

CHAPTER 3 MATERIALS AND METHODS

3.1 Raw materials

Waste dates of "socaria" type which has high sugar content were used in the production of bioethanol under laboratory conditions. Samples were collected from the open fruit market, Barjan, in central Jeddah, Saudi Arabia. The samples of rotten dates were washed under running tap water for five min to remove dust and reduce the number of contaminating microorganisms, particularly fungus which normally grow on the skin of the rotten fruits. After washing, the dates, seeds were separated from flesh.

3.2 Yeast

Yeast (*Saccharomyces cerevisiae* Type II) was used in these experiments from Sigma-Aldrich® Corporation. Properties of yeast are given in the Table 3.1. Before adding to the mashed dates for fermentation, the dried yeast was rehydrated to recover its activity and viability. Rehydration process was done by adding clean filtered tap water to the yeast at 37°C for 15 min. The rehydrated yeast must be used immediately after rehydration process. Yeast used only for flesh which contained only sugar.

Table 3.1: Properties of yeast.

Synonym	Bakers yeast
Type	Type II
Storage temperature	2-8°C
Caution	Only approximately 10% will autolyse in aqueous buffer at 37°C.
Preparation Note	Fast dried to yield 90% active, viable yeast in a convenient solid form.

Source: Sigma chemical company.

3.3 Enzymes

Cellulase and amylase enzymes were used during the experiment. Cellulase (*Aspergillus niger*) from BioChemika with Fluka No. 22180. known as 1,4-(1,3:1,4)- β -D-Glucan 4-glucanohydrolase. The Cellulase ($3\text{U}\cdot\text{mg}^{-1}$) and 1 U corresponds to the amount of enzymes which liberates 1 μmol of glucose from carboxymethyl cellulose per minute at pH 5.0 and 37°C. Based on the manufacturer's guidelines, the enzyme is shown to have an optimum temperature and pH of 40°C and 5.8, respectively. Properties of cellulase and amylase enzymes are given in the Table 3.2. Enzymes were used only for ground seed and seed with peel.

Table 3.2: Properties of cellulase and amylase enzymes.

PROPERTIES	CELLULASE	AMYLASE
Synonym	<i>1,4-(1,3:1,4)-α-D-Glucan 4-glucanohydrolase</i>	<i>1,4-α-D-glucan- glucanohydrolase</i>
CAS Number	9012-54-8	9001-19-8
Enzyme Commission (EC) Number	3.2.1.4	3.2.1.1
EC Number	232-734-4	232-588-1
MDL number	MFCD00081510	MFCD00081319
Product line	BioChemika	Sigma
Form	Powder	Powder
Color	off-white	beige
Storage temperature	2-8°C	2-8°C
Hazard Codes	Xn	X

Risk Statements	42	42
Safety Statements	22-24-36/37	22-24-36/37

Source: Sigma Chemical Company.

3.4 Experimental design

Experimental frame work of the present study is shown in Figure 3.1. As shown in Figure 3.1, production of bioethanol includes two main stages, before and after fermentation. The steps of “before fermentation” are sample collection and pretreatment. However, “after fermentation” processes are filtration, metal analysis and engine running.

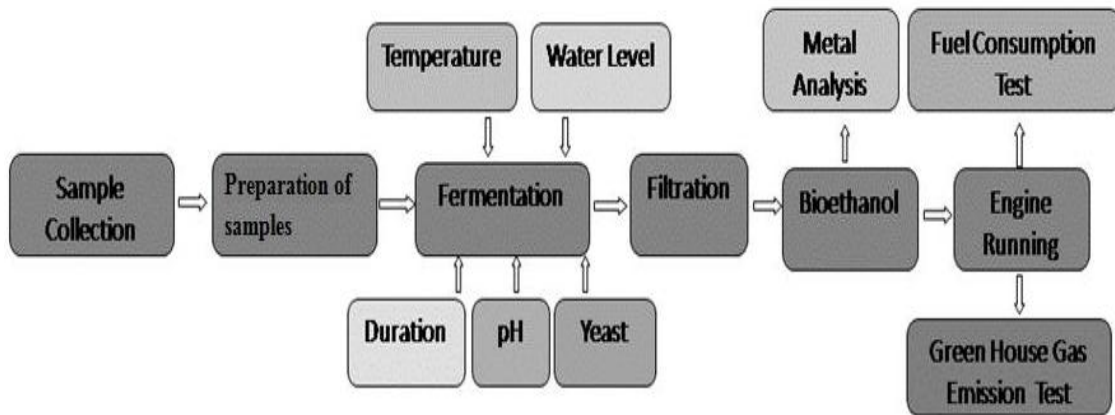


Figure 3.1: Diagram shows the experimental flow chart for production of bioethanol from date.

3.5 Preparation of samples

Sample of 1000 g of flesh (rotten seedless dates, (Figure 3.2a (2))) was ground using Hacte Canada blender until it became liquefied and divided into ten sterile labelled fermentation bottles (Figure 3.2b (2)). pH was adjusted to 5.8 . The working volume of the sample was 125ml (ratio of solid and liquid was 4:1).



Figure 3.2a : 1- dates (sokariah), 2- Seedless dates, 3- Seed covered by dates lift, 4- Mash seedless dates.

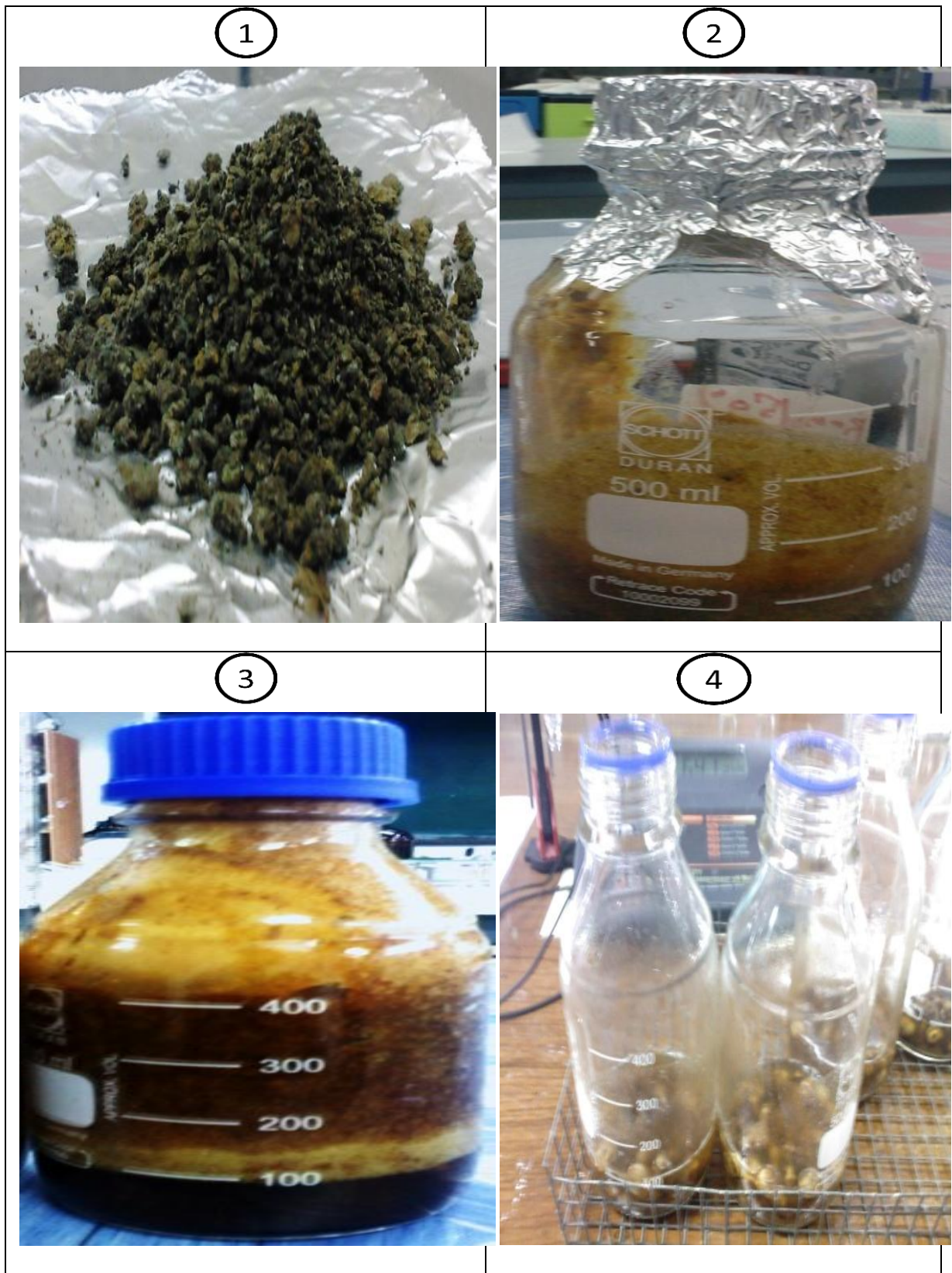


Figure 3.2b: 1- Crush seeds, 2- Sample before fermentation, 3- The sample after fermentation, 4- Seed result.

3.6 Fermentation

A 5 g/L (w/v) of active *Saccharomyces cerevisiae* was added to the bottles. The bottle was close tightly to create micro anaerobic condition. The bottles were then incubated at 28°C for three days. After the fermentation was completed, the product from fermentation was filtered using clean folded cheese cloth. The liquid product was used to measure the volume, pH and TSS while the solid was weighted. The similar method was used for the following experimental replications with different factors were conducted.

3.6.1 Effect of yeast concentration

While the selected physical parameters were kept constant in this experiment, the following of yeast concentration were used (2, 3, 5, 7 and 10 g/L). The effects were studied.

3.6.2 Effect of physical parameters on bioethanol productions from rotten dates

Different physical parameters were applied while the yeast concentration was kept constant.

3.6.2.1 Effect of fermentation time

In this experiment, the physical parameters except the time (water content, pH and temperature) and the concentration of yeast were fixed. The effects were studied. It has been applied the following times in the experiment (2, 3, 4, 5 and 6 days).

3.6.2.2 Effect of initial pH

Different pH was applied and its effects were studied in this experiment while the yeast concentration and the other physical parameters (water content, temperature and the

time) were kept constant of (60%, 28°C and 5 days) respectively. The different pH values were used (5, 5.8, 7 and 7.5).

3.6.2.3 Effect of temperature changing

The effect of different temperatures (28, 35 and 40) °C were investigated using 5g/l of Yeast, 60% of water content and pH 5.8 for 5 days fermentation period.

3.6.2.4 Effect of water content

The water contents of 20, 30, 40, 50, 60 and 80% were studied. The yeast concentration 5g/l, pH 5.8, temperature 28°C and the time were remained at 5 days.

3.6.3 Effect of Different date fruit parts

In this experiment, different parts of fruit flesh (without seed), crashed/ground seed and whole seed with peel were used. The temperature, pH, water content and the time were 28°C, 5.8, 60% and three days respectively. The enzymes were used only with crashed/ground seed and whole seed with peel while the yeast was used with flesh only.

3.7 Filtration

After 5 days, the samples were taken out from the incubator and filtrated by clean folded cheese cloth. The samples were filtrated by using filter paper (Whatman no. 1) and left the samples approximately about 2 h until there was nothing comes out from the samples. The raw bioethanol was distilled by vacuum evaporator to purify the bioethanol. The bioethanol yield was measured by using Atago Refractometer, Japan, shown in Figure 3.2c.

3.8 Analytical methods

Samples obtained from different fermentation process were analysed for the changes of pH, total soluble solids, bioethanol, glucose concentration and residue weight, viscosity and elemental analysis.

3.8.1 pH

The changes in the pH of all fermentations were determined (using pH meter model HANNA instruments). The pH was checked before and after fermentation.

3.8.2 Total Soluble Solids (TSS)

Total soluble solids (TSS) content of all fermentations were determined by using Atago digital refractometer (Tokyo, Japan), shown in Figure 3.2c (4b) with a scale ranging between 0 and 30 °Brix unit. The results were reported as degree Brix (°Brix). Total soluble solids content was checked before and after fermentation process.

3.8.3 Bioethanol concentration (Yield %)

Bioethanol was measured by using Atago Refractometer, Japan, shown in Figure 3.2c (4b).

0.05 ml of the yield was put in the sensor of Refractometer and the Bioethanol percentage was shown in the monitor.

3.8.4 Glucose estimation

Glucose content was determined according to the method of Miller (1959). 1% of dinitrosalicylic acid reagent solution was prepared by added 10 g of dinitrosalicylic (DNS) acid, 2 g of phenol, 0.5 g of sodium sulfite, 10 g of sodium hydroxide and mixed; followed by 1 litre of water and mixed well. 3 ml of DNS reagent was added to 3 ml of glucose sample in a lightly capped test tube. The mixture was then incubated in water bath for 5 to 15 min at 90 °C until the red-brown color appeared. Then, 1 ml of a 40% potassium sodium tartrate (Rochelle salt) solution was added to stabilize the color. The absorbance values of the reducing sugar was measured using spectrophotometer at 575 nm after cooling to room temperature in a cold water bath.

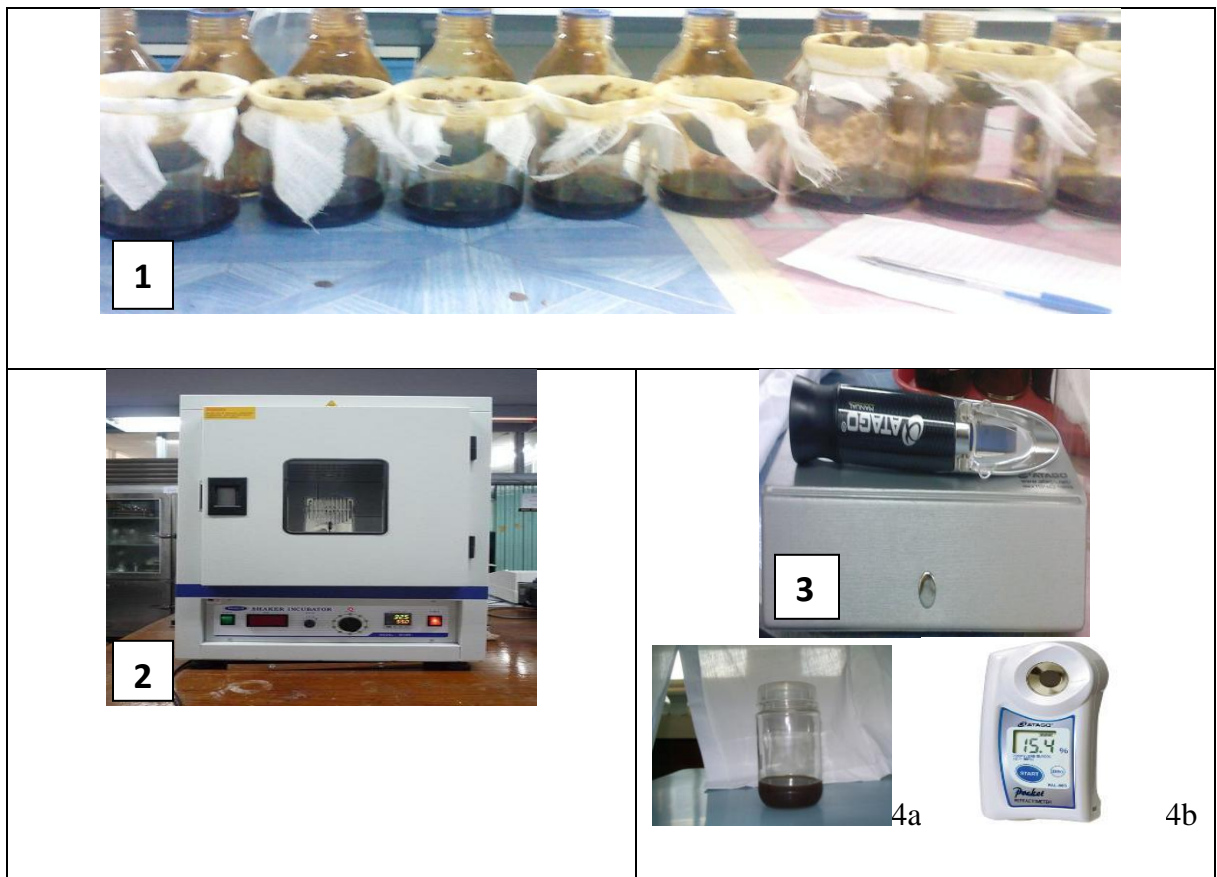


Figure 3.2c: 1) The filtration process, 2) Incubator for fermentation process and bioethanol, 3) The Total Soluble Solid (TSS) device and 4a) Bioethanol and 4b) refractometer ethyl alcohol device, Atago, Japan.

3.8.5 Viscosity analysis

Viscosity analysis of sample fermented at different temperatures was measured at the Faculty of Engineering, University of Malaya. For viscosity test, the samples were put in the beaker and heated up at 40°C and then measured using viscometer. The viscometer was set at 30 rpm (with spindle size of 63). The results were recorded.

3.8.6 Elemental Analysis

Bioethanol from the fermentation of the rotten dates at different pH of rotten dates and different concentrations of yeast was analyzed by using Multi-element Oil Analysis (MOA) Spectrometry at Tribology Laboratory, Faculty of Engineering, University of Malaya, Malaysia. 5 ml of each sample were used for analysis. Data were displayed in part per million (ppm).

3.8.7 Engine test

Three types of blending fuels were used in this study. The first one was pure petrol fuel (called E0). The second one was bioethanol blended with petrol fuel containing 5% bioethanol (E5) and the third one was bioethanol blended with petrol fuel containing 10% bioethanol (E10) [For engine starting, (Gen 2 car engine) 2 litres petrol (synergy, 95), were needed. For E5, it was produced 100 ml and for E10, it was produced 200ml bioethanol]. The multi cylinder hydra spark ignition engine with injection system was used. The tests were performed at 2000 rpm and the test fuels were gasoline (E0) and gasoline ethanol blends E5 and E10, the numbers following E indicate percentage of volumetric amount of ethanol. Fuel consumption was measured using Ohaus GT 8000 model (Gen 2 proton, made by Malaysia) and exhaust emissions were measured using Sun MGA 1200 model emission tester.

3.8.8 Statistical analysis

Statistical analysis was completed by SPSS software. Significant difference among the treatments was evaluated by one way ANOVA and Duncan's multiple range test (DMRT) at $p \leq 0.05$ was calculated using the error mean squares. Flow diagram of bioethanol production process to engine test is shown in Figure 3.1.