# of Glucose to 2,5-diketo-D-gluconic Acid

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In memory of Prof. Emeritus Vera Johanides

citreus is the first step of a two-stage fermentation process to ketogluconic acid. This work presents the optimization of pH and temperature for biomass growth. A two-level factorial experimental design with central point was used. The evolutionary operation method (EVOP) was used to determine optimal operating conditions. A numerical algorithm explained below determined maximum specific growth rate, as a trial function. Optimal operating conditions were found to be pH 5.5 and the temperature 32 °C.

Key words:
Bioconversion process, *Erwinia citreus*, 2,5-diketo-D-gluconic acid, EVOP method

Introduction

Chemical producers of Vitamin C, such as Hoffmann La Roche and BASF-Merck have recently acknowledged the power of biotechnology,[1] replacing a traditional way of obtaining Vitamin C with new direct fermentation route. The traditional route to Vitamin C, named Reichstein-Grüssner synthesis,[2-4] involves the catalytic hydrogenation of glucose to sorbitol followed by the fermentative oxidation of sorbitol to sorbose. Sorbose is then converted by a multistep chemical synthesis to ketogulonic acid, the key Vitamin C intermediate.

Some of the fermentation routes to ketogluconic acid have been already described[5,6] and thoroughly investigated. These investigations resulted in the ongoing construction of the first plant in Germany employing a direct fermentation route from sorbitol to ketogulonic acid.[1]

One of the steps in a development of a fermentation process is its laboratory characterization. A successful study of a complex microbial process requires the optimization of operating conditions. To optimize the operating conditions of any microbial process, the evolutionary operation (EVOP) technique can be applied. EVOP, as an efficient and simple optimization technique, is recommended where a process is not supported by adequate theoretical knowledge. Also, EVOP is very efficient to optimize the combined effect of two or three variables and their interaction on a biological process.[7-9]

We applied this technique to simultaneously optimize the two quantities (pH and temperature), influencing the biomass growth rate in the bioconversion of glucose to 2,5-diketo-D-gluconic acid (2,5-DKG). The biomass specific growth rate μ was selected as an optimization criterion hence the product formation rate depends on biomass concentration as was shown in our earlier paper.[14] In a tandem or a co-fermentation, proposed by Sonoyama et. al,[10-12] glucose is oxidized to 2,5-DKG during the first step. This is followed by the stereospecific reduction of 2,5-DKG to 2-keto-L-gulonic acid (2-KLG).

Batch fermentation were carried out in order to obtain the optimal pH and temperature values for biomass growth when maximum specific growth rate was set as a trial function. Numerical algorithm generated by authors was used to determine the maximum specific growth rate.

Materials and methods

Materials

Chemicals used in this work: glucose (Kemika, Croatia); yeast extract (Difco, USA); peptone (Difco); KH_2PO_4 (Kemika); and $MgSO_4 \times 7H_2O$ (Kemika).

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The microorganism *Erwinia citreus*, strain ATCC 31623 (new name *Pantoea citrea* sp. nov. SHS 2003$^{T\ 13}$), used in this study was obtained from PLIVA, Pharmaceutical industry, incorporated (Croatia), Research Institute, Biotechnology Department. Culture samples were stored at –70 °C in w = 50 % glycerol after freezing in liquid nitrogen.

Fermentation

All batch experiments were carried out in four 300 cm^3 bioreactor (Biostat Q, B. Braun, Germany), containing 250 cm^3 of medium. The bioreactor was equipped with a pH electrode, pH titrator, pO_2 electrode, thermostat and a micro-MFCS data collection and processing system. Fermentation medium for batch experiment contained: glucose 100 g dm^{-3}; yeast extract 5 g dm^{-3}; peptone 3 g dm^{-3}; KH_2PO_4 1 g dm^{-3}; $MgSO_4 \times 7H_2O$ 0.2 g dm^{-3} in distilled water.

The fermentation medium was sterilized at 120 °C, except glucose, which was sterilized at 110 °C. According to experimental plan; the initial pH was adjusted with 2 mol dm^{-3} HCl or 5 mol dm^{-3} NaOH. The pH value of the culture was maintained by the automated titration of 2 mol dm^{-3} HCl or 5 mol dm^{-3} NaOH. Growth temperature was regulated according to experimental plan. Sufficient aeration was obtained by vigorous stirring (600 RPM) and the airflow rate of 1 dm^3 min^{-1}. A reflux cooler condensed the outlet gas stream and the condensate was returned to the medium. The inoculum for all batch experiments were prepared in w = 0.9 % NaCl solution in distilled water.

Analytical methods

In addition to the determination of a dry biomass concentration, the optical density was measured in a double beam spectrophotometer (UV-1601, Shimadzu, Japan) at 660 nm.

Glucose, gluconic acid and ketogluconate were determined by the HPLC (Knauer, Germany), after centrifuging samples at 10000 min-1 for 15 min. A BioRad Aminex column XPX-87H was used. The metabolites were detected by the refraction index with differential refractive index monitor. The temperature during the chromatography was kept constant at 40 °C; approximately 20 nm^3 sample was injected. Each run was carried out by using 5 mmol dm^{-3} H_2SO_4.

Determination of maximum specific growth rate

According to the mass balance for biomass growth in the batch process, changes in the biomass concentration are described as follows:

$$\frac{\mathrm{d}\gamma_X}{\mathrm{d}t} = \mu \cdot \gamma_X \tag{1}$$

Specific biomass growth rate μ is a physiological state value for the growth process and usually a complex function of biological, chemical and physical state values. During the bioprocess in the bioreactor, maximum value of a specific biomass growth rate was obtained in relatively short period of time. This is the exponential growth phase in which the growth rate is under control of metabolic processes in the microorganism cell.

To determine the specific biomass growth rate on the basis of experimental data on biomass concentration changes during a bioprocess, numerical algorithm was set:

– if we suppose that μ = cons., differential equation (1) can be integrated in the equation (2)

$$\ln\frac{\gamma_X}{\gamma_{X_0}} = \mu \cdot t \tag{2}$$

– experimental results for the determination of μ_{MAX} can be described as a set of data pairs t_i, c_{Xi} (i = 1... r). If we use least square fit method with the subset of data pairs, i. e. $t_{j,s}$, $c_{Xj,s}$ (j = 1,..., p; 2 < p < r; s = 1,..., (r – p + 1)) the specific biomass growth rate can be determined according to equation (3):

$$\mu_s = \frac{\sum_{j=s}^{s+p-1}\left(\ln\frac{\gamma_{X_j}}{\gamma_{X_s}}\right)}{\sum_{j=s}^{s+p-1}(t_j - t_s)} \tag{3}$$

– specific biomass growth rate at points t_i (i = p,..., (r – p + 1)) was determined as the arithmetic mean of the all values μ_s for each data subset according to equation (4):

$$\mu_i = \frac{1}{p}\sum_{s=i-p+1}^{i}\mu_s \tag{4}$$

If we increase the p value of the data subset for determination of the specific growth rate, the profile of the changes of the specific growth rate during the process, is more reliable.

Here we used the subset p containing 4 pairs of experimental data to determine the specific growth rate (Figure 1.) If p was smaller than 4, the fitted lines have been not smooth, yielding difficult estimation of maximum. The p values higher than 4 have no influence on the estimation of maximum. The numeric algorithm described in the equation (1) – (4) was implemented in the "Sigma plot" software package.

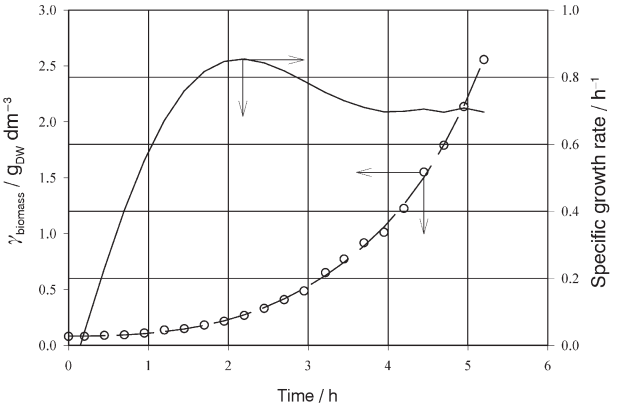

Fig. 1 – *Graphic presentation of determination of maximum specific growth rate; biomass experimental (○), biomass calculated (---), μ (——)*

EVOP methodology

EVOP technique can be used as a multivariable sequential search technique, in which the effects of two or three factors are studied together, and the responses are analyzed statistically to arrive at the decision. In case of experimental design with two variable factors, each factor has to be studied at five levels. These consist of a "center point" (coded as A_1) which is the intermediate level, and the other four levels (coded as A_2, A_3, A_4 and A_5) are suitably selected around the "center point". The responses of experiment at all five levels constituting one cycle of operation are to be recorded for analysis, which needs two repetition of the same experiment at least. For processing the response data to determine the influence of variable quantities and their interaction, a standard calculation procedure for two variable system, which is described elsewhere,[7-9] is proposed.

EVOP decision making procedure

1. To maximize the response, increase (reduction) of variable will help if effect is +(-) R and large compared to error limit and change in mean effect is small.

2. If effect is small compared to error limit while change in mean effect is large, then, if change in mean effect is –R, the maximum has been reached and the EVOP program ends.

Results and discussion

Based on the existing literature data[10-12] and personal observation of this process,[14] the first experimental plan (Table 1.) was defined. Two measurement series were performed for each point of the experimental plan and maximum specific growth rate was determined according to equations (1) – (4). Interestingly, we found out that maximum specific growth rate in all experiments were observed after 2–3 hours of fermentation, probably because of short lag-phase and inhibition of biomass growth by high products concentrations. Differences and average values were calculated accordingly (Table 1.; Figure 2.). Table 2. shows the value of effects and error limits.

Table 1 – *The first experimental plan, results, differences and average values*

Experimental plan	A_1	A_2	A_3	A_4	A_5
Temperature, °C	28	26	30	30	26
pH	4.5	4.0	5.0	4.0	5.0
Results					
μ_{MAX}, h^{-1} (1. cycle)	0.8062	0.7350	0.8501	0.7816	0.7481
μ_{MAX}, h^{-1} (2. cycle)	0.7872	0.7120	0.8383	0.7897	0.7526
Differences and averages					
Differences	0.0190	0.0230	0.0118	-0.0081	-0.0045
μ_{MAX}, h^{-1}	0.7967	0.7235	0.8442	0.7857	0.7504

Based on the analytical results given in Table 2., it is obvious that the effect of average changes is

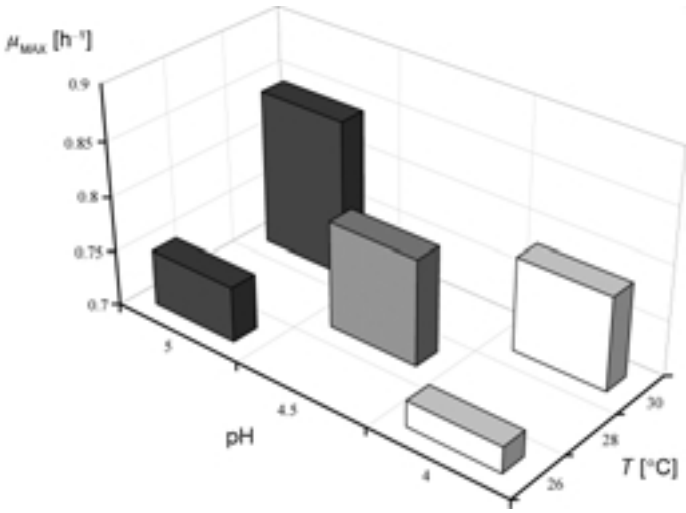

Fig. 2 – *Values of the biomass specific growth rate for the first experimental plan*

Table 2 – *Calculated values for effects and error limits for the first experimental plan*

Temperature effect	0.0780 ± 0.0132
pH effect	0.0427 ± 0.0132
Interaction effect of temperature and pH	0.0158 ± 0.0132
Averages effect	–0.0166 ± 0.0117

negative and higher than error limit. This shows that the optimum obtained in this process was used as a central point. This also means that EVOP method has to be finished in the first plan or a new experimental plan has to be made, keeping the same parameter value in the central point but the step has to be at a higher or lower level. On the other hand, pH, temperature and interaction effects are positive and significantly higher than error limit. A new experimental plan was made in which pH and temperature values were set up at a higher level keeping the same step.

The new (second) experimental plan with its results (Figure 3.) for two measurement series and calculation of differences and average values is shown in Table 3. Table 4. gives the calculated values and error limit for the second experimental plan.

Table 4. clearly shows that only the temperature effect on specific growth rate is positive and

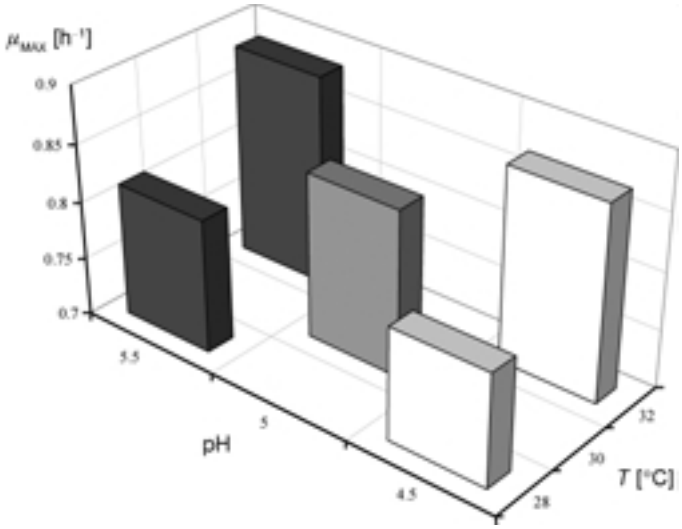

Fig. 3 – *Values of biomass specific growth rate for the second experimental plan*

Table 3 – *The second experimental plan, results, differences and average values*

Experimental plan	A_1	A_2	A_3	A_4	A_5
Temperature, °C	30	28	32	32	28
pH	5.0	4.5	5.5	4.5	5.5
Results					
μ_{MAX}, h^{-1} (1. cycle)	0.8501	0.8062	0.8631	0.8734	0.8153
μ_{MAX}, h^{-1} (2. cycle)	0.8383	0.7872	0.9062	0.8654	0.8550
Differences and averages					
Differences	0.0118	0.0190	–0.0431	0,0080	–0,0397
μ_{MAX}, h^{-1}	0.8442	0.7967	0.8847	0.8694	0.8351

Table 4 – *Calculated values for effects and error limits for the second experimental plan*

Temperature effect	0.0611 ± 0.0263
pH effect	0.0269 ± 0.0263
Interaction effect of temperature and pH	–0.0116 ± 0.0263
Averages effect	0.0018 ± 0.0234

significantly higher than error limit, while the effects of pH, interaction and average changes are lower than error limit. This is why the next (third) experimental plan should be performed so that the temperature at every point of the plan, by keeping the same step, is set to a higher level. pH must be the same as in the second experimental plan (Table 5.). The interesting results from Table 4. show that the optimal direction of the temperature is toward higher temperature as it is shown in the third experimental plan (Table 5.). It was stated[13] that the 2,5-DKG producing strains grew on nutrient agar at 8 – 39 °C but not below 5 and above 41 °C. Therefore, the biosynthesis of 2,5-DKG in medium described above was succesfuly performed at 32 °C.

Table 5 – *The third experimental plan*

	A_1	A_2	A_3	A_4	A_5
Temperature, °C	32	30	34	34	30
pH	5.0	4.5	5.5	4.5	5.5

Conclusion

EVOP method is a simple and practical method for the optimization of operating conditions in the

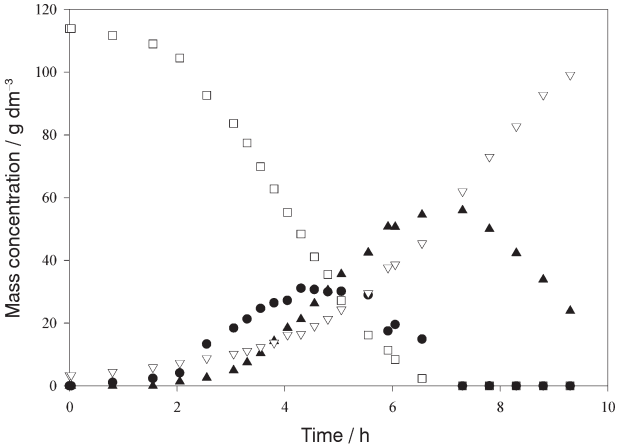

Fig. 4 – *Changes in glucose and ketoacids concentration during the process of bioconversion of glucose to 2,5-diketo--D-gluconic acid; glucose (□), gluconic acid (●), 2-keto-D-gluconic acid (▲), 2,5-diketo-D-gluconic acid (▽)*

process of bioconversion of glucose to 2,5-diketo-D-gluconic acid. It does not require many experiments and permits simultaneous analysis of two quantities. The most reliable conclusion requires more than two measurements in every experimental plan. On the other hand, even a small number of series suffices for preliminary investigation.

The optimal operating conditions for the process of bioconversion of glucose to 2,5-diketo--D-gluconic acid are pH 5.5 and temperature 32 °C, at which the biosynthesis of 2,5-diketo-D-gluconic acid is successfully performed (Figure 4.).

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Symbols

γ_X – biomass concentration at time t, g dm^{-3}
γ_{X0} – biomass concentration at time t = 0, g dm^{-3}
t – time, h
T – temperature, °C
w – mass fraction, %

Greek symbols

γ – mass concentration, g dm^{-3}
μ – specific growth rate at time t, h^{-1}
μ_{MAX} – maximum specific growth rate, h^{-1}

Abbreviations

2,5-DKG – 2,5-diketo-D-gluconic acid
2-KLG – 2-keto-L-gulonic acid

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