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ORIGINAL PAPER

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# Growth of *Bacillus methanolicus* in seawater-based media

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**Abstract** *Bacillus methanolicus* has been proposed as a biocatalyst for the low cost production of commodity chemicals. The organism can use methanol as sole carbon and energy source, and it grows aerobically at elevated temperatures. Methanol can be made available from off-shore conversion of natural gas to methanol, through gas-to-liquid technology. Growth of the organism in seawater-based medium would further reduce the costs of chemical production performed near an off-shore natural gas source. The growth of strain PB1 (ATCC 51375) in shake flask experiments with trypticase soy broth medium showed minimal salt-inhibition at the concentration of NaCl in seawater. The ability of *B. methanolicus* PB1 to grow in Pacific Ocean water using methanol as a carbon and energy source was also tested. Following a simple adaptation procedure, PB1 was able to grow on methanol in semi-defined medium with 100% seawater with good growth yields and similar growth rates compared with those achieved on media prepared in deionized water.

**Keywords** *Bacillus methanolicus* · Salt-tolerant · Seawater · Adaptation

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## Introduction

The chemical industry is continually under pressure to develop processes that are more energy efficient, environmentally friendly, and cost-effective. While many large chemical companies have explored enzymes or microbial processes as a potential route to developing improved processes, it is well established that the key to the success of these processes, often requiring many years of research and development, is their ability to compete financially with their chemical process counterparts [21]. *Bacillus methanolicus* is a thermotolerant, Gram-positive, restrictive faculatative bacterium that shows great promise as an industrial host organism for the production of commodity chemicals. It has been demonstrated to grow in a medium with minimal complex nutrients and methanol as a sole carbon and energy source [1, 2, 7, 17]. The organism has been genetically modified to produce lysine [9, 12, 17] and, in its native form, has been shown to produce glutamic acid [3, 15,16]. The ability to grow in methanol has recently been shown to be encoded on a 19 kb endogenous plasmid [2].

That *B. methanolicus* uses methanol for growth is relevant to the petroleum industry, which is not able to capture and utilize all the natural gas that is produced at off-shore oil wells [6, 8]. At current prices, the revenue from natural gas sales does not offset the cost of transformation and transportation of this feedstock or fuel to end-users. Thus, there may be commercial interest, as well as environmental benefits, in utilizing the methane from off-shore oil rigs for on-site production of commodity and specialty chemicals using microbial technologies. The technology for the conversion of natural gas to methanol is known as gas-to-liquid (GTL) technology [10, 18, 19]. Methanol can serve as the substrate for the production of other higher valued chemicals at off-shore production sites using *B. methanolicus* as a catalyst.

The use of seawater for bacterial growth media has been described for various marine microorganisms as well as for methanol-utilizing yeast [20]; however, no

publications have described the use of seawater as the liquid component of the growth medium for *B. methanolicus*. The salt tolerance of *B. methanolicus* has been described previously [1, 15]. Pluschkell [15,16] found that the growth rate of *B. methanolicus* MGA3 grown in medium containing 25 g/l NaCl, the salt concentration in seawater, was reduced by about 25% compared to that in medium prepared from distilled water. The final dry biomass concentration of the 25 g/l NaCl culture was about one-half that in distilled water. The composition of various seawaters have been determined, and artificial seawater can be made (http://www.stanford.edu/group/Urchin/seawater.htm). In the present report, the growth properties of *B. methanolicus* have been compared in trypticase soy broth (TSB—a complex medium), at a range of added salt concentrations up to the concentration found in seawater (25 g/l). In addition, the growth properties in a semi-defined medium prepared from seawater using methanol as carbon source are reported. *B. methanolicus* strain PB1 can grow with comparable growth rates in both the semi-defined medium prepared with salt levels found in seawater, as well as in media of low salt content, after carrying out a simple adaptation process [13].

## Materials and methods

*B. methanolicus* strain PB1 was purchased from the American Type Culture Collection (ATCC; Rockville, Md.). The PB1 strain was originally deposited in the National Collection of Industrial and Marine Bacteria and ATTC as NCIMB 133113 and ATCC 51375, respectively. TSB (BBL, Cat. no. 211768), was purchased from Fisher (Pittsburgh, Pa.), and vitamin $B_{12}$ was purchased from Alexis Biochemicals (San Diego, Calif.). Trypticase soy agar plates were prepared from TSB with the addition of 1.5% agar (w/v). Deionized water was used to prepare all buffer solutions. Minimal yeast (MY) medium was prepared as previously described [16].

Pacific Ocean seawater was collected from the shoreline of Santa Cruz (California) during a high-tide condition. In order to separate the dirt sediments from Pacific Ocean seawater, the seawater was initially filtered through filter paper (Whatman no. 2, Cat. no. 1001917). The seawater was then filter-sterilized (Corning 0.22 μm bottle filter, Cat. no. 430769; Corning, N.Y.). Methanol was added to the Pacific Ocean seawater at 1% (v/v) as the carbon source for *B. methanolicus*. Yeast extract (0.5 g/l), trace metals, biotin (1.0 μg/l) and vitamin $B_{12}$ (20 μg/l) were added to the seawater as in the MY medium [16]. The composition of the artificial seawater is given in Table 1. As with media prepared from natural seawater, no magnesium salts were added to MY medium due to the high concentration of $Mg^{2+}$ in the sea salts.

Shake flasks were prepared with a liquid volume of 200 ml in 1 l baffled Erlenmeyer flasks with metal vent caps. The culture medium was inoculated with 2% (v/v) 10-h-grown inoculum. The culture medium was pre-warmed to 50°C before the addition of inoculum. The culture conditions were set at 50°C with shaking at 200 rpm in an incubator shaker (Model G25; New Brunswick Scientific, Edison, N.J.).

**Table 1** Formula for artificial seawater (http://www.stanford.edu/group/Urchin/seawater.htm)

| Compound | Concentration (g/l) |
|---|---|
| NaCl | 24.60 |
| KCl | 0.67 |
| $CaCl_2{\cdot}2H_2O$ | 1.36 |
| $MgSO_4{\cdot}7H_2O$ | 6.29 |
| $MgCl_2{\cdot}6H_2O$ | 4.66 |
| $NaHCO_3$ | 0.18 |

Absorbance at 500 nm was determined with a UV/Vis Spectrophotometer (Hewlett Packard Model 8452A) every 60–120 min. Measurements of dry cell weight were correlated to the optical density measurements and found to obey

$$\text{dry biomass} = (0.178 \pm 0.009) \times \text{optical density}$$

Cell pellets were washed twice with ten volumes of deionized water, dried and weighed.

The osmolality, defined as the number of osmoles of solute particles per kilogram of $H_2O$, was determined for all growth media using an Advanced Micro-Osmometer (Model 3MO Plus; Advanced Instruments, Needham Heights, Mass.). The osmometer was calibrated using the 50 mOsmol/kg$H_2O$ and 850 mOsmol/kg$H_2O$ standard solutions (Advanced Instruments, Cat. nos. 3MA005 and 3MA085, respectively) according to the operation manual from manufacturer. The osmolality of samples were measured using the Advanced 20-Microliter Sampling System Kit (Advanced Instruments, Cat. no. 3MO825) in accordance with standard operating procedures.

### Determination of methanol concentration

In order to monitor the substrate (methanol) concentration during growth, the residual methanol concentration in the samples was determined with a membrane probe (Biochemistry Analyzer YSI, Model 2700 Select, Yellow Springs, Ohio). Methanol membranes (YSI, Cat. no. 2725) and carbonate buffer (YSI, Cat. no. 1579) were employed with the Biochemistry Analyzer for this application. Standard operation procedures were followed for the determination of methanol concentration in the samples.

### Adaptation to growth in high salt medium

TSB was used for the adaptation procedure. Salt was added to five flasks of 100 ml TSB at five different concentrations (5, 10, 15, 20 and 25 g/l NaCl). After

growth of strain PB1 for 12 h in TSB without additional salt added, 2 ml of the culture was used to inoculate a flask containing 5 g/l added NaCl. Again, following 12 h of incubation, 2 ml of the 5% added salt culture was used to inoculate a flask containing 10 g/l added NaCl, and so on up to 25 g/l added salt. The cells from this final flask were stored frozen after the addition of 30% glycerol and used for further growth studies.

### Frozen storage of bacterial cultures

The cultures were grown to an optical density of 2 in TSB, and 30% glycerol was added prior to frozen storage. The bacteria were stored at −80°C.

## Results and discussion

Microorganisms that utilize methanol produce about 3.5 times more metabolic heat per gram biomass than organisms that grow on glucose. Because of this, the cost savings from the use of methanol, which is currently comparably priced with glucose on a weight basis [4, 11] will be confounded by the additional cost of cooling water to maintain the optimal process temperature in the reactor. Since *B. methanolicus* is thermotolerant, growing optimally between 50 and 55°C, a dramatic reduction in cooling water is a major cost benefit of employing this organism.

Figure 1 shows a comparison of the cooling water requirements as a function of fermentation temperature and reactor liquid volume during a metabolic heat production of 124 kJ $l^{-1}$ $h^{-1}$ [15]. Data from 11 l fed-batch fermentations of lysine-producing *B. methanolicus* indicated that the heat generated under peak metabolic activity of the organism is 124 kJ $l^{-1}$ $h^{-1}$ [12]. The assumptions for the calculation of the cooling water requirement for large volume fed-batch reactors are given in Table 2.

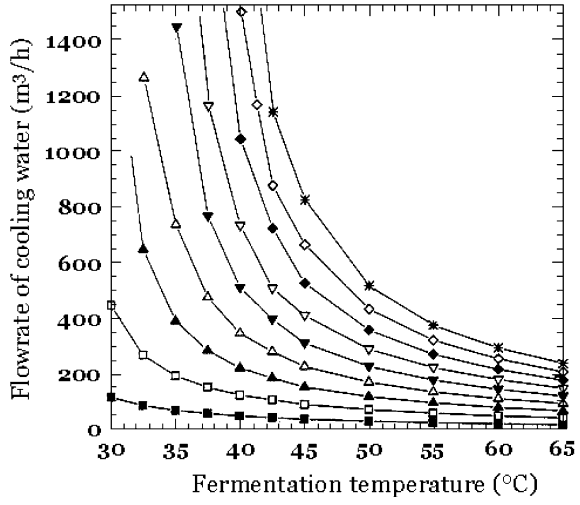


**Fig. 1** Cooling water requirements as a function of fermentation temperature and reactor liquid volume during a metabolic heat production of 124 kJ $l^{-1}$ $h^{-1}$ . The coolant inlet temperature is 10°C. ■ 50 $m^3$, □ 100 $m^3$, ▲; 150 $m^3$, △ 200 $m^3$, ▼; 250 $m^3$, ▽ 300 $m^3$, ◆ 350 $m^3$, ◇ 400 $m^3$, * 450 $m^3$

At about 200 $m^3$ reactor liquid volume, the cooling water requirements for an organism growing on glucose at 35°C and an organism growing on methanol at 50°C are equivalent. At larger reactor sizes, the cost saving at a reaction temperature of 50° becomes very significant.

Initial shake flask experiments compared the growth of *B. methanolicus* in TSB with added salt concentrations varying from 0 to 25 g/l. The osmolality of the broth was found to have a linear relationship with the added NaCl according to the following equation

TSB :

$$\text{osmolality (mOsm/kg H}_2\text{O)} = (33.51 \pm 0.93) \times \text{added salt (g/l)} + 280.57 \pm 14.00$$

The osmolality in MY medium with added salt concentrations of 0–25 g/l likewise obeyed a linear relationship as follows

MY :

$$\text{osmolality (mOsm/kg H}_2\text{O)} = (32.05 \pm 0.28) \times \text{added salt (g/l)} + 178.86 \pm 4.26$$

The effect of NaCl on the growth of *B. methanolicus* in TSB is shown in Fig. 2. For all salt concentrations, the exponential growth phase begins at approximately the same time after inoculation. Without added salt, the specific growth rate and final dry biomass were determined to be $0.32 \pm 0.04$/h and $0.88 \pm 0.09$ g/l, respectively. Up to 10 g/l added salt there was no observed effect on specific growth rate, but at 25 g/l it decreased to $0.21 \pm 0.02$/h. The final concentration of dry biomass was reduced in the presence of 10 g/l or more of added salt. At 25 g/l added salt, the biomass was measured at $0.16 \pm 0.01$ g/l.

The effect of salt was also compared in MY medium (Fig. 3). As in TSB, the growth rate was not affected as significantly as the final biomass concentration. Up to 15 g/l added salt, the specific growth rate was determined to be $0.32 \pm 0.01$/h, and decreased at higher salt concentrations. At 25 g/l added NaCl, the maximum specific growth rate was determined to be $0.18 \pm 0.012$/h. The final dry biomass concentration was slightly higher in MY medium than in TSB. At no added salt, the biomass was determined to be 1.13 g/l and increased slightly to 1.67 g/l at 15 g/l. Further addition of salt reduced the biomass and at 25 g/l added salt the concentration was measured as 0.32 g/l.

Comparing the measurements in TSB and MY medium, the growth and biomass concentration are highest at about 600 mOsmol/kg$H_2O$. However, the osmolality of Pacific Ocean water was determined to be 981 mOsmol/kg$H_2O$. Thus, a method of adaptation

**Table 2** Estimations used for the calculation of the cooling water requirement for large-scale reactors for growth of *Bacillus methanolicus*

| | |
|---|---|
| Reactor | |
| Height to diameter ratio | $H/D = 4$ |
| Cooling coil diameter | $d = 0.1$ m |
| Estimated coil surface area available for heat exchange | $A = (0.8H/2d)(0.8\pi\ D)\pi\ d = 12.63D^2$ |
| Overall heat transfer coefficient | $U = 300$ Btu/h·ft$^2$·F = 6,132.56 kJ/h·m$^2$·°C) [14] |
| Cooling water inlet temperature | 10°C |
| Driving force for heat exchange | $\Delta T$ = logarithmic mean temperature difference between coolant and reactor operating temperature |
| Aeration (dry air) | 0.5 m$^3$ /(min·m$^3_{\text{liquid volume}}$) [12] |
| Heat input | |
| By reaction | |
| Specific oxygen uptake rate | 9 mmol $O_2$/(h·$g_{\text{dry biomass}}$) [12] |
| Heat evolution | 460 kJ/mol $O_2$ [5] |
| Dry biomass concentration | 30 g/l |
| By agitation | |
| Power requirement | 2 kW/m$^3_{\text{liquid volume}}$ |
| Motor/gear box efficiency | 92% |
| Heat loss | |
| By evaporation | This term was estimated by calculating the heat required to saturate dry air with water at the operating temperature [14] |

was employed to enhance the growth properties of *B. methanolicus* in medium with the salt concentration of seawater (http://www.stanford.edu/group/Urchin/seawater.htm).

The shake flask growth experiments with PB1 in TSB with varying NaCl concentrations described above were repeated using cultures (defined as PB1A$^{ST}$) that had been adapted to high salt concentration. A comparison of the final biomass concentrations of PB1 and PB1A$^{ST}$ are shown in Fig. 4. Strain PB1A$^{ST}$ shows similar final biomass concentrations at all salt concentrations tested. At 25 g/l added salt, the final dry biomass was measured as 0.97 g/l. As compared with PB1, these results show approximately a threefold improvement in final biomass concentration for PB1A$^{ST}$.

The adapted strain PB1A$^{ST}$ was tested for growth in natural seawater- and artificial seawater-based media. As can be seen from the composition of the artificial seawater (Table 1), the magnesium concentration is significantly higher than in the MY medium with methanol (48 mM in seawater vs 1 mM molar in MY medium). Therefore, further addition of magnesium was not required for bacteria growing in seawater. The measured osmolality of the artificial seawater, 971 mOsmol/kg$H_2O$, is very similar to the natural seawater (981 mOsmol/kg$H_2O$) used in these experiments. Likewise, the seawater osmolality values are similar to that of the minimal salt solution with 25 g/l added NaCl, which is 979 mOsmol/kg$H_2O$. Figure 5 shows a comparison of growth in artificial and natural seawater-based media. In medium prepared from artificial seawater, the maximum

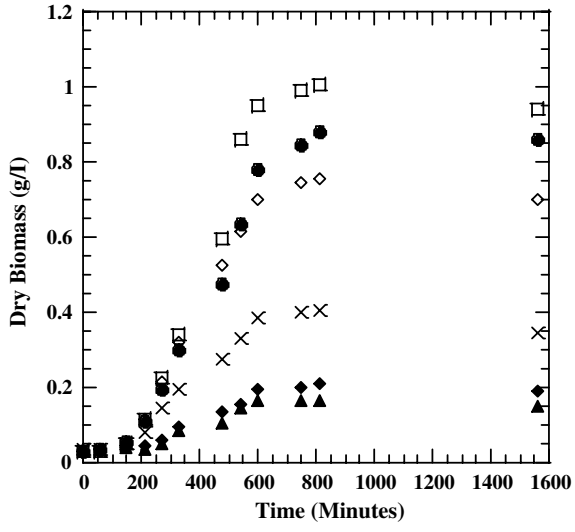


**Fig. 2** The effect of added sodium chloride concentration on the growth of *Bacillus methanolicus* strain PB1 in trypticase soy broth (TSB). The strain had not been adapted for growth in high salt media. ● 0 g/l, □ 5 g/l, ◇10 g/l, x 15 g/l, ◆ 20 g/l, ▲; 25 g/l

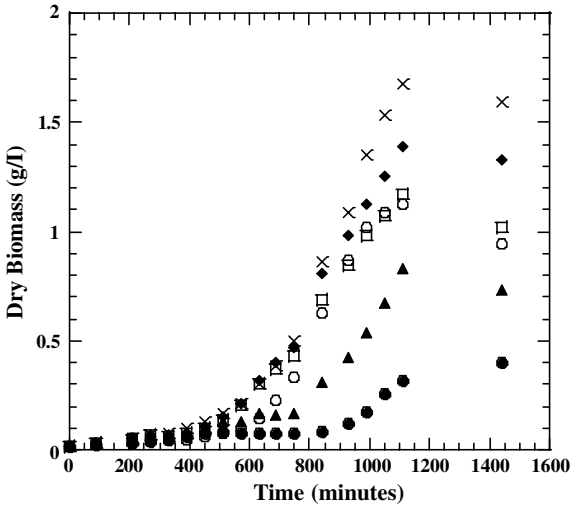


**Fig. 3** The effect of sodium chloride on the growth of strain PB1 in minimal yeast (MY) media. The strain had not been adapted for growth in high salt media. ○ 0 g/l, □ 5 g/l, ◆10 g/l, x 15 g/l, ▲; 20 g/l, ● 25 g/l

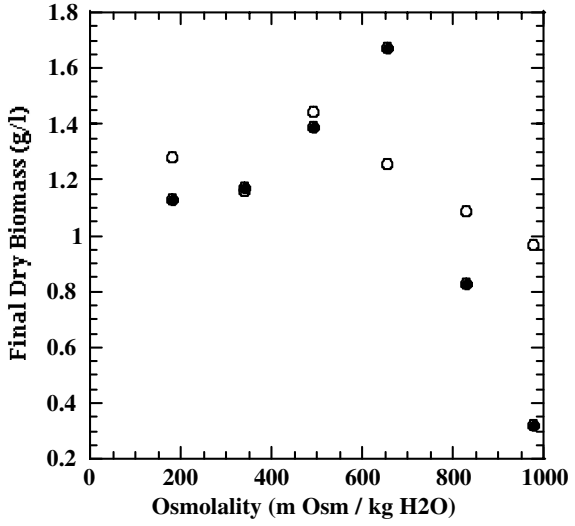


**Fig. 4** Comparison of the final dry biomass concentrations of cultures of PB1 at different concentrations of salt added to MY medium before (●) and after (○) adaptation to high salt media

specific growth rate was measured as $0.33 \pm 0.03$/h and in Pacific seawater, the growth rate was determined to be $0.33 \pm 0.04$/h. As compared with strain PBI in MY medium with 25 g/l NaCl which had a specific growth rate of $0.14 \pm 0.02$/h, (see Fig. 3), this result shows a twofold improvement in specific growth rate for strain PBIA$^{ST}$. The final biomass concentrations in natural and artificial seawater were determined to be 0.44 g/l and 0.40 g/l, respectively.

It is interesting to note that the further addition of yeast extract above 0.5 g/l to the high salt medium can improve the growth characteristics of PB1A$^{ST}$. A comparison of growth in natural seawater-based medium

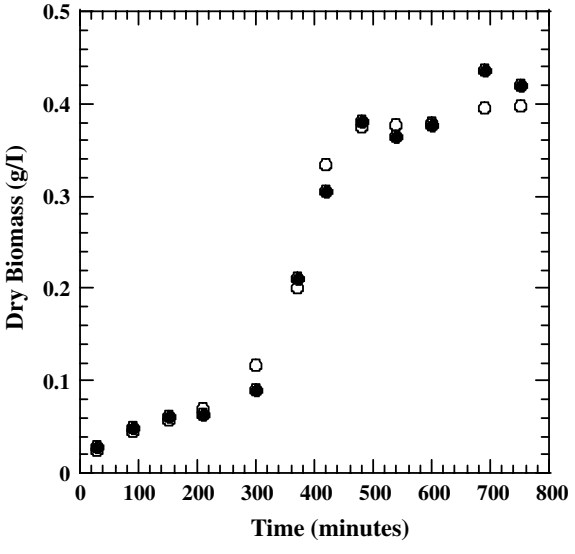


**Fig. 5** Typical growth curves of strain PB1 in artificial seawater-based (○) and Pacific Ocean-based (●) MY medium. Initial methanol concentration 1% v/v

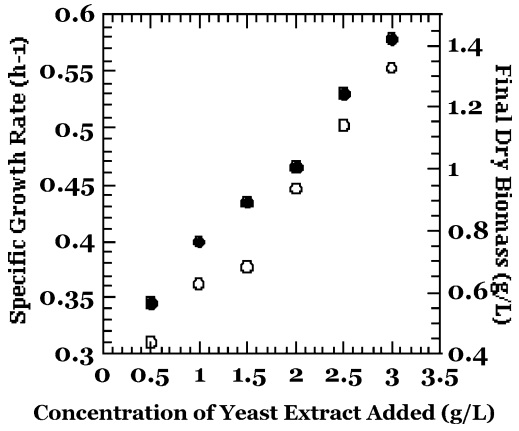


**Fig. 6** The effect of added yeast extract on the specific growth rate (●) and final dry biomass (○) of strain PB1 grown in Pacific Ocean Seawater-based MY media with methanol as growth substrate

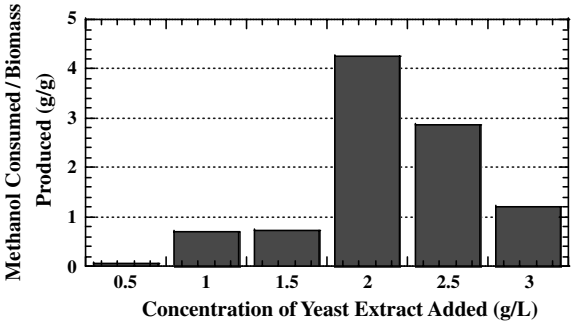


**Fig. 7** The effect of yeast extract concentration on methanol uptake in Pacific Ocean seawater-based medium. Initial methanol concentration 1% (v/v)

with different concentrations of yeast extract is shown in Fig. 6. The ratio of methanol consumed per biomass (g/g) in the flasks during this experiment (Fig. 7) shows that with 2.0 g/l yeast extract in the medium, the PB1A$^{ST}$ cultures utilized the most methanol of any concentration of yeast extract. While the use of yeast extract for a large scale process is not preferred due to cost considerations, this result suggests that further optimization of the medium can result in improved growth characteristics of *B. methanolicus* at high salt concentrations.

## Conclusion

The growth of *B. methanolicus* PB1 in seawater-based medium has been demonstrated. After a simple adaptation of the bacteria to grow in medium with 25 g/l added salt, the bacteria could grow at a similar rate and with similar final biomass titer as with the native strain in medium without added salt. Cultures of strain PB1A$^{ST}$ likewise grew with a similar growth rate in seawater-based medium using methanol as a carbon source. Addition of 2.0 g/l yeast extract further

improved the growth characteristics, suggesting the optimization of the MY medium formulation may result in enhanced growth properties in seawater-based medium. The results of this work contribute to the motivation to explore *B. methanolicus* as a potential biocatalyst for the production of commodity chemicals near offshore oil rigs.

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