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Properties of the β-glucosidase from *Cellulomonas flavigena* and from *Escherichia coli* harboring the recombinant plasmid pJS3

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SUMMARY

Plasmid-coded β-glucosidase produced by *Escherichia coli* was characterized and compared to the enzyme produced by *Cellulomonas flavigena*. Cell-free extracts, non-denaturing PAGE and 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside (X-glu) as substrate were used to compare both enzymes. The β-glucosidase was assayed for cellobiose and *p*-nitrophenyl-glucopyranoside (PNPG). Cellobiose hydrolysis was performed at 50 °C for the enzyme from *C. flavigena* and at 37 °C for that from *E. coli* pJS3, both with an optimal pH of 6.5. For PNPG hydrolysis, the optimal conditions were pH 5.5 and 37 °C for both cell extracts. Most of the β-glucosidase activity was intracellular. When cultures of *C. flavigena* were grown with cellobiose or carboxymethylcellulose (CMC) as inducers, the expression of β-glucosidase was increased considerably. *E. coli* pJS3 produces a cellobiase which hydrolyzes cellobiose and PNPG. The K_m values for cellobiose and PNPG indicated that the β-glucosidase activity of *C. flavigena* had a higher affinity for cellobiose as substrate, whereas the β-glucosidase from *E. coli* pJS3 showed higher affinity for PNPG.

INTRODUCTION

The cellulase system converts cellulose to glucose by a cellulase complex which is composed of three general kinds of enzymes: endo-β-1,4-glucanase (EC 3.2.1.4), exo-β-1,4-glucanase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21). The first two act cooperatively to depolymerize cellulose to cellobiose and oligosaccharides. β-Glucosidases catalyze the hydrolysis of cellobiose and related glycosides [7]. *Cellulomonas flavigena* and *Xanthomonas* sp. are components of a symbiotic bacterial culture used for conversion of sugar cane bagass into single cell protein [6]. The level of the β-glucosidase activity in *C. flavigena* limits the specific growth rate of the mixed culture. Modification of this activity will probably increase specific growth rate. For this reason, we are interested in characterizing the β-glucosidase activity from *C. flavigena*.

Two types of β-glucosidases have been described, the aryl β-D-glucosidase which catalyses the hydrolysis of alkyl and aryl β-glucoside (e.g., methyl-β-D-glucoside and *p*-nitrophenyl-β-D-glucoside) [11,15], and the cellobiase which is active on alkyl- and aryl-β-D-glucosides, as well as glycosides containing only carbohydrate residues (e.g., cellobiose) [1,2,3,12,17].

In a previous paper [13] we reported the cloning of the genes coding for three different cellulolytic enzymes from *C. flavigena* in *E. coli*. Plasmid pJS10 encodes an endo-β-glucanase and the other two plasmids code for β-glucosidases, each with different activities, pJS4 (X-glu$^+$ and MUC$^+$) and pJS3 (X-glu$^+$). The present paper describes some properties of the β-glucosidase from the recombinant clone of *E. coli* pJS3 and *C. flavigena*.

MATERIALS AND METHODS

Bacterial strains

The *Cellulomonas flavigena* strain used in this work was isolated by De la Torre and Campillo [6]. The *E. coli* recombinant strain was DH5-α (F$^-$, *end*A1, *hsd*R17, (r$^-$ k, m$^-$ k), *sup*E44, *thi*-1, lambda, *rec*A1, *gyr*A96, *rel*A1, Φ80, *lac*Z M15). This strain harbored plasmid pJS3 (pUC18 plus 6.5 kb DNA from *C. flavigena*) the construction of which has been described previously [13]. *C. flavigena* was grown at 30 °C in minimal medium as described by Steward and Leatherwood [14], supplemented with 0.2% glucose, 0.2% lactose, 0.2% cellobiose, 1% CMC (low viscosity grade Sigma) or 1% solkafloc. *E. coli* was grown at 37 °C in LB medium (1% bactotriptone, 0.5% yeast ex-

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tract and 1% NaCl) supplemented with 100 μg/ml of ampicillin.

Cell free extracts

The extracts were prepared as described by Wakarchuk et al. [15] with some modifications. The procedure was as follows: cultures were harvested by centrifugation at mid log phase, washed with half the original volume of 100 mM phosphate buffer (pH 6.5) and centrifuged for 10 min at 12000 × *g* at 4 °C. Pellets were resuspended in 1/25 the original volume with the same buffer, sonicated until lysis occurred and centrifuged at 50000 × *g* for 20 min and 4 °C to remove cell debris. To prevent proteolysis, PMSF was added to the supernatant to a final concentration of 50 μg/ml. The β-glucosidase activity of the supernatants was evaluated weekly and remained stable for 1 month when the supernatants were kept at 4 °C.

Gel electrophoresis

Proteins from different extracts were separated by non-denaturing electrophoresis in 7% polyacrylamide gel using 5% polyacrylamide stacking gel and TBE buffer (89 mM Tris-borate, 89 mM boric acid and 0.2 mM EDTA). Samples (50 μg of protein) were loaded into the gel and run at 100 volts at room temperature until the bromophenol blue reached the bottom of the gel. The gel was placed in 100 mM phosphate buffer (pH 6.5) and shaken for 5 min at room temperature. Buffer was removed and the gel was glazed with 20 μg/ml of X-glu. The gel was incubated in a humidity chamber (to prevent it from drying) for 1–2 h at 37 °C, and blue bands of β-glucosidase activity were observed.

Enzyme assays

Enzymatic activity was measured by two methods: first, by measuring the release of *p*-nitrophenol using PNPG as the substrate, as described by Ait et al. [1]. In *C. flavigena* assays, 1 μg protein of crude enzyme preparation, 100 mM phosphate buffer (pH 5.5) and 3 mM PNPG were used. In *E. coli* assays, 5 μg protein of crude enzyme, 0.5 mM PNPG and the same phosphate buffer were used. In both cases the reaction mixtures were incubated for 1 h at 37 °C and stopped by the addition of an equal volume of 1 M Na_2CO_3 solution. The extinction coefficient of *p*-nitrophenol was 18300 l/mol/cm at 400 nm.

In the second assay, the release of glucose from cellobiose hydrolysis was determined. Glucose was measured by using a glucose oxidase and peroxidase diagnostic kit from Merck. Assays were performed according to the manufacturer's instructions. The reaction mixtures consisted of 2 μg of protein from the crude extract, 100 mM phosphate buffer (pH 6.5) and 50 mM cellobiose. The *C. flavigena* and *E. coli* reaction mixtures were incubated at 50 and 37 °C, respectively, for a period of 1 h. The reactions were stopped by heating to 96 °C for 10 min.

Enzyme units are defined as μmol of glucose or *p*-nitrophenol released per minute per milligram of protein.

Protein determination

Protein was measured by the method of Bradford [5] using bovine serum albumin as the standard.

RESULTS

Identification of β-glucosidase activity on non-denaturing PAGE

The construction of the recombinant plasmid pJS3, which expresses a β-glucosidase from *C. flavigena* in *E. coli* DH5-α has been described before [13]. This recombinant strain hydrolyzes X-glu in agar plates, producing blue colonies like those produced by *C. flavigena*.

It is known that some compounds such as lactose, cellobiose, CMC and solkafloc are good inducers of β-glucosidase expression, while glucose is a repressor. In order to determine the effect of these inducers on *C. flavigena* β-glucosidase activity, cell-free extracts from cells grown with different carbon sources were studied, as well as *E. coli* pJS3 grown in LB. These extracts were analyzed by non-denaturing polyacrylamide gel electrophoresis as indicated in Materials and Methods. The blue bands of the gel were visualized (Fig. 1). Only one band showing β-glucosidase activity was detected in each case. A weak blue band was observed (Fig. 1, lane 1) when glucose (a repressor) was used. This may be due to a basal level of expression. Extracts from *C. flavigena* grown in the presence of different inducers were analyzed. A sharp blue band was detected (Fig. 1, lanes 2–5). Bands with the highest intensity were observed in extracts from cells grown on cellobiose and solkafloc as substrates. When the *E. coli* pJS3 extract was analyzed, a blue band was detected with the same mobility as that observed for *C. flavigena*, suggesting that the cloned enzyme corresponds to that expressed in *C. flavigena*. When the negative control was used (i.e., *E. coli* pUC18 extract) the band was absent (data not shown).

Enzymatic characterization of β-glucosidase produced by C. flavigena and E. coli pJS3

The optimal pH and temperature of the *C. flavigena* and *E. coli* pJS3 were determined by using both crude preparations and the two substrates, cellobiose and PNPG. The optimum pH was 6.5 for cellobiose degradation, and 5.5 for PNPG hydrolysis (data not shown). The *C. flavigena* crude preparation showed maximum activity

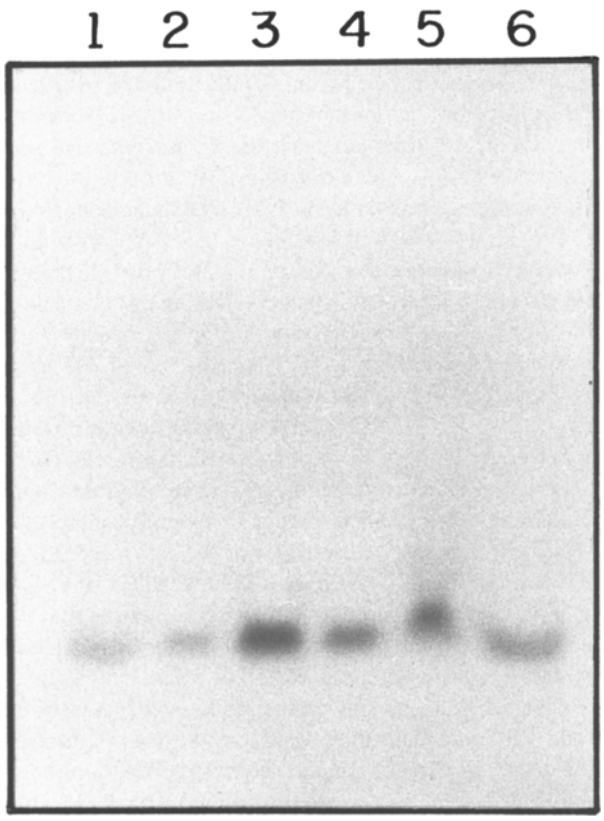

Fig. 1. Detection of β-glucosidase activity in *C. flavigena* and *E. coli* DH5α (pJS3) extracts using X-glu. Extracts were prepared from cells grown on different carbon sources as described in Material and Methods; 100 μg of protein were analyzed by non-denaturing PAGE. Active bands were visualized by spotting 20 μg/ml X-glu as substrate. Extracts from *C. flavigena* were grown in: 1, glucose; 2, lactose; 3, cellobiose; 4, carboxymethyl cellulose; 5, solkafloc; and 6, extract from *E. coli* DH5α (pJS3) grown in LB. The negative control *E. coli* DH5α (pUC18) was not included in the figure.

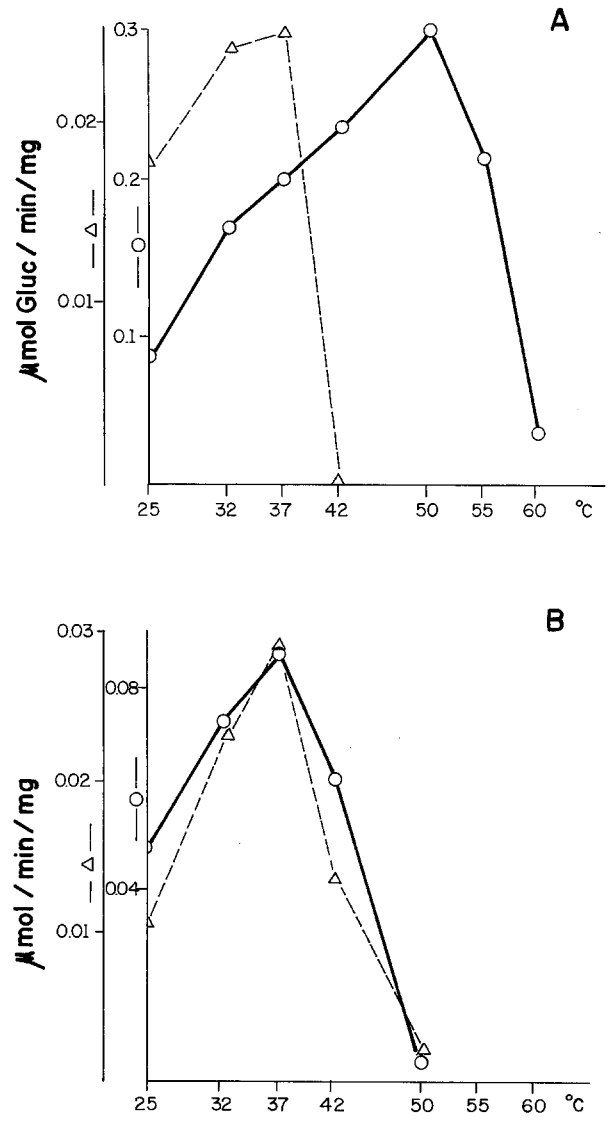

Fig. 2. Temperature profile of β-glucosidase activity of *C. flavigena* and *E. coli* pJS3 using cellobiose and PNPG. β-Glucosidase activity was determined in cell free extracts from *C. flavigena* grown in cellobiose (○) and *E. coli* grown in LB (△) at different temperatures using (A) cellobiose, 100 mM phosphate buffer (pH 6.5) and (B) PNPG, 100 mM phosphate buffer (pH 5.5).

at 50 °C for cellobiose degradation, while the *E. coli* pJS3 enzyme preparation showed maximum activity at 37 °C (Fig. 2A). When PNPG was used as a substrate, the optimum temperature was 37 °C in both crude extracts (Fig. 2B). Temperatures higher than 37 °C caused the β-glucosidase activity to decrease sharply in the two samples. However, for the *C. flavigena* extract using cellobiose as a substrate the loss of activity was above 50 °C.

Localization of β*-glucosidase activity in C. flavigena and transformed E. coli cells*

In order to localize the β-glucosidase activity within the cell, the cytoplasmic fraction was separated from cell debris. *C. flavigena* was grown with different carbon sources, and *E. coli* containing pJS3 and pUC18 plasmids were grown in LB medium. The two main fractions (debris and cytoplasm) were assayed for cellobiose and PNPG hydrolysis. In both cases more than 95% of the activity was found in the soluble fraction (Table 1).

Induction of β*-glucosidase activity*

As mentioned above, cellobiose and CMC were good inducers for β-glucosidase production. In order to measure the β-glucosidase activity, the soluble fraction of cell

TABLE 1

β-Glucosidase activity in fractionated cell extracts

Carbon source	Strain	Cellobiose units		PNPG units	
		soluble	debris	soluble	debris
Glucose	*C. flavigena*	0.112	0.005	0.012	0.015
Cellobiose	*C. flavigena*	0.305	0.031	0.058	0.032
CMC	*C. flavigena*	0.439	0.002	0.089	0.020
	E. coli pJS3	0.032	0.004	0.01	0.001
	E. coli pUC18	0.001	0.006	0.001	0.001

β-Glucosidase activity was determined in the soluble and cell debris fractions from *C. flavigena* grown in glucose, cellobiose and CMC, and *E. coli* grown in LB medium. Reactions were made as indicated in Materials and Methods.

extracts from *C. flavigena* grown on cellobiose and CMC were analyzed and compared with the basal level expression from cells grown under repressed conditions (glucose). When *C. flavigena* was grown on cellobiose and CMC, the production of β-glucosidase increased 2.7- and 3.9-fold, respectively, for cellobiose degradation. When these extracts were assayed with PNPG as a substrate, the activity increased 4.8- and 7.4-fold, respectively, above the basal level. β-Glucosidase activity from the recombinant *E. coli* pJS3 grown in LB, was compared to *C. flavigena* grown in repressed conditions of glucose; for cellobiose degradation the activity was 30% and for PNPG hydrolysis it was 80%. As shown in Table 1, the control (*E. coli* pUC18) was negative in all cases.

K_m values of β-glucosidase for cellobiose and PNPG

Kinetic studies of β-glucosidase were carried out with crude extracts from *C. flavigena* (grown on cellobiose) and *E. coli* pJS3 (grown in LB). The K_m values were determined by two methods: the double-reciprocal plot method of Lineweaver-Burk (Fig. 3) and the computer-based program of Wilkinson [16]. The K_m value (cellobiose) for the extracts of *C. flavigena* and *E. coli* were 1.6×10^{-3} M and 2×10^{-2} M, respectively. The K_m value (PNPG) for the *C. flavigena* extract was 3.3×10^{-3} M and for the *E. coli* extract 1.6×10^{-4} M. In addition, only the *E. coli* pJS3 enzymatic extract showed substrate inhibition with PNPG above 0.5 mM.

DISCUSSION

Two different β-glucosidases have been described in *C. fimi* [15]. One of them was active using PNPG as a substrate, but it was inactive when using cellobiose. This enzyme seems to be an aryl-β-glucosidase. The other β-glucosidase was more active with cellobiose, and appears to be a β-D-glucoside glucohydrolase (or true cellobiase), which has been observed to be slightly inhibited by glucose and induced by cellobiose and Avicel. In this work only one blue band was detected for *C. flavigena* on non-denaturing PAGE and X-glu as a substrate. Although two different β-glucosidases from *C. flavigena* had been cloned [13] in *E. coli* (pJS3 and pJS4), it was only possible to properly express one of them (pJS3). It is possible that the aryl-β-glucosidase activity from *C. flavigena* (and presumably cloned in pJS4) could migrate in the same position as the cellobiase activity (pJS3). Another possibility is that the expression might be induced by different substrates. The synthesis of β-glucosidase was not completely repressed by glucose but it was induced with cellobiose, solkafloc and CMC. In addition, the extract from the recombinant *E. coli* pJS3 showed a blue band with the same mobility on non-denaturing PAGE as that of the β-glucosidase from *C. flavigena*. The higher enzymatic activity was found associated with cells, indicating that it is localized intracellularly; similar observations have been reported for other bacteria [1,4,9].

It is known [1,8] that a number of β-glucosidases degrade PNPG and cellobiose. Extracts from *C. flavigena* and *E. coli* pJS3 were able to hydrolyze PNPG and cellobiose. These results showed that the enzyme cloned in plasmid pJS3 was the β-D-glucoside glucohydrolase (or cellobiase) from *C. flavigena*, because this enzyme was able to hydrolyze either substrate.

The enzymatic activity of β-glucosidase in this work was analyzed using two different substrates, PNPG and cellobiose. The present results are in the range of activities for other β-glucosidases previously reported [8,9,18]. Differences in the optimum pH and temperature between activities from *C. flavigena* and *E. coli* pJS3 extracts could be explained by conformational changes acquired by the enzyme in conjunction with the substrate, as they are expressed in different biological systems. Substrate inhibition with higher concentrations of 0.5 mM of PNPG was observed in *E. coli* pJS3 extract; similar results have been reported for the enzyme from other sources [11,18].

In the crude extract of *E. coli* pJS3, the K_m for PNPG was lower than the K_m for cellobiose; thus, the enzyme has higher affinity for PNPG. The opposite was observed for *C. flavigena*. This difference in affinity has been reported for other β-glucosidases from *Alternaria alternata* [1], *Clostridium thermocellum* [9] and *Caldocellum saccharalyticum* [10].

The best inducers for the expression of β-glucosidase in *C. flavigena* were cellobiose and CMC. This system is not completely repressed by glucose. The recombinant clone of *E. coli* showed expression levels for cellobiose degradation of 30% and 80% for PNPG if compared with

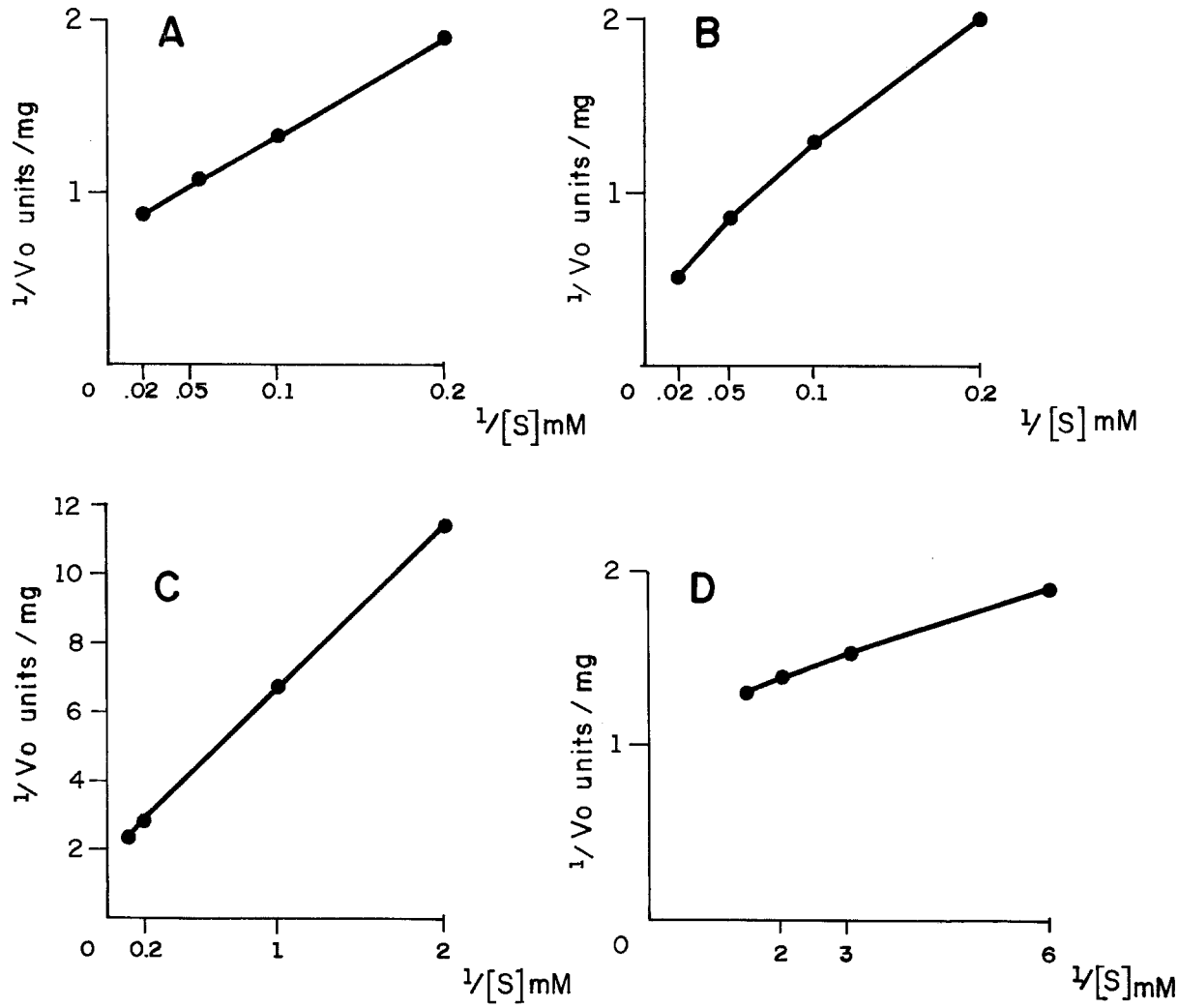

Fig. 3. Degradation kinetics of cellobiose and PNPG for the determination K_m and V_{max} values of the β-glucosidase in *C. flavigena* and *E. coli* (pJS3) extracts. Soluble fractions from *C. flavigena* grown in cellobiose and *E. coli* (pJS3) grown in LB supplemented with ampicillin were assayed with different concentrations of cellobiose and PNPG as substrates. (A) *C. flavigena*-cellobiose; (B) *E. coli* pJS3-cellobiose; (C) *C. flavigena*-PNPG; and (D) *E. coli* pJS3-PNPG.

the repressed conditions in *C. flavigena*. It will be of interest to upgrade these levels by using other vector systems. Also, it will be useful to analyze the nucleotide sequence of this fragment in order to obtain more information for the designing of tailor-made enzymes.

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