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Bioremediation of Dyes using Immobilized Laccase Enzyme

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ABSTRACT

Increased use of synthetic dyes in industries and small-scale laboratories causes water pollutants to rise, which impacts the ecosystem. Because of their high efficiency, selectivity, and environment-friendly reactions, laccase can be very helpful in wastewater treatments. This study aimed to extract and purify laccase from different sources to test its efficiency for decolorization of dyes commonly used in college labs and thus help in bioremediation at the source. Among various sources of laccase enzyme, the P75 fraction of Lemon peel extract showed the highest laccase enzyme activity (0.007 Units/min/ml), followed by the P75 fraction of Pomegranate (0.005 units/min/ml). The enzyme was immobilized using the sodium alginate calcium chloride method. The highest percent decolorization was observed by Pomegranate enzyme extract (87.5%) in 0.025% Crystal violet. P75 fraction of extracts of Pomegranate, Sweet lime, Lemon, and Button Mushroom, when combined in an immobilized form, showed 32.53% dye decolorization of a mixture of three dyes: Safranin, Crystal Violet, and Methylene blue. When tested singly, Crystal Violet showed the highest decolorization of 86.67% by the immobilized mixture of laccase from all sources. The mean percentage decolorization of dyes by immobilized enzyme beads was significantly different as compared to decolorization by enzyme blank (Student's t-test; n=10, $p<0.001$). Kits can be made of immobilized laccase enzyme beads to be used in school and college laboratories for the bioremediation of dyes at the source.

Key words: *Laccases, Bioremediation, Decolorization, Immobilized enzyme, Dyes*

Introduction

Laccases are enzymes that contain 15-30% carbohydrates, having a molecular mass of 60–90kDa. They belong to the multi-copper oxidases (MCOs) enzyme family and are classified as benzenediol oxygen reductases (EC 1.10.3.2). They have low substrate specificity hence they oxidize many phenolic and non-phenolic molecules using oxygen as an electron acceptor and generating water as a by-product (Morozova *et al*. 2007; Upadhyay *et al*. 2016). The substrates of laccases vary from diphenols and polyphenols to diamines, aromatic amines, benzenethiols, and substituted phenols (Kiiskinen 2002).

Laccases are very common enzymes obtained from plants and fungi and could have a wide range of applications in water bioremediation (Arregui, 2019). These enzymes have applications in processes like electrocatalysis, delignification, and ethanol production (Buddolla *et al*., 2014). The enzyme is used in many industries such as the food industry, textile industry, paper and pulp industry (Shraddha *et al*. 2011), wine industry (More *et al*., 2011), biofuel, and pharmaceutical industry (Yang, 2017).

Dyes and reagents used in industries and laboratories are a major concern to the environment and biodiversity. They affect pH, temperature, light, and

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oxygen content of water. They influence aquatic ecosystems, cause soil contamination and pose a risk to public health due to their property of bioaccumulation. Reduced synthetic dyes can be carcinogenic and difficult to remove because of their complex structures in water.

Methods like physical/chemical adsorption, oxidation, and biological treatments are used for dye removal (Akar *et al*., 2013). Laccase can be used to detoxify industrial waste effluents and prevent their harmful effects on the environment (Naz *et al*., 2022). Laccases can be used for wastewater treatment to improve water quality because of their high efficiency, high selectivity, and environmentally benign reactions (Buddolla *et al*., 2014; Alseroury, 2018).

Earlier research on laccase enzyme application catered mostly to industrial and textile dyes, and not to lab dyes. Though on a small scale, laboratories in schools, colleges, and institutes, have the dyes drained out regularly into the water directly.

This study aimed to address the above-mentioned problem. Three dyes that are frequently used in laboratories; Crystal violet (Triphenylmethane type alkaline dye), Methylene Blue (phenothiazine dye), and Safranin (Phenazine), have been targeted in this study. Laccase enzymes from different sources have been extracted and purified (Singh and Kapoor, 2019).

The objectives of this study were to; extract the laccase enzyme from different sources, fractionate and purify the laccase enzyme, determine the protein content and enzyme activity, determine its molecular weight using gel electrophoresis and also to check the property of decolorization of dyes by enzyme extract and by the immobilized enzyme in the form of beads. Immobilized laccase enzyme beads can be used as kits in future for laboratory use.

Materials and Methods

Materials

Pomegranate (*Punica granatum*), Sweet Lime (*Citrus limetta*), Lemon (*Citrus limon*), along with Fungal source Button Mushroom (*Agaricus bisporus*) were collected from the local market.

Methods

Preparation of crude enzyme extract

Fresh fruit peel samples of Pomegranate (*Punica granatum*), Sweet Lime (*Citrus limetta*), Lemon (*Citrus limon*) and Button Mushroom (*Agaricus bisporus*)), were weighed 2 grams each and homogenized by using mortar and pestle in 20mL of 0.1M Sodium acetate buffer, pH 4.5 and filtered through Whatman filter paper. The filtrate was centrifuged at 4000rpm for 15 min. The obtained supernatant was used as crude extract for further studies (Prabhu *et al*., 2019).

Selection of dyes

Methylene blue (0.04%), Safranin (1%), and Crystal violet (0.025%) dyes, commonly used in laboratories, were selected. Their Lambda max (λ max) was determined, which was used for further studies.

Ammonium sulfate precipitation/fractionation of laccase

Initial purification was carried out by salting out proteins by Ammonium Sulfate precipitation. Dialysis was performed to remove ($(NH_4)_2SO_4$) salt from the sample (Duong-Ly and Gabelli 2014).

Protein Estimation

Total protein content in enzyme extract was determined by Lowry's method (Lowry *et al*., 1951). 1.0 ml of sample, 5.0 ml of alkaline copper sulfate reagent were mixed and incubated at room temperature for 20 min. Then 1.0 ml of diluted Folin-Ciocalteu's phenol reagent was added. After 20 min of incubation at room temperature, absorbance was measured at 660 nm. Protein content in the sample was calculated from a standard curve for bovine serum albumin (Prabhu *et al*., 2019).

Estimation of enzyme activity- Laccase Assay

Laccase enzyme activity was measured as O-dianisidine (from High Purity Laboratory Chemicals Pvt. Ltd.) oxidation at 460 nm. The reactive mixture contained per 1 ml: 200 µl citrate (100 mM)-phosphate (200 mM) buffer (pH 5.0), 100 µl o-dianisidine, (1 mM), 600 µl enzyme extract and 100 µl hydrogen peroxide (6%) (Li and Tian, 2007). The reaction mixture was mixed well and incubated for 30 minutes and measured at 460 nm.

Enzyme Immobilization

4% Sodium alginate and purified enzyme extract were mixed in a ratio of 1:1. The beads were formed in a 6% chilled calcium chloride solution (Gao *et al*., 2016).

Dye decolorization experiments

Decolorization experiment was done using the two forms of laccase enzyme (crude extract and immobilized beads). Methylene blue (0.04%), Crystal Violet (0.025%), and Safranin (1%) were prepared. Decolorization was determined by measuring the absorbance of the decolorization medium at wavelengths depending on the dye (Methylene blue 620nm, Crystal Violet 500 nm, Safranin 500 nm). Absorbance was observed at respective Lambda max wavelength for

Test = 1 ml Acetate buffer pH 4.5 + 1ml dye solution + 1 ml enzyme.

Control = 1 ml Acetate buffer pH 4.5 + 1ml distilled water + 1 ml enzyme, and

Enzyme Blank= 1ml Acetate buffer pH 4.5 + 1ml distilled water + 1 ml dye solution. (Sherifah *et al*., 2019).

The decolorization percentage of dyes was calculated by using formula:

Decolorization percentage (%) = $(A_0 - A)/A_0 \times 100$

Where, A_0 = Initial absorbance of dye and A = Absorbance of dye after treatment

Statistical Analysis

Student's t-test was performed for the decolorization of dyes. Two hypotheses were set, Null hypothesis: All sample means are equal and Alternate Hypothesis: At least one sample mean is significantly different (Mahajan, 2008).

Molecular weight analysis

The purified laccase enzyme sample was subjected to Native-PAGE using 10% separating gel and 5% stacking gel (Gaur *et al*., 2018).

100 µl sample was mixed with 100 µl Gel Loading Buffer. The protein markers and samples were loaded on the gel, and run at 50 V initially and later at 100 V. The molecular weight of laccase was determined by comparing against the protein ladder with 12 prestained proteins covering a range of molecular weights from 10 to 245 kDa.

The gel was stained with 0.1% Coomassie brilliant blue for 20 minutes and then destained (40% methanol and 10% glacial acetic acid) overnight (Preissler, 2019; Preissler *et al*., 2015)

Results

Lambda max (λ max) of selected dyes

Lambda (λ) max for Safranin and Crystal violet was 500 nm, for Methylene Blue 620 nm and for the mixture of three dyes it was found to be 500 nm.

Extraction and Purification

Optimum pH 4.5 was maintained for the extraction, purification, and decolorization of dyes (Morsy, 2020). Crude enzyme was then subjected to the purification process shown in Figure 1. As Patel *et al*. (2014) reported, complete precipitation of laccase from crude extract required 75% saturation of supernatant by ammonium sulfate. It was confirmed by Lowry's method for protein estimation and laccase enzyme assay by O-dianisidine. After dialysis, the supernatant was used for further estimation processes.

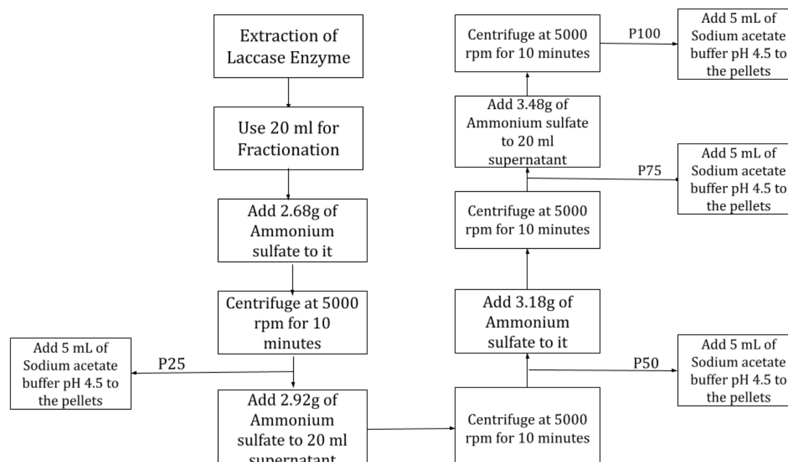

Fig. 1. Procedure for Ammonium sulfate fractionation of laccase

Protein Estimation

Protein content in the fractions was calculated from the standard protein graph shown in Figure 2. As shown in Table 1, P75 fraction for Pomegranate showed 0.424 mg/ml, Lemon 0.09mg/ml, Button mushroom 0.039mg/ml, and the P100 fraction of Sweet Lime showed 0.12mg/ml protein.

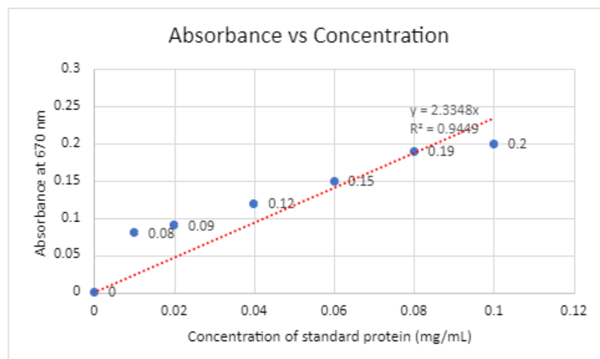

Fig. 2. Standard protein graph by Lowry's Method

Table 1. Protein content in fractions

EnzymeFraction	Absorbance at670 nm (Average)	Calculated Protein Content using equationy =2.3348x (mg/ml)
Pomegranate P25	0.52	0.223
Pomegranate P50	0.53	0.227
Pomegranate P75	0.99	0.424
Pomegranate P100	0.84	0.360
Mushroom P25	0.07	0.030
Mushroom P50	0.08	0.034
Mushroom P75	0.09	0.039
Mushroom P100	0.05	0.021
Sweet Lime P25	0.16	0.069
Sweet Lime P50	0.21	0.090
Sweet Lime P75	0.25	0.107
Sweet Lime P100	0.29	0.124
Lemon P25	0.08	0.034
Lemon P50	0.14	0.060
Lemon P75	0.22	0.094
Lemon P100	0.20	0.086

Laccase Enzyme Assay

As shown in Table 2, the highest laccase enzyme activity was observed in the fractions with the highest protein content of P75 fraction for Pomegranate, Mushroom, and Lemon and the P100 fraction of Sweet Lime, hence these fractions were selected for further dye decolorization experiments.

Table 2. Laccase Enzyme Activity in enzyme fractions

Enzyme Fraction	Absorbance at 460 nm	Enzyme Activity (units/min/ml)
Pomegranate P25	0.03	0.001
Pomegranate P50	0.08	0.0027
Pomegranate P75	0.15	0.005
Pomegranate P100	0.06	0.002
Mushroom P25	0.01	0.0003
Mushroom P50	0.01	0.0003
Mushroom P75	0.05	0.0017
Mushroom P100	0.03	0.001
Sweet Lime P25	0.00	0
Sweet Lime P50	0.01	0.0003
Sweet Lime P75	0.03	0.001
Sweet Lime P100	0.08	0.0027
Lemon P25	0.02	0.0007
Lemon P50	0.06	0.002
Lemon P75	0.21	0.007
Lemon P100	0.19	0.006

Dye Decolorization Experiments

1% Safranin, 0.025% Crystal Violet, and 0.04% Methylene Blue dyes were decolorized by the purified and immobilized laccase enzymes. The observed results in this study were similar to that reported on the purified and immobilized laccasesof *Kluyveromyces dobzhanskii* DW1 and *Pichia manshurica* DW2 by Sherifah *et al*., 2019, which efficiently decolorized two synthetic dyes Malachite green and Methyl red.

As shown in table 3, it was observed that the immobilized enzyme from P75 fraction of Pomegranate extract and P75 fraction of Lemon Extract in the form of sodium alginate beads were efficient ($p<0.001$) in decolorizing the synthetic dye Safranin.

Readings were taken up to day 4. Further readings were found to be stable.

It was observed that the immobilized enzyme from P75 fraction of Pomegranate extract and P75 fraction of Lemon extract in the form of sodium alginate beads were efficient ($p<0.001$) in decolorizing the synthetic dye Crystal Violet as shown in table 4. When all the extracts were mixed and immobilized beads were formed using the combined extract they also showed efficient decolorization of Crystal Violet ($p<0.001$).

It was observed that the immobilized enzyme from P75 fraction of Pomegranate extract and P75 fraction of Lemon extract were efficient ($p<0.001$) in decolorizing the synthetic dye Methylene Blue as shown in Table 5. When all the extracts were mixed

and immobilized beads were formed using the combined extract also showed efficient decolorization of Methylene Blue ($p<0.001$).

Table 6 shows absorbance readings of the mixture of dyes decolorized by immobilized beads. P75 fraction of Pomegranate extract and immobilized form of mixture of enzymes were efficient in decolorizing the mixture of dyes ($p<0.001$).

Molecular weight analysis

Fractions P75 for Pomegranate, Lemon, and Button mushroom and fraction P100 for Sweet lime (fractions having the highest enzyme activity and protein content) were further analyzed by Native-PAGE for molecular weight analysis. As shown in Figure 3, pomegranate fraction P75 showed a band on Na-

Table 3. Immobilized beads assay for 1% Safranin: (Absorbance: 500nm) Buffer used: Sodium Acetate Buffer (pH 4.5)

Time	Blank (Buffer+Dye+ Distilled water)	Buffer+Dye+ P75 Lemon enzyme extract beads	Buffer+ Dye+ P75 Pomegranate enzyme extract beads	Buffer+ Dye+P75 All enzyme extract beads	Buffer+ Dye+ Beads without enzyme (Enzyme Blank)
0 Min	0.73	0.72	0.66	0.72	0.73
60 Min	0.72	0.72	0.60	0.72	0.71
Day 2	0.66	0.64	0.48	0.67	0.64
Day 3	0.57	0.49	0.35	0.55	0.55
Day 4	0.54	0.50	0.28	0.50	0.53
% Decolorization	26.02%	30.55%	57.57%	30.55%	27.39%
Mean % Decolorization(n=10)	26.03%	30.56%	57.58%	30.56%	27.3%
Standard Deviation	1.543	1.941	2.313	1.976	3.014
t-value		5.778	35.88	2.771	
p-value		5.778> 4.781 at p<0.001	35.88> 4.781 at p<0.001	2.771>2.262 at p<0.05	

% Decolorization= (A0 -A)/ A0 x100; Where,
A0= Initial Absorbance of dye taken on 0th minute at 500 nm
A= Absorbance of dye after treatment taken on Day 4 at 500 nm

Table 4. Immobilized beads assay for0.025%Crystal Violet: (Absorbance :500 nm)
Buffer used: Sodium Acetate Buffer (pH 4.5)

Time	Blank (Buffer+Dye+ Distilled water)	Buffer+ Dye+P75 Lemon enzyme extract beads	Buffer+Dye+ P75 Pomegranate enzyme extract beads	Buffer+Dye+ P75 All enzyme extract beads	Buffer+Dye+ Beads without enzyme (Enzyme Blank)
0 Min	0.45	0.41	0.32	0.45	0.45
60 Min	0.44	0.35	0.28	0.39	0.40
Day 2	0.44	0.29	0.19	0.23	0.36
Day 3	0.34	0.25	0.09	0.15	0.32
Day 4	0.33	0.19	0.04	0.06	0.30
% Decolorization	26.67%	53.66%	87.5%	86.67%	33.33%
Mean % Decolorization(n=10)	26.67%	53.66%	87.5%	86.44%	33.56%
Standard Deviation	2.257	4.093	2.099	2.503	3.203
t-value		12.354	62.390	41.129	
p-value		12.354>4.587 at p<0.001	62.39>4.587 at p<0.001	41.129>4.587 at p<0.001	

% Decolorization = (A0-A)/A0 × 100; Where,
A0= Initial Absorbance of dye taken on 0th minute at 500 nm
A= Absorbance of dye after treatment taken on Day 4 at 500 nm

tive-PAGE with MWt 73kDa when compared to the protein ladder. Gaur *et al.* (2018) reported the molecular weight of laccases to be in the range of 60 to 100 kDa.

Discussion

In the current study, Pomegranate extract showed the highest enzyme activity among various sources of laccase enzyme. The enzyme was immobilized using the sodium alginate and calcium chloride method.

Lemon and Pomegranate Enzyme extracts had significant capacity todecolorizedyes.

As a combined source, all four selected sources: Pomegranate, Sweet lime, Lemon, and Button

Table 5. Immobilized beads assay for 0.04% Methylene Blue: (Absorbance: 620nm)
Buffer used: Sodium Acetate Buffer (pH 4.5)

Time	Blank (Buffer+Dye+ Distilled water)	Buffer+Dye+ P75 Lemon enzyme extract beads	Buffer+Dye+ P75 Pomegranate enzyme extract beads	Buffer+Dye+ P75 All enzyme extract beads	Buffer+Dye+ Beads without enzyme (Enzyme Blank)
0 Min	0.31	0.31	0.27	0.29	0.31
60 Min	0.30	0.30	0.26	0.28	0.30
Day 2	0.29	0.26	0.20	0.21	0.26
Day 3	0.26	0.23	0.15	0.17	0.25
Day 4	0.26	0.20	0.13	0.15	0.24
% Decolorization	16.13%	35.48%	51.85%	48.28%	22.58%
Mean % Decolorization (n=10)	16.77%	35.5%	51.9%	48.3%	22.7%
Standard Deviation	3.949	4.467	4.083	5.485	3.624
t-value		9.925	19.532	12.362	
p-value		9.926>4.587 at p<0.001	19.532>4.587 at p<0.001	12.362>4.587 at p<0.001	

% Decolorization= (A0 -A)/ A0 x100; Where,
A0= Initial Absorbance of dye taken on 0th minute at 620 nm
A= Absorbance of dye after treatment taken on Day 4 at 620 nm

Table 6. Immobilized beads assay for Mixture of dyes (1:1:1 Ratio)
Buffer used: Sodium Acetate Buffer (pH 4.5)

Time	Blank (Buffer + Dye+ Distilled water)	Buffer+ Dye + P75 Lemon enzyme extract beads	Buffer + Dye + P75 Pomegranate enzyme extract beads	Buffer+ Dye+P75 All enzyme extract beads	Buffer+ Dye+ Beads without enzyme (Enzyme Blank)
0 Min	0.83	0.85	0.85	0.83	0.83
60 Min	0.82	0.80	0.85	0.83	0.81
Day 2	0.69	0.73	0.63	0.66	0.75
Day 3	0.64	0.68	0.60	0.61	0.70
Day 4	0.61	0.58	0.57	0.56	0.64
% Decolorization	26.51%	31.76%	32.94%	32.53%	22.89%
Mean % Decolorization(n=10)	26.32%	31.68%	32.9%	32.6%	22.79%
Standard Deviation	2.514	3.306	2.679	0.9	2.423
t-value		4.075	5.688	12.052	
p-value		4.075>3.250 at p<0.01	5.197>4.587 at p<0.001	12.052>4.587 at p<0.001	

% Decolorization= (A0 -A)/ A0 x100; Where,
A0= Initial Absorbance of dye taken on 0th minute at 500 nm
A= Absorbance of dye after treatment taken on Day 4 at 500 nm

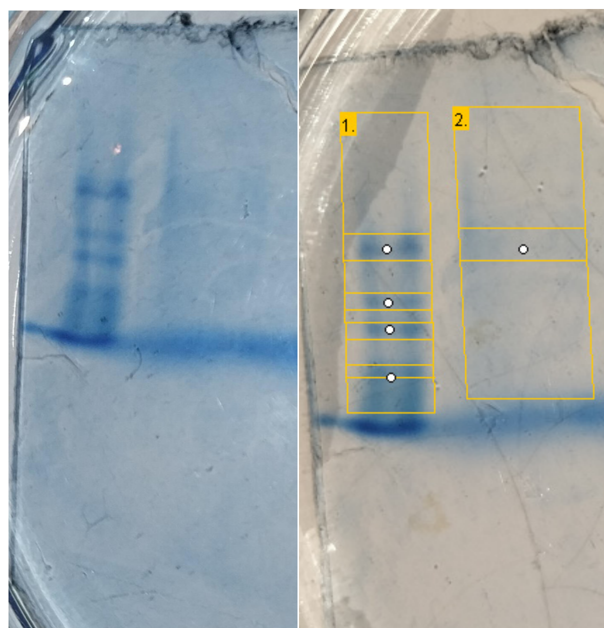

Fig. 3. Molecular weight analysis

Lane #	Band #	Rf	Raw volume	Cal. volume	MW
1.	1.	0.456	8969	-	75
1.	2.	0.631	5446	-	63
1.	3.	0.721	5439	-	48
1.	4.	0.882	4389	-	35
2.	**1.**	**0.485**	**8400**	-	**73**

The data was obtained from GelAnalyzer 19.1 Software. (GelAnalyzer 19.1 (www.gelanalyzer.com) by Istvan Lazar Jr., Ph.D., and Istvan Lazar Sr., Ph.D., CSC)

Mushroom, together in immobilized form, showed 32.53% dye decolorization in a mixture of three dyes: Safranin, Crystal Violet, and Methylene blue. Compared to other dyes, Crystal violet individually showed the highest decolorization of 87.5% by the immobilized mixture of laccase from all sources.It was observed that there is better decolorization of individual dyes by the enzyme beads.

The three dyes selected in this study are the most common laboratory dyes used in staining procedures, and these are then drained out as waste in the water. Hence this study will help in decolorization of these dyes before draining out, thus aiding in bioremediation.Immobilized laccase enzyme beads can be used as kits for small-scale bioremediation of lab effluents, especially in schools and colleges (Nasief and Abd, 2019). Such kits can be distributed in schools and colleges.

Further shelf life of these immobilized beads can be determined by measuring the enzyme activity at time intervals. New sources can be screened for laccase enzyme. Besides these dyes, laccase enzyme can be tested against other commonly used lab reagents and thus widen its scope and utility.

Immobilized laccase beads can be used for bioremediation, at source, of various dyes and reagents which are used in laboratories and as the laccase enzyme is extracted from fruit peels, the study and its application are very sustainable, contributing to waste managementthus helping in achieving the targets of the UN SDG Accord of Waste Management (SDG Goal 11, 12).

Conflict of Interest

The authors declare no conflict of interest.

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