CHAPTER 3: MATERIALS AND METHODS

3.1 MATERIALS

Klebsiella oxytoca NRRL B-199 obtained from Northern Regional Research Laboratory, Peoria, Illinois, USA was used as the microorganism for the production of 2-3 Butanediol from blackstrap molasses. *Klebsiella oxytoca* was maintained on standard nutrient agar (Sigma Chemicals, USA) slants. For the shake flask culture, 200 ml of sterile medium was inoculated from an agar-agar slant. This was incubated in a rotary shaker for 36 hours at 37°C.

Blackstrap molasses was obtained from Liqueur Agency Sdn. Bhd, Selangor, Malaysia. Glucose was obtained from E. Merck, Germany and all the chemicals used for media preparation were supplied by BDH, UK.

3.1.1 Strain

The strain used was *Klebsiella oxytoca*, formerly known as *Aerobacter aerogenes* or *Klebsiella pneumoniae* (NRRL B 199). *Klebsiella oxytoca* was maintained on nutrient agar slants. The slants were stored at 4 °C. The stock culture was subcultured every 2-3 weeks to maintain the potency and metabolic activity of the culture.

3.1.2 Description of fermenter

All experiments were carried out in a 5L Biostat B Fermenter (B. Braun, Germany) with an initial culture volume of 3.5 L and the temperature was maintained at 37^{0}C by thermostatic control. The pH was maintained at 5.8 by automatic addition of 2N NaOH and 1N HCl. Sterile air was supplied continuously at a rate of 1.8 v.v.m. throughout the fermentation run. The dissolved oxygen (DO) was monitored by 'Ingold' oxygen probe. Foaming was controlled by automatic addition of a silicone based antifoam. The stirrer speed was kept constant at 400 rpm. The substrate was saturated with oxygen at 400 rpm by passing sterile air prior to inoculum addition. Fig. 3.1 shows the photograph of the bioreactor.

The blackstrap molasses contained 847 g/L of sugars and was diluted with ultrapure water to the desired concentration for each run. The fermentation runs were carried out with molasses having initial sugar concentrations of 25.0 g/L, 50.0 g/L, 75.0 g/L and 100 g/L respectively. The ammonium salts were prepared separately in desired concentrations and sterilised. It was added to the molasses in the bioreactor by pumping in before innoculation.

Fig. 3.1 Experimental Set-up for Fermentation

3.2 METHODS

3.2.1 Formation of Agar Slant

Klebsiella oxytoca was maintained on nutrient agar of the following composition, supplied by Unipath Ltd., Hampshire, England.

Composition	(g/L)
Lab-Lemco powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
Agar	15.0
pH	7.4 $\pm$ 0.2

28.0 g of nutrient agar was suspended in 1000 mL of distilled water. The suspension was brought to boiling temperature to dissolve the agar completely. After that 5 mL of agar solution was poured into small bottles of 20-mL capacity. The bottles with nutrient agar solution were sterilised by autoclaving at 121°C for 15 minutes. Finally when the agar was still in a liquid state these bottles were put in a slanting position so that the agar would solidify and form a slant.

3.2.2 Culture medium

The composition of the culture medium was as follows (Pirt and Callow, 1958). All the chemicals were of analar grade. The quantity of the various ingredients used in the culture media and their source are listed in Table 3.1.

Table 3.1: Composition of the culture medium

Glucose	50.00 g/L
EDTA (disodium salt)	0.51 g/L
Potassium hydroxide	0.45 g/L
Ammonium sulphate, $(NH_4)_2SO_4$	7.20 g/L
Diammonium hydrogen phosphate	6.00 g/L
Magnesium sulphate, $MgSO_4.7H_2O$	0.30 g/L
Calcium chloride, $CaCl_2$	0.09 g/L
Ferrous sulphate, $FeSO_4.7H_2O$	0.0225 g/L
Zinc sulphate, $ZnSO_4.7H_2O$	0.0375 g/L
Manganese sulphate	0.075 g/L

The medium was prepared and autoclaved in two separate parts: a concentrated solution consisting of ammonium salts, while the rest of the media along with sugar, trace metals and yeast extract in the other part. This was done to prevent caramelising of sugars. After autoclaving, these components were mixed together in sterile conditions to give the concentrations listed above.

3.2.3 Inoculum

From the agar slant, one loop of culture was transferred into a 500-ml shake flask containing 50.0 $g.L^{-1}$ of sugar in 200 ml of the above medium. The shake flask was incubated in a rotary shaker at 37^0 C for 36h with a shaking speed of 200 rpm.

After 36 hrs the nutrient media turned turbid indicating the growth of the microorganism. Then 5 ml of the media was transferred to production shake flasks.

3.2.4 Shake Flask Study

Three sets of shake flasks of 500 mL were used in this experiment. The nutrient media were different for the three sets of flasks. One contained 100 $g.L^{-1}$ glucose, and the others contained 100 $g.L^{-1}$ sucrose and molasses having 100 $g.L^{-1}$ of total sugar. The molasses, which was used in this experiment, contained 847 g of total sugar per 1000 mL. In order to get 100 $g.L^{-1}$ of total sugar in the solution, 11.8 ml of molasses was taken into a flask. Other

trace metals (Fe, Zn, Ca, Mg etc.) and ammonium salts were added to the molasses.

The flasks containing glucose, sucrose and molasses solution were sterilised in an autoclave at 121^0C for 15 min. The ammonium salts solutions were autoclaved separately. The flasks were kept under ultra violet radiation for 24 hrs. Before innoculation, the ammonium salt solution was added to the nutrient media solution containing sugars and other additives. The flasks were innoculated with 5ml of the innoculum by a sterile pippet. The shake flasks were incubated in a rotary shaker at 37^0C with a shaking speed of 200rpm. Samples were drawn at 16 hrs, 32 hrs, 48 hrs, 72 hrs, and 96 hrs respectively. The samples were analysed for biomass, sugar and products i.e., 2,3-butanediol and acetoin.

3.2.5 Total sugar estimation (by phenol-sulphuric acid method):

Reagents:

5% (w/v) aqueous solution of phenol (stored at 4°C)

98% sulphuric acid

Procedure:

Suitably diluted sugar solution (1.0 mL) is pipetted out directly into a test tube. Phenol reagent (1.0 mL) is added in the same way followed by 5 mL of concentrated H_2SO_4, which was pipetted directly onto the sugar solution. The solution was mixed immediately and allowed to cool before the absorbance was read at 490 nm by a UV-Spectrophotometer (Shimadzu, Japan). The absorbance values (after subtraction of reagent blanks) were then translated into glucose equivalent using a standard graph obtained by plotting glucose against absorbance (Fig. 3.2)

3.2.6 Biomass determination

For determination of biomass, 10 mL of fermentation sample was drawn and 1 drop of 10% formaldehyde solution was added to stop cell growth. The cells were centrifuged at 5000 r.p.m. and the supernatant was discarded. The cell mass deposited at the bottom was again centrifuged at 5000 r.p.m. with 10% w/v NaCl solution. The supernatant was again discarded and the cell mass deposited at the bottom was put in previously weighed aluminium cups and dried at 60°C for 24 hrs. The difference in weight was taken as the weight of biomass.

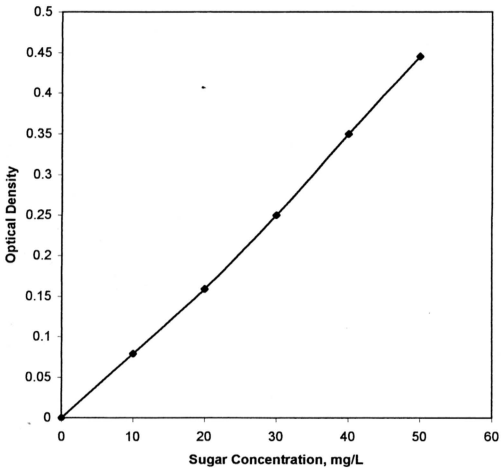

Fig. 3.2 Sugar Concentration vs. Optical Density

3.2.7 Determination of 2,3-butanediol and acetoin

The butanediol and acetoin were determined by a Gas Chromotograph (Varian 3400). A C3-C5 capillary column was used for this purpose and the following conditions were maintained.

Injector temperature	200°C
Detector temperature	220°C
Initial temperature	100°C
Final temperature	170°C
Rate of temperature rise	8°C/minute
Detector	Flame ionisation type
Gases used	Nitrogen, compressed air and hydrogen
Injection mode	Split

Fig. 3.3 and 3.4 show standard chromotograms for 1 μL sample of 1% w/v and 1.5% w/v 2,3-butanediol in ultrapure water respectively. In both the cases there are two peaks, which indicate the presence of two isomers of 2,3-butanediol. Fig. 3.5 shows the standard plot of 2,3-butanediol for 1 μL of sample in the concentration range of 0.1% w/v and 2.0% w/v. The plot area and the concentration shows a linear relationship with r = 0.9973, which indicates excellent linear relationship in this range. Standard chromatogram in Figs. 3.6 and 3.7 indicate acetoin concentration of 0.1% w/v and 0.5% w/v respectively. The standard plot of acetoin is shown in Fig. 3.8. In the concentration range of 0.01% w/v to 0.5% w/v, the plot area vs. concentration of acetoin shows a

VARIAN 3400 GAS CHROMATOGRAPH
METHOD 1 RUN 212
TIME 23:54 21 AUG 99
SAMPLE:
RUN MODE: ANALYSIS
CALCULATION TYPE: PERCENT

PEAK NO.	PEAK NAME	TIME MIN	RESULT	AREA COUNTS
1		2.365	22.5470	112473
2		2.702	77.4529	386363
TOTALS:			100.0000	498836

DETECTED PEAKS: 29 REJECTED PEAKS: 27
AMOUNT STANDARD: 1.0000000
MULTIPLIER: 1.0000000 DIVISOR: 1.0000000
NOISE: 20.2 OFFSET: 30

ERROR LOG:
ANNOTATION OMITTED

Fig. 3.3 Peak area in GC Vs. 1% 2,3-Butanediol .

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VARIAN 3400 GAS CHROMATOGRAPH
METHOD 1                    RUN 211
TIME 23:43                  21 AUG 99
SAMPLE:
RUN MODE: ANALYSIS
CALCULATION TYPE: PERCENT

PEAK  PEAK      TIME      RESULT       AREA
NO.   NAME       MIN                  COUNTS
  1             2.359     22.0056      167185
  2             2.711     77.9943      592553
-------------------------------------------------
   TOTALS:                100.0000     759738

DETECTED PEAKS:   55   REJECTED PEAKS: 53
AMOUNT STANDARD: 1.0000000
MULTIPLIER: 1.0000000  DIVISOR: 1.0000000
NOISE:   20.2          OFFSET: 32
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Fig 3.4 Peak area in GC Vs. 1.5 % 2,3 – Butanediol .

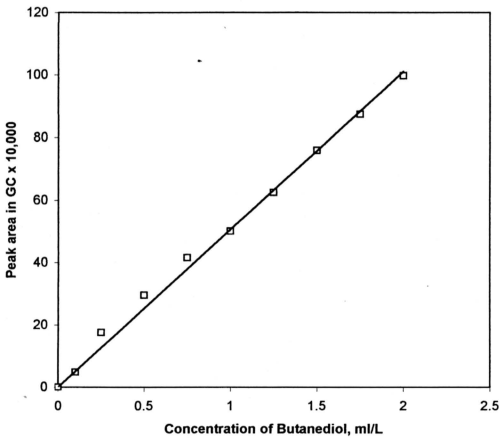

Fig 3.5 Peak area in GC vs. 2,3-Butanediol Concentration

0.1%.

VARIAN 3400 GAS CHROMATOGRAPH
METHOD 1 RUN 173
TIME 20:54 14 JAN 99
SAMPLE:
RUN MODE: ANALYSIS
CALCULATION TYPE: PERCENT

PEAK NO.	PEAK NAME	TIME MIN	RESULT	AREA COUNTS
1		1.911	36.6824	27331
2		1.936	63.3175	47176
TOTALS:			100.0000	74507

DETECTED PEAKS: 2 REJECTED PEAKS: 0
AMOUNT STANDARD: 1.0000000
MULTIPLIER: 1.0000000 BIVISOR: 1.0000000
NOISE: 27.0 OFFSET: 32

Fig. 3.6 Peak area in GC Vs. 1% Acetoin

VARIAN 3400 GAS CHROMATOGRAPH
METHOD 1 RUN 185
TIME 11:50 21 AUG 99
SAMPLE:
RUN MODE: ANALYSIS
CALCULATION TYPE: PERCENT

0.5 %.
i.e 5gm/l

PEAK NO.	PEAK NAME	TIME MIN	RESULT	AREA COUNTS
1		0.878	6.5861	25733
2		1.046	7.5693	29575
3		1.545	68.2238	266567
4		1.773	17.6206	68848
TOTALS:			100.0000	390724

DETECTED PEAKS: 35 REJECTED PEAKS: 31
AMOUNT STANDARD: 1.0000000
MULTIPLIER: 1.0000000 DIVISOR: 1.0000000
NOISE: 20.2 OFFSET: 40

Fig 3.7 Peak area in GC Vs. 1.5 % Acetoin

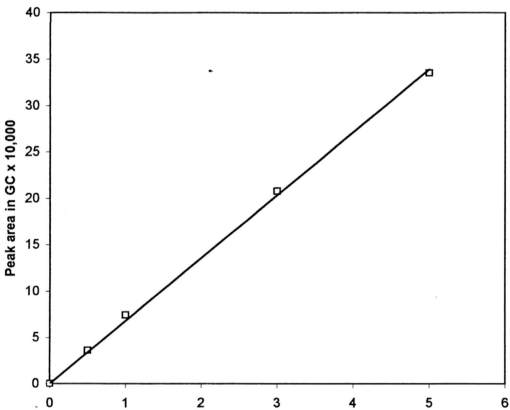

Fig. 3.8 Peak area in GC vs. Acetoin Concentration

linear relationship having r=0.9995, which indicates almost perfect linear relationship in this range.