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2-Ketogluconic Acid Production by *Acetobacter pasteurianus*

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ABSTRACT

Production of 2-ketogluconic acid in batch fermentation was investigated. *Acetobacter pasteurianus* ATCC 6438, which produces selectively 2-ketogluconic acid only, was used. The optimal pH for glucose dehydrogenation to gluconate by resting cells was 5.0 and for gluconate dehydrogenation to 2-ketogluconate 4.25. When glucose medium was used, the 89% yield was achieved after 90 h. For the optimal productivity, medium containing glucose and gluconate with the molar glucose:gluconate ratio 7.4 was proposed, and the yield of 92% after 56 h was achieved. This composition of medium led to the elevation of dissolved oxygen concentration during fermentation. It consequently resulted in elevated gluconate dehydrogenase activity being discussed as the rate-limiting activity of the batch production.

Index Entries: *Acetobacter pasteurianus*; gluconic acid; 2-ketogluconic acid; gluconate dehydrogenase.

INTRODUCTION

The acetic acid bacteria, especially strains from the genera *Gluconobacter*, are known for their ability to oxidize incompletely glucose to gluconic, 2-ketogluconic, 5-ketogluconic, and 2,5-diketogluconic acids (*1*). 2-Ketogluconic acid is produced from glucose via glucose dehydrogenation to gluconic acid, which is sequentially dehydrogenated to 2-ketogluconic acid. Glucose is dehydrogenated to gluconic acid by membrane-bound

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glucose dehydrogenase. This glucose dehydrogenase has pyrroloquinoline quinone (PQQ) as a cofactor, which was claimed to be specific to glucose (*2*). The participation of intracellular coenzyme-dependent glucose dehydrogenase is of minor importance (*3,4*). Almost all *Gluconobacter* strains are able subsequently to oxidize gluconic acid to ketogluconic acids. These dehydrogenations were reported to be catalyzed by specific gluconic acid dehydrogenases. Gluconate dehydrogenase yielding 2-ketogluconate contains flavine covalently bound to the enzyme as a prostethic group, and the substrate specificity of the purified enzyme was found to be very strict for D-gluconate (*5*).

The selectivity of glucose oxidation to 2-keto-, 5-keto-, or 2,5-diketogluconate and ratio of produced acids considerably depend on the particular strain and cultivation conditions (*6,7*). The formation of ketogluconates by the strains frequently cited in the literature was studied (*7*), although it was concluded that both 2-keto- and 5-ketogluconic acid are usually produced simultaneously, and only a few strains are able to produce diketogluconate. It has been proposed that glucose dehydrogenation to gluconic acid is very effective, and high production rates and yields are usually obtained. The second step in the 2-ketogluconate production, i.e., gluconate dehydrogenation to 2-ketogluconate, is more obstructed. The production rates and yields achieved are considerably lower. Furthermore, only a few strains are able selectively to produce only 2-ketogluconate (*6–8*). Metabolism and regulation mechanism of gluconate and ketogluconate formation have been intensively studied (*4,7,9,10*). However, a lot of information has been published, but the regulatory mechanism is still poorly understood.

Influences of such simple parameters as pH and dissolved oxygen concentration are relatively simple to evaluate. The final product of glucose oxidation by *Gluconobacter oxydans* was reported to be dependent on pH (*7*). At pH 5.5, gluconate was further oxidized to ketogluconates, and in a culture without pH control (pH below 3), oxidation stopped at gluconic acid. The influence of pH on gluconate dehydrogenation was especially reported for 2,5-diketogluconate formation via the 2-ketogluconate route by *Gluconobacter* (*6*), and the pH below 4 was reported to be the most favorable condition for further gluconate dehydrogenation. For 2,5-diketogluconate formation via 5-ketogluconate, optimal fermentation pH value at harvest of 2.6 was reported (*11*). For the 5-ketogluconic acid production by *Gluconobacter*, the optimum pH was reported to be in the range of 2.5–3.0 (*8*). Anyway, it cannot be concluded which pH is optimal for particular ketogluconate or gluconate. Furthermore, distinguishing effect of pH during various growth phases seems to be necessary (*6*). The use of a neutralizing agent may also influence the ketogluconate formation. When calcium carbonate was used, favorized production of 5-ketogluconate instead of 2-ketogluconate by the *G. oxydans* strains was observed (*7,12,13*).

Considering already published data, the influence of oxygen concentration is the parameter of the main importance for gluconate dehydrogenation. Intensification of glucose oxidation to 2-ketogluconic acid by *Acetobacter suboxydans* was already reported (*14*). Later intensification of oxidation activity was studied (*11,15*), but effect of oxygen has not been quantified. The positive influence of constant high dissolved oxygen tension on the production of 2,5-diketogluconic acid was published (*16*). The enzyme activities associated with glucose oxidation are substantially elevated when the microorganism is grown under increased dissolved oxygen concentrations (*16,17*). Also, the actual electron transport system associated with membrane-bound dehydrogenases appears to be influenced by an oxygen concentration on the growing culture (*18*). The positive influence of oscillations of dissolved oxygen concentration on overall oxidation capacity of *Gluconobacter melanogenum* was described (*17*). The literature data also suggest limits of optimal oxygen concentration range. The oxygen saturation constant of *G. oxydans* is about 3% of air saturation (*19*), but the threshold limit for the optimum productivity was found to be at 30% of air saturation at 1 bar (*17*). However, high oxygen concentration was observed as being inhibitory to the growth of *G. oxydans* (*19*). At oxygen concentrations over 60% of saturation with pure oxygen, the strongly diminished growth and product formation were observed.

This article presents a method for the batch production of 2-ketogluconic acid. The main aim was to propose a batch procedure diminishing oxygen demand restrictions in 2-ketogluconate production.

MATERIALS AND METHODS

Microorganism

A. pasteurianus ATCC 6438 was used. It was maintained on a solidified medium containing: glucose, 100 g/L; yeast autolyzate (Imuna, Slovak Republic), 5 g/L; $CaCO_3$, 10 g/L; agar, 20 g/L; and tap water.

Media

Glucose and gluconate media were used. The first one consisted of: glucose, 100 g/L; yeast autolyzate 5 g/L; $CaCO_3$, 20 g/L; and tap water. The latter one was composed of: calcium gluconate, 110 g/L; glucose, 12 g/L; yeast autolyzate, 5 g/L; and tap water.

Inoculum for Batch Production

The strain maintained in a slant agar was transferred to 100 mL of glucose or gluconate medium. The culture was cultivated until reaching the exponential phase (15–25 h) corresponding to the medium absorbance

A_{650} = 0.45–0.50. Then three flasks were inoculated (5% vol of 100 mL media). Exponential phase was reached within 10–12 h. Then the culture was used for a bioreactor inoculation. The cultivations were performed in the 500-mL flasks filled with 100 mL of media on a rotary shaker (180 rpm) at 30°C.

Batch Cultivation

The batch production was performed in a 5-L laboratory bioreactor LF-2 (Laboratory Instruments Praha, Czech Republic) filled with 2.7 L of media and 300 mL of exponential-phase inoculum. The batch productions were performed at 30°C, agitation 500 rpm, and aeration 1.0 vvm. During the production on glucose medium, pH was maintained by addition of 10-g portions of calcium carbonate.

Measurement of Substrate-Dependent Oxygen Consumption Rate

For the measurement of pH profile and saturation curves, the cells grown on glucose medium were used. The preparation procedure was the same as for inoculum. The exponential-phase culture was filtered through a paper filter ($CaCO_3$ removing), and the cells for measurement were harvested by centrifugation (20 min at 3000*g*) and resuspended in the same volume of 0.9% sodium chloride solution. After 1 h lasting starvation at 5°C, cells were centrifuged and resuspended in 20-fold reduced volume of 0.9% sodium chloride solution. The biomass concentration of this suspension was determined after its dilution using absorbance measurement. For measurements, 1 mL of this suspension was added to 39 mL of buffer was used. Oxygen consumption rate was monitored polarographically with a Clark-type oxygen electrode (Chemoprojekt Satalice, Czech Republic). The measurement was performed in a 40-mL thermostated cell. For the measurement of glucose-dependent oxygen consumption rate, McIlvaine 0.2*M* buffers containing glucose of appropriate concentration saturated with pure oxygen at 30°C were used. The respiration medium for measurement of gluconate-dependent oxygen consumption rate was prepared by dilution of calcium gluconate in distilled water, and pH was adjusted by addition of concentrated hydrochloric acid. The oxygen consumption rate was expressed per a unit of the biomass dry weight ($mol_{O2}/g_{DW}/s$). From the values presented in this article, endogenous respiration rates were subtracted.

Analytical Methods

The samples for glucose, gluconic, and ketogluconic acid determinations were centrifuged and immediately diluted. Glucose was assayed by standard glucose test (Lachema, Czech Republic). Gluconic, 2-ketoglu-

conic, and 5-ketogluconic acids were measured by Isotachophoretic analyzer ZKI-01 (Labeco, Slovak Republic). Analyses were performed in the capillary 0.8 × 150 at driving current of 250 μA using a conductometric detector. The electrolytes used were: Leading—10 m*M* hydrochloric acid, β-alanine pH 3.0, 0.1% mHEC; Terminator—5 m*M* acetic acid. Biomass concentration was expressed as the dry weight matter, which was estimated as the optical density of the bacterial suspension measured by the following procedure: 1 mL of sample was mixed with 2 mL of 1*M* hydrochloric acid, and after calcium carbonate decomposition, the optical density was measured at 650 nm in 1-cm cell with Shimadzu 2100-S Spectrophotometer (Shimadzu, Japan). As a reference, centrifuged sample was used. Biomass concentration was extracted from the calibration curve. The dry weight matter for the calibration curve construction was determined as follows: 50 mL of sample were filtered through the paper filter, centrifuged (20 min at 3000*g*), resuspended in the same volume of distilled water, and centrifuged again. Sediment was resuspended in a small volume of distilled water and dried to a constant weight at 105°C. Ratio of absorbance (A_{650}) and dry weight matter (g_{DW}/L) was 0.83 in absorbance range 0–0.6. At absorbances over the values of 0.6, the relationship between absorbance and dry weight was nonlinear. Therefore, samples had to be diluted.

RESULTS AND DISCUSSION

For 2-ketogluconate, production is frequently cited as genus *Gluconobacter*. During our experimental work, many strains from the genera *Acetobacter* and *Gluconobacter* were tested. According to our experiences, the gluconate dehydrogenase activities are in general very low (not presented). For further experimental work, the strain *A. pasteurianus* ATCC 6438, which exhibits a favorable ratio of glucose/gluconate dehydrogenase activity, was chosen, although the optimization strategy presented here is applicable also to the other strains.

The study of the glucose dehydrogenation to 2-ketogluconic acid by *A. pasteurianus* ATCC 6438 includes two consequent dehydrogenation steps, which have been studied separately. For the purposes of process characterization, the resting cells were used in our work. The resting cells of bacteria belonging to the genera *Acetobacter* and *Gluconobacter* are capable to dehydrogenate substrates by membrane-bound dehydrogenases without participation of other metabolic pathways (*1*). This phenomenon seems to be common to all substrates dehydrogenated by acetic acid bacteria. The use of resting cells has already been reported for the 5-ketogluconate production. The resting cells have been used also for evaluation of various influences (e.g., pH, temperature) on the production mentioned above (*8*).

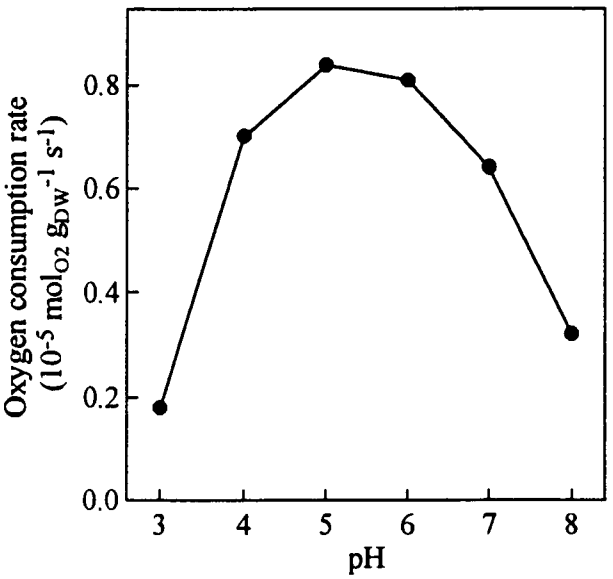

Fig. 1. Influence of pH on glucose-dependent oxygen consumption rate by resting cells. Measurements were performed at the biomass concentration 0.38 g_{DW}/L. D-glucose concentration was 0.02 mol/L.

The first step in formation of 2-ketogluconate from glucose is glucose dehydrogenation to gluconic acid. The influence of pH on glucose dehydrogenation was evaluated by measuring of the glucose-dependent oxygen consumption rate (Fig. 1). The wide optimum was found in the range 4.5–6.5. A narrower pH optimum range of the membrane-bound dehydrogenase studied with the isolated enzyme was reported (*20*). Furthermore, the optimal pH depends on the artificial acceptor used. The pH influence studied in the whole resting cells (Fig. 1) includes both influence on dehydrogenase and respiratory chain as the electron acceptor. Thus, this may be the reason for the wide pH optimum. The glucose dehydrogenation by resting cells followed Michaelis-Menten kinetic (data not shown). The apparent K_s and V_{max} were $3.5/10^3$ mol/L and $0.93/10^5$ $mol_{O2}/g_{DW}/s$. The value of K_s corresponds to the values obtained for other membrane-bound glucose dehydrogenases (*2,10*).

The dehydrogenation of gluconate to 2-ketogluconate (also to the 5-ketogluconate) is usually the limiting step in a batch production. The influence of pH is documented in Fig. 2, and the sharp optimum was found at the value of 4.25. The optimum is at a lower pH value than for glucose dehydrogenase, and the shape of the curve suggests that the protonized form of gluconate is preferably dehydrogenated. The influence of the gluconate concentration was also evaluated (Fig. 3). Optimum concentration for the gluconate dehydrogenation at about 0.4 mol/L gluconate was observed. The data of the oxygen consumption rate presented in the Fig. 3 do not follow the Michaelis-Menten kinetic. Thus, the gluconate dehydrogenation by resulting cells cannot be characterized by its saturation constant. The reason, according to our opinion, may be in

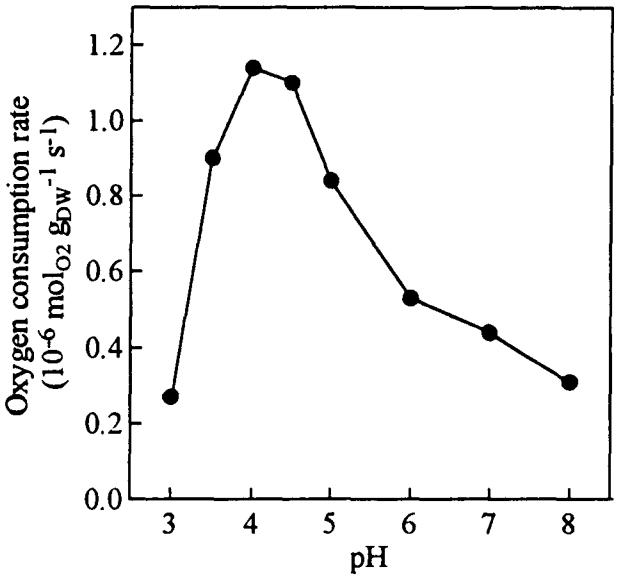

Fig. 2. Influence of pH on gluconate-dependent oxygen consumption rate by the resting cells. Measurements were performed at the biomass concentration 0.38 g_{DW}/L.D-gluconate concentration was 0.3 mol/L.

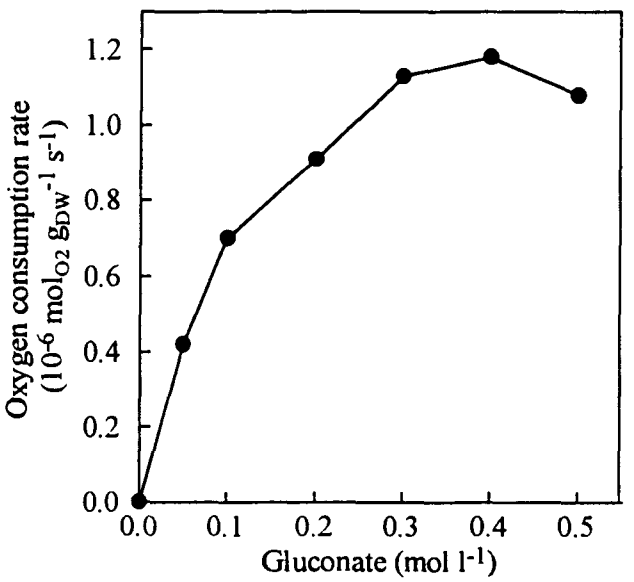

Fig. 3. Influence of D-gluconate concentration on gluconate-dependent oxygen consumption rate by resting cells. Measurements were performed at the biomas concentration 0.38 g_{DW}/L, pH 4.25.

the effect of the high calcium concentration. The maximum gluconate-dependent oxygen consumption rate observed in optimal conditions (pH 4.25, 0.4 mol/L) was $1.2/10^6$ $mol_{O2}/g_{DW}/s$; it is eightfold lower than V_{max} for the glucose dehydrogenase. Thus, it can be concluded that gluconate dehydrogenation to 2-ketogluconate is the rate-limiting step in 2-ketogluconate production by strain *A. pasteurianus* ATCC 6438.

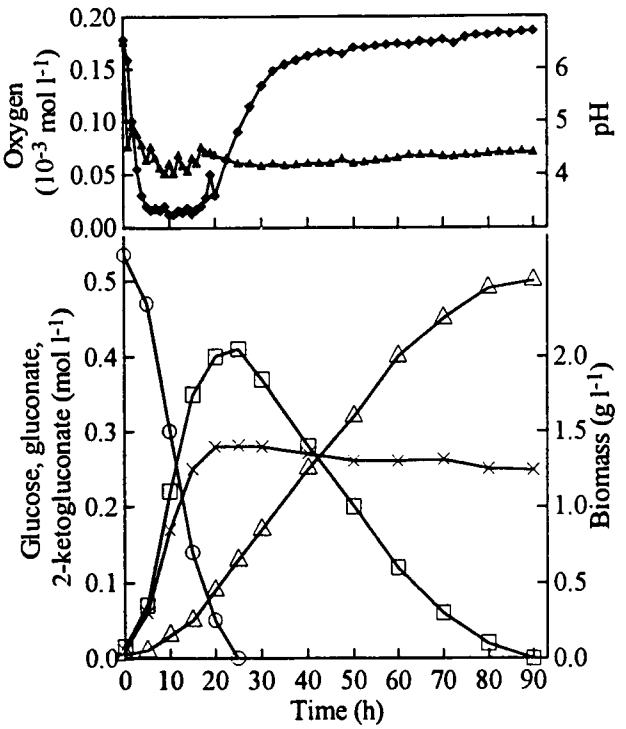

Fig. 4. Batch production of 2-ketogluconic acid by *A. pasteurianus* ATCC 6438 on glucose medium: glucose (-○-), gluconate (-□-), 2-ketogluconate (-△-), biomass (-×-), pH (-▲-), and oxygen concentration (-◆-). The pH was maintained by additions of calcium carbonate accompanied by drops of pH and oxygen concentration. For other experimental details, *see* Materials and Methods.

Considering the findings mentioned above, the pH value of 4.25 was chosen as the optimal one for the batch production. The course of fermentation is presented in Fig. 4. For the purpose of pH regulation, the following procedure was adopted: pH after sterilization was spontaneously decreased to 4.25, and during a further course of fermentation, it was maintained by additions of calcium carbonate. The duration of fermentation was 90 h and the phase of gluconate dehydrogenation was observed to be several times longer than the glucose dehydrogenation phase. It is apparent that during the glucose dehydrogenation period (until the 20th h), the concentration of dissolved oxygen decreases to 0.01/10^3 mol/L (corresponds to 5% of the air saturation). The biomass was grown in the low oxygen concentration. This fact may cause low enzymatic activities associated with glucose dehydrogenation, especially gluconate dehydrogenase activity, which is rate-limiting. The same effect was observed by Buse et al. (*17*), who reported that the oxygen concentration threshold level for optimum productivity was 30% of the air saturation (at 1 bar).

The effect of elevated oxygen concentration during biomass growth was investigated by the experiment presented in Fig. 5. The culture conditions until the 8th h were analogous to batch production documented in

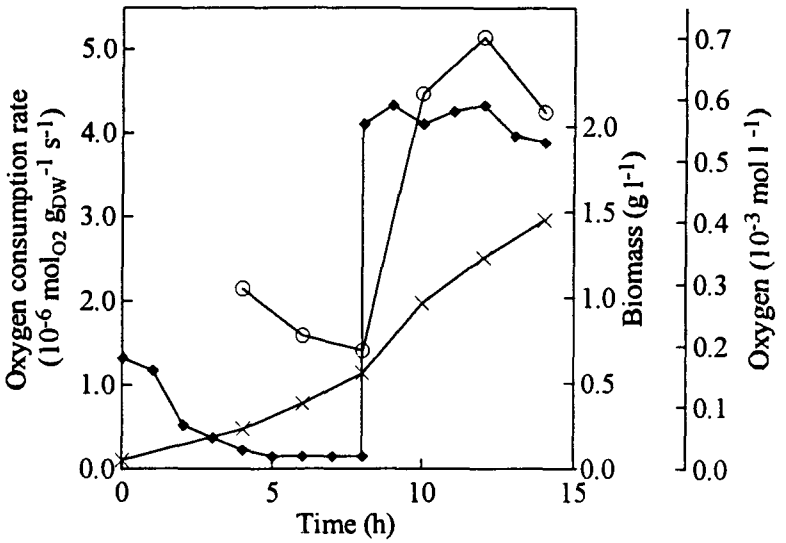

Fig. 5. Effect of elevated oxygen concentration on gluconate-dependent oxygen consumption rate by growing cells: gluconate-dependent oxygen consumption rate (-○-), biomass (-×-), oxygen concentration (-◆-). Since the 8th h, aeration was performed by pure oxygen.

Fig. 4. From the 8th h, oxygenation was performed by pure oxygen. It results in increased actual oxygen concentration and, consequently, in increased gluconate dehydrogenase activity. It suggests the possibility of optimizing productivity by higher oxygenation. Another way is to minimize oxygen demands. The oxygen is intensively consumed for simultaneous glucose and gluconate dehydrogenation. We propose the use of gluconate instead of glucose as the substrate. It will result in the total oxygen consumption being reduced to a half.

The improved batch fermentation procedure for gluconate dehydrogenation to 2-ketogluconate is presented in Fig. 6. If only gluconate was used as the carbon source, insufficient growth was observed (not presented). In order to obtain a good growth, glucose in the mixture with gluconate was used as the assimilatable substrate. The gluconate: glucose molar ratio was chosen at 7.4; it guarantees the pH decrease to 4.25, which is optimal for the rate-limiting gluconate dehydrogenation. The pH regulation by calcium carbonate addition was not performed. During this batch production, it was observed that oxygen concentration decreased only to $0.10/10^3$ mol/L (50% of the air saturation). In Fig. 7, there is a comparison of gluconate dehydrogenase activities, when fermentation with glucose and gluconate-glucose medium was performed. It is evident that the growth on the gluconate medium under elevated oxygen concentration resulted in an increased gluconate dehydrogenase activity. At the end of batch production (Fig. 6), a slight pH decrease was observed, probably because of ketogluconate dissimilation accompanied by yield decrease. Considering the sum of initial glucose and gluconate concentration, the achieved yield of 2-ketogluconate was 92% of the theoretical

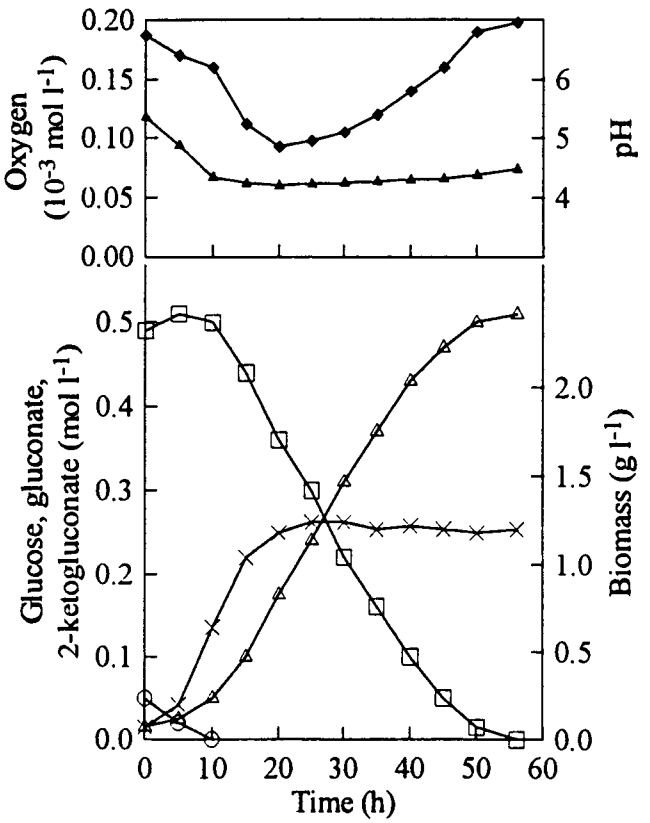

Fig. 6. Batch production of 2-ketogluconic acid by *A. pasteurianus* ATCC 6438 on gluconate medium: glucose (-○-), gluconate (-□-), 2-ketogluconate (-△-), biomass (-×-), pH (-▲-), oxygen concentration (-◆-).

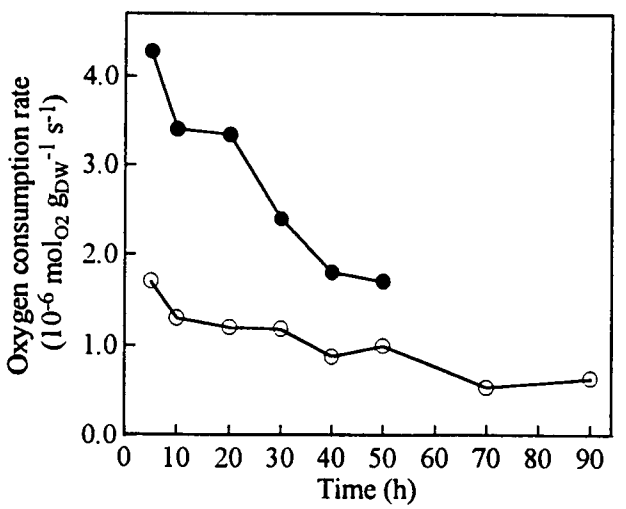

Fig. 7. Comparison of gluconate-dependent oxygen consumption rate during 2-ketogluconate production on glucose medium (-○-) and gluconate medium (-●-) documented in Figs. 4 and 6.

value. At the end of the fermentation, $6.1/10^3$ mol/L of 5-ketogluconate was determined in the broth. This corresponds to the product yield decrease of 1.1%.

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