Chapter III

EXPERIMENT

3.1 Preparation of Pineapple Juice.

The pineapples without crowns and stems were washed in tap water to remove all dirts and contaminants. Then the peel and the core were taken off and washed again with tap water. The fresh meat was cut into small pieces and crushed in blender at high speed for 1 min. The juice was extracted from the pulp by squeezing in the cheese cloth. Sucrose, which used to fortified the juice was added and then the juice was suddenly pasteurized at 70 °C. for 10 min. Then let it become cool in the clean bottle which was washed 3 times with boiling water.

3.2 Method of Analysis.

Some properties of pineapple juice such as degree Brix, acidity, and pH had to be analysed in order to know how the juice should be adjusted to the optimum conditions or desirable levels. After the alcoholic fermentation, the fermented juice had to be analysed for the percent alcohol produced. Growth of the cultures were investigated by using Bausch & Lomb Spectronic 20 Spectrophotometor. Finally, the vinegar produced was determined for percent total acid, volatile acid, and non-volatile acid.

Fig. 1 Flow diagram of juice extraction

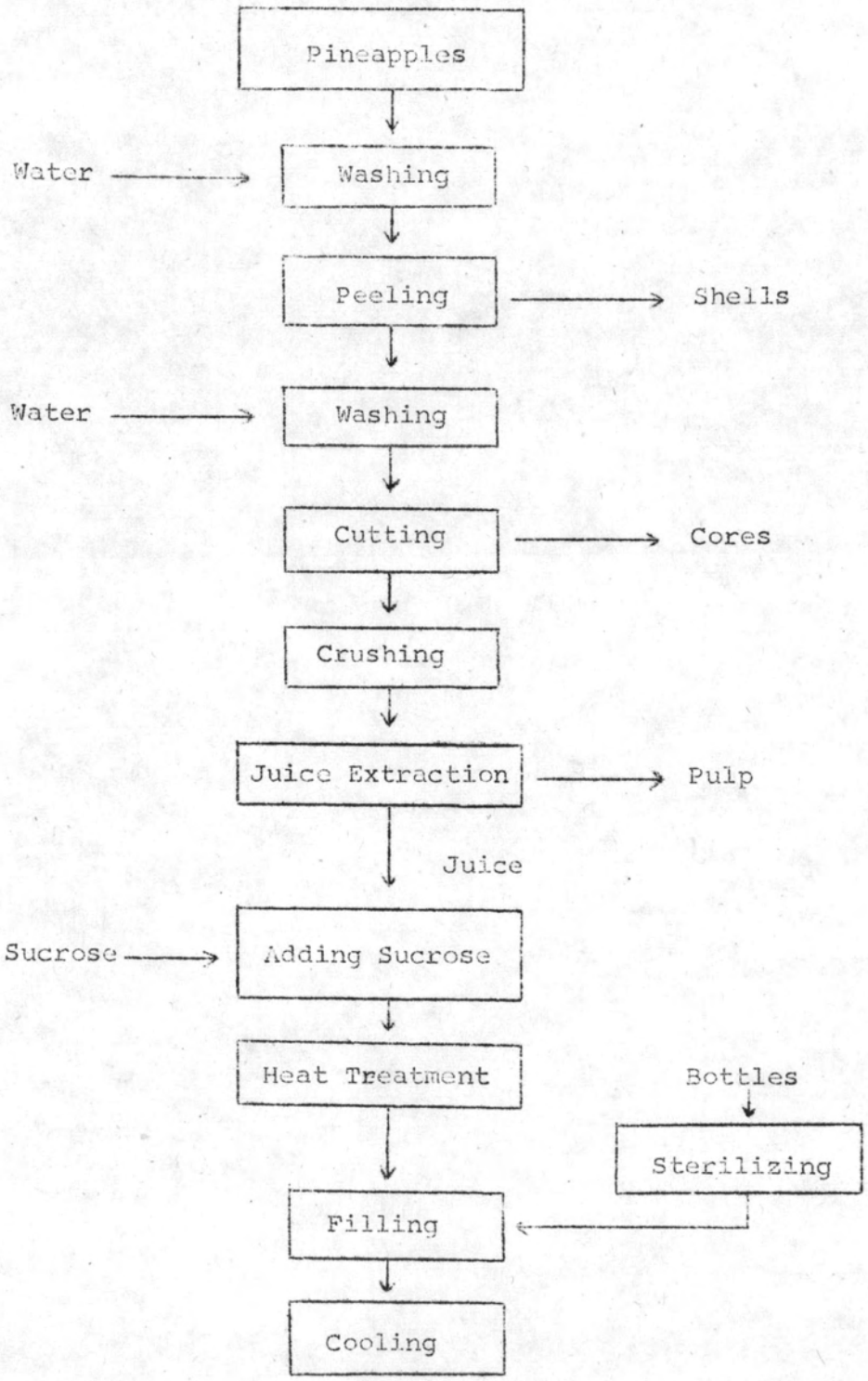
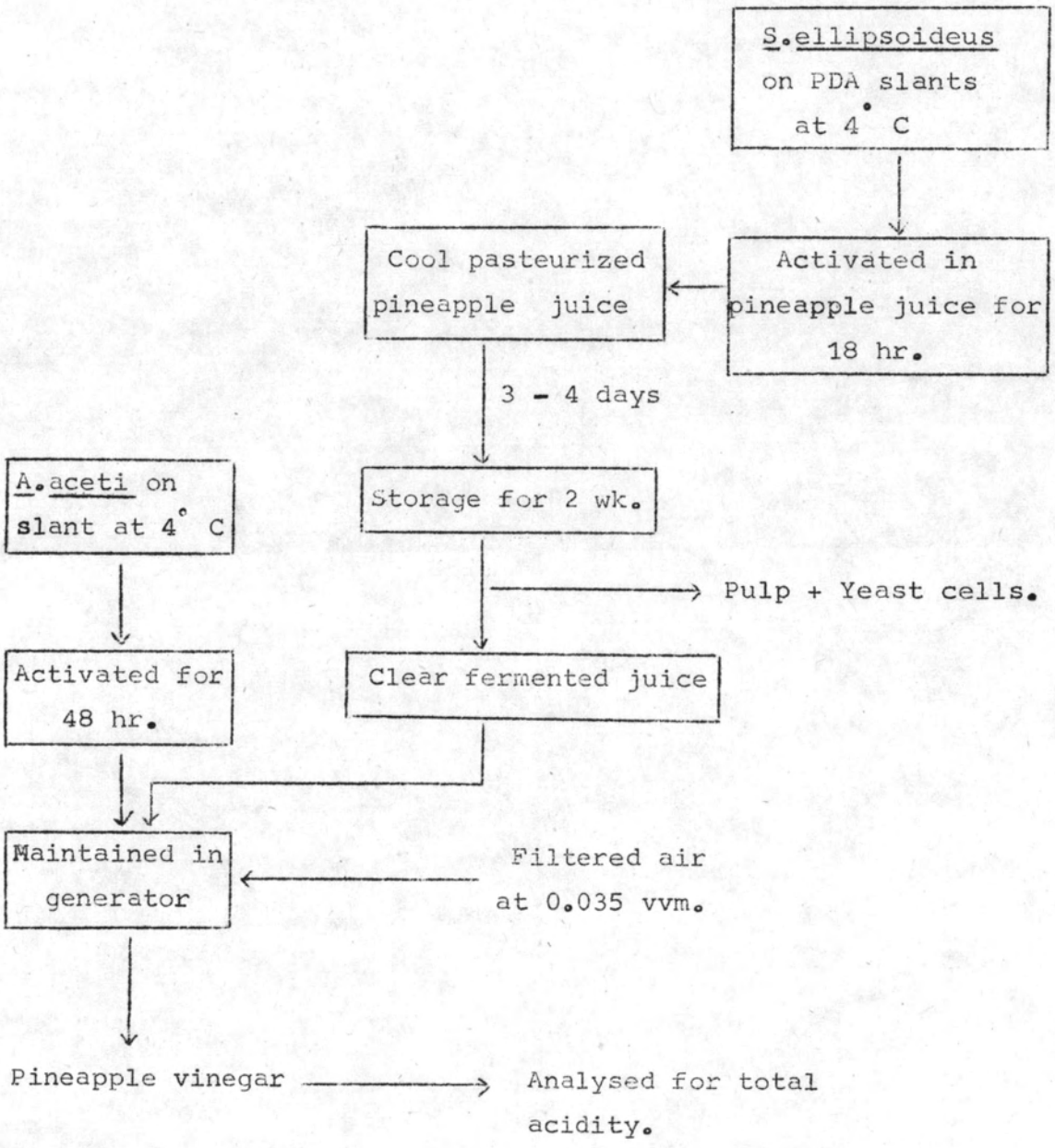


Fig. 2 Flow diagram of vinegar manufacture



3.2.1 Determination of total acidity.

The titration with 0.5 N. NaOH, having phenolphthalein as an indicator, was used.

10 ml of clear sample solution was pipetted into 250 ml flask, added 20 ml of distilled water and shaked it until the solution was well mixed. After adding 2 drops of phenophthalein indicator, the solution was titrated with 0.5 N. NaOH solution. The acidity was calculated from the following formulas (1):

For pineapple juice, 1 ml 0.5 N. NaOH = 0.09606 gm of citric acid. For vinegar, 1 ml 0.5 N. NaOH = 0.0300 gm of acetic acid.

3.2.2 Determination of total soluble solid ($^{\circ}$Brix)

The amount of total soluble solid of pineapple juice and fermented pineapple juice was directly determined by means of Abbé Refractometer.

3.2.3 Determination of pH

The pH meter with glass electrode, Electronic Instruments Limited, Serial No. 7010/582 was used for determining the pH value of juice and fermented product.

3.2.4 Determination of optical density.

Optical density of cell suspension was measured by using Bausch & Lomb Spectronic 20. Spectrophotometer at wavelength 500 nm which gave the maximum absorption value (Appendix A). Sterile pineapple juice was used as blank.

3.2.5 Determination of percent ethyl alcohol.

An aliquot (100 ml) of the clear supernatant of the fermented liquor was diluted with 50 ml, distilled water and distilled. About 90 - 95 ml distillate was collected and made up to 100 ml with distilled water. The alcohol content of the distillate was determined by Abbé refractometer.

The calibration curve of concentration of ethyl alcohol and refractive index was prepared and shown in Appendix B.

3.2.6 Non-volatile and volatile acids determination.

10 ml of vinegar was pipetted into 200 ml porcelain dish, evaporated just to dryness, added 10 ml of distilled water and evaporated again. The evaporation was repeated more than 5 times. The residue was dissolved in 200 ml distilled water. The solution was titrated with 0.5 N. NaOH with phenolphthalein as an indicator. Then the amount of non-volatile acid was calculated (1). (1 ml 0.5 N. NaOH = 0.0300 gm acetic acid or non-volatile acids).

The quantities of volatile acids were the difference between total acids (3.2.1) and non-volatile acid.

3.3 Alcoholic Fermentation.

The pineapple juice from 3.1 was used as raw material in alcoholic fermentation and some important factors affecting alcoholic fermentation were studied.

3.3.1 Preparation of culture.

The culture used in this study was Saccharomyces ellipsoideus. obtained from the Science Department, Ministry of Industry. The culture was kept at 4°C on (Difco) Potato Dextrose Agar slants, activated prior to use by passage 2 times on the same media of agar slants. Each time the slants were incubated at 25 - 30°C for 24 hr. Then it was transfered to a 500 ml shake flask which contained 300 ml sterile pineapple juice and incubated at 25° - 30°C for 18 hr. The speed of the shaking machine was 240 revolution per minute. This mother culture was, thereupon, used as an inoculum for the same medium under the same condition (at 25°- 30°C and pH 4.5), whose volume was 5% that of the total pineapple juice used. Each time the population of the inoculum at optical density value 2.4 was used by diluting it with sterile pineapple juice.

3.3.2 Determination the growth of yeast.

Inoculum of S. ellipsoideus was inoculated into a 500 ml flask containing 350 ml sterile pineapple juice. The mash of pineapple juice was adjusted to pH 4.5 by using 0.5% NaOH sterile solution and fortified with 0.5% dipotassium hydrogen phosphate. This mash was shaken through out this part of the experiment on a shaking machine at 240 revolution per minute.

The cell suspension was removed from the flask by aseptic technique at various intervals of time within 24 hours in order

to determine the number of cells by plate count method and also by measuring in term of optical density, 3.2.4.

The plate count method (32), 1 ml of cell suspension was diluted with 0.1% sterile peptone solution to 1:100, 1:10,000 and 1:1,000,000 dilution. After addition of 1 ml of each dilution to sterile plates, 15 - 20 ml of Potato Dextrose Agar medium was poured into each of them. The plates were gently rotated the distribution of cell suspension throughout the medium. After solidification, the plates were placed, inverted, in a incubator for 24 hours. Then number of colonies on plates which contained from 30 - 300 colonies were counted. The calculation of count was : number of colonies counted on plate X dilution of sample = number of cells per ml.

3.3.3 Effect of some nutrients.

Firstly, the pineapple juice was prepare to the desirable concentration of sugar, 22 ^{o} Brix, by adding some of sucrose as well as the pH of solution had to adjust to 4.5 by adding with dilute NaOH solution. Secondly, 300 ml of prepared pineapple juice was inoculated with 5% inoculum of yeast culture (3.3.1). Finally, 1% $(NH_4)_2$ HPO_4, 1% K_2HPO_4, and 0.5% $(NH_4)_2$ HPO_4 + 0.5% K_2HPO_4 were added into each flask. The fermentation was proceeded for 24 hours.

Each experiment was duplicated and the average value of alcohol was determined as in 3.2.5.

3.3.4 Effect of nutrient concentration.

In order to study the effect and to find a suitable concentration of nutrient on the alcoholic fermentation, the prepared solution from 3.3.3 was added with different concentration of K_2HPO_4. The concentration of K_2HPO_4 was varied from 0.25 to 2.5%

Each experiment was duplicated and the average value of alcohol after 24 hours under anaerobic fermentation was determined.

3.3.5 Effect of sugar concentration.

The way to study the effect of sugar concentration on alcoholic fermentation, the experiment was started with 300 ml of pineapple juice, 0.5% K_2HPO_4, 5% inoculum, and initial pH at 4.5. These mash were added with variety amount of sucrose as follows: 22.5, 30.0, 40.0, 47.5, 60.0, 80.0 and 85.0 gm respectively.

3.3.6 Change of pH, $^{\circ}$Brix and percent alcohol during fermentation.

20 litres of pineapple juice (3.1) in 25 litres glass bottle was fortified with 0.5% K_2HPO_4 and sucrose to give initial degree Brix of 19.6°. The pH of the juice was also adjusted to 4.5 and 5% inoculum (3.3.1) was used.

The pH value, $^{\circ}$Brix, and percent alcohol were measured at various time intervals within 7 days under anaerobic fermentation.

3.3.7 Storage

After 3 - 4 days, the change in pH value, degree Brix and percent alcohol were almost constant and these showed that the fermentation was nearly completed. The yeast cells and the pineapple pulp were allowed to settle for 2 - 3 weeks. Then the clear supernatant fermented juice was removed by siphon and pasteurized at 65 - 70 °C for 10 min. Pasteurized fermented juice was kept in bottles and stored at 4 °C.

3.4 Acetic Acid Fermentation.

A laboratory - scale generator was installed. Pure culture of Acetobacter aciti was used to convert the alcohol in fermented pineapple juice to acetic acid. The effects of number of recycling, of 0.5% K_2HPO_4 and 1% acetic acid, of air flow rates, and of fermented liquor flow rates were studied.

3.4.1 Preparation of culture.

The culture used in this fermentation was Acetobacter aceti obtained from Microbiology Section, Department of Biology, Kasetsart University. The culture was kept at 4 °C on acetobacter agar slant (40) of the following composition: yeast extract 5 gm, peptone 3 gm, agar 15 gm, distilled water 1,000 ml heated to 100 °C, adjusted pH to 4.5 and added mannitol 25 gm.

This culture was activated by passage 2 times on the same media of agar slants and incubated at 24 - 27 °C for 2 days. Then the culture was transfered to 1,000 ml flask which contained

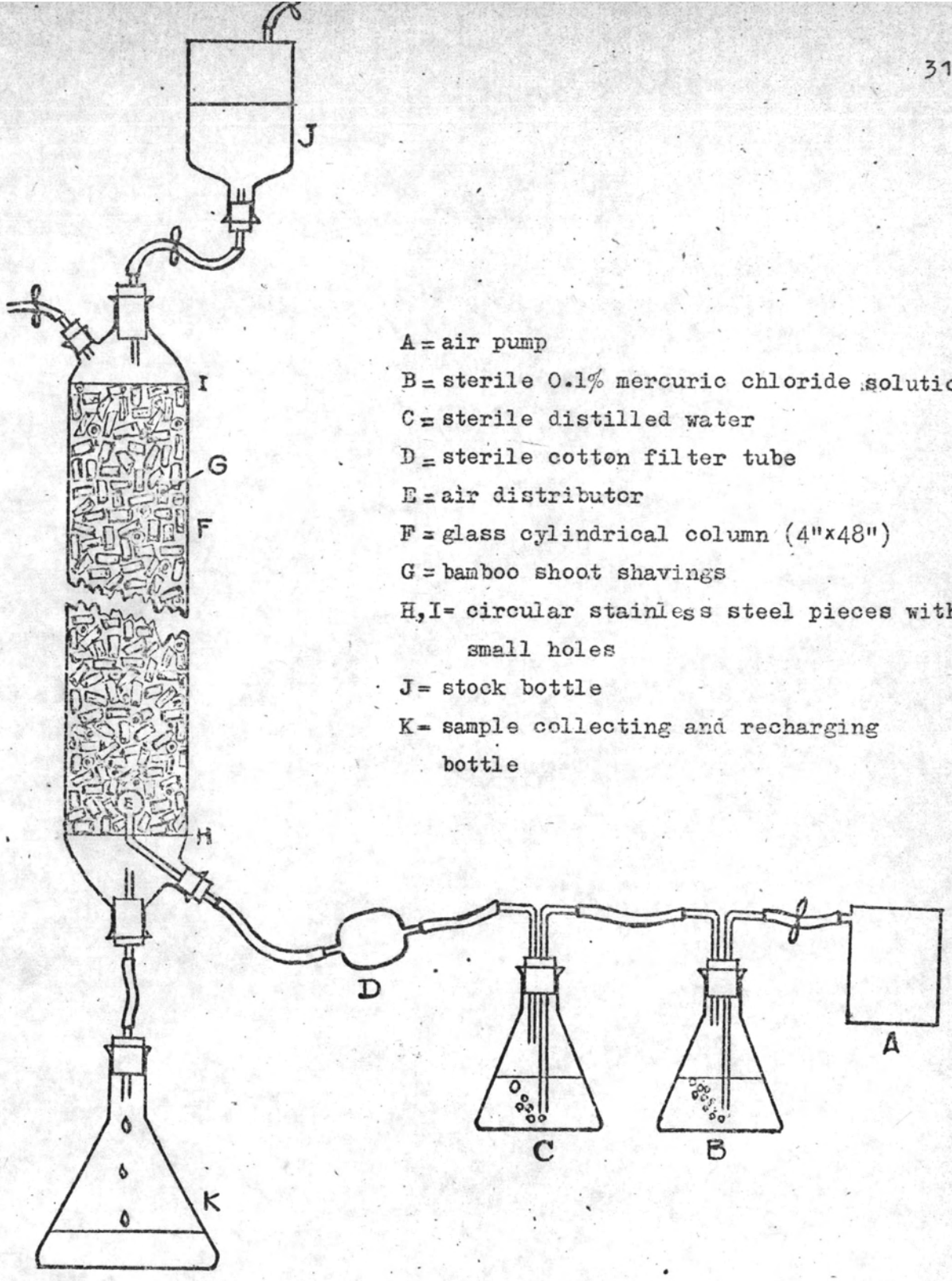
600 ml Acetobacter broth of the following composition : yeast extract 5 gm, peptone 3 gm, distilled water 1,000 ml and mannitol 25 gm (40). The broth was aerated all the time for 2 days by using filtered air from air pump. This culture was used for inoculation of the laboratory-scale generator column for acetic acid fermentation. Each time the population of the inoculum at optical density value 3.0 was used by adjusting with sterile Acetobacter broth.

3.4.2 Determination of the growth of A. aceti.

The active inoculum of A. aceti (3.4.1) was inoculated into 1 litre flask containing 600 ml acetobacter broth. The filtered air from air pump was introduced into the broth through out this step of experiment. The cell suspension was pipetted from the flask by aseptic technique at various time intervals within 2 days in order to determine the number of cells by plate count method (18) and also by measuring in term of optical density as described in 3.3.2. But the cell suspension in this step of experiment had to be diluted to $1:10^7$ dilution for plate count method.

3.4.3 Preparation of packing medium.

Packing medium or shaving in this work was peeled bamboo shoots of 1 - 1.5 cm. in diameter and 2.5 cm. in its length. These shavings were soaked in 5% hydrochloric acid for 72 hours in order to hydrolyze all starchy material. Then they were washed in running tap water before use. These shavings



A = air pump
B = sterile 0.1% mercuric chloride solution
C = sterile distilled water
D = sterile cotton filter tube
E = air distributor
F = glass cylindrical column (4"x48")
G = bamboo shoot shavings
H,I = circular stainless steel pieces with small holes
J = stock bottle
K = sample collecting and recharging bottle

Fig.3 Schematic diagram of the laboratory-type generator column.

were sterilized prior to the experiment by autoclaving for 20 min. at 121 °C and cooled.

3.4.4 Preparation of the generator and method of fermentation.

The generator, Figure 3, was a cylindrical column with 4 "in diameter and 48" in its length. The assembly consisted of an air inlet from air pump (A) passing through 0.1% mercuric chloride (B) to sterile the air and then through a sterile water bottle (C) (21). The flow rate of air was adjusted to 0.035 Vol. of air/Vol. of fermenter/min by a clamp on the rubber tubing connecting A and C flask. The air was then dried by passing through a sterile cotton filter tube (D) which was further connected to an air delivery tube (E) which having a bulb at its end containing small holes for uniform distribution of air through the body of the generator (F). The latter was made of a glass column, 4 inch in diameter and 48 inch in its length, packed loosely with sterile bamboo shoot shavings (G). The shavings were held in the column by a circular stainless-steel pieces (H, I), each having small holes (2/16 inch). (I) at the top was used for uniform distribution of the fermented liquor being trickled through the column supporting the shavings. The fermented liquor from the stock bottle (J) was trickled slowly at a controlled rate of 1,000 ml/12 hours by using a clamp. A 2 litres collecting flask (K) was connected to the column. The flask (K) could be disconnected for sampling and/or recycling from the stock bottle (J).

The shavings, rubber tubes, flasks, and 0.1% mercuric chloride solution were sterilized prior to the experiment by autoclaving for 25 min at 121 °C and cooled. The column was washed 3 times with 90% ethyl alcohol and 3 times with boiling water. The inoculation was accomplished by passing the culture (3.4.1) through the column several times. The fermented pineapple juice was then trickled through the generator. The rate of trickling of the fermented liquor from the stock bottle through the column was adjusted so that it required 12 hours for 1,000 ml of liquor to pass through the column (one cycle) and samples were obtained (after each cycle) for the determination of total acid (3.2.1). The air flow rate in this experiment was 0.035 Vol. of air/Vol. of fermenter/min (vvm).

3.4.5 Effect of recycling.

To study this effect, the procedure was followed as described in 3.4.4.

3.4.6 Effect of adding K_2HPO_4 in fermented liquor.

The fermented liquor was fortified with 0.5% K_2HPO_4 before pasteurization and the procedure was the same as described in 3.4.4.

3.4.7 Effect of adding K_2HPO_4 and acetic acid in fermented liquor.

The fermented liquor was fortified with 0.5% K_2HPO_4 before pasteurization and acetic acid after pasteurization to give 1.33% of initial acidity and the procedure was followed as described in 3.4.4.

3.4.8 Effect of air flow rates.

The air flow rate of 0.035 vvm, 0.053 vvm, and 0.106 vvm. were introduced to the generator in this experiment. The procedure was followed as described in 3.4.4.

3.4.9 Effect of fermented liquor flow rates.

In this experiment the flow rate of fermented liquor was varied from 1,000 ml/12 hr. to 1,500 ml/12 hr. in one cycle. The procedure was the same as described in 3.4.4.