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ISOLATION AND OPTIMIZATION OF POLY β HYDROXYBUTYRATE PRODUCING CYANOBACTERIAL STRAINS

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ABSTRACT: Cyanobacteria have many unexploited potential for natural products with a huge variability in structure and biological activity. Under stress conditions they are reported to produce biopolymers like poly-β-hydroxybutyrate (PHB), which can be produced intracellularly.Cyanobacteria are capable of synthesizing small amount of poly-β-hydroxybutyrate (PHB) under nitrogen and phosphorous starvation conditions. High performance liquid chromatography (HPLC) analysis revealed that about percentage PHB of the cell dry weight (CDW) was accumulated under mixotrophic culture condition. However, Nile red stained cells showed the presence of large quantities of granules in the cell cytoplasm when viewed under fluorescent microscope. The qualitative observation was in contrast to the quantitative HPLC analysis which suggested that the fluorescent granules are PHB. Among the ten different isolates, three strains showed the accumulation of PHB. The amount of PHB produced after 10 days in the depleted conditions are 1.182 mg/l for spirulina, 2.322 mg/l for anabena and 3.741 mg/l. The maximum PHB producer was further studied in detail. The extracted polymer was compared with the authentic PHB and was confirmed to be PHB using FTIR analysis. The present study shows increased PHB accumulation in different species by nitrogen and phosphorous depleted condition and pH concentration in the growth media. **Keywords:** Cyanobacteria, poly-β-hydroxybutyrate, PHB, HPLC, FTIR

INTRODUCTION

Poly-β-hydroxybutyrate is a wide spread intracellular storage compound typically in prokaryotic organisms (Liebergesell, M et al 1994, Mallick, N et al 2007). The properties of pure poly-β-hydroxybutyrate including thermoplastic process ability, absolute resistance to water and complete biodegradability suggest that PHB could be an attractive to common plastics and would fit well with new waste management strategies (Hrabak, O 1992). The use of PHB produced by bacterial fermentation as a commodity polymer is limited by its high production cost compared to some widely used petroleum derived plastics (Arun, A et al 2006, Chien, C.C et al 2007). The number as well as the types and potential qualities have greatly increased producing superior materials such as epoxides, polysulfones, and have become one of the most widely used products all over the globe (Dawes, E.A 1990, Khanna, S et al 2006).

Amongst the 150 different types of polyhydroxyalkanoids identified so far, the homopolymer of hydroxybutyrate like PHB is widespread in different taxonomic group of prokaryotes including cyanobacteria. The properties of pure PHB including thermoplastic processibility, hydrophobicity, complete biodegradability and biocompatibility with optical purity have increasingly become of interest as a raw material for biodegradable plastics (Kim, M.K et al 1996, Braunegg G et al 1998). Cyanobacteria can be considered as an alternative host system due to their minimal nutrient requirements and photoautotrophic nature. Cyanobacterial species have the ability to accumulate the homopolymer of PHB under photoautotrophic condition (Byrom D 1987) Cyanobacteria are capable of accumulating PHB. Industrial utilization of cyanobacteria as PHB producers has the advantage of converting waste carbon dioxide, a green house gas to environmental friendly plastics using the energy of sunlight (Kulaev IS and Vagabov VM 1987, Yan, et al 2005) . Various species of cyanobacteria accumulate considerable amounts of PHB.

The major problem associated with the PHB production is its cultural optimization studies. In this study different cyanobacterial species have been identified, isolated and screened. Nitrogen sources, phosphorous sources and pH were tested to analyze the effect on PHB production by the higher yielding PHB producer. Then the same strain was usedfor further studies. The PHB inclusion in the microbe was identified to be PHB from FTIR analysis by comparing with the standard.

MATERIALS AND METHODS

Collection of samples

Algal species were isolated and purified using basic microbial techniques like serial dilution, spread plating and streak plate methods using BG11 agar medium with streptomycin (400 mg/L antibiotic). The medium pH was maintained at 7.6 in throughout the study. Further, isolated and purified species were preserved in 50 % glycerol as stock at -20 ºC for screening studies.

Isolation and screening of PHB producing cyanobacteria

The isolates were screened for PHB production using Nile red staining method and observed under fluorescent microscope. The selected isolates were then identified on the basis of their morphological features.

Organisms and growth conditions

Axenic pure cultures of cyanobacteria was grown in 250mL Erlenmeyer flasks containing 100ml of BG11 media at 24 ºC under illumination with cool white fluorescent light and light–dark cycle of 14/10 h. It is grown in the flasks till it attains the stationary phase.

Nile red staining

Isolated pure cultures were further used for screening their PHB production using nile red staining method in which 200 ul of algal samples were added with 50 ul of nile red dye (1mg/ml DMSO stock) and incubated for 10 minutes at room temperature followed by thorough washing with double distilled water. Finally the wet mount method was used to prepare the slides with algal culture and observed the slides under fluorescent microscope (Mahishi LH et al 2003) at 465 nm excitation.

PHB production from cyanobacteria

Species which had shown positive results in fluorescent studies were further taken for increasing the production level in N and P neglected BG11 media. For this, cultures were grown till they reach stationary phase and then the algal samples were carefully transferred into minimal media under sterile conditions. The culture flasks were incubated at 28 ^{0}C in throughout the study.

Growing of organisms in nitrogen depleted media

To study the impact of nitrogen deficiency on PHB accumulation, the cells were directly grown in nitrogen deficient medium. For nitrogen deficiency cells were incubated in BG-11 medium devoid of $NaNO_3$. Ferrous ammonium citrate and Co $(NO_3)_2$. $6H_2O$ were also substituted by equimolar concentrations of ferric citrate and $CoCl_2$.6H2O.

Growing of organisms in phosphorous depleted media

To study the impact of phosphorus deficiency on PHB accumulation, the cells were directly grown in phosphate-deficient medium, where K_2HPO_4 of the medium was substituted by equimolar concentrations of KCl.

Interaction of mixotrophy with N/P deficiency

The interactive effects of exogenous carbon supplementation with N or P deficiency were studied in the following ways: (i) The stationary phase cells were subjected to nitrogen or phosphorous deficiency with supplemented carbons, and (ii) cells pre-grown in glucose supplemented BG-11 medium till stationary phase were subjected to N or P deficiency with supplemented carbons. PHB content was analyzed in these depleted conditions.

Growth measurement

Cell growth was monitored by measuring the optical density at 730 nm of the culture broth. Cell pellets obtained from definite volume of broth were dried to obtain the according cellular density based on dry weight.

Extraction of poly-β-hydroxybutyrate

Cells were harvested by centrifugation (10000 rpm, 10min), washed with distilled water and the biomass was suspended in methanol overnight at 4 ◦C for the removal of pigments. The pellet obtained after centrifugation was dried at 60 ◦C and PHB was extracted in hot chloroform. PHB was precipitated from the chloroform solution. Then the polymer was dissolved in chloroform. After evaporation of the solvent, PHB was obtained as a tough, translucent film. Qualitative determination of PHB was done by FTIR analysis.

Fourier transform infrared spectroscopy (FTIR)

KBr pellet was prepared using PHB from sample culture and also prepared the same with standard PHB purchased from Sigma. A PerkinElmer spectrum GX FTIR spectrometer was used with spectral range, 4000–400cm^{-1} to record the IR spectra.

Quantification of PHB using HPLC

From each purified samples (10 days old) 5 ml of culture was taken in 15 ml screw cap centrifuge tubes and centrifuged (MPW-351R, Poland) at 10,000 rpm for 10 minutes. The resulting pellet was dried at 80 ºC at overnight. The dry pellets were boiled in 1 ml of conc. H_2SO_4 at 90 ºC for 30 minutes. Samples were then diluted with 4 ml of 5 mM H_2SO_4 and vortex. From this, 200 µL of sample was taken in a fresh micro centrifuge tube and further diluted 10 times with 5 mM H_2SO_4 followed by membrane filtration using 0.22 µm filters (Millipore). 50 µl of this filtered sample was then analyzed by HPLC with an ROA column 78 X 300 mm (Shimadzu CBM-20A Made in Japan). Commercially available PHB (Sigma-Aldrich) was also processed in parallel with the samples.

RESULTS AND DISCUSSION

Morphological and growth characteristics

Based on microscopic observations, isolates were identified as *Synechocystis*sp, *Oscillatoria*sp, *Lignolella*sp, *Nostoc*sp, *Anabena*sp, *Spirulina*sp,*Alcaligenes*sp. In this *Anabena*sp, *Synechocystis*sp *and Spirulina* sp. These three strains gave the positive result for PHB accumulation through nile red staining method. PHB inclusions were seen as brightorange intracellular granules shown in the figures 1, 2 and 3.

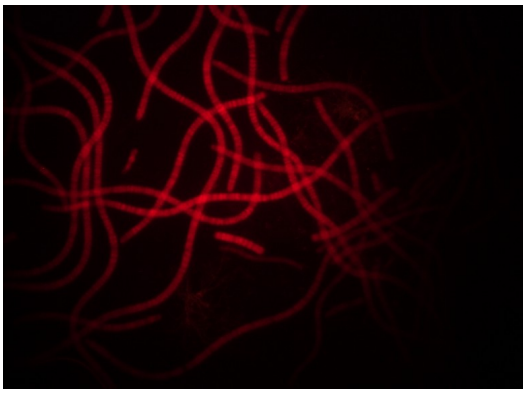

Fig 1: Nile red stained *spirulina*sp containing PHB inclusions

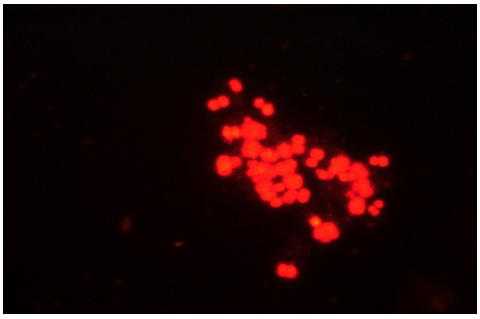

Fig 2: Nile red stained *synechocystis*sp containing PHB inclusions

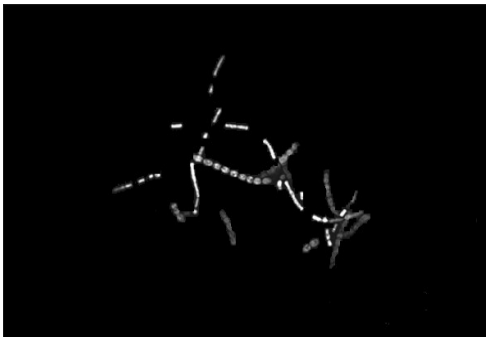

Fig 3: Nile red stained *anabena*sp containing PHB inclusions

Optimization studies

In order to find a better substrate, different carbon sources, nitrogen, phosphorous depleted conditions and pH levels were tested so as to increase the productivity of PHB. The strain *spirulina*sp, *anabena*sp, *synechocystis*sp which exhibited the higher PHB productivity among the other isolates, was selected for the more detailed analysis of the optimization process. The various conditions optimized for the intracellular accumulation of PHB by strains were carbon source nitrogen and phosphorous depleted conditions with pH 7.8. The above mentioned optimized conditions were used for the further studies. Thus, it is shown that depending upon the sources of carbon, nitrogen, pH, PHB synthesis may be selectively induced in these species. The growth pattern of *Anabena* sp, *Synechocystis* sp *and Spirulina* sp in control and depleted nitrogen and phosphorous is shown in the figures 4, 5 and 6. The culture entered the stationary phase after 12 days.

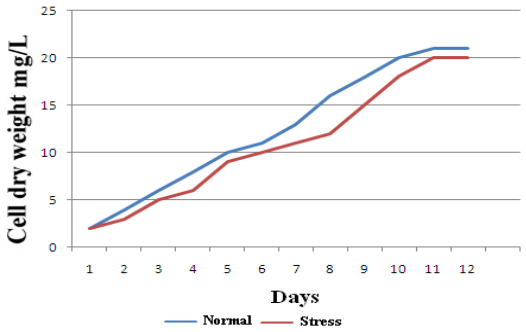

Fig 4: Growth of *spirulina*sp

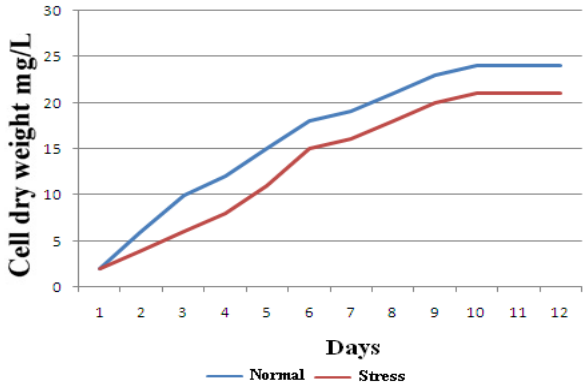

Fig 5: Growth of *anabena*sp

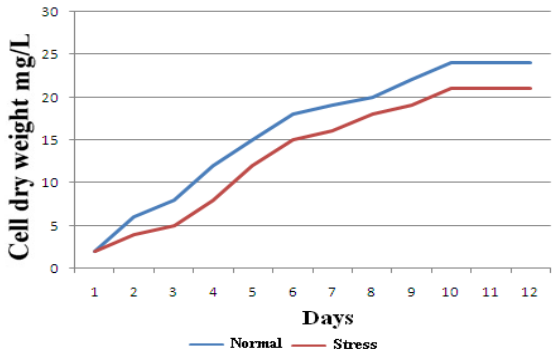

Fig 6: Growth of *synechocystis*sp

Impact of pH and N&P starvation on PHB accumulation

PHB accumulation was found maximum at pH 8.5 followed by pH 7.5. Acidic pHs was not found suitable for PHB accumulation, so also the high alkaline pH is essential. A significant positive correlation between dry weight and PHB accumulation demonstrates that accumulation of PHB in the test organism is growth dependent. Limitations of phosphorus and nitrogen appeared to be suitable stimulants for PHB accumulation. The stationary phase cells when transferred to P-deficient medium for 10 days, PHB accumulation was increased with the percentage of dry cell weight. This affirms the earlier findings results that PHB accumulation was enhanced when growth was restricted due to unavailability of phosphorus (Lee S.Y., et al., 1994). Under nitrogen deficiency a rise in PHB pool up to was observed. Where an increased PHB accumulation was observed under high intracellular concentrations of NADPH or high ratio of NADPH/NADP (Lee et al., 2002). It was observed that cells grown under usual growth conditions, i.e. in control culture the amount of flux into the TCA cycle was almost constant throughout the cultivation period and therefore, the NADPH production during the whole cultivation period did not vary significantly. However, under nitrogen deficiency NADPH consumption was decreased due to limitation of nitrogen sources, which blocks the amino acid synthesis pathways, especially the reaction from α-ketoglutarate to glutamate, thus resulting into accumulation of excess NADPH in the cells. This residual NADPH might be responsible for the enhanced PHB accumulation in nitrogen-deficient cells.

Effect of Nitrogen and phosphorous deficiency on PHB biosynthesis

Effect of Nitrogen and phosphorous deficiency concentration on PHB biosynthesis in *spirulina, anabena*spand*synechocystis*sp has been reported. The culture incubated in nitrogen and phosphorous deficient was compared with the culture incubated in normal condition (control) for PHB production. When the culture was incubated the nitrogen and phosphorous depleted media, the culture showed increased PHB synthesizing ability, which amount to a maximum of PHB.

PHB quantification

Extracted PHB was quantified using HPLC (Shimadzhu) in comparison to standard PHB (Sigma). As a result *Synechocystis*spp was found to produce maximum up to 3.741 mg/L with the retention time of 31 minutes which was confirmed with the standard. Following to *Synechocystis, Anabaena* was found to produce 2.322 mg/L and *Spirulina*spp was able to produce 1.182 mg/L.

Chemical analysis of the PHB produced

The polymer obtained from the strain was characterized by FTIR and it was shown in the figures 7, 8, 9, and 10. Thefigure shows the FTIR spectra of the PHB obtained from *spirulina* strain and compared with the standard PHB from Sigma. The bands found at 1461 cm^{-1} correspond to the asymmetrical C–H bending vibration in CH3 group, while the one found at 1161 cm^{-1} is equivalent to CH_2 asymmetrical bending vibration. The bands at 1377 cm^{-1} correspond to the C–O–H bond whereasthe band found at 1740 cm^{-1} indicated the stretching of C=O bond. The series of bands located at 1000–1200 cm^{-1} correspond to the stretching of the C–O bond of the ester group. The absorption band at and around 3450 cm^{-1} corresponds to the terminal OH group. The obtained IR absorption peaks correlated with the literature value and with the spectrum of pure PHB.

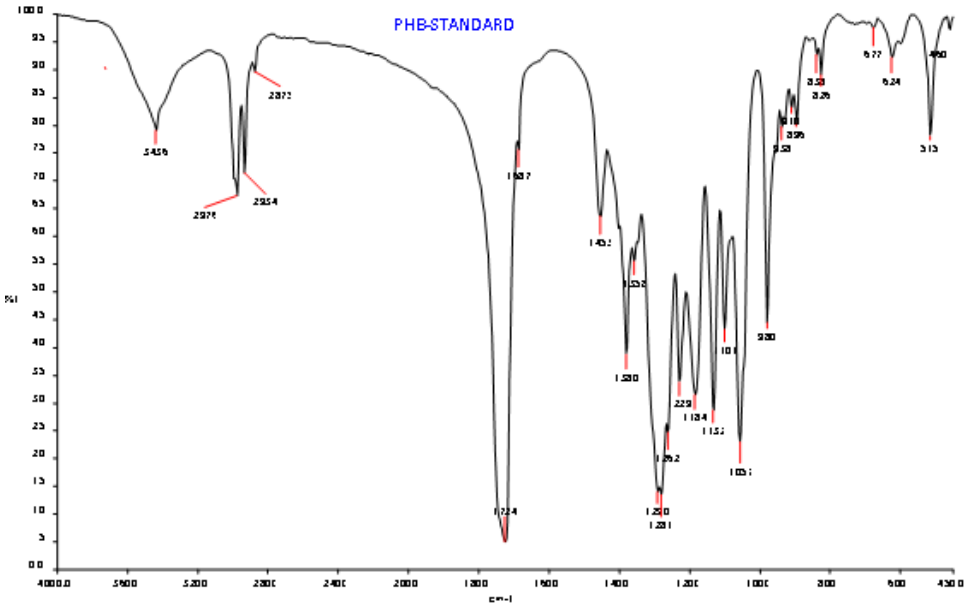

Fig 7: FTIR spectra of standard PHB

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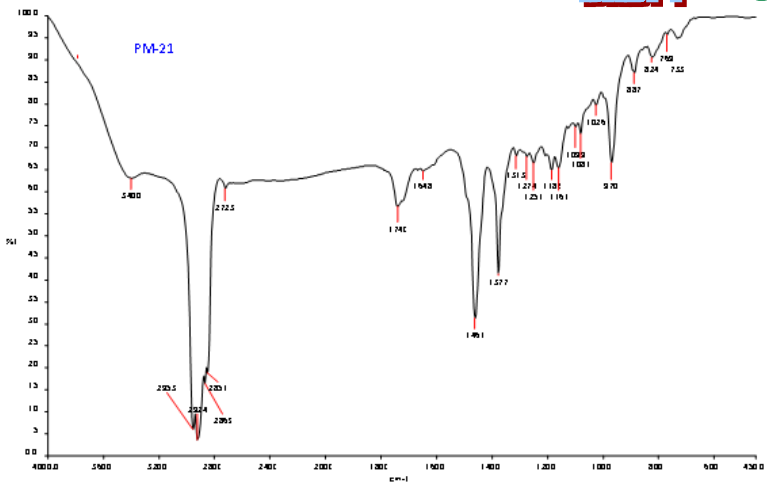

Fig 8: FTIR spectra of *spirulina* species

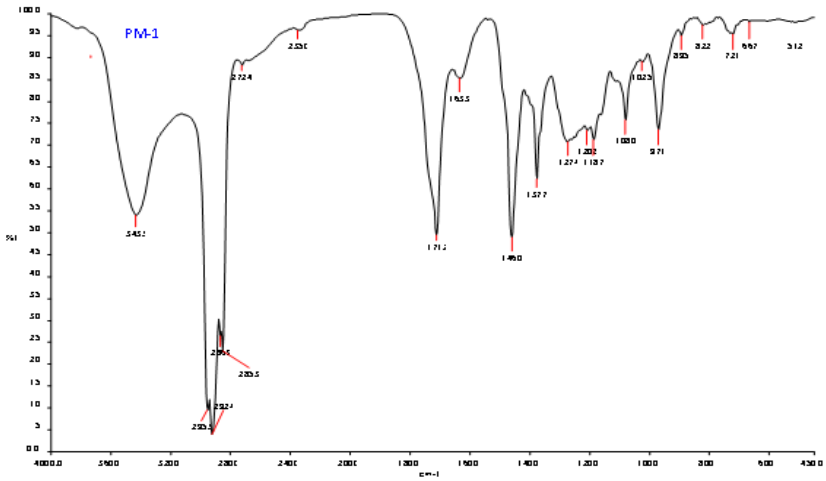

Fig 9: FTIR spectra of *anabena* species

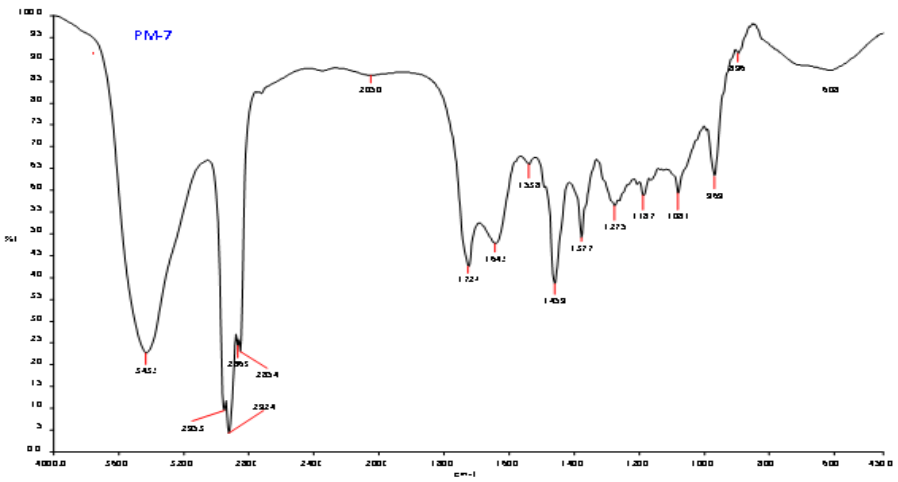

Fig 10: FTIR spectra of *synechocystis* species

The bands found at 1460 cm^{-1} correspond to the asymmetrical C–H bending vibration in CH_3 group, while the one found at 1185 and 1187 cm^{-1} is equivalent to CH_2 asymmetrical bending vibration. The bands at 1377 cm^{-1} correspond to the C–O–H bond whereas the band found at 1712 and 1724 cm^{-1} indicated the stretching of C=O bond. The series of bands located at 1000–1200 cm^{-1} correspond to the stretching of the C–O bond of the ester group. The absorption band at and around 3450 cm^{-1} corresponds to the terminal OH group respectively. Thus, it was identified that the compound was PHB.

CONCLUSION

Cyanobacteria do have the potential to produce biopolymers like PHB from CO_2 as the sole carbon source, and the yield of PHB could be increased by various means such as nutrient limiting conditions, stress conditions, different PHB enhancing precursors in vitro etc. The common practice of exploiting the diversity of bacteria in the environment for the industrial production of novel compounds, few reports have been published for the potential of industrial PHBs production by these bacteria. The present study shows increased PHB accumulation in the strains by altering in the growth media. Characterization of the PHB produced was performed by FTIR which confirmed the chemical structure as compared to the standard PHB (Sigma). The amount of PHB produced after 10 days in the depleted conditions are 1.182 mg/l for spirulina, 2.322 mg/l for anabena and 3.741 mg/l. These three novel organisms which produced highest amount of PHB than compared with the earlier reports. The concentration of PHB produced is relatively lower in phototrophs as compared to heterotrophic bacteria. Due to their minimal nutrient requirement and ability to grow even in wastewaters in the presence of CO_2 and sunlight these phototrophs (cyanobacteria) could be explored as an alternative source for PHB production, as the biomass could be inexpensively converted into biodegradable plastics by solar energy which can aid in overall reduction of the production cost of biodegradable plastics, which is the limiting factor for the replacement of synthetic polymers by such biodegradable biopolymers.

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