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Investigation of hydrogen-producing ability of extremely halotolerant bacteria from a salt pan and salt-damaged soil in Thailand

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ABSTRACT

Extremely halotolerant hydrogen-producing bacteria were investigated, owing to their ability to live in high-salinity conditions. Based on this characteristic, it was hypothesized that extremely halotolerant hydrogen-producing bacteria can tolerate high concentrations of Na^+ ions. To test this hypothesis, we investigated the characteristics of extremely halotolerant hydrogen-producing bacteria obtained from salt-damaged soil in Khon Kaen and a commercial salt pan field near Bangkok (Samut Sakhon), Thailand. The result of this preliminary investigation showed that hydrogen production under saturated conditions of 26% (6 M) NaCl was possible after one year of acclimatization. The extremely halotolerant hydrogen-producing bacteria in this study were also confirmed to have a requirement for Cl^- ions for hydrogen production.

Introduction

The dependence on fossil fuels for energy supply has had great impacts on global warming and climate change [1]. Therefore, the development of alternative renewable energy sources is being pursued globally [2]. Biohydrogen is one of the promising candidates for future use because it is a CO_2-free, clean, and highly efficient energy carrier. Production of biohydrogen can be achieved through bio-photolysis, photo fermentation, and dark fermentation process [3].

Dark fermentation process offers several advantages in industrial biohydrogen production. Among them are high production rates, high yields per mole of substrate, continuous production regardless of solar light condition [4], high variety of carbon sources as substrates, and has no oxygen limitation since the process is fully anaerobic [5,6]. On the other hand, dark fermentation also has several limitations,

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such as thermodynamically unfavorable condition as hydrogen yields increases and carbon dioxide's presence in the produced gas [5].

Among carbon sources to supply fermentable sugars in biohydrogen production, lignocellulosic biomass is a highly considered option. It doesn't compete with food production, available abundantly in nature as grasses and woods, in forestry and agricultural residues, as well as in domestic and industrial wastes. It was estimated that lignocellulosic biomass residue is being produced more than 220 billion tons annually all over the world [7]. However, lignocellulosic biomass requires pretreatment [7] such as alkaline and heat treatment followed by hydrolysis (with enzymes) prior to use as feedstock in fermentative hydrogen production [8]. Most bacteria do not retain their viability after alkaline and heat pre-treatment because of the high concentration of Na^+ ions, as the acidogenesis process in anaerobic digestion is severely inhibited by such conditions [9]. Therefore, an additional step to dilute or neutralize the alkaline conditions is required before proceeding to the next step of fermentative hydrogen production. However, this additional step makes the whole process less economical. One way to overcome this problem is by utilizing extremely halotolerant hydrogen-producing bacteria in dark fermentation process. These bacteria are advantageous for developing 'Next Generation Industrial Biotechnology' (NGIB) as they cut the costs of fresh water, oxygen, and sterilization [10].

In the past few years, several studies have investigated hydrogen production using halotolerant and halophilic bacteria in dark fermentation process. Most of these studies focused mainly on pure cultures in a moderate halophilic environment with a salt concentration of 0.5 M (25 g/L) to 2.5 M (150 g/L), although one study also investigated mixed cultures. Liaw & Mah [11] mentioned production of 144.5 μmol hydrogen from 5 mL medium with 12% NaCl and 0.5% glucose at 37 °C by *Haloanaerobacter chitinovorans* sp. *nov.*, while Mouné et al. [12] found that *Halonanaerobacter salinarius* sp. *nov.* was able to produce 2 mM hydrogen from 4.17 mM glucose substrate in 14–15% NaCl, 45 °C, and pH 7.4–7.8. Matsumura et al. [13] discussed the production of 1.7 mol H_2/mol mannitol by *Vibrio tritonius* strain AM2 at initial 2.25% (w/v) NaCl, pH 6 and 37 °C.

Kivisto et al. [14] reported that *H. saccharolyticum* subspecies *saccharolyticum* produced 0.6 mol H_2/mol glycerol at a salt concentration of 150 g/L (2.6 M), pH of 7.4, temperature of 37 °C, and glycerol concentration of 2.5 g/L, while *H. saccharolyticum* subspecies *senegalensis* produced 1.6 mol H_2/mol glycerol at pH 7.0. Brown et al. [15] found that *H. hydrogeniformans* was capable of producing hydrogen at a pH of 11, 7% (wt./vol.) NaCl, and 33 °C. Pierra et al. [16] described the ability of a mixed culture affiliated to the family of *Vibrionaceae* to produce 0.9 ± 0.02 mol_{H2}/mol $_{glucose}$ at initial pH of 8 and temperature of 35 °C under a moderate halophilic environment (75 g/L NaCl). To date, no studies have investigated hydrogen production under conditions of a high salinity of 26% (6 M or 351.35 g/L NaCl).

This study aims to investigate hydrogen production by extremely halotolerant bacteria. The hypothesis is that extremely halotolerant hydrogen-producing bacteria can tolerate high concentrations of Na^+ ions. Based on this hypothesis, hydrogen production under different salinity concentrations before and after acclimatization was evaluated. The bacteria's requirement for chloride ions in high salinity conditions was also investigated.

Materials and methods

Seed microorganisms and medium

The soil samples were obtained from salt-damaged soil in Khon Kaen, Thailand and a commercial salt pan field near Bangkok (Samut Sakhon), Thailand. The soil samples were mixed with a substrate for cultivation in anaerobic conditions. The composition of the substrate used for biohydrogen production experiments at different salinity concentrations, i.e., between 3–10% and 15–26% NaCl, before the acclimatization experiments was as follows: 2 g/L $NaHCO_3$, 2 g/L K_2HPO_4, 1 g/L yeast extract, 0.7 g/L $(NH_4)_2HPO_4$, 0.75 g/L KCl, 0.85 g/L NH_4Cl, 0.42 g/L $FeCl_3 \cdot 6H_2O$, 0.82 g/L $MgCl_2 \cdot 6H_2O$, 0.25 g/L $MgSO_4 \cdot 7H_2O$, 0.018 g/L $CoCl_2 \cdot 6H_2O$, 0.15 g/L $CaCl_2 \cdot 2H_2O$, and 0.018 g/L $NiCl_2 \cdot 6H_2O$. Glucose concentration were adjusted according to each experiment. All chemicals were purchased from Wako Pure Chemical Industries, Ltd., Japan. The composition of the substrate for the experiments on the bacteria's requirement for chloride ions in high salinity conditions, acclimatization period, and biohydrogen production at 26% NaCl after 2 years acclimatization was the same as that above, except that $NiCl_2 \cdot 6H_2O$ was omitted.

Culture conditions and experimental procedures

The first step of the experiment was to evaluate the hydrogen production in conditions of 3–10% salinity before acclimatization. The experiments were done under six NaCl concentrations of 3%, 3.5%, 5%, 7%, 7.5%, and 10%. The second step of experiment was to evaluate the hydrogen production in conditions of 15–26% salinity before acclimatization and at 26% salinity after an acclimatization period of 2 years. The experiment was done at NaCl concentrations of 15%, 20%, and 26%.

The third step of the experiment was the evaluation of the bacteria's requirement for chloride ions in high salinity conditions. The experiment was done by comparing the culture under Na_2SO_4: NaCl ratios of 1:1 and 4:1. The ratio was prepared by weight to reach 26% (351.35 g/L) concentration of the mix. The third step was done after one year of acclimatization.

The experiments to determine biohydrogen production under salinity concentrations of 3–10% and 15–26% NaCl before acclimatization and various F/M ratio at 15% NaCl were done under the following conditions: 100 mL sealed serum bottles in a nitrogen atmosphere with an initial pH of 6.8 adjusted with 1 M HCl and 1 M NaOH and incubated in a shaking incubator (BT 100 & BT 300; Yamato Scientific Co., Ltd. Japan) at 35 °C and a 100-rpm shaking speed. Biogas samples were periodically collected and the compositions were analyzed via gas chromatography.

The experiment to determine the bacteria's requirement for chloride ions in high salinity conditions followed the same conditions, except that 125-mL serum bottles were used. The main culture bottles with a volume of 500 mL each for the

three different sources of soil were also maintained. These bottles were also used for acclimatization purposes. In acclimatization period of two years, the substrate's NaCl concentration was kept at 26%. Gas production was periodically measured, and substrate was changed after no gas production was detected. Anaerobic condition was maintained under nitrogen atmosphere.

Biohydrogen production at a NaCl concentration of 26% after 2 year period of acclimatization experiments was also studied under the same conditions, except that the initial pH was not adjusted, the serum bottles had a volume of 75 mL, and shaking incubator temperature was at 37 °C.

Analytical method

The initial pH was adjusted by using a pH meter (D-13; Horiba Co. Ltd. Japan). The volume of the produced biogas was measured with a glass syringe. The composition of H_2, N_2, CH_4, and CO_2 was analyzed via gas chromatography (GC-8APT/TCD; Shimadzu Corp. Japan) with a 60/80 activated charcoal mesh column (1.5 m × 3.0 mm internal diameter) and Argon as a carrier gas, with operational temperatures of the injector, column, and detector of 50 °C, 60 °C, and 50 °C, respectively. The compositions of volatile fatty acids (VFAs) were determined by gas chromatography (GC-8APF/FID; Shimadzu Corp. Japan) with a flame ion detector (FID) and a Unisole F-200 30/60 glass mesh column (3 m × 3.2 mm internal diameter). The operational temperatures for the injection port, FID detector, and oven were 250 °C, 140 °C, and 140 °C, respectively.

The water content and total organic matter of the soil was determined via the JIS A 1203 test method for water content of soils and JIS A 1226-2000 test method for ignition loss of soils [17,18]. Sample masses of 20.5506–44.1789 g were used to determine the soil's water content and ignition loss. Volatile Suspended Solid (VSS) was determined according to method 2540 E of *Standard Methods* [19].

Soil salinity was determined by mixing soil and distilled water in 1:2.5 dry soil to water ratio, shaken for 3 h at 180 rpm (Eyela Multishaker MMS; Tokyo Rikakikai Co, Ltd. Japan). After 25 min of settling time, the supernatant is measured by a thermo salinity meter (TS-391; As One Corp. Japan). The measurement results were then multiplied by the dilution factor of water content of soil.

Theoretical hydrogen production and yield

The theoretical hydrogen production was determined using equations (1) and (2) in Table 1. Based on equation (1), 1 mol of glucose will produce 4 mol of hydrogen. Thus, 1 g of glucose at standard temperature and pressure (STP) conditions will produce 498 mL of H_2 via the acetic acid (HAc) pathway. Based on equation (2), 1 mol of glucose will produce 2 mol of hydrogen; hence, 1 g of glucose at STP conditions is required to produce 249 mL of H_2 via the butyric acid (HBu) pathway.

Table 1 – Standard Gibbs energy of formation for glucose fermentation (obtained from Refs. [20,21]).

Equations of fermentative reactions		ΔG^0
$C_6H_{12}O_6 + 4H_2O \rightarrow 2CH_3COO^- + 2HCO_3^- + 4H^+ + 4H_2$	(1)	−206 kJ
$C_6H_{12}O_6 + 2H_2 \rightarrow CH_3(CH_2)_2COOH + 2CH_3COO^- + H^+ + 2H_2$	(2)	−254 kJ
$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COO^- + 2H_2O + 2H^+$	(3)	−358 kJ
$C_6H_{12}O_6 \rightarrow 2CH_3CH(OH)COO^- + 2H^+$	(4)	−198 kJ
$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3CH_2OH + 2HCO_3^- + 2H^+$	(5)	−358 kJ

Table 2 – Biohydrogen production at 3–10% salinity of salt-damaged soil from Khon Kaen.

Salt concentration (%)	Biohydrogen production (ml)	Theoretical H_2 production (%)		Yield ($molH_2/mol_{glucose}$)
		HAc pathway	HBu pathway	
3	10.9	14.7	29.5	0.61
3.5	10.9	14.7	29.5	0.61
5	9.46	12.8	34.6	0.53
7	13.4	18.1	36.2	0.75
7.5	7.43	10	20.1	0.41
10	18.1	24.5	49	1.01

Glucose 0.15 g (5000 mg/L), inoculum 3000 mg/L VSS, F/M ratio 1.5.

Results and discussion

Biohydrogen production at 3–10% salinity

Table 2 compares the biohydrogen production at salinities of 3%–10% for a culture from salt-damaged soil from Khon Kaen, Thailand. Very low hydrogen production was observed at these salinity conditions. No methane was produced at salinities of 7.5% and higher. The experiments were conducted for 15 days with 0.15 g glucose for each 100-mL serum bottle. The maximum theoretical cumulative hydrogen yield was 74 mL H_2 for the acetic acid (HAc) pathway, and 37 mL H_2 for the butyric acid (HBu) pathway. The highest yield of 1.01 mol $H_2/mol_{glucose}$ was achieved at 10% salinity.

This is because the salt-damaged soil in its natural state is always exposed to a high salt concentration; thus, lower salt concentrations might not be suitable for extremely halotolerant anaerobic microorganisms to grow. Another reason for this is that the food to microorganism (F/M) ratio at 1.5 mg/L volatile suspended solids (VSS)/mg/L of the substrate might not be ideal for production for 3–10% salinity conditions.

Loss on ignition (LOI) is one of the most commonly used methods for quantifying soil organic matter [22]. The LOI results in Table 3 correspond to mixed sediment with low organic matter content obtained by Heiri et al. [23]. Microbial biomass is usually low in salt-affected soils [24]. The Khon Kaen salt-damaged soil had a lower moisture content than the soil from the Samut Sakhon salt pan. Water content in soil is an important factor that influences the microbial activity of aerobic and anaerobic bacteria, and it also affects osmotic potentials of saline soils [25].

Although LOI percentage and water content from Samut Sakhon salt pan showed a higher value, but the soil sample from the location gave lower biohydrogen yields, as shown in Tables 5 and 7. One of the possible reason for this, is that high organic contamination doesn't occur in the salt pan since it

Table 3 – Soil characteristics of Khon Kaen salt-damaged soil and soil from Samut Sakhon salt pan.

	Water content (%)	Ignition Loss after 600 °C (g)	Loss on ignition (%)	Salinity (%)
Khon Kaen salt damaged soil				
at the shore	32.94	0.42	2.37	7.74
close to the shore	21.33	0.25	1.57	20.16
farther from the shore	13.56	0.32	3.16	30.26
Samut Sakhon salt pan				
Salt pan surface	77.29	0.15	4.35	1.92
salt pan at 5 cm depth	60.4	0.95	5.41	3.45
Dry salt pan surface	43.26	0.04	0.43	7.80
Dry salt pan at 5 cm depth	40.15	0.29	1.51	6.15

Table 4 – Biohydrogen production at 15–26% salinity of salt-damaged soil from Khon Kaen.

Salt Concentration (%)	Biohydrogen production (ml)	Theoretical H_2 production (%)		Yield ($molH_2$/$mol_{glucose}$)
		HAc pathway	HBu pathway	
15	49.8	67.3	134.6	2.78
20	0.02	0.03	0.05	0.00
26	0	0	0	0.00

Glucose 0.15 g (5000 mg/L), inoculum 3000 mg/L VSS, F/M ratio 1.5.

was under a protected environment to maintain the purity of the salt, unlike the salt damaged soil of Khon Kaen. A major part of the organic matter that contributed to the LOI value in Samut Sakhon salt pan could be refractory organic. In Samut Sakhon salt pan's location, no plants and fish were observed, so not many supply for organic matter were available.

Biohydrogen production at 15–26% salinity

The experiments on biohydrogen production at 15–26% salinity were performed twice. The first was done before acclimatization and the second was after two years of acclimatization. Table 4 compares biohydrogen production at the initial salinity of 15–26% before acclimatization. The highest production of 2.78 mol H_2/$mol_{glucose}$ occurred at 15% salinity. Theoretical hydrogen production value exceeding 100% of HBu pathway at 15% salinity suggested that the HAc pathway took place.

Fig. 1 shows that after a lag phase of 8 days, the cumulative hydrogen production at 15% salinity was significantly higher (49.8 mL (Table 4)) than that at 10% salinity (18.1 mL (Table 2)).

During the initial experiment, almost no hydrogen was produced under conditions of 20% salinity or more. After an acclimatization period of 2 years, confirmation experiments were conducted at 26% salinity (Table 5). The results showed that hydrogen production was possible at this concentration, with hydrogen yields of 0.66–1.15 mol H_2/$mol_{glucose}$.

Khon Kaen salt damaged soil (1) was taken from the shore part of the pond, with finer soil and more water content. Khon Kaen salt damaged soil (2) was taken from the farther part of the pond, approximately 10 m apart toward drier land with coarser soil and less water content. Both samples were taken during the dry season. During the rainy season, the surfaces of both sampling locations are covered with water.

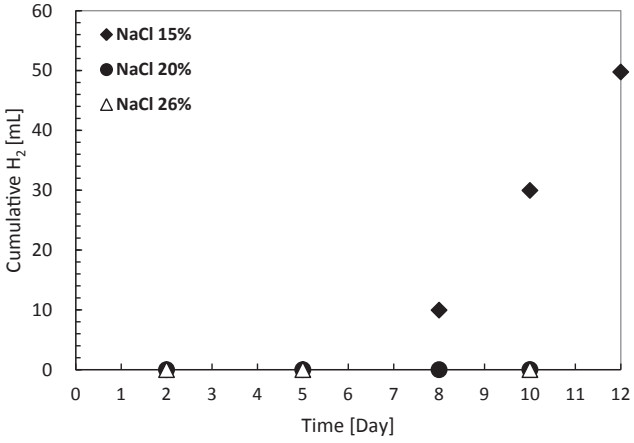

Fig. 1 – Cumulative hydrogen production of salt-damaged soil in Khon Kaen.

In Table 1, equations (1) and (2) show hydrogen production through the acetic acid and butyric acid pathways, respectively. Equations (3)–(5) show the pathways with no hydrogen production. The equations express the pathways of propionic acid fermentation, lactic acid, and alcohol fermentation, respectively.

The possible reason for the hydrogen yield being below the theoretical value is that the low F/M ratio produced conditions that were not optimum for the pathways expressed by equations (1) and (2). From the comparison of the standard Gibbs energy of the formation values, it can be assumed that the pathways for propionic acid fermentation (Equation (3)) and alcohol fermentation (Equation (5)) are more spontaneous than the rest. Thus, they are more likely

Table 5 – Biohydrogen production at 26% salinity after two years of acclimatization.

Soil Sample	Biohydrogen production (ml)	Theoretical H_2 production (%)		Yield ($molH_2$/$mol_{glucose}$)
		HAc pathway	HBu pathway	
Samut Sakhon salt pan	13.44	27.02	54.03	1.08
Khon Kaen salt damaged soil (1)	14.31	28.76	57.55	1.15
Khon Kaen salt damaged soil (2)	8.22	16.53	33.07	0.66

Glucose 0.12 g (5,000 mg/L), inoculum 10 mL (3 mg/L VSS).

Table 6 – Biohydrogen production at 15% salinity experiments for 0.5–2.0 F/M ratio of salt-damaged soil from Khon Kaen before acclimatization.

F/M ratio	Glucose (g)	Glucose (mg/L)	Biohydrogen Production (ml)	Theoretical H_2 production (%)		Yield ($molH_2$/$mol_{glucose}$)
				HAc pathway	HBu pathway	
0.5	0.045	1500	8.26	36.9	73.8	1.48
1	0.09	3000	24.49	54.7	109.3	2.19
1.5	0.15	5000	49.75	67	134.1	2.67
2	0.18	6000	5.31	1.34	2.7	0.24

Inoculum 3000 mg/L VSS.

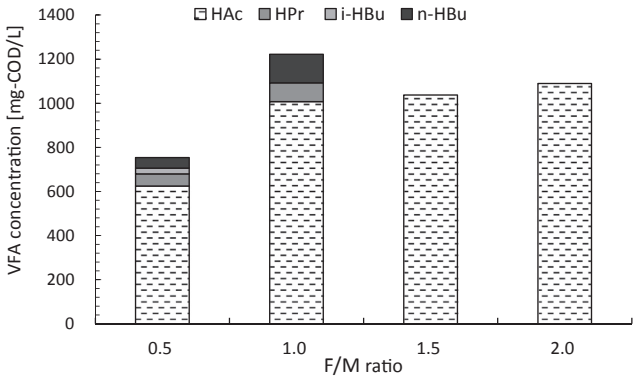

Fig. 2 – VFA composition for 0.5–2 F/M ratio at 15% salinity experiments of salt-damaged soil from Khon Kaen before acclimatization. (HAc = acetic acid, HPr = propionic acid, i-HBu = isobutyric acid, n-HBu = n-butyric acid).

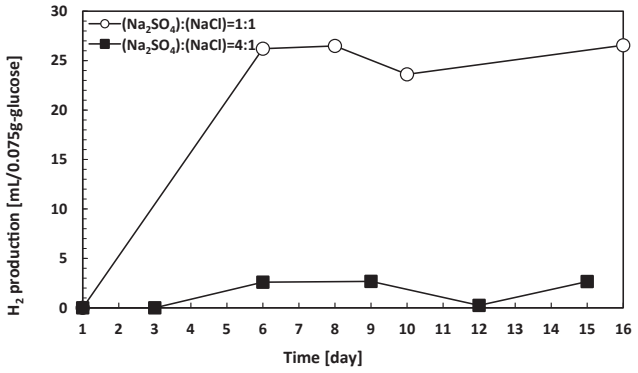

Fig. 3 – Cl^- ion requirement for H_2 production in salt-damaged soil of Khon Kaen.

to occur, as the substrate concentration was low, the reaction rate was high, and the hydrogen recovery rate was low. The production of propionate can decrease the production of hydrogen [26–29]. Very low substrate concentrations can be unsuitable for hydrogen production as shown in Table 6. Fig. 2 showed the composition of VFAs produced for each F/M condition in Table 6. For F/M ratio of 1.5 and 2, almost all propionic acid and butyric acid were transformed to acetic acid.

Evaluation of the bacteria's requirement for chloride ions in high salinity conditions

In this experiment, Na_2SO_4 was used to partially replace NaCl in two different ratios (1:1 and 4:1) to confirm the extremely halotolerant hydrogen-producing bacteria's requirement for Cl^- ions. The substrate was adjusted such that the overall salinity of the mixture of Na_2SO_4 and NaCl was 26%. The bacteria's requirement for Cl^- ions was confirmed by a clear difference in the amount and yield of hydrogen production at different ratios of Na_2SO_4 to NaCl (Fig. 3 and Table 7).

Halophilic archaea and halophilic fermentative bacteria use the 'salt-in' strategy in their survival mechanism in extremely hypersaline conditions [30,31]. To adapt to this condition, cells maintain high salt concentrations at the intracellular level to sustain isosmotic conditions within the cell. Usually, K^+ and Cl^- ion salts are accumulated in molar concentrations at the intracellular level [30]. Cl^- is the preferred anion accumulated in the 'salt-in' strategy and it is possible that it plays critical roles in haloadaptation [31]; however, some halophilic microorganisms also utilize sulfate in high concentrations [30,32].

Table 7 – Biohydrogen production at different ratios of Na_2SO_4 to NaCl.

Soil Sample	(Na_2SO_4): (NaCl)	Glucose (g)	Glucose (mg/L)	Inoculum (mg/L VSS)	Biohydrogen production (ml)	Theoretical H_2 production (%)		Yield (mol H_2/ $mol_{glucose}$)
						HAc pathway	HBu pathway	
Samut Sakhon salt pan	1:1	0.022	7500	5000	1.77	16.0	32.1	0.64
Khon Kaen salt damaged soil (1)		0.0044	2250	1500	1.57	71.2	–	2.85
Khon Kaen salt damaged soil (2)		0.0044	2250	1500	0.26	23.3	46.7	0.47
Samut Sakhon salt pan	4:1	0.075	1500	1000	0	0	0	0.00
Khon Kaen salt damaged soil (1)					2.66	7.12	14.2	0.29
Khon Kaen salt damaged soil (2)					0	0	0	0.00

Conclusions

The experimental results showed that it is possible to produce biohydrogen under high salt concentrations (26% NaCl). This indicates that extremely halotolerant hydrogen-producing bacteria can exist under such concentrations. The extremely halotolerant hydrogen-producing bacteria were also confirmed to require Cl^- ions. Further studies will be made to investigate the microbial community characteristics and their applications for hydrogen production from lignocellulosic biomass.

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