FERMENTATIVE PRODUCTION OF BUTANOL FROM SORGHUM MOLASSES

by

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CHAPTER 1

INTRODUCTION

As the costs of crude oil and natural gas increase and the proven reserves decrease, the search for alternative sources of raw materials (e.g., sorghum molasses, Jerusalem artichoke, and cellulosic materials) for energy production, such as butanol, acetone, and ethanol, becomes increasingly crucial.

Sorghum is a valuable source of grain and sugar. Because of its short growing season and drought tolerance (Creelman et al., 1981), sorghum is gaining attention as a sugar and starch source. So far more sorghum juice has been used to produce sorghum syrup than crystalline sugar. Sorghum juice is usually evaporated and marketed in the name of sorghum molasses as both feed and food. Commercial sorghum molasses varies widely in quality. It appears that no attempt has been made to ferment it for producing butanol-acetone in spite of the fact that molasses from other sources has been widely employed for this purpose.

Besides ethanol fermentation, there are several fermentation processes available for producing suitable substitutes for traditional, petroleum derived liquid fuels. In particular, butanol and acetone can be produced via anaerobic fermentation (see, e.g., Weizmann, 1938; Beesch, 1952; McCutchan and Hickey, 1954). These products may be used as fuel individually or blended together.

Butanol, as a potential agri-fuel, has certain physical-chemical qualities and production attributes, which for blending with conventional petroleum fuels and on-farm use, make it superior to ethanol. The physical characteristics of butanol are more like those of gasoline and diesel fuel; thus it mixes more readily than does ethanol. Butanol is slightly water-soluble, and therefore, will remain mixed with gasoline or diesel fuel even if water is present. Due to its larger molecular size, butanol has a much higher heat of combustion than ethanol, which means a greater percentage of butanol can be mixed with petroleum fuels without engine power loss.

Butanol-acetone fermentation by Clostridium species has been reported by numerous investigators (see, e.g., Abou-Zeid et al., 1978; Gottschal and Morris, 1982; Gottschalk and Bahl, 1981). Two different types of strains have mainly been known as the starchy material fermenting strains; one is of the Fitz type (Fernbach, 1932), which utilizes potato starch effectively, and the other is of the Weizmann type (Weizmann, 1919) which ferments corn starch more effectively than potato starch. Organisms of the Weizmann type frequently have been used commercially because of the superior yield and characteristics favorable to low cost material in the early days of the butanol-acetone fermentation industry.

Because of its relatively low cost and also because of its ease in handling, molasses has attracted attention as a carbon source for butanol-acetone fermentation since the early 1920's. Many attempts were made to ferment molasses by the organism of the Weizmann type; in some attempts, mixtures of grain and molasses were used (Weizmann,

1938), and in others protein was added to the molasses (Hellinger, 1939). It appears, however, that none was overly successful.

The objectives of this research were rejuvenating past butanol-acetone fermentation processes and developing new fermentation technology for eventually using butanol as a potential agri-fuel. These investigations were concerned with identifying several crucial fermentation parameters and determining how improvements in the butanol process can be achieved.

Chapter 2 reviews the published literature on several aspects of butanol fermentation, including strains, raw material, solvent production, gas production, butanol toxicity, and mechanism of solvent formation. The screening of strains is described in Chapter 3. To optimize the fermentation medium, the effects of sorghum molasses concentration, initial pH of medium, and concentrations of ammonium salt, calcium carbonate and phosphate on the solvent yield were investigated; this work is detailed in Chapter 4. The diauxy phenomenon, sugar utilization pattern and solvent production from unhydrolyzed sorghum molasses are described in Chapter 5. Chapter 6 reports the sugar utilization pattern and effects of hydrolysis condition, temperature and agitation on the solvent yield from inverted sorghum molasses.

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CHAPTER 2

LITERATURE REVIEW

The interesting history of the butanol-acetone fermentation process has been described by several authors (Gabriel, 1928; Gabriel and Crawford, 1930; McCutchan and Hickey, 1954; Walton and Martin, 1979). Butanol as a product of microbial action was probably first observed by Pasteur in 1861. There was considerable interest in commercializing the process in 1909 in England primarily as a means to obtain butadiene as a raw material for synthetic rubber.

From its start as a wartime venture in England in 1916 until the establishment of the synthetic butanol process in the late 1930s, the fermentation process was the major method for manufacturing butanol.

Because of the unfavorable economics in comparison to the synthetic process using petroleum-based feed stocks, the fermentation process ceased to operate in the early 1960s in the United States and in most other countries. However, there is renewed interest in examining the fermentation processes as a means of producing all or a portion of our future needs of butanol and acetone.

MICROORGANISMS

The organisms employed for the various butanol fermentation processes are Clostridia species. The strain, Cl. acetobutylicum, is classified as a strain of the Weizmann type according to the description in Bergey's

Manual (Breed et al., 1957). It has been widely employed in the fermentation of cereal grain mashes.

Among the sugar--or molasses--fermenting organisms are, for example, Cl. propyl butylicum alpha (Müler, 1938), Bacillus tetryl (Arroyo, 1938; Owen, 1939). These organisms necessitated the use of invert sugar for best operation since sucrose was not fermented readily. Other cultures have been found which ferment uninverted molasses. Among these are Cl. saccharo-acetobutylicum (Woodruff et al., 1937) and several variants of Cl. saccharo-butyl-acetonicum-liquefaciens (Arzberger, 1938; Carnarius and McCutchan, 1938). A patent was issued to Hall (1939), for a process which employed an organism called Bacillus butacone. The spores were described as being unusually heat resistant, withstanding 100°C for 45 to 190 minutes. Both molasses and starchy media were fermented and complex nitrogeneous nutrients were required.

MATERIALS

Most of the acetone and butanol produced by fermentation has used one or more of three sources of carbohydrates, i.e., corn (Weizman, 1938) blackstrap molasses, or high-test molasses (Paul, 1963). Other available sources of carbohydrates such as grain, milk whey, Jerusalem artichoke, straw, and wood waste can also be used (Pomeranz and Munck, 1981; see Table 2).

Blackstrap molasses is the mother liquor from cane sugar refining. Its total content of sugars is about 52% as sucrose. It actually contains about 30% sucrose and about 22% invert sugar.

When excessive sugar cane is harvested, the juice is expressed from it and concentrated to about 70 to 75% sugar in the presence of a small

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Table 1. Some Butanol-Acetone Cultures and Their Application (McCutchan and Hickey, 1954; Prescott and Dunn, 1959).

Names of bacteria	Substrate	Solvent Ratios, %			
		Butyl alcohol	Ethyl alcohol	Acetone	Isopropyl alcohol
Bacillus saccharobutylicum-beta	Inverted molasses and $CaCO_3$	75	--	3	35
Clostridium saccharobutylicum-gamma	Blackstrap molasses and $CaCO_3$	65-80	--	18-34	1-2
Cl. saccharobutyl-acetonicum	Blackstrap molasses, corn glutten, and $(NH_4)_2SO_4$	64	--	36	
Cl. viscifaciens	Inverted molasses and $CaCO_3$	66	--	3	31
Cl. saccharoacetobutylicum-beta and gamma	Cane molasses and degraded protein, such as ammonia, steep water, or distillery slop	68-73	1-3	26-32	14-28 (mixture isopropyl and ethyl)
Cl. propyl butylicum	Inverted molasses, NH_3, and $CaCO_3$	69-70	--	4-17	14-28 (mixture isopropyl and ethyl)
Cl. invertoacetobutylicum	Louisiana molasses (inverted) and ammonium salts or alkalies	66-70	2-3	27-31	
Cl. saccharoacetobutylicum	Louisiana molasses, $(NH_4)_2SO_4$, and $CaCO_3$	68-73	1-3	26-32	
Cl. saccharobutyl-isopropyl-acetonicum	Inverted molasses and degraded protein	Low pH, 60-70; High pH, 65-80			

(continued)

Names of bacteria	Substrate	Solvent Ratios, %			
		Butyl alcohol	Ethyl alcohol	Acetone	Isopropyl alcohol
Cl. saccharoacetobutylicum--alpha	Cuban molasses, $(NH_4)_2SO_4$, and gluten meal	68-73	1-3	26-32	
B. tetryl		74	6	20	
Cl. propyl butylicum--alpha	Inverted molasses, $(NH_4)_2SO_4$ $CaCO_3$, K_2HPO_4, and $MgSO_4$	65-70	3-4	5-10	16-20
Cl. saccharobutyl-acetonicum-liquefaciens--gamma and delta	Blackstrap molasses $(NH_4)_2SO_4$, $CaCO_3$, and P_2O_5	58-74	2-6	24-36	
Cl. saccharobutyl-acetonicum-liquefaciens--gamma and delta	Cuban molasses, $(NH_4)_2SO_4$, $CaCO_3$ and P_2O_5	60-69	3-4.5	26-35	
B. butacone	Blackstrap molasses and animal and vegetable protein	65	--	28	
Cl. celorifactor	Inverted molasses, ammonia, and $CaCO_3$	60	2	38	
Cl. granulobacter acetobutylicum	Molasses, corn gluten, ammonium salts, and $CaCO_3$	60-75	1-10	25-30	
Cl. saccharobutyl-isopropyl-acetonicum--beta	Cane and beet molasses, $(NH_4)_2SO_4$, and $CaCO_3$	60-85	--	15-40	0.1-4.0
Cl. madisonii	Cuban blackstrap, NH_4OH, $(NH_4)_2SO_4$, and $CaCO_3$	75-76	4-6	17-20	
Cl. amylosaccharobutyl-propylicum	Invert molasses, $(NH_4)_2SO_4$, $CaCO_3$, and P_2O_5, or NH_4OH, and P_2O_5	65-72	Trace	2-4	26-32

(continued)

Names of bacteria	Substrate	Solvent Ratios, %			
		Butyl alcohol	Ethyl alcohol	Acetone	Isopropyl alcohol
Cl. acetobutylicum	Corn hydrol, complex N	60-62	8-10	29-30	
Cl. acetobutylicum	Corn, complex N	60-62	8-10	29-30	
Cl. saccharo-acetobutylicum	Corn, NH_3	70-75	20-25	3-5	

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amount of mineral acid, thus partially inverting sugar and preventing it from crystallization on standing. The resultant mixture contains about 50% invert sugar and 25% sucrose; it is known as high test molasses.

When blackstrap or high-test molasses is used for the fermentation, the media is generally deficient in avilable nitrogen and phosphate. A culture, such as the Cl. saccharo-butyl-acetonicum-liquefaciens type (Arzberger, 1938), can utilize ammonia nitrogen in the medium. It does not need complex nitrogeneous sources, such as those required for growth by some other strains (see Table 1).

Extensive laboratory research has been carried out on the butanol-acetone fermentation of wood sugar obtained as wood hydrolyzate (Prescott and Dunn, 1959) or as waste sulfite liquor (Walton and Martin, 1979). Leonard et al. (1947) found that sugar solutions prepared by the hydrolysis of maple, oak, and fir could be fermented with Cl. acetobutylicum to give a solvents yield of 24.5 to 38.5% based on the sugar utilized. The sugar utilization was about 80-85% with a solvent ratio of approximately 72% butanol, 25% acetone, and 3% ethanol. Further work needs to be done on this raw material since solvent yields fell off when the initial sugar concentration of the mash exceeds 3% (Tsuchiya et al., 1949).

Agricultural residues such as bagasse and rice straw have been hydrolyzed by mixed culture filtrates to obtain fermentable sugars. The hydrolysate was used to produce butanol to the extent of 16 g/l (Soni et al., 1982). The production of butanol and acetone from the sugar present in waste sulfite liquor was studied by Wiley et al. (1941). They heated the liquor first with lime to remove sulfites, then with additional lime to precipitate lignin. This resulted in an initial sugar concentration

of 5.03% and eventually gave rise to a sugar utilization of 90% and a solvent yield of 30.6% based on the sugar used. The ratio of solvents was butanol 61.7%, acetone 31.8%, and ethanol 6.5%.

SOLVENTS

The solvents, butanol, acetone, and ethanol have been produced from starchy or sugary materials by use of the species Cl. acetobutylicum. The resultant solvent ratio is usually 30% acetone, 60% butanol, and 10% ethanol (Gabriel, 1928; Gabriel and Crawford, 1930; McCutchan and Hickey, 1954; Walton and Martin, 1979). Table 3 shows the yield and composition of solvent when molasses is used as substrate for butanol-acetone fermentation.

The ratio of solvents can be modified to some extent by manipulating the temperature of cultivation and the composition of the nutrients in the cultivation media. Tarvin (1941) reported that an addition of nitrates instead of ammonia to a molasses fermentation increased the yield of acetone at the expense of butanol. For example, in a typical experiment the addition of $NaNO_3$ at a concentration of 4.5 g/l increased the final concentration of acetone from 30.7 to 40.9%. Carnarius (1940) found that the butanol ratio could be increased 3 to 5% at the expense of acetone by cooling a molasses mash from 30°C to 24°C 16 hours after inoculation.

Table 3 shows some results on the butanol fermentation of hydrolysates of corncobs and wood. Langlykke et al. (1948) studied the production of butanol and acetone by fermenting xylose and glucose derived from the acid hydrolysis of corncobs. After purification, the hydrolysate with an initial sugar concentration of 5.03% gave a sugar utilization of 90% and

Table 2. Available Carbohydrates for Fermentation Use

Sugars	Cane Beet Grape Process wastes from above Milk (lactose - whey) Molasses Jerusalem artichoke	Surplus production may be temporary and local. Prior treatments minimal. Whey is a clean waste well suited for biomass products. Cane and beet could be grown specifically for fermentation.
Starchy Materials	Cereals Rice Cassava Manioc Potato Process wastes from above Corn	Local surpluses especially of spoiled materials inevitable but seasonal. Prior treatments minimal. Earmarked cultivation possible but may conflict with food uses. Some clean wastes best for biomass.
Cellulosic Materials	Corncobs, oat hulls, etc. Straw Wood waste Sulphite liquor Paper waste, etc.	Large surpluses, but some local and seasonal. Low cost but bulky in transport. Prior treatment maximal. Cheapest when as process waste for on-site use.

a 30.6% yield of solvents based on the sugar used. The ratio of solvents was butanol 61.7%, acetone 31.8%, and ethanol 6.5%.

BY-PRODUCTS

Among by-products of the butanol-acetone fermentation process are the fermentation gases. The amount produced from cultures giving the higher butanol ratios is about 0.29 l/g sucrose. Approximately 60-65% by volume is CO_2, the remainder being H_2. Cultures yielding higher acetone percentage produces a greater proportion of H_2 in the evolved gases. These gases have been used for synthesizing both methanol and ammonia (Moreira et al., 1982). Some interaction may exist between the volume of H_2 evolved during the fermentation and the level of butanol present in the final fermented broth.

Table 3. Butanol-Acetone Fermentation from Molasses. (Prescott and Dunn, 1959; McCutchan and Hickey, 1954; Walton and Martin, 1979).

Culture	Substrate	Yield* (%)	Solvent Composition (%)		
			Butanol	Acetone	Ethanol
SES-4	Molasses sugar conc. 5-6%	33	56.8	33.5	9.6
SES-5	Molasses [6.28% sugar in mash plus rice bran 0.3%, $(NH_4)_2SO_4$ 0.3%], and $CaCO_3$ 0.4%; temperature 37.5°-27°C	32.0			
Cl. acetobutylicum	Molasses plus rye or wheat meal	26.5	66	31	3
Cl. acetobutylicum S_{25}	12% Molasses	24.9			
Cl. acetobutylicum	14% Molasses	28.6	71	29	0

Yield* (%) = solvent produced (g) X 100 / glucose consumed (g)

Table 4. Butanol-Acetone Fermentation of Hydrolysates of Corncobs, Cornstalks, and Wood. (Prescott and Dunn, 1959; McCutchan and Hickey, 1954).

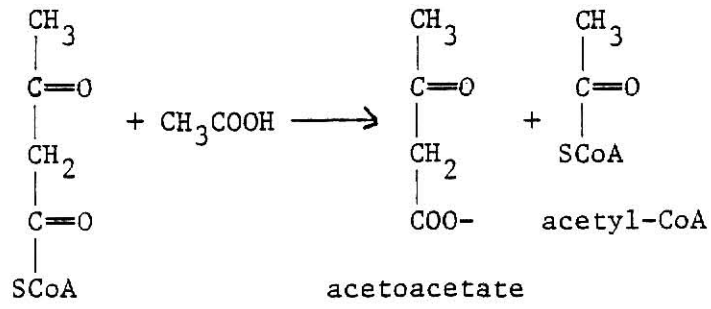
Culture	Substrate	Yield (%)	Solvent Composition (%)		
			Butanol	Acetone	Ethanol
Cl. acetobutylicum S_{25}	Hydrolysate of corncobs, 7% Hydrolysate of sawdust, 7%	26.2 22.2			
Butyl culture	One part hydrolysate of corn-stalks, three parts molasses	31-37			
Butyl culture	Wood and plant hydrolysates, 8%	35.5	62	32	6
Cl. acetobutylicum 314	Corncob hydrolysate, 40-60% Molasses, 60-40% (sugar)	40	67.5		
Butyl culture	Pentoses, 13.5%	25.4	67	33	

MECHANISM OF ACETONE-BUTANOL FERMENTATION

Formation of Acetone

Although the mechanism of alcoholic fermentation of sugar by yeast and that of the lactic acid fermentation by lactic acid bacteria have been well established, the intermediary metabolism of butanol-acetone fermentation has not been elucidated completely. The existing knowledge of the mechanism of the butanol-acetone fermentation was reviewed by Prescott and Dunn (1959), and Doelle (1975). The most probable metabolic reactions involved in glucose utilization by Clostridium organisms are shown in Figure 1 (Prescott and Dunn, 1959; Doelle, 1975; Stanier et al., 1969).

Cl. acetobutylicum possesses a transferase system that diverts acetoacetyl-CoA from the normal cyclic mechanism to produce acetoacetate.



The decarboxylation of acetoacetate to acetone is an irreversible step. The enzyme responsible for this reaction is acetoacetate decarboxylase.

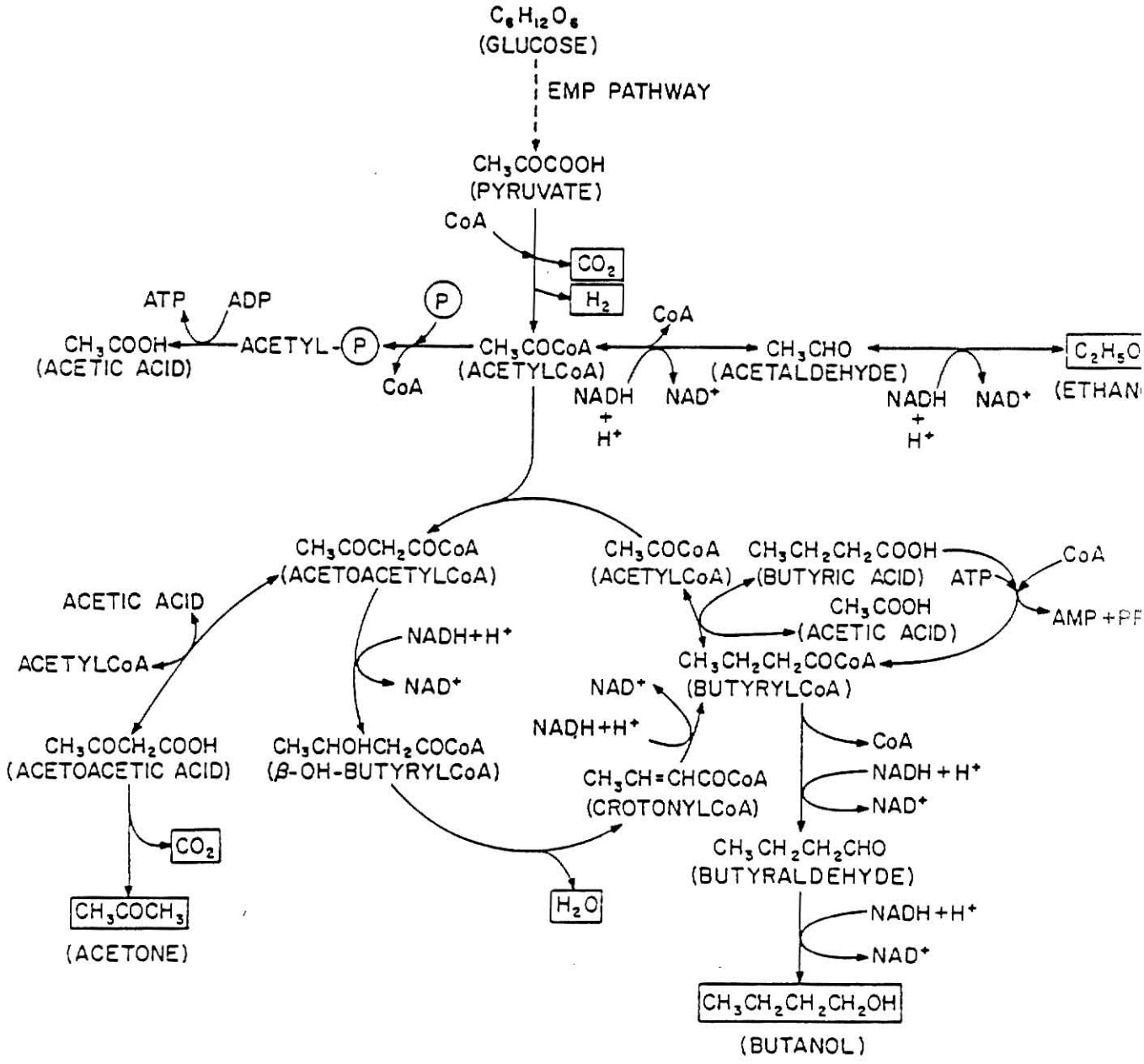
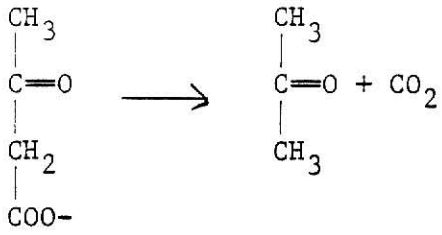


Fig. 1. The formation of acetone, butanol, and butyric acid by species of the genus Clostridium (Doelle, 1975).



acetoacetate acetone

Formation of Butanol

The reduction of butyric acid to butanol is carried out in three consecutive reactions. Butyryl-CoA may be formed differently if the amount of available acetyl-CoA is deficient. The reduction of butyryl-CoA to butyraldehyde is by NAD^+ and catalyzed by the aldehyde dehydrogenase, i.e.,

$$\text{Butyryl-CoA} + \text{NADH} + H^+ \text{ —— } \text{Butyraldehyde} + NAD^+ + \text{HSCoA}$$

The final reduction to butanol is also carried out with a well-known enzyme, NAD^+-linked alcohol dehydrogenase, i.e.,

$$\text{Butyraldehyde} + \text{NADH} + H^+ \text{ — } \text{n-Butanol} + NAD^+$$

The production of butanol occurs after the change to the production of acetone has taken place.

The metabolic pathway is, therefore, glucose to butyrate via pyruvate. The pH of the medium drops to 4.0 due to accumulation of butyrate and acetate, which is produced at the initial stage during the fermentation. At this point, a new enzyme system is activated, leading to the reduction of the accumulated butyrate to butanol.

Formation of Butyric Acid

Saccharolytic Clostridia ferments glucose to butyric acid. The intermediate acetyl-CoA will be taken as the starting point (see Fig. 2). From the point of view of energetics, the production of acetate as the sole end product would not be satisfactory because it becomes more and more difficult to reoxidize NADH + H^+ to NAD^+ + H_2 as the pH falls into the acid region.

As shown in Figure 2, the cyclic mechanism brings about formation of butyric acid, which is much less an acid end product than acetate. Two acetyl-CoA molecules undergo a condensation to form acetoacetyl-CoA, liberating 1 mole of CoA. The next step in the cycle is a reductive step, whereby acetoacetyl-CoA is reduced to β-hydroxybutyryl-CoA and reduced NAD is oxidized to NAD^+. The dehydration of β-hydroxybutytyl-CoA occurs, forming crotonyl-CoA and water. An NAD^+-linked dehydrogenase is responsible for the further reduction to butyryl-CoA. The last step in the cyclic mechanism is a transfer reaction to produce acetyl-CoA and butyric acid.

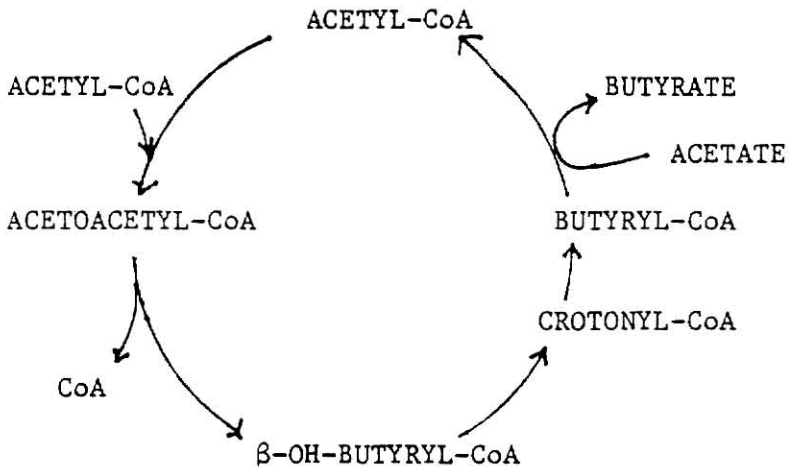


Fig. 2. The formation of butyric acid from acetyl-CoA by Clostridium species (Doelle, 1975).

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CHAPTER 3

SCREENING OF MICROORGANISM

Butanol-acetone fermentation of starchy material by Clostridium species has been reported by numerous investigators (see, e.g., Gabriel, 1928; Peterson and Fred, 1932; Fouad et al., 1976). Mainly, two different types of strains have been known as the starchy material fermenting strains; one is of the Fitz type (Fernbach, 1932), which utilizes potato starch effectively, and the other is of the Weizmann type (Weizmann, 1938), which ferments corn starch more effectively than potato starch. Organisms of the Weizmann type frequently have been used commercially because of the superior yield and characteristics favorable to low cost material in the early days of the butanol-acetone fermentation industry.

Because of its relatively low cost and also because of its ease of handling, molasses has held attention as a carbon source for butanol-acetone fermentation since the early 1920's. Many attempts were made to ferment molasses by the organisms of the Weizmann type; in some attempts, mixtures of grain and molasses were used (Paul, 1963; Taha et al., 1973), and in others, protein was added to the molasses (Doi et al., 1960; Mahmoud et al., 1974). It appears, however, that none was overly successful. Meanwhile, the interests of researchers in this field have shifted to searching for new and effective strains for fermenting molasses, but relatively little effort has been spent in elucidating why the new strains are more effective than those of the Weizmann type.

The molasses fermenting strains, which have been reported, belong to two types, one fermenting inverted molasses (Stiles, 1937; Hildebrandt and Erb, 1939) and the other fermenting molasses directly (Sherman, 1935; Arzberger, 1938; Carnarius and McCutchan, 1938). The latter has been used widely in commercial plants; it gives relatively high yield, and using it eliminiates the necessity to invert sugar in molasses. Despite the fact that this type of strain ferments molasses effectively, it ferments starchy material poorly. In fact, butanol-acetone fermentation plants traditionally had to maintain several types of strains to utilize different feedstocks. Unfortunately, this complicates the operation and management of such plants.

Some investigators (see, e.g., Compere and Griffith, 1979) reported the fermentation capability of several butanol-acetone producing strains on different kinds of sugar as the sole carbon source. The objective of this work was to select a strain from a collection of five American Type Culture Collection's (ATCC) strains, which are known butanol-acetone producers. The strains were tested for their sugar utilization capabilities with a sorghum molasses medium and a synthetic medium which contained glucose, fructose, or sucrose as the sole carbon source.

MATERIALS AND METHODS

Procedure

Microorganism. A strain suitable for fermenting sorghum molasses was screened from butanol-acetone producers obtained from the American Type Culture Collection (ATCC). The cultures screened were Clostridium acetobutylicum ATCC 824, ATCC 4259, and ATCC 10132, Cl. saccharoperbutyacetonicum ATCC 27022, and Cl. butyricum ATCC 860.

The cultures were maintained in screw-capped tubes (16 x 150 mm) with 10 ml of the 5% (w/v) corn meal medium at 5°C after cultivation for 72 hr at 37°C, and were transferred routinely once a month.

Medium. A basal medium with the composition as shown in Table 1 was used for screening strains. A 25 g/l of glucose, fructose, or sucrose was added to the basal medium as the sole carbon source. The sorghum molasses medium comprised of 100 g/l sorghum molasses, 6 g/l $(NH_4)_2SO_4$, and 2 g/l KH_2PO_4 was also used for the screening. Sorghum molasses used for this study was obtained from a farm co-op (Alma, Kansas). The sugar composition of the molasses was: sucrose, 28% (w/w); glucose, 14% (w/w) and fructose, 15% (w/w).

Cultivation. For each strain, a 10 ml of corn meal medium was used to activate the maintenance culture stored at 5°C. The inoculated corn meal medium was heat-shocked to germinate the spores in boiling water for 60 seconds, and was immediately cooled in a water bath. The culture was then incubated at 37°C for 18 hours. The growing culture on corn mash was inoculated to 140 ml of the molasses medium or synthetic medium in a 300 ml screw-capped flask equipped with a 14 mm screw-capped cleanout and a 130 mm depressed arm (Bellco Glass Inc., Vineland, New Jersey). The screw cap of the flask's cleanout was replaced by a rubber cap through which a syringe needle was inserted to permit fermentation gases to escape prior to autoclaving.

Anaerobic conditions for the cultures were maintained by sparging nitrogen gas through the media. No sparging was needed to maintain an anaerobic atmosphere after growth began.

Fermentation was performed for 72 hr at 37°C. The initial pH was adjusted at 6.0. The pH was checked and adjusted to not drop below pH 4.5 by adding 5N sodium hydroxide during fermentation.

Analytical Methods

Optical density. In screening strains, no attempt was made to measure the biomass concentration. Cell growth was monitored for reference simply by measuring the optical density of the medium at 560 nm using a Bauch and Lomb Spectronic 20 spectrometer.

Sugar concentration. Sucrose, glucose, and fructose were monitored using a Varian model 5000 high pressure liquid chromatograph (HPLC), equipped with a column for sugar analysis with packings, an Aminex ion exclusion HPX-87H (Bio-Rad Laboratories). For protection, the column was preceded by a Micro-Guard column packed with ion exclusion resin, Aminex HPX-85H (Bio-Rad Laboratories). The guard column was replaced routinely every 50 injections. A detecting system used for sugar analysis was a differential refractometer, Waters model 401. The column was heated to 45°C, and 0.01N sulfuric acid was used as the mobile phase at a flow rate of 0.9 m./min. Samples were centrifuged for 15 min at 3000 rpm in a model IEC-HN-SII centrifuge (International Equipment Co). n-Propanol was used as the internal standard.

Solvent and organic acid. Solvents and organic acids were assayed using a Hewlett Packard Model 5710A gas chromatograph. A glass column, 1.82 m by 2 mm, was filled with packings (Chromosorb W-AW, 80/100 mesh), coated with 10% AT-1000 (Alltech Associates, Arlington Heights, Illinois). The operating conditions were: injector temperature, 200°C; flame ionization detector temperature 250°C; column temperature, 80°C to 160°C.

Helium was used as the carrier gas at a flow rate of 30 ml/min. Samples were treated by the same methods as those employed for the sugar analysis and were acidified by adding sulfuric acid to transform the salts of organic acids to the free acid form so that samples contained 1% (w/v) sulfuric acid.

RESULTS AND DISCUSSION

Microorganisms suitable for sorghum molasses fermentation were screened from a collection of five American Type Culture Collection's (ATCC) strains, which are known butanol-acetone producers. As indicated in Table 2, two strains, Cl. acetobutylicum ATCC 824 and Cl. acetobutylicum ATCC 4259, were superior to the others in fermenting sorghum molasses. Both strains showed a similarity in utilizing sugar and in producing solvent. Glucose was consumed more easily than other sugars by these two strains. The remaining three strains consumed sugars, especially glucose and sucrose, without appreciable competition. Most strains, except Cl. acetobutylicum ATCC 4259, consumed sucrose more easily than fructose.

The strains were tested for their sugar utilization capabilities with a synthetic medium which contained glucose, fructose, or sucrose as the sole carbon source. As shown in Tables 3 and 4, both strains, ATCC 824 and ATCC 4259, exhibited comparable capabilities for utilizing glucose and fructose.

As indicated in Table 5, the strain, Cl. acetobutylicum ATCC 824, could not compete with the strain eventually selected for use in this work, Cl. acetobutylicum 4259, in utilizing sucrose. However, the sucrose

Table 1. Composition of the basal medium for screening of strains.

K_2HPO_4	0.75 g
KH_2PO_4	0.75 g
$MgSO_4$	0.2 g
$FeSO_4 \cdot 7H_2O$	0.01 g
$MnSO_4 \cdot H_2O$	0.2 g
NaCl	1.0 g
Cystine	0.5 g
Yeast Extract	5.0 g
Asparagine	0.5 g
$(NH_4)_2SO_4$	2.0 g
Resazurin	0.3 g
Distilled Water	1000 ml

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Table 2. Comparison of growth and solvent production by several Clostridium species in the sorghum molasses medium.

Strain	Growth (OD at 560nm)	Sugar Consumption (g/100ml)	Production (g/100ml) Acetone	Ethanol	Butanol	Total	Yield (%)
Cl. acetobutylicum ATCC 824	3.90	glucose 1.00 fructose 0.45 sucrose 0.60 Total 2.05	0.05	0.04	0.32	0.41	20.0
Cl. acetobutylicum ATCC 4259	4.03	glucose 1.30 fructose 0.76 sucrose 0.70 Total 2.76	0.08	0.06	0.43	0.57	20.1
Cl. acetobutylicum ATCC 10132	1.56	glucose 0.53 fructose 0.38 sucrose 0.43 Total 1.34	0	0.01	0	0.01	0.7
Cl. saccharoperbutylacetonicum ATCC 27022	2.08	glucose 0.75 fructose 0.40 sucrose 0.57 Total 1.72	0	0.01	0.22	0.23	13.4
Cl. butyricum ATCC 860	1.82	glucose 0.60 fructose 0.27 sucrose 0.68 Total 1.55	0	0.01	0	0.01	0.6

a) The molasses medium used in this test was analyzed as containing 2.5% (w/v) sucrose, 1.3% (w/v) glucose and 1.14% (w/v) fructose.

Table 3. Comparison of growth and solvent production by several Clostridium species in the glucose medium.

Strain	Growth (OD at 560nm)	Sugar Consumption (g/100mℓ)	Production (g/100mℓ) Acetone	Ethanol	Butanol	Total	Yield (%)
Cl. acetobutylicum ATCC 824	0.96	1.22	0.01	0.02	0.24	0.27	22.1
Cl. acetobutylicum ATCC 4259	1.52	2.27	0.10	0.12	0.39	0.61	26.8
Cl. acetobutylicum ATCC 10132	1.69	0.20	0.01	0.01	0	0.02	10.0
Cl. saccharoperbutylacetonicum ATCC 27022	2.08	0.82	0.01	0.01	0.14	0.16	19.5
Cl. butylicum ATCC 860	2.34	0.18	0.01	0.01	0	0.02	11.1

a) Initial glucose concentration was 2.5 g/100 mℓ.

Table 4. Comparison of growth and solvent production by several *Clostridium* species in the fructose medium.

Strain	Growth (OD at 560nm)	Sugar Consumption (g/100mℓ)	Production (g/100mℓ) Acetone	Ethanol	Butanol	Total	Yield (%)
Cl. acetobutylicum ATCC 824	2.73	1.21	0.01	0.02	0.18	0.21	17.3
Cl. acetobutylicum ATCC 4259	3.90	1.83	0.04	0.02	0.26	0.32	17.5
Cl. acetobutylicum ATCC 10132	1.56	0.63	0.01	0.02	0	0.03	4.7
Cl. saccharoperbutylacetonicum ATCC 27022	2.08	0.46	0.01	0.02	0.06	0.09	19.5
Cl. butyricum ATCC 860	2.34	0.10	0.01	0.02	0	0.03	30.0

a) Initial fructose concentration was 2.5 g/100 mℓ.

Table 5. Comparison of growth and solvent production by several Clostridium species in the sucrose medium.

Strain	Growth (OD at 560nm)	Sugar Consumption (g/100ml)	Production (g/100ml) Acetone	Ethanol	Butanol	Total	Yield (%)
Cl. acetobutylicum ATCC 824	1.95	0.71	0.01	0.01	0.13	0.15	20.0
Cl. acetobutylicum ATCC 4259	3.51	1.99	0.03	0.03	0.26	0.32	16.0
Cl. acetobutylicum ATCC 10132	1.25	0.21	0.01	0.01	0	0.02	14.2
Cl. saccharoperbutylacetonicum ATCC 27022	2.86	1.60	0.02	0.02	0.08	0.12	7.5
Cl. butyricum ATCC 860	1.76	1.11	0.01	0.01	0	0.02	18.0

a) Initial sucrose concentration was 2.5 g/100 ml.

utilizing capability of the strain, Cl. saccharoperbutyacetonicum ATCC 27022, was almost comparable to the latter, but its solvent producing capability was not.

It is worth drawing attention to the strain, Cl. acetobutylicum ATCC 4259 (Bacillus butylicus), with respect to its capability for utilizing sucrose; it consumes as much as 80% of sucrose contained in the medium. This implies that this strain is highly capable of producing invertase. From the results of the test for sugar utilization, it was chosen for subsequent experiments.

The strain, Cl. acetobutylicum ATCC 4259, should be classified as a strain of the Weizmann type according to the description in Bergey's Manual (1948). The elucidation of its characteristics, namely, different rates of consuming different kinds of sugar, seems to be important for solving difficulties encountered by a strain of the Weizmann type in fermenting molasses in earlier days.

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CHAPTER 4

MEDIA OPTIMIZATION

The commonest of the carbohydrate raw materials used in the acetone-butanol fermentation are molasses and corn. Cane or beet molasses, which is sugary, was used as substrate for the butanol-acetone fermentation in earlier days. Molasses are classified into two types, blackstrap molasses and high test molasses, according to the manner by which they are manufactured. Blackstrap molasses is a type of the mother liquor of crystalline sugar. On the other hand, high test molasses is a kind of concentrated juice from sugar cane or sugar beet. The concentrated juice is partly inverted by acid to prevent it from forming sugar crystal during storage. Strictly speaking, high test molasses is not molasses but inverted syrup.

Sorghum is a valuable source of sugar and also grain. Recently, sorghum is gaining attention as a sugar and starch source because of its short growing season and drought tolerance (Chubey and Dorrell, 1974). So far, more sorghum juice has been used to produce sorghum syrup than crystalline sugar. Sorghum juice is usually evaporated and marketed in the name of sorghum molasses for both feed and food. Commercial sorghum molasses vary widely in quality. So far no attempt has been made to ferment it for producing butanol-acetone in spite of the fact that molasses from other sources have been widely employed for this purpose.

It is, however, justified to supplement the molass medium with ammonium sulphate and phosphate as sources of nitrogen and phosphorous, respectively. Grain was often used as a carbohydrate source during the early work on the acetone-butanol fermentation. Since the strains of Cl. acetobutylicum are able to hydrolyse starch and other polysaccharides, the grain mashes do not need to be hydrolyzed. The use of sugar materials in the acetone-butanol fermentation has been discussed (Beesch, 1952, 1953).

The objective of this study was to increase the solvent yield by optimizing fermentation medium. The experimental parameters are as follows: sorghum molass concentration, effect of initial pH of medium, concentrations of ammonium salt, calcium carbonate and phosphate, and effect of temperature.

MATERIALS AND METHODS

Procedure

Microorganism. The selected strain, Cl. acetobutylicum ATCC 4259, was used throughout the study.

Medium. A medium, containing 140 g/l sorghum molasses, 6 g/l $(NH_4)_2SO_4$, 2 g/l $CaCO_3$, and 2 g/l KH_2PO_4, was used as a basal medium for optimization.

Cultivation. The same as those listed in Chapter 3.

Analytical Methods

Sugar Concentration . The same as those listed in Chapter 3.

Solvent and organic acid. The same as those listed in Chapter 3.

RESULTS AND DISCUSSION

Sorghum Molasses Concentration

A series of experiments with different sorghum molasses concentrations were carried out to examine the effect of concentration on the extent of sugar utilization and subsequent solvent production. The nutrients were added to the medium in proportion to the sorghum molasses concentration. The range of molasses concentration, namely, 10-18% (w/v), was chosen so that the effect of solvent inhibition is minimum.

As indicated in Figure 1, glucose was consumed completely independent of the molasses concentration; however, the relative consumption of sucrose decreased and that of fructose increased with an increase in the molasses concentration. As also indicated in Figure 1, the final solvent concentration increased in proportion with an increase in the molasses concentration. The results imply that fructose and sucrose compensate each other to render the relative or fractional sugar consumption essentially constant. The results also suggest that fructose is more easily consumed than sucrose when the sugar concentration is high. It appears, however, that when the concentration of sugar is low, sucrose may become relatively more easily consumed.

Effect of the Initial pH of Medium

The pH of the molasses medium was adjusted by the addition of sulfuric acid or sodium hydroxide solution to a level of 5.0, 5.2, 5.4, 5.6, 5.8, 6.0, 6.2, 6.4, 6.6, 6.8, or 7.0 before inoculation; the optimum pH ranged between 5.6 and 6.6 (see Figure 2). At any initial pH value over or below this optimum range, the production of butanol, acetone, and

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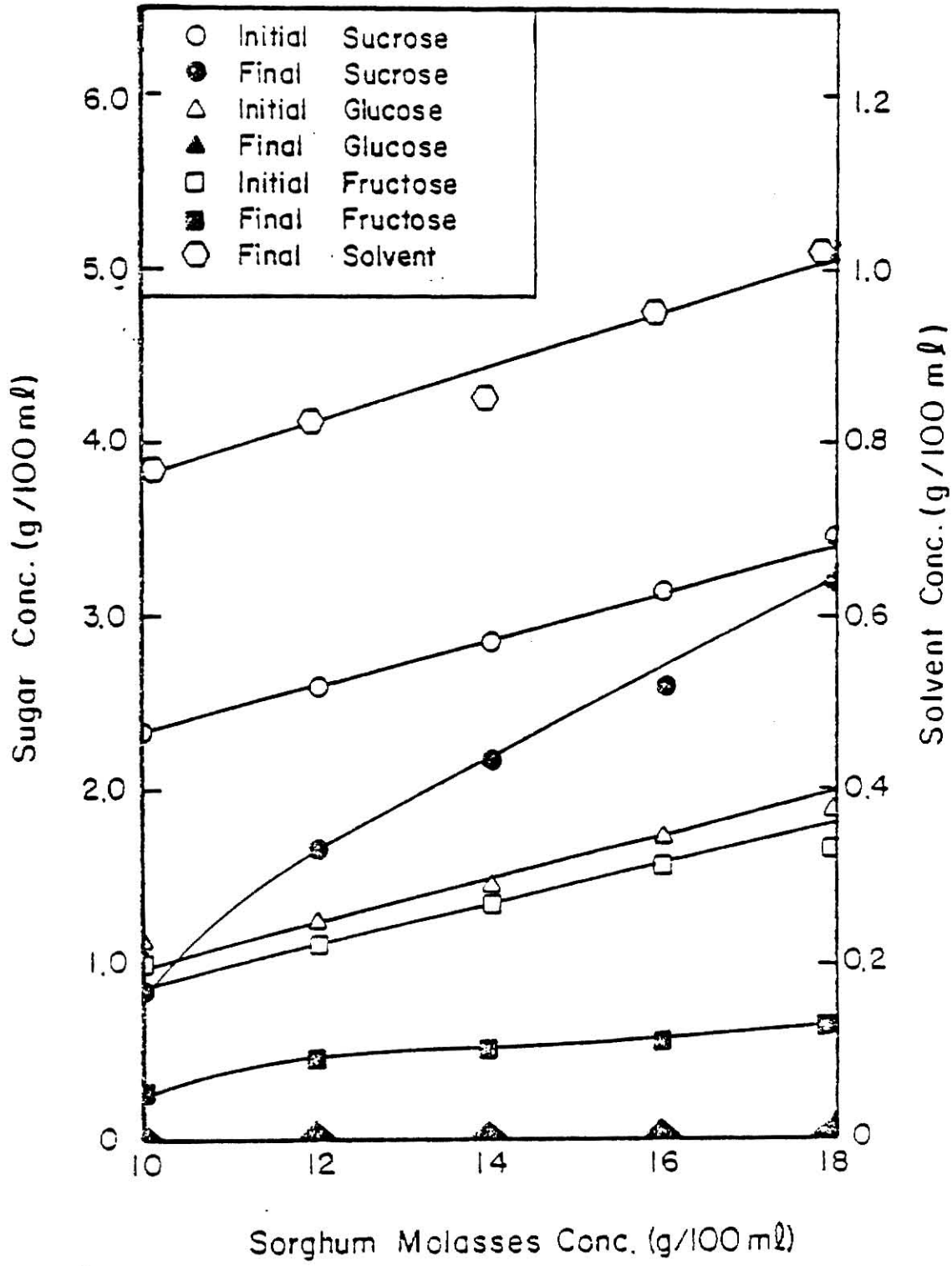

Fig. 1. Effect of the sorghum molasses concentration on sugar utilization and solvent yield.

ethanol dropped sharply. The range of optimum initial pH was slightly wider and lower than those reported by other investigators (Janke and Siedler, 1937; Kovats, 1960; Senkevich et al., 1960; Taha et al., 1973), whose values ranged between 6.0 and 6.7 for almost all substrates, including sugar cane molasses. As also indicated in Figure 2, within the range of optimum initial pH, the pH of the medium was highly dependent on the age of culture; more specifically, the pH at the final stage was appreciably higher than that at the early stages of fermentation. The maximum solvent production was obtained at the initial pH of 5.8. The butyric acid concentration in the final broth increased gradually with an increase in the initial pH of the molasses medium.

The pH of the medium plays an important role for the production of solvent. Toward the middle of the fermentation when the pH of the medium drops to 4.5 two new products are formed: acetone and butanol. Their formation coincides with a decrease in the concentration of butyrate. This is also indicated by the observation that solvent formation is largely reduced when the pH of the medium is kept above 5 (Andersch et al., 1982; Gottschalk and Bahl, 1981).

Ammonium Salt Concentration

Ammonium sulfate in the basal medium was replaced with different amounts of ammonium sulfate. Sterilization of a resultant medium was carried out in two ways. In one, ammonium sulfate was added before sterilization, and in the other, it was added after sterilization. As shown in Figures 3 and 4, independent of the sterilization method, the optimum concentration was 0.2%. Above this point, total solvent

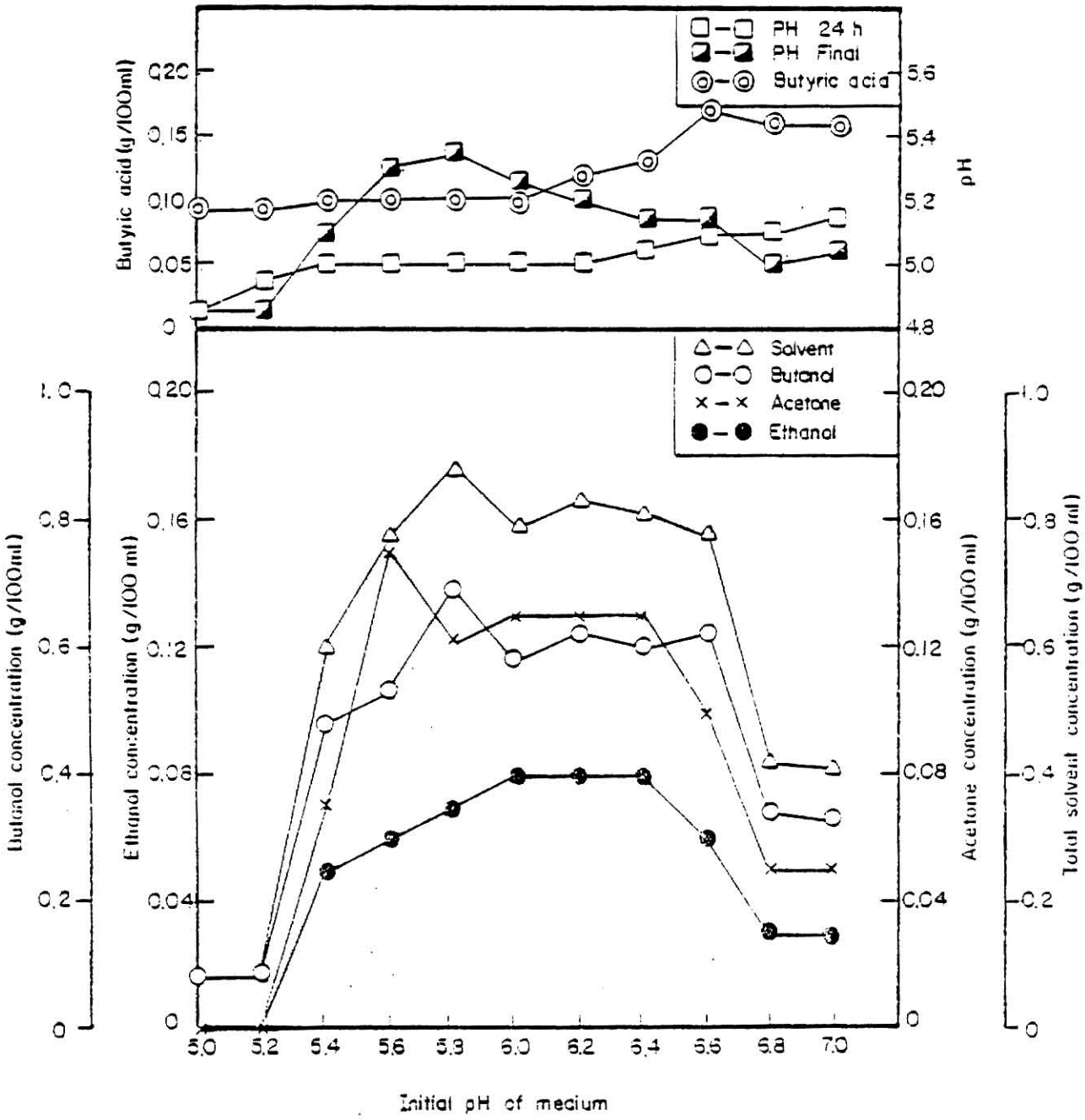

Fig. 2. Effect of the initial pH of medium on solvent production by C. acetobutylicum ATCC 4259.

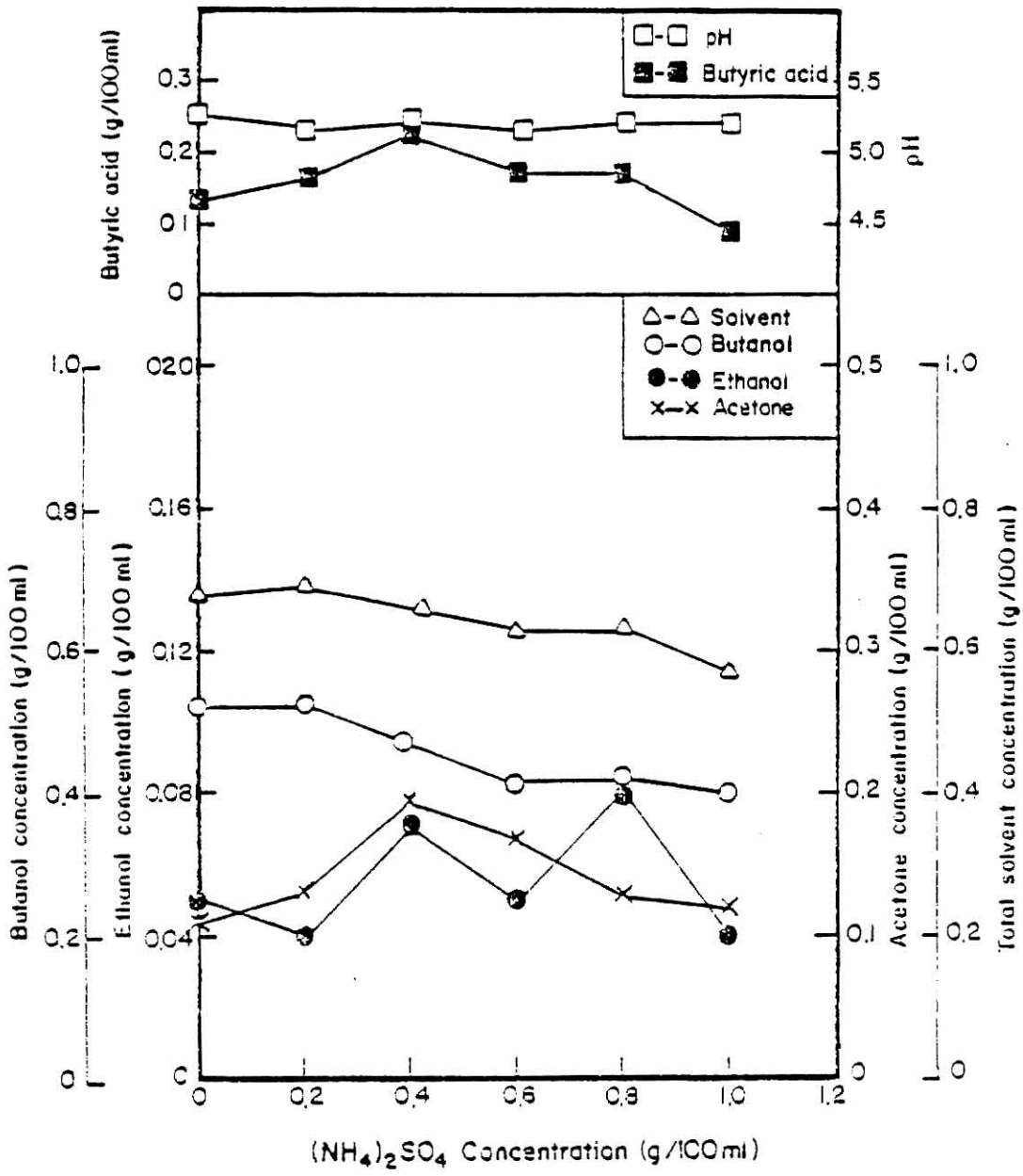

Fig. 3. Effect of the concentration of ammonium sulfate (added before sterilization) on solvent production by C. acetobutylicum ATCC 4259.

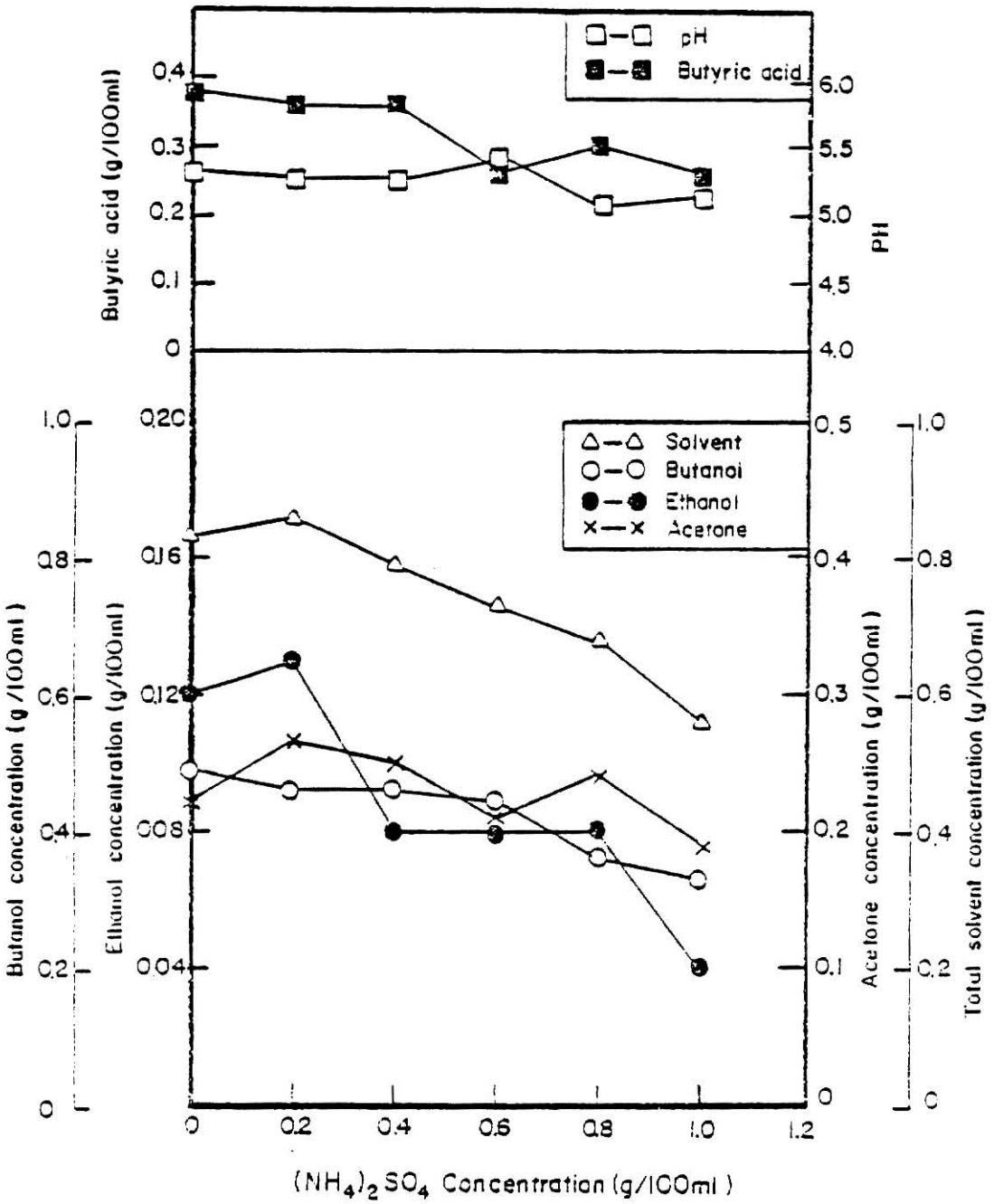

Fig. 4. Effect of the concentration of ammonium sulfate (separate sterilization) on solvent production by *Cl. acetobutylicum* ATCC 4259.

production decreased with a decrease in the ammonium sulfate concentration. Previous workers (McCutchan and Hickey, 1954; Abou-Zeid et al., 1976, 1978) stated that the suitable amount of ammonia to be added to the medium was about 0.7 to 1.7 wt. % of sugar contained in the molasses medium. The amount of ammonia used in this work was in the range of 0.75 to 3.75 wt. % of sugar contained in the medium. The optimum was approximately at 0.8 wt. % which was close to the low end of the reported range, i.e., 0.7 wt. %.

Sterilizing ammonium sulfate separately from the medium gave a remarkable increase in the concentrations of acetone and ethanol. Notice that the butyric acid concentration attained in this scheme was twice as high as that attained when ammonium sulfate was sterilized together with the medium. A higher concentration of butyric acid suggests a potential increase in butanol via a reduction process. It appears that ammonium reacts with reducing sugars during sterilization, and that the product could inhibit fermentation.

Nitrogen is supplied through the use of degraded protein nitrogen, ammonium sulfate, and the use of corn-steep liquor, yeast water, or distillation slops (McCutchan and Hickey, 1954). Although some ammonia may be added to the mash initially to bring the pH to 5.7 to 6.7, most of it is added after the fermentation has proceeded for about 16 hr.

Calcium Carbonate Concentration

The standard basal medium contained 0.2% calcium carbonate; however, various quantities of calcium carbonate were added to create media with different concentrations. As shown in Figure 5, the concentrations of

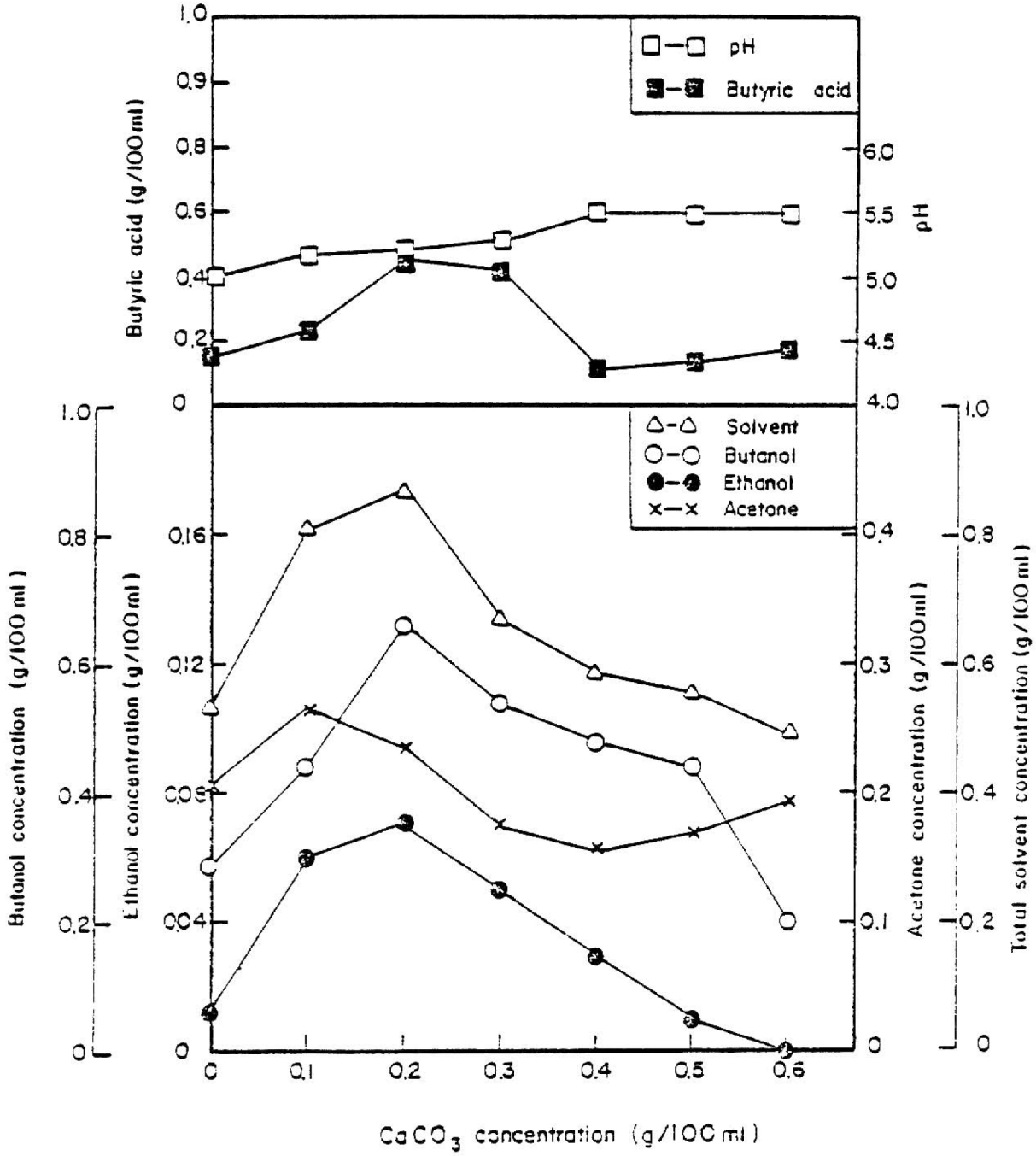

Fig. 5. Effect of $CaCO_3$ concentration on solvent production by C. acetobutylicum ATCC 4259.

solvents and butyric acid increased with an increase in the calcium carbonate concentration, reaching the optimum at 0.2% of the medium; this optimum concentration of calcium carbonate was lower than the published value. The presence of calcium carbonate in the fermentation medium buffered the acidic organic compounds liberated during the fermentation production of acetone-butanol and this led to an increase in the total yield of the solvents. An earlier worker (Fouad et al., 1976) reported that the suitable amount of calcium carbonate in the molasses medium was 5 to 7 wt. % of sugar contained in the medium when ammonium sulfate was also used as nutrient. The amount of calcium carbonate used in this work ranged from 2.6 to 7.9 wt. % of sugar.

Phosphate Concentration

Different amounts of phosphate in the form of KH_2PO_4 were added to the medium. Concentrations were expressed as the equivalent quantities of phosphorus pentoxide; they ranged from 0.05 to 0.3 wt. % of sugar in the medium. No response was detected in this series of experiments. An earlier investigator (Walton and Martin, 1979) reported that 0.2% phosphorus pentoxide was suitable for high test molasses; however, a very small amount of phosphate or none was needed for blackstrap molasses. Sorghum molasses used in this work was found to contain phosphorus pentoxide in the amount of 1.33% of the weight of sugar. According to these results sorghum molasses employed in this work contained sufficient phosphate for the butanol-acetone fermentation.

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CHAPTER 5

FERMENTATION OF UNHYDROLYZED SORGHUM MOLASSES

Incomplete consumption of sugar is often encountered in fermenting multisubstrate media such as molasses and wood hydrolyzates (Chiang et al., 1981; Hsiao et al., 1982). When microorganisms are grown in a mixture of two or more types of sugar, the growth curve may exhibit more than one growth phase, sometimes separated by a distinct lag period, due to an adaptation phase of cells to one of the substrates, if its metabolism requires induced enzyme synthesis (Standing et al., 1972; McGinnis and Paigen, 1969; Stumm-Zollinger, 1966). This phenomenon was first reported by Monod (1947) and was termed as diauxic or polyauxic depending upon the types of sugar present in the mixture.

MATERIALS AND METHODS

Procedure

Microorganism. The strain, Cl. acetobutylicum, was used.

Medium. The optimized medium, containing 140 g/l sorghum molasses, 2 g/l $(NH_4)_2SO_4$, and 2 g/l $CaCO_3$, was used throughout this study.

Cultivation. Fermentation was performed for 1/2 hr at 37°C using a 5 l jar fermentor (New Brunswick Scientific Co., Inc. Model 19) containing 2.85 l of medium and 150 ml of inoculum. The initial pH was adjusted at 6.0. The pH was maintained at 5.0 by adding 5N sodium hydroxide during fermentation.

Analytical Methods

Biomass. 10 ml of broth were centrifuged for 15 min at 3000 rpm in a model IEC-HN-SII centrifuge (International Equipment Company, Needham Heights, Mass.). The supernatant of the culture medium was removed and the cells were resuspended in 10 ml of 2N H_2SO_4 and centrifuged for 15 min at 3000 rpm. The cells were washed again with distilled water, transferred to a filter paper under vacuum condition, and dried overnight at 105°C. Prior to weighing, the filter paper was placed in a desiccator until it attained a constant weight.

Sugar concentration. The same as those listed in Chapter 3.

Solvent and organic acid. The same as those listed in Chapter 3.

Gas flow rate and composition. The gas flow rate was measured using a flowmeter with a micrometervalve (Gilmont Instruments, Inc., Great Neck, New York), having a measuring range between 0.02 and 15 ml/min. Composition of the gas was analyzed by means of a Packard Model 427 gas chromatograph with a column, 1.82 m x 6.3 mm, filled with packings, Porapak N (Waters Associates). The gas was analyzed under the operating conditions of: injector temperature, 100°C; thermal conductivity detector temperature, 100°C; column temperature, 100°C; and carrier gas (nitrogen) flow rate, 80 ml/min.

RESULTS AND DISCUSSION

Diauxy Phenomena and Sugar Utilization

Sorghum molasses contains three different types of sugar, sucrose, glucose and fructose. The sugar composition of blackstrap molasses depends naturally on its origin and source; reportedly (Creelman et al.,

1981), it has approximately the following composition: sucrose, 30% (w/v); glucose, 11% (w/v) and fructose, 11% (w/v). Sorghum molasses used in this work contained 28% (w/v) sucrose, 14% (w/v) glucose and 15% (w/v) fructose. Thus far, the pattern of sugar utilization in molasses by Clostridium acetobutylicum has not been reported, while the solvent yield based on the weight of sugar consumed during fermentation has been given in numerous publications.

Figure 1 shows the sugar utilization and cell growth pattern of Clostridium acetobutylicum ATCC 4259 grown in the sorghum molasses medium. As indicated in this figure, the three types of sugar contained in the medium were consumed sequentially. Glucose was consumed most rapidly and sucrose was slowest. The pattern of cell growth indicates that growth is closely related to the sugar consumption; sucrose was not consumed until glucose disappears completely, and the cell growth was divided into two cycles by a period in which the growth rate was essentially zero or negative. Apparently this pattern of growth was influenced by the diauxie phenomenon reported by Monod (1947); the first exponential phase of cell growth ended almost simultaneously with exhaustion of glucose. This fermentation system was controlled by the so-called catobolite regulatory mechanisms (Russell and Baldwin, 1978).

When microorganisms are grown in multisubstrates, such as molasses and wood hydrolyzate, the phenomenon of diauxic growth and sequential substrate utilization is often encountered. Epps and Gale (1942) first noticed this effect in bacteria in 1942; it was termed the "glucose effect" by Monod (1947) in the late 1940's after he had investigated this phenomenon further. Magasanik (1961) invented the term "catabolite

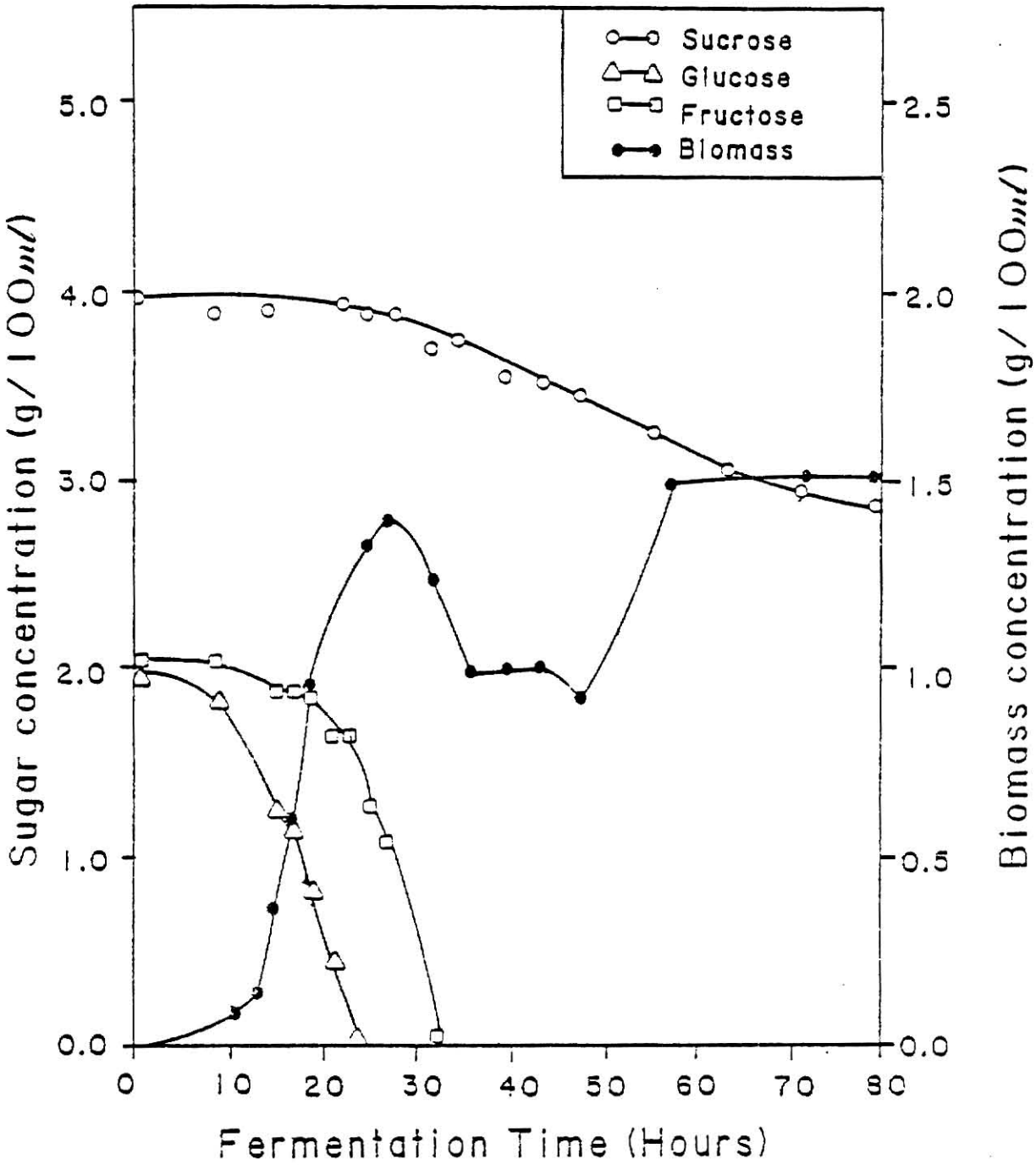

Fig. 1. Relationship between the pattern of sugar consumption and biomass concentration during butanol-acetone fermentation of the sorghum molasses by *C. acetobutylicum* ATCC 4259.

repression" to describe it when he discovered that substrates other than glucose could produce the same effect.

Glucose and fructose were consumed in parallel after the middle stage of exponential growth phase. However, sucrose started to be consumed almost immediately after the disappearance of fructose. The biomass concentration also decreased immediately after the fructose was consumed completely.

The results described in the above paragraph indicate that diauxie phenomenon can be clearly identified between the consumption of inverted sugar and that of sucrose, but it is not the case between the consumption of glucose and that of fructose. Therefore, apparently the second exponential growth of Clostridium acetobutylicum is effected purely at the expense of sucrose. The results also seem to suggest that the sucrose consumption system of the cell is inhibited by the presence of glucose and fructose, presumably more strongly by the former than by the latter, and sucrose, glucose and fructose compete with each other during their transport across the cell membrane (McGinnis and Paigen, 1969). The reduction in the biomass concentration during the transitional period between the two exponential growth cycles is possibly due to cell autolysis caused by the inability of the microorganism to consume sugar in the medium.

Solvent Production

The solvent production pattern of sorghum molasses fermentation was closely related to the cell growth pattern, as shown in Figure 2. However, it is worth noting that the rate of solvent production recovered slightly ahead of the rate of cell growth after termination of the transitional phase.

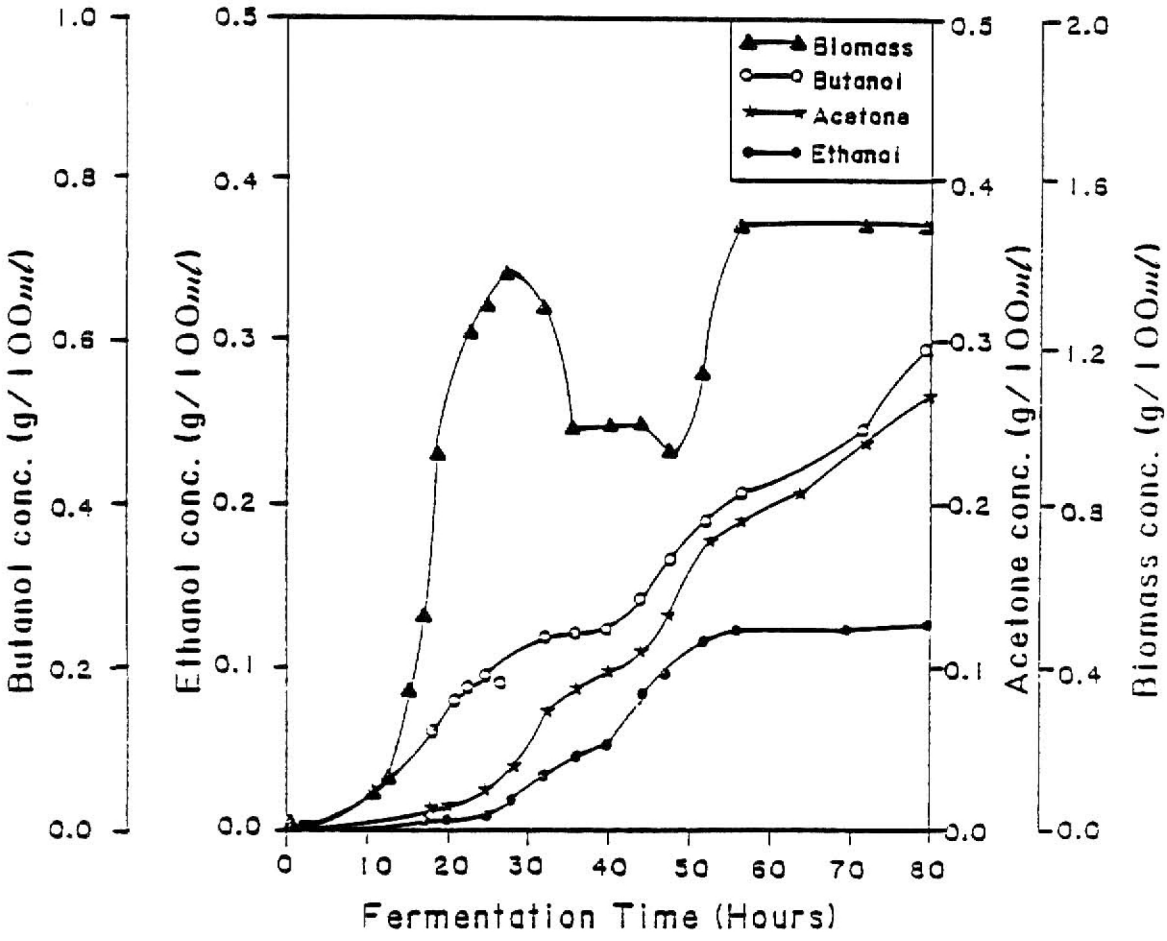

Fig. 2. Biomass and solvent concentrations during butanol-acetone fermentation of the sorghum molasses medium by *C. acetobutylicum* ATCC 4259.

The development of butanol-acetone fermentation from molasses has apparently been influenced by the diauxie phenomenon. Earlier investigators (Ono, 1943; Toi, 1943) reported that the intermediate or side products from sugar refining, e.g., white sugar, raw sugar, sugar juice (sugar cane or sugar beet), inverted molasses and blackstrap molasses, gave rise to different solvent yields. The solvent yield increased with an increase in the extent of purification; white sugar, being the most purified, gave the highest yield and blackstrap molasses, with the lowest extent of purification, gave the lowest yield.

The composition of sugar juice is very different from that of inverted molasses. While sucrose is the major component in sugar juice, it is only a minor component of inverted molasses. From the standpoint of the diauxie phenomenon, it appears that in the production of butanol-acetone the role of the minor sugar component in sugar juice is different from that in inverted molasses. The minor component in sugar juice, inverted sugar, is consumed prior to the major component, sucrose; this implies that the sugar juice medium is a simple substrate system, namely, a non-competitive system, from the standpoint of the diauxie phenomenon, in a comparatively short time, the viability of the cell to remain high when it starts consuming sucrose. However, because inverted sugar is the major component in inverted molasses, this medium remains a multiple substrate system, namely, a competitive system, for a relatively long time when inverted molasses is used as substrate; this elongation of the competitive period makes the cell less vital, and consequently, its comsumption rate of less-competitive sugar becomes low when inverted sugar is largely digested.

The final solvent concentrations in the medium were: butanol, 0.59% (w/v); acetone, 0.28% (w/v) and ethanol, 0.13% (w/v). The solvent yields based on the weight of sugar consumed were: butanol, 11.8%; acetone, 5.6%; and ethanol, 2.6%. The total solvent yield was 20.0%.

Gas Production

Hydrogen and carbon dioxide are produced in the butanol acetone fermentation (Doelle, 1975; Moreira, 1981). Gas production from the sorghum molasses medium, realized in this work is illustrated in Figure 3. The total gas yield in terms of the volume was almost nineteen times the volume of broth. At the early stage of fermentation, the cumulative volume of hydrogen gas produced was larger than the cumulative volume of carbon dioxide. Subsequent to 18 hours, however, the latter exceeded the former. The ratio of hydrogen and carbon dioxide in the cumulative total gas production was 48/52. The cumulative total gas production or yield and the ratio between hydrogen and carbon dioxide were close to those from the corn meal medium, as reported by an earlier investigator (Peterson and Fred, 1932).

As can be seen in Figure 4, certain relationships apparently exist between the gas production rate and biomass concentration, and between the gas production rate and sugar consumption pattern. The curve for the gas production rate ran four to ten hours ahead of that for the biomass concentration up to 44 hours of fermentation. Therefore, the two curves ran in parallel. Furthermore, the curve for the gas production rate exhibited a significant change at the time each kind of sugar disappeared.

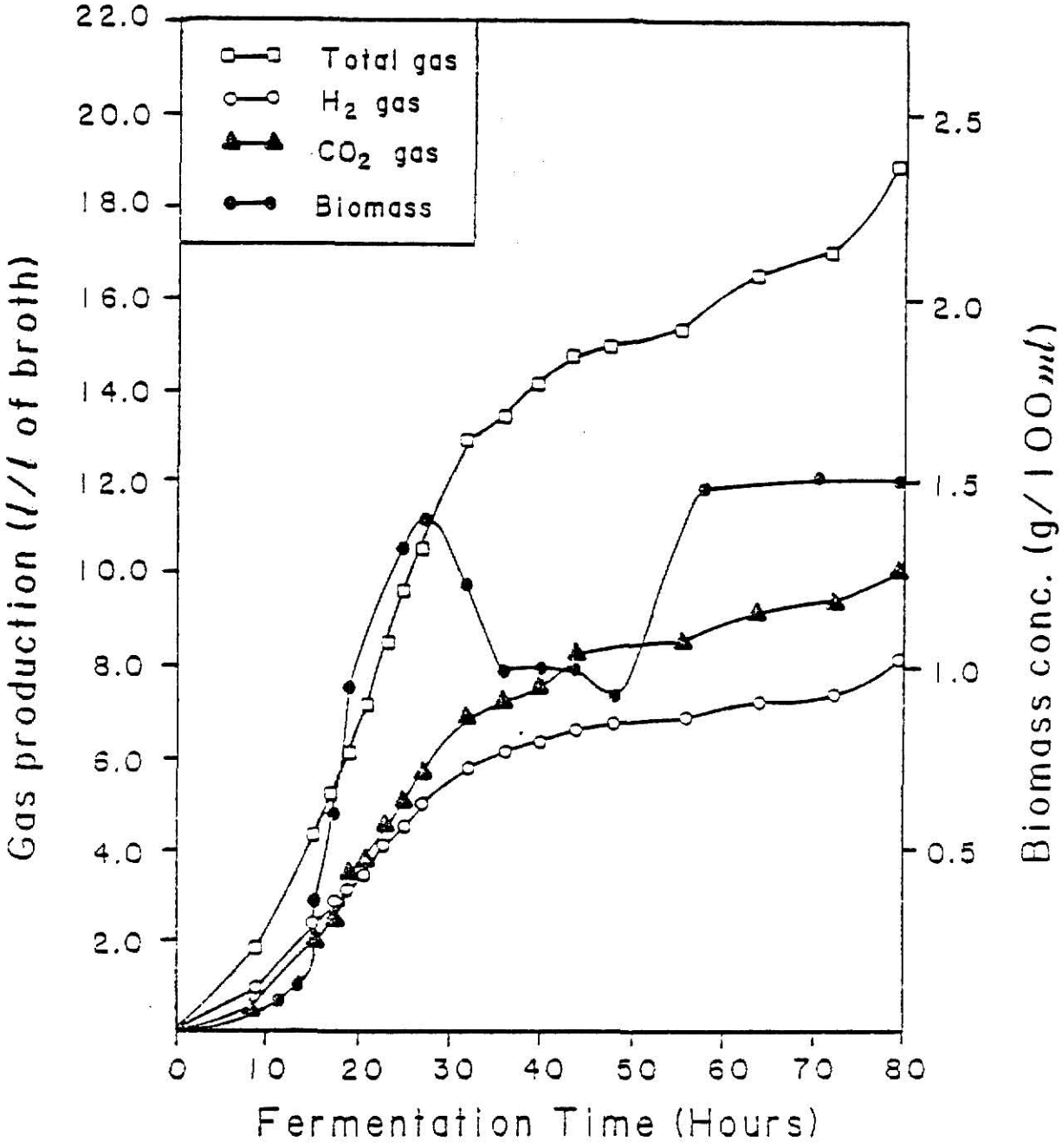

Fig. 3. Cumulative gas production and biomass concentration during butanol-acetone fermentation of the sorghum molasses medium by *Cl. acetobutylicum* ATCC 4259.

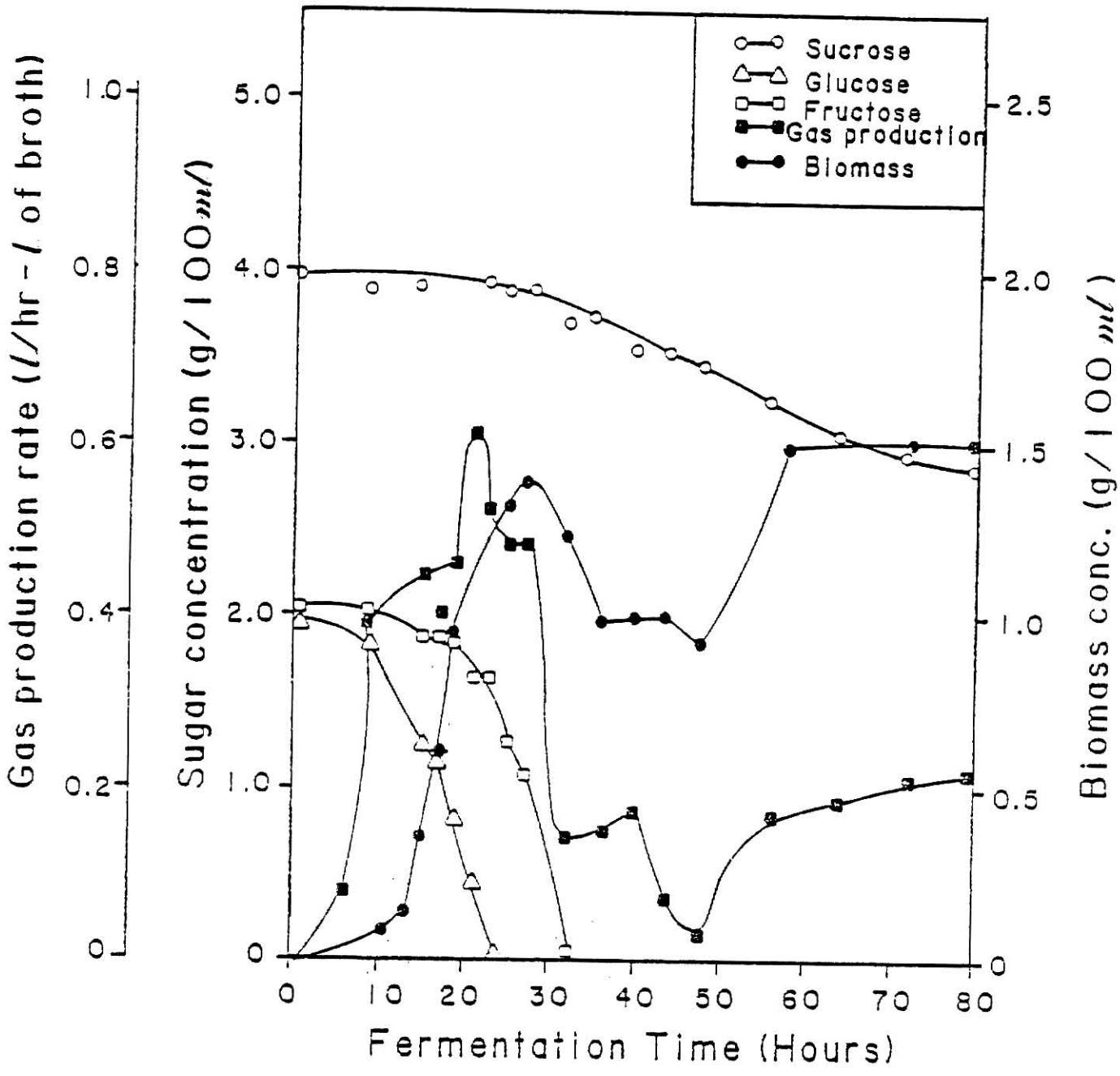

Fig. 4. Relationship among the biomass concentration, sugar consumption and gas production rate during butanol-acetone fermentation of the sorghum molasses medium by C/. acetobutylicum ATCC 4259.

The present results indicate that it is possible to predict the biomass concentration or cell growth rate from the gas production rate and to detect the time when a particular type of sugar is consumed completely in a multiple substrate system, e.g., molasses. The present results also suggest that control of the sugar concentration in such a multiple substrate system can be achieved through measurements of the gas production rate in fed batch fermentation. So far not much has been published on the instantaneous flow rate measurement with a "microflowmeter" during butanol-acetone fermentation in a flask or small scale fermentor.

Organic Acid Production

It has been known (Wood et al., 1945; Twarog and Wolfe, 1962) that butyric acid and acetic acid play an important role in producing butanol-acetone by the strain Clostridium acetobutylicum. The Weizmann type microorganism has been reported to produce a high concentration of butyric acid and that of acetic acid in the corn meal medium up to 20 hr of fermentation; between 20 and 30 hr of fermentation the acid concentration decrease sharply (Davies, 1942); and they increase again slowly from the minimum point at around 30 hr to the end.

In the present work, as shown in Figure 5, the concentrations of both acetic and butyric acids increased sharply until 23 hr of fermentation when glucose was consumed completely; thereafter, the acid concentrations continued to increase, but with decreasing rates. However, no distinct breakpoint could be detected in either acid concentration-time curve, as indicated in the references cited in the preceding paragraph. It has been reported (Gottschalk and Bahl, 1981) that the accumulation

of organic acids is indicative of a rapid increase in the solvent concentration that will follow. Our results, as given in Figure 5, appear to suggest that the diauxie phenomenon affects largely the concentrations of acetic and butyric acids; the concentration-time curves of both acids seem to be affected by the complete consumption of glucose. The final concentrations of organic acids in sorghum molasses were: butyric acid, 0.75% (w/v) and acetic, 0.34 (w/v).

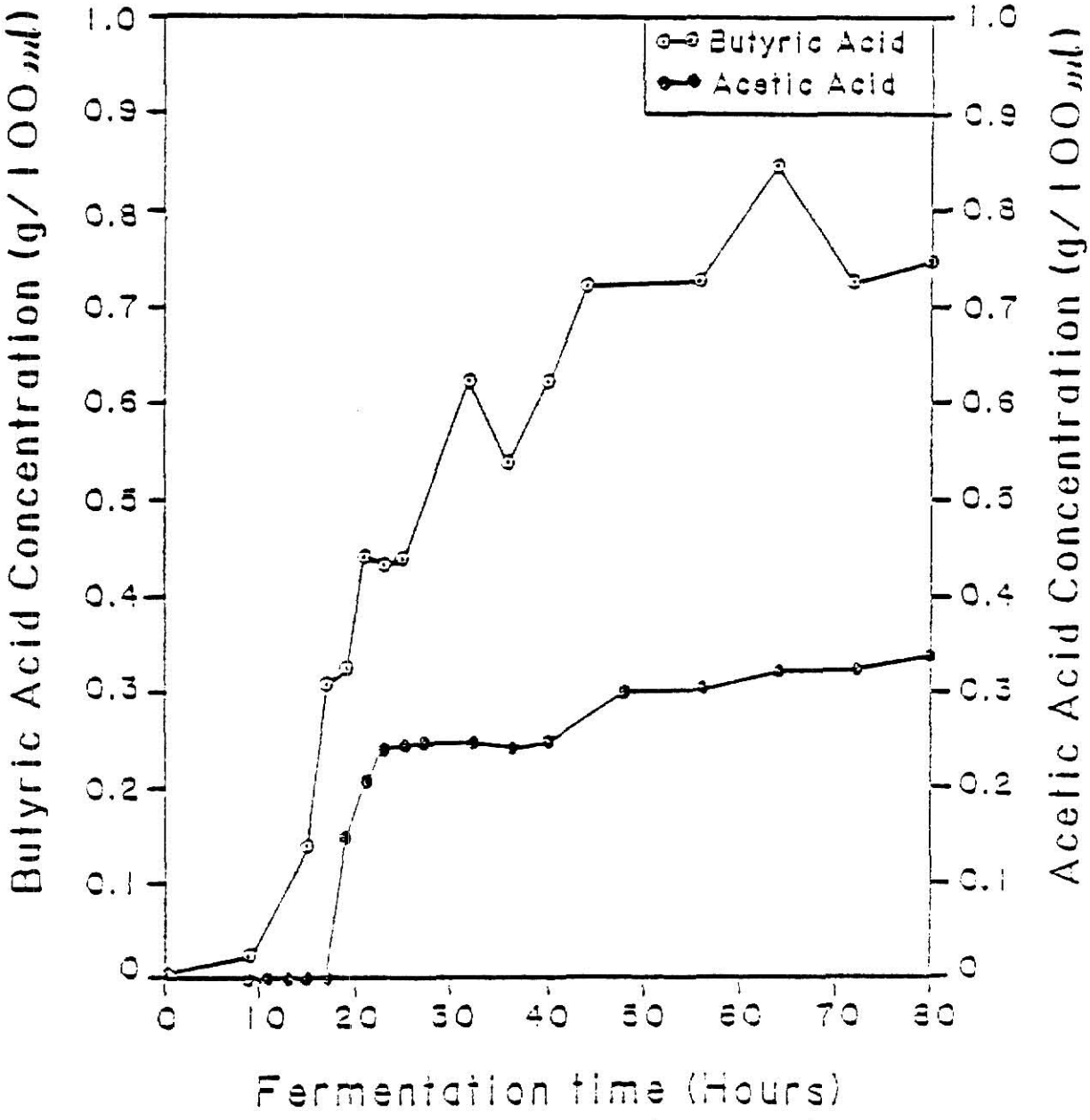

Fig. 5. Organic acid production during butanol-acetone fermentation of the sorghum molasses by *C. acetobutylicum* ATCC 4259.

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CHAPTER 6

FERMENTATION OF INVERTED SORGHUM MOLASSES

The results presented in the preceding chapters indicate that butanol-acetone fermentation of sorghum molasses is inhibited by a diauxie phenomenon, i.e., leaving sucrose in the medium incompletely consumed, and that the effect of this phenomenon can be minimized by inverting sorghum molasses by means of acid hydrolysis.

Acids have widely been employed for hydrolysis of sucrose contained in sugar juice (Olbrich, 1963) or molasses (Junk and Pancoast, 1973). Inverted molasses, the so-called high test molasses, has been produced from evaporating sugar juice previously inverted by acid. In this case the hydrolysis temperature is comparatively mild (83-95^{o}C); however, the hydrolysis time is relatively long (90 min). The content of inverted sugar in acid-hydrolyzed sugar juice is only two thirds of the total sugar contained in the unhydrolyzed sugar juice.

When inverted sugar juice is employed as the substrate for butanol-acetone fermentation, it should be sterilized. During sterilization, inverted sugar is partly converted into other types of sugars through a mutual conversion process, and simultaneously it possibly is decomposed into pigment compounds, mostly caramel and melanoidines; the pigment hinders the butanol-acetone fermentation.

In the present work, the simultaneous process, involving hydrolysis and media sterilization, has been designed to protect the fermentation media from forming substances detrimental to the fermentation.

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MATERIALS AND METHODS

Procedure

Microorganism. The strain, Cl. acetobutylicum, was used.

Medium. The medium, containing 140 g/l sorghum molasses, 2 g/l $(NH_4)_2SO_4$, and 2 g/l $CaCO_3$ was hydrolyzed. The hydrolysis of sorghum molasses was carried out at 120°C for 30 min, and was performed simultaneously with the media sterilization. Sulfuric acid and hydrochloric acid were used for this purpose. The pH of both acids were between 1.0 and 4.0. Sodium hydroxide was added to the medium to neutralize the acids. The initial concentrations of different kinds of sugar in the resultant medium were: sucrose, 0.6% (w/v); glucose, 3.1% (w/v); and fructose, 3.7% (w/v).

Cultivation. The same as those listed in Chapter 5.

Analytical Methods

The same as those listed in Chapter 5.

RESULTS AND DISCUSSION

Sugar Utilization and Cell Growth

In the fermentation of sorghum molasses described in the preceding subsection, it was found that the diauxie phenomenon exerted a significant influence on the pattern of sugar consumption and cell growth. However, as illustrated in Figure 1, there was no indication of the existence of the diauxie phenomenon in the fermentation of inverted sorghum molasses, even though the biomass concentration decreased immediately after glucose was completely consumed. No transitional phase could be identified in the pattern of biomass concentration. The biomass

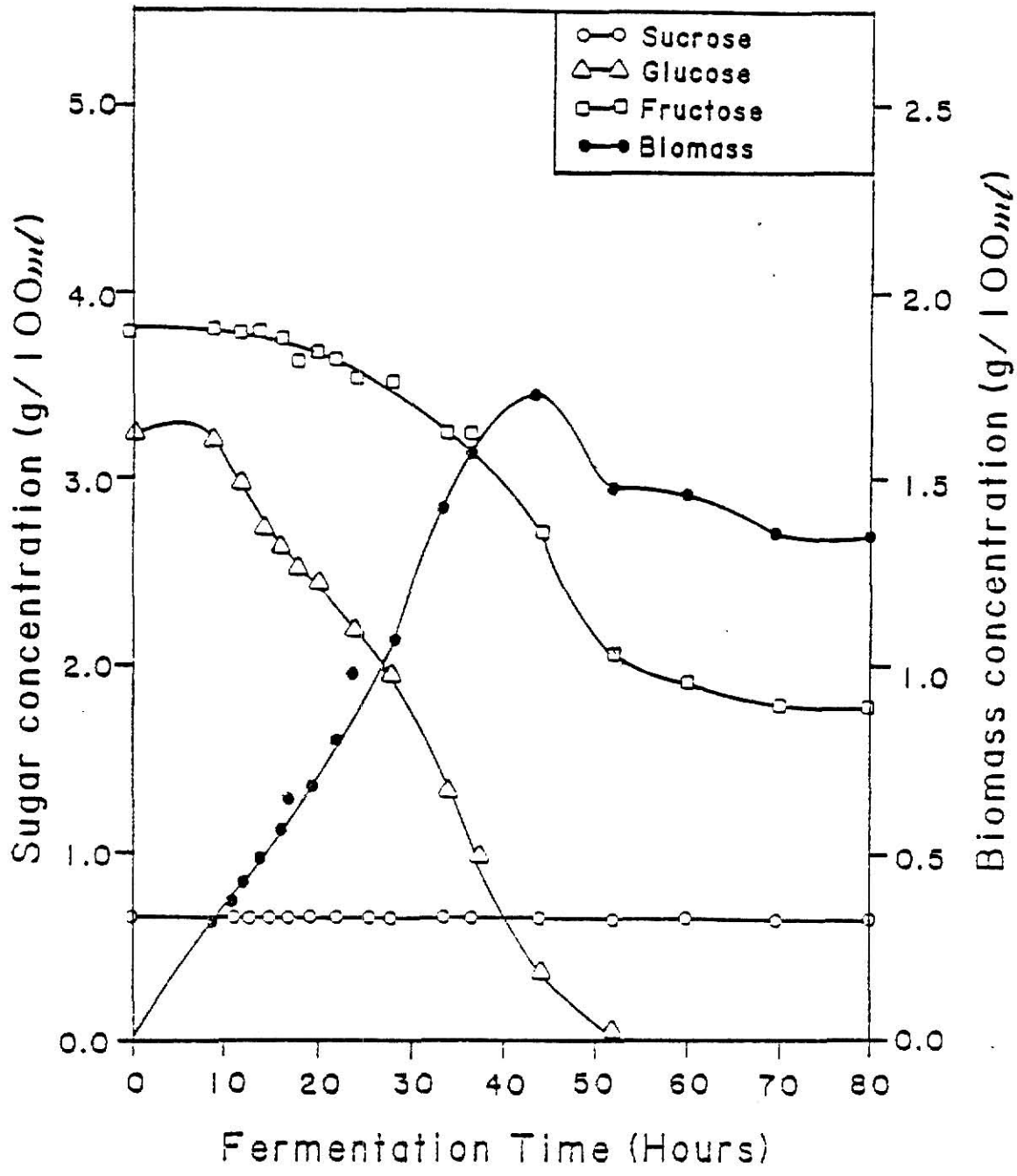

Fig.1. Relationship between the pattern of sugar consumption and biomass concentration during butanol-acetone fermentation of the inverted sorghum molasses by C. acetobutylicum ATCC 4259.

concentration in the inverted sorghum molasses medium increased with a decrease in the concentration of glucose remaining in the medium up to 44 hr of fermentation, indicating that the exponential growth time of the cell in the inverted sorghum molasses medium was much longer than that in the sorghum molasses medium, and that the biomass concentration in the former was much higher than that in the latter. This pattern of cell growth and sugar consumption is apparently beneficial to the butanol-acetone fermentation.

The apparent lack of a diauxie phenomenon between glucose and fructose was also indicated by the fact that fructose was consumed simultaneously with glucose. If the diauxie phenomenon occur during fermentation, the less favored sugar, in this case fructose, is not consumed during the consumption of the more favored sugar, in this case glucose. The consumption of the former should begin only after the complete consumption of the latter.

In spite of the apparent lack of a diauxic phenomenon between the two monosaccharides, glucose and fructose, the results, as presented in Fig 1, strongly indicate that competition for consumption indeed exists between the monosaccharides, and sucrose contained in the medium is hardly consumed during the fermentation, even after one of the monosaccharides, glucose, is essentially completely utilized. The results depicted in the preceding paragraph imply that competition exists between sucrose and fructose, and from the standpoint of consumption by microorganism, fructose is more favorable than sucrose after the total removal of glucose from the medium. Even though competition for consumption between sucrose and fructose is active in the multiple substrate system

under discussion, i.e., inverted sorghum molasses, it will not affect the solvent yield significantly because the sucrose concentration is comparatively low in such a medium. In fact, the remaining sucrose can be completely inverted into monosaccharides if the hydrolysis is optimized, so that products of acid hydrolysis will not detrimentally affect the outcome of fermentation. On the other hand, a decrease in the rate of fructose consumption immediately after removal of glucose, concomitant with a decrease in the biomass concentration, is probably affected by the existence of another control mechanism, most likely the end product inhibition during fermentation.

Solvent Production

Unlike the fermentation of sorghum molasses, as shown in Figure 2, no transitional phase existed in the solvent production pattern when inverted sorghum molasses was fermented. The rate of solvent production was almost in parallel to the rate of biomass production up to the point where the biomass concentration attained its peak. Subsequently, the rate of solvent production decreased with a decrease in the biomass concentration. As stated in the preceding paragraph, the end product inhibition was probably responsible for a gradual decrease in the solvent production rate immediately after glucose was completely consumed.

The final solvent concentrations in the inverted molasses medium were: butanol, 1.5% (w/v); acetone, 0.37% (w/v); ethanol, 0.18% (w/v); and the total solvent 1.55% (w/v). The solvent yield based on the weight of sugar consumed was: butanol, 19.6%; acetone, 7.2%; ethanol, 3.4%; and the total solvent 30.3%.

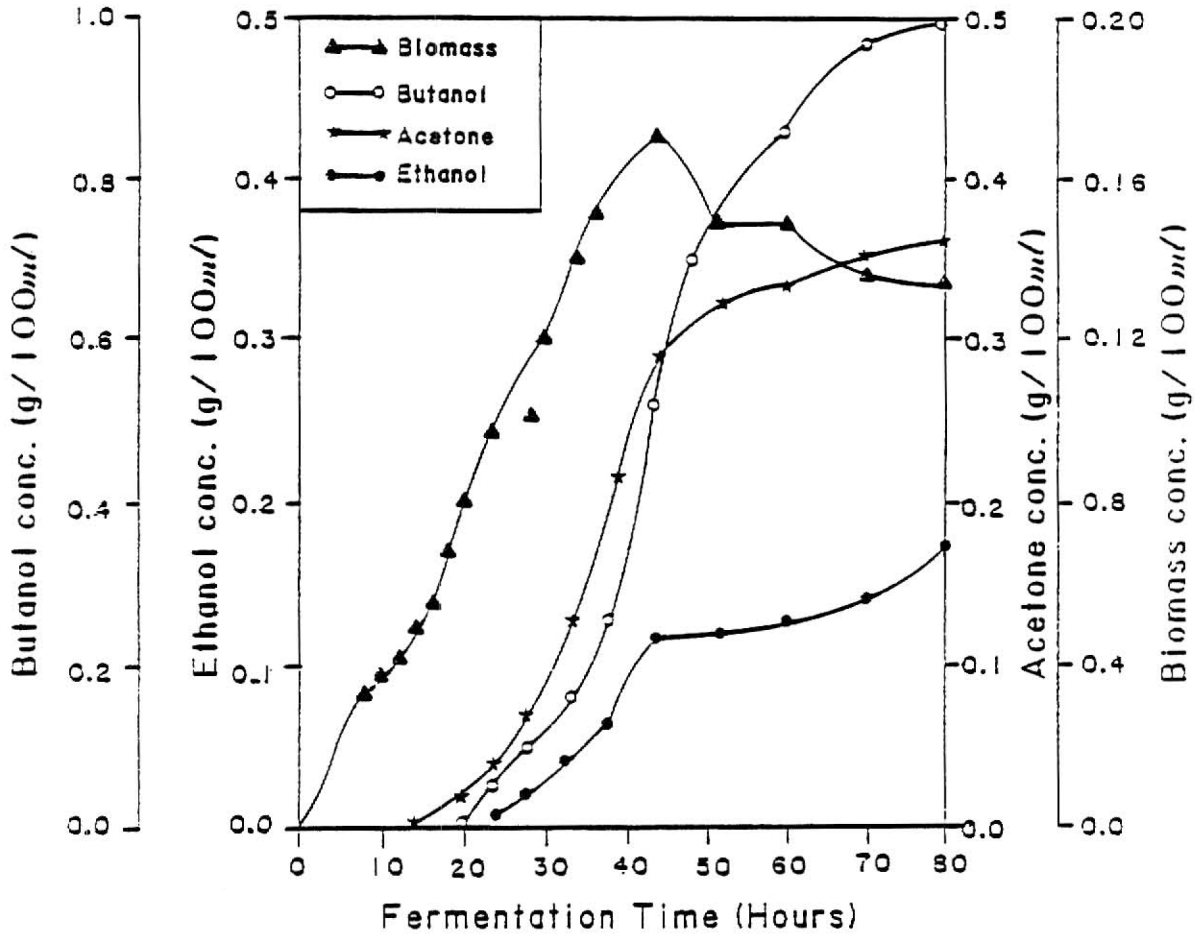

Fig. 2. Biomass and solvent concentrations during butanol-acetone fermentation of the inverted sorghum molasses medium by *Cl. acetobutylicum* ATCC 4259.

The final solvent concentration and solvent yield based on the weight of sugar consumed in the inverted sorghum molasses medium were more than 50 percent higher than those in the sorghum molasses medium. The solvent yield of inverted sorghum molasses was almost the same as that of high test molasses employed in the past (McCutchan and Hickey, 1954); however, the concentration of total solvent was about 80 percent of that reported for high test molasses. The difference in the solvent concentration between sorghum molasses and high test molasses was probably due to the fact that the latter gave rise to a higher extent of sugar consumption than the former. Apparently, the consumption could be increased by optimizing the sugar concentration in the inverted sorghum molasses medium and by completely inverting sucrose in the medium.

Gas Production

Gas production from the inverted sorghum molasses medium is illustrated in Fig 3. The total gas yield in terms of volume was 18.4 times of the broth and the cumulative volume of carbon dioxide exceeded that of hydrogen at 28 hr of fermentation. This point was 10 hr behind the corresponding point for the sorghum molasses. The ratio of cumulative volumes of hydrogen and carbon dioxide at the end of fermentation was 4/6. Note that even though the total cumulative volumes of the gases produced from sorghum molasses and inverted sorghum molasses were very similar, it is highly probable that the ratio of the cumulative volumes of hydrogen to carbon dioxide from each of these media is closely related to the solvent yield. It has been suggested (Gavard et al., 1957; Twarog and Wolfe, 1962; Valentine and Wolfe, 1960; Zerner et al., 1966)

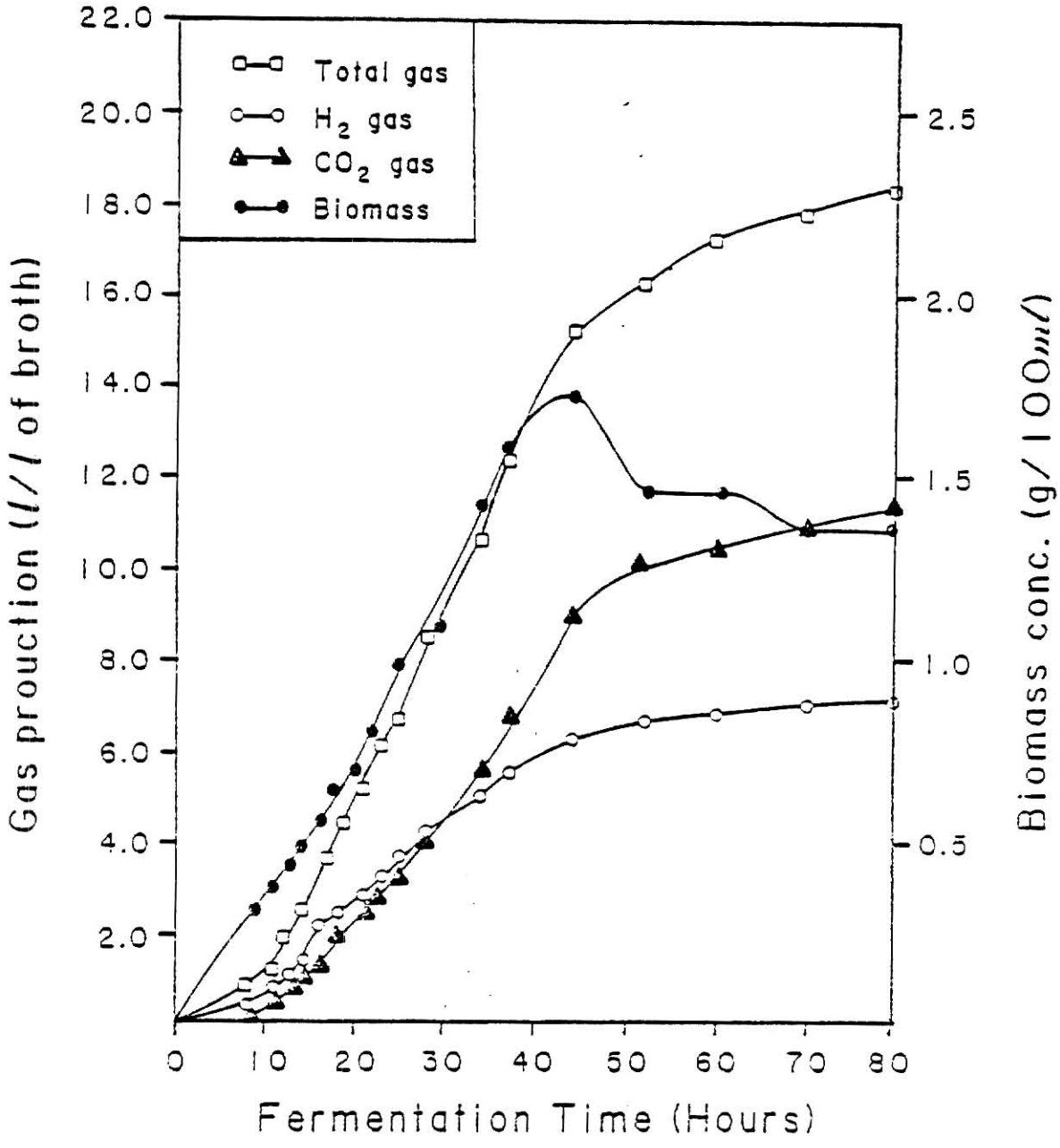

Fig. 3. Cumulative gas production and biomass concentration during butanol-acetone fermentation of the inverted molasses medium by *Cl. acetobutylicum* ATCC 4259

that hydrogen plays the role of an electron donor in converting butyric acid to butanol. Therefore, it could be expected that the sorghum molasses medium would produce more hydrogen gas than the inverted sorghum molasses medium. As indicated earlier in connection with Fig 3 in Chapter 5, the total cumulative volume gradually decreased with a decrease in the biomass concentration.

The gas production rate from the inverted sorghum molasses medium is shown in Fig 4. As mentioned in the preceding paragraph in connection with the sorghum molasses fermentation, the gas production rate moved ahead of the biomass concentration up to around 50 hr of fermentation. The gap between the gas production rate and biomass concentration gradually decreased with the maximum gap to about 8 hr occurring at the time when the gas production rate moved ahead of the biomass concentration. Subsequently, the gas production rate and biomass concentration were almost synchronous. Similar to the sorghum molasses medium, the gas production rate from the inverted sorghum molasses medium also drastically decreased around the point where glucose was consumed completely.

These results suggest that it is possible to predict the biomass concentration or cell growth rate from the gas production rate and to detect the time when a particular type of sugar disappears not only in the sorghum molasses medium but also in the inverted sorghum molasses medium.

Organic Acid Production

Figure 5 illustrates the organic acid production pattern in the inverted sorghum molasses medium. A comparison of this figure with

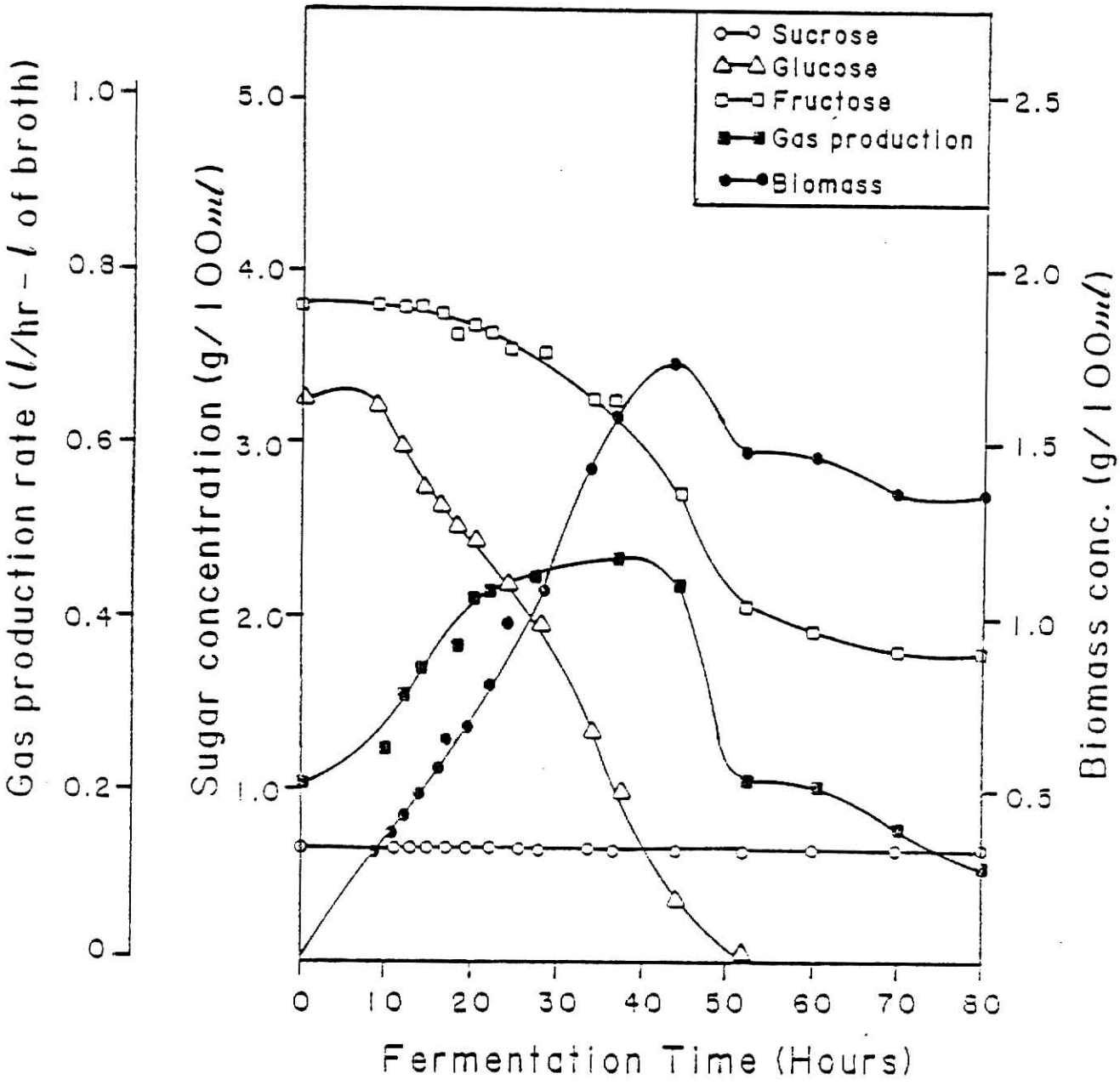

Fig. 4. Relationship among the biomass concentration, sugar consumption and gas production rate during butanol-acetone fermentation of the inverted sorghum molasses medium by *C. acetobutylicum* ATCC 4259.

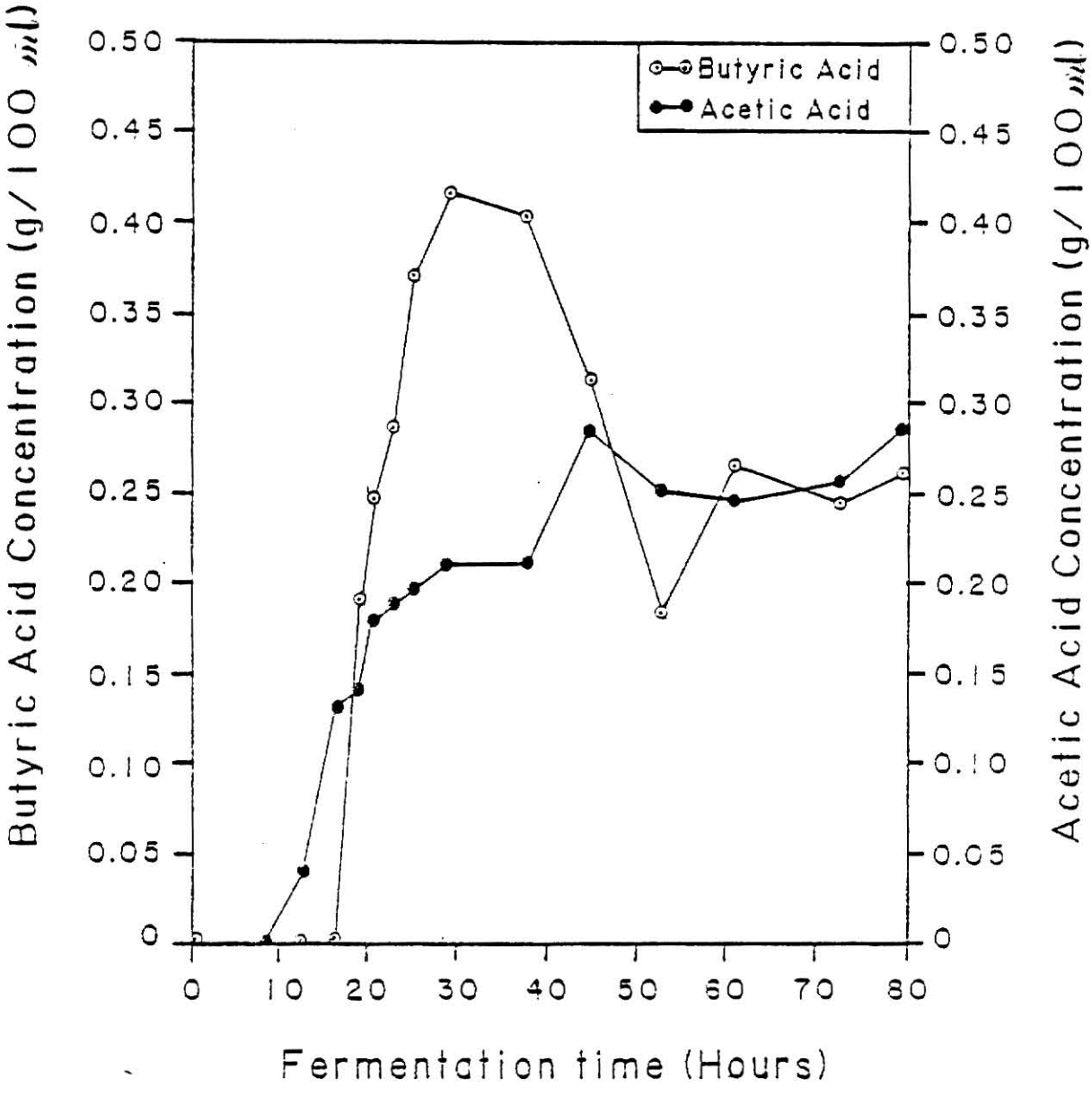

Fig. 5. Organic acid production during butanol-acetone fermentation ofthe inverted sorghum molasses by Cl. acetobutylicum ATCC 4259.

Fig 5 in Chapter 5 shows that there is a significant difference between the pattern of butyric acid production rate from the sorghum molasses medium and that from the inverted sorghum molasses medium. In the sorghum molasses medium, both butyric acid and acetic acid concentrations increased sharply at around 20 hr of fermentation; then they increased gradually. In the inverted sorghum molasses medium, however, the butyric acid concentration-time curve exhibited a distinct peak at about 28 hr of fermentation, and subsequently it declined to 53 hr, and then it increased again slightly to the end. The highest butyric acid concentration in the inverted sorghum molasses medium was 0.42% (w/v). These results seem to suggest that the peak in the butyric acid concentration-time curve is closely related to the production of butanol as reported by earlier investigators (Beesch, 1953; Davies and Stephenson, 1941). Their works indicate that at the later stage of fermentation, the rate of conversion of butyric acid into butanol becomes higher than the rate of production of butyric acid. Consequently, the increase in the butyric acid concentration ceases; it eventually decreases giving rise to a peak. The acetic acid concentration-time curve in the inverted sorghum molasses medium is very similar to that of the sorghum molasses medium. The final concentration of acetic acid in inverted sorghum molasses was 0.28% (w/v), which was lower than the 0.34% (w/v) of sorghum molasses.

Effect of Hydrolysis Condition

As described previously, sorghum molasses differs from inverted sorghum molasses in the pattern of sugar utilization by Clostridium

acetobutylicum. Figures 6 and 7 show the pattern of sugar utilization at different pH in the sorghum molasses medium inverted by sulfuric acid and hydrochloric acid, respectively. The two acids exhibited very similar patterns of sugar consumption except at a relatively low pH. The amount of sugar consumed by the organism between pH 2.0 and 3.0 was relatively high. The results indicate that no appreciable differences exist in the acids' hydrolyzing capacities at the same pH. The extent of hydrolysis ranged from 17% at pH 4.0 to 80% at pH 1.0. Much of the sucrose contained in sorghum molasses was hydrolyzed.

The substances detrimental to the fermentation could possibly be produced during acid hydrolysis at low pH. Moreover, it is possible that sugar utilization by the microorganism would be influenced by the concentration of salt produced by hydrolysis and neutralization. No sucrose contained in the unhydrolyzed sorghum molasses medium was consumed; apparently this was caused by the diauxie phenomenon. As indicated in Table 1, the solvent yield from molasses inverted by hydrochloric acid was far less than that inverted by sulfuric acid; however, more butyric acid were produced from the former than the latter. The solvent yields were comparatively high in the pH range between 2.0 and 3.0 for both acids. Note that not only the concentration, but also the type of salt could influence the solvent yield.

In spite of the fact that remarkable increases in the sugar utilization and solvent yield have been attained in the inverted molasses medium, much remains to be done to improve them; while the solvent yield based on the sugar consumed is reasonably high, it is rather low on the basis of the initial sugar available in the medium (see Table 1). These results

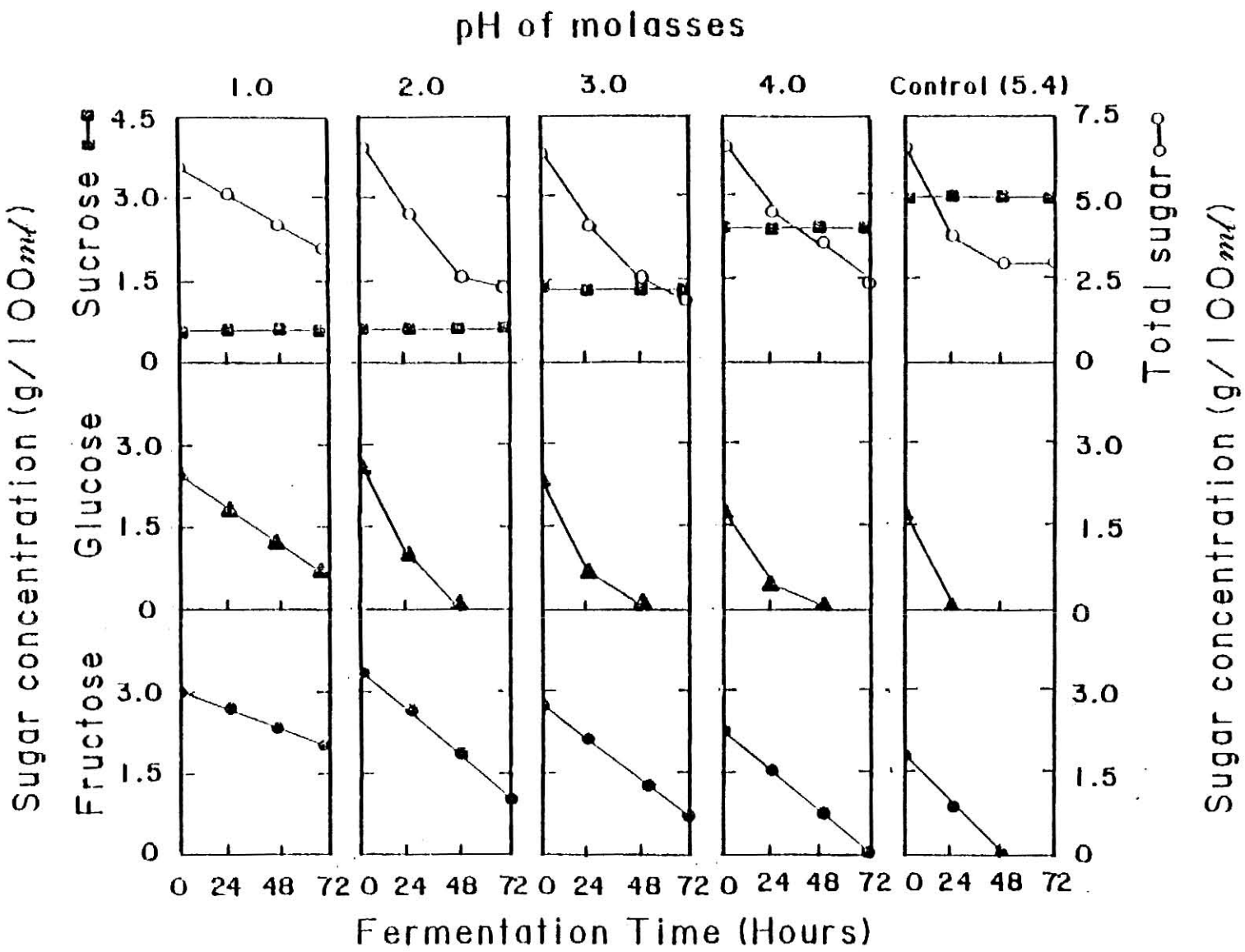

Fig. 6. Effect of pH of sorghum molasses during acid hydrolysis under the sterilization condition on sugar consumption pattern in butanol-acetone fermentation by *Cl. acetobutylicum* ATCC 4259 (Molasses was acidified with conc. H_2SO_4).

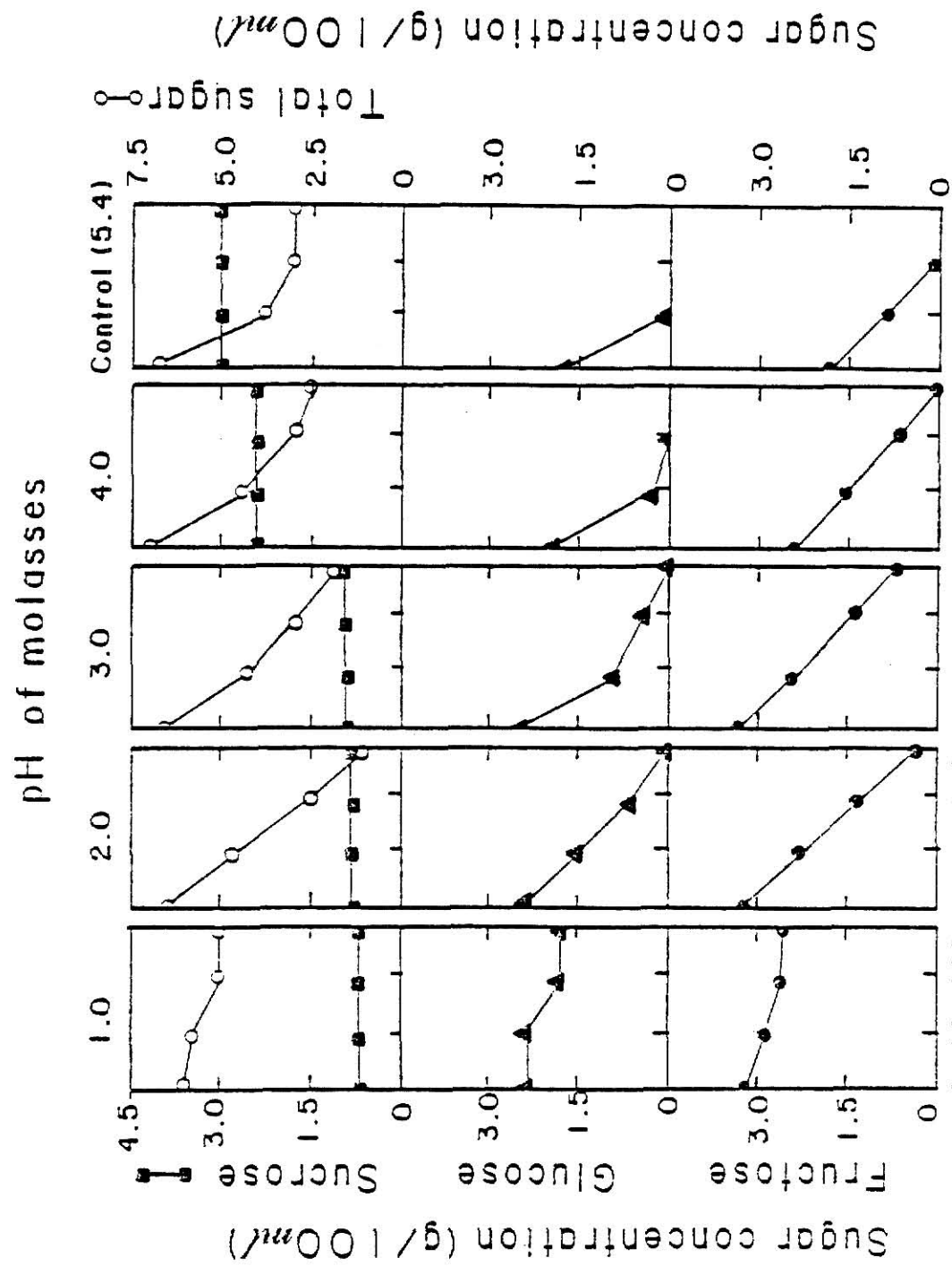

Fig. 7 Effect of pH of sorghum molasses during acid hydrolysis under the sterilization condition on sugar consumption in butanol-acetone fermentation by *Cl. acetobutylicum* ATCC 4259

show that the solvent yield of inverted sorghum molasses by Cl. acetobutylicum can be increased by carefully selecting a proper hydrolysis condition, e.g., pH, temperature, or time. From the standpoint of osmotic pressure, ammonium hydroxide, and calcium carbonate, which are related to the nutrient of the medium, can be used as neutralizing agents to prevent formation of excessively concentrated salt solution.

Effect of Temperature

Table 2 shows the effect of temperature on solvent yield in the inverted molasses fermentation. It can be observed that the optimum fermentation temperature is in the range of 30°C to 35°C. Our results are in good agreement with available information (Walton and Martin, 1979).

Effect of Agitation on Solvent Yield

Fermentation experiments were carried out under different agitation rates of 0 RPM, 100 RPM, and 200 RPM (O'Brien and Morris, 1971). The jar fermentor scale fermentation was performed as described in the Materials and Methods section. Figure 8 shows the total volume of gas evolved during the fermentation experiments at different agitation rates as functions of time. Note that the increase in the rate of agitation tends to enhance gas production appreciably. The total volume of gas released varied from 18.4 liters for 0 RPM to 24.0 liters for 200 RPM. The available information (Walton and Martin, 1979) indicates that the total gas production per gram of glucose can be as high as 0.44 liters which is equivalent to 22 liters for the condition of 0 RPM. This is comparable to our result. The corresponding levels of butanol and butyric acid observed in the fermentation broth at 0 and 200 RPM are

Table 1. Effect of pH of sorghum molasses during the hydrolysis by two different acids on solvent and butyric acid concentration, and yield.

Productivity	Acid	pH 1.0	pH 2.0	pH 3.0	pH 4.0	No Hydrolysis
Total Solvent Concentration (g/100mℓ)	H_2SO_4	0.75	1.10	1.15	0.97	0.63
	HCℓ	0.10	0.78	0.79	0.77	
Yield (%) $\frac{\text{Solvent Produced (g)}}{\text{Sugar Consumed (g)}} \times 100$	H_2SO_4	25.8	26.2	26.1	23.9	21.2
	HCℓ	10.5	15.0	15.5	18.0	
Yield (%) $\frac{\text{Solvent Produced (g)}}{\text{Sugar (g)}} \times 100$	H_2SO_4	11.5	16.9	17.6	14.9	9.6
	HCℓ	1.5	12.0	12.1	11.8	
Butyric Acid Concentration (g/100 mℓ)	H_2SO_4	0.38	0.33	0.34	0.22	0.33
	HCl	0.26	0.65	0.62	0.43	

Table 2. Effect of the temperature on the solvent production and yield during butanol-acetone fermentation of the inverted sorghum molasses.

Fermentation temperature (°C)	Production (g/100 mℓ)				Yield based on Sugar(%)
	Acetone	Ethanol	Butanol	Total	
25	0.30	0.21	0.85	1.36	26.7
30	0.35	0.18	1.0	1.52	29.8
35	0.37	0.18	1.0	1.55	30.3
40	0.28	0.17	0.79	1.24	24.3

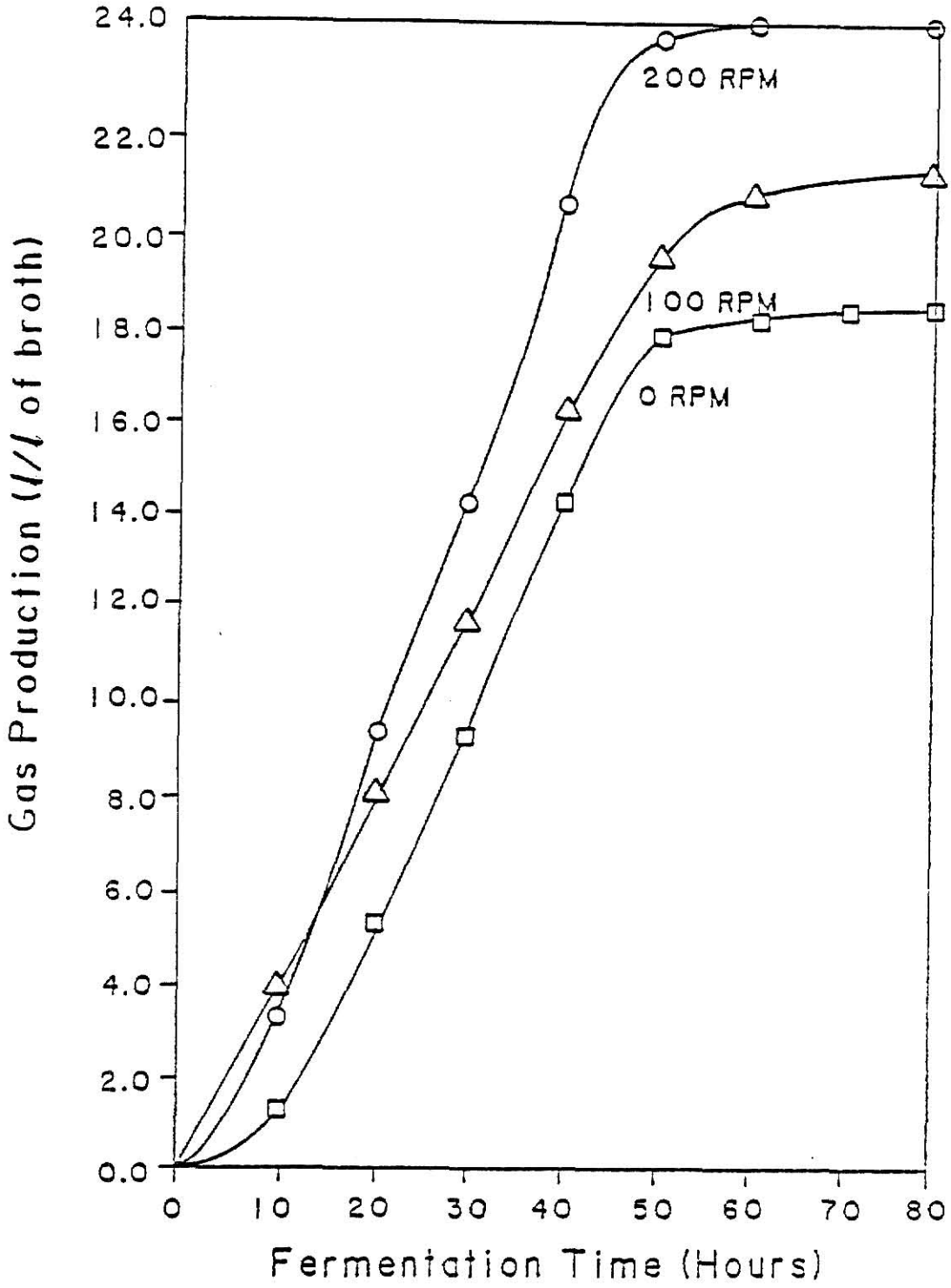

Fig. 8. Cumulative gas production under different agitation rates during butanol-acetone fermentation of the inverted sorghum molasses medium by C. acetobutylicum ATCC 4259.

shown in Figs. 9 and 10, respectively. It is important to note that the highest concentration of butanol occurred at the 0 RPM which produced the lowest amount of H_2 in the off-gas stream; however, for the run with 200 RPM the highest level of butyric acid was obtained. These data indicate that high agitation rate tends to drive hydrogen away from aqueous phase and this lost hydrogen is no longer available for the reduction of butyric acid to butanol during the final stage of fermentation.

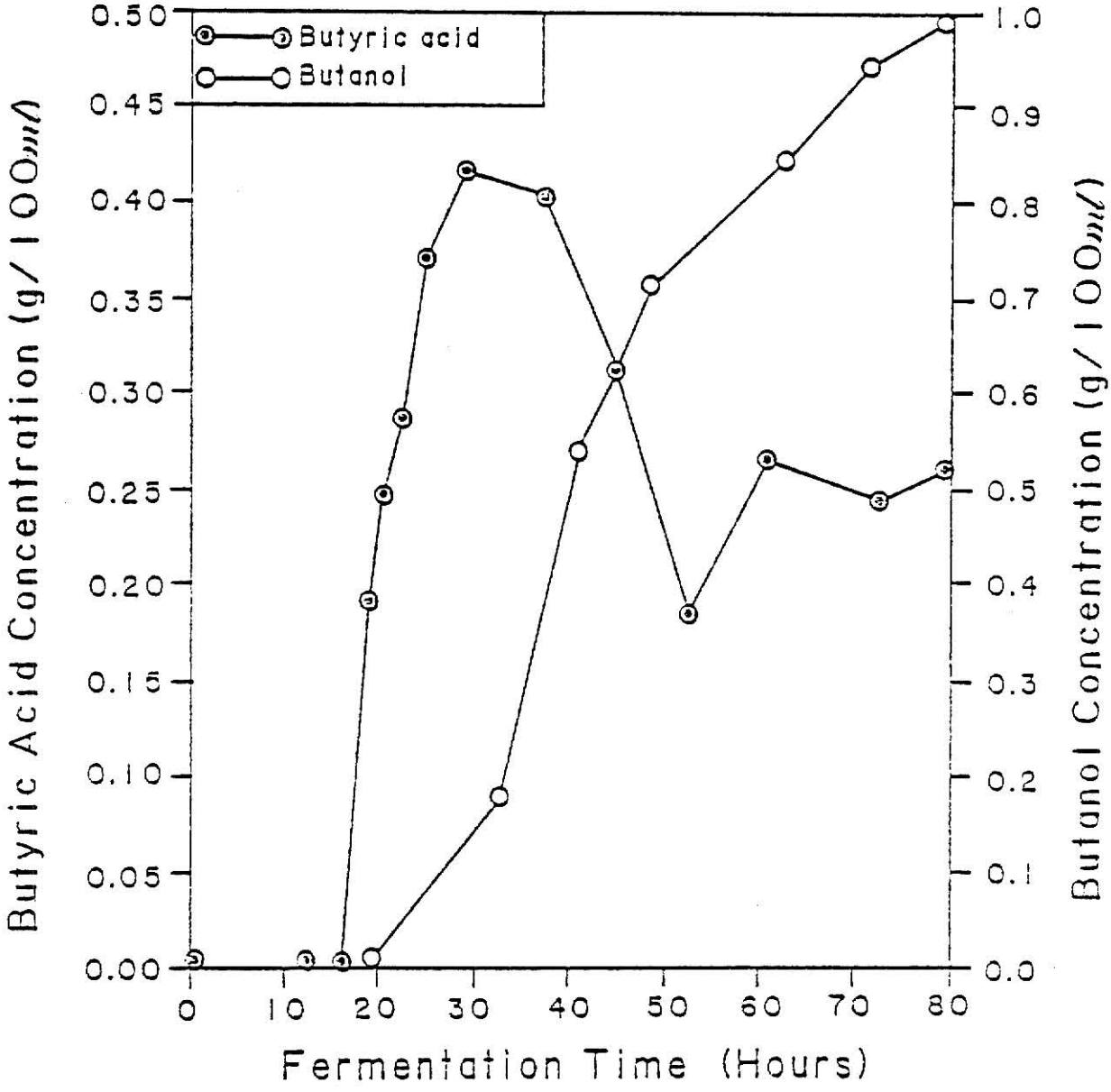

Fig. 9. Butanol and Butyric acid production during butanol-acetone fermentation of the inverted sorghum molasses by C/. acetobutylicum ATCC 4259; 0 RPM.

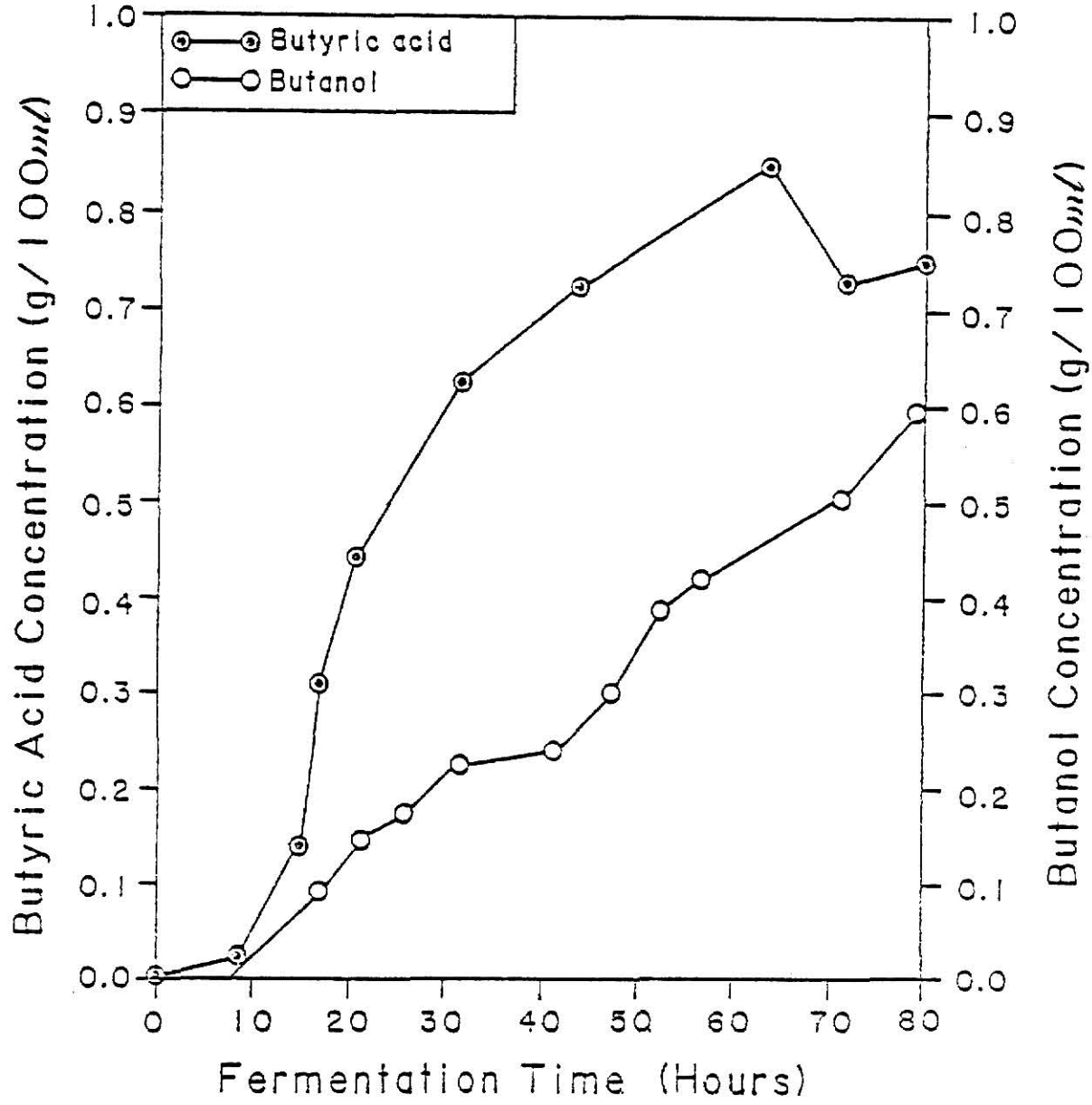

Fig.10. Butanol and Butyric acid production during butanol-acetone fermentation of the inverted sorghum molasses by C/. acetobutylicum ATCC 4259; 200 RPM.

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CHAPTER 7

CONCLUSIONS AND RECOMMENDATION

The strain, Clostridium acetobutylicum ATCC 4259, was selected after testing several strains for their capabilities to utilize sorghum molasses and individual sugar found in the sorghum molasses. Even though the capability of this strain to consume sorghum molasses and individual sugar is better than those of other strains, it has difficulty in consuming sucrose contained in the sorghum molasses medium. This is apparently due to the diauxic effect between inverted sugar and sucrose.

The diauxie phenomenon can be minimized by inverting sorghum molasses by acid; in fact, the solvent yield can be increased by as much as 50 percent. Methods besides acid hydrolysis may also be used to minimize the diauxic effect. If the medium contains predominately one type of sugar, the diauxie phenomenon will be dormant. Therefore, one approach is to use sorghum juice as the substrate because relatively little inverted sugar is contained in it; sucrose is the dominant component in the juice.

The fermentation of sorghum molasses has shown that the consumption of sucrose can be increased by lowering its concentration. This implies that a fed batch approach may be advantageous for fermenting sorghum molasses directly.

The strain, Clostridium acetobutylicum ATCC 4259, seems to belong to the Weizmann type, which can utilize starchy material effectively.

This fact in conjunction with the findings of the present work, indicates that it may be advantageous to produce butanol-acetone commercially not only from starchy material but also from sugary material by solely employing this strain.

FERMENTATIVE PRODUCTION OF BUTANOL FROM SORGHUM MOLASSES

by

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B.S., Korea University, 1977
M.S., Korea University, 1979

AN ABSTRACT OF A MASTER'S THESIS

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A strain, Clostridium acetobutylicum ATCC 4259, suitable for butanol-acetone fermentation of sorghum molasses was selected from several strains of the American Type Culture Collection (ATCC). It was cultivated in the composition-optimized sorghum molasses medium. The microbial growth and sugar consumption pattern in the sorghum molasses medium exhibited a typical diauxie phenomenon. The results strongly suggest that the difficulty encountered by the Weizmann type of organisms in butanol-acetone fermentation of molasses is due to the diauxie phenomenon causing a significant decrease in the solvent production rate. Acid hydrolysis of sorghum molasses minimizes the occurrence of the phenomenon, thereby remarkably increasing the solvent yield. The final solvent concentrations in the inverted molasses medium were butanol, 1.0% (w/v); acetone, 0.37% (w/v); ethanol, 0.18% (w/v); and total solvent, 1.55% (w/v). The total solvent yield in the inverted sorghum molasses medium was 30.3% based on the weight of sugar consumed. Effects of the temperature, agitation and heat-shocking were also investigated.