inoculated and plugged with air locks. Three flasks without the supplementation of ammonium dihydrogen phosphate were control. Three more flasks were aseptically supplemented with ammonium dihydrogen phosphate of 700 ppm at the beginning of fermentation. The other three flasks were aseptically supplemented with ammonium dihydrogen phosphate of 700 ppm after 17 h of fermentation. All the flasks were weighed before keeping in the controlled room at 20 $^{o}$C for wine fermentation. Sampling times were similar to 2.3.1.3

### 2.3.3 The Suitable Concentration of Ammonium Dihydrogen Phosphate in the Must as Nitrogen Sources for *Saccaromyces cerevisiae*

2.3.3.1 Starter preparation

It was similar to 2.3.1.1

2.3.3.2 Must preparation

It was similar to 2.3.1.2, but 450 mL of grape juice was aseptically filled into a 1000 mL flask. Eighteen flasks were prepared.

2.3.3.3 Wine fermentation

Fifty mL of starter (10 per cent by volume) was aseptically added into 450 mL of must in a 1000 mL flask. All flasks were inoculated and plugged with air locks. Three flasks without supplementation of ammonium dihydrogen phosphate were control. The serial of three flasks were aseptically supplemented with ammonium dihydrogen phosphate of 100, 300, 500, 700, and 1000 ppm after 17 h of fermentation, respectively. The flasks were weighed before keeping in the controlled room at 20 $^{o}$C for wine fermentation. Sampling times were similar to 2.3.1.3

**2.3.4 The Suitable Concentration of Ammonium Sulfate in the Must as Nitrogen Sources for *Saccaromyces cerevisiae***

2.3.4.1 Starter preparation

It was similar to 2.3.1.1

2.3.4.2 Must preparation

It was similar to 2.3.3.2

2.3.4.3 Wine fermentation

It was similar to 2.3.3.3



**2.4 Analysis**

2.4.1 pH Determination

pH of the sample was determined by using pH meter. It was calibrated with standard buffer solutions pH 7.0 and 4.0 before measuring the sample.

2.4.2 Total Soluble Solid Determination

Total soluble solid of the sample was determined by using hand refractrometer 0-32 $^{o}$ Brix. It was calibrated with deionized water before use.

2.4.3 Reducing Sugar Determination

Reducing sugar of the sample was determined by DNS method (Appendix A). One mL of the sample was mixed with 1 mL of DNS solution, boiled for 10 min. Then, it was cool down in the water. Absorbance of the sample was measured at 540 nm. The amounts of reducing sugar were read out from the standard curve using glucose as the reference.

2.4.4 Alcohol Determination

Alcohol content in the sample was determined by Ebulliometer. It was calibrated with deionized water before measuring the sample.

2.4.5 Biomass Determination

Biomass of the sample was determined at 550 nm. (Appendix B). Standard curve for the biomass concentration was also carried out.

2.4.6 Carbon Dioxide Concentration Determination

Carbon dioxide concentration of the sample was determined by weighing the flask before the start of wine fermentation and record as the initial weigh. After that, the flask was weighed at the time interval along the wine fermentation and record as the weight at that time. Then, the initial weight of the flask minus by the weight of the flask at that time. The different weight was the amount of carbon dioxide concentration.



**2.5 Fermentation Kinetics**

During wine fermentation, parameters of wine such as reducing sugar, ethanol and biomass concentrations were determined. Then, graphs of these parameters along with fermentation time were plotted. Fermentation kinetic parameters; the specific

growth rate ($\mu$), the specific rate of substrate consumption ($q_s$), the specific rate of product formation ($q_p$), the yield coefficient of biomass from substrate ($Y_{x/s}$), and the yield coefficient of product from substrate ($Y_{p/s}$) were calculated out as follows:

| | | |
|---|---|---|
| Specific growth rate ($\mu$) | $= (1/x)(dx/dt)$ | ($h^{-1}$) |
| Specific rate of substrate consumption ($q_s$) | $= (1/x)(ds/dt)$ | (g/g-h) |
| Specific rate of product formation ($q_p$) | $= (1/x)(dp/dt)$ | (g/g-h) |
| Yield coefficient of biomass from substrate ($Y_{x/s}$) | $= \mu / q_s$ | (g/g) |
| Yield coefficient of product from substrate ($Y_{p/s}$) | $= q_p / q_s$ | (g/g) |