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Simultaneous Saccharification and Extractive Fermentation for Acetone/Butanol Production from Pretreated Hardwood

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ABSTRACT

An integrated bioreactor-extractor was investigated for applicability in simultaneous saccharification and extractive fermentation (SSEF), and production of acetone/butanol from pretreated hardwood by *Clostridium acetobutylicum* and cellulase enzymes. The SSEF system was constructed such that products of fermentation were extracted from the broth through a semipermeable membrane. *In situ* removal of inhibitory products was found to be beneficial in sustaining cell viability, thus allowing fed-batch operation of the bioreactor over a period of several weeks. Hardwood chips were pretreated by monoethanolamine in such a way that hemicellulose and cellulose were retained in high yield. The feed material thus prepared was readily converted by SSEF. The ability of *C. acetobutylicum* to ferment both glucose and xylose was a major factor in simplifying the overall process into a single-stage operation.

Index Entries: *Clostridium acetobutylicum;* simultaneous saccharification and fermentation; butanol fermentation; extraction.

INTRODUCTION

Simultaneous saccharification and fermentation (SSF) is a viable bioprocess applicable for production of acetone and butanol from lignocellulosic materials. The SSF offers important technical advantages; it reduces

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the inhibition of cellulase by sugars and eliminates the need for a separate reactor for saccharification (*1*). Yet, adaptation of SSF for acetone-butanol-ethanol (ABE) production by *Clostridium acetobutylicum* still faces an obstacle of end-product inhibition on the microorganism by butanol. The product inhibition on the growth of *C. acetobutylicum* and ABE production is rather acute, the effect of which begins to show at 4 g/L and increases further with butanol concentration (*2*). The butanol is also known to inhibit cellulase enzymes during the SSF process (*3*). For these reasons, in the normal batch SSF process, low product concentration is prevalent, and higher enzyme loadings are required for complete conversion of cellulose.

As a means of alleviating the product inhibition problem, various *in situ* product separation schemes have been proposed. In the case of the ABE fermentation, extraction appears to be promising because of low energy requirements (*4–6*). Extraction has been proven to improve solvent productivity and yield (*7*). Extractive fermentation in general offers an economic advantage over straight batch fermentation (*7,8*) The combination of extractive fermentation and SSF has the potential of improving the longevity of both the microorganism and cellulase enzymes.

Membrane-assisted extractive fermentation carries a unique feature in that it prevents emulsion formation, toxic effects, and loss of extractant, which normally occur when the extractant is brought directly into contact with the broth (*9*). Semipermeable membrane (*9*) and hydrophobic microporous hollow fibers (*10*) have been used to alleviate these technical difficulties.

Incorporation of membrane-assisted extraction into SSF brings about an additional benefit. Cellulase enzymes and the microorganism are retained in the bioreactor at all times. Cellulase enzyme represents a significant fraction (14%) of total production cost in SSF (*11*). It is essential to retain the enzyme in the bioreactor for reuse. Retention of cells also improves the productivity. Use of a membrane inside the bioreactor can facilitate integration of product recovery with SSF. A semipermeable membrane is better suited for SSF because it is less prone to fouling by either cells or fibers of the wood chips.

This study was undertaken to assess the performance of an integrated bioreactor-extractor in simultaneous saccharification and extractive fermentation of pretreated hardwood. Our particular interest was on long-term behavior of this reactor system operated under fed-batch mode.

MATERIALS AND METHODS

Microorganism and Medium

Clostridium acetobutylicum, ATCC 824, was used throughout this research. It was stored in sporulated form at 4°C on the medium containing 5% (w/v) corn mash and 0.5% (w/v) glucose. The growth media con-

tained the following components in 1 L of distilled water: KH_2PO_4 0.75 g, K_2HPO_4 0.75 g, $MgSO_4 \cdot H_2O$ 0.4 g, $MnSO_4 \cdot H_2O$ 0.01 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, cysteine 0.5 g, yeast extract 5 g, asparagine·H_2O 2 g, $(NH_4)_2SO_4$ 2 g.

Substrates and Enzyme

Aspen chips of 0.066–0.25 in nominal size were used as initial substrate. Aspen chips were pretreated with 20% aqueous monoethanolamine (MEA) solution at 186°C for 3 h. The ratio of MEA solution/dry wood was 2. The detailed procedure of this pretreatment is given elsewhere (*12*). Cellulase enzyme (85 IFPU/mL), a product of Genencor, Inc., San Francisco, CA, was used without β-glucosidase supplementation.

Acetone/Butanol Inhibition on Enzyme

Inhibitory action of acetone and butanol on enzyme was tested by carrying out enzymatic hydrolysis in the presence of varying amount of these chemicals. The hydrolysis of pretreated chips at the level of 40 g/L was carried out at 36°C in citrate buffer (0.01*M*) of pH 4.5. Butanol at 6 and 12 g/L, and acetone at 8 g/L were added to separate reactors. Control experiment with no added chemical was used for comparison. Enzyme loading was kept at 16 IFPU/g. The samples were taken at the end of 3 d to determine the extent of hydrolysis. The samples were heated to 70°C for 20 min to deactivate the enzyme (*13*) before they were subjected to HPLC analysis.

Simultaneous Saccharification and Extractive Fermentation

Bioreactor-Extractor

An integrated bioreactor-extractor system consisted of a 750-mL bioreactor (New Brunswick Scientific Co., New Brunswick, NJ), a silicone membrane tubing immersed into the broth, an extractant container, and a recirculation pump. Figure 1 shows schematics of the apparatus. The silicone tubing (R6411-64, Cole-Parmer Instrument Co., Chicago, IL) of 5 m length, 4.76 mm od, and 3.18 mm id was used as a semipermeable membrane. It was wound against the wall of the bioreactor in a helical pattern. Oleyl alcohol was chosen as an extractant because it is nontoxic to *C. acetobutylicum* and has a favorable distribution coefficient for butanol (*9,14*).

Experimental Procedure

The bioreactor containing growth medium and pretreated chips was autoclaved at 121°C for 15 min. Nitrogen was sparged into the reactor to create anaerobic conditions. Inoculum (10% v/v) and enzyme were then added to the reactor to start SSF. Working volume of broth was 400 mL. Temperature was maintained at 36°C, and pH was controlled at 4.5. Oleyl alcohol from previous experiments, which was saturated with butyric

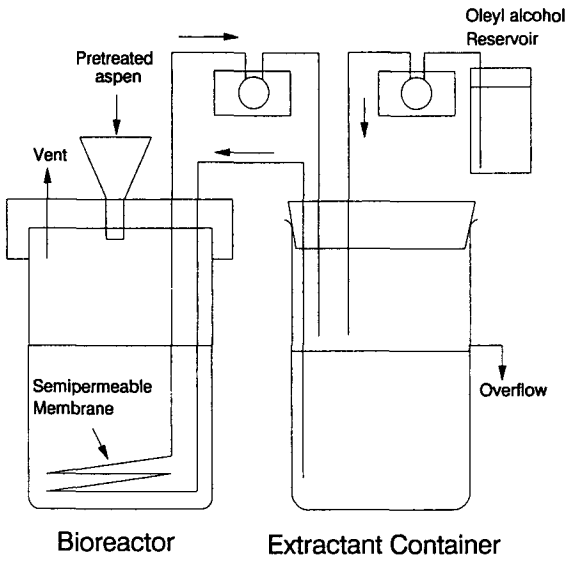

Fig. 1. Schematics of apparatus for simultaneous saccharification and extractive fermentation (SSEF).

acid, was regenerated by vacuum evaporation and used for extraction of products in the present study. Most of the butyric acid remained in regenerated oleyl alcohol. Extraction was started when butanol concentration in the reactor reached 7 g/L (at 100 h) by recirculating oleyl alcohol (10 mL/min) through the membrane tubing. Total extractant vol was 410 mL. To maintain slight negative pressure inside the tube, extractant was circulated by withdrawing it from the outlet end of the tube as shown in Fig. 1. This procedure prevented the extractant from permeating to the broth. The permeation of the broth into the extractant stream did not occur regardless of the pressure differential. When butanol concentration in the extractant reached 10 g/L (at 200 h), continuous addition and withdrawal of extractant ($D = 0.01\ h^{-1}$) was initiated to maintain steady-state concentration of products in the extractant. Pretreated chips (dried and sterilized) were added to the reactor intermittently with a 30–40 h interval. Dried chips were fed into the bioreactor to keep the liquid volume essentially constant. Cumulative input of pretreated chips during the entire operation was 243 g/L. The buildup of solid feed in the bioreactor was insignificant because the substrate was continuously dissolved by hydrolysis. Cellulase enzyme at the loading of 16 IFPU/g chips were added whenever chips were fed to the bioreactor. Thus, the enzyme loading on the basis of dry substrate input was kept constant (16 IFPU/g chips). Concentrated growth medium was also added with the chips to maintain its concentration in the bioreactor constant. Volume of broth was maintained at a constant level by bleeding small amount of broth equal to that of growth medium added.

Table 1
Composition of Aspen Before and After Monoethanolamine Pretreatment[a]

Aspen	Solids yield %	Glucan %	Xylan %	Lignin %
Untreated	100.0	47.6	18.4	18.4
Treated	63.1	39.0	16.3	4.6

[a]Based on dry wt of untreated aspen; other minor sugars (galactose, mannose, and arabinose) and extractives not included in this analysis.

Analytical Methods

Fermentation products were analyzed by Gas Chromograph (GC) equipped with chromosorb 101 column and FID. Samples were acidified using 3*N* HCl (*15*), and *n*-propanol was used as the internal standard. Partition coefficients and concentrations of products in the extractant were estimated according to the procedure described elsewhere (*16*). Lignin content in pretreated chips was determined by the TAPPI standard method T236 os-76. The amounts of glucan and xylan in the pretreated chips were determined by enzymatic hydrolysis. Sugars were analyzed by HPLC using a column packed with Bio-Rad, Aminex Q15S resin in Ca^{2+} form.

RESULTS AND DISCUSSION

Pretreatment

Table 1 shows the average composition of sugars and lignin in aspen chips before and after pretreatment. Composition of untreated aspen was estimated from Chum et al. (*17*). It is seen that a majority of the lignin was removed, whereas most of the xylan fraction remained intact in the chips. Amounts of glucan and xylan reported here are those actually converted to glucose and xylose, respectively, with enzymatic hydrolysis at 50°C. High enzyme loading of 300 IFPU/g was applied to ensure maximum saccharification of carbohydrates.

Inhibition of Enzyme by Acetone/Butanol

Enzymatic hydrolysis experiments were carried out with and without addition of acetone and butanol to determine the inhibitory effects these chemicals might have on cellulase enzyme. Reduction in sugar production at or below 6 g/L butanol was insignificant. Butanol at a concentration of 12 g/L reduced total sugar production by 16%. Acetone at 8 g/L also

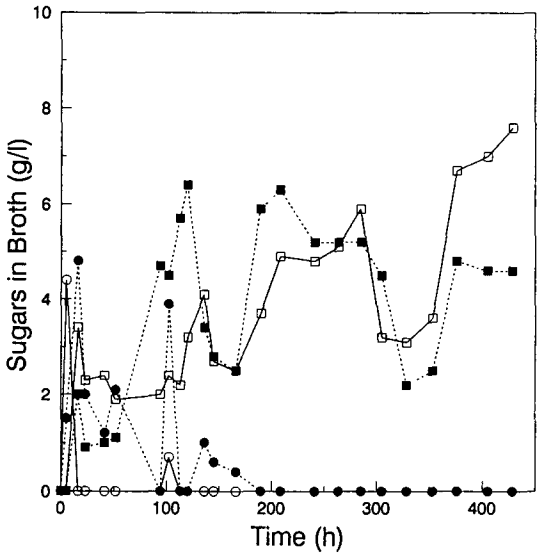

Fig. 2. Concentration profile of sugars in the broth during SSEF. --●-- Cellobiose, —○— glucose, --■-- xylobiose, —□— xylose.

reduced total sugar production by 7%. During straight batch ABE fermentation, butanol and acetone concentration can reach up to 14 g/L and 7 g/L, respectively. The fact that acetone and butanol in the bioreactor are maintained at a low level during SSEF is particularly beneficial from the standpoint of enzyme inhibition.

Simultaneous Saccharification and Extractive Fermentation

Fermentation products were continuously extracted through a semipermeable membrane after butanol concentration reached 7 g/L in the bioreactor. Continuous removal of products lowered the butanol concentration in the bioreactor, which in turn enabled the microorganism to sustain the viability for considerably longer periods of time (428 h) than is possible with batch SSF (100–150 h).

Enzyme, at a loading of 16 IFPU/g dry substrate, efficiently hydrolyzed both the cellulose and xylan fractions of aspen. The "cellulase" enzyme undoubtedly exhibits xylanase activity. Concentration profiles of various sugars are presented in Fig. 2. During the initial phase of the SSF, a substantial amount of glucose and cellobiose was accumulated. Glucose then disappeared completely at the end of first day, and fermentation proceeded under glucose limitation thereafter. The cellobiose level gradually decreased to zero at 200 h (Fig. 2) and was not detected for the remainder of the operation. This is positive proof that *C. acetobutylicum* ferments cellobiose. Xylose and xylobiose were present in the reactor at the level of 2–6 g/L for the entire duration of the experiment (Fig. 2). If xylose and xylobiose were not utilized at all, their accumulation in the bioreactor

Table 2
Extent of Hydrolysis and Utilization
of Carbohydrates in Fed-Batch Operation of SSEF

Carbohydrate	Total charged g/L	Total hydrolyzed g/L	Total utilized g/L
Cellulose	150	134	134
Xylan	62.5	52	37

Table 3
Comparison of Saccharification Rates
in Enzyme Hydrolysis and SSEF

Process	Carbohydrate addition rate g/L/h	Carbohydrate hydrolysis rate g/L/h
Enzyme hydrolysis	0.5	0.28
SSEF	0.47	0.43

would have been much higher, eventually reaching a level as high as 52 g/L (*see* Table 2). This indicates that xylose and xylobiose were consumed simultaneously with glucose, but at a slower rate.

Table 2 summarizes carbohydrates hydrolysis and utilization in fed-batch operation. Overall enzyme loading was 16 IFPU/g pretreated substrate. Cumulative addition of cellulose was 150 g/L, and that of xylan was 62.6 g/L. About 4% of cellulose and 8% of xylan were lost in sampling and bleeding. Percent conversion of carbohydrates was estimated after accounting for these losses. The overall cellulose conversion to glucose was 93%, and it was completely consumed by *C. acetobutylicum*. The extent of xylan conversion to xylose and xylobiose was 90%, from which 71% of the xylose was utilized by the microorganism. Xylose and xylobiose (29%) remained in the bioreactor at the end of the run (Fig. 2).

Presented in Table 3 is the comparison of the hydrolysis rate in SSEF and that of control saccharification run without the presence of microorganism. The control run in which the enzyme loading was 16 IFPU/g yielded the rate of 0.28 g/L/h conversion to monomers or dimers. The extent of conversion was 56%. In case of SSEF, with the same enzyme loading, the rate was substantially higher at 0.43 g/L/h, and percent conversion reached 92%.

Improvement in enzyme efficiency in SSEF resulted from two factors. First, in SSEF, inhibition of cellulase enzymes by sugar was significantly reduced because sugars were consumed as they were produced. Second, the enzyme was retained in the bioreactor at all times while the substrate

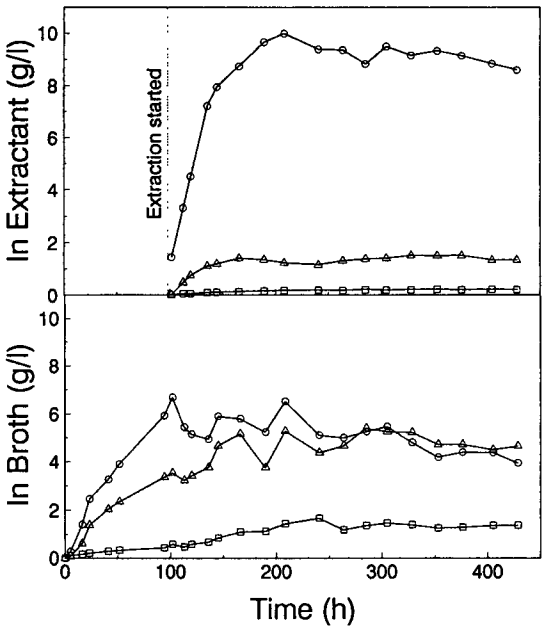

Fig. 3. Concentration profile of solvents in the extractant and in the broth during SSEF. —□— Ethanol, —△— acetone, —○— butanol.

depleted continuously. The effective enzyme loading per unconverted polysaccharides was therefore increased with the progress of fed-batch operation.

Utilization of xylose and glucose was an important feature of this process. Furthermore, both sugars were consumed concurrently, not exhibiting a diauxic consumption pattern. The overall process is therefore greatly simplified by converting the cellulose and hemicellulose fractions of wood in a single step.

Figure 3 shows the concentration profiles of acetone, butanol, and ethanol in the fermentor broth and in the extractant. After extraction was started, the butanol concentration in the broth remained below 6 g/L. This level of butanol was below the inhibition limit (14 g/L) at which ABE production ceases (2). The acetone concentration in the bioreactor steadily increased, since its rate of removal by oleyl alcohol was lower than the rate of production by fermentation. Ethanol also accumulated in the bioreactor for similar reasons. Concentrations of acetone and ethanol in the broth eventually leveled off at 4.7 and 1.4 g/L, respectively (Fig. 3). The levels of each solvent in the extractant also remained constant following the commencement of continuous extraction at 200 h. Acetone and ethanol have low partition coefficients in oleyl alcohol (Table 4). Therefore, concentrations of these two components in the extractant remained at lower levels than in the broth (Fig. 3).

Table 4
Product Recovery and Yield in SSEF

Product	Partition coefficient in oleyl alcohol	% Recovery by extraction[a]	Overall yield[b]
Acetone	0.34	57.8	8.6
Butanol	3.56	81.3	18.1
Ethanol	0.2	33.5	1.5
Acetic acid	0.3	8.7	3.1
Butyric acid	2.87	25.5	2.0

[a]Recovery in 428 h of fed-batch SSEF.
[b]g Product formed/100 g (glucose+xylose) consumed.

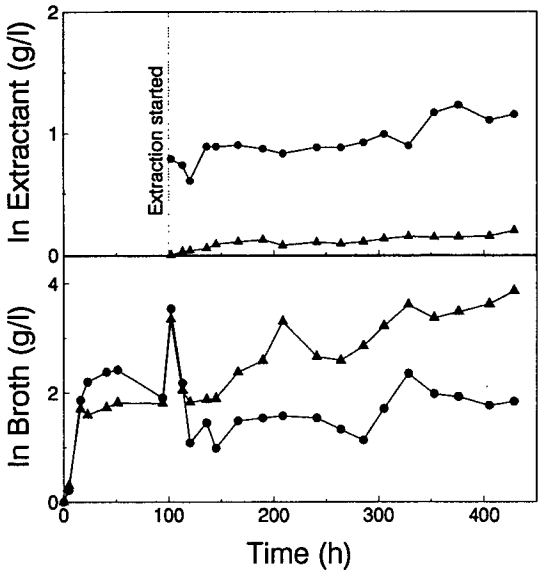

Fig. 4. Concentration profile of acids in the extractant and in the broth during SSEF. —▲— Acetic acid, —●— butyric acid.

The data on product recovery by extraction are shown in Table 4. The extraction recovery for butanol was 81.3%, reflecting its high partition coefficient in oleyl alcohol. On the contrary, acetone and ethanol extractions were much lower at 57.8 and 33.3%, respectively. Percent of solvent recovery by extraction would undoubtedly increase upon extended fed-batch operation (beyond 428 h).

Acetic acid and butyric acid are intermediate products in ABE fermentation, and they play a key role in solventogenesis. Acetone and butanol are produced only when the amount of undissociated butyric acid reaches a certain level in the fermentor (*18*). Acetic acid also accumulates along with butyric acid before the onset of solvent production. Figure 4 shows

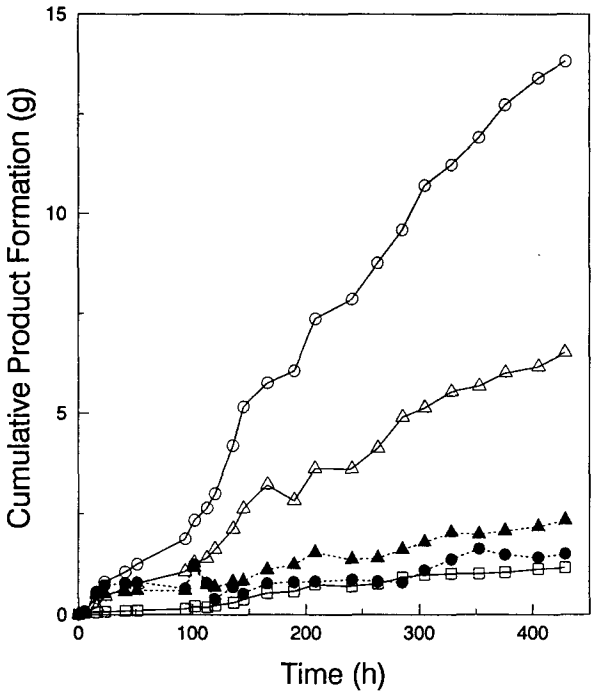

Fig. 5. Cumulative product formation during SSEF. (Broth vol: 400 mL, extraction dilution rate: 0.01 h^{-1}). —□— Ethanol, —△— acetone, —○— butanol; --▲-- acetic acid, --●-- butyric acid.

the concentration of acids in the broth and in the extractant. During the initial phase of the operation, concentrations of both acids reached 3.5 g/L and then dropped significantly thereafter. The level of butyric acid remained between 1-2 g/L, whereas the level of acetic acid kept increasing until the end of operation.

If formation of acids were to be avoided, it would be desirable to retain them in the bioreactor, since they are intermediate products. In the SSEF process, the quantity of acids extracted from the bioreactor is considerably lower than the amount of the solvents extracted. The partition coefficient of acetic acid in oleyl alcohol is low. Small quantities of acetic acid (8.7% of total) were therefore extracted (Table 4). A high partition coefficient of butyric acid in oleyl alcohol (Table 4) could have resulted in transfer of a substantial amount of butyric acid to the extractant. To prevent such losses, regenerated oleyl alcohol containing butyric acid at the level of 0.7 g/L was used. Consequently, the butyric acid concentration in the extractant remained below 1 g/L (Fig. 4). Therefore, only 25.5% butyric acid was removed by extraction despite its high partition coefficient (Table 4).

The cumulative production of acids and solvents is shown in Fig. 5. Solvents were produced at a steady rate until the experiment was terminated at 428 h. This clearly demonstrated that removal of inhibitory

products plays an important role in keeping *C. acetobutylicum* viable over a long period of time. The average volumetric productivities for acetone, butanol, ethanol, acetic acid, and butyric acid were 0.038, 0.081, 0.0068, 0.014, and 0.0088 g/L/h, respectively.

The overall yield of products for the fed-batch run is presented in Table 4. Combined solvent yield of 28.2 g/100 g sugars consumed was lower than that attainable with pure glucose. High acid production in the latter phase of fermentation is believed to be the main reason for the low solvent yield. Total acid yield of 5.1 g/100 g sugars consumed (Table 4) was higher than that normally observed in straight batch fermentation. In fed-batch fermentation using mixed sugars, acid production was found to increase with a decrease in the sugar feeding rate (*19*). The high acid production in SSEF may be associated with low sugar production rates imposed by the rate-limiting hydrolysis of wood chips. It has been reported that the acids also inhibit the production of solvents by *C. acetobutylicum* (*2*). In this study, however, the acids did not reach the inhibitory level.

CONCLUSIONS

In SSEF process, *C. acetobutylicum* cells remained viable for several weeks. Removal of inhibitory products from the system made the fed-batch operation feasible. A relatively constant solvent productivity was observed throughout the fed-batch operation. Acids, the intermediate products, were retained in the bioreactor, whereas solvents were extracted by oleyl alcohol. Cellulase enzyme was efficiently utilized in the bioreactor-extractor system giving a high saccharification rate. Under the glucose-limited condition in SSEF, the microorganism simultaneously consumed glucose and xylose. The overall process was simplified by converting xylose and glucose to fermentation products in a single bioreactor. The solvent yield was lower than that attainable in the glucose fermentation because of the glucose-limited condition prevalent in the SSEF process.

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