A new and effective approach to boron removal by using novel boron-specific fungi isolated from boron mining wastewater

Burcu Ertit Taştan, Dilara Nur Çakir and Gönül Dönmez

ABSTRACT

Boron-resistant fungi were isolated from the wastewater of a boron mine in Turkey. Boron removal efficiencies of *Penicillium crustosum* and *Rhodotorula mucilaginosa* were detected in different media compositions. Minimal Salt Medium (MSM) and two different waste media containing molasses (WM-1) or whey + molasses (WM-2) were tested to make this process cost effective when scaled up. Both isolates achieved high boron removal yields at the highest boron concentrations tested in MSM and WM-1. The maximum boron removal yield by *P. crustosum* was 45.68% at 33.95 mg l^{-1} initial boron concentration in MSM, and was 38.97% at 42.76 mg l^{-1} boron for *R. mucilaginosa*, which seemed to offer an economically feasible method of removing boron from the effluents.

Key words | biological uptake, boron, *Penicillium crustosum*, *Rhodotorula mucilaginosa*, wastewater treatment

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INTRODUCTION

Boron is found in rocks, soil, and water; in boron-rich areas its concentration can be as high as 100 ppm (Woods 1994). Several industries such as ceramics, glass and frit, detergents and soaps use boron in their processes. According to the Royal Society of Chemistry, boron has been used for different applications such as in rocket fuel igniter, pyrotechnic flares, eye drops, mild antiseptics, washing powders, tile glazes, pyrex, fiberglass textiles, neutron absorber in nuclear reactors, foliar fertilizers and food preservatives. As reported by the US Department of Health and Human Services Agency for Toxic Substance & Disease Registry (ATSDR) boron is also used in agriculture as a micronutrient, and there are 189 pesticide products registered in the United States that contain boric acid or one of its salts as an active ingredient (ATSDR 2010).

As for all essential trace elements, boron in excess becomes toxic to organisms, and the reported high boron concentrations in surface waters ranges from 15 mg l^{-1} to 360 mg l^{-1} (ATSDR 2010). General clinical effects of boron in rats, mice and rabbits given 0.2–2 g boron kg^{-1} body weight per day doses are depression, ataxia, decreased body temperature, observed testicular lesions and changed skin color. The reported lethal doses of boron are 0.03–20 g kg^{-1} body weight for human (WHO 2009). According to Gunes *et al.* (2006) 10–30 mg kg^{-1} boron concentrations significantly reduced leaf and root growth of grapevine.

According to the EPA (Environmental Protection Agency) the world's largest boron minerals are in the United States and Turkey (Moore & Expert Scientific Committee 1997). It is anticipated that boron pollution is an important threat for these countries. Turkey also is a major importer of borates, supplied by Argentina, Chile, Russia and some other countries.

Existing methods for boron removal include chemical oxo-precipitation (Shih *et al.* 2014), electrocoagulation (Isa *et al.* 2014), nanofiltration/reverse osmosis (Huertas *et al.* 2008) and immobilization on boron selective resins (Yu *et al.* 2013; Sasaki *et al.* 2014). Although bioremoval processes are known as economical and eco-friendly methods, the number of studies on boron remediation is very limited (Del-Campo Marín & Oron 2007; Sasmaz & Obek 2009; Taştan *et al.* 2012a).

The main objective of this study is to investigate the boron removing performance of novel high-boron-tolerant fungi isolated from wastewater from Eti Mine General Directorate – Emet Boron Works, the largest boron processing facility in the world with its 120,000 t y^{-1} capacity.

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Factors affecting and increasing boron removal by the novel fungal isolates were comprehensively evaluated.

MATERIALS AND METHODS

Isolation and culture conditions

Fungi were isolated from the wastewater of Eti Mine General Directorate – Emet Boron Works, Kütahya (Turkey). Aliquots (0.1 ml) of the water samples were spread on Petri dishes containing 3 mg l^{-1} boron dissolved in Minimal Salt Medium (MSM). The medium was composed of 1.7 g l^{-1} KH_2PO_4, 2.69 g l^{-1} $(NH_4)_2SO_4$, 0.2 g l^{-1} $CaCI_2$ and 0.03 g l^{-1} $MgSO_4$ (Afzal *et al.* 2007). Agar (15 g l^{-1}) and 1 g l^{-1} glucose were added to each of the samples and their pH was adjusted to 7 with 0.01 M sulfuric acid or 1 M sodium hydroxide solutions. To select and isolate the most boron-resistant microorganisms in the wastewater samples, a small amount of boron (6 mg l^{-1}) was added in the Petri dishes and was increased subsequently to 9, 12 and 20 mg l^{-1} respectively. The cultures were incubated at 25 ± 2 °C for 7 days. Cells from micro-colonies on these dishes were isolated by micromanipulation and inoculated into media with 9 mg l^{-1} boron. The same procedure was repeated for 12 and 20 mg l^{-1} boron concentrations and the living cells at 20 mg l^{-1} level were isolated. The isolated pure colonies that appeared on the agar dishes, including the one with the highest boron concentration (20 mg l^{-1}), were kept at 4 °C. They were transferred to fresh MSM containing 20 mg l^{-1} boron every 3 months.

Polymerase chain reaction and sequencing

Whole cells taken from one of the exponentially growing culture of the isolates were used for 5.8S rRNA gene amplification. The 5.8S rRNA region was amplified with primers forward F ITS1: 5′-TCCGTAGGTGAACCTGCGG-3′ and reverse R ITS4: 5′-TCCTCCGCTTATTGATATGC-3′ as described by Hutchens *et al.* (2010) and Martinson *et al.* (2012). Polymerase chain reactions (PCRs) were carried out in 50 μL reaction mixtures with 0.4 μM of each primer, 0.2 mM of each dNTP, 1.5 mM of $MgCl_2$ and 1× *Taq* buffer (thermo). *Taq* polymerase (thermo) (1.25 U) was used in the amplification, which was carried out by an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 45 s.

Boron solution

Stock solution of boron was prepared by dilution of boric acid (H_3BO_3) (Carlo Erba) (99%) to a final concentration of 10 g l^{-1} of boron. Appropriate volumes of the stock solution were added to the media.

Boron removal process in MSM

To determine the optimum conditions of the boron removal process, pH and boron concentration-rate experiments were carried out. First, to investigate the effect of pH, the experiments were performed at pH 4, 5, 6 and 7 at 20 mg l^{-1} initial boron concentration for 6 days. Secondly, the experiments were performed at 20, 40, 60 and 80 mg l^{-1} boron concentrations at pH 4 for 6 days to determine the effect of increasing boron concentrations, by inoculating 100 mL MSM with 0.4 g l^{-1} dry weight of fungal biomass. Uninoculated Erlenmeyer flasks containing boron were used as control samples to detect any reactions between media and boron.

Boron removal process in waste media

To decrease the cost of the MSM culture medium used, molasses (WM-1) and molasses + whey powder (WM-2) media were prepared. The composition of WM-1 was 8 ml molasses solution (approximately equivalent to 4 g l^{-1} sucrose), 1.0 g l^{-1} $(NH_4)_2SO_4$, and 0.5 g l^{-1} KH_2PO_4 (Aksu & Dönmez 2000). Why powder (0.16 g l^{-1}) was included in addition to the above in WM-2. The experiments were performed in both of these media at the appropriate pH value found and the presence of 75 mg l^{-1} boron concentration by incubating the samples for 6 days. Uninoculated Erlenmeyer flasks containing boron were used as control samples to detect any reactions between media and boron.

Analytical methods

During the incubation period, 3 ml samples were taken daily from each of the flasks. The boron concentration was determined by measuring the absorbance at 585 nm with a Shimadzu UV 2001 model spectrophotometer, by using carmine as the complexing reagent (Adams 1990). The percentage removal of boron and the maximum specific boron uptake were calculated from Equations (1) and (2),

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respectively:

$$\text{Removal}\,(\%)\ Y = (C_0 - C_t)/C_0 \times 100 \quad (1)$$

$$\text{Uptake}\,(\text{mg g}^{-1})\ q_m = (C_0 - C_t)/X_m \quad (2)$$

In the study, q_m represents the maximum specific boron uptake per unit dry weight of fungal cells (mg g^{-1}), X_m the dried cell mass per volume, C_0 the initial concentration of boron (mg l^{-1}) and C_t the final concentration of boron (mg l^{-1}).

The dried cell mass was obtained by measuring the ultimate weight of the pellets which were dried at 80 °C overnight (Nüve FN 400 model sterilizator) after centrifugation at $3{,}421 \times g = 5{,}000$ rpm for 10 min (Hettich EBA 12 model centrifuge).

All of the experiments were performed in triplicate. The standard error (SE) of the data was calculated according to Equation (3) formulated by Kenney & Keeping (1951); where σ represents the square root of the estimated error variance of the quantity.

$$\text{SE} = \sqrt{\sigma^2} \quad (3)$$

RESULTS AND DISCUSSION

As a result of 5.8S rRNA gene sequencing, the fungal isolates were identified as *Penicillium crustosum* and *Rhodotorula mucilaginosa*.

Boron removal process in MSM

pH experiments

Both of the fungal isolates showed their maximum boron removal efficiency at pH 4 (Table 1). *P. crustosum* reached its maximum boron removal yield, which was 28.58%, at this pH, whereas *R. mucilaginosa* showed a bit higher removal efficiency than *P. crustosum* with the yield of 31.86% at pH 4, which was 11.5% higher. There are no studies on boron bioaccumulation by fungus; the results of constructed wetlands and microalgae, however, have indicated that boron removal was mainly related to the pH ranges (Taştan *et al.* 2012a; Türker *et al.* 2014). Some researchers tested the effective boron removal process in acidic wastewater by using different organisms and at different conditions (Allende *et al.* 2012; Allende *et al.* 2014) and Bursali *et al.* (2009) found lower boron removal yields by the invasive marine seaweed *Caulerpa racemosa* var. *cylindracea* at acidic pH values. The reason for such variations may be due to differences in the natural growth conditions. As both of the fungal isolates showed the maximum boron removal efficiency at acidic pH levels, it can be concluded that fungal boron removal is associated with optimum pH for microbial growth. This is in accordance with the earlier article by Türker *et al.* (2014) who reviewed the relation between pH of the media and boron removal, and stated that pH substantially affected the efficiency of the removal since boron was mainly present in boric acid form at acidic pH levels, borate concentrations were increasing at higher pH values and at pH 9–10 high concentrations of OH^- were present in the solutions.

Concentration experiments

Experiments to find the optimum initial boron concentrations were carried out in MSM at the optimum pH values found. At the third day of incubation period, the maximum removal yield for *P. crustosum* was 19.97% at 33.95 mg l^{-1} boron concentration, and 19.76% at 42.76 mg l^{-1} boron concentration for *R. mucilaginosa*. Boron removal yields increased to 45.68% and 38.97% respectively at these boron concentrations on the sixth day of the experiments (Figure 1(a)). *R. mucilaginosa*, on the other hand, reached 36.08% removal yield of boron at

Table 1 | The effect of initial pH on boron removal yields (Y %) of *P. crustosum* and *R. mucilaginosa* after 4 days

	pH 4	pH 5	pH 6	pH 7
P. crustosum	28.58 ± 3.22	19.49 ± 3.59	16.09 ± 0.68	10.46 ± 1.56
R. mucilaginosa	31.86 ± 4.68	22.04 ± 0.30	16.64 ± 1.26	18.64 ± 1.34

T: 25 ± 2 °C.
Stirring rate: 100 rpm.
C_0: initial boron concentration 20 mg l^{-1}.
Y%: percentage of boron removal.

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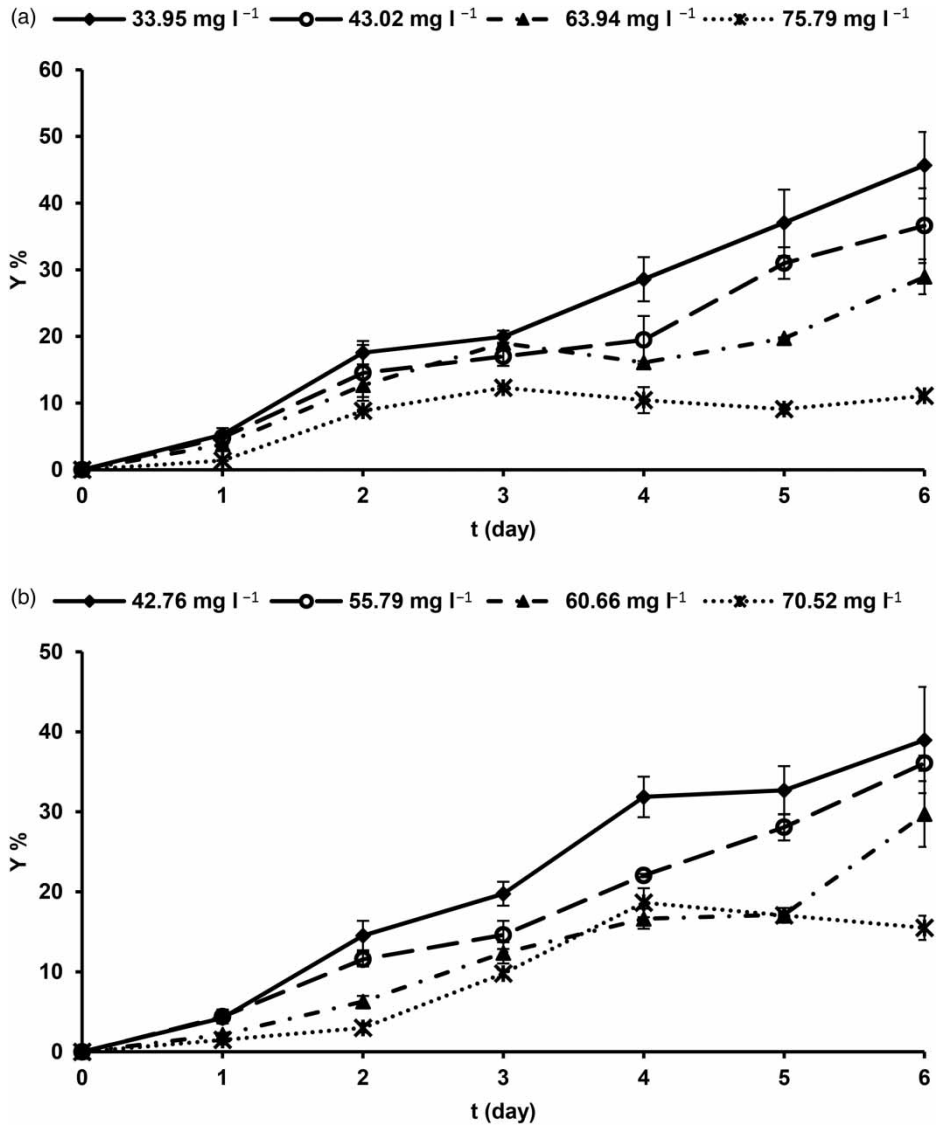


Figure 1 | The effect of increasing boron concentrations on the removal yields (Y%) of *P. crustosum* (a) and *R. mucilaginosa* (b) after 6 days of incubation period. (T: 25 ± 2 °C; stirring rate: 100 rpm; pH: 4).

55.79 mg l^{-1} and 29.72% at 60.66 mg l^{-1} initial concentrations (Figure 1(b)). Although the yields obtained at the sixth day of the experiments decreased in terms of percentage at higher initial concentrations, the absolute values measured on different days of the incubation periods showed that the maximum yield was 11.11% at the highest boron concentration (75.79 mg l^{-1}) for *P. crustosum* and 15.49% (70.52 mg l^{-1}) for *R. mucilaginosa*. It is clearly shown that boron removal yields decreased when the concentrations were increased. The minimum removal rate was obtained at the highest boron concentration for both of the fungi. On the other hand removal rates generally increased during the incubation period.

There is no detailed research available about boron toxicity to fungi. Acute toxicity of boron nanoparticles to *Daphnia magna* and *Vibrio fischeri* was researched (Strigul *et al.* 2009). According to their findings boron nanoparticles were classified as harmful to aquatic microorganisms in the EC50 range of 10–100 mg l^{-1}. In another study toxicity of boron to rainbow trout was evaluated (Loewengart 2001). It was found that low concentrations of boron stimulate embryonic growth, while higher doses lead to an adverse response that increases with increasing dose.

Although there are no studies on boron bioaccumulation by fungi, a biosorption study on marine seaweed, indicated that boron removal by *Caulerpa racemosa* var.

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cylindracea was about 63% at optimum conditions (pH 7.5), and the initial boron concentration was only 8 ppm (Bursali *et al.* 2009). In the present study, both of the fungal isolates showed high removal efficiency even at the range of 30–80 mg l^{-1} boron concentrations, proving the efficiency of these innovative boron-specific fungal isolates in boron bioaccumulation.

A comparison of the maximum amount of boron removal per unit dry weight of the fungal cells (q_m) used in our study is shown in Table 2. In general, the q_m values were decreasing with increasing boron concentrations up to a certain level. At the lowest (33.95 mg l^{-1}) and at the highest boron concentrations (75.79 mg l^{-1}) the q_m of *P. crustosum* was 21.60 and 7.13 mg g^{-1}, respectively. The q_m values measured in *P. crustosum* and *R. mucilaginosa* samples were 9.72, 15.56, 15.10 and 11.47 mg g^{-1} at 42.76, 55.79, 60.66 and 70.52 mg l^{-1} boron concentrations respectively.

The presence of high concentrations of boron in the culture media would have an effect on boron uptake if the uptake process was solely determined by the species; in fact the other potential factors were stable in this study. Boron removal yields and q_m values decreased when initial boron concentrations were increased up to the highest concentrations tested.

Boron removal process in WM-1 and WM-2

Considering the possibility of detecting an interactive relation between media composition and fungal boron removal efficiency, which could offer a more economical process, a series of experiments were performed at the highest boron concentration tested. In accordance with the previously reported results on some other pollutants, molasses was used as a low-cost carbon source for this purpose (Dönmez 2002; Taştan *et al.* 2012b; Michailides *et al.* 2015). In the present work whey powder was added into molasses to compose a new medium.

The first waste material medium (WM-1) was molasses and the second was molasses + whey powder (WM-2). As presented in Table 3, *P. crustosum* achieved 24.40% boron removal yield at 81.97 mg l^{-1} boron concentration in WM-1. It is clearly seen that fungal boron removal efficiency was generally higher than in MSM for both of these media, with the exception of WM-2 for *P. crustosum,* where there was not an important removal yield. The maximum removal yield obtained was 14.49% at the fourth day of incubation; on the other hand, there were important differences between the effects of MSM and both of these waste media on the performance of *R. mucilaginosa*. The boron removal yields were nearly the same in MSM and WM-1, but removal yield decreased when fungus was incubated in WM-2; the maximum boron removal yield was 9.75% in this medium at 68.86 mg l^{-1} boron concentration. *R. mucilaginosa* did not show satisfactory stimulation of boron removal efficiencies in both of these waste media.

The q_m values, which are also presented in Table 3, showed that boron uptake capacities of *P. crustosum* were lower than those of *R. mucilaginosa*. The highest fungal boron removal yield measured was 24.40% at the highest initial concentration tested (81.97 mg l^{-1}) in WM-1. It is worth mentioning here that, according to our best knowledge, this level is the highest boron concentration that was tried on any microorganism taxa in the literature. The related q_m value was 3.44 mg g^{-1}, lower than the rate obtained in MSM. The removal yield was higher in WM-2 than MSM; thus the related q_m value, 2.17 mg g^{-1}, was lower. On the other hand q_m levels of *R. mucilaginosa* were 11.94 mg g^{-1} in WM-1 and 4.36 mg g^{-1} in WM-2 at about 70 mg l^{-1} initial boron concentrations. These rates were lower than the rate that was obtained in MSM. The lowest removal yield (9.75%) and the lowest q_m value (4.36 mg g^{-1}) were recorded in WM-2 for this fungus.

Table 2 | The effect of initial boron concentrations on the maximum specific boron uptake (q_m) of *P. crustosum* and *R. mucilaginosa* after 6 days

P. crustosum	C_o	33.95	43.02	63.94	75.79
	q_m	21.60 ± 4.72	20.42 ± 2.55	16.35 ± 1.09	7.13 ± 0.59
R. mucilaginosa	C_o	42.76	55.79	60.66	70.52
	q_m	9.72 ± 1.51	15.56 ± 2.90	15.10 ± 6.20	11.47 ± 0.51

T: 25 ± 2 °C; pH: 4.
Stirring rate: 100 rpm.
C_0: initial boron concentration.
q_m: maximum specific boron uptake (mg g^{-1}).

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Table 3 | The effect of different media compositions on boron removal yields and the maximum specific boron uptake of *P. crustosum* and *R. mucilaginosa* after 4 days

	Co	Y %	q_m
P. crustosum			
MSM	75.79	10.46 ± 1.56	3.45 ± 1.23
WM-1	81.97	24.40 ± 1.81	3.44 ± 0.39
WM-2	71.00	14.49 ± 3.80	2.17 ± 0.64
R. mucilaginosa			
MSM	70.52	18.64 ± 1.34	13.16 ± 0.75
WM-1	74.70	19.47 ± 2.69	11.94 ± 2.80
WM-2	68.86	9.75 ± 0.71	4.36 ± 0.64

T: 25 ± 2 °C; pH: 4.
Stirring rate: 100 rpm.
C_0: initial boron concentration (mg l^{-1}).
Y%: percentage of boron removal.
q_m: the maximum specific boron uptake (mg g^{-1}).
MSM: Minimal Salt Medium.
WM-1: Molasses medium.
WM-2: Molasses + whey powder medium.

When all of the results are compared with standard errors, preference of *P. crustosum* can be recommended as the more suitable microorganism for the boron bioremoval process, due to the higher boron removal rate per unit dry weight of the cells. As a matter of fact, in another study, chelating resins were used to remove boron from aqueous solutions, and the maximum boron sorption capacity of these resins was found as 4.54 mg g^{-1} at 25 °C (Wang *et al.* 2014). It is worth mentioning here that the maximum q_m value found in the present work was 21.60 mg g^{-1} for *P. crustosum*.

Reports on the use of biological materials in boron removal studies are still limited. Some investigators used fungal isolates as a biomaterial for effective removal process for chromium(VI) (Kumar *et al.* 2008), reactive dye, chromium(VI), copper(II), nickel(II) (Taştan *et al.* 2010) and dye (Wang *et al.* 2015), but according to our best knowledge not for boron.

Although several genes have been associated with boron tolerance in plants, the tolerance mechanisms in other organisms are still unclear. Kaya *et al.* (2009), however, identified *ATR1* as a major boron resistance gene in yeast, which supports the growth of *Saccharomyces cerevisiae* yeast cells in the presence of high concentrations of boric acid. In our previous work the highest boron removal yields of all the aquatic organisms that were previously tested in the literature were achieved by *Chlorella* sp.; the maximum removal yield found was 32.95% at 5.45 mg l^{-1} boron concentration (Taştan *et al.* 2012a). In the present work we aimed to increase the boron removal yield, considering the continuous increase in boron concentrations in the environment (ATSDR 2010). According to this ATSDR report, boron concentrations in ground water ranged from 0.14 mg l^{-1} to 120 mg l^{-1} and ranged from 15 mg l^{-1} to 360 mg l^{-1} in surface waters. In order to cope with this tendency, we attempted to isolate high-boron-tolerant novel microorganisms from boron mining wastewater, and succeeded in showing that the novel fungal isolates could serve as effective bio-materials for bioremediation of boron-polluted wastewaters containing relatively high boron concentrations.

CONCLUSIONS

The ultimate aim of this study was to investigate the boron removal efficiency of high-boron-tolerant novel fungal isolates obtained from boron mining wastewater. Compared to traditional boron removal processes developed for high levels, the fungal boron removal process can be offered as an eco-friendly and economical method. In this study the maximum boron removal yields (45.68%) and the maximum specific boron uptake (21.60 mg g^{-1}) were achieved by *P. crustosum*. These are the highest boron removal yields according to our best knowledge. Both of the fungal isolates can be introduced as effective biomaterials for the boron removal process in desalinated waters.

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