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Formation of Succinic Acid by *Klebsiella pneumoniae* MCM B-325 Under Aerobic and Anaerobic Conditions

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Abstract The present study describes the formation of succinic acid by a nonvirulent, highly osmotolerant *Klebsiella pneumoniae* strain SAP (succinic acid producer), its profile of metabolites, and enzymes of the succinate production pathway. The strain produced succinate along with other metabolites such as lactate, acetate, and ethanol under aerobic as well as anaerobic growth conditions. The yield of succinate was higher in the presence of $MgCO_3$ under N_2 atmosphere as compared with that under CO_2 atmosphere. Analysis of intracellular metabolites showed the presence of a smaller PEP pool than that of pyruvate. Oxaloacetate, citrate, and α-ketoglutarate pools were considerably larger than those of isocitrate and fumarate. In order to understand the synthesis of succinate, the enzymes involved in end-product formation were studied. Levels of phosphoenolpyruvate carboxykinase, fumarate reductase, pyruvate kinase, and acetate kinase were higher under anaerobic growth conditions. Based on the profiles of the metabolites and enzymes, it was concluded that the synthesis of succinate took place via oxaloacetate, malate, and fumarate in the strain under anaerobic growth conditions. The strain SAP showed potential for the bioconversion of fumarate to succinate under N_2 atmosphere in the presence of $MgCO_3$. At an initial fumarate concentration of 10 g/l, 7.1 g/l fumarate was converted to 7 g/l succinate with a molar conversion efficiency of 97.3%. The conversion efficiency and succinate yield were increased in the presence of glucose. Cells grown on fumarate contained an 18-fold higher fumarate reductase activity as compared with the activity obtained when grown on glucose.

Key words: Succinic acid, anaerobic bacteria, *Klebsiella pneumoniae*, TCA cycle, metabolites

Many anaerobic and facultative anaerobic Gram-negative bacteria ferment carbohydrates to a mixture of organic acids [30]. Recently, the fermentative production of succinic acid from carbohydrates by microbial process has become of applied significance for C_4 chemical feed stocks used in oxychemical manufacture. Succinic acid, a four-carbon aliphatic dicarboxylic acid, has applications in the manufacture of specialty chemicals, agriculture, food, medicine, textiles, plating, and waste gas scrubbing [27]. Although many bacteria produce succinate as an anaerobic end product, only few species make it as the major end product [30]. Several succinate-forming mesophilic bacteria have been isolated and their biochemical pathways have been elucidated [31, 8]. Phosphoenolpyruvate (PEP) is one of the central intermediates during the mixed acid fermentation, and it is converted either into pyruvate, resulting in the formation of fermentation products lactate, acetate, formate, and ethanol, or into oxaloacetate (OAA), resulting in the formation of succinate and propionate [18, 5]. Under anoxic conditions, the flux of PEP towards either oxaloacetate or pyruvate greatly varies among the different mixed acid-fermenting bacteria [26].

Recently, we have isolated a nonvirulent, highly osmotolerant, facultative anaerobe, *Klebsiella pneumoniae* strain SAP (succinic acid producer), from buffalo rumen using anaerobic techniques [13]. The strain SAP carries out both aerobic and anaerobic metabolism and produces succinic acid from glucose under aerobic as well as anaerobic conditions. Under N_2 atmosphere in the presence of $MgCO_3$, the strain SAP produced about 2 g/l of succinic acid from 15 g/l glucose as one of the major end products. Moreover, preliminary studies with the strain SAP showed higher yields of succinic acid when supplemented with fumarate under similar growth conditions. These observations encouraged us to explore the potential of the strain for bioconversion of fumarate to succinate, since very few strains have been shown to have the ability to convert fumarate to succinate [23]. None of the *Klebsiella pneumoniae* strains has been reported to produce succinic acid from glucose or fumarate [8] in significant amount, as compared with the strain SAP. These peculiar characteristics of the strain SAP prompted us to

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study the profile of metabolites and enzymes involved in the synthesis of succinate. This is the first report on the measurement of intracellular metabolite concentration and elucidation of the enzyme profile of succinate synthesis in *Klebsiella pneumoniae*.

MATERIALS AND METHODS

Materials

All chemicals and reagents were of analytical grade. Coenzymes and enzymes were purchased from Sigma Chemicals (U.S.A.) or Boehringer Mannheim (Germany).

Organism and Cultivation Conditions

Klebsiella pneumoniae strain SAP (MCM B-325) was obtained from the MACS Collection of Microorganisms (MCM, Pune, India). The composition of the medium, (hereafter referred to as ANS-1) employed for the cultivation of the strain was (g/l): glucose, 15; polypeptone, 5; yeast extract, 2.5; K_2HPO_4, 3; NaCl, 1; $(NH_4)_2SO_4$, 1; $CaCl_2 \cdot 2H_2O$, 0.07; $MgCl_2 \cdot 6H_2O$, 0.2; and $MgCO_3$, 15. The pH was adjusted to 7.0. The strain was anaerobically grown under nitrogen atmosphere in 500 ml serum bottles containing 250 ml of ANS-1 medium. For aerobic growth, the culture was grown in 500 ml Erlenmeyer flasks containing 150 ml of ANS-1 medium at 37°C on a shaking incubator (Gyromax, U.S.A.) at 150 rpm.

Analysis of Metabolites

Fermentation products in the culture supernatant were analyzed by high-performance liquid chromatography (Dionex, Germany) equipped with an Aminex HPX-87H column (300×7.8 mm, Bio-Rad Laboratories, Hercules, CA, U.S.A.) and a Refractive Index (RI) detector (Shodex, Japan). The column was isocratically eluted with 5 mM H_2SO_4 at a flow rate of 0.5 ml/min. The column temperature was maintained at 45°C. Intracellular metabolites were isolated and determined as described by Banul *et al.* [2]. Cell pellet was collected by centrifugation of 5 ml of culture broth at 12,000 ×*g* at 4°C for 10 min and then resuspended in a tube containing 0.28 ml of 11.7 M perchloric acid plus 20 mM Na_2EDTA. The cell suspension was vigorously mixed for 1 min and then left in an ice-bath for 2 h before neutralization with 0.78 ml of 3 M $KHCO_3$. Salts and cell debris were removed by centrifugation at 12,000 ×*g* at 4°C for 10 min. Enzymatic determination of metabolites was performed at 37°C by coupling appropriate enzymes with spectrophotometric determination of NADH, NADPH, or related metabolites using a Shimadzu double beam spectrophotometer UV-2501 PC.

Preparation of Cell-Free Extracts

Cells of *Klebsiella pneumoniae* strain SAP were grown aerobically as well as anaerobically as described above and were harvested during the late exponential phase of growth. The cells were centrifuged at 8,000 ×*g* for 35 min at 4°C and washed twice with 100 mM phosphate buffer, pH 7.5. The washed cell pellet was suspended in 50 mM phosphate buffer (pH 7.5) containing 5 mM dithiothreitol (DTT), and immediately sonicated at 4°C. The cell debris was removed by centrifugation for 20 min at 20,000 ×*g* and 4°C and the supernatant was saved as the enzyme suspension. Anoxic conditions were maintained throughout the preparation of cell extracts. Extracts were used always on the same day of preparation.

Enzyme Assays

All enzyme assays were performed in both aerobic and anaerobic conditions as described previously [24]. All buffers and substrates were prepared in glass vials sealed with rubber stoppers and rendered anaerobic by the above procedure. For enzyme assays under anaerobic conditions, glass cuvettes (1.7 ml total volume) were sealed with grey butyl rubber stoppers and made anaerobic by repeatedly evacuating and flushing with N_2 gas. All additions to the cuvettes were made with a microliter syringe, to give a final liquid volume of 1 ml. Enzyme activities were calculated from the linear part of the reaction, and values for activities were determined by a minimum of three separate measurements each on two different cell extract preparations. All assays were performed at 37°C. Enzymes were assayed by coupling the particular step to the appropriate NAD^+- or $NADP^+$-linked reaction, with the use of commercially available crystalline enzymes as coupling enzymes. The rate of production or disappearance of reduced nucleotides was followed continuously on a Shimadzu double beam spectrophotometer UV-2501 PC. The wavelength and millimolar extinction coefficient for NAD, NADH, NADP, and NADPH were 340 nm and 6.22 cm^{-1} mM^{-1}, respectively, and 578 nm and 8.65 cm^{-1} mM^{-1} for benzyl viologen. Protein was determined by the Biuret method [6]. One milliunit (mU) of enzyme activity was defined as the amount of enzyme to produce 1 nmol of product per min. Enzyme specific activities are presented in mU per mg of protein.

RESULTS AND DISCUSSION

Formation of Succinic Acid by Strain SAP

Table 1 summarizes the product yields of the strain SAP in aerobic and anaerobic conditions (N_2 atmosphere) at 9 h of growth. The strain SAP fermented glucose to succinate as an end product, along with ethanol, acetate, and lactate in aerobic as well as in anaerobic conditions (N_2 atmosphere in the presence and absence of $MgCO_3$). In the presence or absence of $MgCO_3$, under aerobic condition, 90 mmol of cell carbon was formed per 100 mmol of glucose consumed, whereas it was 61.32 mmol in the same fermentation

Table 1. End products of glucose fermentation by strain SAP at 9 h of growth under aerobic and anaerobic conditions (N_2 atmosphere).

End products	Concentration (mmol/100 mmol of consumed glucose)		
	Aerobic growth with $MgCO_3$ (15 g/l)[c]	Anaerobic growth (N_2 atmosphere)	
		With $MgCO_3$ (15 g/l)[c]	Without $MgCO_3$
Cell carbon[a]	90	61.32	45.30
Succinic acid	6.80	18.70	6.20
Lactic acid	3.40	12	15.80
Acetic acid	22	26	29.50
Formic acid	ND	13	ND
Ethanol	8.20	50	28.75
Carbon recovery	0.31	0.56	0.39
Electron recovery[b]	0.31	0.61	0.43

ND, Not detectable.

[a] Cell carbon was calculated with $CH_2O_{0.5}N_{0.2}$ [24].

[b] Electron recovery was calculated by using the difference in hydrogen contents between substrate and product [19, 20].

[c] End products were determined following complete dissolution of $MgCO_3$

period in the presence of $MgCO_3$ under N_2 atmosphere. In aerobic condition, 100 mmol of glucose consumption resulted in the formation of 6.8 mmol of succinate with an efficiency of 3.4%, assuming that 2 mol of succinate can theoretically be formed homofermentatively from 1 mol of glucose [26], whereas the production of succinic acid was much higher under N_2 atmosphere in the presence of $MgCO_3$. The efficiency increased to 9.3% under this anaerobic condition, which was about 2.7 times higher than that of aerobic condition. It is of interest to note that the less cell mass developed in anaerobic condition showed a 2.7-fold higher production of succinate. The yields of lactate, acetate, and ethanol as well as carbon and electron recovery were also considerably higher in anaerobic condition than those of aerobic condition (Table 1).

This possibly indicates that the phosphoenolpyruvate (PEP) was used mainly towards the synthesis of succinate via oxaloacetate, whereas pyruvate was converted to lactate, acetate, and ethanol in anaerobic condition. It is also interesting to note that the presence of $MgCO_3$ (a source of slow release of CO_2) in anaerobic conditions played a dramatic role in the production of succinic acid. In the absence of $MgCO_3$ under N_2 atmosphere, the yield of succinic acid was only 6.2 mmol per 100 mmol of glucose consumed, which was 3 times less than that of $MgCO_3$-supplemented. The formation of lactic acid and acetic acid was higher in the absence of $MgCO_3$ (Table 1).

It is of interest to note that supplementation of fumarate as the only carbon source resulted in a 2.5-fold higher production of succinate than that of glucose as the sole carbon source, when the strain SAP was grown under N_2 atmosphere in the presence of $MgCO_3$. The yield increased by 3.6-fold, when fumarate was supplemented along with 1.5% glucose (Table 2), possibly indicating that fumarate was directly converted to succinate. Succinate formation from fumarate was favored in the presence of glucose, during which available glucose was used for building of cell mass. This resulted in excess cell mass formation. The yield of succinate and other end products by the strain SAP was compared with published data of other succinogens (Table 2). After 24 h of incubation, the yield of succinic acid by the strain SAP was 17.3 mmol as compared with 6.60, 133, 41.25, and 55.50 mmol by *Escherichia coli* K-12, *Anaerobiospirillum succiniciproducens*, *Actinobacillus* sp. 130Z, and *Mannheimia succiniciproducens* MBEL55E, respectively. The yield of succinate by the strain SAP was low as compared with that of *A. succiniciproducens*, *Actinobacillus* sp. 130Z, and *M. succiniciproducens* MBEL55E. However, it was about 2.6-fold higher than that of *E. coli* K-12. When the strain SAP was grown on malate or fumarate as the sole carbon source under similar conditions, the yield of succinic acid was found to be higher. This result suggests the possibility of succinate synthesis mainly via malate and fumarate by the reductive pathway of the TCA cycle. The strain did not grow on succinate (data not shown); however, it showed poor growth on other substrates such as isocitrate, α-ketoglutarate, glutamate, and GABA. Succinic acid production with lower yields was observed with these supplemented intermediates, which are the precursors of different possible pathways of succinic acid synthesis (Table 2). It appeared that there was a high yield of succinic acid after supplementation of only malate or fumarate. This indicates that the synthesis of succinic acid took place mainly from fumarate by a reaction catalyzed by fumarate reductase.

Effect of Carbon Dioxide on Fermentation Products of Strain SAP

The formation of succinate as a fermentation product requires CO_2 fixation [26]. Therefore, the effect of CO_2 on

Table 2. Comparison of fermentation end products of strain SAP with those of other succinate forming-organisms.

Organism	Substrate	Dry cell weight (g/l)	mmol of fermentation products/l						Succinate productivity (g/l/h)	Reference
			Succinate	Lactate	Acetate	Formate	Malate	Ethanol		
Strain SAP[a]	Glucose (1.5%)	1.51	17.30	14.20	59.65	41.30	0	52.30	-	Present study
	Fumarate (1%)	0.65	43.50	0	0	0	4.80	0	0.29	
	Fumarate (1%)+ glucose (1.5%)	1.86	62.25	3.70	33.20	0	6	19	0.42	
	Malate (1%)	0.84	22.40	0	10.50	0	0	0	-	
	Isocitrate (1%)	0.60	2.80	2.20	6.30	0	0	0	-	
	α-KGA (1%)	0.59	2.90	2.10	5.70	0	0	10	-	
	Glutamate (1%)	0.79	2.70	2.20	5.80	0	0	9	-	
	Fumarate+ glutamate (1% each)	0.68	32	0	0	0	3.40	0	-	
	GABA (1%)	0.70	2.70	2.40	5.90	0	0	0	-	
E. coli K-12	Glucose (1%)	NA	6.60	17	42.90	83.60	0	29.10	-	[28]
E. coli CA79	Fumarate (4%)+ glucose (1%)	NA	17.60	0	2.65	0	39.46	0	0.11	[9]
Enterococcus faecalis RKY1	Fumarate (10%)+ glucose (1.5%)	NA	548	NA	NA	NA	NA	NA	4.45	[22]
Anaerobiospirillum succiniciproducens	Glucose (2%)	NA	133	ND	73.70	13.20	0	ND	-	[24]
Actinobacillus sp. 130Z	Glucose (1%)	NA	41.25	0	48.40	42.90	0	3.80	-	[26]
	Fumarate (0.8%)	NA	34	0	7.0	5.50	0	0	0.23	
Mannheimia succiniciproducens MBEL55E	Glucose (1%)	NA	55.50	2.22	38.85	50.50	0	0	-	[15, 12]

NA, Not available; ND, Not detectable.

[a] Cells of strain SAP were grown in ANS-1 medium supplemented with $MgCO_3$ (1.5%) under N_2 atmosphere for 24 h.

Table 3. Influence of availability of CO_2 in the form of $MgCO_3$ and $CaCO_3$ on the end products of glucose fermentation by strain SAP.[a]

(A) Effect of $MgCO_3$

End products[b] (mmol/l)	100% N_2							100% CO_2	
	$MgCO_3$ (g/l)							$MgCO_3$ (g/l)	
	0	5	10	15	20	25	30	0	15
Succinate	7.03	15.08	16.28	17.30	17.11	16.86	17.03	6.18	7.03
Formate	0	9.56	34.80	41.30	60.25	63.03	65.56	91.68	125.57
Lactate	20.87	21.33	17.13	14.20	13.66	11.06	9.33	9.76	29.41
Acetate	32.47	41.33	56.83	59.65	64.30	68.16	72.50	55.62	77.76
Ethanol	36.30	41.73	47.47	52.30	57.17	61.74	64.30	72.39	63.47

(B) Effect of $CaCO_3$

End products[b] (mmol/l)	100% N_2						
	$CaCO_3$ (g/l)						
	0	5	10	15	20	25	30
Succinate	7	13.72	14.37	14.28	13.88	13.98	13.38
Formate	0	3.28	4.17	4.5	4.25	4.0	5.69
Lactate	20.87	18.0	16.76	16.11	16.11	15.77	15.55
Acetate	32.47	32.43	33.83	31.22	34.33	33.83	32.28
Ethanol	36.3	54.56	56.08	55.43	56.71	59.10	60.65

[a] Cells of strain SAP were cultivated in ANS-1 medium under N_2 or CO_2 atmosphere at 37°C for 24 h.

[b] End products were determined following complete dissolution of $MgCO_3$/$CaCO_3$.

glucose fermentation was studied. Carbon dioxide was supplied in the form of poorly soluble $MgCO_3$, which generates CO_2-HCO_3 during fermentation. Results showed that the yield of succinate was poor under N_2 and CO_2 atmosphere or when a combination of CO_2 atmosphere and $MgCO_3$ was used. However, the yield increased 2.5 fold when $MgCO_3$ was supplemented under N_2 atmosphere (Table 3). This suggests that the yield of succinate increased under N_2 atmosphere only in the presence of $MgCO_3$. It was reported that *Clostridium thermosuccinogenes* grew optimally and produced succinate from inulin at 58°C in the presence of 85% N_2–15% CO_2 atmosphere [25]. Samuelov *et al.* [24] showed that succinate production by *A. succiniciproducens* could be controlled by the availability of CO_2 as $MgCO_3$. At high CO_2-HCO_3 levels, succinate was the major product and neither lactate nor ethanol was detected, whereas succinate yield was lower and lactate was a major end product along with acetate, formate, and ethanol under low CO_2-HCO_3 levels. Van der Werf *et al.* [26] showed a direct relationship between the amounts of CO_2 in the medium and the succinate production by *Actinobacillus* sp. 130Z: With increasing concentrations of CO_2 in the medium as $MgCO_3$, succinate yield increased. The amount of acetate was independent of the CO_2 concentrations, whereas formate and ethanol production decreased with an increase of CO_2 in the medium.

In order to study the role of CO_2 in end-products formation by the strain SAP, $MgCO_3$ and $CaCO_3$ at various concentrations (0 to 30 g/l) were separately supplemented in ANS-1 medium under N_2 atmosphere. With increasing initial concentrations of $MgCO_3$ upto 15 g/l, succinate production was also increased and thereafter remained almost constant. Yields of formate, acetate, and ethanol were also increased with increasing concentration of $MgCO_3$ in the medium, whereas that of lactate was decreased. In contrast, when increasing concentrations of $CaCO_3$ were supplemented, succinate production at only the initial stage was increased and remained almost constant thereafter. A similar pattern was observed in the case of formate and ethanol. Acetate production remained almost constant, whereas lactate production was decreased. The amounts of succinate and other end products formed were largely affected by the availability of CO_2 as $MgCO_3$. A direct relationship was observed between the amounts of CO_2 in the medium as $MgCO_3$ at an initial concentration of 15 g/l and the production of succinate. On the other hand, lactate production decreased with an increase of CO_2 availability. In the case of $CaCO_3$ supplementation, similar results with lower yields of succinate were obtained. The formation of succinate requires fixation of 1 mol of CO_2 per mol of succinate [26], and this explains the higher yields of succinate in the presence of CO_2 as $MgCO_3$ in the medium.

Bioconversion of Fumarate to Succinate by Strain SAP

Since the strain SAP showed succinate production by using fumarate as the only carbon source, we examined the

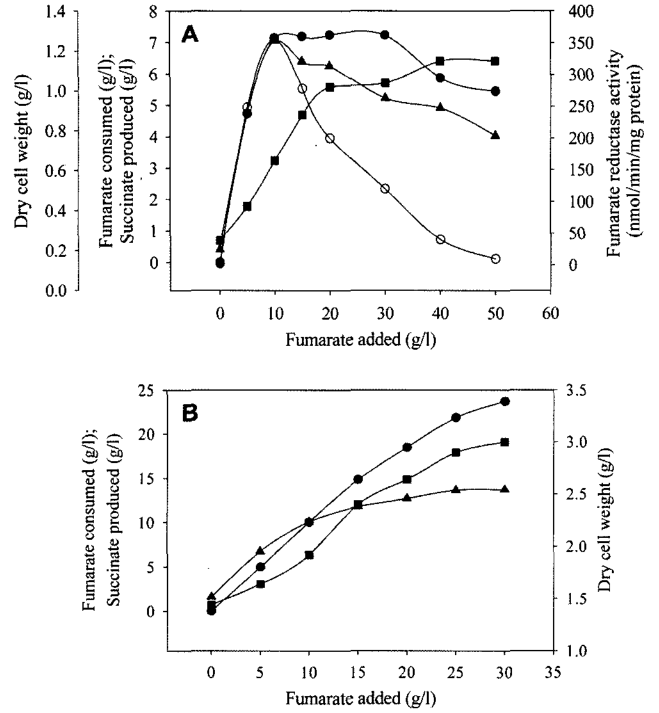

Fig. 1. Effect of fumarate supplementation on succinate production in absence (**A**) and presence (**B**) of glucose (15 g/l).
(●) Fumarate consumed (g/l); (▲) succinate produced (g/l); (■) dry cell weight (g/l); (○) fumarate reductase activity (nmol/min/mg protein).

potential of the strain to bioconvert fumarate to succinate and also the fumarate reductase activity with varying concentrations of fumarate. As shown in Fig. 1A, the maximum amount of succinate was obtained at an initial fumarate concentration of 10 g/l. Out of 10 g/l fumarate, 7.1 g/l of fumarate was converted to 7 g/l succinate with a molar conversion efficiency of 97.3%, and 3 g/l fumarate remained unutilized. Higher concentrations of sodium fumarate inhibited succinate production. The specific activity of fumarate reductase increased in the presence of fumarate, and it was the highest at 10 g/l fumarate concentration and it gradually decreased with an increase of fumarate concentrations (Fig. 1A). Cells grown on 10 g/l fumarate contained 18-fold higher fumarate reductase activity as compared with the activity of cells grown on 15 g/l glucose. In contrast, fumarate reductase activity of *Actinobacillus* sp. 130Z remained constant even in the presence of fumarate [26]. It was observed that, in the presence of glucose (15 g/l), succinate production by the strain SAP increased with increasing concentrations of fumarate (Fig. 1B).

Based on these results, the potential of the strain SAP for the production of succinate was compared with published data (Table 2), and the results showed that succinate production by the strain SAP after supplementation of fumarate was higher than that of *Actinobacillus* sp. 130Z and *E. coli* CA79. However, it was lower than that of *Enterococcus faecalis* RKY1. The experiment conducted with 11% cell suspension under N_2 atmosphere in 1.5% $MgCO_3$ containing 100 mM phosphate buffer (pH 7.0) showed that the production of succinate was considerably higher from fumarate: The molar yield of succinate was about 80% with a productivity of 1.03 and 1.35 g/l/h, when cells were supplemented with fumarate or fumarate along with glucose, respectively (data not shown).

Succinate is a highly reduced fermentation product using four electrons per molecule formed [5]. Therefore, the effect of hydrogen gas as electron source on fumarate was also studied during the growth. To our surprise, we observed that the yield of succinate did not change in the presence of hydrogen atmosphere, even though significant growth was observed (data not shown). In contrast to this, Van der Werf *et al.* [26] examined the growth of *Actinobacillus* sp. 130Z on fumarate plus hydrogen as electron source and showed that the strain grew anaerobically on fumarate in the presence of hydrogen and produced succinate. However, no growth was observed in the absence of hydrogen.

These interesting observations of the fermentation pathway encouraged us to study the metabolic and enzyme profiles of the strain to understand the synthesis of succinic acid.

Intracellular Metabolites of the Strain SAP

Table 4 summarizes the profile of intracellular metabolites of the strain SAP grown for 9 h on glucose under aerobic condition as well as N_2 atmosphere in the presence of $MgCO_3$. The PEP pool was smaller than that of pyruvate, indicating that PEP was rapidly converted to pyruvate, which was further degraded to lactate, acetate, and ethanol. Furthermore, considerable amount of oxaloacetate accumulated,

Table 4. Intracellular metabolite profile of strain SAP at 9 h of growth on glucose under aerobic and anaerobic conditions.

Metabolites	Concentration (μmol/g dry cell weight)	
	Aerobic growth[b]	Anaerobic growth[b]
Phosphoenolpyruvate	3.18	0.81
Pyruvate	5.73	3.40
Oxaloacetate	0.34	0.44
Citrate	0.56	0.38
Isocitrate	0.14	ND
α-Ketoglutarate	0.36	0.33
Malate	ND	ND
Fumarate	0.14	ND
Lactate	0.43	0.38
Succinate[a]	106.13	122.25
Acetate[a]	270.25	372.63

ND, Below detectable level.
[a] Analyzed by HPLC.
[b] Cells were grown in ANS-1 medium supplemented with (1.5%) $MgCO_3$ under aerobic condition as well as under anaerobic condition (N_2 atmosphere).

which was further converted to succinate in both cases. However, accumulation of pyruvate suggests that the rate of formation of these end products was slower than that of formation of pyruvate, or PEP was rapidly converted to oxaloacetate with a higher rate. The oxaloacetate produced was further converted to succinic acid via malate and fumarate by the reductive TCA pathway, resulting in accumulation of considerable amount of succinic acid and its excretion in the broth. This effect with a higher rate was observed under N_2 atmosphere in the presence of $MgCO_3$. It is also interesting to note that oxaloacetate, citrate, and α-ketoglutarate pools were considerably larger than those of isocitrate and fumarate. There is a possibility of conversion of isocitrate as well as fumarate to succinate. The concentration of malate was below detectable levels in both fermentation conditions, which indicated its rapid conversion to fumarate. The higher production of succinate, when fumarate and malate were supplemented as the sole carbon source (Table 2), indicates biosynthesis pathway of succinic acid by fumarate reductase.

Enzyme Activities Involved in End-Product Formation

The profile of enzyme activities responsible for end-product formation showed that most of the related enzymes were present in the extracts of glucose-fermenting cells of the strain SAP under aerobic as well as anaerobic conditions (Table 5). Prominently high activities of fumarate reductase, malate dehydrogenase, pyruvate kinase, PEP carboxylase, PEP carboxykinase, lactate dehydrogenase, and acetate kinase were detected under aerobic and anaerobic fermentation conditions. However, the PEP carboxykinase (1.2-fold), fumarate reductase (1.5-fold), fumarase (1.6-fold), pyruvate kinase (2.8-fold), and acetate kinase (2-fold) activities observed were much higher under anaerobic growth conditions. In contrast, the activities of PEP carboxylase (1.2-fold), malate dehydrogenase (1.2-fold), and lactate dehydrogenase (4.3-fold) were lower in anaerobic condition than those of aerobic condition. A detectable amount of formate dehydrogenase activity was also observed under both conditions. The activities of pyruvate formate lyase and NADH-dependent alcohol dehydrogenase were below the detectable level under aerobic growth conditions, but their activities were detectable under anaerobic growth conditions. In the case of the present strain, aerobic and anaerobic conditions were chosen to distinguish the changes of carbon flow to end products. Under both conditions, succinate was produced as one of the fermentation products along with lactate, acetate, and ethanol. However, the yields of succinate, lactate, acetate, formate, and ethanol were significantly higher under anaerobic condition than those of aerobic condition. These observations indicated that the synthesis of succinate by the strain SAP took place mainly by a fumarate reductase catalyzed reaction, whereas

Table 5. Profile of enzyme activities in strain SAP and other succinate forming organisms.

Enzyme (EC number)	Assay reference	Specific activity (nmol/min/mg protein)					
		Strain SAP[a]		*E. coli* K-12[b]	*Actinobacillus* sp. 130Z[b]	*A. succiniciproducens*[c]	
		Aerobic growth	Anaerobic growth			Low CO_2 pH 7.2	High CO_2 pH 6.2
Phosphoenolpyruvate carboxylase (EC 4.1.1.31)	[25]	14.51	11.94	140	10	NA	NA
Phosphoenolpyruvate carboxykinase (EC 4.1.1.49)		5.80	6.99	140	4,700	10	356
Malate dehydrogenase (EC 1.1.1.38) (NADH-dependent)		16.68	13.51	160	2,100	18,800	20,900
Fumarase (EC 4.2.1.2)		1.92	3.18	450	8,600	NA	NA
Fumarate reductase (EC 1.3.1.6)		12.54	18.98	1,100	1,200	380	118
Pyruvate kinase (EC 2.7.1.40)		8.92	24.88	1,400	1,100	9	8
Pyruvate formate lyase (EC 2.3.1.54)		ND	<1	1,400	390	NA	NA
Formate dehydrogenase (EC 1.2.1.2)		2.68	1.79	5	12	NA	NA
Lactate dehydrogenase (EC 1.1.1.28) (NADH)		43.29	10.13	560	9	27	ND
Acetate kinase (EC 2.7.2.1)		20.96	40.70	6,500	3,200	1,370	3,320
Alcohol dehydrogenase (EC 1.1.1.1) (NADH)		ND	6.51	75	17	44	ND

ND, Not detectable; NA, Not available.

[a] Extracts were prepared from cells grown in ANS-1 medium in the presence of 1.5% $MgCO_3$ under aerobic or anaerobic condition (N_2 atmosphere) and harvested in the late exponential phase of growth.

[b] Cells were grown in a medium (pH 7.0) containing glucose (10 g/l) with CO_2 as the gas phase [26].

[c] Extracts were prepared from fermenter cells grown at a constant pH with an initial glucose concentration of 10 g/l and harvested in the late exponential phase of growth. Carbon dioxide was supplied with (1.6%) Na_2CO_3 for low CO_2 condition, whereas a high CO_2 condition was maintained by (4.7%) Na_2CO_3 and continuous gassing with CO_2 (1 l/min) [24].

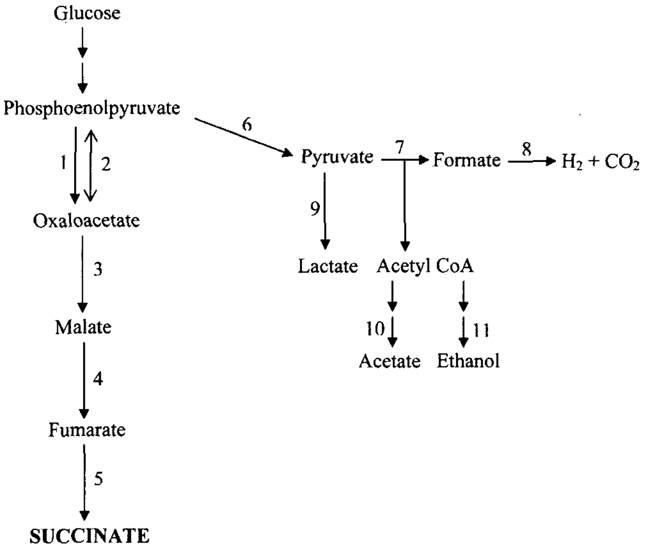

Fig. 2. Fermentative pathway for the formation of succinate in strain SAP under anaerobic growth conditions.

The numbers represent the reactions of the enzymes as follows: 1, PEP carboxylase; 2, PEP carboxykinase; 3, Malate dehydrogenase; 4, Fumarase; 5, Fumarate reductase; 6, Pyruvate kinase; 7, Pyruvate formate lyase; 8, Formate dehydrogenase; 9, Lactate dehydrogenase; 10, Acetate kinase; 11, Alcohol dehydrogenase.

the contribution of the other enzyme-catalyzed reactions was negligible. Based on the end products formed and enzyme activities detected under anaerobic fermentation of glucose, the major fermentative pathway for the formation of succinic acid in the strain SAP appears to be via fumarate (Fig. 2).

To gain insight as to which enzyme activities are the most important for the formation of succinate, we compared the enzyme activity levels of the strain SAP with those of *E. coli* K-12 [11, 3, 17], *A. succiniciproducens* [24], and *Actinobacillus* sp. 130Z [26] (Table 5). It can be seen in Table 5 that the strain SAP, *E. coli* K-12, and *Actinobacillus* sp. 130Z contained almost the same set of enzymes for the formation of end products from PEP. The formation of oxaloacetate (OAA) from PEP can be catalyzed by two enzymes, PEP carboxykinase and PEP carboxylase. PEP carboxykinase plays a key role in the CO_2 fixation necessary for succinate formation. Whereas the PEP carboxykinase activity is 470-fold higher in *Actinobacillus* sp. 130Z than the PEP carboxylase activity, the levels of these two enzyme activities are similar in *E. coli* K-12, and it is 1.7-fold lower in the strain SAP. Moreover, PEP carboxykinase activity in *Actinobacillus* sp. 130Z is about 34-fold and 670-fold higher than that in *E. coli* K-12 and the strain SAP, respectively. The levels of other enzyme activities varied greatly in these strains: In *Actinobacillus* sp. 130Z, much higher enzyme activities involved in the conversion of PEP to succinate (PEP carboxykinase, L-malate dehydrogenase, fumarate reductase, and fumarase) are present than in *E. coli* K-12 and the strain SAP. Furthermore, the activity of formate dehydrogenase in *Actinobacillus* sp. 130Z was higher than these two strains. The activity of L-malate dehydrogenase was found to be 10-fold lower in *Actinobacillus* sp. 130Z than in *A. succiniciproducens* (high CO_2, pH 6.2). Fumarate reductase activity was much lower in the strain SAP than in *Actinobacillus* sp. 130Z and *E. coli* K-12, whereas the activity was comparable in extracts of these two strains. The activity of fumarate reductase was about 10-fold higher in *Actinobacillus* sp. 130Z than in *A. succiniciproducens* (high CO_2, pH 6.2). The activity level of acetate kinase was almost similar in *Actinobacillus* sp. 130Z and *A. succiniciproducens.* On the other hand, much higher levels of enzyme activities (pyruvate formate lyase, lactate dehydrogenase, acetate kinase, and alcohol dehydrogenase) involved in the formation of pyruvate-derived end products were observed in *E. coli* K-12 than in the strain SAP, *Actinobacillus* sp. 130Z, and *A. succiniciproducens.* The pyruvate kinase and PEP carboxylase activities were much higher in *E. coli* K-12 than in the strain SAP, *Actinobacillus* sp. 130Z, and *A. succiniciproducens.*

In conclusion, efforts were made in the present study to study the profile of metabolites and enzymes to elucidate the pathway of succinic acid synthesis in the strain SAP. When the strain was grown under nitrogen atmosphere in the presence of $MgCO_3$ with glucose as the carbon source, a significant amount of succinic acid was produced. The succinic acid yield was increased by the supplementation of fumarate. Therefore, the TCA cycle in the strain SAP under anaerobic condition appears to be directed towards the production of biosynthetic intermediates more than metabolic energy with the production of succinate by the reductive TCA cycle. This conclusion is supported by the presence of fumarate reductase and its induction by supplementation of fumarate. Results obtained during fermentation indicated that succinate production was controlled by the availability of CO_2. In the presence of $MgCO_3$, succinate was produced in significant amount. The levels of PEP carboxykinase, fumarase, and fumarate reductase were high in the strain SAP under anaerobic condition than those under aerobic condition. This resulted in rapid conversion of PEP to succinate via oxaloacetate, malate, and fumarate under anaerobic condition. The ratio of glucose carbon used for cell mass production to that used for end products [C (cells)/C (succinate)+C (lactate)+ C (acetate)+C (formate)] was 0.22 and 0.24 in the presence and absence of $MgCO_3$ under anaerobic conditions, respectively, whereas it was 0.92 under aerobic condition. This indicates that carbon flow is mainly towards the formation of succinate as a major end product in the presence of $MgCO_3$ under N_2 atmosphere, which is in accordance with higher levels of PEP-carboxykinase present under anaerobic growth conditions.

The presence of PEP-carboxylase and PEP-carboxykinase indicates the possibility of the presence of a mechanism of converting a three-carbon glycolytic intermediate PEP to a four-carbon TCA cycle intermediate OAA by the fixation of CO_2, as well as formation of a three-carbon metabolite PEP from a four-carbon compound OAA, respectively. The partitioning of PEP is highly regulated: *E. coli* elaborates two enzymes capable of carboxylating PEP to produce OAA, and PEP carboxylase functions aerobically to replenish OAA consumed in biosynthetic reactions [1]. Under fermentative conditions, PEP-carboxylase also has a catabolic function; it directs a portion of the PEP to succinate [10]. PEP-carboxykinase functions physiologically in gluconeogenesis, catalyzing the nucleotide triphosphate-dependent decarboxylation and phosphorylation of OAA to yield PEP [4].

Kim *et al.* [14] investigated succinate fermentation in *E. coli* strains overexpressing *Actinobacillus succinogenes* PEP-carboxykinase. In *E. coli* K-12, PEP-carboxykinase overexpression had no effect on succinate fermentation. In contrast, in the PEP-carboxylase mutant *E. coli* strain K-12 *ppc*::*kan*, PEP-carboxykinase overexpression increased succinate production. In *E. coli*, however, PEP-carboxylase is the enzyme that carboxylates PEP during growth on glucose. However, PEP-carboxykinase seems to be better suited than PEP-carboxylase for succinate production, because it generates ATP-conserving energy during glycolysis, whereas PEP-carboxylase dissipates that energy. The present results showed that PEP-carboxykinase can replace PEP-carboxylase as the PEP-carboxylating enzyme in *E. coli*, and that PEP-carboxykinase overexpression results in a significant increase of succinate production. In this respect, since PEP-carboxykinase's K_m for HCO_3^- is two orders of magnitude higher than that of PEP-carboxylase, optimization of the HCO_3^- concentration might be a critical step towards increasing succinate production through PEP-carboxykinase overexpression [14]. Yang *et al.* [29] observed that PEP-carboxykinase showed significant activity in glucose-grown *E. coli* cultures, in which it decarboxylates OAA back to PEP. A detailed metabolic flux analysis suggested that the opposing fluxes generated by PEP-carboxylase and PEP-carboxykinase activities are controlled at the kinetic level by PEP and OAA concentrations. Gokarn *et al.* [7] reported slightly higher production of succinate in a modified *E. coli* strain JCL1242/pTrc99A-*pyc* than by cells that overproduced PEP-carboxylase (JCL1242/pPC201, ppc^+), even though the PEP-carboxylase activity in cell extracts of JCL1242/pPC201 (ppc^+) was 40-fold greater than pyruvate carboxylase activity in extracts of JCL1242/pTrc99a-*pyc*.

Magnesium ion plays an important role in maintaining cellular metabolism, and is especially important since it is a cofactor for PEP-carboxykinase, a key enzyme in succinic acid production [16]. Podkovyrov and Zeikus [21] also reported that the *in vitro* activity of PEP-carboxykinase purified from *A. succiniciproducens* was significantly enhanced by magnesium ion. It is of interest to note that $MgCO_3$ plays an important role in the formation of succinic acid by the strain SAP. The presence of $MgCO_3$ also helps maintain the pH during the fermentation process as well as provide CO_2 for the synthesis of succinic acid, where PEP-carboxykinase plays a key role in the CO_2 fixation.

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