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Characterization of recombinant *E. coli* ATCC 11303 (pLOI 297) in the conversion of cellulose and xylose to ethanol

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Abstract This work describes the characterization of recombinant *Esherichia coli* ATCC 11303 (pLOI 297) in the production of ethanol from cellulose and xylose. We have examined the fermentation of glucose and xylose, both individually and in mixtures, and the selectivity of ethanol production under various conditions of operation. Xylose metabolism was strongly inhibited by the presence of glucose. Ethanol was a strong inhibitor of both glucose and xylose fermentations; the maximum ethanol levels achieved at 37° C and 42° C were about 50 g/l and 25 g/l respectively. Simultaneous saccharification and fermentation of cellulose with recombinant *E. coli* and exogenous cellulose showed a high ethanol yield (84% of theoretical) in the hydrolysis regime of pH 5.0 and 37° C. The selectivity of organic acid formation relative to that of ethanol increased at extreme levels of initial glucose concentration; production of succinic and acetic acids increased at low levels of glucose (<1 g/l), and lactic acid production increased when initial glucose was higher than 100 g/l.

Introduction

Ethanol promises to be a renewable and environmentally clean alternative to petroleum as a fuel (Lynd et al. 1991). Lignocellulosic biomass, available in abundance as waste products or crops, could be used in the future for ethanol production. The economical production of ethanol from lignocellulosic materials depends on efficient conversion of both cellulose and hemicellulose components to ethanol. A wide range of yeast and bacterial species are capable of producing ethanol from fermentation of glucose (Gottschalk 1986). Only a few naturally occurring microorganisms, however, can metabolize pentose sugars to ethanol (Jeffries 1990; McMillan 1993). The development of genetically engineered bacteria to achieve high selectivity of ethanol production from both hexose and pentose sugars (Ingram et al. 1991) offers an opportunity to convert both hexose and pentose fractions of biomass efficiently to ethanol. Recent reports (Hahn-Hägerdal 1993; McMillan 1993) provide comprehensive reviews on the fermentation of pentose sugars to ethanol.

Fermentation of glucose and xylose by *Escherichia coli* ATCC 11303 (pLOI297) has been studied previously by Ingram and coworkers (Ohta et al. 1990; Beall et al. 1991; Lawford and Rousseau 1991). More recent studies have used strains of recombinant *E. coli* and *Klebsiella oxytoca* with the foreign genes integrated into the host chromosome (Ohta et al. 1991a, b). Ethanol yields greater than 95% of theoretical values have been reported from the pure sugars. A number of studies have also focused on fermentation of sugar mixtures that are more representative of pretreated biomass substrates. Mixtures of glucose, arabinose and xylose were studied by Bothast et al. (1994) with recombinant *K. oxytoca* and by Takahashi et al. (1994) with *E. coli* 11303 (pLOI297). Actual substrate studies based on pine hydrolyzates (Barbosa et al. 1992) and corn hull hydrolyzates (Beall et al. 1992) demonstrate preferential utilization of glucose and incomplete xylose conversion in sugar mixtures, a phenomenon that needs to be addressed in process design. Lawford and Rousseau (1993, 1994), on the other hand, have reported no adverse interactions between glucose and xylose either in synthetic media or in spent sulfite liquor.

The simultaneous saccharification and fermentation (SSF) process has been shown to be an efficient method for ethanol production from cellulose (Emert and Katzen 1980; Wright 1988). Doran and Ingram (1993) have shown that operation of SSF at a low pH is important to achieve high product yields with *K. oxytoca* P2. Grohmann (1993) provides a review of microorganisms

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and operating conditions of importance to efficient SSF design.

Our studies of pure sugar fermentations were directed toward further characterizing the behaviour of *E. coli* 11303 (pLOI297) as a test recombinant microorganism in the conversion of glucose, xylose, and cellulose to ethanol.

Materials and methods

Growth media and techniques

E. coli ATCC 11303 (pLOI 297) was obtained from Prof. L.O. Ingram at the University of Florida (Alterthum and Ingram 1989). The growth medium used was Luria broth (LB), which consisted of 20 g/l tryptone, 10 g/l yeast extract, 10 g/l NaCl, and 0.2 M phosphate buffer (K_2HPO_4 + KH_2PO_4), supplemented with the appropriate carbon source. Tetracycline was added at 15 mg/l to ensure plasmid retention by the recombinant microorganism.

Fermentation studies

Fermentations were started with an inoculum of about 0.33 g/l (initial absorbance 1.0 at 550 nm) similar to the procedure adopted by Beall et al. (1991). The inoculum for fermentation was grown overnight in LB medium and a 40-g/l concentration of the carbon source being studied (glucose was used in the inoculum preparation for SSF of cellulose). D-Xylose and D-glucose were obtained from Sigma Chemicals. Experiments were carried out in duplicate 250-ml shake flasks with a working volume of 200 ml. Microaerophilic conditions were maintained by connecting a water trap to the outlet gas port of the flask. The flasks were sampled about every 4 h during xylose and glucose fermentations by a quick removal of the stopper. The standard conditions for fermentation were pH 6.5, 37° C, and 150 rpm, unless stated otherwise (chosen from previous studies of Beall et al. 1991). Separate experiments were conducted with LB medium in the absence of a carbon source to determine the amount of ethanol produced from its complex ingredients. Ethanol yield values reported in the results have been corrected for the contribution by the LB medium (approx. 1.6 g/l ethanol) and carryover of ethanol from the inoculum. Fermentor experiments were carried out in a New Brunswick Bioflo III reactor with a working volume of 2 l. The buffer was absent in the reactor fermentations where pH was controlled with 5 M NaOH.

In the SSF experiments, Sigmacell 50 (Sigma Chemicals) and Laminex BG (Genencor International) were used as cellulose and cellulase respectively. The Laminex enzyme showed a cellulase activity of 64 FPU/ml and a β-glucosidase activity of 80 IU/ ml. The standard conditions, unless stated otherwise, for SSF of cellulose was 40 g/l cellulose, 37° C, pH as stated, and an enzyme loading of 25 FPU Laminex/g initial cellulose. The phosphate buffer was absent in the growth medium, and the pH was controlled by periodic manual addition of NaOH (for shake flasks) or by automatic addition of NaOH (for fermentor runs). Tetracycline was added to a final concentration of 15 mg/l every 3 days in these prolonged runs.

Analytical methods

Cellulase assays were conducted as described by Ghose (1987). The residual sugars and products from fermentation were measured by HPLC (Hewlett Packard Series II 1090) equipped with an organic acids column (BioRad HPX-87H). Analyses of mixed sugars were performed similarly with a carbohydrate column (BioRad HPX-87P).

Results

Sugar fermentation by recombinant *E. coli*

Individual fermentation of glucose and xylose

The ethanol yield from 40 g/l initial xylose was observed to be about 90% of the theoretical value at 37° C and pH 6.5 with an average volumetric productivity of about 0.82 g/l-h. The productivity was calculated as the net final ethanol concentration divided by the time required for completion of fermentation. Similar ethanol yields were obtained from glucose at standard conditions although the volumetric productivity was about 30% higher. Organic acids, predominantly succinic, were produced as byproducts of the fermentation to a total final level of about 4 g/l (from 40 g/l initial sugar).

Ethanol inhibition of metabolism

Ethanol is well known as an inhibitor of bacterial growth (Ingram and Dombek 1989). We conducted standard fermentations of 40 g/l xylose in the presence of exogenous ethanol varying from 0 to 50 g/l. As shown in Fig. 1A, the final cell mass concentration (measured as absorbance at 550 nm) declined progressively with increasing initial ethanol level. However, this effect represents cumulative effect of the initial ethanol and the product of fermentation. If the initial linear rates of cell mass production are examined, Fig. 1A indicates a significant 60% decline at 25 g/l of initial ethanol compared to the control containing no initial ethanol. The effect of ethanol on net product yield was not as prominent as that on cell mass production (Fig. 1B). At 25 g/l initial ethanol, the yield was about 88% of the theoretical value with a twofold lower productivity (0.4 g/l-h) compared to the control. At 50 g/l initial ethanol, both cell growth and product formation were barely perceptible suggesting that this concentration is close to the tolerance level of this bacterium at 37° C.

To find the maximum achievable ethanol level by the recombinant *E. coli*, fermentations were carried out at high initial glucose levels. Substrate inhibition of cell growth was observed at 150 g/l (not shown); this observation corresponds well with previous results of Beall et al. (1991). At 37° C, the fermentation of 125 g/l glucose showed complete substrate conversion and a final ethanol concentration of 52 g/l, which is estimated to be the ethanol tolerance limit at 37° C. At 42° C, the fermentation of 100 g/l glucose terminated at a final ethanol concentration of 25 g/l, resulting in a low product yield of 49% of the theoretical value. The twofold reduction in the ethanol tolerance limit of the bacterium by only a 5° C temperature increase is indicative of the strong influence of temperature on ethanol inhibition.

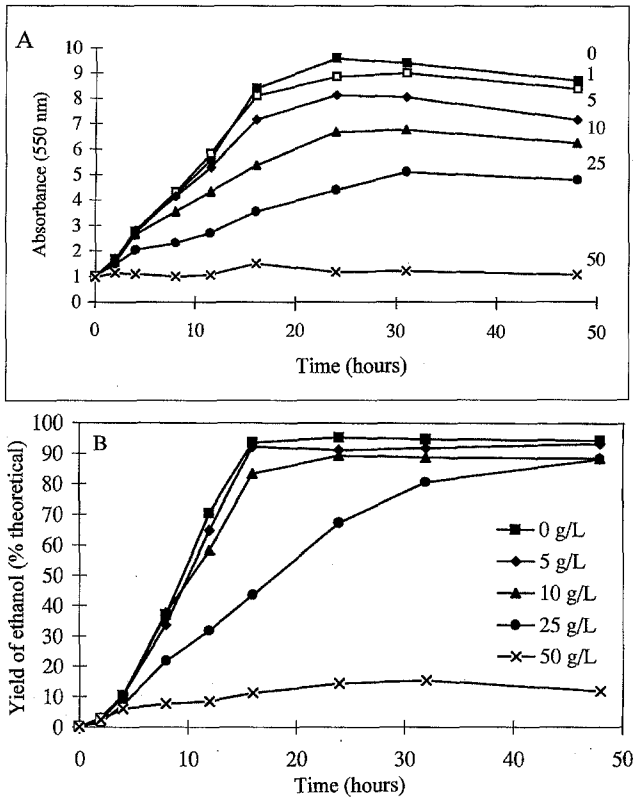

Fig. 1A, B Effect of ethanol on cell growth and product yield. Fermentation was carried out with 40 g/l xylose at 37° C and pH 6.0 **A** Cell growth. Numbers on the *right* of the plot show concentration of ethanol added exogenously at the start of the fermentation. **B** Product yield. The different symbols show the initial ethanol concentration

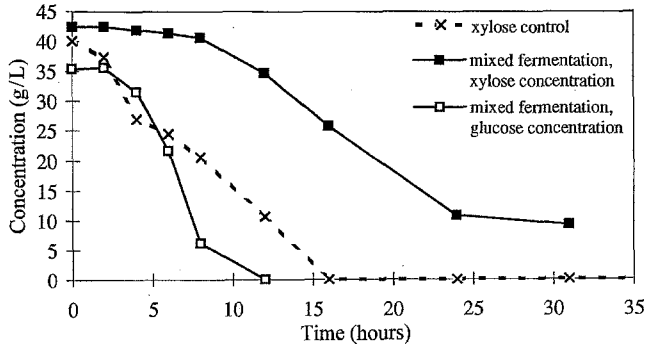

Fig. 2 Fermentation of glucose and xylose mixture, and a control fermentation containing only 40 g/l xylose, at 37° C and pH 6.0. Glucose (□) and xylose (■) concentrations in a mixed sugar fermentation with a roughly 1:1 ratio of the two sugars; xylose (×) concentration in control

Mixed sugar fermentation: effect of glucose on xylose metabolism

We studied fermentation of 40 g/l xylose in the presence of glucose varying from 0 to 40 g/l. The ethanol yield, based on total initial sugars, remained unaffected at 90% of theoretical up to an initial glucose concentration of 20 g/l. At 40 g/l initial glucose, however, the yield was limited to about 78% of the theoretical value, possibly because of inhibition by the high levels of product ethanol. Figure 2 shows a representative plot of substrate concentrations in the mixed-sugar fermentation with a 1:1 initial ratio of glucose and xylose (40 g/l each). The uptake of xylose was very slow until glucose was completely metabolized; a comparison with the control (xylose with no glucose) clearly suggests a strong inhibition of xylose metabolism in the presence of comparable levels of glucose.

SSF of cellulose to ethanol

The SSF of cellulose to ethanol combines the action of two biocatalysts – the cellulase enzyme and the microorganism. The cellulase, Laminex, is reported to show peak cellulase activity in the pH range of 4.8–5.0 and at a temperature of about 50° C (Genencor International, 1993). Because these operating conditions are believed to be inhibitory to growth of *E. coli*, we conducted SSF experiments separately, first in the near-neutral pH range, and then under lower pH conditions favoring cellulose hydrolysis.

The SSF experiments conducted at pH 6.0 and 37° C produced an ethanol yield of 42% of the theoretical value after 7 days (168 h) (Fig. 3). The concentration of glucose was below detectable levels throughout the SSF, indicating a rapid fermentation of the sugar and a limitation of rate by the enzyme under these conditions. At the hydrolysis-favoring conditions of pH 5.0 and 37° C, the ethanol yield obtained after 6 days (144 h) was about 84% of the theoretical value (Fig. 3). The succinic acid production was 2.0 g/l, and acetic acid and lactic acids were below detectable levels. On the basis of total products comprising acids and ethanol (cell mass increase was assumed to be small under these low-glucose conditions), the cellulose conversion was estimated at 92%.

Dependence of product selectivity on operating conditions

High-glucose fermentations

The fermentations at high initial glucose concentrations showed an increased selectivity of lactic acid production. At 125 g/l glucose, about 8 g/l lactic acid was produced compared to 0.9 g/l in the reference case of 40 g/l initial glucose. Although substrate conversion was complete, the ethanol yield was only 80% of the theoretical value. Other acids such as succinic and acetic acids were close to their expected levels of production. A similar result was obtained with the 100 g/l

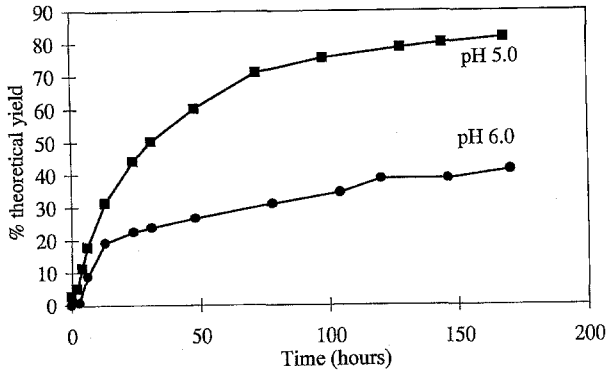

Fig. 3 Effect of pH on simultaneous saccharification and fermentation (SSF) of cellulose with *E. coli* and Laminex enzyme. The SSF was performed in a bench-scale reactor as described in Materials and methods with 40 g/l cellulose, 25 FPU/g cellulose, 37° C. ■ pH 5.0, ● pH 6.0

Table 1 Dependence of product selectivity on operating conditions

Regime	Operating conditions	Product ratios relative to ethanol (g/g ETOH)		
		Succinic	Acetic	Lactic
I	40 g/l glucose, pH 6.0, 37°C	0.100	0.035	0.03
II	125 g/l glucose, pH 6.0, 37°C	0.100	0.019	0.15
III	40 g/l cellulose, pH 6.0, 37°C 25 FPU Laminex/g cellulose	0.920	0.305	0.00
IV	40 g/l cellulose, pH 5.0, 37°C 25 FPU Laminex/g cellulose	0.100	0.000	0.00

glucose run at 37° C. The mass ratio of final ethanol to lactic acid (g/g) in these high-glucose runs was observed to be 8.3, about fivefold lower than that in the reference case (see Table 1). The fermentations at 42° C showed a similar trend in increased lactic acid production.

SSF of cellulose

The SSF experiments at pH 6.0 and 37° C showed an increased selectivity of succinic and acetic acid production (Table 1). The ratio of final ethanol to succinic acid (g/g) was 1.1 compared to the reference value of 10.0. Similarly, the acetic acid production yielded an ethanol-to-acid ratio of 3.3 compared to the reference of 40.0. Lactic acid production, in this case, was undetectable. In a separate experiment, the ethanol yield at an enzyme level of 50 FPU/g cellulose was about 60% of the theoretical value, an enhancement from the 42% achieved at the standard level of 25 FPU/g. The production of succinic and acetic acids decreased correspondingly. In a second experiment, an SSF run at pH 6.0, 37° C, and 25 FPU/g loading was supplemented with exogenous glucose, after 1 day and 5 days, each time to a final glucose concentration of 20 g/l.

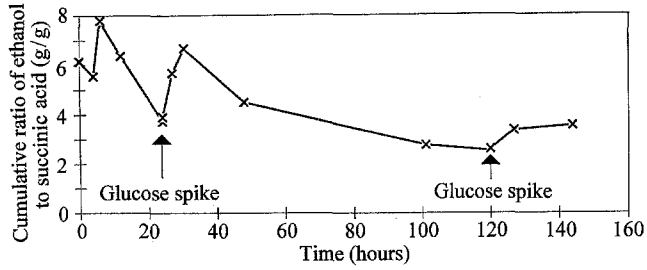

Fig. 4 Effect of glucose addition during SSF on succinic acid production. SSF was started with 40 g/l cellulose, 25 FPU/g cellulose, 37° C and pH 6.0. Glucose was added to a final concentration of 20 g/l at times indicated by the *arrows*

Figure 4 shows that the production of ethanol relative to that of succinic acid increased immediately after the spikes when free glucose was present. When the free glucose was exhausted, the relative acid production increased. A similar trend was observed with acetic acid production (not shown).

Discussion

Our studies have focussed on three important aspects of ethanologenic fermentations by *E. coli* 11303 (pLOI 297): ethanol inhibition, glucose inhibition of xylose fermentation, and dependence of product selectivity on operating conditions.

The inhibition by ethanol is not surprising; however, the quantitative characterization of the effect of ethanol on cell production and product yield in the concentration ranges of 2%–5% (w/v) is useful for the design of continuous processes where the microorganisms are constantly exposed to the effluent product level. The inhibition also constrains the initial substrate loading to be below 8%–10% (w/v) carbohydrate (with a theoretical yield of 0.511 g ethanol/g sugar) to achieve efficient conversion. Fermentations can be carried out at lower temperatures such as 30–35° C to alleviate ethanol inhibition; Doran and Ingram (1993) show high ethanol yields at 32° C and pH 5.2–5.5 from 100 g/l initial cellulose. The development of a process for simultaneous product recovery with fermentation could yield considerably higher productivities if the ethanol concentration in the fermentor could be maintained below inhibitory levels.

Our observations of glucose inhibition of xylose agree well with those reported earlier by Takahashi et al. (1994) for the same *E. coli* strain. Lawford and Rousseau (1993, 1994), however, report a simultaneous conversion of glucose and xylose in their mixtures. It should be noted, however, that the sugar concentrations used in the studies of Lawford and Rousseau were considerably lower than those of Takahashi et al. (1994) and the current study. Bothast et al. (1994) and

Barbosa et al. (1992) have reported high yields of ethanol from hydrolyzates of corn hull and pine respectively. However, both studies have clearly demonstrated a preference for glucose and the rate of xylose utilization to be slower in the presence of glucose. The preferential uptake of glucose in mixed-sugar fermentations suggests that glucose concentration should be kept low in order to achieve simultaneous conversion of glucose and xylose, or sufficient reaction time should be provided for the individual fermentation of the two sugars.

Our results of cellulose conversion agree with those reported by Doran et al. (Doran and Ingram 1993; Doran et al. 1994) for *K. oxytoca* P2. Efficient SSF is possible only when both hydrolysis of cellulose and fermentation of glucose can be achieved. Since cellulose hydrolysis is generally slower than glucose fermentation, conditions that favor the former play a key role in efficient SSF. High temperatures, which increase the rate of hydrolysis, can cause increased product inhibition of fermentation as observed by Doran and Ingram (1993). A low pH in the range of 5.2–5.5 at an appropriate temperature can provide the desired high product yields. Doran and Ingram (1993) achieved 85.6% of the theoretical ethanol yield at 32°C and pH 5.5 with 100 g/l cellulose, while our studies, which use a lower cellulose concentration, could achieve similar yields at a higher temperature of 37°C with *E. coli* 11303 (pLOI 297). The maximum yields (percentage of theoretical) from bagasse reported by Doran et al. (1994) are similar to those above. It should be noted that the enzyme loading used by Doran et al. (Doran and Ingram 1993; Doran et al. 1994) is about 2.5-fold lower than that in the present study presumably because of the ability of *K. oxytoca* to convert cellobiose directly to ethanol (Ohta et al. 1991b).

The nature of the product distribution from glucose fermentation in this recombinant *E. coli* strain suggests four distinct regimes of product formation as described in Table 1. In the first regime (standard conditions of fermentation), ethanol is the main product with succinic acid as the predominant byproduct. Acetic and lactic acids are also produced at low levels. In the second regime, where initial glucose levels are above 100 g/l, ethanol is still the predominant fermentation product. However, the byproduct pattern is different; lactic acid production is roughly fivefold higher relative to regime I. In regime III, SSF at pH 6.0 and 37°C where glucose levels are extremely low, the production of succinic and acetic acids is significantly enhanced and the ethanol yield is correspondingly lower. In regime IV – cellulose conversion at pH 5.0 and 37°C – the ethanol selectivity is high; succinic acid is the only byproduct formed at levels comparable to those in pure sugar fermentations.

In wild-type *E. coli*, the glycolytic pathway metabolizes through phospho*enol*pyruvate (*p*pyr) to pyruvate, which is subsequently converted predominantly to acids under anaerobic conditions (Gottschalk 1986). Under standard conditions, the pyruvate is converted to ethanol at high selectivity owing to predominance of the plasmid-expressed enzymes pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adh*), and to the lower K_m value of the *pdc* enzyme relative to those in the acidogenic pathways (Ingram et al. 1987; Ingram and Conway 1988). In the analysis of competing metabolic pathways, however, it is important to include *p*pyr carboxylase – the first enzyme in the conversion of *p*pyr to succinic acid – and pyruvate kinase, which converts *p*pyr to pyruvate. The low glucose concentrations present during SSF clearly make this interaction an important one. Ohta *et al.* (1991a) discuss mutated strains of recombinant *E. coli* with reduced levels of acid formation; however, an inverse correspondence is suggested between the expression level of *pdc* and *adh*, and the production of acidic byproducts. Ohta et al. (1991b) suggest a lack of direct competition between *pdc* and *p*pyr carboxylase in *K. oxytoca* since different levels of *pdc* expression do not change succinate levels. However, the constancy of succinate levels cannot be taken as conclusive proof of a lack of competition.

A thorough explanation of the product distribution requires a knowledge of the metabolic fluxes, enzyme reaction rates, and enzyme concentrations; we speculate that, at the low levels of the pathway intermediates present during SSF of cellulose, the conversion of *p*pyr to succinic acid competes significantly with that of pyruvate to ethanol. At very high substrate loadings, pyruvate probably accumulates even at the maximum rate of ethanol formation, resulting in its conversion by lactate dehydrogenase (the lactic acid pathway). Other factors such as the energy charge of the cell and enzyme regulation under different operating conditions are also likely to play key roles in the selectivity of product formation. Two factors may be at play in providing the high ethanol yields from SSF at pH 5.0. First, the low pH would enhance enzymatic activity resulting in higher rates of glucose production from cellulose and, therefore, a high selectivity for ethanol production. Second, the acidic operating conditions may inhibit further production of the organic acids from fermentation of glucose.

More work needs to be done to elucidate the mechanisms involved in product selectivity in recombinant *E. coli*. A metabolic model based on carbon fluxes through the various pathways and on the dependence of enzymatic rates on external conditions would be useful in achieving this understanding. The recombinant *E. coli* strain used in this study can certainly be used to design a process for achieving high yields of ethanol from both hexose and pentose sugars. However, the ethanol inhibition, glucose inhibition of xylose, and compatibility of the microorganisms with enzymatic hydrolysis of cellulose would be important factors in the design of an efficient, cost-effective

process for conversion of both cellulose and xylose to ethanol.

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