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Glucose oxidation by *Gluconobacter oxydans*: characterization in shaking-flasks, scale-up and optimization of the pH profile

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Abstract In this study, the advantage of a novel measuring device for the online determination of oxygen and carbon dioxide transfer rates in shaking-flasks is reported for glucose oxidation by *Gluconobacter oxydans*. In this fermentation process, this device was used for the characterization of the oxidation pattern of different strains. *G. oxydans* NCIMB 8084 forms 2,5-diketogluconate from D-glucose in a multi-stage process via three different membrane-bound dehydrogenases. This strain was chosen for a scale-up of the process from shaking-flasks to a 2-l stirred vessel. An enhancement of 2,5-diketogluconate production was realized by controlling the pH at different levels during the fermentation.

Introduction

A main characteristic of *Gluconobacter* sp. is its ability to rapidly oxidize many organic compounds to the corresponding acids and ketones (Asai et al. 1968). The accumulation of products in the medium was exploited to establish several industrial processes using strains of this genus, e.g. the production of L-sorbose from D-sorbitol, the production of dihydroxyacetone from glycerol and the production of D-gluconate from D-glucose (Lusta and Reshetilov 1998; Macauley et al. 2001). D-Glucose can be oxidized by two different metabolic pathways: one includes uptake, intracellular oxidation and further dissimilation via the pentose phosphate pathway; and the other consists of the direct oxidation by membrane-bound pyrroloquinoline quinone (PQQ)-dependent glucose de-

hydrogenase. The latter one was identified as the main pathway during typical batch fermentations with *G. oxydans* on media containing D-glucose (Levering et al. 1988; Pronk et al. 1989). Many strains of this genus show the property of 2-keto-D-gluconate and/or 5-keto-D-gluconate production from D-gluconate by membrane-bound gluconate dehydrogenases (Asai 1968). Weenk et al. (1984) described a characteristic oxidation pattern caused by sequential production of ketogluconic acids from glucose in pH-controlled batch fermentations. Beschkov et al. (1995) investigated 5-keto-D-gluconate production from D-glucose by *G. oxydans* NBIMCC 1043 by membrane-bound NADP-independent dehydrogenases. An intracellular, 5-keto-D-gluconate-producing enzyme in *G. oxydans* DSM 3503, gluconate:NADP-5-oxidoreductase, was characterized by Klasen et al. (1995). During fermentation of *G. oxydans* subsp. *melanogenes* under similar conditions, 2-ketogluconate formation was followed by its oxidation to 2,5-diketogluconate as the final product (Weenk et al. 1984).

The individual product yields vary widely among different strains. Additionally, they depend on the medium and on the particular conditions used for cultivation (Asai 1968; Olijve and Kok 1979). The influence of pH value on product formation is significant. De Ley and Swings (1984) found a pH optimum at 5.5 for growth of *G. oxydans* cells and Qazi et al. (1991) described one optimal pH of 5.5 for the activity of the glucose dehydrogenase and another below 3.5–4.0 for the activity of gluconate- and ketogluconate-forming dehydrogenases. In batch fermentations with *G. oxydans*, the pH of the medium was often buffered by the addition of CaCO₃ (e.g. Beschkov et al. 1995). As a result, the pH decreased steadily with increasing acid production. At no time of the fermentation was the pH kept at a constant and optimal value for the ongoing enzymatic reactions. Other groups adjusted the pH value by titration at a constant level (e.g. Olijve and Kok 1979). Qazi et al. (1991) controlled the pH at a constant level until the glucose concentration reached a value of about 0.1 mol l⁻¹. Afterwards, they

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stopped the pH control to let the pH value decrease by microbial acid formation.

The objective of this study was to demonstrate the advantage of the measurement of oxygen and carbon dioxide transfer rates in shaking-flasks. These experiments aimed to optimize a batch fermentation process with regard to 2,5-diketogluconate production.

Materials and methods

Microorganisms and media

G. oxydans NCIMB 8084 was obtained from the National collections of industrial, food and marine bacteria, UK. The Institute for Biotechnology at the Juelich research center, Germany, kindly put *G. oxydans* DSM 3503 at our disposal. Stock cultures were prepared by growth at 30 °C and a shaking speed of 240 rpm on mannitol medium, containing (per liter): 25 g mannitol, 5 g yeast extract and 3 g peptone. The cultures were incubated in 500-mL Erlenmeyer shaking-flasks with an operating volume of 50 ml. The initial pH of the medium was adjusted to 6.0 by the addition of 1 M H₂SO₄. The liquid culture samples were stored at -20 °C after addition of 4% (v/v) DMSO.

Conditions of biotransformation in shaking-flasks

Experiments in shaking-flasks were carried out at 30 °C, at a shaking speed of 240 rpm, using the novel online measuring device for oxygen and carbon dioxide transfer rates, the Respiration activity monitoring system (RAMOS; Hitec Zhang, Germany) in combination with a laboratory shaker (Kühner, Switzerland) with a shaking diameter of 50 mm. The set-up and principles of function were the same as described by Anderlei and Büchs (2001, 2002). All media contained (per liter): 90 g glucose, 5 g glycerol, 10 g yeast extract and 10 g CaCO₃. For the determination of substrate and product concentration, 8–12 parallel 250-ml Erlenmeyer flasks were prepared and cultivated under the same conditions. For each sample, the broth of one of these parallel flasks was used for the analysis described below. The design of RAMOS ensures equivalent gas concentrations in the measuring flasks of RAMOS and in the parallel Erlenmeyer flasks (Anderlei et al. 2002), thereby enabling the proposed sampling method.

Conditions of biotransformations in stirred-tank fermenters

Fermentations were carried out at 30 °C in a 2-l stirred-tank fermenter (Visual safety fermenter; Bioengineering, Switzerland) with an operating volume of 1.4 l. The media used contained (per liter): 90 g glucose, 5 g glycerol and 10 g yeast extract. To neutralize the acid formed during the process, either 10 g CaCO₃ l⁻¹ was added at the beginning, or 3 M NaOH was used for automated titration. Sufficient aeration was guaranteed by a constant aeration rate of 1 vvm and a stirrer speed varying between 600 rpm and 1,600 rpm. The minimum pO₂ of the fermenter was set to 30%. Precultures were prepared from frozen DMSO stock in 500-ml shaking flasks filled with 50 ml of medium containing 10 g glycerol l⁻¹ and 10 g yeast extract l⁻¹. Conditions were as described in the previous section.

Analytical methods

The amount of biomass was quantified as the optical density at 600 nm. In experiments in which CaCO₃ was used for pH regulation, insoluble compounds in the medium prevented measurement. Even the addition of 2 M HCl did not clarify the samples satisfactorily. Substrate and product concentrations were measured

in culture supernatants which were obtained after centrifugation at 18,000 g for 15 min. Quantitative analysis of glucose, gluconate (GA), 2-keto-D-gluconate (2-KGA) and 5-keto-D-gluconate (5-KGA) were performed by HPLC. An organic acid design HPLC column (CS-Chromatografie, Germany) was used for the resolution of organic compounds. H₂SO₄ (1 mM) was used as the eluent and the column temperature was maintained at 30 °C. The quantitative analysis of 2,5-diketogluconate (2,5-DKGA) was difficult, as no pure standard material is commercially available. A semi-quantitative analysis was performed by Nelson's arsenomolybdate reagent method, according to Perlman (1954). Because this photometric method is selective for both 5-KGA and 2,5-DKGA, the test was calibrated with 5-KGA. The total content of 2,5-DKGA was calculated by subtracting the result of the HPLC analysis of 5-KGA.

During fermentations in stirred-tank fermenters, the pH value of the culture broth was measured online by a pH electrode (Ingold, Germany). Oxygen and carbon dioxide transfer rates (OTR and CTR, respectively) were calculated by an exhaust gas oxygen analyzer (Magnos 3; Hartmann und Braun, Germany) and a carbon dioxide analyzer (Unor 6N, Maihak, Germany). In addition to this, the pO₂ value was measured by a pO₂ electrode (Ingold, Germany).

Results

Strain selection in shaken culture

During cultivation of *G. oxydans* strains in shaking-flasks, useful data concerning the actual status of fermentation can be obtained by applying the novel measuring device, RAMOS. In modified Erlenmeyer shaking-flasks, which allow the installation of an inlet valve, an outlet valve, an oxygen gas sensor and a pressure sensor, RAMOS determines the OTR and the CTR. The following experiments show that the online data given by RAMOS provide significant information for the oxidation of D-glucose to keto-D-gluconates by *G. oxydans*.

Figure 1 shows the results of an experiment with *G. oxydans* DSM 3503. The data obtained from the two parallel experiments show nearly the same course. After inoculation, the OTR increased quickly to 0.03 mol l⁻¹ h⁻¹. At this point, an oxygen limitation of the system occurred, as indicated by the constant OTR level. Then, the glucose oxidation stopped and the OTR decreased. The CTR course represents the dissolution of CaCO₃, as the result of acid formation: CO₂ was released into the medium and caused an increase in the CTR. A continuous decrease in the pH value from pH 6.0 to pH 3.8 was obtained.

The HPLC data in Fig. 1 show that *G. oxydans* DSM 3503 produced D-gluconate as the main product. Only minor concentrations of 2-KGA and 5-KGA were formed. The 2,5-DKGA concentration was negligible. The total oxygen consumption over the course of the experiment can be calculated by integration of the OTR over time. In the shown experiment, a total consumption of 0.26 mol oxygen l⁻¹ was calculated.

As can be seen from the data presented in Fig. 2, *G. oxydans* NCIMB 8084 showed a different mode of biotransformation under the same culture conditions. Similar to strain DSM 3503, the OTR increased quickly after a short lag phase. On reaching an OTR value of

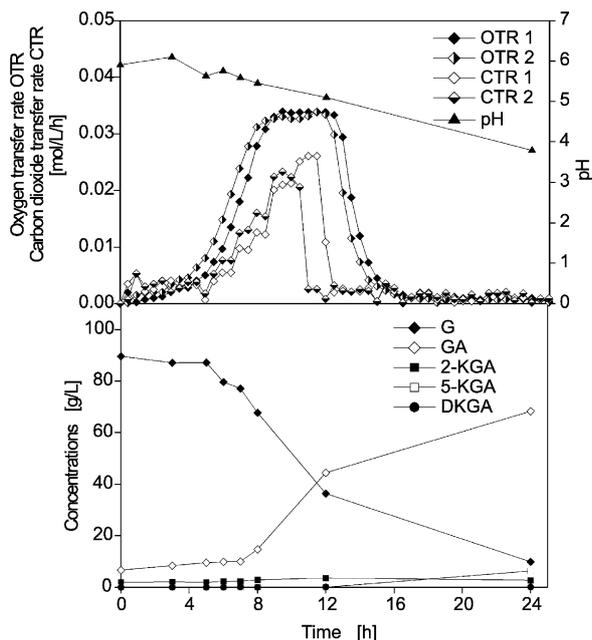


Fig. 1 Time-course of the biotransformation of glucose by *Gluconobacter oxydans* DSM 3503 in shaking-flasks at a shaking speed of 240 rpm. OTR 1, 2 and CTR 1, 2 represent the results of two parallel cultivation experiments done in the Respiration activity monitoring system (RAMOS). The offline samples used for pH measurement and HPLC analysis were taken from Erlenmeyer shaking-flasks which were cultivated under the same conditions. G Glucose, GA gluconate, 2-KGA 2-keto-D-gluconate, 5-KGA 5-keto-D-gluconate, DKGA 2,5-diketogluconate, L liters

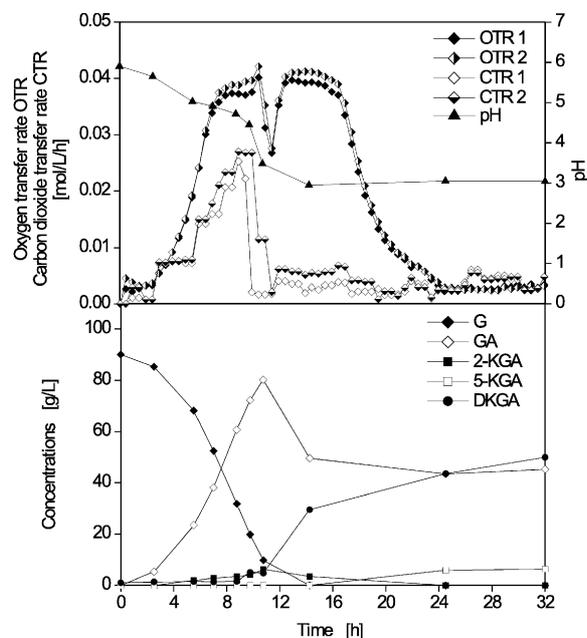


Fig. 2 Time-course of the biotransformation of glucose by *G. oxydans* NCIMB 8084 in shaking-flasks at a shaking speed of 240 rpm. OTR 1, 2 and CTR 1, 2 represent results of two parallel cultivation experiments using the RAMOS. The offline samples used for pH measurement and HPLC analysis were taken from Erlenmeyer shaking-flasks which were cultivated in parallel at equivalent operating conditions

about $0.04 \text{ mol l}^{-1} \text{ h}^{-1}$, an oxygen limitation occurred and prevented a further increase. The OTR level remained constant, until it decreased simultaneously with the pH value. In contrast to strain DSM 3503, the OTR only decreased to a value of $0.03 \text{ mol l}^{-1} \text{ h}^{-1}$ before a second increase occurred, indicating further dehydrogenase activity. In the following paragraphs, the glucose oxidation is divided in two phases, to organize the discussion. The time-span between the start of the fermentation and the first drop in the measured OTR is referred to as the first oxidation phase; and the subsequent time-span between the second increase in the OTR and the end of the fermentation is referred to as the second oxidation phase.

A decrease in pH as a result of intensive acid formation characterized the first phase of biotransformation. When the CaCO_3 added to the medium was totally exhausted, the CO_2 production and therefore the CTR decreased immediately. At this point, the ongoing acid production led into a sharp drop, from pH 4.5 to pH 3.3. The HPLC data clearly show that, in the first oxidation phase, glucose is almost completely oxidized to gluconate. The second oxidation phase started with another increase in the OTR, in which ketogluconate and diketogluconate formation began. During the first phase of fermentation, only small amounts of 2-KGA and 2,5-DKGA were formed. 5-KGA production could not be observed until glucose was totally oxidized to gluconate. The total oxygen consumption of this phase was 0.32 mol l^{-1} . Over the whole fermentation, *G. oxydans* NCIMB 8084 consumed $0.57 \text{ mol oxygen l}^{-1}$ for the oxidation of glucose.

Scale-up from shaking-flask into a stirred-tank fermenter

For further experiments in batch culture, the process of 2,5-DKGA fermentation by *G. oxydans* NCIMB 8084 was scaled-up in a 2-l stirred-tank fermenter (Fig. 3).

A comparison of Figs. 2 and 3 shows that the courses of all parameters were similar. In the fermentation conducted in the stirred-tank, an oxygen limitation could be prevented by an increase in the agitation rate. The OTR therefore reached a maximum value of $0.96 \text{ mol l}^{-1} \text{ h}^{-1}$. Each time the agitation rate was increased, a shift in the OTR value up to a higher level was observed. Under these conditions, a complete biotransformation from D-glucose to D-gluconate took place in the first oxidation phase. In the second oxidation phase, oxidation activity was much slower than in the previous oxidation phase and the maximum OTR value of the first phase could not be reached. A total of 73% of the formed gluconate was further oxidized to 5-KGA and, via 2-KGA, to 2,5-DKGA. The total oxygen consumption of both oxidation phases amounted to $0.57 \text{ mol oxygen l}^{-1}$.

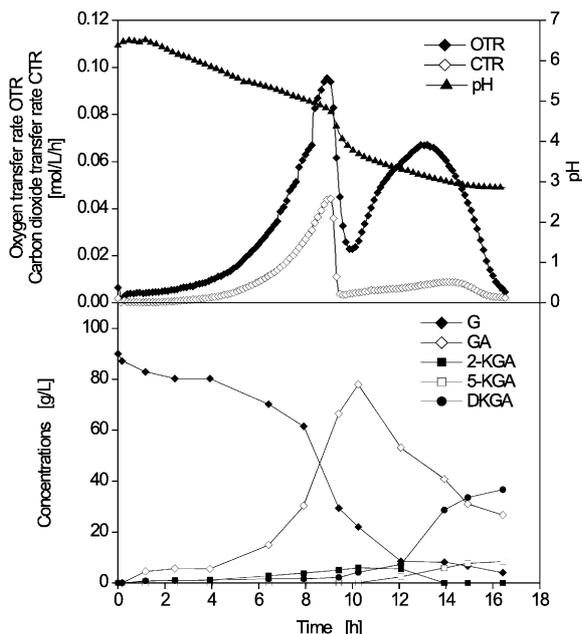


Fig. 3 Time-course of the biotransformation of glucose by *G. oxydans* NCIMB 8084 in 2-l batch culture with $10 \text{ g CaCO}_3 \text{ l}^{-1}$ at an aeration rate of 1 vvm and a stirrer speed between 600 rpm and 1,400 rpm

Optimization of the pH profile in batch fermentations

After scale-up to the 2-l fermenter, the pH course optimization of the batch fermentation was attempted. Each enzyme involved in the biotransformation of glucose to 2,5-DKGA has a specific pH optimum, which must be considered during process improvement. Hence, a pH profile consisting of different pH stages during the course of the fermentation was developed.

In the early phase of the fermentation process, a first stage of pH 5.5 was chosen because this is optimal for cell growth and for glucose dehydrogenase activity. The following pH stages should guarantee good conditions for the *Gluconobacter* dehydrogenases involved in D-glucose oxidation. Since three distinct enzymes are involved, but only two oxidation phases were observed from the OTR course, the pH optima listed in literature were not used (Qazi et al. 1991; Stadler-Szöke et al. 1980). The activity of gluconate- and 2-ketogluconate-dehydrogenases could not be differentiated from each other during the fermentation courses. Therefore, the implementation of different pH stages for these two enzymes did not appear to be advantageous. The fermentation shown in Fig. 3 served as a basis for the calculation of the empirical pH optima of the two oxidation phases. Figure 4 shows the OTR as a function of the corresponding pH value. There is an empirical activity maximum for the oxidation from D-glucose to D-gluconate at pH 4.8 and another for the further oxidation to 2,5-DKGA at pH 3.15. These results were used for the design of a pH profile. As shown in Fig. 5, three different pH stages were established. The first one, at pH 5.5, was chosen for optimal cell growth

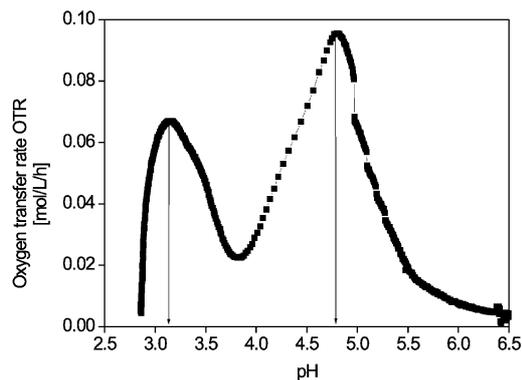


Fig. 4 OTR course of the CaCO_3 -buffered fermentation with *G. oxydans* NCIMB 8084 shown in Fig. 3 as a function of the corresponding pH value. OTR maxima are marked with arrows

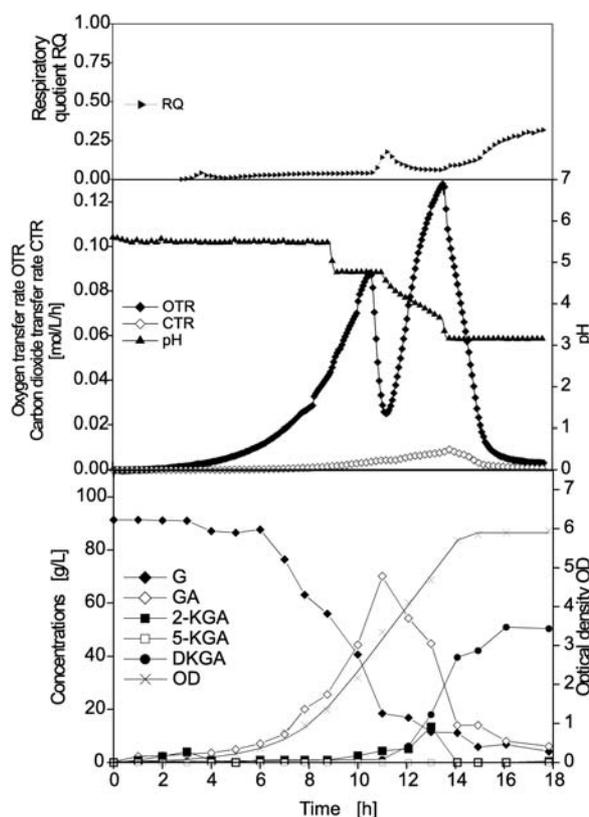


Fig. 5 Time-course of the biotransformation of glucose by *G. oxydans* NCIMB 8084 in 2-l batch culture with automated titration of 3 M NaOH at an aeration rate of 1 vvm and a stirrer speed between 600 rpm and 1,600 rpm

and the second and third ones, at pH 4.8 and pH 3.15, were the observed optima for the dehydrogenation of glucose to 2,5-DKGA.

From the start of the fermentation, the pH of the medium was adjusted to pH 5.5 by automated titration with 3 M NaOH (Fig. 5). This pH was maintained until OTR reached about $0.04 \text{ mol l}^{-1} \text{ h}^{-1}$. Afterwards, a lower pH value, the first empirically derived value of pH 4.8, was chosen for the duration of the first oxidation phase.

The yield of gluconate was nearly quantitative. The determination of the biomass through the measurement of the optical density of the culture broth was possible, because the pH control by CaCO_3 was replaced by titration with NaOH. The optical density increased until the oxidation of glucose, GA and 2-KGA stopped. At the end of the fermentation, the optical density reached a value of 6.0.

At the beginning of the second oxidation phase, the pH control stopped, resulting in a decrease in pH by ongoing acid formation. As expected, the main reaction was the oxidation of gluconate to 2,5-DKGA via 2-KGA. This seemed to be strongly influenced by the introduction of the pH stages. In comparison to the CaCO_3 -buffered fermentation shown in Fig. 3, the introduction of pH stages led to the following results: (1) the total maximum of the OTR of the second oxidation phase reached at least $0.13 \text{ mol l}^{-1} \text{ h}^{-1}$, instead of $0.067 \text{ mol l}^{-1} \text{ h}^{-1}$, (2) the maximum increase in OTR per time unit was nearly 2.8 times higher, (3) the total oxygen uptake during the second oxidation phase (11–18 h) increased up to 0.32 mol l^{-1} and (4) because of this, the 2,5-DKGA yield increased. Interestingly, the OTR decreased immediately after the adjustment of the pH value with 1 M HCl to the second empirical optimum for the dehydrogenase reactions after 13.7 h.

Discussion

This research demonstrates the advantage of RAMOS during the characterization of different *Gluconobacter* strains in shaking-flasks. On the basis of these experiments, one of the tested strains was chosen for scale-up into a 2-l stirred vessel. The novel measuring device, RAMOS, allows the online determination of the OTR and CTR in shaking-flask experiments. Especially during the cultivation of *G. oxydans* strains, the OTR and CTR are useful data concerning the actual status of fermentation. During growth on glucose-containing media, nearly all oxygen is consumed for the membrane-bound oxidation of glucose and the amount of oxygen needed for growth is comparatively low (Levering et al. 1988). Therefore, we can draw conclusions directly from the course of OTR on the present product formation. As shown in Figs. 1 and 2, the online data given by RAMOS provide significant information for this specific process.

In Fig. 1, the cultivation of *G. oxydans* DSM 3503 with glucose as the single carbon source was shown. The HPLC data prove that the given substrate was mainly oxidized to gluconate in one phase. Only minor amounts of keto-D-gluconic acids were formed. The tendency of this strain to oxidize the main part of the D-glucose no further than to D-gluconate is indicated by the OTR course. The total oxygen consumption over the course of the experiment was calculated by integration of the OTR over time. This allows an estimation of the concentration of produced acids by stoichiometric analysis. For the oxidation of the amount of glucose given in the medium

(0.5 mol l^{-1}) to D-gluconate, *G. oxydans* theoretically needs $0.25 \text{ mol oxygen l}^{-1}$. A total oxidation from $0.5 \text{ mol D-gluconate l}^{-1}$ to keto-D-gluconates additionally requires $0.25 \text{ mol oxygen l}^{-1}$ and, for the formation of $0.5 \text{ mol 2,5-DKGA l}^{-1}$ from 2-KGA, the same amount of oxygen is necessary. In the shown experiment, a total consumption of $0.26 \text{ mol oxygen l}^{-1}$ indicates that the main amount of D-glucose was oxidized no further than to D-gluconate.

The oxidation pattern of a second *G. oxydans* strain, *G. oxydans* NCIMB 8084, was investigated under the same culture conditions (Fig. 2). A comparison of the OTR courses obtained by RAMOS shows prominent differences. Whereas *G. oxydans* DSM 3503 formed gluconic acid from glucose in one step, the oxidation of glucose by *G. oxydans* NCIMB 8084 was divided into two oxidation phases. In the first oxidation phase, the activity of the glucose dehydrogenase led to a nearly quantitative formation of gluconate. After this phase, the OTR level decreased simultaneously with the pH value. At this point, the CaCO_3 added to the medium was totally exhausted. The CO_2 production, which is expressed as the CTR, decreased immediately. The sharp pH drop from pH 4.5 to 3.3 was a result of the ongoing acid production. In contrast to Fig. 1, a second increase in the OTR course occurred. Due to further dehydrogenase activity during this oxidation phase, keto-D-gluconates were formed from gluconate. As can be seen from the HPLC data in Fig. 2, the main product of *G. oxydans* NCIMB 8084 was 2,5-DKGA. A calculation of the total oxygen uptake between the start of fermentation and the end of the first OTR decrease (0.0–11.5 h) confirmed these findings. A total oxygen consumption of 0.25 mol l^{-1} agrees well with the expected value for the complete oxidation of $0.5 \text{ mol glucose l}^{-1}$ to gluconate. The membrane-bound, PQQ-dependent glucose dehydrogenase catalyses this reaction. Its optimal pH is 5.5 (Qazi et al. 1991; Stadler-Szöke et al. 1980) and, according to Pronk et al. (1989), its K_s value is $0.36 \text{ g glucose l}^{-1}$. The OTR decreased at the same time as the pH drop was observed, although the substrate concentration was still above $10 \text{ g glucose l}^{-1}$. This indicates that the pH of the medium had fallen below the optimum of the active enzyme. In this case, regulation of the activity level of this enzyme due to the pH changes would be responsible for the observed change in enzyme activity. In fact, regulation of the expression level in response to changing external factors could not be excluded as an explanation for our findings.

The second oxidation phase started with another increase in the OTR, in which ketogluconate and diketogluconate formation began. The optimal pH of the enzymes involved in vivo is below pH 3.5–4.0 (Qazi et al. 1991), so their activity did not increase until the pH drop occurred. The fact that the 2-KGA concentration was always below 10 g l^{-1} and decreased in the second oxidation phase shows that the enzyme activity of the 2-ketogluconate dehydrogenase was comparatively high under the chosen culture conditions. The total oxygen consumption in this phase was 0.32 mol l^{-1} . Over the whole fermentation, *G. oxydans* NCIMB 8084 consumed

0.57 mol oxygen l^{-1} for the oxidation of glucose, which was more than twice the amount consumed by strain DSM 3503. Even without looking at the HPLC data, we can conclude from the OTR data and the total oxygen consumption on a shaking-flask level that this *Gluconobacter* strain at least formed ketogluconates from glucose. Our data clearly show that the novel measuring device, RAMOS, facilitated the comparison of different *G. oxydans* strains and the characterization of their metabolic reactions.

For the further scale-up in a 2-L stirred-tank reactor and the further optimization of the pH control, the strain *G. oxydans* NCIMB 8084 was chosen (Figs. 3, 4, 5). A comparison of Figs. 2 and 3 shows that the courses of all parameters were similar. The OTR reached a maximum value of $0.96 \text{ mol } l^{-1} \text{ h}^{-1}$. Each time the agitation rate was increased, a shift in the OTR value up to a higher level was observed. A higher value of dissolved oxygen partial pressure had a positive influence on the activity of the extremely oxygen-dependent glucose dehydrogenase (Buse et al. 1990, 1992). Similar to oxidation in shaking-flasks, a complete biotransformation from D-glucose to D-gluconate took place in the first oxidation phase. In the second oxidation phase, oxidation activity was much slower and the maximum OTR value of the first phase could not be reached. Then, 73% of the formed gluconate was further oxidized to 5-KGA and, via 2-KGA, to 2,5-DKGA.

A closer look at the total oxygen uptake of *G. oxydans* NCIMB 8084 shows a contradiction in the carbon balance. According to stoichiometric calculations based on the HPLC and the Nelson's arsenomolybdate reagent test data (Fig. 3), the total oxygen consumption (in both oxidation phases) should amount to $0.45 \text{ mol } l^{-1}$. In contrast to this calculation, the microorganism actually needed $0.57 \text{ mol oxygen } l^{-1}$ for product formation. Glucose was oxidized completely to gluconate within the first oxidation phase; and the expected amount of $0.27 \text{ mol oxygen } l^{-1}$ was consumed for this biotransformation. In the second phase, starting at 10 h, the formation of 2,5-DKGA via 2-KGA increased. A total of $0.30 \text{ mol } l^{-1}$, much higher than the calculated amount of oxygen, was consumed in the second oxidation phase. This implies that more than the detected 2,5-DKGA must have been formed. As already pointed out in Materials and methods, Nelson's arsenomolybdate reagent method was obviously insufficient for the quantitative detection of 2,5-DKGA. The online determination of the OTR and CTR proved to be very helpful in uncovering these problems with the evaluation of 2,5-DKGA.

After scale-up to the 2-l fermenter, the pH course optimization of the batch fermentation was attempted (Fig. 5). As described in the Results section, the specific pH optimum of cell growth (pH 5.5; De Ley and Swings 1984) and the empirically determined pH optima for the first and second oxidation phases (pH 4.8 and pH 3.1, respectively, see Fig. 4) were considered in process improvement. This resulted in a pH profile consisting of three different pH stages.

Figure 5 shows a fermentation in which the three different pH stages were established. The course of the OTR of the experiments in Fig. 3 (without pH control) and Fig. 5 (with controlled pH stages) was very similar. The glucose supplied in the medium was completely oxidized to gluconate in the first oxidation phase. At the beginning of the second oxidation phase, stopping the pH control resulted in a decrease in pH. As expected, the main reaction was oxidation of gluconate to 2,5-DKGA via 2-KGA. It was strongly influenced by the introduction of the pH stages. Whereas in the CaCO_3 -buffered fermentation, the total maximum of the OTR of the second oxidation phase reached a value of $0.067 \text{ mol } l^{-1} \text{ h}^{-1}$, the introduction of pH stages led to a doubling-up to $0.13 \text{ mol } l^{-1} \text{ h}^{-1}$. Obviously, the use of different pH stages improved the conditions for gluconate oxidation to 2,5-DKGA. This can also be proved by the comparison of the total oxygen uptake during the second oxidation phase (11–18 h). In CaCO_3 -buffered medium, it amounted to $0.3 \text{ mol oxygen } l^{-1}$ whereas, with the design of pH stages, $0.32 \text{ mol oxygen } l^{-1}$ were consumed. The oxidation process from D-gluconate to 2,5-DKGA was much faster using the pH stages. Additionally, the maximum increase in the OTR per time unit was nearly 2.8 times higher than in the CaCO_3 -buffered fermentation. All the mentioned effects of the introduction of pH stages to the fermentation of *G. oxydans* led to an increased yield of 2,5-DKGA.

Interestingly, the OTR decreased immediately after the adjustment of the pH value with 1 M HCl to the second empirical optimum for the dehydrogenase reactions (pH 3.15) after 13.7 h. It is not clear whether this decrease was caused by the reduction in the pH value, indicating that the empirical optimum differs widely from the real enzyme optimum, or whether the oxidation stopped as a result of another factor.

In our experiments, the use of NaOH for pH control did not affect the conversion efficiency from gluconate to ketogluconates, which was postulated by Beschkov et al. (1995). The lower conversion efficiency they found in their experiments may have resulted from the pH titration at a constant pH level of 5.5, which lies above the optimal level for keto-D-gluconate formation. By using pH stages, the pH conditions provided at the beginning of the second oxidation step were better for 2,5-DKGA production than in CaCO_3 -buffered medium. This led to a remarkable increase in enzyme activity, as can be seen from the OTR course. It was shown that, with the design of a more suitable pH profile, 2,5-DKGA production by *G. oxydans* NCIMB 8084 in batch culture can be optimized. The adjustment of the pH value in different pH stages influenced the second oxidation step from gluconate to 2,5-DKGA, leading to a higher oxygen demand as a result of higher enzyme activity.

The results discussed above clearly demonstrate the advantage and the value of online data acquisition in shaking-flasks during the early stages of the glucose oxidation process development by *Gluconobacter oxydans*. This is now possible, using RAMOS. In contrast to the standard offline sample analysis, RAMOS allows

online monitoring of the fermentation process in shaking-flasks by delivering the additional process information of OTR and CTR. After the strain selection in shaking-flasks, the process was scaled-up to a 2-l stirred-tank fermenter. With regard to the high dependence of cell growth and dehydrogenase activity on actual pH conditions, the fermentation process was optimized by the introduction of different pH stages as an alternative form of pH control for fermentations with *G. oxydans*. The product yield for 2,5-diketogluconate was clearly influenced. A significant improvement in enzyme activity was a result of the adaptation of the pH value to the optima of the distinct enzyme reactions. In our further studies, we are focusing on the establishment of a fermentation process with *G. oxydans* NCIMB 8084 under elevated headspace pressure.

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