

Biominerlization and Possible Endosulfan Degradation Pathway Adapted by *Aspergillus niger*

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Endosulfan is a chlorinated pesticide; its persistence in the environment and toxic effects on biota are demanding its removal. This study aims at improving the tolerance of the previously isolated fungus *Aspergillus niger* (*A. niger*) ARIFCC 1053 to endosulfan. Released chloride, dehalogenase activity, and released proteins were estimated along with analysis of endosulfan degradation and pathway identification. The culture could tolerate 1,000 mg/ml of technical grade endosulfan. Complete disappearance of endosulfan was seen after 168 h of incubation. The degradation study could easily be correlated with increase in released chlorides, dehalogenase activity and protein released. Comparative infrared spectral analysis suggested that the molecule of endosulfan was degraded efficiently by *A. niger* ARIFCC 1053. Obtained mass ion values by GC-MS suggested a hypothetical pathway during endosulfan degradation by *A. niger* ARIFCC 1053. All these results provide a basis for the development of bioremediation strategies to remediate the pollutant under study in the environment.

Keywords: Biodegradation, endosulfan, *Aspergillus niger*

Introduction

In agricultural practices, better harvests require intensive cultivation, irrigation, use of fertilizers, and most importantly the use of chemicals to protect plants from pests and diseases. In India, 15–20% of all produce is destroyed by pests; this fact makes the use of synthetic pesticides unavoidable [7]. Presently, unbelievable amounts of synthetic chemicals in the form of pesticides and fertilizers are passing through the soil and contributing to environmental pollution. The extensive use of pesticides results in a widespread occurrence of pesticide residues in the environment, crops, and food products. Chlorinated organic pesticides are one of the major groups of chemicals that are responsible for environmental pollution.

Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,3,4-benzo(e) dioxathiepin- 3-oxide) is a broad-spectrum insecticide. It is persistent in nature and is a US-EPA priority pollutant [15]. As endosulfan is extensively used in agriculture, its residues have been detected in the atmosphere, soils, sediments, surface, rain waters, and foodstuffs [28, 44]. It is moderately persistent in soil, where

it is converted into sulfate, which is highly persistent. Endosulfan remains in the soil and water for 3–6 months or more [2, 18] and has been detected in different components of the environment [43]. Endosulfan gets sorbed to soil and sediments by virtue of its hydrophobic nature, making it persistent in soil and sediments [25, 34, 36] and water [10, 46]. Contamination and persistence of endosulfan in aquatic and soil environments lead to its accumulation in crop wastes [16], macrophytes [27], phytoplankton [11], fishes [33], vegetables, and milk and milk products [21]. Because of its ubiquitous nature and environmental persistence, the presence of endosulfan residues were traced in surface water, groundwater, atmosphere, and water bodies by many researchers [8, 5, 9, 13, 39]. Many countries imposed a ban on endosulfan production and/or its usage, but it is still one of the priority chemicals used extensively for pest control in many of the developing countries [20].

In light of the above facts, detoxification, degradation, and mineralization of endosulfan through biological means are receiving serious attention as an alternative to existing methods, such as incineration and landfill [38]. Several researchers described the biodegradation of endosulfan

under aerobic and anaerobic conditions with bacterial and fungal cultures [1, 19, 30, 37, 38, 41]. Past investigations on the microbial degradation of endosulfan have revealed various intermediates of metabolism, including endosulfan-sulfate, -diol, -ether, -lactone, -hydroxyether, and -dialdehyde [22, 26]. To date, the conversion of endosulfan to endosulfan sulfate during its degradation is the major concern of endosulfan degradation research as this metabolite is more toxic, persists longer in soils, and has bioaccumulation potential [40]. Degradation of endosulfan in solutions and soils is reported using bacterial species like *Klebsiella oxytoca* [22], *Bacillus* spp. [3], *Pandora* sp. [38], and *Micrococcus* spp. [15]. and Many fungi like *Aspergillus niger* [30], *Aspergillus terreus* and *Cladosporium oxysporum* [31], *Mucor thermohyalospora* [37], *Fusarium ventricosum* [38], *Phanerochaete chrysosporium* [19], and *Trichoderma harzianum* [17] have also been tested for their ability to degrade endosulfan. *Anabaena* spp. [24], *Chlorococcum* spp. and *Scenedesmus* spp. [35] are the photosynthetic microorganisms applied in endosulfan degradation studies. Complete mineralization of endosulfan by using bacterial or fungal isolates is always a desirable property. The analysis at regular intervals during the degradation experiments gives an idea about the generation of metabolic intermediates, ultimately for the understanding of the possible degradation pathway adopted by the microbes. Kumar and Philip [20] have proposed the possible pathway of endosulfan by bacterial isolates anaerobically. The present study reports the aerobic degradation of endosulfan by a previously isolated fungal strain (*A. niger* ARIFCC 1053) at higher concentration of endosulfan (1,000 mg/l), identification of the degradation intermediates, and the possible degradation pathway.

Materials and Methods

Chemicals

Technical grade endosulfan, a 35% emulsified preparation (Excel Industries Ltd., Mumbai, India), was used in all experiments. All other reagents were of high purity and analytical grade. Working standard solutions of these compounds were prepared by appropriate dilution of stock solutions using *n*-hexane or acetone.

Endosulfan Degradation Studies

The fungal isolate *A. niger* ARIFCC 1053 was previously isolated from pesticide-contaminated soil by an enrichment culture technique [6]. Tolerance to endosulfan by *A. niger* ARIFCC 1053 was again checked for higher concentration (500 to 1,000 mg/l) in a stepwise manner using broth assay. A series of 250 ml Erlenmeyer flasks containing 100 ml of Czapek-Dox medium amended with increasing concentrations of endosulfan from 500 to 1,000 mg/l were inoculated

with 1 ml of spore suspension (10^8 spores/ml) prepared in 0.1% Triton X-100 and incubated at $30 \pm 2^\circ\text{C}$ on a rotary shaker at 120 rpm for 15 days. Mycelial mass from each flask was separated by filtration using Whatman filter paper No. 1 and washed with deionized water and its dry weight was estimated. Samples from the flasks containing 1,000 mg/l endosulfan with fungal culture *A. niger* ARIFCC 1053 were taken at 24 h intervals up to 192 h and processed for analysis of estimation of released chloride dehalogenase activity and released protein. A separate set of uninoculated flasks was maintained as the reference. All experiments were performed in triplicates. The released chloride was estimated by the mercuric thiocyanate method reported by Bergmann and Sanik [4]. A standard graph of sodium chloride (0.014 N) was prepared by using a series of concentrations to determine the amounts of released chloride during endosulfan degradation by *A. niger* ARIFCC 1053.

Dehalogenase activity was assayed as per the procedure described by Okoh *et al.* [32]. The crude samples were harvested by centrifugation for 20 min at $11,000 \times g$ and suspended in 10 mM Tris- SO_4 buffer (pH 7.5), and enzyme assays were done within 6 h after preparation of the extracts to prevent loss of activity. Dehalogenase assays were carried out by incubating 0.1 ml of crude extract or an adequate dilution thereof at 35°C with 3 ml of 5 mM endosulfan in 50 mM Tris- SO_4 (pH 7.5) and glycine NaOH (pH 9). The reaction mixture was mixed with mercuric thiocyanate and ferric ammonium sulfate. Chloride liberation was measured spectrophotometrically at 460 nm. Dehalogenase specific activity was expressed as $\mu\text{g/ml}$ chloride release/ mg protein / h. The samples collected at different time intervals were further analyzed for released protein. The concentrations of protein were determined by Lowry's method with bovine serum albumin as the protein standard.

Identification of Endosulfan Degradation Intermediates

In order to analyze the residual endosulfan and metabolites formed, cell-free culture broth was acidified to pH 2.0 with 6.0 M hydrochloric acid and extracted three times with acetonitrile. Acetonitrile fractions from each flask were pooled and aliquots were analyzed by HPLC, IR, and GC-MS for quantitative analysis for detecting the intermediates during degradation. HPLC analysis of samples was performed with a UV detector (Chemito LC 6600 series model Japan). For analyzing degradation products, 20 μl final extract was injected, and C18 analytical column and mobile phase consisting of 70% acetonitrile in water (v/v) with a flow rate of 1 ml/min were used. The column temperature was maintained at 40°C and UV absorption at 214 nm was recorded [25].

Infrared spectra of the technical grade endosulfan and the samples collected after fungal degradation were recorded at room temperature (25°C) in the frequency range of $4,000\text{--}400\text{ cm}^{-1}$ with a Fourier Transform Infrared (FTIR) spectrophotometer (8400 Shimadzu, Japan with Hyper IR-1.7 software for Windows) with a Helium Neon laser lamp as a source of infrared radiation. Aqueous samples (after 168 h of incubation) from endosulfan degradation flasks were extracted with ethyl acetate and the solvent was

evaporated under vacuum. The contents were re-dissolved in acetone. A drop of this sample in acetone was placed in between two sodium chloride discs, prior cleaned with ethyl acetate. The background spectrum for acetone was corrected from the sample spectrum.

The GC-MS data were obtained on a Shimadzu QP-2000 instrument at 70 eV and 250°C (courtesy of Central Drug Research Institute, Lucknow, India). The GC-MS system was equipped with an ULBON HR-1 equivalent to OV-1 fused silica capillary column with dimensions of 0.25 mm × 50 m with film thickness of 0.25 µm. Other conditions include the initial temperature of 100°C for 6 min followed by heating at the rate of 10°C/min to 250°C. Helium was the carrier gas passed with a flow rate of 2 ml/min.

Results and Discussion

The fungal isolate *A. niger* ARIFCC 1053 demonstrated luxuriant growth up to 1,000 mg/l of endosulfan. In the present study, during the tolerance capacity assay, endosulfan at higher concentration (1,000 mg/l) was not a sole source of carbon/energy, as the interest was to enhance the endosulfan tolerating capacity of cultures. *A. niger* ARIFCC 1053 demonstrated considerably high potential to tolerate endosulfan (1,000 mg/l).

Endosulfan Degradation Studies

There was an increase in chloride content, dehalogenase activity (Figs. 1 and 2), and released proteins (Figs. 1 and 2) of broth as a function of incubation time. The amount of chloride released at particular intervals in the endosulfan degradation ranged from 38 µg/ml after 24 h to 120 µg/ml after 168 h after inoculation by *A. niger* ARIFCC 1053 (Fig. 2). The significant increase of free chloride from endosulfan in the medium clearly indicates degradation of endosulfan [45], and the release of chloride ions probably led to the

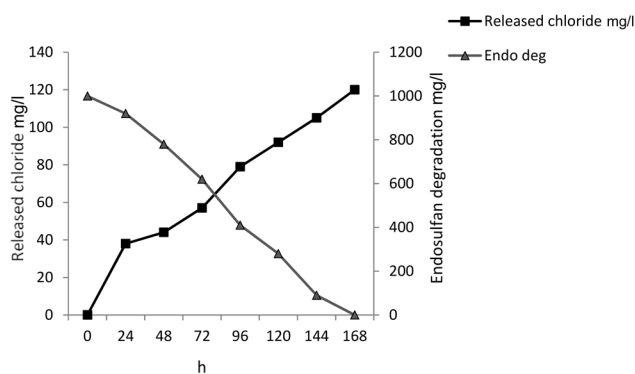


Fig. 1. Amount of chloride released during biodegradation of endosulfan by *A. niger* ARIFCC 1053.

formation of HCl and reduced the pH of the culture medium [38]. The rise in the dehalogenase activity was in accordance with the increase in released protein and chlorides (Fig. 2). In the present study, dehalogenase activity was increased with time during endosulfan degradation by *A. niger* ARIFCC 1053. At 24 h it showed 0.17 µg/ml chloride release/ mg protein / h, at 72 h it was almost double at 0.31 µg/ml chloride release/ mg protein / h, and at 168 h it reached up to 0.87 µg/ml chloride release/ mg protein / h (Fig. 2). The release of chloride ions was observed in correlation with dehalogenation and it is a part of degradative activity [1]. Dehalogenase plays a central role in the biodegradation of many chlorinated compounds [12] and hence its activity could be an appropriate indicator for the assessment of the rate of initial phase of biodegradation of organochlorine pesticides [42].

Confirmation of Endosulfan Degradation by Using HPLC and Infrared Spectroscopy

A HPLC method was established for detecting the endosulfan during the course of degradation. Almost half of the initial endosulfan was degraded by the isolate under study (*A. niger* ARIFCC 1053) within 96 h. Gradual disappearance of endosulfan from culture media was observed up to 144 h. However, the presence of endosulfan was undetectable at the end of 168 h (Fig. 1). Only 3% endosulfan was observed to be degraded in the control flask at 168 h. The detection of endosulfan at regular intervals (every 24 h) using HPLC analysis confirms the potential of *A. niger* (ARIFCC 1053) to degrade endosulfan.

The FTIR spectroscopy gave more insight into structural changes of the endosulfan during the process of biodegradation by *A. niger* ARIFCC 1053. The IR spectra of technical grade

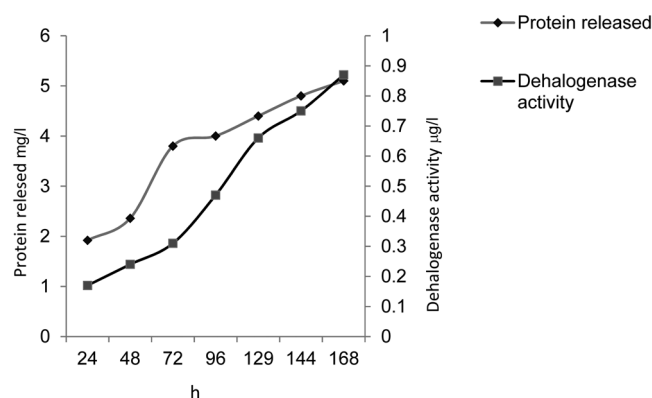


Fig. 2. Amount of protein released and dehalogenase enzyme activity in the broth by *A. niger* ARIFCC 1053 grown in the presence of endosulfan.

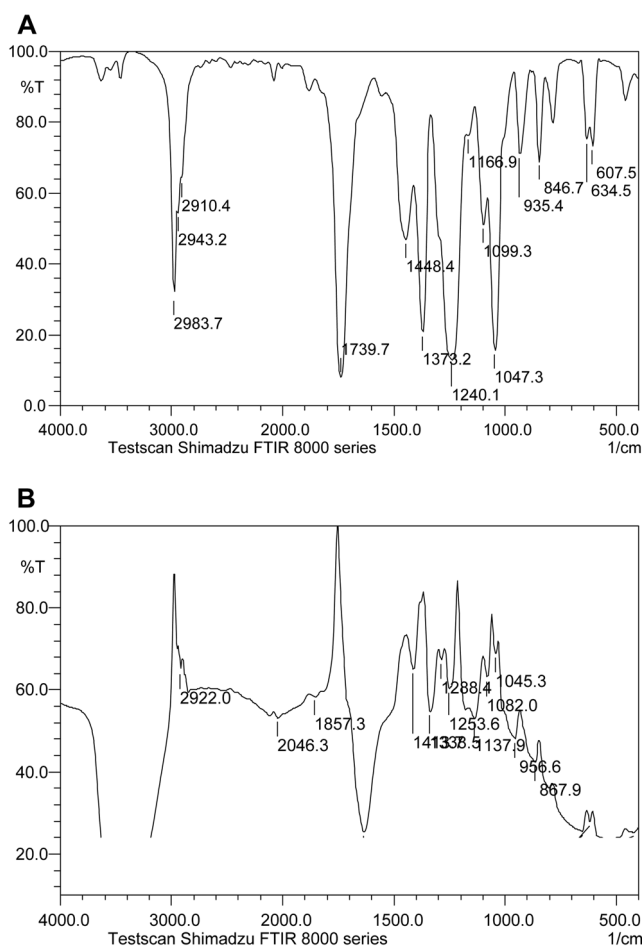


Fig. 3. FTIR spectra of (A) the ethyl acetate extracted endosulfan before degradation by *A. niger* ARIFCC 1053 and (B) of the ethyl acetate extracted sample of degraded endosulfan by *A. niger* ARIFCC 1053 withdrawn at 168 h.

endosulfan and the sample at 168 h after fungal degradation are depicted in Fig. 3. The spectral characteristics of standard endosulfan and that of sample after 168 h treatment differed significantly with each other. The assignments of the different absorption bands and the corresponding functional groups of endosulfan (control) and that of degraded product of endosulfan are given in Tables 1a and 1b. The infrared spectrum of endosulfan showed absorption bands at 2,983, 2,945, and 2,910 cm^{-1} , which were reduced in the spectrum of degraded compound. The appearance of absorption band for -OH (3,431 cm^{-1}) and change in position of absorption band of C=C (1,637) were major changes in the infrared spectrum of the degraded product. The addition of bands for C-O and S-O (1,258, 1,288, and 1,137 cm^{-1}) 621 cm^{-1} for R SO_3 peaks of sulfuric acid, and bands at 434 and 543 cm^{-1}

Table 1. a. Infrared spectral data of technical grade endosulfan.

Band (cm^{-1})	Assignment
2,983, 2,945, and 2,910	=C-H stretching
1,749 (actual 1,650-1,630)	Conjugated C=O
1,450, 1,371, 848	C-O stretching
1,218, 1,166, and 609	S-O and C-O-S stretching
1,099, 1,043	C-O stretching
786, 754, and 632	C-Cl stretching

b. Infrared spectral data for degraded product compound of endosulfan.

Band (cm^{-1})	Assignment
3,431	-O-H stretching
1,637	Conjugated C=C
1,288, 1,253, and 1,137	S-O and C-O- stretching
621	R-SO_3 sulfuric acid
424, 543	SO_4^- (sulfonate)
786, 754, and 632	C-Cl stretching

for sulfonates suggested that the molecule of endosulfan was degraded efficiently by *A. niger* (Fig. 3).

Identification of Endosulfan Degradation Intermediates

Use of GC-MS in the detection of metabolites of endosulfan biodegradation by microorganisms has been reported by many authors [15, 35, 41]. GC-MS spectral details of sample degraded after 168 h are shown in Figs. 4 and 5. Different ion mass values (m/z) 43, 83, 47, and 58 were obtained at different retention times (Fig. 4). The mass spectra peaks at specific retention times helped us in establishing a hypothetical pathway of endosulfan degradation by *A. niger* ARIFCC 1053. The peak of m/z 58 indicated that glyxal was produced as an intermediate compound during endosulfan degradation, and peak 43 did not match with any of the related products. At retention time 8.05 min, only one peak of m/z 83 was observed, which could be assigned to protonated sulfurous acid. The peaks of m/z 83 and 47 suggested the generation of sulfurous acid and formic acid, respectively. In environmental degradation, formic and sulfurous acids are generally converted into water, CO_2 , and SO_2 . This observation has been supported by GC-MS findings. Based on these results, the hypothetical pathway for the degradation of endosulfan by *A. niger* ARIFCC 1053 (Fig. 5) was established. Endosulfan degradation by *A. niger* ARIFCC 1053 endosulfan sulfate was observed by oxidation [6]. After a few hours, endosulfan sulfate was metabolized further and observed to be converted into

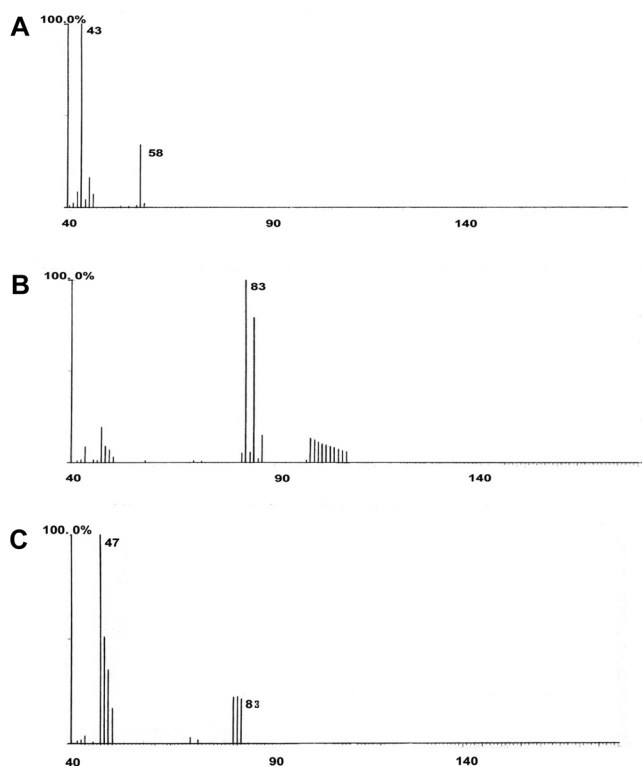


Fig. 4. GC-MS data for product compound of endosulfan degradation by *A. niger* ARIFCC 1053.

endosulfan diol by hydrolysis. Endosulfan sulfate, a major persistent metabolite [21], gets converted into less toxic endosulfan diol by *A. niger* ARIFCC 1053, which in our opinion is a very important feature of this pathway. The other intermediates (*viz.*, 1-chloro-1, 3-propanediol and 2-chloro 4 hydroxyl butanol) may be formed in upcoming degradation stages. Formation of sulfurous acid and formic acid are detectable (Fig. 5). Thereafter, no peak was observed, which might be due to the low stability of the formed compounds in the solvent / liquid medium used for the extraction / analysis or the compound possibly converted into simple carbon dioxide or water molecule. However, this particular observation needs further study for confirmation of this pathway.

The present investigation revealed that indigenous fungal isolate *A. niger* (ARIFCC 1053) could tolerate and utilize higher concentration (1,000 mg/l) of endosulfan. *In vitro* degradation could easily be correlated with increase in the amount of released chlorides, dehalogenase activity, and released proteins. The organism (*A. niger* ARIFCC 1053) was able to degrade half of the initial endosulfan within 96 h of inoculation. Complete degradation of endosulfan was achieved after 168 h of inoculation, as evident from the

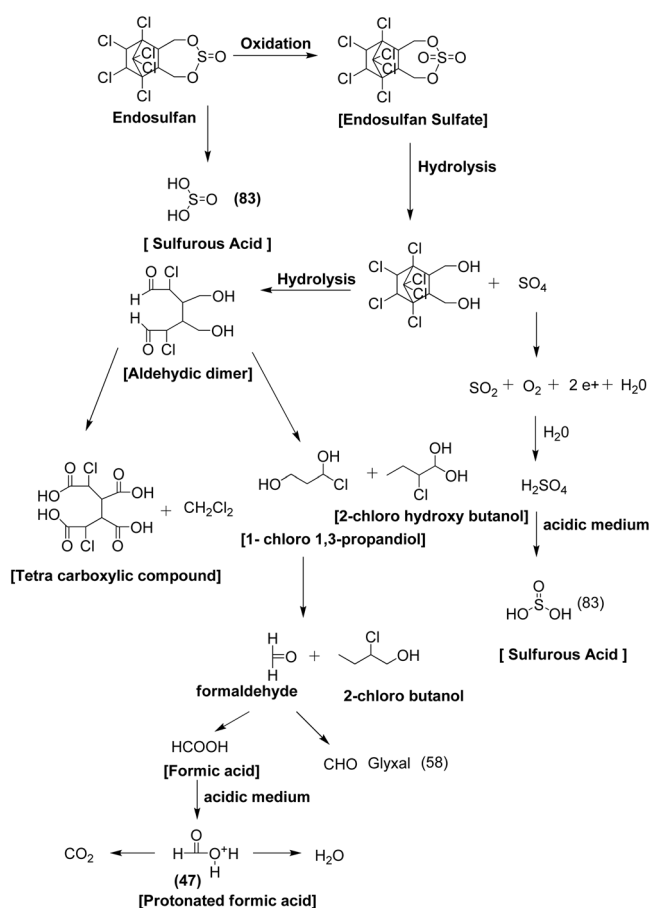


Fig. 5. Proposed pathway for the metabolism of endosulfan by *A. niger* ARIFCC 1053.

HPLC analysis. The spectral comparison of infrared spectroscopy of standard and treated endosulfan also suggested the structural changes, confirming the biological degradation *in vitro*. GC-MS analysis of the treated sample enabled us to propose a hypothetical pathway of endosulfan degradation. The study could identify sulfurous acid, glyxal, and protonated formic acid, which in the environment are generally converted to CO_2 , SO_2 , and H_2O . The present study, in our opinion, is probably the first to report the complete mineralization of endosulfan within 168 h at much higher concentration. On the basis of these results, the fungal strain *Aspergillus niger* ARIFCC 1053 could prove valuable for bioremediation of endosulfan-contaminated soils and waters.

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