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Two-Stage Fermentation for 2-Ketogluconic Acid Production by *Klebsiella pneumoniae*

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2-Ketogluconic acid production by *Klebsiella pneumoniae* is a pH-dependent process, strictly proceeding under acidic conditions. Unfortunately, cell growth is inhibited by acidic conditions, resulting in low productivity of 2-ketogluconic acid. To overcome this deficiency, a two-stage fermentation strategy was exploited in the current study. During the first stage, the culture was maintained at neutral pH, favoring cell growth. During the second stage, the culture pH was switched to acidic conditions favoring 2-ketogluconic acid accumulation. Culture parameters, including switching time, dissolved oxygen levels, pH, and temperature were optimized for the fed-batch fermentation. Characteristics of glucose dehydrogenase and gluconate dehydrogenase were revealed *in vitro*, and the optimal pHs of the two enzymes coincided with the optimum culture pH. Under optimum conditions, a total of 186 g/l 2-ketogluconic acid was produced at 26 h, and the conversion ratio was 0.98 mol/mol. This fermentation strategy has successfully overcome the mismatch between optimum parameters required for cell growth and 2-ketogluconic acid accumulation, and this result has the highest productivity and conversion ratio of 2-ketogluconic and produced by microorganism.

Keywords: 2-Ketogluconic acid, *Klebsiella pneumoniae*, two-stage fermentation, erythorbic acid

Introduction

2-Ketogluconic acid is used for the synthesis of erythorbic acid (isoascorbic acid), an antioxidant used in the food industry [4]. Many bacteria, including *Acetobacter pasteurianus* [13], *Enterobacter intermedium* [7], *Gluconobacter oxydans* [17], *Klebsiella pneumoniae* [10], *Pseudomonas aeruginosa* [4], *Pseudomonas fluorescens* [12], and *Serratia marcescens*, convert glucose to 2-ketogluconic acid [9]. Among these species, *P. fluorescens* is often used in industry.

K. pneumoniae CGMCC 1.6366 (TUAC01) is a strain isolated for the production of 1,3-propanediol [5]. Efficient gene replacement in *K. pneumoniae* has been exploited, and several mutants derived from *K. pneumoniae* CGMCC 1.6366 have been constructed [18,19]. The *budA* mutant that has disrupted the gene encoding alpha-acetolactate

decarboxylase, a key enzyme in the 2,3-butanediol synthesis pathway, was found to accumulate 2-ketogluconic acid in cultures exposed to acidic conditions [20].

The metabolic pathway of 2-ketogluconic acid synthesis and utilization in *Pseudomonas putida* has been deduced from gene annotations [3]. The structure of the 2-ketogluconate utilization operon in *K. pneumoniae* CGMCC 1.6366 (Gene ID KF640649) is homologous to that in *Pseudomonas aeruginosa* PAO1 [14]. Hence, the metabolic pathway of 2-ketogluconic acid synthesis and utilization in *K. pneumoniae* might be similar to that of *Pseudomonas* sp.

2-Ketogluconic acid is not accumulated in the broth of *K. pneumoniae* when cultured in neutral medium. 2-Ketogluconic acid accumulation only occurs under acidic culture conditions (around pH 5). However, cell growth is inhibited in acidic conditions, and subsequently leads to low productivity of

2-ketogluconic acid [20]. To overcome this problem, we developed a two-stage fermentation strategy. During the first stage, the culture pH was kept at neutral conditions, favoring cell growth. During the second stage, the culture pH was switched to acidic conditions optimal for 2-ketogluconic acid accumulation.

Materials and Methods

Strains

K. pneumoniae ΔbudA is a truncated mutant lacking the *bud*A gene that encodes alpha-acetolactate decarboxylase. *K. pneumoniae* ΔbudA was constructed as described previously [20].

Culture Conditions and Analytical Methods

The fermentation medium, culture conditions, and analytical methods were as described previously [20]. For the fed-batch fermentation, sterilized glucose solution was fed into the medium when glucose levels had decreased to 20 g/l, maintaining glucose levels within the range of 20–50 g/l.

Enzyme Assays

Glucose dehydrogenase and gluconate dehydrogenase activity assays were performed as described by Tlemcani *et al.* [15]. Acetate buffer and phosphate buffer with different pH values were used to evaluate the effect of pH on the enzyme activity. The effect of reaction temperature on the enzyme activity was determined by keeping the reaction mixture in a water-bath before measure, and setting the cuvette temperature at the same temperature; the reaction buffer used was a pH 7.0 phosphate buffer.

Optimization of Fermentation Parameters

Unless otherwise stated, for the first stage of fermentation, the culture was maintained at pH 7.0, the agitation kept at 500 rpm, and the pH adjusted for the second stage at 4 h. For the second stage, the culture was agitated at 800 rpm and the pH set at 5.0. The culture pH was gradually dropped from 7.0 to 5.0 by the organic acids (lactic acid, acetate acid, and 2-ketogluconic acid) produced by the cell. No external acid was added in this process. Once the culture reached pH 5.0, NH_3 solution was used to maintain this pH. The conversion ratio of glucose to 2-ketogluconic acid was calculated by their concentrations in the culture broth, and at the same time, the change of culture medium volume was also taken into account.

For studies on the effect of dissolved oxygen concentration, agitation was maintained at 500 rpm during the first stage of fermentation. Changes in dissolved oxygen concentration during the second stage of fermentation were achieved by automatic adjustment of the agitation speed. A 0% dissolved oxygen level was attained by constant agitation at 450 rpm.

For studies on the effect of pH, cultures were maintained at pH 7.0 during the first stage of fermentation, and different pHs assessed during the second stage of fermentation.

Similarly, fermentation temperature was kept constant at 37°C during the first stage of fermentation, and the temperature adjusted during the second fermentation stage.

Results

Effect of Switching Time on 2-Ketogluconic Acid Production

During the first stage, culture pH was maintained at pH 7.0, whilst during the second stage, the culture pH had decreased to pH 5.0. Four runs of fermentation with pH switching times of 2, 3, 4, and 5 h were conducted (Fig. 1).

Fig. 1 demonstrates that cell growth and 2-ketogluconic acid production were separated into two stages. Cell growth proceeded during the first stage, in the absence of 2-ketogluconic acid accumulation. During the second stage, accumulation of 2-ketogluconic acid was initiated because of acidification of the culture broth. Once the culture had reached pH 5.0, cell growth was completely inhibited.

During the second stage, the productivity of 2-ketogluconic acid was closely related to cell density, albeit a nonlinear relationship. 2-Ketogluconic acid productivity had tripled between runs A and B, and this increase in productivity coincided with the increase in cell density (Table 1). However, at a cell density of 3.0 g/l (run C), the increase in 2-ketogluconic acid productivity was no longer consistent with the increase in cell density. Furthermore, when the cell density reached 4.0 g/l (run D), a slight decline in

Table 1. Fermentation results for *K. pneumoniae* ΔbudA cultures at different switching times.

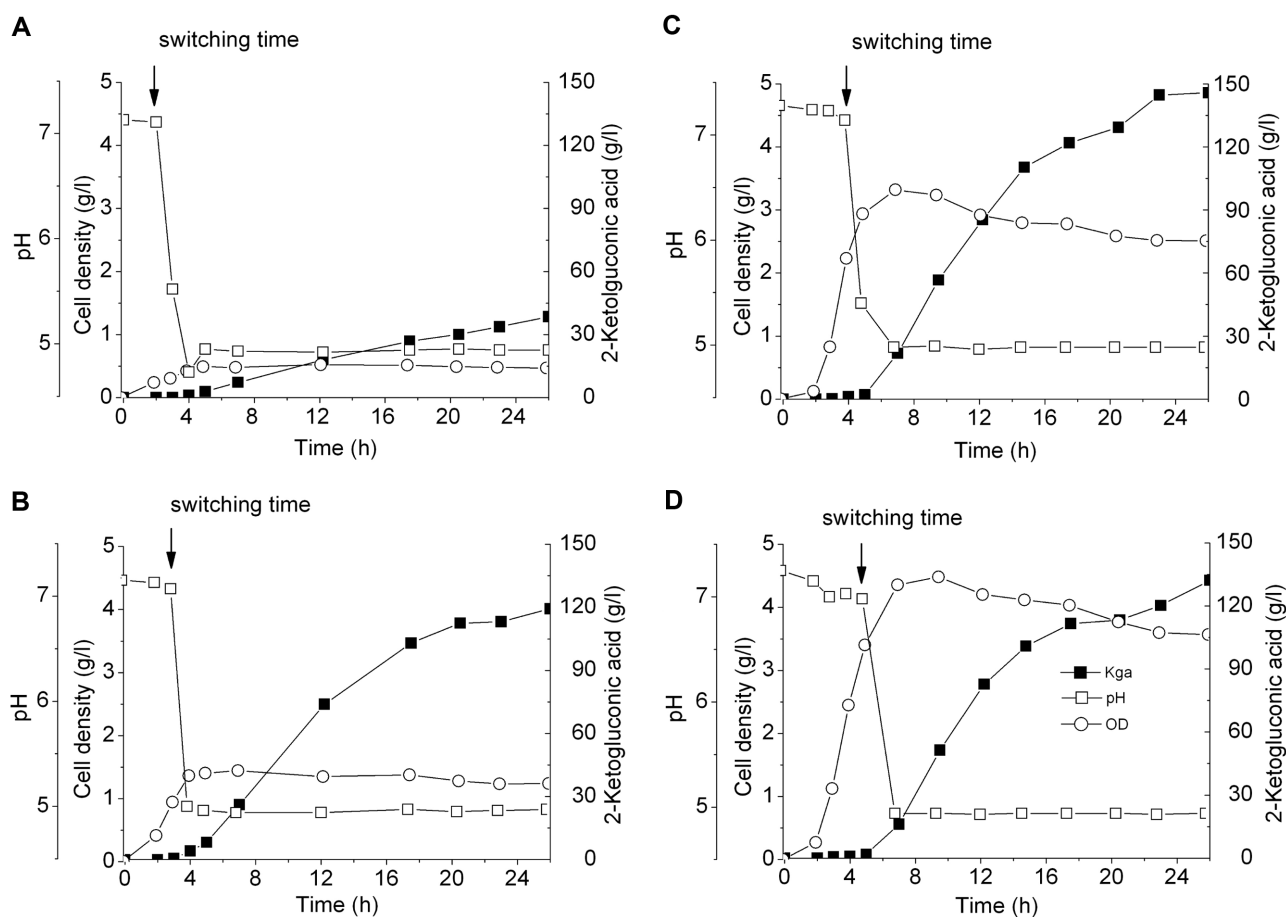| Switching time (h)	Cell density (g/l)	2-Ketogluconic acid productivity (g/lh)	Glucose consumed in the first stage (g/l)	Conversion ratio (g/g)
2	0.5	1.6	0.5	0.86
3	1.4	5.2	1.9	1.00
4	3.0	6.6	7.2	0.93
5	4.0	6.3	9.9	0.86

Fig. 1. Two-stage fermentation of 2-ketogluconic acid production by *K. pneumoniae* ΔbudA at different switching times. (**A**) 2 h; (**B**) 3 h; (**C**) 4 h; (**D**) 5 h. 2-Ketogluconic acid (■); pH (□); cell density (○).

2-ketogluconic acid productivity was observed compared with run C.

During the first stage of fermentation, glucose is used for cell growth. The increase in biomass during the growth stage coincided with a greater consumption of glucose (Table 1), affecting the conversion ratio of glucose to 2-ketogluconic acid. Taking into consideration both productivity and the conversion ratio, the optimum switching time from growth stage to productivity was determined as 4 h, and the optimum cell density was approximately 3g/l.

Effect of Dissolved Oxygen Concentration on 2-Ketogluconic Acid Production

The conversion of glucose to 2-ketogluconic acid proceeds through two oxidative steps; hence the dissolved oxygen level is a key parameter in the fermentation process. Nominated concentrations of dissolved oxygen were achieved by the automatic control of agitation speed. The results are presented in Fig. 2A and Table 2.

Fig. 2A demonstrates the positive relationship between 2-ketogluconic acid productivity and dissolved oxygen level. The glucose to 2-ketogluconic acid conversion ratios increased marginally with increases in dissolved oxygen levels from 0 to 20%, but were lower at 50% dissolved oxygen than at 20% (Table 2). Based on the productivity and conversion ratios, the agitation speed was set at 800 rpm, generating a dissolved oxygen level between 30% and 60%.

Table 2. Substrate conversion ration of *K. pneumoniae* ΔbudA cultures at different dissolved oxygen levels, culture pHs, and culture temperatures.

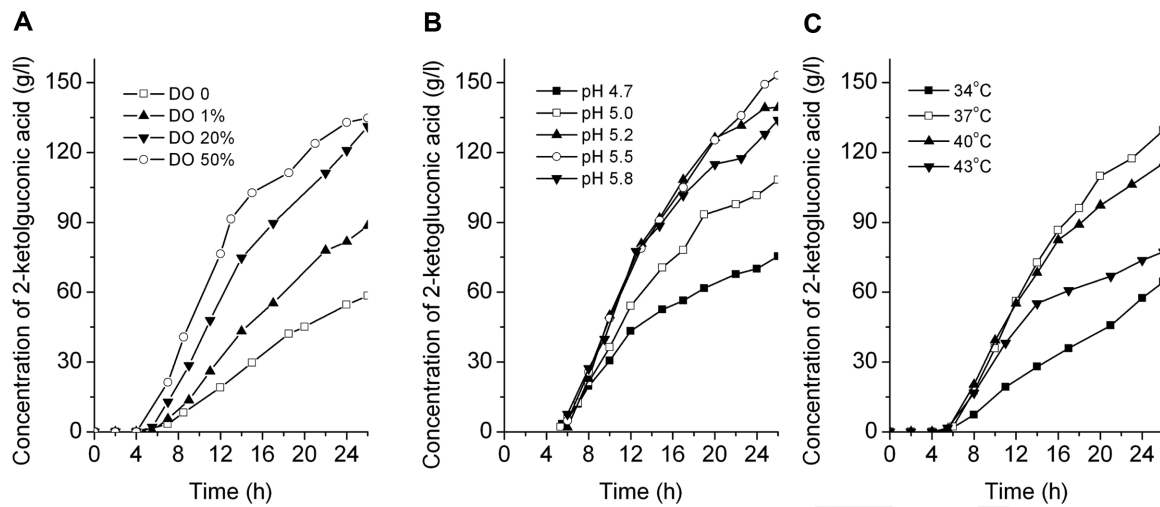| Dissolved oxygen level (%)	0	1	20	50
Conversion ratio (g/g)	0.83	0.95	1.05	1.00

Fig. 2. Effect of dissolved oxygen, culture pH, and culture temperature on 2-ketogluconic acid production.
(**A**) Dissolved oxygen; (**B**) Culture pH; (**C**) Culture temperature.

Effect of pH on 2-Ketogluconic Acid Production

Culture pH has a critical effect on *K. pneumoniae* glucose metabolism. Previous studies have shown that 2-ketogluconic acid accumulation only occurs in broths of low pH [20]. In the current study, we investigated the effect of pH (4.7–5.8) on 2-ketogluconic acid production during the second stage of fermentation.

As shown in Fig. 2B, 2-ketogluconic acid productivity increased with increasing pH from 4.7 to 5.5, but decreased at pH 5.8. The highest conversion ratio of glucose to 2-ketogluconic acid was obtained at culture pH 5.0 to 5.2 (Table 3). Taking into consideration the productivity and the conversion ratio achieved in the fermentation, the culture pH for the second stage was set at pH 5.2.

Effect of Culture Temperature on 2-Ketogluconic Acid Production

Culture temperature is a key parameter for all bioprocesses. Documented culture temperatures for 2-ketogluconic-acid-producing strains, excluding *Klebsiella pneumoniae*, are between 28°C and 30°C, whereas *Klebsiella pneumoniae* NCT418 (*Klebsiella aerogenes* NCT418) is cultured at 35°C [4, 9, 10, 12, 13, 17]. Commonly, *K. pneumoniae* CGMCC 1.6636 and the *budA* mutated strain are cultured at 37°C [18, 19].

Here, we investigated the effect of temperature (34–43°C) on 2-ketogluconic acid productivity during the second stage of fermentation.

Fig. 2C illustrates that the highest productivity of 2-ketogluconic acid was for cultures at 37°C, whereas the lowest productivity was at 34°C. The conversion ratio was influenced by culture temperatures, whereby higher temperatures yielded correspondingly higher conversion ratios (Table 4). Based on the results obtained, the optimum temperature for 2-ketogluconic acid production (stage 2) was 37°C.

Characteristics of Glucose Dehydrogenase and Gluconate Dehydrogenase

Glucose dehydrogenase and gluconate dehydrogenase are the two key enzymes involved in 2-ketogluconic acid synthesis. The effects of pH and temperature on the activities of the two enzymes were determined, and the results are shown in Fig. 3.

The highest activity of glucose dehydrogenase and gluconate dehydrogenase in acetate buffer was both at pH 5.4 to 5.8. In phosphate buffer, glucose dehydrogenase had the highest activity at pH 6.2–7.0, and the activities were similar to that at pH 5.4 to 5.8 in acetate buffer. The activity

Table 3. Substrate conversion ration of *K. pneumoniae* ΔbudA cultures at different culture pHs.

Culture pH	4.7	5.0	5.2	5.5	5.8
Conversion ratio (g/g)	0.90	0.94	0.93	0.85	0.87

Table 4. Substrate conversion ratio of *K. pneumoniae* ΔbudA cultures at different culture temperatures.

Culture temperature (°C)	34	37	40	43
Conversion ratio (g/g)	0.73	0.92	1.02	1.01

A pH

B Temperature

Fig. 4. 2-Ketogluconic acid production by *K. pneumoniae* ΔbudA under optimum conditions.

2-Ketogluconic acid (■); cell density (○). Each datum point represents the mean of three independent experiments. Error bars represent the standard deviation.
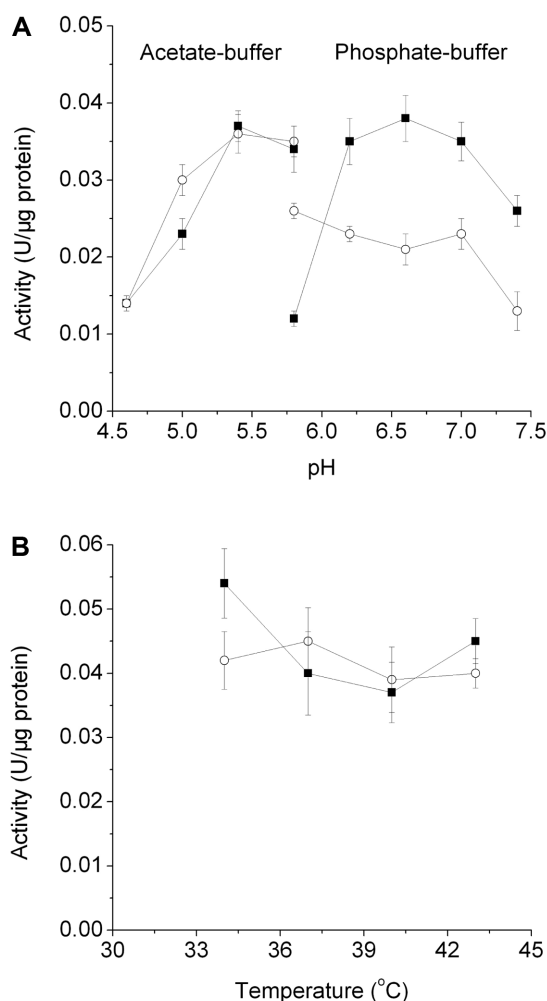
Fig. 3. Effects of reaction pH and temperature on the activities of glucose dehydrogenase and gluconate dehydrogenase.

(**A**) pH; (**B**) Temperature. Glucose dehydrogenase (■); gluconate dehydrogenase (○). Each datum point represents the mean of three independent experiments. Error bars represent the standard deviation.

of gluconate dehydrogenase had the highest value at pH 5.8 and decreased with increasing pH in phosphate buffer. Temperature had no obvious effect on the activities of the two enzymes in the ranges of 34°C to 37°C.

Production of 2-Ketogluconic Acid Under Optimum Conditions

The switching time, agitation speed, culture pH, and temperature were set at 4 h, 800 rpm, pH 5.2, and 37°C, respectively. Three parallel runs of the experiment were performed (Fig. 4).

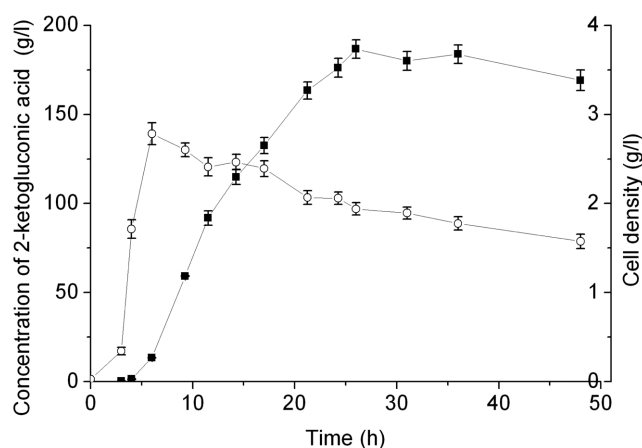Cell density reached to 3 g/l in the growth stage, with 5 g/l of glucose consumed. 2-Ketogluconic acid production proceeded very quickly during the period from 6 to 12 h. The maximum concentration of 2-ketogluconic acid (186 g/l) was achieved at 26 h. The conversion ratio of glucose to 2-ketogluconic acid totaled 1.07 g/g (~ 1 mol/mol) during the second stage of fermentation (up to 26 h), and the total conversion ratio, accounting for glucose consumption in the first stage, was 1.05 g/g (0.98 mol/mol).

Discussion

In a previous work on 2-ketogluconic acid production by *K. pneumoniae* ΔbudA, there did not implement a separate stage for cell growth. Therefore, both the cell density (~ 0.2 g/l) and the 2-ketogluconic acid productivity (~ 0.5 g/lh) were comparatively low [20]. In the current study, a high cell density was achieved in the first stage of fermentation, and 2-ketogluconic acid productivity reached a high rate during the second stage of fermentation. The 2-ketogluconic acid productivity increased more than 10-fold compared with that in the previous work.

In the 2-ketogluconic acid synthesis pathway, two electrons produced from the oxidation of glucose to gluconic acid are transferred to cofactor pyrroloquinoline quinone [2]. Similarly, electrons produced from the oxidation of gluconic acid to 2-ketogluconic acid are received by another cofactor, FAD [16]. Pyrroloquinoline quinone and FAD enter the electron transport chain where they transfer their electrons, *via* electron donors cytochrome *c* or cytochrome *b*, to the final electron acceptor, oxygen. Pyrroloquinoline quinone and

FAD are regenerated to the oxidized form [11]. The positive relationship between 2-ketogluconic acid productivity and dissolved oxygen level shows the available oxygen is the bottleneck of this electron transport chain.

Cytoplasmic pH is stable in bacteria, where the homeostasis system of *E. coli* maintains the cytoplasmic pH between 7.5 and 7.9, even when the pH of the growth medium varies between 5.5 and 8.5. However, the pH of the periplasmic space is most variable, reflecting the pH of the surrounding medium. This is because the outer membrane is permeable to ions and low-molecular solutes that exist in the extracellular medium [1]. Productivity of 2-ketogluconic acid is related to the activity of two enzymes, glucose dehydrogenase and gluconate dehydrogenase, located within the periplasmic space. The optimum pH for glucose dehydrogenase and gluconate dehydrogenase measured *in vitro* were between pH 5.4 and 7.0 and pH 5.4 and 5.8, respectively. These results are consistent with the observed data reported by Hommes *et al.* [6], which present the optimum pH of the two enzymes as between pH 5 and 6. The optimum pHs of the two enzymes are in good agreement with the higher 2-ketogluconic acid productivity observed for cultures at pH 5.2 to 5.8 in the current study.

The conversion ratio of glucose to 2-ketogluconic acid decreased from pH 5.2 to 5.8. This may be attributable to gluconic acid and/or 2-ketogluconic acid being used by the cell to synthesize other metabolites for their growth. Increasing the culture pH from 5.2 to 5.8, may activate the transport of gluconic acid and/or 2-ketogluconic acid to the cytoplasm, and/or facilitate glucose transport to the cytoplasm.

From the *in vitro* experiment, temperature had no obvious effect on the activities of the two key enzymes in 2-ketogluconic acid synthesis pathway. The optimum temperature for 2-ketogluconic acid production might be attributed to the activity of the whole cell, and this temperature is consistent with that selected for 1,3-propanediol and 2,3-propanediol production by other strains of *K. pneumoniae* [8, 21]. This temperature is higher than that used for 2-ketogluconic acid production by other microorganisms. In industrial conditions, cold water is used to keep the bioreactor temperature stable, and high culture temperature has an economic advantage.

Under optimum conditions, both 2-ketogluconic acid productivity and the conversion ratio reached high levels. The 2-ketogluconic acid productivity and the conversion ratio achieved in the current study were 6% and 13% higher than that observed for *P. fluorescens* (2-ketogluconic

acid productivity, 6.74 g/lh; conversion ratio, 0.93 g/g) in a recent study by Sun *et al.* [12].

Using the two-stage fermentation strategy, high rates of 2-ketogluconic acid productivity and substrate conversion ratio were obtained. This strategy has solved the mismatch between the optimum culture parameters for cell growth and 2-ketogluconic acid accumulation. The two-stage fermentation of *K. pneumoniae* presents an attractive alternative for the industrial production of l 2-ketogluconic acid.

Acknowledgments

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