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Thermoascus aurantiacus CBHI/Cel7A Production in *Trichoderma reesei* on Alternative Carbon Sources

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Abstract

To develop functional enzymes in cellulose hydrolysis at or above 70°C the cellobiohydrolase (CBHI/Cel7A) of *Thermoascus aurantiacus* was cloned and expressed in *Trichoderma reesei* Rut-C30 under the strong *cbh1* promoter. Cellulase production of the parental strain and the novel strain (RF6026) was examined in submerged fermentation experiments using various carbon sources, which were lactose, Solka Floc 200 cellulose powder, and steam pretreated corn stover. An industrially feasible production medium was used containing only distiller's spent grain, KH_2PO_4, and $(NH_4)_2SO_4$. Enzyme production was followed by measurements of protein concentration, total cellulase enzyme activity (filter paper activity), β-glucosidase activity, CBHI activity, and endogenase I (EGI) activity. The *Thermoascus* CBHI/Cel7A activity was taken as an indication of the heterologous gene expression under the *cbh1* promoter.

Index Entries: Cellulose; fermentor; lactose; shake flask; Solka Floc 200; steam pretreated corn stover.

Introduction

Currently, the world's fuel ethanol production is dominated by the United States, Canada, and Brazil. Their contribution to the global production is estimated to be around 90%, whereas the European fuel ethanol production accounts for 7% *(1)*. About 14 billion L of ethanol are produced in Brazil annually from sugar cane. Because of the controlled sugar prices in the United States, sugar cane is too expensive raw material for fuel ethanol production. Therefore, corn and other starch-crops are being used in commercial ethanol production in this country. In Europe, significant amount of ethanol is being produced from sugar beet and

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wheat-starch *(1)*. Utilization of lignocellulosic biomass for the production of fuel ethanol could be an interesting option all over the world as lignocellulosic byproducts are generated in huge amounts for example by forestry (wood chip, saw dust, and logging waste), agriculture (various straws), and pulp and paper industry *(2–5)*. In 2005, 280 million t of corn was harvested in the United States and 50 million t in the EU of which 9 million t in Hungary *(6)*. In whole corn plant, corn-stover weighs about 1.5-times as much as corn kernels, thus about 420 million t of corn-stover in the United States and 75 million t in the EU are generated annually. Although, fuel ethanol derived from lignocellulosic biomass can be produced cost competitively with corn-starch based ethanol, the competitiveness with gasoline still remains an issue, which calls for further improvement of such a technology.

Essentially, lignocellulose-to-ethanol processes include three main technological steps. First the polysaccharide content of the feedstock is hydrolyzed to fermentable sugars, which are then converted to ethanol by yeast. In the third step ethanol is refined from the fermentation broth. There are basically three technological concepts available for the hydrolysis of lignocellulosics. These are the one-step concentrated acid hydrolysis, the two-step dilute acid hydrolysis, and the enzymatic hydrolysis, of which the latest seems to have advantages over the former two concepts. The fermentability of hydrolysates obtained in the enzymatic process is significantly better owing to milder processing conditions, resulting in better ethanol yields. On the other hand, for efficient cellulose hydrolysis, high enzyme loading is required making the process economically less favorable because of the high market price of cellulase. Furthermore, the technological margin for economic ethanol distillation has been reported to be 5% ethanol in the fermentation broth, which requires high-density cellulose hydrolysis.

One approach to make the enzymatic process more attractive is to use thermostable cellulases active at high temperatures, thus making the cellulose hydrolysis go faster. Furthermore, the processibility of high-density cellulose slurry could be possible at elevated temperatures. To reach this goal, genetic engineering of different strains has already been carried out *(7,8)*. *Thermoascus aurantiacus* was found to produce thermostable endoglucanase and β-glucosidase in both submerged and solid state cultivation *(9)*.

For economical, large-scale bioethanol production from lignocellulosic material, integrated production plants having the enzyme production on site may be the option. It would be advantageous if the same material could be used for the enzyme production. In the present study, the heterologous production of *T. aurantiacus* cellobiohydrolase (CBHI/Cel7A) in *Trichoderma reesei* RF6026 and the general cellulase enzyme production of the Rut-C30 were investigated and compared on different carbon sources, i.e., on water-soluble (lactose) and water-insoluble carbon sources (cellulose and steam pretreated corn-stover).

Materials and Methods

Carbon Sources for Cellulase Production

Solka Floc 200 (SF200) cellulose powder (International Fiber, New York, NY), lactose-L-hydrate (SPECTRUM-3D, Hungary) and steam pretreated Italian corn-stover (SPCSI, obtained from ENEA, Italy) were used as carbon sources for cellulase production. Different batches of corn-stover were steam pretreated at 190°C and 210°C for 5 min in continuous reactor, and analyzed for carbohydrate content using the Hägglund method *(10)*. The composition of SPCSI batches obtained after pretreatment at different temperatures were different. The batch pretreated at 190°C contained 36.7% glucan, 15.8% xylan, and 1.0% arabinan on dry matter (DM) basis. The DM content after the treatment was 38.7%. The other batch pretreated at 210°C, contained 32.5% DM glucan, and 2.9% DM xylan. The DM content after the treatment was 59.5%.

Microorganisms

Two different *T. reesei* strains were used in cellulase production experiments, *T. reesei* RF6026 carrying the *T. aurantiacus cbh1/cel7A* under the strong *T. reesei cbh1/cel7A* promoter and the parental strain Rut-C30. In the RF6026 strain the native *T. reesei cbh1/cel7A* gene has been deleted. Freeze-dried conidia of *T. reesei* Rut-C30 (ATCC56765) were obtained from the American Type Culture Collection. Culture of *T. reesei* RF6026 was prepared and kindly supplied from ROAL Oy, Finland.

Inoculum Preparation

The stock culture of the fungus *T. reesei* Rut-C30 was maintained on agar slants containing 50.0 g/L of malt extract, 5.0 g/L of glucose, 1.0 g/L proteose peptone, and 20.0 g/L of bacto agar. *T. reesei* RF6026 was maintained on Potato-dextrose agar slants containing 15.0 g/L starch, 20.0 g/L glucose, and 18.0 g/L bacto agar. After 14 d of incubation at 30°C, the greenish conidia were suspended in 5 mL of sterile water and 1.5 mL of this suspension was transferred to 750-mL Erlenmeyer flask containing 150 mL of sterile and pH adjusted (5.6–5.8) modified Mandels' medium *(11)* in which the concentration of nutrients was added as follows: 0.4 g/L urea, 1.87 g/L of $(NH_4)_2SO_4$, 2.67 g/L of KH_2PO_4, 0.53 g/L of $CaCl_2{\cdot}2H_2O$, 0.81 g/L of $MgSO_4{\cdot}7H_2O$, 0.33 g/L of yeast extract, 1.0 g/L of proteose peptone, and 10.0 g/L of lactose. Furthermore, the medium was supplemented with the following trace elements: 6.6 mg/L of $FeSO_4{\cdot}7H_2O$, 2.1 mg/L of $MnSO_4{\cdot}H_2O$, 1.9 mg/L of $ZnSO_4{\cdot}7H_2O$, and 26.7 mg/L of $CoCl_2$. The shake flasks were incubated at 30°C for 3 d on an orbital shaker (350 rpm).

Enzyme Production in Shake-Flasks

An aliquot of 15 mL 3 d old mycelium suspension obtained from inoculum cultures was used to initiate growth in a 750-mL Erlenmeyer flask containing 150 mL of the Technical Research Centre of Finland (VTT) medium *(8)* in which the concentration of nutrients were 10.0 g/L carbon source (lactose, SF200, and SPCSI), 5.0 g/L distiller's spent grain, 0.83 g/L KH_2PO_4, and 0.83 g/L $(NH_4)_2SO_4$. After inoculation, the flasks were incubated for 7 d at 28°C and 200 rpm. Samples were withdrawn daily at the same time and when necessary, the pH in the flasks was adjusted to 5.6 using sterile 10 wt% solutions of NaOH or H_2SO_4. Aseptically taken samples were centrifuged at 9000 rpm for 5 min. The collected supernatants were assayed for enzyme activities such as filter paper, β-glucosidase, CBH I, and enduglucanase I, respectively.

Enzyme Production in Laboratory-Scale Fermentor

Cellulase production was also performed in a 31-L (20 L working volume) double-walled stainless steel laboratory fermentor (Biostat CDCU-3, B Braun Biotech, Germany). The production medium was made up of 60.0 g/L carbon source (lactose, SF200, and SPCSI), 30.0 g/L distiller's spent grain, 5.0 g/L KH_2PO_4, and 5.0 g/L $(NH_4)_2SO_4$ *(8)*. The medium components required for 20 L production medium were dissolved in 18 L tap water and sterilized at 121°C for 20 min. After sterilization, 2 L of inoculum culture was aseptically added to initiate growth and enzyme production. During 92 h of fermentation, the temperature was maintained at 28°C and the pH was automatically kept at 5.5 by addition of 10 wt% solutions of either phosphoric acid or ammonium hydroxide. The dissolved oxygen level in the fermentor was controlled to 30% of saturation by cascade controlling first the airflow rate (between 1 and 12 L/min) and agitation speed (300–650 rpm). To avoid foam formation, silicon oil-based Sigma Aldrich Antifoam A (Munich, Germany) in 30% ionic emulsion was added manually. Samples were withdrawn three times a day and centrifuged at 9000 rpm for 5 min. The collected supernatants were assayed for enzyme activities (filter paper activity [FPA], β-glucosidase, CBHI, and EGI).

Analysis

Reducing sugar (RS) concentration, enzyme activities, and soluble protein content were assayed in sample collected during fermentation. All samples were analyzed in triplicates and the main value was calculated. Relative standard deviation of all measurements was less than 5%. The RS content was measured using 3,5-dinitrosalicylic-acid reagent *(12)*. For fermentation runs carried out using lactose carbon source, the calibration of 3,5-dinitrosalicylic-acid reagent was made using lactose standard, whereas glucose calibration was used for the other two carbon sources. The overall

cellulase activity was measured by FPA *(13)*. In FPA measurements the background RS content was taken into account by subtracting the absorbance of the blank from the absorbance of the activity assay. β-Glucosidase activity was determined against 4-nithrophenyl-β-(D)-glucopyranoside substrate *(14)*. CBHI and EGI activities were assayed using 4-methylumbelliferyl-β-D-lactoside substrate *(15)* in the presence of 100 m*M* glucose to inhibit β-glucosidase. As the reaction is not strictly for CBHI (EGI can also hydrolyze the substrate), for EGI activity measurement 5 m*M* cellobiose is added to inhibit CBHI/Cel7A in the mixture. CBHI activity can be calculated by substracting EGI from the total enzyme activity. Enzyme activities were expressed in international units (IU) except for FPA, wherein filter paper unit (FPU) was used. Enzyme activity measurements from samples collected during the fermentation were carried out at 50°C, if otherwise not indicated. Incubation times and pHs were as following: 60 min and 4.5 for FPA, 10 min and 4.5 for β-glucosidase, and 10 min pH 5.0 for CBHI and EGI activities. Protein content of the samples was also assayed *(16)*.

Results and Discussion

Cellulase Production of T. reesei *Rut-C30 in Shake-Flask*

In shake flask experiments, cellulase production of *T. reesei* Rut-C30 was examined using the VTT medium in which the carbon source concentration was set to 10.0 g/L. The reason for reducing carbon source concentration from the original 60.0 g/L was owing to the fact that SPCSI at this DM content could not be mixed efficiently in shake-flask cultures. The enzyme production profile obtained on SPCSI carbon source, a potential substrate for ethanol making, was compared with the enzyme profiles obtained on lactose, an industrial standard carbon source, and on purified cellulose powder (SF200), which is regarded as the most effective cellulase inducing carbon source.

The overall cellulase activities were in the range of 1.3–1.9 FPU/mL after 7 d of cultivation. There was no significant difference between the level of cellulase activities on media containing either lactose or SF200, and about 1.3 FPU/mL cellulase titers were reached in both cases. However, when SPCSI carbon source was added to the production medium the overall cellulase activity was 1.9 FPU/mL, which was 45% higher than those obtained for the other two carbon sources. On lactose-containing medium about 1.2 IU/mL β-glucosidase titer was reached in the fermentation; however, the level of this enzyme was considerably lower, around 0.9 IU/mL on solid carbon sources, i.e., SF200 and SPCSI. Levels of CBHI and EGI in the fermentation broth did not show significant variation with different carbon sources. For all three carbon sources the CBHI and EGI activities were around 0.7 and 0.2 IU/mL, respectively (Table 1).

Table 1
Summary of *T. reesei* Rut-C30 Cellulase Production Using Different Carbon Sources

	Carbon source		
Enzyme activity	Lactose	SF200	SPCSI
FPA (FPU/mL)	1.35	1.25	1.9
β-Glucosidase (IU/mL)	1.24	0.86	0.86
CBHI (IU/mL)	0.66	0.72	0.75
EGI (IU/mL)	0.22	0.28	0.22

Enzyme activities obtained in shake flask experiments after 7 d of cultivation.

It has been proven in shake-flask experiments, that SPCSI, a potential substrate for fuel ethanol production, can be efficiently used for cellulase production as well. In spite of the lower carbohydrate content of SPCSI, the overall cellulase activity was increased by 40% compared with lactose. On the other hand the enzyme mixture produced on SPCSI was less balanced in term of the FPA to β-glucosidase ratio. Whereas on lactose carbon source, this proportion was almost 1/1, which is close to the optimal ratio for cellulose hydrolysis, the enzyme cocktail produced on SPCSI was deficient in β-glucosidase enzyme. It is noteworthy to mention that, although significantly higher FPA was measured in case of SPCSI carbon source, individual cellulase (CBHI and EGI) activity assays did not reflect this observation. Unfortunately, with the present set of data and methodology used in this research plausible explanation cannot be provided.

Cellulase Production of T. reesei *Rut-C30 in Fermentor*

Scaled-up cellulase production of *T. reesei* Rut-C30 using the VTT medium supplemented with the previously mentioned carbon sources at 60.0 g/L was performed in a 31 L total volume lab-scale fermentor. Furthermore, with the cultivation of *T. reesei* RF6026 on the same carbon sources the heterologous expression of *T. aurantiacus cbh1/cel7A* was also investigated. Enzyme activities and RS concentration in samples withdrawn during the course of cultivation are summarized in Fig. 1.

Cultivation of *T. reesei* Rut-C30 on medium containing 60.0 g/L lactose resulted in an overall cellulase activity of 3.4 FPU/mL. Significant cellulase production was observed after 20 h of fermentation (Fig. 1A). The activity of β-glucosidase, CBHI, and EGI were quite low at this time, and detectable amounts were only observed in the fermentation broth after 30 h. At end of the fermentation the β-glucosidase, CBHI, and EGI activities were 1.0, 1.5, and 0.4 IU/mL, respectively. About 35% higher, 4.6 FPU/mL FPA was reached at the end of *T. reesei* Rut-C30 cultivation when the cellulase production was induced by SF200 cellulose powder (Fig. 1B).

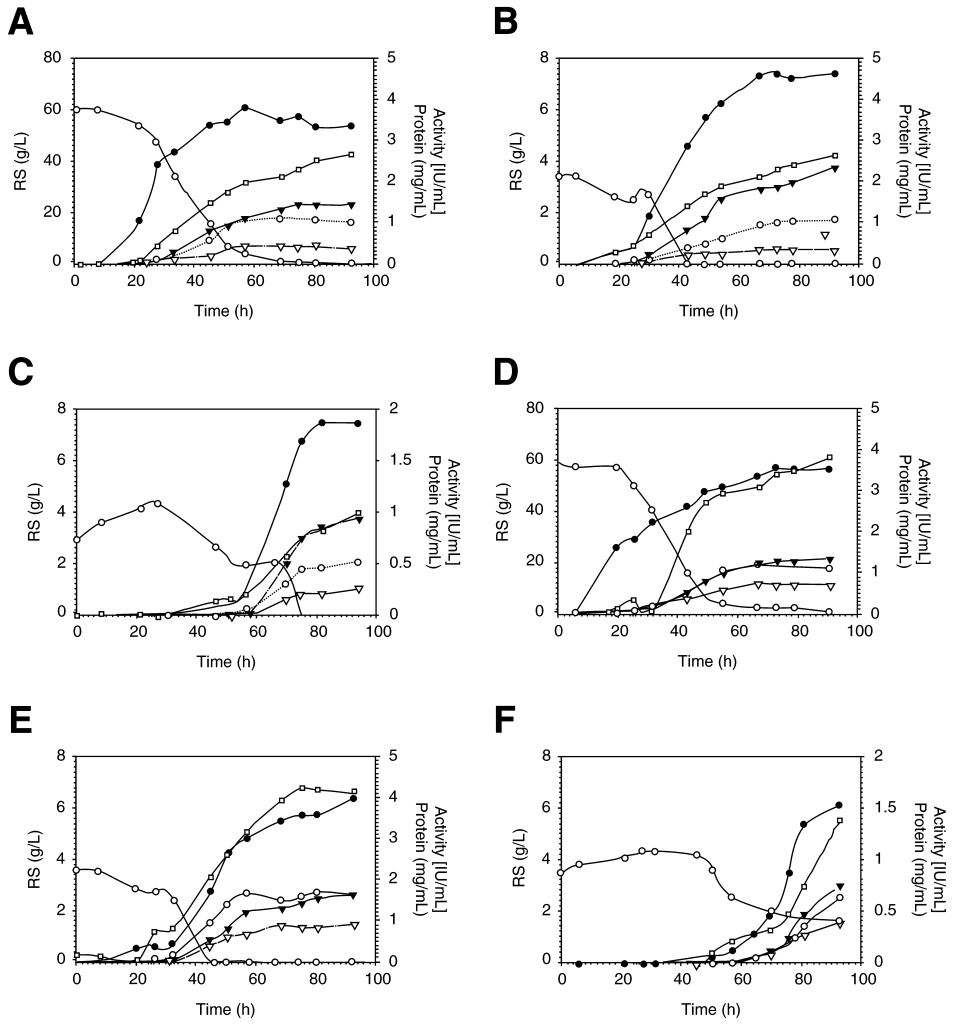

Fig. 1. Enzyme production of *T. reesei* strains in lab-scale fermentor using different carbon sources. **(A)** Lactose Rut-C30, **(B)** SF 200 Rut-C30, **(C)** SPCSI Rut-C30, **(D)** Lactose RF6026, **(E)** SF 200 RF6026, and **(F)** SPCSI RF6026; (●) RS, (●) FPA, (○) (β-glucosidase, (▼) CBHI, (▽) EGI, and (□) protein content.

However, the level of β-glucosidase enzyme stayed as low as 1.1 IU/mL. As expected, the expression of CBHI enzyme was clearly higher on SF200 than on lactose carbon source, resulting in a CBHI activity of 2.3 IU/mL at the end of the experiment. On the other hand, the expression of the other cellulase component, EGI, was not influenced by the carbon source at all, and the same level of activity was reached on the strong cellulase inducer as with lactose (Fig. 1 panel A,B). When SPCSI carbon source was used to produce cellulases in the concentration of 60.0 g/L, there was no enzyme

production observed at all. The RS content in the medium (data not shown) increased continuously indicating that cellulase added with the inoculum started to hydrolyze the cellulose fraction of the carbon source, but glucose liberated was not taken up by the fungus. Same phenomenon has been observed elsewhere *(17)*, where the lack of microbial growth and enzyme production was explained by the presence and inhibitory effects of compounds, such as furfural and acetic acid, generated during steam pretreatment of lignocellulosics. The experiment with this carbon source was therefore repeated; however, with reduced amount of SPCSI at 30.0 g/L. As shown in Fig. 1C, even at this lowered SPCSI concentration, a long lag phase could be seen during which the RS concentration was first increasing and then slowly declining as microbial activity picked up. Cellulase production started rather late, at the fortieth hour of the cultivation, compared with the enzyme production carried out with the other two carbon sources. Final enzyme titers were also significantly lower in this case, 1.9 FPU/mL, 0.5 IU/mL, 1.0 IU/mL, and 0.3 IU/mL for FPA, β-glucosidase, CBHI, and EGI activities, respectively. Though it has to be kept in mind that these results were obtained at considerably lower carbon source concentration.

CBHI/Cel7A Production of T. reesei *RF6026 in Fermentor*

Heterologous production of *T. aurantiacus* CBHI in *T. reesei* RF6026 strain was examined by measuring the CBHI activity during the growth of the fungus on lactose, SF200, and SPCSI. Lactose and SF200 were applied in 60.0 g/L concentrations, whereas the concentration of SPCSI in the production medium was 30.0 g/L. Besides FPA, CBHI, EGI, and β-glucosidase activities were also measured. However, when judging the FPA results it should be kept in mind that the CBHI enzyme of *T. aurantiacus* does not have a cellulose binding domain. The enzyme activity and RS curves were very similar to what has been seen during the cultivation of the parental strain on the same carbon sources, except the level of CBHI activities, which were always lower to the same degree as activity measurement was carried out at suboptimal condition of the thermoactive *T. auranticus* CBHI enzyme, for example, 50°C. The enzyme production on lignocellulosics, for example, SF200 and SPCSI, did not seem to be influenced by the facts that:

1. The produced *T. auranticus* CBHI lacks the cellulose binding domain, which would facilitate better hydrolysis of the carbon source thereby supporting growth of the fungus in greater extent.
2. The cultivation of the mesophilic *T. reesei* was performed at 28°C, a definitely suboptimal temperature for the thermo-active CBHI enzyme.

In order to obtain an accurate number on the amount of thermoactive CBHI activity measurement of samples taken at the end of every fermentation have been done at 70°C as well as shown in Fig. 2, where

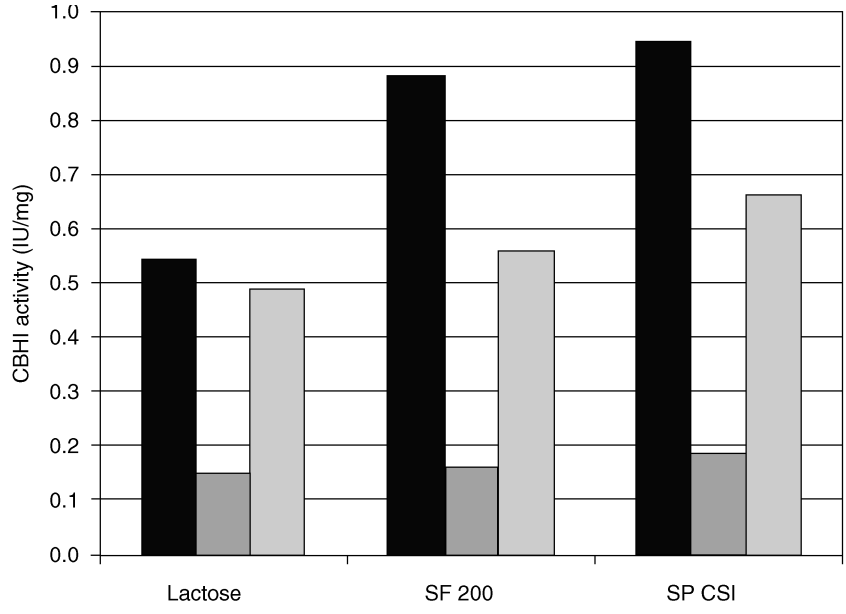

Fig. 2. CBHI activities of *T. reesei* Rut-C30 measured at 50°C (■), Rut-C30 measured at 70°C (■) and RF6026 measured at 70°C (■), produced on different carbon sources.

CBHI specific activities of both endogeneous and cloned CBHI activities measured at optimal temperatures are compared for different carbon sources (CBHI activity of Rut-C30 on elevated temperature is also included for comparison). As expected the protein production pattern under *T. reesei* cbh1 promoter was similar for both strains on cellulosic carbon sources. However, the parental strain loses its CBHI activity at 70°C, whereas CBHI of RF6026 strain still performs well, providing the possibility to work at elevated temperatures.

Conclusions

The main objective of the present study was primarily to examine the heterologous protein production in a model strain, *T. reesei* RF6026, under the *T. reesei cbh1* promoter. It has been established by CBHI activity measurements at optimal temperatures for both CBHI enzymes that the implementation of the thermo-active enzyme was successful and the target protein was produced. The second objective of this work was to examine the feasibility of utilizing the same material, for example, steam pretreated corn-stover for cellulase enzyme production that could be used as a substrate for fuel ethanol production. It was concluded that the application of SPCSI for enzyme production is limited probably because of the presence of microbial growth inhibiting compound that are liberated during pretreatment of corn-stover.

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