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Effect of pH on Cellulase Production of *Trichoderma reesei* RUT C30

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Abstract

Currently, the high market price of cellulases prohibits commercialization of the lignocellulosics-to-fuel ethanol process, which utilizes enzymes for saccharification of cellulose. For this reason research aimed at understanding and improving cellulase production is still a hot topic in cellulase research. *Trichoderma reesei* RUT C30 is known to be one of the best hyper producing cellulolytic fungi, which makes it an ideal test organism for research. New findings could be adopted for industrial strains in the hope of improving enzyme yields, which in turn may result in lower market price of cellulases, thus making fuel ethanol more cost competitive with fossil fuels. Being one of the factors affecting the growth and cellulase production of *T. reesei*, the pH of cultivation is of major interest. In the present work, numerous pH-controlling strategies were compared both in shake-flask cultures and in a fermentor. Application of various buffer systems in shake-flask experiments was also tested. Although application of buffers resulted in slightly lower cellulase activity than that obtained in non-buffered medium, β-glucosidase production was increased greatly.

Index Entries: Cellulase production; *Trichoderma reesei* RUT C30; pH profiling; β-glucosidase; shake flask; fermentor.

Introduction

Because of the environmental considerations that has emerged during the past two decades, biomass-originated alternative fuels have gained remarkable attention. Ethanol, owing to its advantageous physical properties, seems to be a potential substitute for gasoline in internal combustion engines in the near future. The obvious choice of raw material that could support large-scale fuel ethanol production would be lignocellulo-

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sics because of their widespread availability. One of the process alternatives suggested for the production of fuel ethanol from lignocellulosic materials is based on the enzymatic saccharification of cellulose. For efficient conversion of the cellulose fraction, a large enzyme dosage per unit of raw material must be applied, which, owing to the high market price of cellulases, significantly increases the overall production cost of ethanol.

Trichoderma reesei RUT C30 is known to be one of the best hyperproducing cellulolytic fungi. Several factors, such as the amount and quality of carbon source, temperature and pH of the cultivation, and aeration, influence enzyme production of this strain. It has been indicated in previous studies that pH and the pH-controlling strategy have a great effect on the amount of cellulase produced *(1–9)*.

It has been shown that depending on the nature of the carbon source used to induce the cellulase production of *Trichoderma* strains, different initial pH values of the cultivation may be optimal for maximum cellulase yield. Ryu and Mandels *(1)* have reported that a pH range of 3.0 to 4.0 was optimal for pure cellulose carbon source, but a higher initial pH was recommended for lignocelluloses. For medium containing sugarcane bagasse (free of water-soluble sugars), a pH between 5.0 and 6.0 was observed to be optimal using *T. reesei* QM 9123 (NRRL 3653) *(2)*. In other studies, maximum yield of cellulases was obtained in the range of pH 3.0–5.0 *(3–5)*.

Tangnu et al. *(6)* studied the influence of pH on cellulase production of *T. reesei* RUT C30 in a pH-controlled fermentor. In the pH range of 4.0–6.0, no significant effect on the production rate and final cellulase yield was observed, but β-glucosidase production was affected to a large extent. At pH 4.0 and 5.0, β-glucosidase activity gradually increased until it reached its maximum value by d 8 of cultivation. Controlling the pH in the fermentor to 6.0 increased the production rate of β-glucosidase considerably, and an approx 30% higher enzyme yield was achieved by d 4 than at lower pH values. However, during the final stage of the fermentation the level of β-glucosidase activity decreased to the same value as measured at lower pH levels. Hendy et al. *(7)* reported that performing the fermentation above pH 5.0 resulted in a significant loss of cellulase activity. Instead of keeping the pH at a constant value during the whole fermentation process, Doppelbauer et al. *(8)* recommended using pH profiling. For the growth phase of *T. reesei*, the pH of cultivation was suggested to be maintained at 4.0, while in the later stage of production and secretion of cellulases, an elevated pH level of 5.0 was recommended. In another study, pH cycling coupled with temperature profiling increased the amount of cellulases by 13% compared to the control case during which the pH was maintained at a constant value *(9)*.

In shake-flask cultures, pH control is usually limited to either addition of buffering salts such as phosphates, or periodic manual pH adjustment, which is obviously tedious and less effective. The use of ammonium sulfate as the major nitrogen source in Mandels' medium *(10)* requires a more compelling buffering system. Without buffering, the pH drops quickly

during the first stage of the fermentation owing to the depletion of ammonia and liberation of protons *(11–13)*. Application of a KH_2PO_4-K_2HPO_4 buffer system for controlling the pH in shake flasks proved to be inefficient in compensating acidification. However, higher cellulase activity was obtained in the buffered system than in the basal Mandels' medium *(14)*. Kadam and Keutzer *(5)* investigated several organic acid buffer systems—acetate, succinate, phthalate, and citrate; unfortunately, they did not report the efficiency of the various buffer systems.

In the present study, pH-controlling strategies were executed in both shake-flask cultures and a fermentor. Furthermore, various organic acid buffer systems were studied and their efficiency was evaluated in shake-flask cultures.

Materials and Methods

Inoculum Preparation

Freeze-dried conidia of *Trichoderma reesei* RUT C30 (ATCC 56765) were obtained from the American Type Culture Collection (ATCC). The stock culture of the fungus was maintained on agar slants containing 20 g/L of malt extract, 5 g/L of glucose, 1 g/L of proteose peptone, and 20 g/L of bacto agar. After 14 d at 30°C, the greenish conidia were suspended in 5 mL of sterile water, and 1 mL of this suspension was transferred aseptically to a 750-mL Erlenmeyer flask containing 150 mL of sterile and pH-adjusted (5.5) Mandels' medium in which the concentrations of nutrients were as follows: 0.3 g/L of urea, 1.4 g/L of $(NH_4)_2SO_4$, 2.0 g/L of KH_2PO_4, 0.3 g/L of $CaCl_2$, 0.3 g/L of $MgSO_4$, 0.25 g/L of yeast extract, 0.75 g/L of proteose peptone and 7.5 g/L of Solka Floc 200 (International Fiber, New York, NY) cellulose powder. Furthermore, the medium was supplemented with the following trace elements: 5 mg/L of $FeSO_4 \cdot 7H_2O$, 20 mg/L of $CoCl_2$, 1.6 mg/L of $MnSO_4$, 1.4 mg/L of $ZnSO_4$. Preparation of the inoculum was completed by 4 d of cultivation at 30°C on an orbital shaker (350 rpm) *(10)*.

Enzyme Production in Shake Flasks

A 15-mL mycelium suspension obtained from the inoculum cultures was used to initiate growth in a 750-mL Erlenmeyer flask containing 150 mL of a modified Mandels' medium in which the concentration of the carbon source (i.e. Solka Floc 200), was increased to 10.0 g/L. After inoculation, the Erlenmeyer flasks were incubated on an orbital shaker at 30°C and 350 rpm for 7 d. Samples were withdrawn daily at the same hour of the day, and when necessary, the pH in the flasks was manually adjusted using sterile, 10 wt% solutions of NaOH or H_2SO_4. Aseptically taken samples were centrifuged at 3400*g* for 5 min. The collected supernatants were analyzed for enzyme activities. In shake-flask cultures, basically three pH-controlling strategies were applied: (1) the pH was adjusted to its initial value (5.0 or 6.0), thereby obtaining a sawtooth-like pH profile; (2) starting from pH values of 5.0 or 6.0, the adjustment was restricted to

those cases when the pH dropped below 4.0; (3) by applying various buffers in the production medium, the pH was kept at a constant value of 5.0 or 6.0. Each experimental condition was performed in triplicate, and the average of measured parameters (pH, enzyme activities) was calculated.

Enzyme Production in Laboratory-Scale Fermentor

Upscale enzyme production experiments were performed in a 31-L double-walled stainless steel laboratory fermentor (Biostat CDCU-3; B Braun Biotech, Germany). Fermentation of *T. reesei* RUT C30 was performed in modified Mandels' medium as described in the previous section. Prior to sterilization at 121°C for 20 min, the nutrients, carbon source, and trace elements required for 20 L of production medium were dissolved in 19.5 L of tap water. During sterilization, vapor equivalent to about 500 mL of water was bled through the gas exhaust system in order to achieve sterile conditions. After sterilization, the temperature of the fermentor was decreased to 30°C and 1 L of starter culture was aseptically added to initiate growth and enzyme production. Cultivation of *T. reesei* RUT C30 was carried out with an agitation rate of 250 rpm for 72 h. Samples were withdrawn regularly and centrifuged at 3400*g* for 5 min. Supernatants were collected and enzyme activities were measured. Four different experimental conditions were performed applying two pH-controlling strategies: (1) from the initial pH (5.0 or 6.0), the pH was allowed to drop to 3.5, after which it was adjusted to and further controlled at the starting pH; (2) no pH shift was allowed and the pH was continuously set to the starting pH of 5.0 or 6.0. In both strategies the pH was controlled by automatic addition of sterile, 10 wt% solutions of either H_2SO_4 or NaOH. The dissolved oxygen (DO) level in the production medium was kept at 30% of saturation value for the medium throughout the fermentation by controlling the flow rate of the air supply. To avoid the formation of foam, silicon oil–based Sigma-Aldrich Antifoam A (Munsch, Germany) in 30% ionic emulsion was added manually four times a day at about 6-hour intervals.

Analysis

Cellulase activity of the samples was determined as filter paper activity (FPA) expressed in filter paper units (FPU) using Mandels' procedure *(15)*, and β-glucosidase activity was assayed using 4-nitrophenyl-β-D-glucopyranoside substrate according to Berghem and Petterson's *(16)* method. All samples were analyzed in triplicate and the mean values were calculated. The relative standard deviation of enzyme activity measurements was always below 5%.

Prior to high-performance liquid chromatography (HPLC), samples were filtered through a 0.2-μm pore size mixed cellulose ester filter (Schleicher & Schuell, Dassel, Germany). Buffer components such as acetic acid, citric acid, maleic acid, and succinic acid were separated on an Aminex HPX-87H (Bio-Rad, Hercules, CA) organic acid column at 65°C using a

Table 1
Application of Organic Acid Buffer Systems in Shake-Flask Cultures

Buffer system	Cellulase	β-Glucosidase	pH stability
0.1 *M* Citric acid–citrate, pH 5.0	Poor	Poor	Poor
0.1 *M* Acetic acid–acetate, pH 5.0	—	—	Very good
0.1 *M* Succinic acid–NaOH, pH 5.0	High	High	Poor
0.1 *M* Tris–maleic acid, pH 6.0	Good	Good	Very good
0.1 *M* Maleic acid, pH 6.0	Good	High	Very good

5 m*M* H_2SO_4 mobile phase at a flow rate of 0.5 mL/min. The analytical column was protected with a Cation-H (Bio-Rad) precolumn. For detection of organic acids separated on the analytical column, a Shimadzu RID-10A refractive index detector (Kyoto, Japan) was used.

Results and Discussion

Shake-Flask Cultivations

The aim of the first set of experiments performed in shake-flask cultures was to develop a buffer system with a sufficiently high buffering capacity to compensate the rapid pH drop typically observed on Mandels' medium during the first stage of the cultivation process. Inorganic phosphate salts were excluded during the selection of potential buffer systems, because they have already been proved to be insufficient *(14)*. The choice of organic acid buffer systems seemed to be promising for two reasons: (1) organic acids were believed not to be utilizable by the microorganism, and, therefore, they could be applied in relatively small concentrations compared to phosphate salts; and (2) most of the organic acid buffer systems are capable of controlling the pH in the range of our interest. Although citric acid and acetic acid have already been examined in a previous study, the pH range in which they were applied was much lower than our primary interest *(5)*. Thus, these organic acids were also tested in our experimental series along with Tris–maleic acid, maleate, and succinate as shown in Table 1. All buffer systems were applied in a 0.1*M* concentration. The concentration of various organic acids in fermentation samples was determined using HPLC. The acetic acid–acetate buffer system applied at pH 5.0 kept the pH constant throughout the fermentation. No changes in acetic acid concentration could be observed in the medium. However, no enzyme activities were detected after 7 d of cultivation. Inhibition of microbial growth by weak acids in pH intervals below or close to the pK_A of the acid used (4.8 in this case) could be a reasonable explanation for the poor performance of *T. reesei* RUT C30. Although employing succinic acid in the fermentation medium at pH 5.0 resulted in rather high enzyme activities compared to the other buffer systems used,

Table 2
pH-Controlling Strategies Followed in Shake-Flask Cultivation of *T. reesei* RUT C30

Condition	pH-controlling strategy
A	Daily pH adjustment to 5.0
B	pH adjustment in case pH drops below 4.0, starting pH: 5.0
C	Daily pH adjustment to 6.0
D	pH adjustment in case pH drops below 4.0, starting pH: 6.0
E	0.1 *M*, Tris–maleic acid buffer, pH 6.0, no manual adjustment necessary
F	0.1 *M*, Maleic acid–NaOH buffer, pH 6.0, no manual adjustment necessary

the pH fluctuated over a wide range. At the beginning of the cultivation, buffering capacity was not high enough to prevent acidification caused by ammonia depletion, whereas in the final stage, succinic acid was taken up by the fungus and a dramatic pH shift to the alkaline region was observed. In contrast to previous results reported by Kadam and Keutzer *(5)*, citric acid buffer could not control the pH at 5.0. Similarly to the succinic acid buffer system, citric acid was also consumed by *T. reesei* RUT C30, and an extensive basification of the medium was observed. In this case, cellulases and β-glucosidase enzymes were produced in moderate amounts. Cultivation of *T. reesei* RUT C30 in Tris–maleic acid, and maleic acid–NaOH buffer systems was quite promising. Both FPA and β-glucosidase activities were considerably high in the fermentation broth using these systems. Furthermore, these buffer systems managed to maintain the pH around the desired value with great stability.

In another set of shake-flask experiments, Tris–maleic acid and maleic acid–NaOH buffer systems were quantitatively compared with two other pH-controlling strategies. All together six experimental setups were tested against each other, as shown in Table 2. No significant difference was observed when final FPA activities were compared. Cellulase activites of about 1.2–1.4 FPU/mL were measured on the d 7 of cultivation in each condition. In conditions A and B, the cellulase activity increased gradually, reaching a maximum value of 1.4 FPU/mL on the last day of cultivation. However, when the initial pH of the cultivation was 6.0 without buffer addition (conditions C and D), the cellulase activity reached its maximum of 1.5 FPU/mL on d 4, while was followed by a steady decrease until the end of fermentation. As for the two sets of buffered conditions, a cellulase activity of 1.3 FPU/mL was achieved by the d 4. Moreover, the cellulase activity remained at this level for the rest of the cultivation. In terms of enzyme production rates (*see* Table 3), the highest value of 15.8 FPU/(L·h) was reached with non-buffered conditions C and D, whereas the value was slightly lower, 13.4 FPU/(L·h), for the buffered conditions E and F. It seems

Table 3
Cellulase Activities and Cellulase Formation Rates (r_c) in Shake-Flask Experiments by d 4 of Cultivation

Condition	FPA (FPU/mL)	r_c (FPU/[L·h])
A	1.034	10.8
B	1.054	11.0
C	1.520	15.8
D	1.520	15.8
E	1.282	13.4
F	1.282	13.4

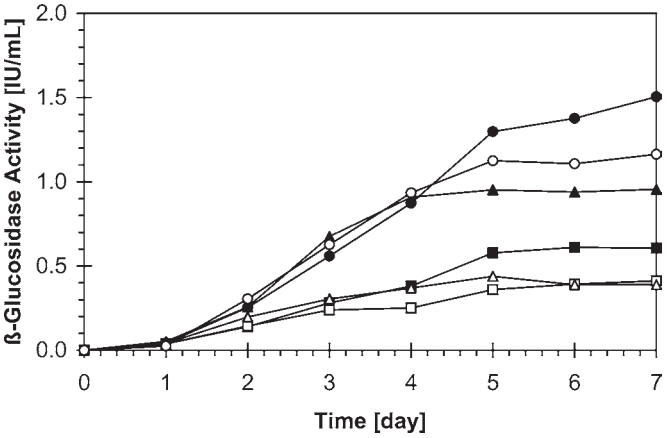

Fig. 1. β-Glucosidase activity vs time for shake-flask cultivations using different pH-controlling strategies. (—■—), Condition A; (—□ —), condition B; (—▲ —), condition C; (—△—), condition D; (—● —), condition E; (—○—), condition F.

that in non-buffered conditions it is only the starting pH that influences cellulase production with *T. reesei* RUT C30, since similar results were obtained with two different pH-controlling strategies (A, B or C, D).

Both the starting pH of cultivation and pH-controlling strategy followed during the fermentation process had a synergistic effect on the amount of produced β-glucosidase enzyme. To obtain some exact data for the pH representative for a certain pH-controlling strategy, time-averaged pH values were calculated from the daily measured pH values for each condition. These averaged pH values were the highest, pH 6.0, for the buffered conditions E and F. The best β-glucosidase activities, 1.5 and 1.2 IU/mL, were also obtained for condition E and F, respectively (Fig. 1).

Table 4
pH-Controlling Strategies Followed in Fermentor

Condition	pH-controlling strategy
I	pH continuously controlled to 5.0
II	pH allowed to drop down to 3.5 from initial 5.0, and further controlled to 5.0
III	pH continuously controlled to 6.0
IV	pH allowed to drop down to 3.5 from initial 6.0, and further controlled to 6.0

An approximate β-glucosidase activity of 1.0 IU/mL was reached when condition C was applied. In this case, the time-averaged pH was calculated to be 5.7. With daily pH adjustment to 5.0 (condition A), a β-glucosidase level as low as 0.6 IU/mL was reached. On the other hand, the time-averaged pH of cultivation, 4.7, was much lower than in previously mentioned cases. The lowest β-glucosidase activities of about 0.4 IU/mL were obtained with conditions B and D, in which cases the time-averaged pH values were also the lowest, 4.1 and 4.6, respectively. In most cases, except for conditions, B and D, in which the β-glucosidase activity was steadily increasing throughout the whole fermentation, maximum β-glucosidase activities were obtained on d 5 of cultivation.

Cellulase Production in Laboratory-Scale Fermentor

Four different pH-controlling strategies were subjected for further investigation in a laboratory-scale fermentor. Based on the results of the shake-flask trials, it seemed that keeping the pH at a constant value could be advantageous for both cellulase and β-glucosidase production. Although slightly faster cellulase production was observed in nonbuffered cultures with the pH-adjusted daily to 6.0, the 50% higher β-glucosidase activity obtained applying the Tris–maleic acid buffer system was appealing for constant pH regulation strategy. Table 4 summarizes the pH-controlling strategies followed in the fermentor. Hereafter these conditions are referred to by roman numerals.

Andreotti et al. *(17)* reported that the growth of *T. reesei* was more rapid at high pH ranges, while the optimum pH interval for enzyme production was between 3.0 and 4.0 *(1,17)*. Cultivation of *T. reesei* RUT C30 using control strategies I and II is summarized in Fig. 2. Throughout the fermentation process, the DO was kept at a constant value of 30% of saturation for the medium; therefore, the airflow rate could be used as a representative measure of microbial growth (Fig. 2B). Although the growth of *T. reesei* RUT C30 was faster when the pH was regulated to 5.0 (Fig. 2B), the formation of cellulases was slower than for condition II (Fig. 2A), as we had expected based on literature data. However, in the case of condition II,

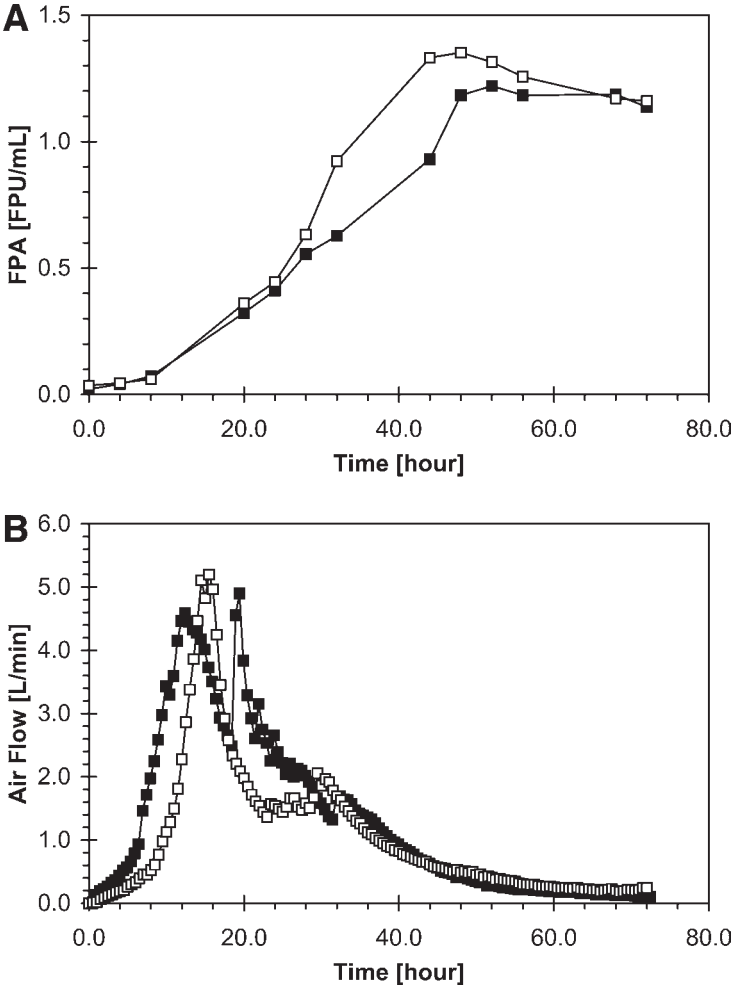

Fig. 2. Cultivation of *T. reesei* RUT C30 in laboratory fermentor using pH-controlling strategies I and II: **(A)** cellulase activity; **(B)** air flow rate. (—■ —), Condition I; (—□ —), condition II.

in which the pH was allowed to drop to 3.5, contrary to our expectation, the cellulase production rate was similar to that of condition I, until the pH was shifted back and regulated to 5.0 for the rest of the fermentation. For cellulase production, an immediate response to the pH shift was observed. About 40% higher cellulase activity was achieved by h 44 of the fermentation, when compared with that obtained with constant pH (condition I). Unfortunately, during the later phase of the fermentation, deactivation of cellulases was observed.

In the case of pH strategies III and IV, no remarkable differences in the growth and production rate of cellulases were seen up to h 52 of fermentation (data not shown), after which deactivation of cellulases was detected.

At a constant pH of 6.0 (condition III), this deactivation occurred to a greater extent than for that seen in the case of pH shift applied (condition IV).

The amounts of β-glucosidase were below the expected values and an activity of about 0.4 IU/mL was achieved in all cases. In each experiment, the amount of β-glucosidase increased steadily throughout the whole fermentation, reaching a maximum at h 60. No noteworthy differences in the production rates were seen.

Conclusions

Six pH-controlling strategies were compared in shake-flask experiments using *T. reesei* RUT C30. The initial pH of the cultivation did not influence the final cellulase activity remarkably, but the cellulase production rates and β-glucosidase activities were affected to a great extent. Regardless of the pH-controlling strategy applied, at pH 6.0, a maximum cellulase activity was achieved by d 4 of cultivation; however, during the final stage of the fermentation, deactivation of cellulases was observed. At pH 5.0, cellulase activity gradually increased throughout the whole fermentation process. A correlation between time-averaged pH values of the cultivations and the β-glucosidase activity was observed. Higher pH is favorable for β-glucosidase production of *T. reesei* RUT C30. Organic acid buffer systems were evaluated for their potential to keep the pH of the cultivation constant. Most of the buffer systems tested could not regulate the pH throughout the fermentation because they were possibly metabolized by the fungus. Tris–maleic acid and maleic acid–NaOH systems performed very well in shake-flask cultures, and a noteworthy improvement in β-glucosidase production was achieved. It was assumed that regulating the pH at a constant level would be favorable for β-glucosidase production. However, laboratory-scale fermentor experiments performed in non-buffered but pH-regulated medium did not confirm this theory. In fact, significantly lower β-glucosidase production was seen in the fermentor, which may suggest that one of the buffer components affected the cellulase production. However, based on these results, it would be a rather premature conclusion to claim any of the buffer components responsible for the higher cellulase and β-glucosidase activities obtained in the shake-flask experiments. A sudden pH shift after allowing acidification of the production medium during the initial phase of the cultivation increased the rate and titer of the cellulases at pH 5.0. However, at pH 6.0 no difference was observed.

Acknowledgments

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