Chapter 4: RESULTS AND DISCUSSION

4.1 Shake flask studies

Shake flask studies were conducted to assess the suitability of molasses as a substrate for fermentation of 2,3-butanediol. Molasses, diluted to a total reducing sugar content of 100 g/L was used for the studies. For comparison purposes, same concentration of glucose and sucrose (100 g/L) were fermented separately. In all cases the conditions were as follows.

Table 4.1: Conditions of shake flask culture

Temperature	37°C
pH	5.8
Quantity of media	100 mL
Flask type	Conical, 500 mL
Shaking speed	200 rpm

All the experiments were carried out in triplicate and the mean values were calculated. The composition of the media is given in Table 3.1. The run time was 96 h in all cases and the samples were drawn at 16, 36, 48, 72 and 96 hrs. The samples were analysed for sugar content, biomass and product (acetoin and 2,3-butanediol). The method of analysis is described in Chapter 3.

Figs. 4.1-4.3 indicate the biomass, product and substrate concentrations with respect to time. The product consisted of acetoin and 2,3-butanediol and the substrates were glucose, sucrose and molasses as indicated in Fig. 4.1, 4.2 and

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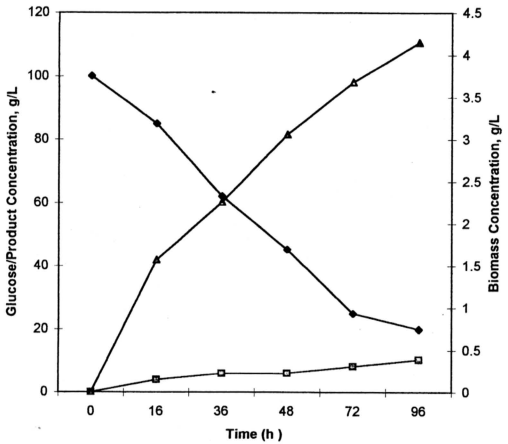

Fig. 4.1 2,3- Butanediol fermentation in shake flask with glucose as the carbon source

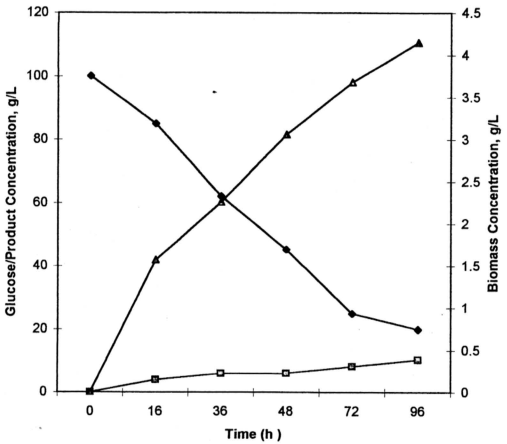

Fig. 4.1 2,3- Butanediol fermentation in shake flask with glucose as the carbon source

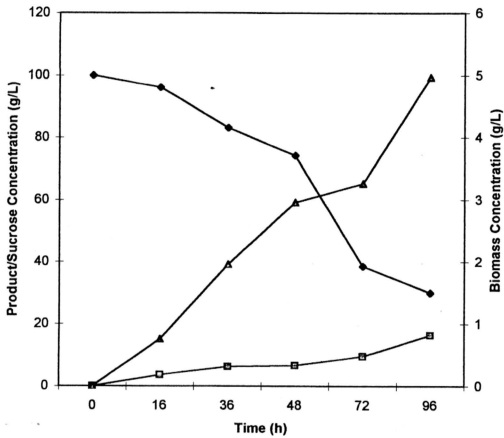

Fig. 4.2 2,3-Butanediol fermentation in shake flask with sucrose as the carbon source

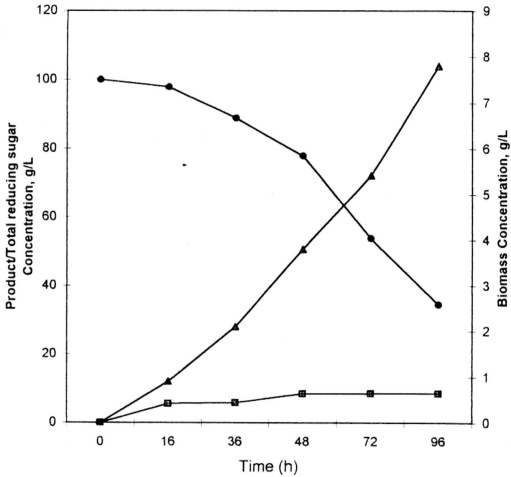

Fig. 4.3 2,3-Butanediol fermentation in shake flask with total reducing sugar in molasses as the carbon source

4.3 respectively. In all cases, the substrate concentration dropped steadily with respect to time. However, the fall in glucose concentration was almost linear with time while sucrose and molasses concentrations dropped slowly in the first 16 hrs. and more rapidly in the remaining period of time. The total product formation was maximum for sucrose, followed by that of glucose and molasses. The maximum product concentrations were 16.4 g/L, 10.4 g/L and 8.5 g/L respectively for sucrose, glucose and blackstrap molasses. The biomass concentration for molasses was 7.8 g/L, while those of glucose and sucrose were 4.15 g/L and 4.97 g/L respectively. Considering the fact that blackstrap molasses is not a pure carbon source and is a mixture of various sugars, carbohydrates and impurities, the shake flask yield is reasonable and further studies in fermenter can be undertaken.

4.2 Fermenter runs

The fermentation experiments were carried out with different molasses concentration in a 5-litre Biostat-B (B. Braun, Germany) fitted with temperature, pH, dissolved oxygen (DO) probes and stirrer speed controllers. The blackstrap molasses was diluted with ultrapure water to achieve the desired sugar concentration. For all the fermenter runs, the culture was grown at 50 g/L of glucose (as in Table 3.1). The initial sugar concentrations in four different runs were 25.0, 50.0, 75.0 and 100.0 g/L respectively. The residual sugar and product concentrations were determined as described in Chapter 3. All fermentation

studies were carried out at 37°C and pH 5.8 by addition of 2N HCl or 2N NaOH. In this work, sterile air supply and stirrer speed were maintained at 1.8 litres per minute and 400 rpm respectively. Dissolved oxygen (DO) was monitored using an oxygen probe. Figures 4.4-4.12 indicate the sugar, biomass, DO and product concentration with respect to time for various initial sugar concentrations. The representative nature of substrate utilization, product (butanediol and acetoin) and cell mass formation, and dissolved oxygen with respect to time are shown in Figs. (4.8–4.10) for an initial sugar concentration of 75.0 g/L. It can be seen from Fig. 4.9 that the organism started growing approximately after 8 h of lag phase. This lag phase is due to the change in the nature of the carbon source from shake flask to production medium. In shake flask the organism was grown in glucose but in production medium, blackstrap molasses was used as the carbon source. Since molasses contains sucrose, the cells needed to produce invertase, which can break sucrose to glucose and fructose for cells to metabolise. This adaptation period was reflected as the lag phase. For an initial sugar concentration of 75.0 g/L, DO started dropping sharply after 8 h as microorganisms entered the growth phase and exerted a high demand for oxygen for their respiration and growth. This is evident from Fig. 4.9, which shows the growth in biomass and the corresponding change in DO with time. In this case, the DO dropped to 0 after 16 h. Near the end of fermentation, DO increased sharply as the sugar concentration depleted to less than 10% of the

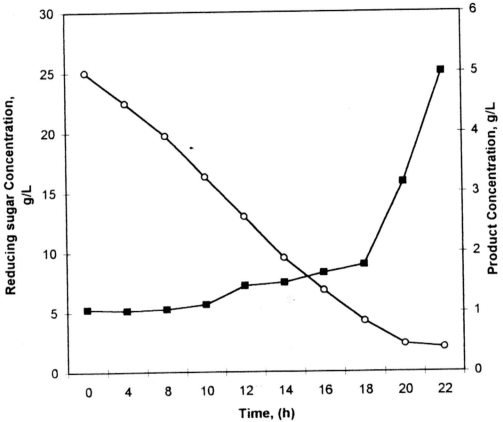

Fig. 4.4 Sugar and Product concentration versus Time

2,3-Butanediol fermentation in a 5-L fermenter with molasses as the carbon source. Conditions: pH 5.8; Temp 37^0C;
Initial total sugar concentration, 25 g/L

—○— sugar —■— product

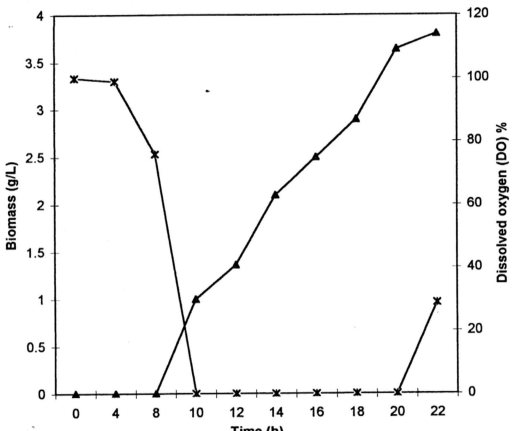

Fig. 4.5 Biomass and Dissolved Oxygen (DO) % versus time

2,3-Butanediol fermentation in a 5-L fermenter with molasses as the carbon source. Conditions: pH 5.8; Temp 370C; Initial total sugar concentration. 25 g/L

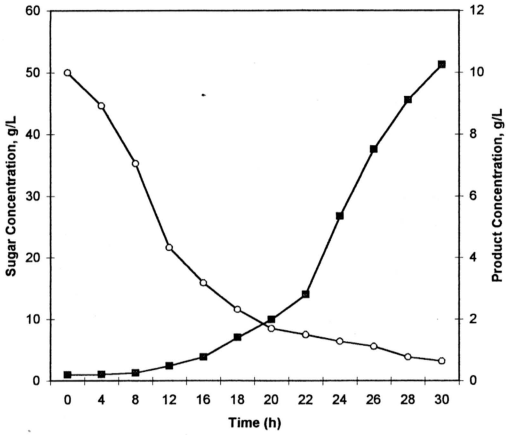

Fig. 4.6 Sugar and Product concentration versus Time

2,3-Butanediol fermentation in a 5-L fermenter with molasses as the carbon source. Conditions: pH 5.8; Temp. 37^{0}C; Initial sugar concentration 50 g/L

—○— sugar —■— product

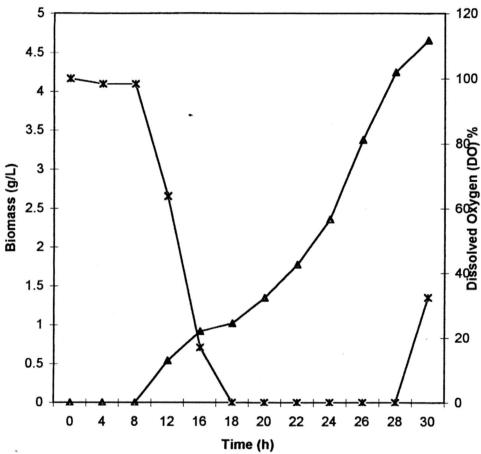

Fig. 4.7 Biomass and Dissolved Oxygen (DO) versus Time

2,3-Butanediol fermentation in a 5-L fermenter with molasses as the carbon source. Conditions: pH 5.8; Temp 37^{0}C; Initial sugar conc. 50 g/L

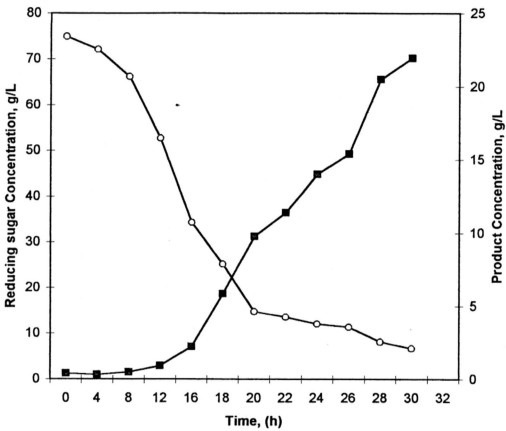

Fig. 4.8 Sugar and Product Concentration versus Time

2,3-Butanediol fermentation in a 5-L fermenter with molasses as the carbon source. Conditions: pH 5.8; Temp 37°C; Initial sugar concentration, 75.0 g/L

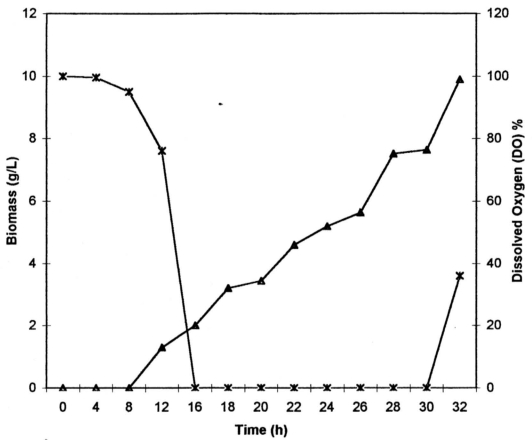

Fig. 4.9 Biomass and Dissolved Oxygen versus Time

2,3-Butanediol fermentation in a 5-L fermenter with molasses as the carbon source. Conditions : pH 5.8; Temp 37°C;
Initial sugar concentration 75.0 g/L
—▲— biomass —✱— DO %

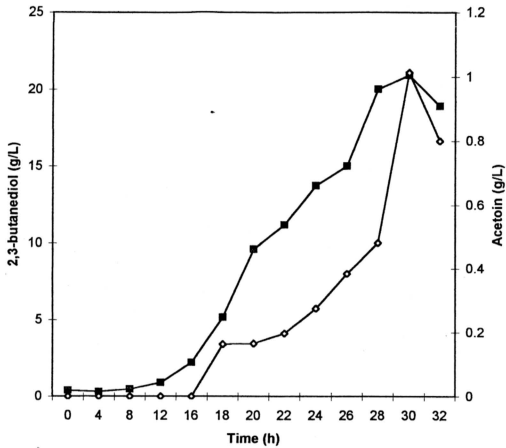

Fig. 4.10 2,3-butanediol and acetoin versus time

2,3-Butanediol fermentation in a 5-L fermenter with molasses as the carbon source. Conditions: pH 5.8; Temp 37°C; Initial sugar conc. 75 gm/L

—■— 2,3-butanediol —◇— Acetoin

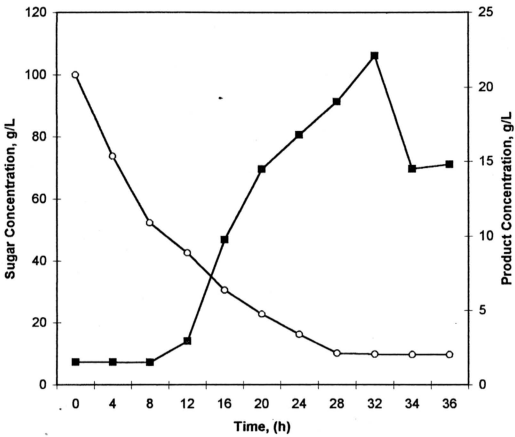

Fig. 4.11 Sugar and Product Concentration versus Time

2,3-Butanediol fermentation in a 5-L fermenter with molasses as the carbon source.Conditions: pH 5.8; Temp 37°C;Initial sugar concentration, 100.0 g/L

—○— sugar —■— product

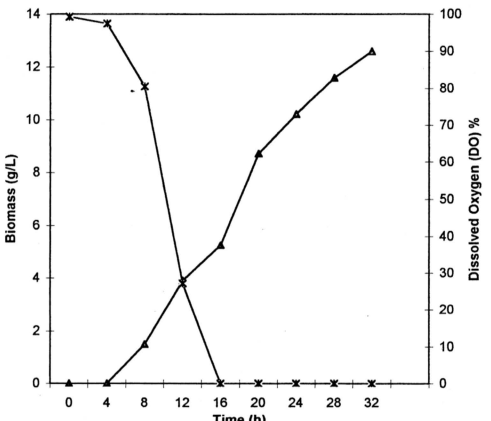

Fig. 4.12 Biomass and Dissolved Oxygen versus Time

2,3-Butanediol fermentation in a 5-L fermenter with molasses as the carbon source. Conditions: pH 5.8; Temp 37^{0}C; Initial sugar concentration 100 g/L

initial value, indicating the onset of the death phase of the microorganisms. The product concentration increased concurrently with the increase in biomass concentration between 12 h and 30 h as shown in Fig. 4.8. The product concentration, however, declined after 30 h due to rise in DO and oxidation of the product (Fig. 4.9). Butanediol is formed by reduction of acetoin. In this case, the product comprises about 95% butanediol and 5% acetoin as shown in Fig. 4.10. A similar trend was observed in case of initial sugar concentration of 50.0 and 100.0 g/L as shown in Figs. 4.7 and 4.12. In these cases, DO fell sharply after 8 h and took nearly 8 more hours to attain zero value. However, the DO dropped to zero in 10 h for the initial sugar of 25.0 g/L.

This indicates that a longer period of acclimatisation and slower growth of microorganism occurred when an initial sugar concentration is more than 25g/L. A zero value of DO also indicates the onset of the growth phase. This growth behaviour is shown in Figs. 4.5, 4.7, 4.9 and 4.11. The cell concentration increased linearly because oxygen was limiting during this period. The DO started increasing after 20 h for an initial sugar concentration of 25.0 g/L while it took 28h, 30h and 32 h respectively for sugar concentrations of 50.0, 75.0 and 100.0 g/L. The rise in DO reflected the initiation of death phase of the microorganism and consequently a fall in oxygen consumption. This also marked the onset of product oxidation due to high DO.

The runs were terminated at this stage to ensure maximum yield. In all cases, appreciable product synthesis started after 16 h. This period lasted until 22 h., 30 h., 30 h. and 32 h. respectively for initial sugar concentrations of 25.0, 50.0, 75.0 and 100.0 g/L as shown in Figs. 4.4, 4.6, 4.8 and 4.11. For initial sugar concentrations of 75.0 and 100.0 g/L, the product concentration fell after 30 h. and 32 h. respectively as shown in Figs. 4.8 and 4.11. The oxidation of the products was 17 w/w% and 37 w/w% respectively for initial sugar concentrations of 75.0 and 100.0 g/L. From the growth and product formation profiles, it can be seen that 2,3-butanediol synthesis is growth associated. The maximum yield (%), which is defined as the [maximum total product (butanediol and acetoin)/ total sugar] x 100%, was found to be 20.93, 20.50, 24.30 and 22.29 for initial sugar concentration of 25.0, 50.0, 75.0 and 100.0 g/L respectively.

Table 4.1 summarises the product attributes in fermentation of 2,3-butanediol from blackstrap molasses having different initial sugar concentrations.

It can be seen that the maximum cell concentration increased with the increasing sugar concentration (S_0). The final cell concentration (X_{max}) was found to be 3.8 g/L at a substrate concentration of 25 g/L whereas, the cell concentration reached a maximum value of 4.66 g/L when the substrate concentration was 50g/L. In comparison to the final product, the substrate concentration of 50g/L

Table 4.2 Yields and productivities obtained with different initial Substrate concentrations.

S_0 (gl^{-1})	t (hr)	t_{lag} (hr)	P_L (hr)	X_I (gl^{-1})	X_{max} (gl^{-1})	Y_p gg^{-1})	P_I (gl^{-1})	P_{max} (gl^{-1})	Y_p (gg^{-1})	R_p ($gl^{-1}h^{-1}$)	Substrate consumption (%)
25	22	12	12-20	1.36	3.8	0.167	1.0497	5.232	0.167	0.19	91.96
50	30	16	16-24	0.54	4.66	0.201	0.199	10.252	0.201	0.34	93.6
75	36	12	12-20	1.3	9.89	0.238	0.393	18.224	0.238	0.495	93.6
100	36	20	20-32	1.5	14.2	0.127	1.465	14.13	0.127	0.352	92.5

S_0 = Initial substrate concentration (gl^{-1})
Total run = t (hr)
t_{lag} = lag phase (hr)
P_L =Linear phase (hr)
X_i= initial dry cell mass (gl^{-1})
X_{max} = Final dry cell mass (gl^{-1})
Y_p = Cell yield (gg^{-1})
P_i= Initial product concentration(gl^{-1})
P_{max}= Product Concentration (gl^{-1})
Y_p = Yield (gg^{-1})
R_p =Productivity ($gl^{-1}h^{-1}$)

produced almost double the amount of butanediol and acetoin (P_{max}) than the concentration of 25g/L. It is assumed that more sucrose was diverted to the 2,3-BD fermentation due to oxygen limitation. From Table 4.1, it is also observed that the final cell concentration increased with the increasing substrate concentration from 50 g/L to 100 g/L. At highest substrate concentration of 100g/L, the final product concentration was 14.13 g/L with productivity (R_p) of 0.352g/L/h. The maximum product concentration and yield (Y_p, g of product per g of sugar) increased with the increase of substrate concentration from 25g/L to 75 g/L. However, both P_{max} and Y_p declined when the initial sugar was 100 g/L . This is perhaps due to substrate inhibition beyond an initial sugar level of 75.0 g/L. The overall sugar utilization increased from 91.96% (for S_0=25.0 g/L) to 93.6% for both 50.0 and 75.0 g/L of initial sugar (S_0) and declined to 92.5% for 100 g/L of S_0.

It is difficult to compare the batch yield data with other published work (Wheat, 1953; Afschar et al. 1993). Wheat (1953) obtained 7.25% butanediol in batch fermentation of sugar beet molasses having a sugar content of 51.0 to 57.0 % (approximately 0.17 g diol / g of sugar). Afschar et al. (1993) reported 0.5 g diol / g of sugar with high-test molasses (inverted cane syrup) in fed batch mode. Hence, only the results of Wheat (1953) form the basis of comparison in this case. The present yield, which ranged from 20.93 to 24.30, is higher than that reported by Wheat (1993). The merit of the present work also lies in the fact

that blackstrap molasses being a waste product of sugar refinery is a cheaper source of sugar than other types of molasses.

4.3 Fed batch study

Fed batch culture may be defined as a batch cultures which is continuously fed with a growth limiting substance. Fed batch operations are suitable for those fermentations which exhibit growth rate inhibition due to high initial sugar concentration but desired metabolic product has little inhibitory effect on its own production rate. The purpose of fed batch operation for fermentation of 2,3-butanediol is as follows.

(i)To increase the final diol concentration in the broth.

(ii)To improve reactor productivity.

2,3-Butanediol has no inhibitory effect on its own production rate even at concentrations higher than 100 g/L and is an ideal candidate for fed batch fermentation (Maddox, 1996). Available reports (Afschar et al. 1991, 1993) indicate that excellent yield of butanediol could be obtained from starch, high test molasses (HTM) or invert sugar. Afschar et al. (1993) used saccharified starch to achieve 55 g/L of initial glucose for fermenting butanediol. They added more starch after 16 and 32 h to raise the glucose concentration to 90 g/L. The purpose of having a relatively low initial sugar is to ensure there is no significant

substrate inhibition during the growth phase of the microorganism. A higher substrate concentration at a later stage helped achieve a product yield of 0.5 g diol / g. of starch. However, it took 150 h to accomplish the fermentation. They also used high test molasses (HTM) as substrate with an initial concentration of 200 g/L to produce 95-99 g/L of 2,3-butanediol and 2.5-4.3 g/L of acetoin. They recycled the cells and the yield reported above was achieved with a cell concentration of 16 g/L. While starch and HTM (pure unbleached sugar) have predictable substrate characteristics, blackstrap molasses being the residue of sugar production contains a variety of other substances depending on the nature of raw material and processing. Moreover, mixing and pumping of blackstrap molasses is difficult due to the presence of non-sugar substances. It was found in laboratory scale trial that the broth turned too viscous at the later stage of fermentation (after 24 h) when the initial sugar content in feed exceeded 100 g/L. To overcome this problem, the stirrer speed had to be more than 400 rpm, which in turn triggered excessive foaming despite normal dosage of antifoam. It was possible to achieve a complete off bottom suspension of biomass with 400 rpm stirrer speed and initial sugar concentration of 100 g/L.

Since the fed batch experiment was commenced with *K. oxytoca* inoculum, it was necessary to check on the substrate inhibition on the growth of microorganism. Shake flask studies with initial sugar concentration of 100 g/L showed that after 16 h, the biomass concentration was 1.57, 0.76 and 0.9 g/L

respectively for glucose, sucrose and molasses. This result shows that molasses with an initial sugar concentration of 100 g/L does not exert any significant growth inhibition. Thus, the following operational strategy was adopted in this work.

1. The total sugar input during the course of fermentation was kept at 100 g/L, which would ensure that the biomass and sparingly soluble compounds in blackstrap molasses remain suspended until the end of the run at a constant stirrer speed of 400 rpm.
2. In order to facilitate the biomass formation, the initial sugar concentration was kept at 40 g/L and the remaining sugar (60 g/L) was fed after the microorganism had entered the growth phase.
3. Once the sugar had depleted to approximately 20% of the initial value, the substrate was fed at a constant rate of 28.8 g/h for 8.33 h and the feeding was suspended thereafter. Thus, 60 g/L of sugar was introduced over a period of 8.33 h.

The above feeding strategy is a modification over the step input method adopted by Afschar et al. (1993). In their work, the initial sugar was 55 g/L and substrate was fed after 16 h when the residual sugar concentration had fallen to 2.0 g/L. The feed was again introduced after 32 h when the residual sugar concentration had dropped to 38 g/L. In both cases, the sugar level rose to

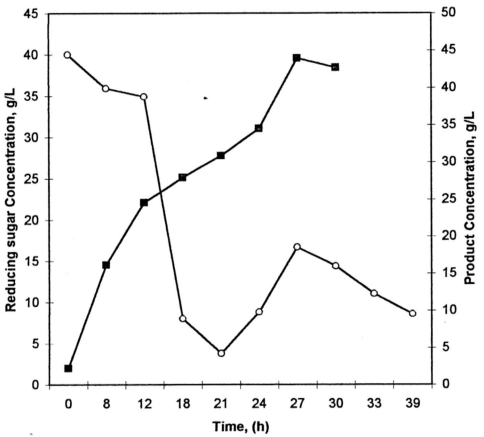

Fig. 4.13 Reducing sugar and Product Concentration, g/L

Fed batch fermentation of 2,3-Butanediol in a 5-L fermenter with initial sugar concentration of 40g/L. The total sugar into the fermenter was 100g/L.

Conditions: pH 5.8; temp 37°C.

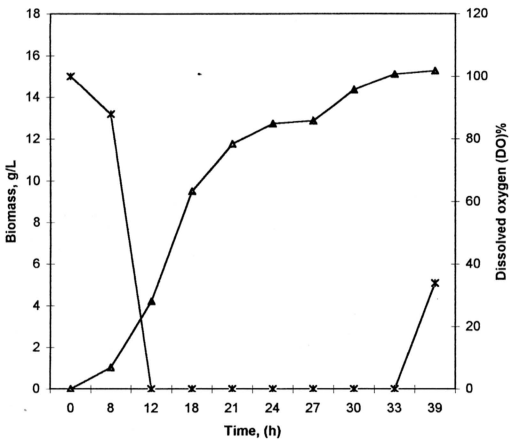

Fig 4.14 Biomass and DO versus Time

Fed batch operation with 40 g/L of initial sugar

85 g/L after the substrate input. Afschar et al. (1993) did not assign any reason for such a feeding strategy. However, saw tooth type feed profile caused rapid changes in substrate concentration, such as a sharp change in sugar level from 2.0 g/L to 85.0 g/L after 16 h. Afschar et al. (1993) also used *K. oxytoca* and blackstrap molasses to produce 2,3-butanediol. They started the fermentation with 135 g/L of sugar and fed additional molasses after 20 and 70 h when the sugar concentrations had dropped to 75 g/L and 30 g/L respectively. The sugar concentrations increased to 140 g/L and 70 g/L respectively due to addition of molasses in those two cases, and the final sugar concentration was about 2.5 g/L in 125 h. It is difficult to draw any logical conclusion from their work as the feeding policies are widely different for different substrates and no scientific explanations are provided for such operational strategies. Afschar et al. (1993) required 125 h to ferment 2,3-butanediol from blackstrap molasses as mentioned above with a total sugar input of 200 g/L in three stages. The productivity of 2,3-butanediol was 0.5 g/g of reducing sugar. They have reported a long lag phase in this experiment but did not provide any data on cell growth.

A much better feed regulation was adopted in this work. In order to iron out the substrate shock, the feeding was commenced after 18 h at a sugar level of 8.0 g/L and at a constant rate of 7.2 g/h/(L of working volume). The sugar and product concentrations have been shown in Fig. 4.13. Since sugar utilisation

rate differed to some extent from the feed rate, the transient sugar concentration oscillated between the terminal values, 8.0 g/L (after 18 h) and 8.6 g/L (after 39 h). The lowest and the highest sugar concentration observed during this period were 3.8 (after 21 h) and 16.7 g/L (after 27 h) respectively. The biomass concentration increased steadily (Fig. 4.14) over the entire fermentation time and reached the maximum value of 15.28 g/L on a dry basis at the end. The yield in the present case was 0.44 g of diol per g of sugar as against 0.42 g of diol per g of sugar reported by Afschar et al. (1993). The average productivity of diol in this work was 1.13 g/L in comparison with 0.75 g/L reported by Afschar (1993). The higher productivity obtained in this work was due to low initial sugar concentration that reduced the lag phase and a discerning feeding protocol based on the substrate consumption pattern of earlier batch experiments.