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FERMENTATION AND RECOVERY OF L-GLUTAMIC ACID FROM CASSAVA STARCH HYDROLYSATE BY ION-EXCHANGE RESIN COLUMN

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ABSTRACT

Investigations were carried out with the aim of producing L-glutamic acid from *Brevibacterium* sp. by utilizing a locally available starchy substrate, cassava (*Manihot esculenta* Crantz). Initial studies were carried out in shake flasks, which showed that even though the yield was high with 85-90 DE (Dextrose Equivalent value), the maximum conversion yield (~34%) was obtained by using only partially digested starch hydrolysate, i.e. 45-50 DE. Fermentations were carried out in batch mode in a 5 L fermenter, using suitably diluted cassava starch hydrolysate, using a 85-90 DE value hydrolysate. Media supplemented with nutrients resulted in an accumulation of 21 g/L glutamic acid with a fairly high (66.3%) conversation yield of glucose to glutamic acid (based on glucose consumed and on 81.74% theoretical conversion rate). The bioreactor conditions most conducive for maximum production were pH 7.5, temperature 30°C and an agitation of 180 rpm. When fermentation was conducted in fed-batch mode by keeping the residual reducing sugar concentration at 5% w/v, 25.0 g/L of glutamate was obtained after 40 h fermentation (16% more the batch mode). Chromatographic separation by ion-exchange resin was used for the recovery and purification of glutamic acid. It was further crystallized and separated by making use of its low solubility at the isoelectric point (pH 3.2).

Key words: *Brevibacterium* sp., L-glutamic acid, cassava hydrolysate, batch and fed-batch process, ion-exchange resin, purification

INTRODUCTION

L-amino acids have a wide spectrum of commercial use as food additives, feed supplements, infusion compounds, therapeutic agents and precursors for the synthesis of peptides or agrochemicals (14). Monosodium glutamate (MSG), the sodium salt of glutamic acid is used commercially as a flavour enhancer, usually in combination with nucleotides inosinate to provide an expansion and extension of taste in processed food such as soups, biscuits, noodles, Chines foods, meat and vegetable processing etc. (12). Glutamic acid mother liquor in MSG production is being used in the manufacture of Sauce and as soil conditioner, fertilizer etc. Several strains of *Corynebacterium* and *Brevibacterium* are used as cost effective bioconverters, which have been exploited by the fermentation industry to provide various amino acids, including L-glutamic acid (1).

Owing to the importance of the particular industrial fermentation, much efforts were still going

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on to improve the glutamic acid fermentation process especially from the standpoint of savings in production cost (3, 10). Cassava *(Manihot esculenta* Crantz*)* popularly known as tapioca, is one of the major tuber crops of the world, being cultivated extensively in tropical countries and obviously provides a major source of calories to millions of the people in the world. On dry weight basis, tapioca contains about 80-82% starch (fresh roots have about 22-30% starch) of which 55-60% is recoverable as starch. It is an excellent substrate for the reducing sugars such as (maltose, glucose etc), especially because of its ease of liquefaction.

The present study was undertaken with an aim to test the efficiency of cassava starch to be used as the substrate for L-glutamic acid production in batch- and fed-batch processes. The optimized parameters in the fermentation using pure glucose (11) were applied to the cassava starch hydrolysate for the production of glutamic acid. Attempts were also made to isolate and purify the L-glutamic acid using chromatographic techniques with ion-exchange resin.

MATERIALS AND METHODS

Microorganism and cultivation

A strain of *Brevibacterium* sp. (DSM 20411) was used in the present study. The growth medium, culture preservation conditions and inoculum preparation were same as mentioned elsewhere (8).

Preparation of cassava starch

Fresh cassava tubers obtained locally were washed and peeled. The process of starch extraction from these tubers consisted of wet milling the washed cassava roots followed by washing the starch from the fibrous mass. Starch was sedimented in settling containers and air-dried.

Hydrolysis of cassava starch

Liquefaction of the starch slurries (5%, w/v) was carried out using a thermostable α-amylase (Termamyl 120, Novo Industries, Bagsvared, Denmark) produced from a strain of Bacillus licheniformis. The enzyme was having 120 KNU/g activity (one Novo α-amyl ase unit (NU) is defined as the amount of enzyme that hydrolyses 5.26 g starch/h under Novo's standard conditions). The pH of the slurry was adjusted to 6.0 and reaction was carried out in a stirred reactor with enzyme at 85°C for 2 h. The resulting solution was cooled to 60°C and after adjusting the pH 4.8-5.0 (with 1 N HCl), saccharification was carried out with a fungal glucoamylase (AMG 300, Novo industries, which has an activity of 300 AGU/ml) (one Novo amyloglucosidase unit is defined as the amount of enzyme that splits one micromole of maltose per minute at 25°C). Reaction was carried out for 18-24 h at 60°C and was then stopped by heating to 90°C for 10 minutes. The hydrolysate was double filtered using a nylon cloth and later by using Whatman Nº 1 filter paper so as to get the clear hydrolysate. Hydrolysate of different dextrose equivalent (DE) values containing different concentrations of sugars were prepared by the analysis of hydrolysate at different time intervals for the reducing sugars.

Fermentation

Batch process in flasks. Initial studies were carried out by taking 50 ml media in 250 ml Erlenmeyer flasks to study the effect of starch hydrolysate with different DE values on the growth and activity of *Brevibacterium* sp. Media were prepared by taking hydrolysate of different DE values and supplemented with $NaNO_3$ 0.7 g, KH_2PO_4 0.12 g, 1 ml mineral solution ($FeSO_4.7H_2O$, $MnSO_4$, $MgSO_4.7H_2O$, $ZnSO_4.6H_2O$ and NaCI, each 1 mg), 100 µl corn steep liquor and one drop of Tween 80 in 100 ml starch hydrolysate (pH 7.2). After autoclaving, the media were inoculated with 5% v/v suspension (10^8 cells/ml) of 20 h old *Brevibacterium* sp. (9). The optimum parameters obtained in earlier studies were maintained throughout the period of fermentation such as pH 7.5, temperature 30°C, and agitation speed 180 rpm (8-10). Samples were withdrawn as whole flask at desired time intervals for analysis. The results reported are the average of three sets of experiments.

Batch process in fermenter. Cassava starch hydrolysate (85-90 DE) was diluted to 5% initial sugar concentration and was supplemented with $NaNO_3$ 0.7 g, KH_2PO_4 0.12 g, 1 ml mineral solution ($FeSO_4.7H_2O$, $MnSO_4$, $MgSO_4.7H_2O$, $ZnSO_4.6H_2O$ and NaCI, each 1 mg), 100 µl corn steep liquor and one drop of Tween 80 in 100 ml starch hydrolysate (pH 7.2). Fermentation was carried out with a

working volume of 2.5 L in a 5 L fermenter (BIOFLO III, New Brunswick Scientific, Edison, N, J., USA). Dissolved oxygen was maintained at 60% of air saturated medium.

Fed-batch process in fermenter. Fed-batch process was also carried out in the fermenter. The initial concentration of reducing sugars in the medium was 5%, and at the stages, where the concentration fell to 2% (as determined by the analysis of fermentation medium), starch hydrolyzate solution containing 10% reducing sugars, was added to bring the sugar concentration of fermenting medium as 5%. Fermentation conditions were the same as for batch process.

Filtration and Centrifugation of Broth

Two batches, each consisting of 2.5 L fermented broth (obtained from batch fermentation in fermenter) was filtered using a microfiltration unit (Millipore, USA), fitted with a 50 mm membrane diameter with a pore size of 45 μm under vacuum using a pump. Both the filtrates were combined and then centrifuged at 10,000 rpm for 10 minutes to get the supernatant, which was then used for the recovery of the product.

Preparation of Resin

Spherical particles of cation exchange resin, Amberlite IR 120 (Hi-media) was used. Prior to use, the resin was pre-conditioned according to the method of Moore and Stein (6). The resin (100 g) was washed thoroughly two times with 4 N HCI. After two washes with distilled water, the resin was then washed with 2 N NaOH until the filtrate was alkaline. The resulting material (sodium salt of the resin) was suspended in 3-times its volume of 1 N NaOH and heated over a steam bath for 2 h with occasional mixing. The supernatant fluid was decanted after 30 minutes of settling and replaced with fresh hot 1 N NaOH. The procedure was repeated two times. The resin was filtered and washed with 2 L of distilled water to make it free of alkali. The resin was finally stored as the moist sodium salt.

Packing the Column

Resin (as above) was placed in a column (3 cm × 50 cm) containing distilled water and filled upto 25 cm^3. The excess water was removed using a siphon.

Separation Process by Ion-Exchange Column

The chromatographic conditions were selected to minimize the inhibitory effect of co-existing inorganic ions on the adsorption of amino acids by ion-exchange resins, following the method of Samejima (13). Removal of impurities from the broth was done by filtering and centrifuging the broth. The pH of the broth dropped from 7.5 to 4 with 1 N HCl, which was the most important factor affecting the adsorption of glutamic acid on the resin because the ionic forms vary with the pH.

The processes involved in the column were adsorption and elution. In the adsorption process, the broth used was adjusted to a suitable pH of 1.8-2.0, using 1 N HCl to charge the glutamic acid so that the ion exchange between the glutamic acid and the resin could occur. The broth was continuously recycled at a flow rate of 20 ml/minute (retention time 50 minutes) until glutamic acid was fully adsorbed in the column, leaving other ions. In the elution process, the pH was increased to 3.8-4.0 by treating the broth with urea and sodium hydroxide. This was done to release the glutamic acid bound on the resin by changing the glutamic acid charge.

Crystallization

After adsorption and elution, the eluent containing a high amount of glutamic acid was acidified to pH 3.2, the isoelectric point of glutamic acid with 1 N HCl. Storage at 20°C for 48 h resulted in the formation of crystals of glutamic acid. After evaporation of the eluent, the dry solid crystals were obtained.

Analytical Methods

Bacterial growth was determined by measuring the optical density (OD) of the culture broth at 610 nm using an UV-visible spectrophotometer (UV-160 A Shimadzu, Japan). Total carbohydrates in the samples were detected by phenol-sulphuric-acid method (4). Starch content was determined by hydrolyzing the substrate with 10% HCl and estimating the glucose content by DNS reagent (5). pH measurements were made by a standard pH meter (model 361 μ, Systronics, Ahmedabad, India). Thin layer chromatography (TLC) was used (Silica gel G, Solvent mixture-n butanol/glacial acetic acid/water 4:1:1, v/v) for the qualitative detection of L-glutamic acid (2). The TLC plate with ninhydrin showed only

one spot, which was identical with authentic sample of L-glutamic acid, hence ninhydrin colour reaction method was used for quantitative estimation of L-glutamic acid (15). Product purity was reconfirmed by IR-spectrum (Perkin Elmer Model 882) using pure L-glutamic acid as a standard (3).

RESULTS AND DISCUSSION

Growth and glutamic acid production based on the hydrolysate having different DE values. Fig. 1 shows the growth pattern of *Brevibacterium* sp. on cassava starch hydrolysate. In general, higher DE hydrolysate supported better growth of the culture, which was maximum with the 85-90 DE hydrolysate (OD 1.92 at 18 h). Apparently, the rate of cell growth was directly related with the DE values of the hydrolysate, as higher the DE value, lesser was the time to achieve maximum cell growth in hydrolysate of different DE values. In case of 15-20 DE hydrolysate, the growth was slowest and it took 30 h for the cells to achive the maximum growth.

Fig. 2 shows the consumption of reducing sugars by *Brevibacterium* sp. at different DE values. The pattern was similar in all cases with a consumption of more than 85% sugars (a maximum of 94% with DE 85-90). Fig. 3 shows the glutamate production at different time intervals. A maximum of 8.8 mg/ml

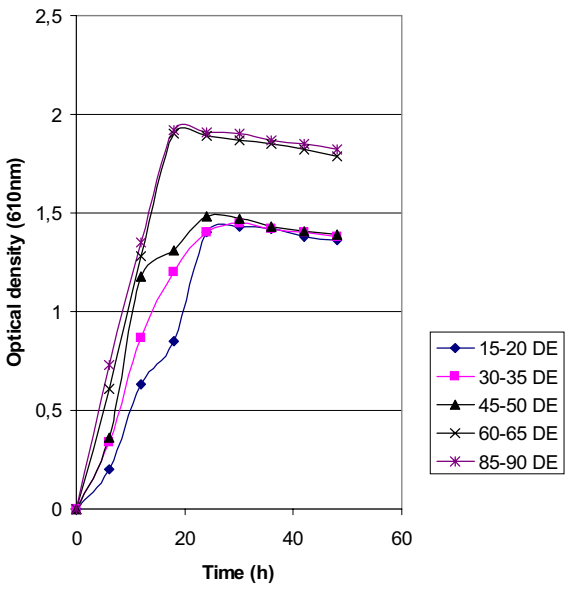

Figure 1 – Growth pattern of *Brevibacterium* sp. in cassava starch hydrolysate medium of different DE values.

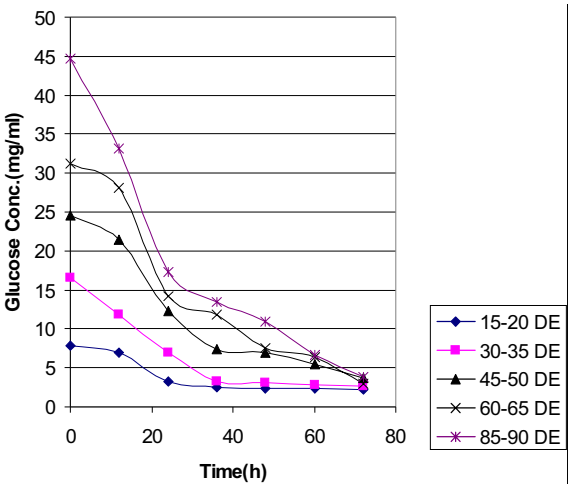

Figure 2 – Consumption of sugars by *Brevibacterium* sp. in different DE starch hydrolysate.

L-glutamic acid was obtained after 60 h fermentation with the medium having 85-90 DE. While considering the percentage conversion of sugars to L-glutamic acid (based on glucose consumed and 81.74% as the theoretical conversion rate) (8); it was maximum (34%) with the hydrolysate having DE value 45-50 as shown in Fig. 4. On the other hand, with 85-90 DE hydrolysate, the conversion was lowest (~27%). Thus, if conversion factor has to be considered as a major criterion, a low DE value hydrolysate, i.e. 45-50 DE would be sufficient for L-glutamic acid production. There are reports in the literature where a wide variety of applications have been mentioned for low DE starch hydrolysate (7).

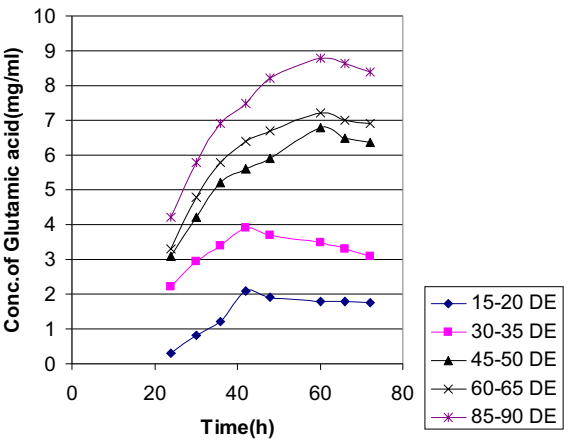

Figure 3 – Yields of L-glutamic acid by *Brevibacterium* s. in different DE value starch hydrolysate.

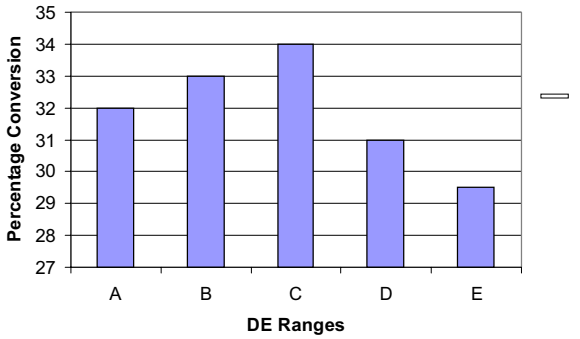

Figure 4 – Percentage conversion of cassava starch hydrolysate of L-glutamic acid.

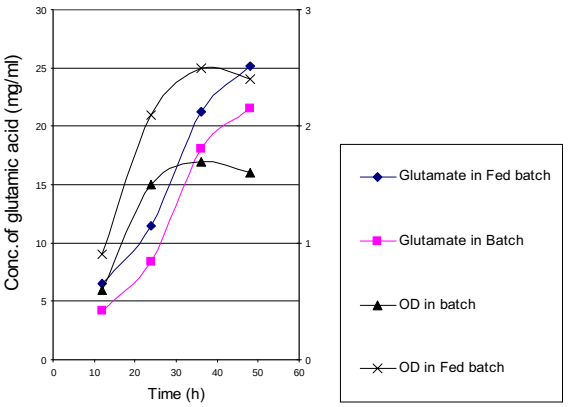

Figure 5 – Comparison of the growth profile and glutamic acid production in batch and fed batch processes.

In this context, our findings are significant. Yet, another advantage of partially hydrolyzed starch was that the glucose could be made available in a kind of controlled release process, which avoided the kind of repression, which normally one faces with fermentation feed stocks.

Studies in fermenter. Table 1 shows the summary of the fermentation process (batch mode) such as cell growth, substrate consumption and the corresponding product fermentation. From the data it was evident that more than 95% of the reducing sugars were consumed and within 40h fermentation the accumulation of glutamic acid was nearly 21 g/L, which was approximately two and half fold more than what was obtained in shake flask studies. Based on the glucose consumed and also by assuming 81.7% as the theoretical conversion, we got conversion of about 66.3%.

Fig. 5 shows a comparison of bacterial growth and L-glutamic acid production in batch and fed-batch process. A maximum of 25 g/L of glutamate was obtained in fed-batch process, which was 16% more than the batch mode. Hence, the maintenance of an active biomass constantly for a long period could enhance the accumulation of glutamate to a certain extent only.

Recovery of glutamic acid. The fermented broth contained various impurities such as bacterial cells, macromolecules, pigments, inorganic substances, organic substances etc., which were removed by filtration and centrifugation. Glutamic acid was purified from cation exchange resin. The elution profiles of the glutamic acid concentration from the column are shows in Fig. 6. Symmetrical peaks of glutamic acid concentration and absorbance were obtained. Glutamic acid was recovered for the highest

Table 1 – The overall changes during L-glutamic acid fermentation (in 5 L fermenter) using *Brevibacterium* sp.

Time	Optical density (610 nm)	Conc. Of glutamic acid (mg/ml)	Glucose consumption (%)
12	1.65	4.15	21.84
24	2.12	8.35	42.64
36	2.07	4.70	89.78
48	1.98	25.20	92.73
60	1.95	25.14	93.10

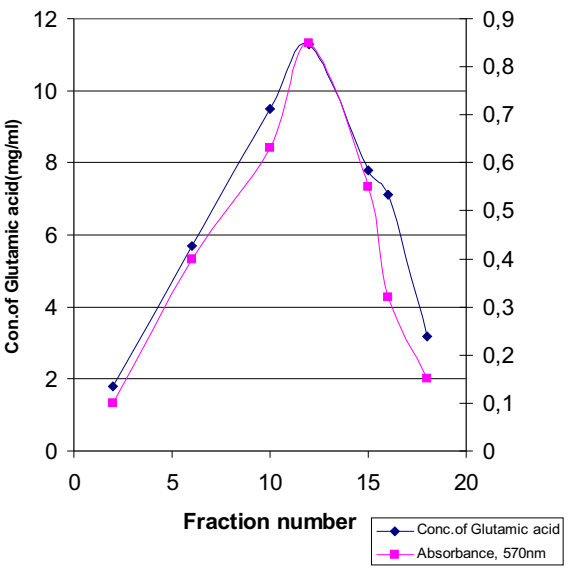

Figure 6 – Glutamic acid recovered at different elution volumes through ion-exchange resin column.

yield, taking the fractions of 8 to 17 (total elution volume of 200 ml) through the ion-exchange column. By changing the pH to the isoelectric point (3.2) and by the subsequent cooling of the eluent, glutamic acid was crystallized out. The purity of the final product, which was reconfirmed using IR spectrum, showed similar peak at the same frequencies. All functional groups of the product (NH_2 COOH, and CH_2) showed a frequency similar to standard glutamic acid as shown in Fig. 7.

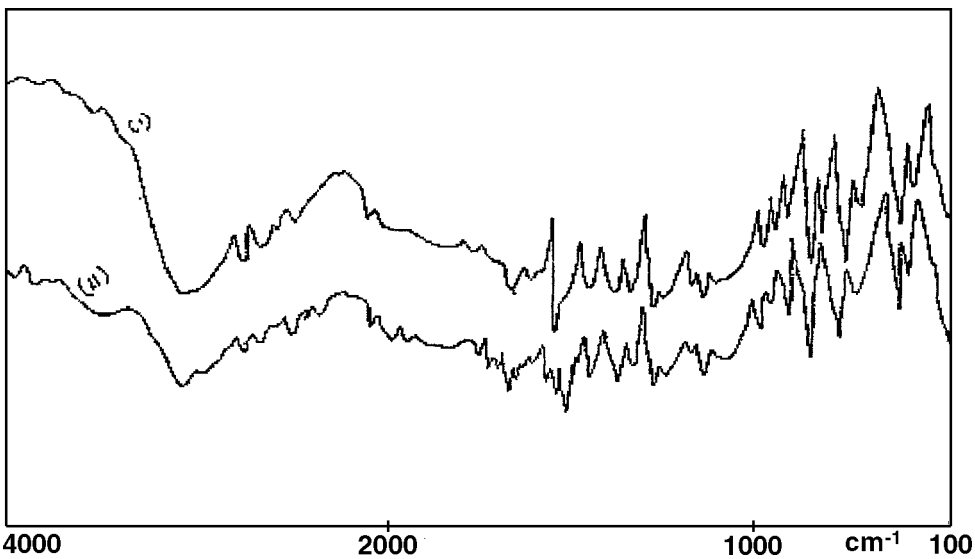

Figure 7 – IR-spectra of standard (1) and purified (II) samples of L-glutamic acid.

CONCLUSIONS

The results of the present study have indicated the possibility of utilizing starchy tubers as a raw material for L-glutamic acid production. It further indicated that as a soluble non-sweet, easily digestible carbohydrates, the low DE starch hydrolysate could find a potent industrial application (as a low cost raw material for amino acid fermentation). By making use of various combinations of environmental factors, ion-exchange resins could effectively be utilized for concentration and separation of glutamic acid.

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RESUMO

Produção de ácido L-glutâmico a partir de um hidrolisado de amido de mandioca usando resina de troca iônica

Pesquisas foram realizadas com o objetivo de produzir ácido glutâmico a partir de *Brevibacterium* sp. utilizando um substrato disponível na região, a mandioca (*Manihot esculenta* Crantz). Estudos iniciais, desenvolvidos em shaker, demonstraram que mesmo obtendo elevado rendimento com 85-90 DE (Dextrose Equivalent value), a taxa de conversão máxima (~34%) foi obtida usando um hidrolisado de amido parcialmente digerido, i.e. 45-50 DE. As fermentações foram realizadas em um fermentador de 5 L, usando um hidrolisado de amido de mandioca adequadamente diluído, preparado à partir de um valor DE de 85-90. O meio enriquecido com nutrientes resultou em um acúmulo de 21 g/L de ácido glutâmico, com uma elevada (66,3%) taxa de

conversão da glicose em ácido glutâmico (baseada em glicose consumida e em uma taxa de conversão teórica de 81,74%). As condições mais favoráveis, levando à uma máxima produção, foram pH 7.5, temperatura 30°C e agitação de 180 rpm. Quando a fermentação foi conduzida em um reator do tipo descontinuo alimentado, onde a concentração de açúcares redutores era mantida em 5% w/v, foram obtidos 25.0 g/L de glutamato após 40 h (16% a mais do que no modo descontinuo). Para a recuperação e purificação do ácido glutâmico, foi utilizada a separação por cromatografia com resina de troca inônica. O ácido foi posteriormente cristalizado e separado, levando-se em consideração a sua baixa solubilidade no ponto isoelétrico (pH 3.2).

Palavras-chave: *Brevibacterium* sp, L-ácido glutâmico, hidrolisado de mandioca, processo batch e fed-batch, resina de troca iônica, purificação

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