Chapter III

MATERIAL AND METHODS

3.1 Microorganism Used

The strain of Candida utilis used in this study was obtained from Department of Science, Ministry of Industries. The stock cultures were carried on potato dextrose agar (Difco Manual, 1953). This medium was composed of potatoes, infusion form 200 g, bacto-dextrose 20 g and bacto-agar 15 g were added and continued heating until they dissolved completely. The solution was adjusted to pH 5.0 with tartaric acid and then divided into tubes and sterilized by autoclaving at 15 pounds pressure, 121° C for 15 minutes. The culture slants were held at the refrigeration tamperature (10° C) and transferred regularly to maintain viability.

3.2 Pineapple Juices

Pineapple were peeled to remove the skin, comminuted by a commercial blender at medium speed to small pieces, and pressed with a commercial juice-making apparatus to obtain pineapple juice. Sugar in the pineapple juice was determined as percent invert sugar by Lane-Ennon method (Pearson, 1970). The concentration of the invert sugar in pineapple juice varied from 12 to 16%.

3.3 Preparation of Medium

The pineapple juice was filtered through coarse filter paper by means of suction and diluted with water to the desired concentration. The clear solution was sterilized by autoclaving at 121° C for 15 minutes. Ammonium sulfate 0.5, potassium dihydrogen phosphate 0.5, yeast extract 0.1, or urea 0.03% (w/v) of the medium were added, when required, as supplementary ingredients. was seperately prepared by dissolved in small amount of distilled water, autoclaved at the same condition, and added to the pineapple juice subtrate solution in order to obtain the desired composition of each formulae. The initial pH of the medium was between 4-5.

3.4 Preparation of Inoculum

The culture slants were removed from the refrigerator and kept at room temperature (25° C) for 48 hours. They were then transferred to new potato dextrose agar slants twice at 24 hours interval and kept at the same temperature. The inoculum was prepared by transferring the yeast culture from agar slants into 500 ml-Erlenmeyer flasks containing 50 ml medium. The flasks were closed with cotton plug and shaked on a reciprocal shaker at the vibrating speed of 240 rpm. After 12 hours of shaking, additional medium (50 ml) was added to each flask and continued shaking for another 12 hours. The absorbance reading

(described in section 3.7.1) was employed to determine the concentration of yeast cells in the inoculum. The inoculum of known concentration was diluted, if necassary, so that the final absorbance of the medium after inoculating into the medium was about 1.

3.5 Size of Inoculum

Twenty percent of inoculum, based on final volume of the medium, was used for each run in both the shake flasks and stirred-vessel fermenter.

3.6 Cultivation Condition

3.6.1 Shake-flask experiments

The purpose of these experiments was to determine some of the nutritive requirement for the growth of C. utilis on the pineapple juice. Individual or combinations of ingredients as listed in Table 2 were supplemented in the pineapple juice. Duplicate were made on each run for each medium studied. The prepared inoculum was used to inoculate 200 ml of the medium in 500 ml-Erlenmeyer flasks. The flasks were shaken on a reciprocal shaker at the vibrating speed of 240 rpm. and at room temperature (25° C). The pH during cultivation was maintained at 4.0 by the addition of 1 N sodium hydroxide solution over 4 hours interval. The yeast growth was estimated both by measuring absorbance of

Table 2 Formulation of nine fermentation media in the shake-flask experiments

Ingredients	Medium								
	1	2	3	4	5	6	7	8	9
Pineapple juice (2% w/v invert sugar)	+	+	+	+	+	+	+	+	+
Ammonium sulfate, $(NH_4)_2SO_4$ (0.5% w/v)	+	-	+	-	+	-	+	+	-
Potassium dihydrogen phosphate, (KH_2PO_4) (0.5% w/v)	+	+	+	+	+	+	+	-	-
Peptone (0.5% w/v)	-	+	-	-	-	+	+	-	-
Urea, $(NH_2)_2CO$ (0.03% w/v)	-	-	-	+	+	-	+	-	-
Yeast extract (0.1% w/v)	-	-	+	-	+	+	+	-	-

\+ added in the medium.

\- not added in the medium.

the cultures with the spectronic 20 spectrophotometer (Bausch and Lomb, VWR Scientific Inc. N. Y.) at 500 mm wavelength, and by determining dry weight of yeast cells at every 4 hours cultivation period (described in section 3.7). The supernatant obtained from the determination of dry weight was collected for analysing sugar consumption. At the end of cultivation, a quantity of yeast culture was centrifuged by garver electrifuge (Garver Manufacturing Co. Union City Indiana. U.S.A.) at the speed of 740 rpm. for 30 minutes. The quantity of centrifuged yeast cell was washed with distilled water, recentrifuged, dried in an oven at 100°C, and kept for determination of protein content. Fresh medium was added to compensate for the samples taken and loss due to evaporation.

3.6.2 Stirred-vessel fermenter experiments

The growth of C. utilis on selected medium obtained from the shake flask experiments was further studied in fermenter. The fermenter is made of stainless steel and has a viewing panel constructed of plastic, together with agitation and aeration devices. The impeller on the agitator shaft is an open turbine type (Solomons, 1969). The dimension of the fermenter was designed according to Finn (1954). The diagrammetric of the fermenter is shown in Figure 1. The entire fermenter and accessories and tubing, were sterilized by 5% (v/v) phenol solution. The steriled medium and the inoculum were then added into

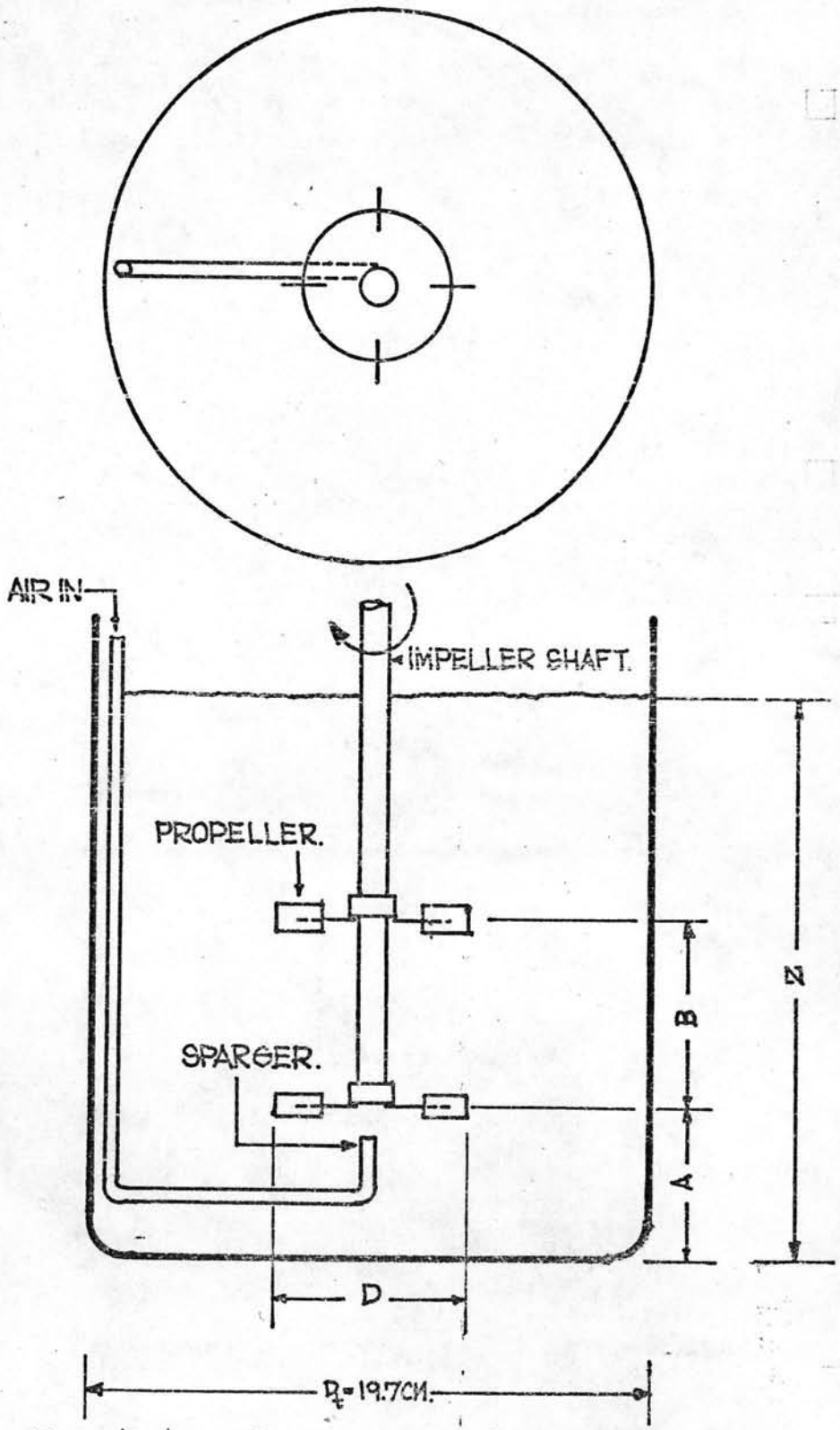

Figure 1. Typical dimension of a stirred fermenter : $Z/D_t = 1.0$ $D/D_t = 0.34$; $A/D = 0.8$ to 1.0; $B/D = 1.0$ to 1.2.

the fermenter. The concentration of sugar in the medium was 2% throughout the entire batch cultivation studied. The total volume of the medium was 6 litres for each run. The culture was aerated with compressed air sterilized by filtration through a packed column (diameter-2.2 cm., length-3.5 cm.) of steriled cotton filter. The air was introduced through this filter to the sparger located under the impeller. The sparger directed the air up into the impeller through 0.1 cm. hole. The agitation was kept constant at the maximum speed of 1400 rpm. The aeration was controlled by the rotameter (Rotameter Manufacturing Co., Ltd., Croydon, England) at the rate of 0.5, 1, and 1.5 volume of air/volume of culture medium/minutes (VVM). Foaming during yeast cultivation was controlled when required by manual addition of steriled silicone solution 10% (v/v). The pH during cultivation was maintained at 4.0 by manual addition of 6 N sodium hydroxide over 2 hours interval. Growth of C. utilis during cultivation in fermenter was measured every hour as described in section 3.7. Two 25 ml of samples of the culture were withdrawn from the fermenter at every two hours for determination of dry weight of yeast cells. A quantity of centrifuged yeast cells was washed with distilled water, recentrifuged, dried in oven at 100°C, and kept for determination of protein content. The supernatant obtained from the determination of dry weight was collected for analysing sugar consumption and chemical oxygen demand (COD). Fresh medium was added to compensate for the samples taken.

3.7 Measurement of Growth

The growth of yeast during cultivation was measured by either by absorbance reading or dry weight determination (Mateles and Tunnenbaum, 1968).

3.7.1 Measurement of absorbance

Growth response was measured with the spectronic 20 spectrophotometer at the wavelength of 500 mm (Vananuvat and Kinsella, 1975). The growth was expressed as absorbance using the steriled medium as blank. Concentrated cell suspension was diluted with distilled water to an absorbance between 0.2 and 0.6 prior to absorbance measurement.

3.7.2 Measurement of dry weight

Dry weight of yeast cell was determined from 25 ml aliquots of culture medium. The aliquots were first centrifuged by garver electrifuge at the speed of 740 rpm. for 30 minutes. The supernatants were collected and the residues were resuspended in 10 ml distilled water. The suspension was transferred quantitatively to aluminium weighing dish which was dried and tared, then dried at 100°C for 2 hours to constant weight (Vananuvat and Kinsella, 1975).

3.8 Yeast yield

Percent yield or conversion ratio was defined as dry weight of yeast cells obtained based on the actual sugar consumption in the medium (Prescott and Dunn, 1959).

3.9 Analyses

3.9.1 Invert sugar

The concentration of sugar in the pineapple juice was estimated by the method of Lane-Ennon (Pearson, 1970) and expressed as percent invert sugar. Reagent included : Fehling's solution, 1N hydrocholic acid, sodium hydroxide 10% (w/v), phenolpthalein 1% (w/v), methylene blue solution 1% (w/v), starch solution 1% (w/v). Felhing's solution was composed of Fehling's solution A and Fehling's solution B mixing together. Fehling's solution A was prepared by dissolving 69.278 g copper sulfate ($CuSO_4 \cdot 5\ H_2O$) in water and made up to 1 litre. Fehling's solution B was prepared by dissolving 100 g sodium hydroxide and 340 g potassium tartrate in water and made up to 1 litre. Starch solution was prepared as described by the method of the A.O.A.C. (1975). One gram of soluble starch was mixed with enough distilled water to make a thin paste, a 100 ml of boiling water was then added and boiled while stirring for approximately 1 minute.

The sample was first hydrolyzed to invert the sucrose by addition of 15 ml of 1 N hydrocholic acid and diluted to 150 ml

with distilled water. The solution was covered with clock glass and heated to boiling. The boiling was held for two minutes. After cooling, it was neutralized with sodium hydroxide solution and diluted to 200 ml with distilled water.

The analysis was divided into two steps, the first step was preliminary titration. A 25 ml portion of mixed Fehling's solution was titrated with 15 ml of the boiling sugar solution using the methylene blue as indicator. Accurate titration was done by repeating the titration, adding before heating almost all of the sugar solution required to effect reduction of the copper in the first step. The sample was boiled gently for two minutes, added 3-5 drops of the methylene blue indicator and then completed the titration within a total boiling time of 3 minutes. The proportions of the various sugar, equivalent to 25 ml of Fehling's solution are given in Appendix 1.

The method described above was used to determine the sugar content in the pineapple juice before cultivation of C. utilis. During cultivation, the sugar in the media was utilized rapidly by the yeast so it was not practical to use this method as it was very difficult to read the value of the invert sugar from the Table in Appendix 1. Another method of measuring the sugar content in the media (Stiles et al., 1926) during cultivation was tried and found to be a better choice. Reagent included : combined microreagent, 1 N sulfuric acid, 0.002 N sodium thiosulfate, and starch solution 1.0% (w/v). The combined micro-

reagent composed of copper sulfate, sodium potassium tartrate, sodium carbonate (anhydrous), potassium iodide, potassium iodate and potassium oxalate at concentrations of 0.5, 7.5, 40.0, 10.0, 0.7, and 18.4 g/l, respectively. The sample was hydrolyzed to invert sugar before determination. For this inversion, 1 ml of concentrated hydrocholic acid was added to 15 ml of the sample and the mixture was heated at 70°C for 10 minutes. After cooling, the solution was neutralized with sodium hydroxide 10% (w/v). Five ml of the microreagent was measured into 25 ml pyrex test tube and 5 ml sample containing 30 to 70 mg/ml of invert sugar was added to this. The tube was stoppered with loose-fitting to minimized oxidation by air and heated for 15 minutes in a boiling water bath, cooled in cold water, and added 5 ml of 1N sulfuric acid. The content in the tube was mixed well, and after one minute it was titrated with 0.005 N thiosulfate using starch as an indicator. The starch solution, prepared by the method described earlier, was added when the solution had turned a light straw color which indicated that only a trace of iodine remained. The titration with thiosulfate was continued until the blue color of the starch-iodine complex completely disappeared. A blank was prepared by substituing 5 ml of water for the sample in the above procedure. The difference in titration of thiosulfate between the blank and the sample were used to determine the amount of invert sugar in the sample as described by Stiles et al. (1926).

3.9.2 Kjeldahl protein (K-protein)

The Kjeldahl nitrogen was determined by the method of A.O.A.C.(1975). Protein was calculated from the amount of nitrogen multiplied by 6.25 (Worgan, 1973). A sample size of 10 to 20 mg protein was used for digestion, 1.94 g of catalyst (1.9 g K_2SO_4 and 40 mg HgO) was added and the digestion was done in micro-Kjeldahl digestion apparatus until a clear solution was obtained. Distillation with 10 ml of sodium hydroxide-sodium thiosulfate solution (dissolved 60 g NaOH and 5 g $NaS_2O_3 \cdot 5\ H_2O$ in water and diluted to 100 ml) was carried out in distillation apparatus until the distillate received in 10 ml of 4% (w/v) boric acid attained a total volume of 50 ml. The quantity of nitrogen was determined by titration with 0.02 N hydrocholic acid. Four drops of methylene blue-methyl red (mix 2 parts of 0.2% alcoholic methyl red solution with 1 part 0.2% alcoholic methylene blue solution) was used as an indicator, giving a violet end point.

The Kjeldahl protein of yeast cells includes nitrogen contained in proteins, nucleic acids, cell walls, in addition to other inorganic nitrogen compounds (Vananuvat and Kinsella, 1975).

3.9.3 Chemical oxygen demand (COD)

The COD of the medium was determined in the supernatant liquor by the method of Porges et al. (1950). Dichromate

oxidizing agent was prepared by dissolving 2.5 g of potassium dichromate in a mixture of 500 ml each of concentrated sulfuric acid and 85% phosphoric acid. The solution was filtered through glass wool before use. Potassium iodide solution was prepared by dissolving 12.41 g in water and made up to 1 litre. Soluble strach solution was made according to the method described earlier.

A 50 ml of dichromate oxidizing solution was placed in a 500 ml-Erlenmeyer flask to which a 5 ml sample containing approximately 100 ppm. COD was added. The flask was placed on a hot plate with magnetic stirrer. The content was heated uniformly and when a temperature of 165° to 175° C was reached in 5 to 6 minutes, the flask was immediately removed from the heat, and cooled to room temperature (25° C) in a water bath. Ten ml of potassium iodide was added and the content was titrated immediately with 0.05 N thiosulfate solution, adding the starch solution near the end point. The color change was from dark blue to pale blue-green. A blank determination using 5 ml water was run at the same time. All determinations was made in duplicate. The chemical oxygen demand (COD), in ppm., is equal to 80 times the difference of thiosulfate solution (in ml) used by the water blank and sample :

$$\frac{D \times 0.05 \times 8 \times 100}{5} = D \times 80$$

In which D is the difference in reading of thiosulfate solution between the blank and the sample; 0.05 is the normality of the thiosulfate used; 8 is the milli-equivalent of oxygen; 5 is the volume of sample taken; and 1000 represents the conversion to one litre, giving mg/litre or ppm.