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 MgCO₃ under N₂ atmosphere as compared with that under CO₂ 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 N₂ atmosphere in the presence of MgCO₃. 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 C₄ 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 N₂ atmosphere in the presence of MgCO₃, 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
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; K₂HPO₄, 3; NaCl, 1; (NH₄)₂SO₄, 1; CaCl₂·2H₂O, 0.07; MgCl₂·6H₂O, 0.2; and MgCO₃, 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 H₂SO₄ 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 ANS-1 medium at 37°C on a shaking incubator (Gyromax, U.S.A.) at 150 rpm.

**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 N₂ 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 (N₂ 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 (N₂ atmosphere in the presence and absence of MgCO₃). In the presence or absence of MgCO₃ 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
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 homfermentatively 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

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**Table 1.** End products of glucose fermentation by strain SAP at 9 h of growth under aerobic and anaerobic conditions (N$_2$ atmosphere).

<table>
<thead>
<tr>
<th>End products</th>
<th>Concentration (mmol/100 mmol of consumed glucose)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aerobic growth with MgCO$_3$ (15 g/l)$^a$</td>
</tr>
<tr>
<td></td>
<td>With MgCO$_3$ (15 g/l)$^a$</td>
</tr>
<tr>
<td>Cell carbon$^b$</td>
<td>90</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>6.80</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>3.40</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>22</td>
</tr>
<tr>
<td>Formic acid</td>
<td>ND</td>
</tr>
<tr>
<td>Ethanol</td>
<td>8.20</td>
</tr>
<tr>
<td>Carbon recovery</td>
<td>0.31</td>
</tr>
<tr>
<td>Electron recovery$^b$</td>
<td>0.31</td>
</tr>
</tbody>
</table>

ND, Not detectable.

$^a$Cell carbon was calculated with CH$_3$O$_2$N$_4$ [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$_3$. 

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[26] Thakker et al.
Table 2. Comparison of fermentation end products of strain SAP with those of other succinate forming-organisms.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Substrate</th>
<th>Dry cell weight (g/l)</th>
<th>mmol of fermentation products/l</th>
<th>Succinate productivity (g/l/h)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain SAP</td>
<td>Glucose (1.5%)</td>
<td>1.51</td>
<td>17.30 14.20 59.65 41.30 0 52.30 0</td>
<td>-</td>
<td>Present study</td>
</tr>
<tr>
<td></td>
<td>Fumarate (1%)</td>
<td>0.65</td>
<td>43.50 0 0 0 4.80 0 0.29</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fumarate (1 %)+ glucose (1.5%)</td>
<td>1.86</td>
<td>62.25 3.70 33.20 0 6 19 0.42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malate (1%)</td>
<td>0.84</td>
<td>22.40</td>
<td>0 10.50 0 0 0 0.09</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Isocitrate (1%)</td>
<td>0.60</td>
<td>2.80</td>
<td>2.20 6.30 0 0 0 0.04</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>α-KGA (1%)</td>
<td>0.59</td>
<td>2.90</td>
<td>2.10 5.70 0 0 10 0.11</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Glutamate (1%)</td>
<td>0.79</td>
<td>2.70</td>
<td>2.20 5.80 0 0 9 0.23</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Fumarate+ glutamate (1% each)</td>
<td>0.68</td>
<td>32</td>
<td>0 0 3.40 0 0.00</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>E. coli K-12</td>
<td>Glucose (1%)</td>
<td>NA</td>
<td>6.60 17 42.90 83.60 0 29.10 -</td>
<td>[28]</td>
<td></td>
</tr>
<tr>
<td>E. coli CA79</td>
<td>Fumarate (4%)+ glucose (1%)</td>
<td>NA</td>
<td>17.60 0 2.65 0 39.46 0 0.11</td>
<td>[9]</td>
<td></td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>Fumarate (10%)+ glucose (1.5%)</td>
<td>NA</td>
<td>548 0 0 0 0 0.45</td>
<td>[22]</td>
<td></td>
</tr>
<tr>
<td>RKY1</td>
<td>Glucose (2%)</td>
<td>NA</td>
<td>133 0 73.70 13.20 0 ND -</td>
<td>[24]</td>
<td></td>
</tr>
<tr>
<td>Anaerobiospirillum</td>
<td>Glucose (1%)</td>
<td>NA</td>
<td>41.25 0 48.40 42.90 0 3.80 -</td>
<td>[26]</td>
<td></td>
</tr>
<tr>
<td>succiniciproducens</td>
<td>Fumarate (0.8%)</td>
<td>NA</td>
<td>34 0 7.0 5.50 0 0 0.23</td>
<td>[15, 12]</td>
<td></td>
</tr>
<tr>
<td>Actinobacillus sp. 130Z</td>
<td>Glucose (1%)</td>
<td>NA</td>
<td>55.50 2.22 38.85 50.50 0 0 -</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NA, Not available; ND, Not detectable.

*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 CO₂ in the form of MgCO₃ and CaCO₃ on the end products of glucose fermentation by strain SAP.

(A) Effect of MgCO₃

<table>
<thead>
<tr>
<th>End products (mmol/l)</th>
<th>100% N₂ MgCO₃ (g/l)</th>
<th>100% CO₂ MgCO₃ (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate</td>
<td>0        5     10    15    20    25    30</td>
<td>0        15</td>
</tr>
<tr>
<td>Formate</td>
<td>0        9.56  34.80 41.30  60.25  63.03  65.56</td>
<td></td>
</tr>
<tr>
<td>Lactate</td>
<td>20.87    21.33 17.13  14.20  13.66  11.06  9.33</td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>32.47    41.33 56.83  59.65  64.30  68.16  72.50</td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>36.30    41.73 47.47  52.30  57.17  61.74  64.30</td>
<td></td>
</tr>
</tbody>
</table>

(B) Effect of CaCO₃

<table>
<thead>
<tr>
<th>End products (mmol/l)</th>
<th>100% N₂ CaCO₃ (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formate</td>
<td>0       3.28   4.17   4.5    4.25   4.0    5.69</td>
</tr>
<tr>
<td>Lactate</td>
<td>20.87   18.0   16.76  16.11  16.11  15.77  15.55</td>
</tr>
<tr>
<td>Acetate</td>
<td>32.47   32.43  33.83  31.22  34.33  33.83  32.28</td>
</tr>
<tr>
<td>Ethanol</td>
<td>36.3    54.56  56.08  55.43  56.71  59.10  60.65</td>
</tr>
</tbody>
</table>

*Cells of strain SAP were cultivated in ANS-1 medium under N₂ or CO₂ atmosphere at 37°C for 24 h.
*End products were determined following complete dissolution of MgCO₃/CaCO₃.

Glucose fermentation was studied. Carbon dioxide was supplied in the form of poorly soluble MgCO₃, which generates CO₂-HCO₃⁻ during fermentation. Results showed that the yield of succinate was poor under N₂ and CO₂ atmosphere or when a combination of CO₂ atmosphere and MgCO₃ was used. However, the yield increased 2.5 fold when MgCO₃ was supplemented under N₂ atmosphere (Table 3). This suggests that the yield of succinate increased under N₂ atmosphere only in the presence of MgCO₃. It was reported that Clostridium thermosuccinogenes grew optimally and produced succinate from inulin at 58°C in the presence of 85% N₂-15% CO₂ atmosphere [25]. Samuelov et al. [24] showed that succinate production by A. succiniciproduces could be controlled by the availability of CO₂ as MgCO₃. At high CO₂-HCO₃⁻ 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 CO₂-HCO₃⁻ levels. Van der Werf et al. [26] showed a direct relationship between the amounts of CO₂ in the medium and the succinate production by Actinobacillus sp. 130Z. With increasing concentrations of CO₂ in the medium as MgCO₃, succinate yield increased. The amount of acetate was independent of the CO₂ concentrations, whereas formate and ethanol production decreased with an increase of CO₂ in the medium.

In order to study the role of CO₂ in end-products formation by the strain SAP, MgCO₃ and CaCO₃ at various concentrations (0 to 30 g/l) were separately supplemented in ANS-1 medium under N₂ atmosphere. With increasing initial concentrations of MgCO₃, succinate production was also increased and thereafter remained almost constant. Yields of formate, acetate, and ethanol were also increased with increasing concentration of MgCO₃ in the medium, whereas that of lactate was decreased. In contrast, when increasing concentrations of CaCO₃ 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 CO₂ as MgCO₃. A direct relationship was observed between the amounts of CO₂ in the medium as MgCO₃ at an initial concentration of 15 g/l and the production of succinate. On the other hand, lactate production decreased with an increase of CO₂ availability. In the case of CaCO₃ supplementation, similar results with lower yields of succinate were obtained. The formation of succinate requires fixation of 1 mol of CO₂ per mol of succinate [26], and this explains the higher yields of succinate in the presence of CO₂ as MgCO₃ 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
FORMATION OF SUCCINIC ACID BY KLEBSIELLA PNEUMONIAE 875

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 N₂ atmosphere in 1.5% MgCO₃ 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 N₂ atmosphere in the presence of MgCO₃. 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.

![Fig. 1. Effect of fumarate supplementation on succinate production in absence (A) and presence (B) of glucose (15 g/l).](image)

![Fig. 1. Effect of fumarate supplementation on succinate production in absence (A) and presence (B) of glucose (15 g/l).](image)

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

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Concentration (µmol/g dry cell weight)</th>
<th>Aerobic growth</th>
<th>Anaerobic growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphoenolpyruvate</td>
<td>3.18</td>
<td>0.81</td>
<td></td>
</tr>
<tr>
<td>Pyruvate</td>
<td>5.73</td>
<td>3.40</td>
<td></td>
</tr>
<tr>
<td>Oxaloacetate</td>
<td>0.34</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>Citrate</td>
<td>0.56</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>Isocitrate</td>
<td>0.14</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>0.36</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>Malate</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Fumarate</td>
<td>0.14</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Lactate</td>
<td>0.43</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>106.13</td>
<td>122.25</td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>270.25</td>
<td>372.63</td>
<td></td>
</tr>
</tbody>
</table>

ND, Below detectable level.

*Analyzed by HPLC.

*Cells were grown in ANS-1 medium supplemented with (1.5%) MgCO₃ under aerobic condition as well as under anaerobic condition (N₂ 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 N₂ atmosphere in the presence of MgCO₃. 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 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 aerobic 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.

<table>
<thead>
<tr>
<th>Enzyme (EC number)</th>
<th>Assay reference</th>
<th>Specific activity (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Strain SAP&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aerobic growth</td>
</tr>
<tr>
<td><strong>Phosphoenolpyruvate carboxylase (EC 4.1.1.31)</strong></td>
<td>[25]</td>
<td>14.51</td>
</tr>
<tr>
<td><strong>Phosphoenolpyruvate carboxykinase (EC 4.1.1.49)</strong></td>
<td></td>
<td>5.80</td>
</tr>
<tr>
<td><strong>Malate dehydrogenase (EC 1.1.1.38)</strong> (NADH-dependent)</td>
<td></td>
<td>16.68</td>
</tr>
<tr>
<td><strong>Fumarase (EC 4.2.1.2)</strong></td>
<td></td>
<td>1.92</td>
</tr>
<tr>
<td><strong>Fumarate reductase (EC 1.3.1.6)</strong></td>
<td></td>
<td>12.54</td>
</tr>
<tr>
<td><strong>Pyruvate kinase (EC 2.7.1.40)</strong></td>
<td></td>
<td>8.92</td>
</tr>
<tr>
<td><strong>Pyruvate formate lyase (EC 2.3.1.54)</strong></td>
<td>ND</td>
<td>&lt;1</td>
</tr>
<tr>
<td><strong>Formate dehydrogenase (EC 1.2.1.2)</strong></td>
<td>2.68</td>
<td>1.79</td>
</tr>
<tr>
<td><strong>Lactate dehydrogenase (EC 1.1.1.28) (NADH)</strong></td>
<td>43.29</td>
<td>10.13</td>
</tr>
<tr>
<td><strong>Acetate kinase (EC 2.7.2.1)</strong></td>
<td>20.96</td>
<td>40.70</td>
</tr>
<tr>
<td><strong>Alcohol dehydrogenase (EC 1.1.1.1) (NADH)</strong></td>
<td>ND</td>
<td>6.51</td>
</tr>
</tbody>
</table>

ND, Not detectable; NA, Not available.
<sup>a</sup>Extracts were prepared from cells grown in ANS-1 medium in the presence of 1.5% MgCO₃ under aerobic or anaerobic condition (N₂ atmosphere) and harvested in the late exponential phase of growth.
<sup>b</sup>Cells were grown in a medium (pH 7.0) containing glucose (10 g/l) with CO₂ as the gas phase [26].
<sup>c</sup>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%) Na₂CO₃ for low CO₂ condition, whereas a high CO₂ condition was maintained by (4.7%) Na₂CO₃ and continuous gassing with CO₂ (1 l/min) [24].
Carboxykinase plays a key role in the CO₂ fixation necessary for succinate formation. The 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*. 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 MgCO₃ 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 CO₂. In the presence of MgCO₃, 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, lactate, 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 MgCO₃ 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 MgCO₃ under N₂ 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 CO₂, 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*::lacI, 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 is a cofactor for PEP-carboxykinase, a key enzyme in succinic acid production [16]. Podkobyrov 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 MgCO₃ plays an important role in the formation of succinic acid by the strain SAP. The presence of MgCO₃ also helps maintain the pH during the fermentation process as well as provide CO₂ for the synthesis of succinic acid, where PEP-carboxykinase plays a key role in the CO₂ fixation.

**References**


FORMATION OF SUCCINIC ACID BY KLEBSIELLA PNEUMONIAE


