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Phenol removal by soluble and alginate entrapped turnip peroxidase

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Abstract

This paper is a comparative study of phenol biodegradation by soluble and alginate entrapped turnip *(Brassica rapa)* peroxidase. The effects of relevant factors on the process such as pH, temperature, concentration of H_2O_2, phenol concentration, enzyme activity and contact time were evaluated in order to optimize the conditions for maximum phenol removal. Results showed that the obtained average removal yield under optimal conditions was 93%. The process duration was 3 hours. The reaction is conducted in aqueous medium under optimal pH 7 and temperature of 40 °C. The highest removal percentage was obtained for phenol concentrations of 80 and 46 mg L^{-1} with soluble and entrapped enzyme respectively.

Keywords: Biodegradation, Immobilization, Turnip Peroxidase, Phenol.

Introduction

Phenols are present in a number of industrial wastewaters, such as petrochemical plants (2.8–1220 mgL^{-1}) and oil refineries (6–500 mg L^{-1}), coal processing (9–6800 mgL^{-1}). Other phenol-containing wastewaters are pharmaceutical, wood, paint, pulp, paper and plastic industries (0.1–1600 mgL^{-1}) (Busca et al. 2008). Phenolics are toxic to fish even at 1–2 ppm and to most of the aquatic organisms in the range from 10–100 ppm due to negative impacts for ecosystems and humans (toxicity, carcinogenic and mutagenic properties) (Babuponnusami et al. 2012).

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Thus, the removal of phenols from industrial wastewater is a problem of great concern. When it comes to removing phenol from wastewater, there are several methods that can be used including; adsorption, incineration, chemical oxidation, irradiation methods and microbial degradation (Mingliang et al. 2013).

These methods frequently present disadvantages, such as low efficiency or could generate products which are even more toxic than phenols (Gómez et al. 2006).

On the other hand, high production costs inhibit the widespread use of these techniques for remediation in industrial scale (Del Castillo et al. 2012). However, enzymatic treatment strategies look to be an environmental-friendly, cheaper, abundant, more reliable and simpler to implement (Busca et al. 2008; Karthik et al., 2008). Horseradish, soybean and turnip peroxidases are among the most studied enzymes for phenol enzymatic degradation (Mohsina et Rehman 2009).

In this study, the comparative study of phenol biodegradation in batch experiments by soluble and alginate entrapped turnip peroxidase was investigated. Effects of factors such as pH, temperature, substrate concentration, enzyme activity and contact time were studied in order to determine optimal conditions under which maximum phenol removal could be achieved.

Materials and Methods

Material

Turnip Peroxidase (TP) was isolated from turnip. Hydrogen peroxide (30 % w/v) was purchased from Panreac. Phenol crystallized was purchased from Panreac; Sodium alginate from Lamirania hyperborean and hexahydrated calcium chloride were obtained from BDH (UK); Potassium Chloride buffer (10 mM, pH 2); Potassium biphthalate buffer (10 mM, pH 3.0–5.0); Potassium phosphate buffer (10 mM, pH 6.0 and 7.0); Potassium chloride and boric acid buffer (10 mM, pH 8.0–10.0) Sigma-Aldrich; 4-aminoantipyrine and potassium ferricyanide were purchased from Fluka chemika. All other

chemical were of analytical grade and were used without further purification.

Extraction and partial purification of peroxidase from turnip roots

Fresh turnip roots (100 g) are peeled and minced, then mixed with 200 mL of distilled water. The mixture was filtered using a cotton gauze filter to remove large poorly ground particles. The filtrate was labeled as turnip peroxidase crude extract and then precipitated using cold acetone (-20 °C; 2:1 ratio acetone/extract). The precipitate was collected by centrifugation at 6,000 rpm for 10 min, redisolved in 10mM potassium phosphate buffer (pH 7.0).
It was stored at 4 °C and warmed to room temperature immediately prior to use.

Immobilization by entrapment method

Immobilization was achieved by dissolving 0.15 g of sodium alginate in 10 mL of soluble TP. The mixture was agitated for 1 to 1.5 hours until obtaining a homogeneous viscous mixture. Calcium alginate capsules were prepared by extrusion using a simple one step process similar to that described by Nigma et al. (1988). The alginate-enzyme mixture was added dropwise using a syringe needle (20 G) into 50 mL of a gelling agent solution of $CaCl_2$ (0.05M) under constant agitation. Beads were left in the $CaCl_2$ solution for 1 hour, then washed with distilled water and stored in a buffer solution (pH 7.0) in a refrigerator (4 °C).

Activity measurement of soluble and immobilized enzyme

The TP activity was analyzed by the 4-aminoantipyrine (Am-NH_2) method (Nicell et wright 1997). For this, 0.2 mL of a soluble enzyme or approx. 25 beads of entrapped enzyme were added to 4 mL of the former mixture. The mixture consisted of 1 ml of a 0.1 M phenol solution, 1 mL of a 0.01 M Am-NH_2 solution, 1 ml of a 0.1mM H_2O_2 solution and completed by potassium phosphate buffer (10 mM, pH 7.0) in a 50 mL flask. The enzyme active concentration is proportional to the color development rate measured at 505 nm, during a period of time in which the substrate concentration is not significantly reduced (Sadasivam et Manickan 2004).

Phenol degradation

Experiments were carried out in a closed assay tube under constant temperature. The reaction mixtures contained 1 mL of hydrogen peroxide, 1 mL of phenol, 2.8 mL of buffer solution and 0.2 mL of soluble enzyme or 25 of alginate beads. One parameter was varied at a time and the optimal value was selected for the following experiment. Mixtures were kept for a fixed duration without agitation. Details are presented in table 1.

Table 1: Ranges of experimental parameters.

Parameters	Range
pH	2-10
T (°C)	20-80
Phenol concentration (mg L^{-1})	20-140
H_2O_2 concentration(mM)	4-24
Enzyme activity(UmL^{-1})	1-7
Contact time (min)	0-210

Phenol analysis

Phenol concentration was determined using Am-NH_2 colorimetric method (Alemzadeh et Nejati 2009). Absorbance was measured at 505 nm against a blank with a UV–Vis spectrometer (UVmini-1240, Shimadzu). The percentage of phenol removal was defined as:

$$\text{Phenol Removal (\%)} = \frac{A(0)-A(t)}{A(0)} \times 100 \quad (1)$$

Where A(0) was the initial absorbance of untreated solution and A(t) the absorbance of the treated solution at a given time.

Results and Discussion

Effect of pH

This parameter acts both on the enzyme (Fersht 1984; Whitaker 1994; Seyhan et al. 2002) and on phenol ionization state (Siva et al. 2009). The effect of this parameter at fixed activity of soluble or alginate entrapped TP (2.3U mL^{-1}) were measured in buffer solutions of various pH values (2.0–10.0) at room temperature (25 °C).The molarity of each buffer was 0.01 $molL^{-1}$. Phenol and peroxide hydrogen initial concentrations were, respectively, 60 mgL^{-1} and 2 mM. Maximum phenol removal (43.6%) was obtained when pH was maintained at a sharp value of 7 with soluble enzyme. A dramatic decrease in the removal yield was observed when pH was shifted by more than a unit. While in the case of alginate entrapped TP, the pH tolerance was larger, between 5 and 9.

The highest yield (29.5%) was noticed for pH values between 6 and 8. Similar types of observations were also reported by Alemzadeh and Nejati (2009) with Horseradish peroxidase (HRP). Contrariwise, Yasha and Qayyum 2006 have found an optimal pH at 5.0 by soluble and cellulose bound TP which as due to the existence of isoenzymes as confirmed by exclusion chromatography. The optimum pH of peroxidases from various vegetable sources was recorded in the acidic range (Vamos-vigyazo 1981). *Cucumissativus* peroxidases show a bell-shaped pH dependence on catalytic activity, which reaches a maximum between 5.5 and 7.5 and sharply decreases outside the interval (Duarte-Vazquez et al. 2000). A change in pH would affect the extent to which each functional group ionizes, in turn affecting the extent of their interactions with each other, and thus the conformation of the enzyme. A change in the structural conformation will inevitably affect the shape of the active site, and thus either increase or decrease the enzyme affinity for substrate molecule (Dunford 1999). More alkaline optimal pH (9.0) was detected for peroxidases from buck wheat (Suzuki et al. 2006).

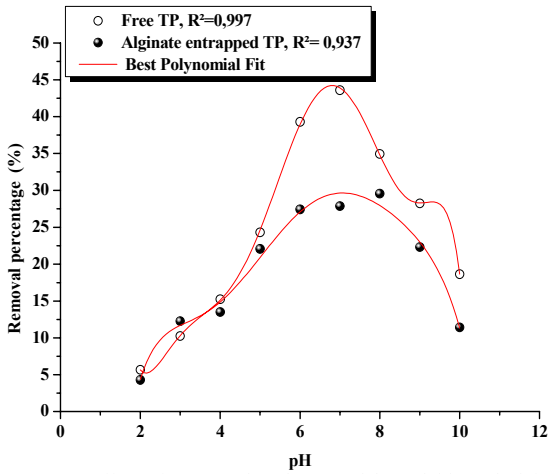

Figure 1: Effect of pH on phenol removal by soluble and alginate entrapped TP.

Effect of temperature

Series of phenol degradation assays were conducted under selected temperatures (20 to 80°C) by using an incubator under the same initial concentrations of phenol (60 mg L^{-1}), Hydrogen peroxide(2 mM) and enzyme activity (2,3 U mL^{-1}). Solutions were prepared in buffers at pH7. Results are shown in figure 2. Enzyme activity increases with increasing temperature, which affects positively the yield of phenol removal. Under optimal temperature of 40°C, maximum removal yields were attained 48.4 and 35.7% by soluble and alginate entrapped TP respectively. Above this temperature, a dramatic decrease occurred due to partial enzyme denaturation. These results could be compared to those by Yasha and Qayyum (2006). The catalytic activity of both soluble and immobilized cellulose bound TP showed same temperature optima at 30°C. In general, the temperature for maximum activity of peroxidases is very broad in the interval 30 –70 °C. An activation up to 60 °C was marked and a strong inhibition at 80 and 90 °C in grapes and apple (Dubey et al. 2007), papaya (Silva and al. 1990), orange (Mohamed et al. 2008) and castor (Kumar et al. 2008).

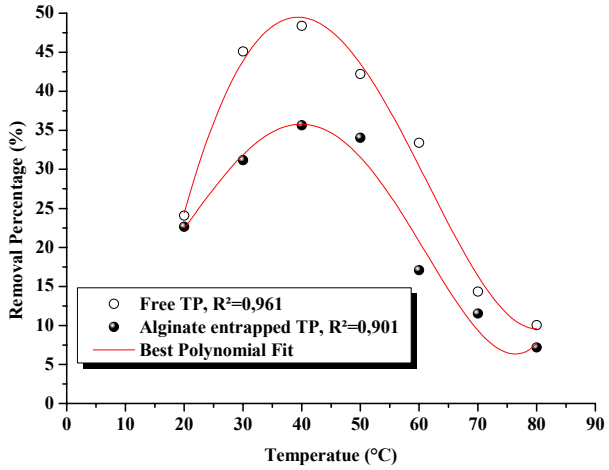

Figure 2: Effect of temperature on phenol removal by soluble and alginate entrapped TP.

Effect of phenol concentration

The experiments were carried out under the same previous conditions with hydrogen peroxide concentration of 2 mM and enzymatic activity of 2.3 U mL^{-1}. Contact time was fixed (2 hours) under optimal pH (7.0) and temperature (40°C). Initial phenol concentration was varied from 20 to 140 mg L^{-1} with an increment of 20 mg L^{-1}. Figures 3 and 4 show the variations of enzymatic degradation rate and yield with initial phenol concentration by soluble and entrapped TP. For both cases, the removal rate increased and the removal yield decreased with increasing phenol concentration. The highest rate is reached at phenol concentration of 80 mg L^{-1}, above which no further significant changes were noticed. The highest removal yield and rate were determined at phenol concentrations of 80 and 45 mg L^{-1} in the case of soluble and alginate entrapped TP respectively. The effect of diffusional limitations in alginate beads was also remarkable when comparing removal rates in both cases (Moon et al. 2005). This phenomenon could be well showed by calculating Michaelis–Menten kinetic parameters, V_{max} and K_m, from Lineweaver–Burk presentation as illustrated in figure 5 and table 2.

Optimal [H_2O_2]/[Phenol] molar ratio

Experiments were carried under optimal conditions of pH (7.0), temperature (40°C), activity (2.3 U mL^{-1}) and phenol concentration (80 mg L^{-1}). To study the effect of hydrogen peroxide concentration (the co-substrate) on the removal capacity, hydrogen peroxide was varied from 1 to 25 mM. Results are shown in figure 6.

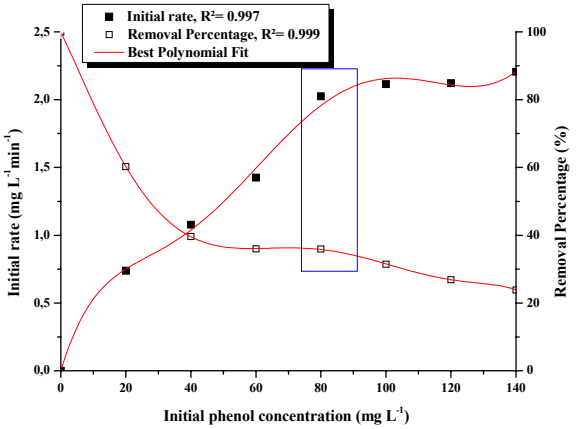

Figure 3: Effect of phenol concentration on initial rate and yield of phenol removal by soluble TP.

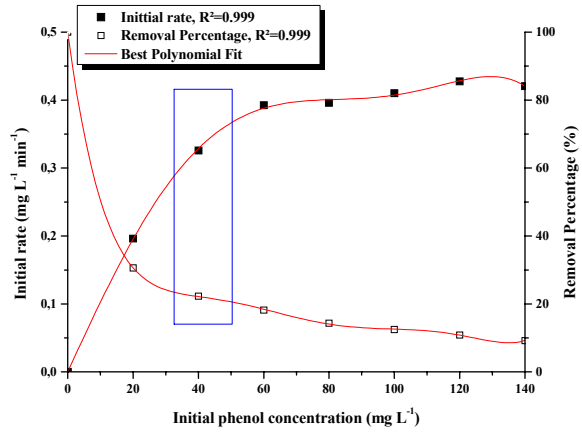

Figure 4: Effect of phenol concentration on initial rate and yield by entrapped TP.

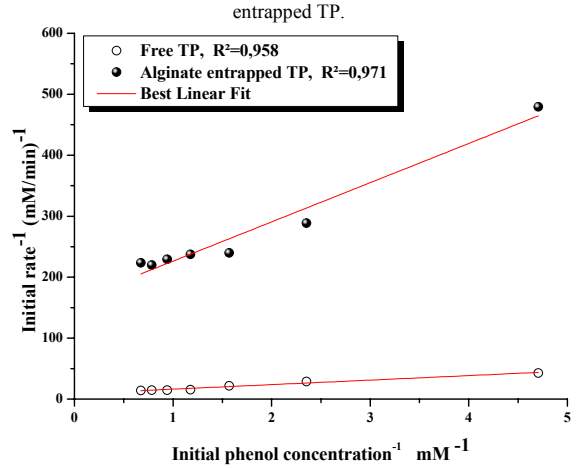

Figure 5: Lineweaver-Burk representation for phenol biodegradation by soluble and entrapped TP.

Table 2: Apparent kinetic parameters for the reaction of phenol removal of native and alginate entrapped TP.

TP preparation	V_{max} (µM min^{-1})	K_m(mM)	R^2
Native	37.170	0.826	0.971
Alginate-entrapped	6.170	0.397	0.958

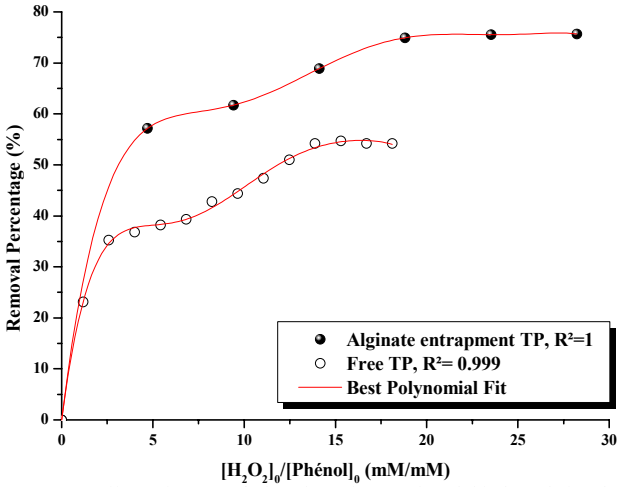

Figure 6: Effect of $[H_2O_2]_0/[Phenol]_0$ ratio on phenol biodegradation by soluble and entrapped TP.

From figure 6, it was noticed that phenol removal increased with hydrogen peroxide concentration. When the ratio of peroxide to phenol was about 20, which corresponds to an initial peroxide concentration of 24 and 13mM in the cases of free and entrapped TP respectively, a maximal yield (76 and 55%) was attained.

Effects of TP concentration

As for any enzymatic reaction, the removal of phenol by TP is dependent on the amount of catalyst added. The influence of this parameter on the phenol removal was investigated by varying catalyst concentration from 1 to 7 U mL^{-1}. Experiments were conducted under optimal conditions established previously by keeping the contact time (2 hours). Figure 7 depicts the effect of enzyme dose on phenol removal.

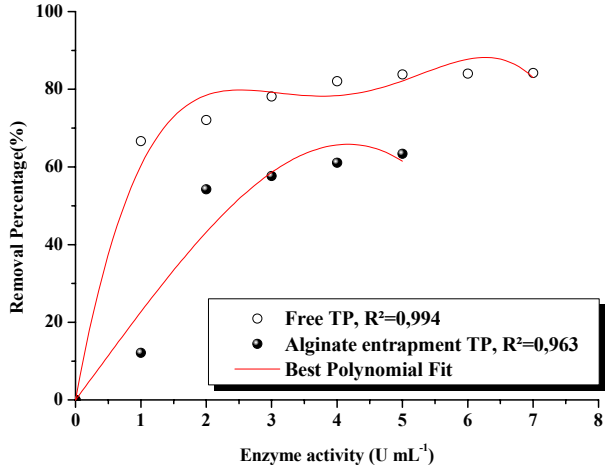

Figure 7: Effect of enzymatic activity on phenol biodegradation by soluble and entrapped TP.

According to the results shown in figure 7, phenol removal increases with increasing enzyme activity. This result was predictable since the availability of enzymatic sites induce the attraction with substrate molecules and subsequently the increase in the number of molecules converted per time. For an activity value between 3 and 4 U mL^{-1}, 84 % of phenol was removed by free TP, while in the case of immobilized enzyme the yield was only 61 %.

Effect of contact time

Initial experiments were performed in order to assess the optimum contact time required for phenol removal. Series of test tubes each one containing phenol and hydrogen peroxide at optimal concentrations, 2.8 mL of buffer solution (pH 7) and 4 U mL^{-1} of soluble or entrapped TP, were incubated at constant temperature of 40 °C. Every 15 minutes, a tube was centrifuged and the supernatant solution was analyzed for the residual phenol concentration. Figure 9 shows the effect of contact time on phenol removal by free and encapsulated enzyme. It was shown that duration between 140 and 180 min was necessary to reach maximum phenol removal. Table 3 resumes the conditions under which maximum phenol removal was achieved by soluble and immobilized TP. The limited value of removal yield in the case of entrapped TP (64%) when compared to soluble enzyme could be due to diffusional limitations causing the accumulation of degradation by products inside the beads and therefore limiting substrate penetration.

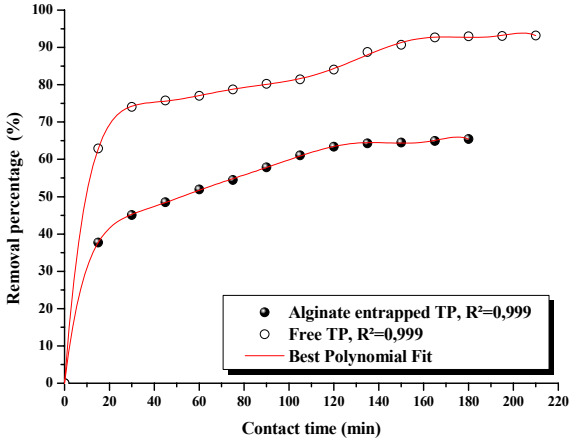

Figure 8: Biodegradation kinetics of phenol under optimal conditions.

Table 3: Optimal values for phenol biodegradation by soluble and alginate entrapped TP.

Parameters	Soluble TP	Alginate entrapped TP
pH	7.0	8.0
T(°C)	40	40
Initial rate (µM mn^{-1})	37.17	6.17
$[Phénol]_0$ (mg L^{-1})	80	45
$[H_2O_2]_0$ (mM)	24	13
$[H_2O_2]_0/[phenol]_0$ (mM/mM)	28	27
Enzymatic activity (U mL^{-1})	7	5
Contact time (min)	180	135
Removal percentage (%)	93	64

Reusability of alginate entrapped TP in phenol removal

The reusability of entrapped TP could be easily assessed for its remained catalytic activity. To demonstrate the reusability of encapsulated enzyme, capsules were separated after 135 min of reaction time and then rinsed with distilled water. The capsules were used for subsequent batches. After five repeated tests, phenol removal efficiency was reduced by more than 90% of its initial value (figure 9). This could be due to the accumulation of degradation byproducts into the beads (figure10), which caused a barrier to phenol penetration. Similar results were reported by Quintanilla-Guerrero and al. (2008) which the phenol removal down to 10% after the fifth cycle using calcium alginate-entrapped TP activity of

1.2 U ml^{-1}, 0.8 mM of hydrogen peroxide, 0.5 mM of phenol concentration and with a cycle contact time of 10 min. Alemzadeh and al. (2009) also reported the decrease in phenol removal capacity to more than 50% after 5 repeated batches using alginate entrapped HRP (7.5 U mL^{-1}) with 0.02 mM of hydrogen peroxide concentration during 100 min of contact time.

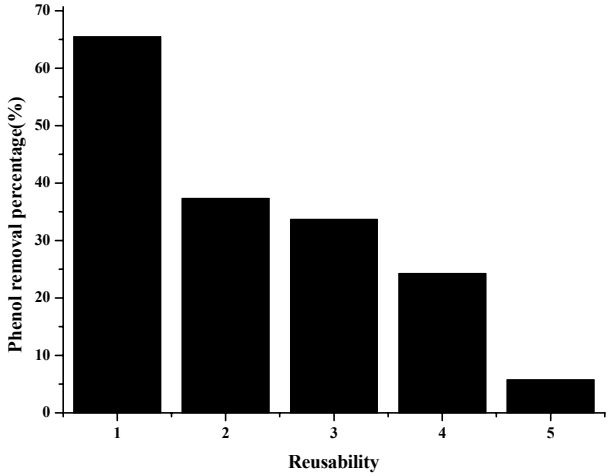

Figure 9: Reusability of alginate entrapped TP, [Phenol] =45 mgL^{-1}; [enzyme] = 5 U mL^{-1}.

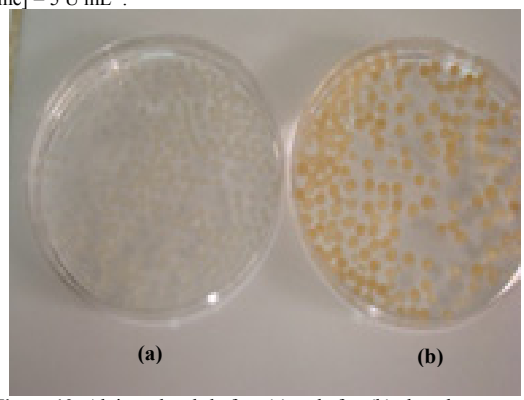

Figure 10: Alginate beads before (a) and after (b) phenol treatment.

Conclusions

The degradation of phenol by soluble and immobilized *Brassica rapa* reactions are affected by many factors (temperature, pH, initial concentrations of phenol and hydrogen peroxide, enzyme activity and contact time. Optimal conditions were determined by one factor at a time approach. Diffusional limitations in alginate beads affected dramatically the reaction kinetics which led to a limited degradation yield when compared to the one obtained with soluble enzyme.

The process seems to be a valuable low cost alternative for phenol removal from industrial waste waters. Peroxidase in the soluble form showed higher degradation efficiency when compared to the immobilized form.

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