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Research Article

BIODEGRADATION OF AMARANTH BY LIGNINOLYTIC CULTURE OF *PHANEROCHAETE CHRYSOSPORIUM*

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ABSTRACT
White rot fungi such as *Phanerochaete chrysosporium* degrade insoluble, recalcitrant polymer of lignin under conditions of nutrient limitation. The ability of *P. chrysosporium* to degrade aromatic xenobiotic compound, namely, Amaranth was explored under current investigation. The biodegradation assay was performed in liquid batch culture medium with the Amaranth as single substrate at increasing substrate concentrations over a period of 30 days. The biodegradation studies were based on dye removal monitored by measuring decrease in absorbance at its respective λ_{max} using spectrophotometer. During biodegradation studies, sulfate release could be observed in Amaranth samples. The degradation of Amaranth followed first order kinetics with half-life of 10 days. The present study revealed the capability of *P. chrysosporium* as potential fungal culture for degradation of Amaranth.

Keywords: *Azo Dyes, Amaranth, Decolorisation, Phanerochaete chrysosporium*

INTRODUCTION
The production and usage of chemicals in industry has led to the entry of many xenobiotic compounds into the environment. Dyes emanating from textile, food, pharmaceutical, electronic industries are major sources of environmental pollution. During dyeing process, a substantial amount of azo dye is lost in wastewater (Ollgaard *et al.,* 1998). Azo dyes, which represent about one-half of all dyes in common use, are employed as coloring agents in the food, pharmaceutical, and textile industries. With the increased use of a wide variety of dyes, pollution by dyes wastewater is becoming increasingly alarming (Zollinger *et al.,* 1987). Azo dyes are by far the most important and versatile class of dyes which have been studied and used more than any other class (Stolz, 2001). Some of the dyes, their precursors, or their biotransformation products such as aromatic amines, have been shown to be carcinogenic (Razo-Fores *et al.,* 1997).
Amaranth is a dark red to purple azo dye, which in addition to coloring in food, is used in dyeing and color photography. Amaranth may cause skin rash and is suspected to be carcinogenic. It is recommended that people who suffer from asthma or aspirin intolerance avoid it.
Due to rapid industrialization and consequent degradation of environment, clean up and protection is crucial for achieving sustainable development. A number of biotechnological approaches have the potential of combating environmental pollution in an eco-efficient manner. A wide variety of microorganisms are capable of decolorization of a wide range of dyes some of them are as **bacteria:** *Escherichia coli* NO3 *Pseudomonas luteola*, *Aeromonas hydrophila*; **fungi:** *Aspergillus niger*, *Phanerochaete chrysosporium*, *Aspergillus terricola*, yeasts: *Saccharomyces cerevisiae*, *Candida tropicalis*, *C. lipolytic*; **algae**: *Spirogyra* species, *Chlorella vulgaris*, *C.sorokiniana*, *Lemna minuscula*, *Scenedesmus obliquus*, *C. pyrenoidosa* and *Closterium lunula* (Ponraj *et al.,* 2011).
In most cases, bacterial anaerobic degradation is initiated by the reduction of the azo linkage to generate aromatic amines which may be strongly carcinogenic and are themselves a disposal problem (Spadaro *et al.,* 1992). Thus a problem emerges when anaerobic bacteria are used in degradation of azo dyes. Some aerobic bacterial strain showed strict specificity towards single dye which organism had adapted (Kulla, 1981; Zimmerman *et al*., 1982). Because of specificity exhibited by bacteria, they are not considered to be of practical value for degrading the waste water and synthetic dyes which often contain substituent such as azo, sulfo-, nitro- groups (Klausener *et al.,* 1983; Mileski *et al.,* 1988; Pagga *et al.*,1986; Shaul *et al.*,

1991). Consequently, the isolation of soil bacteria which utilize these dyes as a sole source of carbon has proved difficult.

The use of a fungal culture for bioremediation has initiated considerable research efforts. By far the single class of microorganisms most efficient in breaking down synthetic dyes is white rot fungi (Wesenberg *et al.*, 2003). The nonspecific nature of the lignin-degrading systems of white rot fungi is a potential advantage for biotreatment of textile effluents, since a mixture of dyes, surfactants and other compounds exist in the wastewater.

The basidiomycete, *P. chrysosporium*, belongs to the white rot class of wood-rotting fungi. White-rot fungi in particular produce lignin peroxidase, manganese dependent peroxidase and laccase that degrade many aromatic compounds due to their non-specific enzyme systems (Stolz, 2001). Other white rot fungi, such as *Trametes versicolor*, *Bjerkandera adusta*, and *Thelephora sp*., have also been shown to be efficient in decolorizing of different azo dyes (Selvam *et al.*, 2003; Heinfling *et al.*, 1997). *P. chrysosporium* has been shown to be effective in removing color from textile-dye effluents of wastewater (Ashoka *et al.*, 2000).

The main objective of this study was to observe the degradation of Amaranth in a liquid batch culture using *P. chrysosporium* (Figure 1).

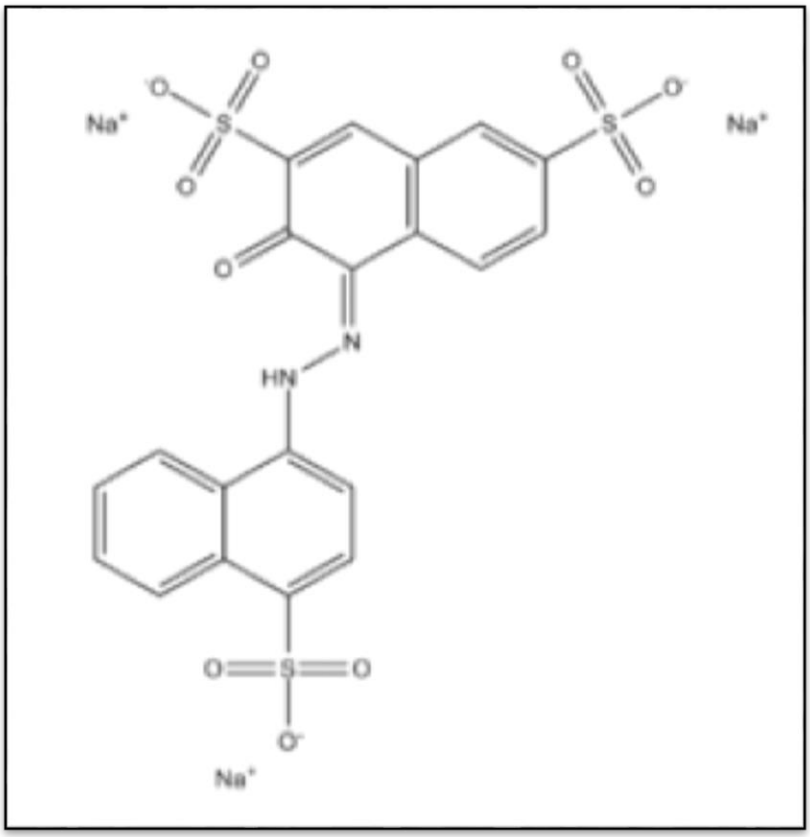

Figure 1: Structure of Amaranth

MATERIALS AND METHODS

Culture: *P. chrysosporium* (MTCC-787) was procured from the Institute of Microbial Technology (IMTECH), Chandigarh, India. The organism was maintained on 2% malt agar slants at 4˚C and sub cultured after every 30 days.

Media: *P. chrysosporium* was grown in liquid medium containing (gL^{-1} Distilled water):Glucose,2; KH_2PO_4, 2 ;$MgSO_4.7H_2O$,0.5 ;$CaCl_2.H_2O$,0.1 ; NH_4Cl, 0.12 and Thiamine ,0.001.The medium was

buffered with 10Mm Sodium acetate which is more economic and equally efficient in lignin peroxidase production than commonly employed 2,2-Dimethyl Succinate buffer (Kang and Stevens,1994).The final pH of the growth medium was adjusted to 4.5.

Substrate: Amaranth was studied for aerobic degradation in liquid batch culture.

Analyses

Reducing Sugars: The total reducing sugars were estimated colorimetrically using 3, 5 - Dinitrosalicylic acid (DNS reagent) with glucose used as a standard as described by Miller. (1859).

Sulfate Analysis The sulfate estimation was done by Standard Turbidometric Method (APHA, AWWA, WPCF, 1995).

Degradation Efficiency: Degradation was calculated as, Degradation (%) = $\frac{(Co - C)}{Co} \times 100$

Where, **Co** is the initial concentration of dye (mgL^{-1}) and **C** is the residual concentration (mgL^{-1}).

UV Spectrophotometer Analysis

Amaranth as mono substrate, was analyzed by UV-VIS Spectrophotometer (UV-1800 Shimadzu) by taking absorbance at its absorption maxima (λ_{max}) by scanning in the UV and visible region. Distilled water was used as a reference. The concentration of Amaranth was calculated from standard calibration curve obtained by plotting Absorbance v/s Substrate concentrations.

Reaction Rate Kinetics

The maximum rate of biodegradation i.e., V_{max} and Michaelis- Menten Constant i.e., K_m was determined by using Lineweaver-Burk's double reciprocal plot. The graph was obtained by plotting reciprocal of substrate removal rate on Y-axis v/s reciprocal of remaining substrate concentration on X-axis.

Determination of Half-life

The graph of log of concentration on Y-axis against time on X-axis was plotted for the same. The half-life **($t_{1/2}$)** in days was calculated by following equation

($t_{1/2}$) / K = 0.693 where **K** is the reaction rate constant

MATERIALS AND METHODS

Biodegradation Studies

Biodegradation of Amaranth was studied in a mono substrate system in Erlenmeyer's flasks of 500ml capacity. The flasks contained 150 ml of nitrogen and carbon limiting growth medium to stimulate ligninolytic activity. The flasks were aseptically inoculated with 200µl spore suspension containing a concentration of 1 x 10^6 spores per ml in sterile growth medium. The spore suspension was added in all the flasks except controls used for study of abiotic loss. The flasks were incubated at 30˚C under static conditions to avoid loss of activity of ligninolytic enzymes that are susceptible to mechanical agitation. The pH of the medium was maintained at 4.5. After an incubation period of 7 days, when mycelia growth appeared in the medium, filtered sterilized stock solution of Amaranth in water was added to different flasks to achieve varying concentrations of 50mgL^{-1},100mgL^{-1}, 150 mgL^{-1}, 200 mgL^{-1}, 250 mgL^{-1}, 300 mgL^{-1}, 400 mg L^{-1} and 500 mgL^{-1}. All experiments were run in duplicates.

Biosorption Studies

After the end of biodegradation studies, the mycelium in the medium containing dyes was transferred aseptically to a fresh medium and placed on shaker condition for 8 hours to remove any adsorbed substrate on to the mycelium. The medium was filtered through Whatmann filter paper 42. The filtrate was analyzed spectrophotometrically.

RESULTS AND DISCUSSION

Results

Degradation Studies

UV spectrophotometric analysis of Amaranth was done to assess their biodegradation.

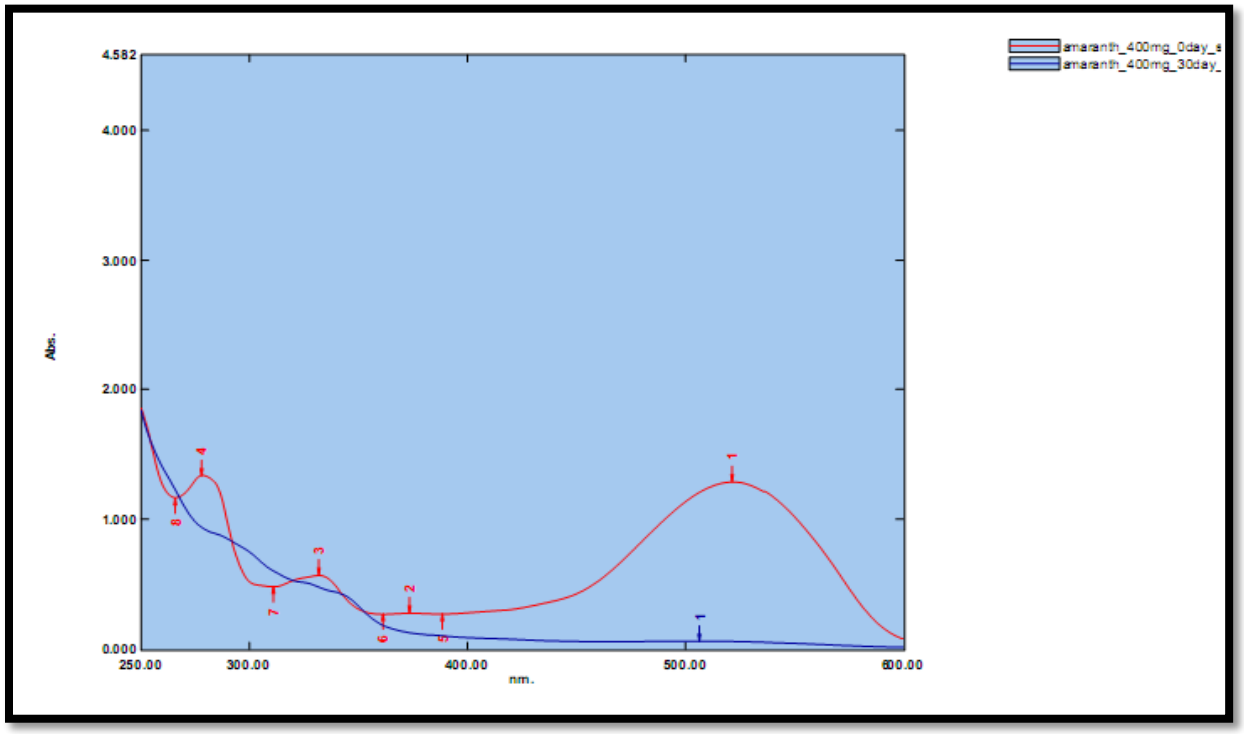

Figure 2: UV Spectrum Showing Amaranth Degradation

Amaranth shows presence of two peaks at 521nm and 331 nm. Effect of varying concentrations of dye, (ranging from 50mgL^{-1}- 500 mgL^{-1}) on dye removal was investigated keeping the other parameters constant.

For 50mgL^{-1} and 100mgL^{-1} concentration of Amaranth dye removed was to the extent of 98%. But with increase in the concentration of Amaranth in the medium, decrease in degradation efficiency was evidenced. After 30 days of incubation, two peaks of Amaranth disappeared completely and a new peak emerged indicating biotransformation (Figure 2). The biodegradation medium showed an increase in sulfate concentration with time. Sulfate release was also upto 45 days of incubation. This further substantiated biodegradation of Amaranth (Figure 3). The rate of degradation increased with concomitant increase in glucose utilization, which indicated viability of mycelium (Figure 4).

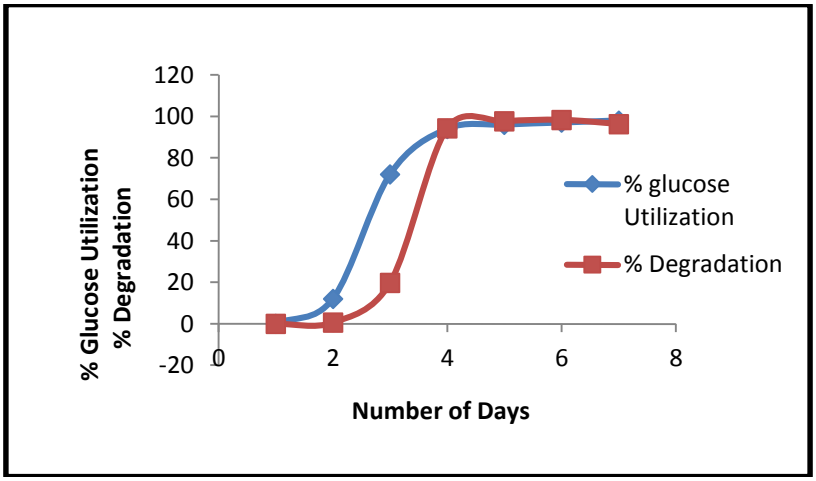

Figure 3: Degradation of Amaranth and Concomitant Utilization of Glucose in liquid batch culture by *P.chrysosporium*

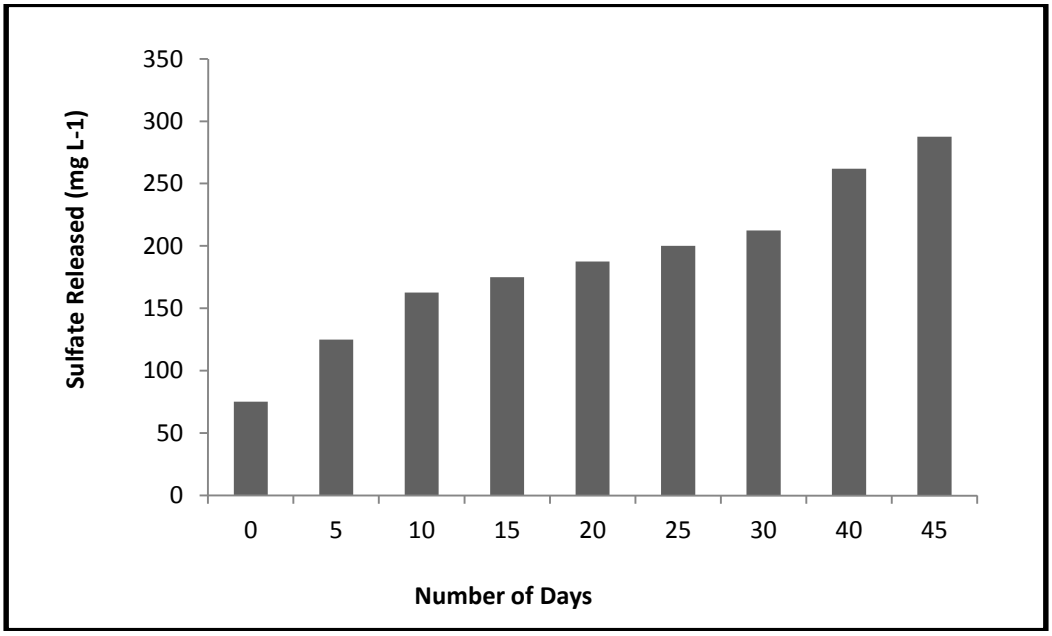

Figure 4: Sulfate Release during Amaranth Degradation

The results of biodegradation of Amaranth in liquid batch culture of *P. chrysosporium* were used for determination of degradation rate constant and consequently half-life.

Kinetics Studies

The maximum rate of biodegradation i.e., $\mathbf{V_{max}}$ and Michaelis-Menten Constant i.e., $\mathbf{K_m}$ was determined for the three xenobiotic compounds, i.e., Amaranth under investigation by using Lineweaver-Burk's double reciprocal plot. The $\mathbf{V_{max}}$ values calculated for Amaranth was 8.8173 mg substrate removed litre^{-1}day^{-1} The K_m values for Amaranth was found to be 4. 00703 mgL$^{-1.}$

The degradation reaction of Amaranth undergoes first order reaction kinetics. A graph of log of concentration v/s time was plotted to determine half life of Amaranth and was found to be 10.34 days.

Biosorption Studies

The mycelium obtained after biodegradation studies was transferred aseptically to fresh medium and kept on shaker conditions under earlier specified conditions. This treatment removes any adsorbed particles from the mycelium. The mycelium was then separated using by filtration. The filtrate was colorless and when analyzed spectroscopically. There was no peak observed at the λ_{max} of Amaranth. This suggests that no adsorption takes place in case of amaranth as the mycelium appears colorless. Thereby we can conclude that decolourisation is purely due to biodegradation of amaranth.

Discussion

P. chrysosporium has been known to mineralize a wide variety of structurally diverse, environmentally significant organic compounds including chlorinated phenols, PCB, dioxins, mono- and polyaromatic hydrocarbons. The ability of *P. chrysosporium* to degrade a wide variety of structurally diverse range of organic compounds is attributed to lignin degrading system of this fungus that is expressed under nutrient- limiting conditions. It requires static culture conditions, high O_2 partial pressure, and addition of veratryl alcohol and detergents, all of which favor the expression and activity of ligninolytic enzymes of this organism (Archibald, 1992) and Dodson *et al.*, (1987). Sulfonated azo dyes, the largest class of dyes have great structural differences and consequently offer a variety of colors. Both sulfonic acid and azo groups are rare among natural products and thus confer a xenobiotic character to sulfonated azo dyes. In our study, we have shown that sulfonated azo dye Amaranth is degraded almost completely by *P. chrysosporium*. It was found that degradation of Amaranth at a concentration of 50mgL^{-1} and 100mgL^{-1} was achieved to the extent of 98% within 30 days after addition of dyes. The compound underwent extensive degradation which was evidenced by substantial decrease in concentration of dye in culture

medium and an increase in sulfate concentration with further incubation. Goszczynski *et al.,* (1994), investigates mineralization of water soluble sulfonated azo dyes by microbial peroxidases of *Phanerochaete chrysosporium* and *Streptomyces chromofuscus.* As permeation through cell membrane is the rate limiting step during bacterial reduction, especially in the case of azo dyes (Haug *et al.,* 1991; Klausener *et al*., 1983; Nortemann *et al.,* 1986) the extracellular enzyme system of *P. chrysosporium* is more effective in this case. *Acinetobacter calcoaceticus* NCIM 2890 was found to decolorize 20 different textile dyes of various classes. Decolorization of an azo dye Amaranth was observed effectively (91%) at static anoxic condition, whereas agitated culture grew well but showed less decolorization (68%) within 48 h of incubation (Ghodake *et al.,* 2011). The effect of various nutritional elements on Amaranth degradation by genus *Trametes* over extended periods of time was investigated. *Trametes meyenii* in particular, did not require nutrient deprivation (Chenaux *et al.,* 2014). In our studies, Amaranth degradation was supported by concomitant utilization of glucose under limited carbon and nitrogen supply. It has been shown that despite high levels of extracellular enzymes, decolorization diminishes as glucose is depleted and is restored by its replenishment (Swamy and Ramsay, 1999a). This could act as a general fixed carbon source required for the production of factors needed by the enzymes or more specifically, it could be a direct precursor of H_2O_2 via glucose-2-oxidase (Champagne *et al.,* 2005; Sen *et al.,* 2012). Release of sulfate in the medium also substantiated biological degradation Amaranth.

Up-flow bioreactor studies with alginate immobilized cells proved the capability of strain to degrade Amaranth in continuous process at 20 ml h^{-1} flow rate. Various analytical studies viz. HPLC, HPTLC, and FTIR gave the confirmation that decolorization was due to biodegradation. Toxicity studies carried out demonstrated that oxidative stress was generated by Amaranth (Jadhav *et al.,* 2013). In our findings also as the concentration of Amaranth was increased from 100mgL^{-1} upto 500mgL^{-1}, a substantial decrease in dye removal was accompanied with decrease in mycelium growth which can be attributed toxic effect of Amaranth at higher concentration.

It has been reported by that much of the initial, rapid loss of dye from culture fluid appears to be due primarily to adsorption upto 30% to the mycelia (Cripps *et al.,* 1990). We therefore conducted adsorption studies on Amaranth, Crystal Violet, Malachite Green, Methylene Blue and Congo Red. All the compounds under study were found not to adsorb onto the mycelia except Congo Red dye. Swamy and Ramsay (1999) reported that white rot fungus, *Trametes versicolor*, degraded Amaranth with no visible sorption.

In *P. chrysosporium*, methylation of phenolic compounds has reported to form corresponding anisoles (Valli and Gold, 1991). These chlorinated anisoles would be expected to be metabolically more stable and this may at least partially account for the comparatively slow rate of metabolism exhibited by *P. chrysosporium*. Sparado *et al., (*1992) established that *P. chrysosporium* was capable of mineralizing a variety of azo dyes and the mineralizaion of aromatic rings of azo dyes was dependent on nature of ring substituents like hydroxyl, an amino, an acetamido, or a nitro substituents. The main reactions that are catalyzed by the ligninolytic enzymes include depolymerization, demethoxylation, decarboxylation, hydroxylation and aromatic ring opening. Many of these reactions result in oxygen activation, creating radicals that perpetuate oxidation of the organopollutants (Reddy *et al.,* 2001).

Kinetics studies revealed that Amaranth showed first order kinetic reaction as most of the organic pollutants are known to undergo first order reactions.

Bioremediation of azo dyes in textile waste effluents by fungi is an alternative to conventional methods for relatively involving low expense and environmental friendly nature. Hence, it has been proposed that *P. chrysosporium* can be useful in color removal and fungal biomass can efficiently and economically be used in bioremediation technologies.

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