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Effect of Dissolved Oxygen Concentration on the Metabolism of Glucose in *Pseudomonas putida* BM014

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The effect of dissolved oxygen concentration on the metabolism of glucose in *Pseudomonas putida* BM014 was investigated. Glucose was completely converted to 2-ketogluconate *via* extracellular oxidative pathway and then taken up for cell growth under the condition of sufficient dissolved oxygen concentration. On the other hand, oxygen limitation below dissolved oxygen tension (DOT) value of 20% of air saturation caused the shift of glucose metabolism from the extracellular oxidative pathway to the intracellular phosphorylative pathway. Specific activities of hexokinase and gluconate kinase in intracellular phosphorylation pathway decreased as the DOT increased, while 2-ketogluconokinase activity in extracellular oxidative pathway increased under the same condition. This result can be usefully applied to microbial transformation of glucose to 2-ketogluconate, the synthetic precursor for iso-vitamine C, with almost 100% yield *via* extracellular oxidation by simple DOT control.

Key words: *Pseudomonas putida* BM014, 2-ketogluconate, extracellular oxidation, dissolved oxygen concentration, glucose metabolism.

There are two routes of glucose assimilation depending on physiological conditions: intracellular phosphorylative pathway and extracellular oxidative pathway [1-3]. Glucose can be degraded to glyceraldehyde-3-phosphate and pyruvate *via* the Entner-Doudoroff pathway, intracellular phosphorylation pathway. Pseudomonads, such as *P. aeroginosa*, *P. fluorescence* and *P. putida*, can also metabolize glucose *via* extracellular direct oxidative pathway [4]. In this pathway, glucose is oxidized extracellularly by glucose dehydrogenase and gluconate dehydrogenase, and the products of these enzymes, gluconate and 2-ketogluconate, are taken up by specific transport systems for intracellular phosphorylation. In this work, the effect of dissolved oxygen tension (DOT) on glucose metabolism in *Pseudomonas putida* BM014 was investigated to examine that DOT can be used as a practical control variable to switch the glucose metabolism from intracellular phosphorylative pathway to extracellular oxidative pathway and *vice versa*.

The microorganism used in this study was *Pseudomonas putida* BM014, a mutant strain which originated from the wild-type strain of *Pseudomonas putida* HL06-3 isolated from soil samples [5], and could accumulate *cis,cis*-muconic acid from benzoic acid [6, 7].

Batch culture of *P. putida* BM014 was carried out in a NO3 minimal medium of the following compositions: $Na_2HPO_4 \cdot 12H_2O$ 18 g/l, KH_2PO_4 13.6 g/l, glucose 10 g/l, $(NH_4)_2SO_3$ 2.25 g/l, $MgSO_4 \cdot 7H_2O$ 3 mM, $CaCl_2$ 0.3 mM, and $FeSO_4 \cdot 7H_2O$ 0.03 mM, and if necessary, the NO3 medium was supplemented with appropriate amount of sodium benzoate. For continuous cultures to examine the activities of glucose metabolizing enzymes, NO3 minimal medium with 20 g/l of glucose and 4.5 g/l of ammonium sulfate to prevent carbon and nitrogen limitation was used as a feed solution. Dilution rate was adjusted to 0.2 h^{-1} and DOT was controlled by air flow rate and/or agitation speed.

Enzymes of glucose metabolism, hexokinase (EC 2.7.1.1) and gluconate kinase (EC 2.7.1.12), were assayed under the conditions described by Ng and Dawes [8]. The combined activity of 2-ketogluconate kinase and 2-keto-6-phosphogluconate reductase (referred to as 2-ketogluconokinase) was assayed by the method of Whiting *et al.* [4]. The protein contents of crude cell extracts were measured by the procedure described by Lowry *et al.* [9] using bovine serum albumin as a standard.

Glucose was determined with a glucose oxidase kit (Young Dong Pharmaceuticals, Korea) and 2-ketogluconate using method described by Frampton and Wood [10]. Gluconate was assayed with Boehringer test-kit 428191.

Silica gel 60 F_{254} (Merck) was used as a TLC plate and a mixture of 2-propanol, acetone, and 0.1 M lactic acid in the volume ratio of 2:2:1 was employed as a running solution. For developing reagents, aqueous silver nitrate (reagent 1) and 0.5 N aqueous methanolic sodium hydroxide (reagent 2) was used. Spraying was carried out with reagent 1 and then reagent 2, and the TLC plate was finally heated at

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100℃ for 1-2 min.

Dissolved oxygen (DO) profile and biomass concentration were studied during the batch culture of *P. putida* BM014 in a NO3 minimal medium supplemented with 10 mM sodium benzoate. During the early part of batch culture, the DOT decreased gradually down to 50% of air saturation until 9 h and then increased abruptly until 11 h (Fig. 1). On the other hand, biomass concentration remained almost constant until 11 h (Fig. 2), implying that glucose was not utilized as a growth carbon source. After this change, DOT continued to decrease, although oxygen supply was enhanced by increasing air flow rate and agitation speed (Fig. 1). The results shown in Fig. 1 and 2 reveal that glucose is converted to unknown substances and this conversion reaction requires a lot of oxygen.

For identification of the unknown substances in glucose conversion reaction, thin layer chromatography (TLC) analysis was performed during the lag phase of cell growth. As shown in Fig. 3, the migration distances of samples on the thin layer plate coincided with those of the standard samples of glucose, gluconate and 2-ketogluconate, implying that the unknown substances might be gluconate and 2-ketogluconate. During the time course of cell growth, the spot area of glucose became smaller, those of gluconate and 2-ketogluconate grew larger, and finally only 2-ketogluconate could be detected. It is inferred that glucose is oxidized to 2-ketogluconate *via* gluconate intermediate and 2-ketogluconate is the final extracellular metabolite which can be taken up for cell growth. Another quantitative analyses are shown in Fig. 2. At 12 h, concentration of 2-ketogluconate was equivalent to that of glucose contained in the initial medium and glucose or gluconate was not detected. After this lag period, exponential cell growth was initiated at the expense of 2-ketogluconate consumption and DO profile gradually decreased. Therefore, it can be concluded that only 2-ketogluconate, the metabolite in extracellular oxidation of glucose, is utilized for the growth of *P. putida* BM014.

Since the extracellular oxidation reaction consumes a lot of oxygen as shown in Fig. 1, oxygen limitation may cause the shift of glucose metabolism from the extracellular direct oxidative pathway to

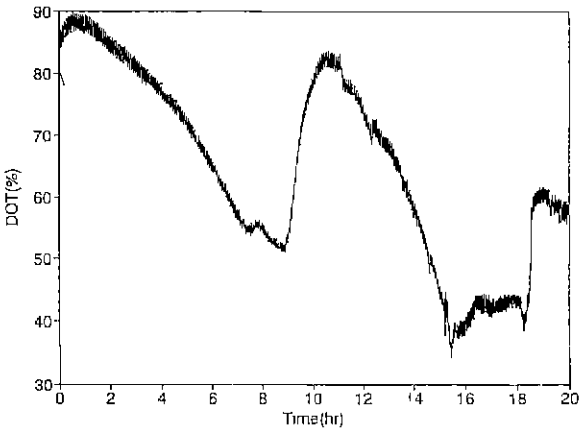

Fig. 1. DO profile during the batch culture of *P. putida* BM014.

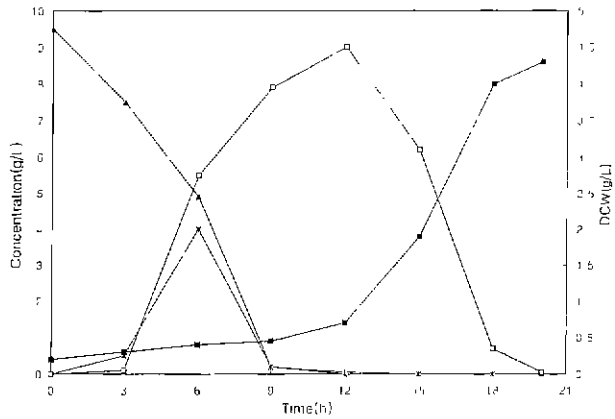

Fig. 2. Biomass concentration (■) and quantitative analysis for glucose (▲), gluconate (✳) and 2-ketogluconate (□) during the batch culture of *P. putida* BM014.

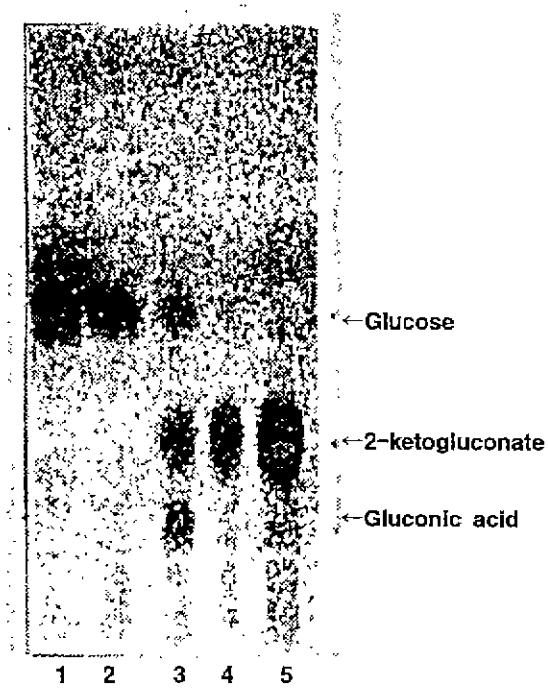

Fig. 3. TLC analysis of culture broth (1-5: samples at 0, 3, 6, 9, 12 h).

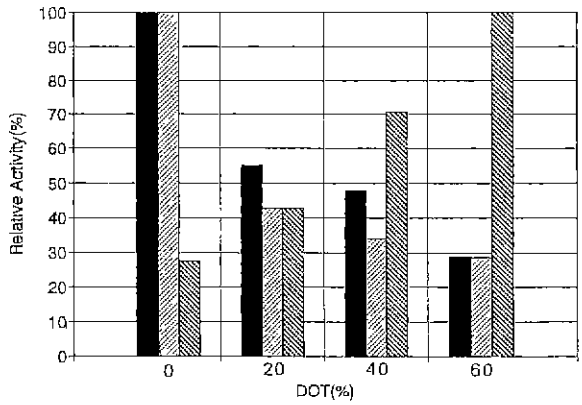

Fig. 4. Effect of dissolved oxygen concentration on the activities of hexokinase (▮), gluconate kinase (▨), and 2-ketogluconokinase (▩) during continuous culture. Relative activities of 100% for hexokinase, gluconate kinase and 2-ketogluconokinase mean 1.14, 0.372, and 0.378 μmol h^{-1} (mg protein)$^{-1}$, respectively.

Table 1. Residual glucose, gluconate, 2-ketogluconate and biomass concentrations in experiments shown in Fig. 4.

DOT (% of air saturation)	Glucose (g/l)	Gluconate (g/l)	2-ketogluconate (g/l)	Biomass (dry wt/l)
0	4.5	0	0	6.8
20	0.1	1.1	3.8	7.5
40	0	0	3.2	7.8
60	0	0	2.8	8.1

the intracellular phosphorylative pathway. Thus, it is of interest to examine the effect of dissolved oxygen concentration on the metabolism of glucose. Specific activities of hexokinase, gluconate kinase, and 2-ketogluconokinase were used as determinants to represent whether glucose was metabolized *via* extracellular oxidative pathway or intracellular phosphorylative pathway.

Specific activities of hexokinase and gluconate kinase in intracellular phosphorylative pathway decreased as the DOT increased, while 2-ketogluconokinase activity increased under the same condition (Fig. 4). When the NO3 medium was supplemented with 10 mM sodium benzoate, similar results were observed (data not shown). These results are quite different from the report described by Mitchell and Dawes [3]. There was an initial increase in the activity of hexokinase at low DOT, and then followed by decrease in activity at high DOT. In case of gluconate kinase, any response of activity change to the changes in DOT could not be observed in their report.

Quantitative analysis for residual glucose, gluconate, 2-ketogluconate and biomass concentration are shown in Table 1. Glucose was not oxidized to gluconate at DOT of 0% of air saturation, but converted to gluconate and 2-ketogluconate at higher DOT. Gluconate was converted to 2-ketogluconate at the DOT of 20% of air saturation. From the above results, it is concluded that glucose is converted to 2-ketogluconate *via* extracellular oxidative pathway and then taken up for cell growth under the conditions of sufficient dissolved oxygen concentration of more than 20% of air saturation. On the other hand, oxygen limitation causes the shift of glucose metabolism from the extracellular oxidative pathway to the intracellular phosphorylative pathway. This result can be usefully applied to microbial transformation of glucose to 2-ketogluconate using pseudomonads with almost 100% yield *via* extracellular oxidative pathway by simple DOT control. 2-ketogluconate is a commercially important precursor for the synthesis of iso-vitamine C which can be used as an antioxidant in food [11].

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