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Modulation of Phosphoenolpyruvate Metabolism of *Anaerobiospirillum succiniciproducens* ATCC 29305

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Modulation of the catabolic PEP-pathway of *Anaerobiospirillum succiniciproducens* was tried using some enzymatic inhibitors such as gases and chemicals in order to enhance succinic acid production. 10% CO increased the succinic acid/acetic acid (S/A) ratio but inhibited growth as well as production of succinic and acetic acid. Hydrogen gas also increased the S/A ratio and inhibited the synthesis of pyruvate: ferredoxin oxidoreductase when used in mixture with CO_2. Catabolic repression by acetic, lactic and formic acid was not recognized and other modulators such as glyoxylate, pyruvate derivatives, arsenic salt, phosphate and sulfate were shown not to be effective. Magesium carbonate was shown effective for repressing acetate production. Palmitic acid, myristic acid and phenylalanine did not affect acetate production but carprylic acid completely inhibited growth.

Anaerobiospirillum succiniciproducens is a Gram-negative, spiral, fermentative, chemoorganotrophic nonsporeformer affilliated with the Family of Bacterioidaceae, that produces succinic acid and acetic acid from glucose as major metabolites, and lactic and formic acid in small amount (9). The habitat of the bacterium was reported to be the gastrointestinal tract of humans and animals (5, 17) and is reported to cause diarrhoea and septicemia (13, 14). This organism attracted attention since succinic acid was known to have potential in the production of value-added C_4- and C_6- chemicals which have large world-wide markets in the plastics, electronics, paper and paint industries (8). Previous papers reported that succinic acid production could be enhanced by introducing large amount of carbon dioxide and through pH control of the fermentation system but the process also increased acetic acid production (18). In this study, we tried to find a way of switching the carbon flux of the catabolic routes from acetic acid to succinic acid.

MATERIALS AND METHODS

Microrganism and Culture Condition

A. succiniciproducens ATCC 29305 was from the American Type Culture Collection. The culture was rehydrated and inoculated into a anaerobic glucose-peptone medium which had been previously sterilized. The culture medium contained the following composition per liter of distilled water: glucose, 20 g; peptone, 10 g; K_2HPO_4, 3 g; NaCl, 1 g; $(NH_4)_2PO_4$, 1 g; yeast extract, 5 g. The medium was steam-sterilized at 121°C for 40 minutes. pH was adjusted to 6.2 with anaerobic sulfuric acid before inoculation. Sterile gases and sodium sulfide were added to maintain a strict anaerobic condition. The bacterium was cultured in pressure tubes (26 ml) or vials (50 ml) in static conditions or in culture vessels with agitation and gassing (500 ml).

Analytical Methods

Organic acids were analyzed using a Perkin-Elmer Liquid Chromatograph (6). Culture broth was spun to remove the cells (14,000 rpm, 15 minutes, Sorvall Centrifuge, U.S.A.) and the supernatant was acidified with sulfuric acid and directly injected. Respective components were separated using an ion exchange column (Biorad Aminex HPX-87 H, 300 mm × 7.8 mm, 35°C, flow rate, 0.5 ml/min) and detected with a UV monitor (Isco Inc. V4 model, 214 nm). The internal standard used was butyric acid.

Ethyl alcohol was analyzed using a Hewlett Packard Gas Chromatograph 5890 A with a Flame ionization detector and Chromosorb 101 (80/120 mesh).

Enzyme Assay

Preparation of the Cell-free Extract (11): Culture

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broth was anaerobically dispensed under nitrogen gas into centrifuge tubes and spun to get cell pellets. The pellets were washed with anaerobic water three times. The cells thus obtained were suspended in anaerobic water and disrupted with a French Press (American Instrument Co., Silver Spring, Md., 2000 lb/in^2). Cell debris was removed and supernatant was stored under nitrogen gas at -70°C.

Protein Assay (2): Protein content was determined using a Gilford spectrophotometer by protein-dye assay. The reaction mixture contained Coomassie Brilliant Blue, methyl alcohol and phosphoric acid. The standard used was bovine serum albumin.

Pyruvate: Ferredoxin Oxidoreductase (PFOR) Activity (10): Pyruvate: ferredoxin oxidoreductase activity was assayed by reading the reduction of methyl viologen using a Varian CARY spectrophotometer (Model 219). The reaction system contained 0.1 M Tris HCl buffer (pH 7.8), 10 mM Na-pyruvate, 2 mM methyl viologen, 2 mM dithiothreitol, 2 mM sodium dithionite, 0.2 mM CoA and cell extract. The reaction was performed at 37°C under nitrogen gas.

Uptake Hydrogenase Activity (3): Uptake hydrogenase activity was assayed by reading the reduction of methyl viologen, or neutral red, under hydrogen gas. The reaction system contained 0.1 M Tris-HCl buffer (pH 7.8), 2 mM dithiothreitol, 2 mM sodium dithionite, 2 mM methyl viologen, or 0.5 mM neutral red, and cell extract.

RESULT AND DISCUSSION

Effect of Types of Gas and Concentration

CO is known as an inhibitor which influences the nickel- or Fe- containing enzymes (1, 16, 21) such as hydrogenase that produce hydrogen gas from reduced nucleotides and PFOR that is a key enzyme on catabolic pathway to acetic acid production by *A. succiniciproducens* (18). Therefore, it was postulated that carbon flow could be switched to succinate production by inhibiting the PFOR activity of the catabolic path for acetate production.

Carbon monoxide was added at the proper concentration into the headspace of a pressure tube (20 ml) that contained 10 ml sterile glucose-peptone medium and carbon dioxide in the headspace. Cell growth was enhanced in a culture with more than 10% CO in the headspace. Maximum OD_{660} was 0.6-0.7 after 12 h. Pressurizing to 10 psi with complete CO did not induce growth. Specific growth was affected at a similar ratio and the maximum specific growth rate at more than 10% CO was 0.25 h^{-1} (Fig. 1 and 2).

After 12 h metabolites were analyzed and the succinic acid/acetic acid ratio (S/A) was plotted against the CO concentration (Fig. 3). The ratio increased when 70% or

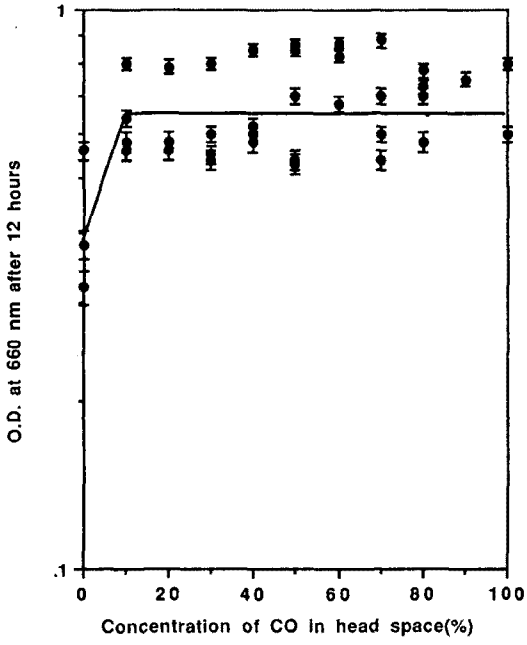

Fig. 1. Effect of CO concentration on the growth of *A. succiniciproducens* ATCC 29305 (Culture was performed in a pressure tubes containing 10 ml of medium and the headspace was balanced with CO_2).

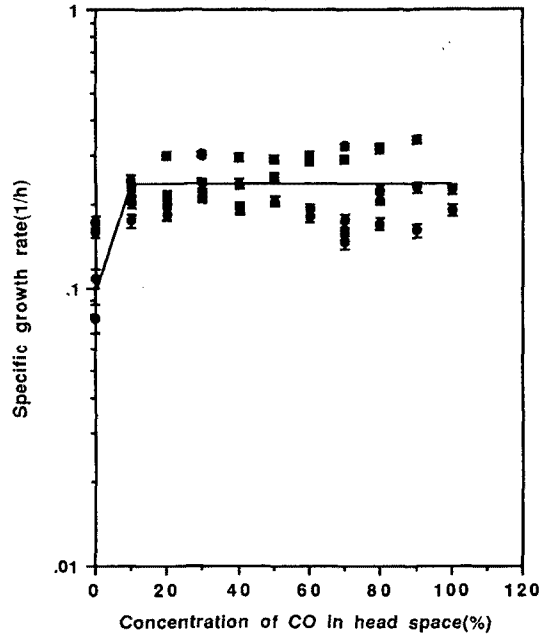

Fig. 2. Specific growth rate of *A. succiniciproducens* ATCC 29305 as affected by CO concentration (Culture was performed in a pressure tubes containing 10 ml of medium and the headspace was balanced with CO_2).

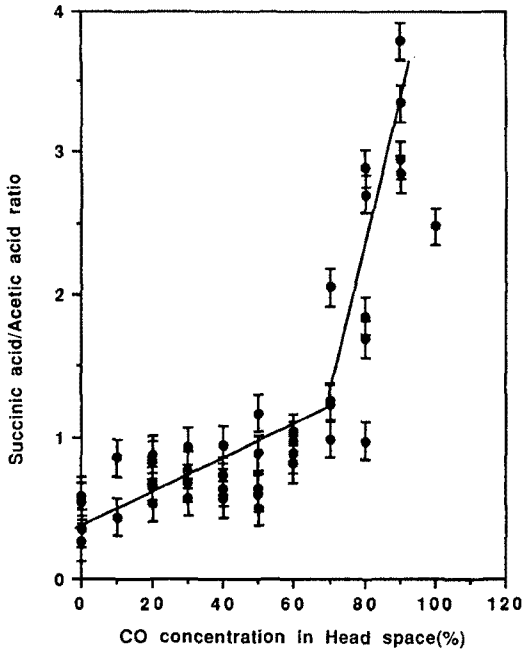

Fig. 3. Effect of CO concentration on the succinic acid production by *A. succiniciproducens* ATCC 29305 after 24 h of incubation at 37°C (Culture was performed in a pressure tubes containing 10 ml of medium and the headspace was balanced with CO_2).

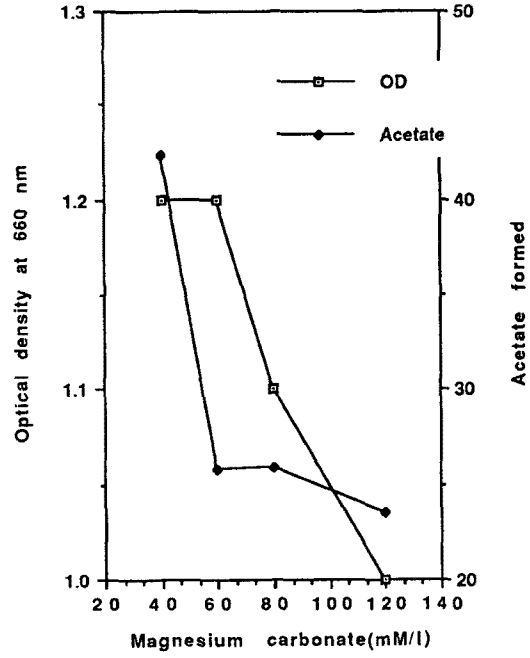

Fig. 4. Effect of CO concentration on the succinic acid production by *A. succiniciproducens* ATCC 29305 after 24 h at 37°C (Culture was performed in 58 ml serum bottle containing 20 ml medium and the headspace was balanced with CO_2. $MgCO_3$ was added on initial stage).

higher CO was introduced into the headspace. The ratio at 70 and 90% CO concentration was found to be 1.2 and 3.3 in this experiment. The reason for the S/A ratio to rise with the increased CO, despite the repression of production of both acids, is that succinic acid production was not so sensitive to CO concentrations as acetic acid. Fig. 4 shows the result in a culture using 50 ml vials fortified with magnesium carbonate. The trend was similar but not remarkable. This might be due to the effect of carbon dioxide released from magnesium carbonate by acids formed during fermentation.

Table 1 shows the effect of types of gases on the fermentation profile after 12 h in a pressure tube. Complete hydrogen gas (10 psi) in the headspace suppressed growth but induced higher succinic acid production when compared to the result in the culture with complete carbon dioxide. The S/A ratio was found to be high in the culture with 10 psi hydrogen or atmospheric CO. The ratios were 2.32 and 2.48, respectively. However, succinic acid and acetic acid concentrations were very low in the CO culture. From these results, we can see that hydrogen gas in the head space is a concern because succinic acid concentrations and S/A ratio were very high. Hydrogen gas can function as an electron donor when synthesizing succinic acid from fumarate (7). According to this assumption, cells grown in hydrogen gas

Table 1. Effect of gas in the head space on the succinic acid production by *A. succiniciproducens* ATCC 29305.

	Hydrogen (10 psi)	CO (atm)	CO_2 (atm)	CO_2 (10 psi)
OD_{660}	0.47	0.80	0.56	0.56
Succinic acid	70	28	27	43
Lactic acid	-	-	-	-
Formic acid	28	13	8	18
Acetic acid	35	11	78	76
S/A ratio	2.32	2.48	0.35	0.56

Cultures were performed using 26 ml pressure tube. Unit, mM/l.

must show uptake hydrogenase activity. Therefore, the hydrogenase activity in whole cell and cell-free extracts were assayed, however, it was not detectable. Indirect method was used to detect negative pressure in the pressure tube after cultivation. But negative pressure was not detected.

Table 2 shows the result after 12 h of fermentation when various types and pressures of gases were used. *A. succiniciproducens* produced succinic acid in large quantities in the cultures with hydrogen gas at 10 psi. The system using successive exchanges of carbon dioxide (5 psi) and additional hydrogen (10 psi) and the system with atmospheric levels of CO and additional carbon dioxide (10 psi) yielded higher succinic acid than those cultures with

complete carbon dioxide (10 psi). This result implies the effect of CO and hydrogen gas on the glucose catabolism by *A. succiniciproducens*.

In order to elucidate the effect of hydrogen gas, batch fermentation was performed by sparging with different ratios of gases and enzyme activity was monitored (Table 3). Cell growth was low when CO_2 and H_2 (20/80) was used compared to complete CO_2. Acetic acid accumulation was also reduced by 40% and therefore S/A ratio increased and vice versa. It is postulated that the main reason for this is the suppression of synthesis of key catabolic enzymes. The cell-free enzyme extracts from the respective culture broths were assayed for PFOR activity. The PFOR activity in the cell extract with CO_2/H_2 (20/80) was reduced by about 90%. Murray *et al.* (15) studied acetic acid fermentation from *Clostridium saccharolyticum* and reported that hydrogen gas decreased the acetic acid formation and enhanced ethanol production.

Table 2. Effect of combination of gas in the headspace of pressure tube on the succinic acid production by *A. succiniciproducens* ATCC 29305.

	Hydrogen (10 psi)	CO_2/H_2[a]	CO/CO_2[b]	CO_2 (10 psi)
OD_{660}	0.3	0.2	0.3	0.2
Succinic acid	150.7	137.3	137.7	118.7
Formic acid	29.3	30.7	31.0	21.1
Acetic acid	10.2	8.2	8.5	10.0
Ethanol	1.1	1.4	1.3	2.5

[a]Pressurized with CO_2 at 5 psi and additional H_2 to 10 psi.
[b]Atmospheric pressure with CO and additional CO_2 to 10 psi.
Data are from 27 h of fermentation. Unit, mM/l.

Table 3. Effect of sparging gas on the enzyme activity and succinic acid production by *A. succiniproducens* ATCC 29305.

Gas Phase	CO_2	CO_2/H_2 (20/80)
OD_{660}	1.2	0.7
Ferredoxin oxidoreductase (nmol/mg/min)	170	14
Succinic acid (mM/l)	209.0	170.9
Formic acid (mM/l)	16.97	28.40
Acetic acid (mM/l)	46.5	27.7
Ethanol (mM/l)	0.5	0.3
S/A ratio	4.76	6.17

Sparging, 0.4 vvm. Working volume, 500 ml. 37°C, 12 h.

Effect of Various Metabolic Modulators

A. succiniciproducens was known to form acetic acid, formic acid and lactic acid via catabolic pathways from glucose. In this pathway, succinic acid was synthesized through a reductive pathway from fumaric acid and acetic acid was produced from pyruvate (18). Therefore, the catabolic flow is thought to be diverted to succinic acid production as a result of catabolite repression when large quantities of acetic acid accumulate in the culture system. To induce a repression effect, these fermentation products were added before fermentation was initiated and then the metabolites were analyzed (Table 4). However, catabolites added neither affected the growth of *A. succiniciproducens* nor induced repression but did increase acetic acid production. Glyoxylate was reported as inhibiting TPP-dependent enzymes such as pyruvate synthase, PFOR and pyruvate decarboxylase (20). Pyruvate oxidation by PFOR results in acetyl-CoA, which is metabolized to acetic acid. 2-10 mM/l of glyoxylate was added to the fermentation system to inhibit the PFOR. Table 5 shows that glyoxylate did not repress the acetic acid formation.

Pyruvate derivatives are likely to react competitively on PFOR, the component of a pyruvate decarboxylase

Table 5. Effect of glyoxylate on the succinic acid production by *A. succiniproducens* ATCC 29305.

	Control	Glyoxylate (mM)			
		2	5	7	10
OD_{660}	0.62	0.53	0.57	0.49	0.50
Succinic acid	64	49	48	44	44
Lactic acid	-	-	-	-	-
Formic acid	17	13	13	12	11
acetic acid	63	73	113	135	161
S/A	1.02	0.68	0.43	0.32	0.27

Cultures were performed using 26 ml pressure tube. Head space was carbon dioxide at 10 psi. Unit, mM/l.

Table 4. Effect of the addition of some catabolic products on the succinic acid production.

	Control	Acetic acid (mM)		Formic acid (mM)		Lactic acid (mM)	
		50	100	20	50	20	50
OD_{660}	0.46	0.40	0.60	0.50	0.42	0.37	0.40
Succinic acid	89	89	61	51	49	39	36
Lactic acid	-	-	-	-	-	25	39
Formic acid	29	25	7	27	54	26	10
Acetic acid	136	458	397	132	176	172	163
S/A	0.60	0.22	0.21	0.39	0.28	0.23	0.22

Head space was carbon dioxide at 10 psi. Unit, mM/l. Cultures were performed using 26 ml pressure tube.

Table 6. Effect of bromo-pyruvate on the succinic acid production by *A. succiniciproducens* ATCC 29305.

Concentration of Br-pyruvate (mM)	1.0	0.5	0.2	0.15	0.10	0.05
OD_{660}	0.02	0.02	0.06	0.07	0.26	0.45
Succinic acid	60.4	94.3	103.3	114.2	122.5	136.8
Formic acid	9.1	20.0	23.1	27.4	26.3	36.7
Acetic acid	2.9	2.8	3.5	2.1	8.2	18.7
Ethanol	0.7	0.6	1.3	1.7	1.7	0.5

Working volume, 500 ml batch culture. Unit, mM/l. Gas, 0.4 vvm CO_2.

Table 7. Effect of sodium arsenate on the succinic acid production by *A. succiniciproducens* ATCC 29305.

Concentration (mM)	5.0	1.0	0.5	0.3	0.1
OD_{660}	-	0.1	0.15	0.26	0.40
Succinic acid	-	111.4	112.8	124.8	137.5
Formic acid	-	24.2	26.1	29.2	30.9
Acetic acid	-	9.4	9.7	14.7	14.6
Ethanol	-	1.7	1.7	1.7	0.6

Working volume, 500 ml batch culture. Unit, mM/l. Gas, 0.4 vvm CO_2.

Table 8. Effect of KH_2PO_4 on the succinic acid production by *A. succiniciproducens* ATCC 29305.

Concentration (mM)	0	17	35	50
OD_{660}	1.1	1.2	1.0	0.2
Succinic acid	199.1	209.0	220.5	98.9
Formic acid	18.0	17.0	29.5	18.0
Acetic acid	41.9	46.5	40.7	9.20
Ethanol	0.5	0.5	0.5	0.7

Working volume, 500 ml batch culture. Unit, mM/l. Gas, 0.4 vvm of CO_2.

complex (12) and the addition of pyruvate derivatives can enhance succinic acid production and repress acetic acid formation by switching the carbon flow in PEP-metabolism to reductive catabolism and succinic acid. Thus 0.05-1 mM bromopyruvate was added to the medium and *A. succiniciproducens* was grown for 12 h. Growth was severely inhibited by about 50% at more than 0.05 mM/l of concentration. Succinic acid and acetic acid production were similarly reduced and the concentrations were 136.8 and 18.7 mM/l, respectively. The reduction of succinic acid was 35%. Fluoropyruvate showed a similar trend. OD_{660}, succinic acid and acetic acid produced were 0.6, 132.85 mM/l and 16.69 mM/l, respectively (Table 6).

Arsenate forms acetyl arsenate in pyruvate metabolism and represses phosphoclastic reaction reducing the oxidation of nucleotides (19). The reduced nucleotide thus conserved can be used for producing other important products such as succinic acid. This idea leads to the introduction of arsenate in the fermentation system. Table 7 shows the result when 0.1-5 mM/l of sodium arsenate was added. Growth of *A. succiniciproducens* was inhibited even at a concentration of 0.1 mM/l of sodium arsenate. OD_{660} after 12 h was 0.4. Succinic acid and acetic acid production were slightly repressed and they were 137.5 mM/l and 14.6 mM/l, respectively.

Table 9. Effect of ammonium sulfate on the succinic acid production by *A. succiniciproducens* ATCC 29305 at 37°C after 12 h of fermentation.

Concentration (mM)	0	1	3	6	9
OD_{660}	1.1	1.1	1.2	1.2	1.1
Succinic acid	206.7	199.1	225.6	238.7	204.5
Formic acid	22.5	18.0	27.2	25.1	27.2
Acetic acid	53.5	41.9	54.0	59.8	52.1
Ethanol	2.8	0.5	1.7	1.7	1.4

Unit, mM/l. Working volume, 500 ml batch culture. Gas, 0.4 vvm of CO_2.

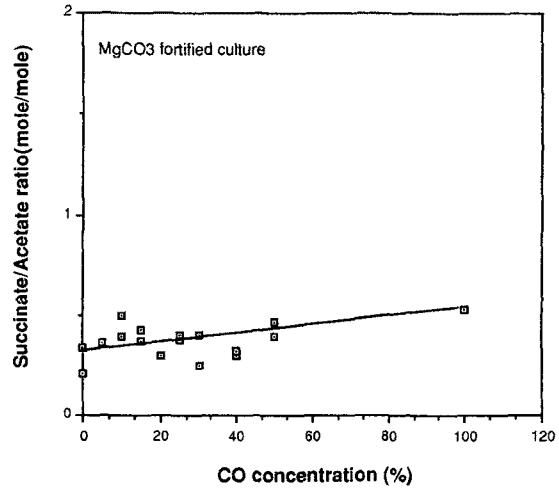

Fig. 5. Effect of magnesium carbonate on the acetic acid formation by *A. succiniciproducens* ATCC 29305.

Pyruvate kinase of *Streptococcus lactis* has also been reported to be repressed by potassium phosphate and this might affect acetate production and increase succinic acid. Table 8 shows the effect of phosphate salt on succinic acid fermentation in the range of 0-50 mM/l concentration. Potassium phosphate did not affect growth and succinic acid production at 35 mM/l of concentration but were severely influenced by 50 mM/l of concentration. Sulfate ions were another factor inhibiting pyruvate kinase (4). Growth was not inhibited at all but succinic acid was increased slightly when 6 mM/l of ammonium sulfate was added but at more than this concentration, no effect was observed (Table 9).

Magnesium ion is known as an inhibitor against pyruvate kinase (22). Fig. 5 shows the effect of magnesium

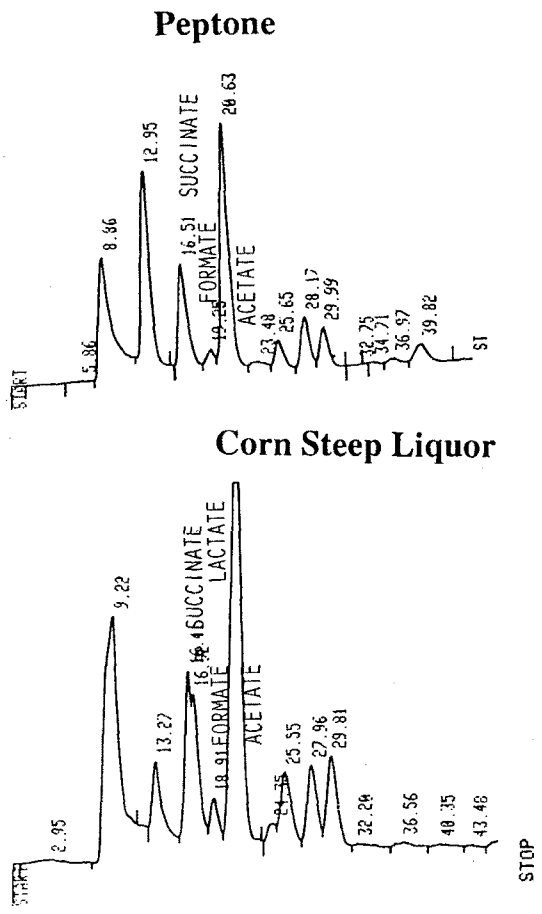

Fig. 6. HPLC profile of metabolites in 12 h cultures of *A. succiniciproducens* ATCC 29305 as affected by nitrogen sources (Injection volumes were same).

carbonate (0-100 mM/l). In this case, 60-80 mM/l of magnesium did not affect growth but severely decreased acetate production. Other candidates for modulating glucose metabolism are fatty acid (23), phenylalanine and alanine (22). Fatty acids affect the key enzymes of glycolysis by feedback inhibition and fatty acid formation itself can be repressed while acetyl Co A will be final catabolic product of fatty acid. This inhibits the action of glucokinase and pyruvate kinase, and thus represses the lactic acid formation. When 10 mM of fatty acids were added to the respective systems, caprylic acid completely inhibited the growth of *A. succiniciproducens* but did not affect acetic acid production. Palmitic and myristic acid did not influence growth and acetic acid production. Phenylalanine was also tried for its modulating effect but showed no effect (Data not shown)

Comparison of Peptone and Corn Steep Liquor as Nitrogen Source

In a previous paper (18), corn steep liquor was chosen to formulate a rich medium for succinic acid fermentation using glucose as a carbon source and a CO_2 as catabolic stimulator. But the nitrogen source produced a particulated medium in which it is very difficult to monitor cell growth. Therefore, peptone was tried as a substitute for corn steep liquor. Fig. 6 showed the analytical HPLC profile after 12 h. It was observed that more succinic acid was produced in a culture with peptone and more lactic acid was formed in a culture with corn steep liquor. The concentration in peptone and CSL medium were 209 and 50 mM/l, respectively.

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