Control Strategy of Fed-Batch Cultivations of Yeasts

[a,b]M. RYCHTERA, [a]L. PAULOVÁ, [c]J. NÁHLÍK, [a]K. MELZOCH, and [a,d]J. VOTRUBA

[a] *Department of Fermentation Chemistry and Bioengineering, Faculty of Food and Biochemical Technology, Institute of Chemical Technology Prague, CZ-166 28 Prague*

[b] *Department of Food Chemistry and Biotechnology, Faculty of Chemistry, Technical University, CZ-637 00 Brno*

[c] *Department of Computer and Control Engineering, Faculty of Chemical Engineering, Institute of Chemical Technology Prague, CZ-166 28 Prague*

[d] *Institute of Microbiology, Academy of Sciences of the Czech Republic, CZ-142 20 Prague*

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Efficient and reliable on-line substrate feed control is of great importance for any type of cultivation of microorganisms. Our work was oriented to the search for criteria of physiological state of microorganisms which can be employed as an appropriate and auxiliary tool for selection of a control strategy. *Saccharomyces cerevisiae* culture used in fed-batch cultivations of baker's yeasts was taken as a model case. Another task was to test several control algorithms with the aim of finding the most suitable one satisfying not only the process parameters themselves but also quality of the product (yeast biomass).

Fed-batch cultivations of yeasts were performed in laboratory fermentor LF-2 of effective volume 1500 cm^3 and several feeding strategies of molasses medium were tested. The feeding rate of this medium was controlled either automatically or manually according to several measured signals: concentration of produced ethanol, concentration of carbon dioxide, and dissolved oxygen concentration.

When considering productivity of biomass very good results were achieved with carbon dioxide and ethanol concentration control with values of 1.91 and 1.65 kg m^{-3}, respectively. As far as biomass yield is concerned the control of dissolved oxygen and ethanol control gave very high values (in both cases yield coefficient $Y_{X/S}$ equals 0.65).

Most of the methods for control of the baker's yeast fed-batch process already published are based on controlling the main production characteristics. Parameters such as dissolved oxygen and ethanol concentrations, respiration quotient are mostly maintained constant [1], while carbon dioxide concentration in gas phase must form an increasing function due to increasing biomass concentration. *Albrecht et al.* [2] studied the impact of ethanol formation coefficient on the fermentation activity of the baker's yeasts in computer-controlled experiments. The authors came to the conclusion that a quite low ethanol formation coefficient is sufficient to secure a good fermentation activity of yeast (raising power). If the ethanol formation rate coefficient is constant it leads to an increase of the ethanol concentration at the end of the fed-batch which brings about a loss in biomass. Authors of paper [2] suggested that the ethanol production rate coefficient had to be a variable parameter so as to obtain the maximum biomass yield. Consequently the ethanol concentration shows a profile characterized by an increase during the first process phase followed by a gradual decrease until the end of the process preventing the above stated economic loss due to excess of ethanol formed. Similar conclusions with practical control procedure were also claimed by *Náhlík* [3].

Yeasts *Saccharomyces cerevisiae* belong to facultative anaerobic microorganisms which possess a typical character of response to lack of oxygen and to surplus of carbohydrate under conditions of optimal aeration. The former phenomenon is known as alcoholic fermentation and the latter as Crabtree effect [4]. Under the condition of Crabtree effect (characterized by a critical D-glucose concentration) there appears an accumulation of ethanol and unutilized D-glucose. This phenomenon has been studied and described by many authors since its first disclosure in 1929 [5]. The theory of Crabtree effect is summarized by *Sonnleitner* and *Käppeli* [6] and occurrence of ethanol and changes of respiration quotient RQ were widely used in many

control strategies for aerobic cultivation of yeasts *S. cerevisiae*.

In order to achieve maximum of production parameters a series of different controllers were used. The most simple one is the PID controller [7, 8]. These papers show that it is difficult to obtain good regulation during the total duration of the cultivation. The results with the PID controller demonstrate that this technique is limited which is caused by the changing dynamics during the microbial process. *Mészáros* and *Báleš* [9] suggested both optimal feedforward and feedback control for a fed-batch cultivation. Numerous techniques utilizing an adaptive controller have been reported trying to compensate the dynamics and nonlinearities during the growth of yeasts [10—12]. *Keulers* [1] stressed importance of the specific growth rate for the control strategy but to overcome the problem of this nonmeasurable variable a simple observer was developed on the basis of on-line measurement. This observer is able to estimate the specific growth rate and the cell concentration. For reaching the maximum yield it is essential to maintain the substrate concentration below its critical level which according to *Enfors et al.* [13] is about 11 g dm^{-3}, according to *Wang et al.* [14] 13 g dm^{-3}, and according to *Dellweg et al.* [15] 20 g dm^{-3}. Further, the dissolved oxygen level should be kept above a critical level, which is around 18 % of saturation value. Nevertheless, in a number of industrial fed-batch productions the substrate feed rate profile is conventionally controlled on the basis of empirical knowledge, although recently a lot of papers on the application of artificial intelligence techniques such as neural [16, 17], expert [18], fuzzy [19], fuzzy knowledge-based [20], and hybrid neuro-fuzzy systems [20, 21] have been suggested for state estimation, variable prediction and control.

In our paper we present comparison of several control strategies based on ethanol, dissolved oxygen, and carbon dioxide concentrations (controlled variables) where these concentrations were either set constant or variable. The main intention of our future research is to control the specific growth rate, the parameter which is responsible for the activity of the culture and its final quality. Molasses medium flow rate was always a manipulated variable.

EXPERIMENTAL

Fed-batch cultivations of *Saccharomyces cerevisiae* yeast culture were performed in laboratory fermentor LF-2 (manufactured in the Workshops of the Czechoslovak Academy of Sciences) in an effective volume of 1500 cm^3 and several feeding strategies of molasses medium were tested.

Yeast strain: *Saccharomyces cerevisiae* var. *Hansen 03/2* from the Culture Collection of Microorganisms of the Department of Fermentation Chemistry and Technology, Institute of Chemical Technology Prague (ICTP). The strain was kept on malt agar slants at 4 °C and monthly reinoculated onto the new ones.

Medium composition: sugar beet molasses was diluted to 45 mass % by a tap water and then to 1 dm^3 of this solution 20.4 cm^3 of 26 vol. % NH_4OH, 5.1 cm^3 of 30 mass % H_3PO_4 and 7.2 cm^3 of 25 mass % H_2SO_4 were added. Solution was clarified, filtered, pH adjusted to the value 5.2 and sterilized in an autoclave at 120 °C for 20 min (medium A). This concentrated solution was sequentially added to the basic solution in a fermentor. One dm^3 of this medium contained: 0.2 g of $MgSO_4 \cdot 7H_2O$, solution was sterilized and after sterilization 0.3 mg of biotin was supplemented to the cool medium (medium B). 1.5 dm^3 of this solution in the fermentor was inoculated by a yeast culture. Concentrated medium A was then added to the medium B in a fermentor according to a control algorithm. The first dose of medium A was given manually to initiate the yeast growth. Initial concentration of dry biomass was 3—4 g dm^{-3}.

Cultivation temperature was 30 °C, pH value was kept in the range 4.8—5.0, air flow rate was between 1.5 and 2.0 dm^3 min^{-1} (1.0—1.3 VVM), volume of medium A added changed according to control algorithm used and its maximum value was 300 cm^3. Initial volume of medium B in the fermentor was 1.4 dm^3. Total cultivation time was always 10 h. Rotation frequency of impeller was 10 Hz (600 min^{-1}).

The feeding rate of substrate was controlled either automatically or manually according to several measured signals, *e.g.* concentration of produced ethanol, concentration of dissolved oxygen, concentration of carbon dioxide, and respiration quotient RQ. For comparison of the results there was one experiment performed without computer control but feeding doses were calculated on the basis of exponential incremental feeding. Fermentor was linked to the control system based on PC-AT. Basis of the program facilities is a multiprogram operational system SMR (system of measuring and control) which operates in a real time.

The standard part of this system is a block of classical PID controller and block of developed controllers, *e.g.* adaptive PID controller (ADA), controller ETREG (ethanol controller), and controller CO2REG (carbon dioxide controller) [22]. These are adaptive controllers which determine the average supply of molasses medium from the Metrex signal (ETREG) or from the Infralyt signal (CO2REG). Molasses medium was fed into the reactor by a programmable pump PD-2000 (New Brunswick Scientific, USA) linked to a control unit. INFRALYT 5 analyzer (VEB JUNKALOR, Dessau, manufactured in former GDR) monitored concentration of carbon dioxide in a gas phase, PERMOLYT 2 (VEB JUNKALOR, Dessau) was used for measuring the oxygen concentration in a gas phase. For monitoring of ethanol concentration analyzer METREX-ELD designed and manufactured at

the Department of Physics and Measuring Technique, ICTP, was used. Here ethanol vapours are burnt catalytically and the thermoeffect is evaluated as the change of electric resistance of the measuring fibre. Values obtained from gas phase analysis are automatically transformed to data expressing ethanol concentration in a liquid phase. Disadvantage of this measurement is determination of oxidizable volatile substances such as aldehydes, ketones, esters, and other alcohols together with ethanol. Therefore the values obtained from the measurement are higher than those obtained from the GC analysis of collected samples.

To verify control algorithms we carried out 23 fed-batch experiments with yeast cells *S. cerevisiae.*

Biomass concentration was determined gravimetrically after centrifugation and washing with distilled water. Ethanol concentration (together with D-glucose, D-fructose, and saccharose) was determined by using HPLC (column filled with catex Ostion LGKS 0800 in the Ca^{++} form) [23] and detected by a flow refractometer. Concentration of dissolved oxygen was measured continuously by a DOC electrode which was a part of PD 4-44-0 controller (Workshops of the Czechoslovak Academy of Sciences).

RESULTS

All 23 experiments were carried out to obtain a necessary knowledge of different control strategies (controllers and controlled variables) as a basis for knowledge analysis and later on for knowledge-based control. In this paper we present the first part of our research describing the effect of several controlled variables (ethanol concentration, dissolved oxygen concentration, and carbon dioxide concentration in gas phase). The first doses of the medium had to be added manually since the concentration of controlled variables must attain a certain value. In this chapter we present four selected cultivation courses which in all respects fulfilled our requirements for a good feeding control. They had almost the same starting conditions such as concentration of feeding medium, concentration of seed yeasts, cultivation temperature, impeller speed, air flow rate, initial medium volume in the fermentor, and total cultivation time. Concentration of dissolved oxygen was always at the beginning set to 100 % of the saturation value. Differences in cultivation courses resulted mainly from control algorithms and obviously also from small differences in the physiological state of inoculum. With respect to withdrawal of samples the final volume of cultivation medium was higher only by about 10—80 cm^3, volume of molasses medium A added into the fermentor was changed according to control algorithm and its maximum value was 300 cm^3. Examples of three main feeding controls are shown in Figs. 1—4 and described in the following paragraphs.

Example I

Carbon dioxide concentration as a controlled variable. Concentrated molasses medium A was discontinuously fed into the bioreactor (filled at the beginning with medium B and the seed culture) as a result of a signal from CO_2 gas analyzer by means of control program CO2REG. A slope of the straight line was given initially to the controller. The equation of this straight line was: $\varphi(CO_2) = 0.395t + 0.08$ ($\varphi(CO_2)$ – vol. % of CO_2 in gas phase, t – fermentation time). For the last hour of the cultivation a new equation had to be applied. Final results of the cultivation (specific growth rate – μ, yield coefficient for biomass – $Y_{X/S}$, and total productivity – p) are shown in Table 1. Relationship

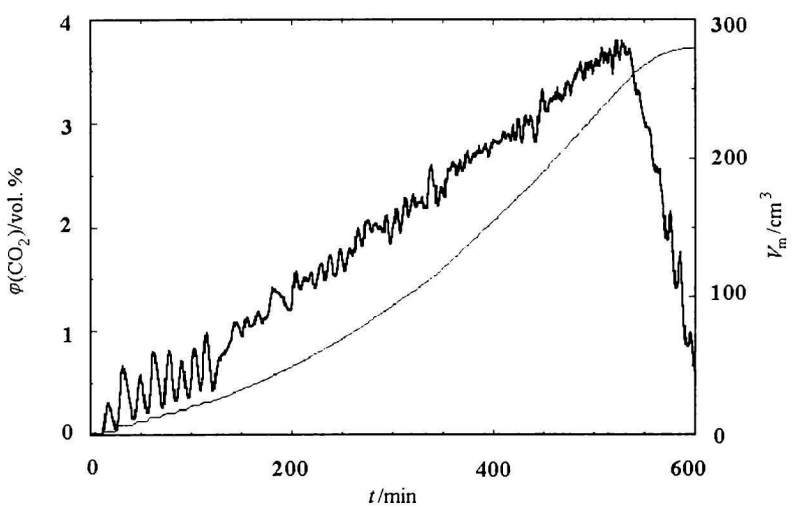

Fig. 1. Carbon dioxide concentration as a controlled variable: time dependence of cultivation medium volume added into the bioreactor and the course of the controlled variable concentration. V_m – total volume of the medium A (cm^3) fed into the bioreactor (——), $\varphi(CO_2)$ – vol. % of CO_2 in gas phase (——).

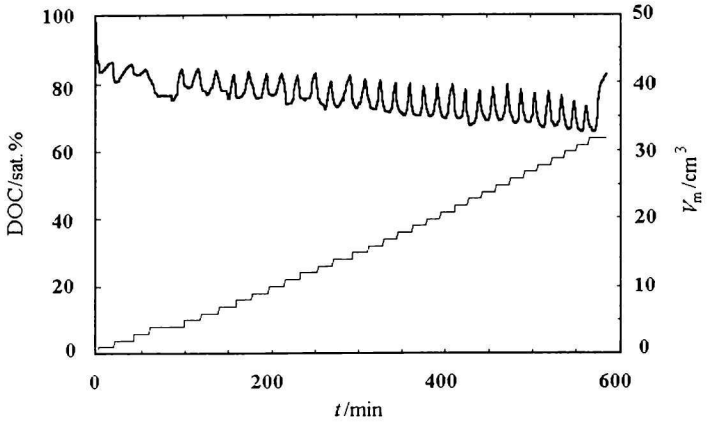

Fig. 2. Dissolved oxygen concentration as a controlled variable: time dependence of cultivation medium volume added into the bioreactor and the course of the controlled variable concentration. DOC – dissolved oxygen concentration (saturation %) (——), V_m as in Fig. 1.

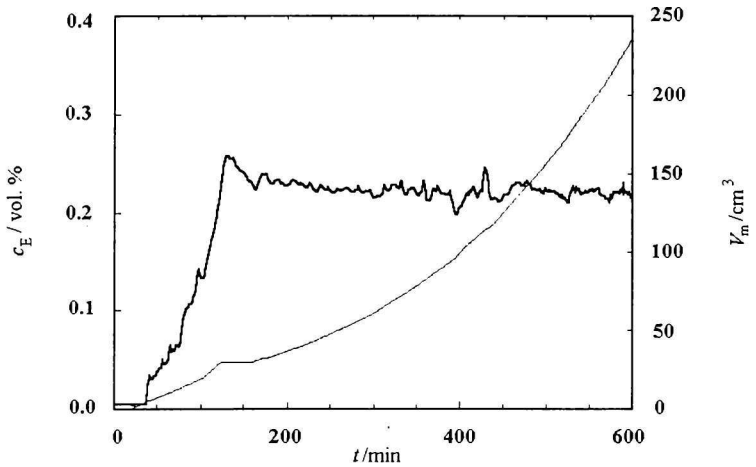

Fig. 3. Ethanol concentration as a controlled variable: time dependence of cultivation medium volume added into the bioreactor and the course of the controlled variable concentration. c_E – ethanol concentration in cultivation medium (vol. %) (——), V_m as in Fig. 1.

between the controlled value of CO_2 concentration in gas phase and volume of the media in the fermentor is shown in Fig. 1.

Example II

Dissolved oxygen concentration (DOC) as a controlled variable. The objective of this type of cultivation was to find out relationship between the magnitude of molasses doses and the profile of DOC response. According to *Miskiewicz* and *Miszak* [24] it is assumed that in aerobic metabolism of cell population oxygen and sugar substrate are exploited not only in parallel but also in the same ratio. It is very important to apply such a dose of sugar substrate which does not bring about the DOC excess. Another criterion for the feeding strategy is not to cause the Crabtree effect. This is not directly given by the DOC level. This level is recommended to be maintained in the range from 20 to 60 % of the saturation value. Economical values are shifted to lower values. Relationship between DOC controlled level and total volume of the medium in the fermentor is shown in Fig. 2. Additions of the medium were carried out manually from the key-board of the computer. The minimal medium pulses were 0.1 cm^3 which corresponds to 30 mg of saccharose. The optimal dose was such an amount of sucrose medium at which the decrease of DOC smoothly changed into its increase without any time lag. In our case it was 1.5 cm^3 which corresponds to concentration of sucrose equal to 0.26 g dm^{-3} and is in agreement with the results of *Miskiewicz* and *Miszak* [24]. The course of DOC values has an oscillating character.

Example III

Ethanol concentration as a controlled variable. Addition of molasses medium was carried out automatically by means of control program ETREG and signal from the ethanol analyzer METREX-ELD. The

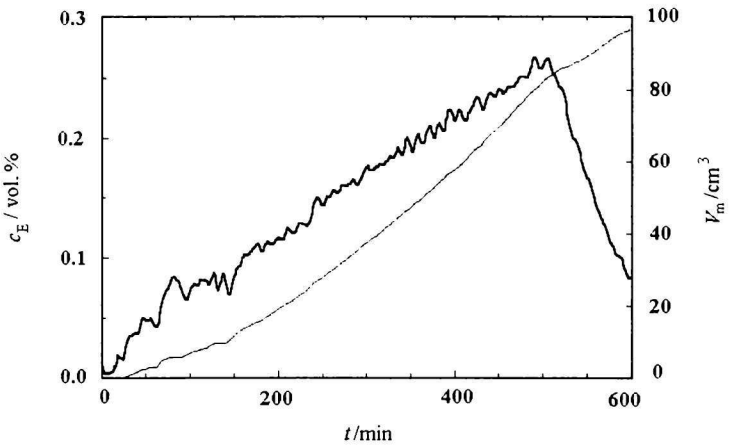

Fig. 4. Ethanol concentration as a controlled variable: time dependence of cultivation medium volume added into the bioreactor and the course of the controlled variable concentration. c_E as in Fig. 3, V_m as in Fig. 1.

Table 1. Kinetic and Stoichiometric Values of Selected Cultivations

Parameter	Unit	Controlled variables Type of control			
		CO_2 increasing profile	DOC constant value	Ethanol constant value	Ethanol profile[a]
Specific growth rate μ	h^{-1}	0.22	0.08	0.19	0.17
Yield coefficient $Y_{X/S}$	–	0.58	0.65	0.65	0.53
Productivity p	g dm^{-3} h^{-1}	1.91	0.42	1.65	1.22

a) Increasing/decreasing profile of the feeding trajectory.
Controlled variables: carbon dioxide concentration – see Fig. 1
DOC – Fig. 2
Ethanol concentration (constant value) – Fig. 3
Ethanol concentration (increasing/decreasing profile) – Fig. 4

aim of these series of experiments was to find out the optimal profile of ethanol concentration (expression ethanol concentration means METREX reading which is discussed earlier). During the first hour of cultivation the molasses medium feeding was controlled manually from the computer key-board. Several possibilities were tested: *a*) constant level of ethanol 0.15 vol. % (1.2 g dm^{-3}), *b*) 0.2 vol. % (1.6 g dm^{-3}), *c*) control of the medium feeding according to the predicted profile of ethanol concentration. Situations *b*) and *c*) are demonstrated in Figs. 3 and 4, respectively. In all three alternatives the feeding pump was controlled by the control program ETREG. In the last case the required value of ethanol concentration ("set-point") was changing along the straight lines: $c_E = 0.03t + 0.005$ (applied till 8.5 h from the beginning of the cultivation when the ethanol concentration 0.25 vol. % was reached) and $c_E = -0.06t + 0.68$ applied for the last 1.5 h; t is time of cultivation from its beginning. In the first hour the feeding was carried out manually. Example of this control strategy is demonstrated in Fig. 4. Various combinations of straight lines were tested in other experiments not presented here.

DISCUSSION

Results obtained during our investigation of various control strategies have been applied for the knowledge-based analysis and formulation of mathematical model of the process, for its application in process simulation and its verification in selected experiments [25]. A part of our research will be presented later [26]. This paper presents summarization and generalization of our initial studies.

The most important parameters for the evaluation of the cultivation are specific growth rate – μ, biomass yield coefficient – $Y_{X/S}$, and biomass productivity – p. Comparison for all types of controls demonstrated in Figs. 1—4 is shown in Table 1. As the goal of the industrial cultivations are the quantity and quality of the biomass, we have tried to find such a control strategy which could justify higher productivity (linearly depending on specific growth rate and biomass concentration) and high yield coefficient. Concerning

yield coefficient the best results were found for ethanol and DOC control ($Y_{X/S} = 0.65$), DOC control however gave the lowest productivity ($p = 0.42$). As far as productivity is concerned carbon dioxide control demonstrated the highest value (1.91 g dm^{-3} h^{-1}), ethanol concentration control showed also high productivity (1.61 g dm^{-3} h^{-1}).

Metabolism of yeast culture grown in bioreactor under conditions of an aerobic "fed-batch" culture is affected by the feeding profile and by the rate of feeding. During the cultivation five metabolic stages could be demonstrated by:

a) Balanced aerobic growth when the rate of feeding of sucrose medium is directly equal to the rate of its uptake; cells do not form ethanol as a by-product and the residual concentration of sugar is virtually zero. Specific growth rate equals the value of 0.2 h^{-1}.

b) Growth under conditions of substrate (sugar) limitation when quantity of saccharidic substrate coming into bioreactor is lower than it is necessary for maximal growth. Residual substrate concentration is therefore zero, ethanol is not formed and specific growth rate is very low.

c) Growth under aerobic fermentation (Crabtree effect) when specific rate of the substrate flow into the fermentor is higher than the specific rate of its uptake. Concentration of sugar exceeds therefore its critical value (*Paulová* [25] found experimentally the value of 0.26 g dm^{-3}) and brings about production of ethanol, higher RQ as a result of "overflow" mechanism [6]. Specific growth rate is higher than 0.21 h^{-1}.

d) Simultaneous growth on sucrose and ethanol when specific rate of the substrate flow into the bioreactor is lower than is the requirement for an uptake of the culture but at the same time there is some quantity of ethanol formed earlier by yeasts. This is a typical case appearing after control failure or after changing control strategies during the fed-batch process. Concentration of the sucrose is lower than its critical value. Part of respiration capacity can be employed for aerobic degradation of ethanol. There is no diauxic lag phase. Specific growth rate is a little lower than for balanced aerobic growth (around 0.18 h^{-1}).

e) Aerobic growth on ethanol as a C-source. This type of metabolism appears in final stages of cultivations controlled by carbon dioxide or ethanol concentrations when supply of molasses medium was stopped. It must be pointed out that from utilization of sucrose and ethanol there is a significant adaptation lag the length of which depends on the cultivation conditions and physiological state of yeasts. Specific growth rate is lower than corresponds to the growth on sugar substrate (0.16 h^{-1}).

During the course of cultivation it is possible to detect directly or indirectly all five metabolic stages as a result of substrate feeding. Type *b)* is characteristic mostly of DOC controlled feeding as can be seen from Table 1. In most of aerobic cultivations of yeasts we have to consider mixed type of metabolism because controllers commonly used do not analyze the real physiological state of the culture. That would need more sophisticated controller based on the artificial intelligence.

Our experimental results can be compared with those published by only a few authors. The main problem of comparison is that one should compare all control strategies studied as one set of data. One strategy can only be qualitatively evaluated but not compared in the whole context since experimental conditions, microorganisms, bioreactors, and many other factors are different. For example, our data can be compared with data published by *Keulers* [1], *Miskiewicz* and *Miszak* [24], and *Williams et al.* [27]. Authors of the last paper carried out similar set of experiments using adaptive controller but their evaluated results with respect to specific growth rate and yield coefficient were much lower (*e.g.* for ethanol concentration control was $\mu = 0.13$ h^{-1} and $Y_{X/S} = 0.4$).

CONCLUSION

For attaining the high yield coefficient of yeast biomass and its productivity algorithms based on ethanol concentration control (ETREG) and carbon dioxide concentration control (CO2REG) have shown advantages over DOC controlled feeding. Control based on ethanol and carbon dioxide profiles could be therefore a good start for further laboratory and large-scale optimizations. Having used all controlled variables we could not simply change the specific growth rate since it is not directly measurable variable. This type of control is a goal of our future investigation.

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