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Adsorption and Removal of Lead (Pb) by Wildtype and Lead Resistant, PbR-101 Cell Line of *Chlorella* sp.

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Abstract

The cell line, PbR-101 isolated from Chlorella sp. by EMS (Ethylmethane sulphonate) mutagenesis was compared against the wild type (WT) Chlorella sp. for study of mechanisms confirming resistance to Pb toxicity. Growth experiments showed that both the tested algal cells, PbR-101 and WT (control) were found to be retarded with increasing Pb^{2+} concentrations in the liquid growth medium. It was found that the higher ID_{50} value of PbR-101 cell line exhibited some degree of resistance to Pb toxicity. When exposed to the liquid medium containing 50 µM Pb^{2+}, kinetic experiments revealed rapid removal and adsorption of Pb^{2+} in both the algal cells during the first few hours. Compared to WT, the PbR-101 cell line showed significantly higher percentage removal and adsorption of Pb^{2+} at 15 minutes and 48 hours interval of time respectively. Extracellular Pb^{2+} adsorption was found significantly higher than intracellular uptake in both the tested algal cells. Total Pb^{2+} accumulation and distribution between the external and internal cell fractions of the PbR-101 were significantly higher to that of the WT. Thus, the PbR-101 cell line appeared more resistant to Pb toxicity and hence may be used for remediation of metal contaminated sites.

Key words: *Chlorella* sp., EMS mutagenesis, adsorption, uptake, lead toxicity.

Introduction

Heavy metal pollution in the environment has become a burning issue over the past several decades as a result of anthropogenic activities[1]. Among the metal pollutants, lead has become a serious worldwide environmental problem since its toxic effect to human and environment has been well recognized[2]. The metal has no specific role in living organisms and is more toxic even at very low concentrations[3]. In human, lead causes neurological, cardiovascular, renal, gastrointestinal, haematological and reproductive effects[1].

Heavy metals can neither be degraded or nor destroyed by any biological or chemical means. Therefore, they are the persistent environmental contaminants. Many conventional physico-chemical methods such as excavation, precipitation with lime, adsorption, flocculation, filtration etc., are costly and inefficient for remediation of toxic metals from contaminated sites[4]. Therefore, there is a growing realization to clean up the metal contaminated soil and water bodies using microorganisms, algae or plants since they are cost effective, efficient and eco-friendly in nature[5].

Algae are capable of accumulating heavy metals to concentrations several orders of magnitudes higher than in the surrounding medium[6] and have therefore been used for their removal from contaminated sites[7,8]. Their high accumulating potential can even be used for the enrichment, recovery or recycling of traces of valuable metals like uranium, gold and silver from nature[9,10]. Many algae growing in metal-polluted environments display an ability to tolerate high concentrations of toxic metals[11].

It is reported in literature that different *Chlorella* species have been isolated from highly polluted domestic, industrial and metal contaminated lakes[10,12]. Many studies have been addressed on metal detoxification of various *Chlorella* spp. However, there is limited information regarding lead toxicity and resistance mechanisms in *Chlorella* sp. Therefore, the present study addresses on isolation of lead resistant *Chlorella* cell lines from the wild type (WT) culture and characterization of mechanism(s) confirming resistance to lead toxicity.

Material and Methods

Isolation of Pb-resistant cell lines and growth conditions: Isolation of lead resistant cell lines from the wild type *Chlorella* sp. was carried out by EMS (Ethylmethane Sulphonate) mutagenesis by following the standard method[13]. All the cell lines were designated as *PbR* with Arabic numerals. They were maintained in modified BG-11 liquid mineral medium containing 25 µM Pb-salt solution. The cultures were continuously exposed to a light intensity of 20–50 µmol by cool white fluorescent lamps while incubated in a gyratory shaker (180 rev./min) at 27 °C. The growth of the algal cells was monitored time to time. When the cultures reached the stationary phase of growth, they were further inoculated into fresh liquid medium to keep them growing. For the present study, one of the lead resistant cell lines, PbR-101 was selected.

Preparation of (0.1 M) $Pb(NO_3)_2$ solution: A stock solution of 0.1 M lead nitrate was prepared by dissolving a calculated amount of $Pb(NO_3)_2$ in ultra pure water and then filter sterilized.

Calibration of absorbance (optical density) vs. cell numbers: Growth of the algal cultures was monitored by measuring absorbance (optical density) in spectrophotometer at 540 nm and counting cell numbers by a Hemacytometer as well. The absorbance was calibrated against the cell numbers.

Growth experiments: The effect of lead on growth of PbR-101 and *Chlorella* sp. (WT) was monitored at the exponential phase of growth. In this experiment, sterilized Erlenmeyers each containing 100 ml of BG-11 medium were added with the prepared lead salt solution in calculated amount such that they maintained concentrations of 0, 1, 10, 50 and 100 µM respectively. The algal cultures were then inoculated to the medium in series in such a way that the initial cell densities were in the range of 5.0 - 5.5 x 10^5 cells/ml of the liquid medium. The growth was then monitored by measuring the change in absorbance of the algal cells at 540 nm. The measurement was taken at the time of inoculation and each day thereafter until it reached the stationary phase. Cells were also counted using a Hemacytometer. The growth rate of the algal cultures was determined between the 2nd and the 6th days by the following equation[1]:

$\mu = (\ln X_6 - \ln X_2)/(T_6 - T_2)$

where, μ = Specific growth rate of the algal culture, X_6 = Absorbance, $A_{540\ nm}$ of the algal culture at time T_6, X_2 = Absorbance, $A_{540\ nm}$ of the algal culture at time T_2

All the experiments were carried out in triplicate.

Adsorption of Pb by the WT and PbR-101 cultures (Kinetic experiment): Firstly, the PbR-101 culture initially maintained in the medium containing 25 µM Pb^{2+} was inoculated into the fresh liquid medium without lead and allowing them to grow for 5 days. The process was repeated three times to ensure that the cells were completely free from the metal ions. Then, the PbR-101 cells at the stationary phase of growth were collected by centrifugation (10^0 C, 8000 g for 15 min). The pelleted cells were re-suspended in 10 ml of fresh liquid medium and counted in the Hemacytometer to note the actual cell numbers. Three flasks containing fresh liquid medium were inoculated with the dense cell suspension such that each flask contained 10^9 cells per hundred milliliters. Similar experiment with the WT culture as control was also carried out in parallel.

To study the adsorption kinetics at different time intervals, the metal solution was added to each of the flasks maintaining a final concentration of 50 µM. From each of the metal added flasks, a 10-ml sample was drawn immediately in order to represent a zero hour sampling, however it took 15 minutes to proceed through a complete treatment. Hence, the sample at 15 minute was regarded as the zero hour sample in each case. In a similar way, the samples were drawn at 0.5, 1.0, 2.0, 4.0, 8.0, 12.0, 24.0 and 48.0 hours respectively. The flasks were placed back to the shaker after each sample drawn. The samples, at each of these time intervals were spun down in a bench centrifuge (3500 rpm, 10 min) and the supernatants collected separately for metal analysis. This supernatant yields the residual metal left over the medium. The cell pellets were then washed with 5-ml of EDTA (10 g/lit.) three times[14]. Each time, the cells were spun down (3500 rpm, 10 min) and the supernatants containing EDTA were collected for metal analysis. The experiment was carried out in triplicate.

Digestion of sample and Pb determination: After EDTA treatment, the cell pellets were subjected to the experiments involving the intracellular Pb^{2+} uptake. For this, each of the cell pellet samples was re-suspended in 10 ml of double distilled water by gentle vortex. The cells were spun down and re-suspended in distilled water repeatedly for three times following the process of washing. Each of the washed cells was then re-suspended in 1 ml of double distilled water and transferred to digestion tubes. The cell suspensions in the digestion tubes were treated with 2 ml of conc. nitric acid. The mixture was placed in a chemical hood overnight so as to ensure a complete dissolution and prevent foaming during subsequent digestion processes. The sample was digested at 100^0C for 1 hour followed by gradually increasing the temperature up to 230 ^{0}C. The digestion was carried out for approximately 3 h until the solution became completely clear and transparent. After the digestion was completed, the digest was cooled, diluted and adjusted to a final volume of 5 ml with double distilled water. Then, atomic absorption spectrophotometer (Perkin Elmer 1100 B) was used to determine Pb concentrations in the samples.

Results and Discussion

Effect of Pb^{2+} on growth of the WT and PbR-101 cell line: Table 1 shows that the effect of Pb^{2+} on growth of the WT and PbR-101 at different concentrations. It was observed that the growth of both the algal cells was gradually arrested by increasing the concentration of Pb^{2+}. Under similar experimental condition, the growth of both the algal cells remained almost unaffected by the concentration of 1µM Pb^{2+} while growth inhibition of the WT and PbR-101 was observed by 35% and 24% respectively in presence of 10µM Pb^{2+}. Similarly, the PbR-101 sustained less inhibitory effect (54%) compared to that of the WT (78%) in the presence of 50µM Pb^{2+}. The presence of 100µM Pb^{2+} had significantly arrested the growth rate of both the algal cells. Under the condition, the PbR-101 sustained inhibitory effect by 88% while the WT was by 94%. This shows that the PbR-101 could survive to some extent even at higher Pb concentration compared to the WT. The results are in agreement with several findings that also responded inhibitory effects in order of increasing metal concentration[15,16] (Macfie and Welbourn, 2000; Wong and Wong, 1990;). However, the comparatively better growth of the PbR-101 may plausibly be due to the EMS mutagenesis.

Table-1
Percentage growth rate of WT and PbR-101 at different Pb^{2+} concentration

Culture	Pb^{2+} concentration (µM)				
	0 (control)	1	10	50	100
WT	100	93	65	22	6
PbR-101	100	95	76	46	12

Based on the growth rates, inhibition of 50% growth rate (ID_{50}) was also calculated. The ID_{50} value in the PbR-101 (36 µM) and WT (18 µM) reflect that the PbR-101 cell line exhibited a certain degree of resistance to Pb toxicity.

Cell counts of the WT and PbR-101 cell line: Table 2 shows the initial and final cell counts of the WT and PbR-101 in the liquid growth medium. Both the cultures were exposed to the medium containing 50 µM Pb^{2+} up to 48 treatment hours. The final cell numbers of both the cultures even after their exposure to 48 hours in 50 µM Pb^{2+} did not show significantly different compared to that of the initial cell counting. Besides, the difference of initial and final (48 hours of treatment with Pb^{2+}) cell counting between the tested cell lines was not found significant (t-test, $p>0.05$). Therefore, the same cell numbers were presumed for all time intervals throughout the kinetic experiment and for further calculations as well.

Table-2
Cell numbers per 100 ml of liquid growth medium at initial (before addition of Pb^{2+}) and final (48 hours after addition of Pb^{2+}) hours of treatment; the WT and PbR-101 were treated with 50 µM Pb^{2+}

Culture	Cell numbers per 100 ml of growth medium	
	Initial	Final
WT	6.2×10^9	6.4×10^9
PbR-101	7.0×10^9	7.5×10^9

Kinetics of Pb^{2+} removal and adsorption from the growth medium by WT and PbR-101 cell line: The kinetics of Pb^{2+} extracellular adsorption and removal by the WT and PbR-101 is shown in figures 1 and 2 (A and B). On exposure to 50 µM Pb^{2+}, the rate of the metal ion removal was rapid during the first few hours, increased gradually until 12 hours and then reached a steady state thereafter (figure 1B). Decreasing residual Pb^{2+} concentration in the medium with time indicated the amount of Pb^{2+} being removed from the medium simultaneously (figure 1A). During the first hour of treatment, Pb^{2+} removal from the medium was 45% (figure 1B). Correspondingly, extracellular adsorption of Pb^{2+} occurred side by side, being rapid in the first half-hour and then remained unaltered until 2 hours (figure 1A). Lead adsorbed by the WT cell surfaces during the first hour was 37%. Further, a gradual increase in Pb^{2+} adsorption was observed until 12 hours and then reached the equilibrium point after this treatment hour. Initially, the removal of Pb^{2+} from the medium was 27% at 15 minutes. Finally 72% of the total lead supplemented was found to be removed at 48 hours of which 56% was externally bound to the cell walls.

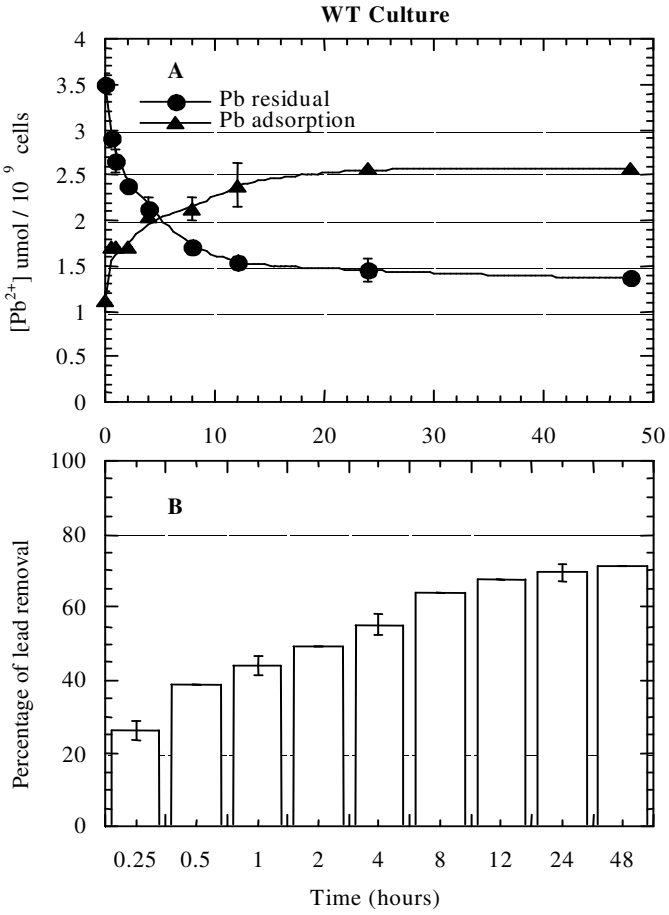

Figure-1
Extracellular adsorption and residual concentration of Pb^{2+} at different time intervals (A) and percentage removal of Pb^{2+} from the medium (B) in WT culture; mean (standard deviation; n=3)

Apparently, PbR-101 exhibited a distinct kinetics of Pb^{2+} extracellular adsorption and removal (figure 2A and B) compared to the WT. On exposure to 50 µM Pb^{2+}, the rate of Pb removal from the medium was very rapid during the first few hours unlike that of the WT. The removal was more than 80% within few hours. In other words, residual concentration of Pb^{2+} in the medium was less than 20% within the first few hours of treatment (figure 2A). Until 48 treatment hours, the cell line showed 96% removal of the total metal ions from the medium (figure 2B). Correspondingly, a very rapid increase in the metal adsorption was found in the cell line unlike the WT. The adsorption of Pb^{2+} to the cell surface was very rapid during the first few minutes, remained almost constant until 2 hours and gradually increased up to 8 hours (figure 2A). The adsorption attained a point of saturation after 8 hours. At 15 minutes, Pb^{2+} removal from the growth medium was 67% contributing 50% to the extracellular adsorption alone. This figure shows difference significantly to that of the WT in terms of Pb^{2+} removal and adsorption at 15 minutes. Similarly, of the 96% Pb^{2+} removal at 48 treatment hours, 66% was found externally adsorbed to the cell walls of the cell line, which is higher to that of the WT.

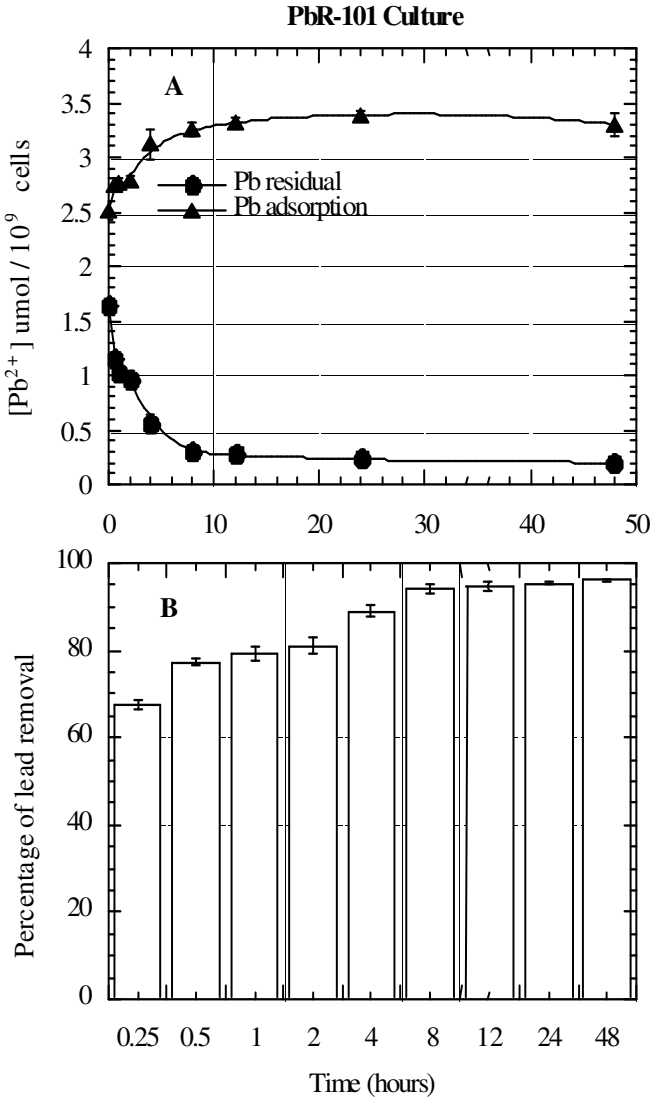

Figure-2
Extracellular adsorption and residual concentration of Pb^{2+} at different time intervals (A) and percentage removal of Pb^{2+} from the medium (B) in PbR-101 culture; mean (standard deviation; n=3)

The present study is in agreement with several findings as reported in literatures[17,18]. Accordingly, two mechanisms are taken into account for the removal of metal ions. One is metabolically independent passive surface adsorption or biosorption while the other, active uptake of the metal ions into the cells, is metabolically dependent. Both mechanisms work simultaneously in algal cells in which adsorption is very rapid and occurs in few minutes. Other possible mechanisms that govern heavy metal resistance are reduced transport across the cell membrane, active efflux, compartmentalization and chelation[19]. The rapid adsorption to the algal cell surface may be due to the availability of specific binding sites to which the metal ions are bound until all the sites are saturated followed by a slow intracellular uptake[20]. Furthermore, the difference in the magnitude of metal binding capacity to the external cell fractions between the WT and PbR-101 at different time intervals may be due to different affinities of the algal cells towards the metal ion[21].

Total lead accumulation and distribution in external and internal cell fractions of the WT and PbR-101 cell lines: Tables (3 and 4) show total Pb^{2+} accumulation and distribution between external and internal cell fractions of the WT and PbR-101 cell lines at different time intervals. It was found that the WT showed a gradual accumulation of Pb^{2+} with increasing time of exposure to the medium containing 50 µM Pb^{2+} (table 3). Total Pb^{2+} accumulation reached a steady state after about 12 hours while the accumulation was rapid during the first few hours. The distribution of Pb^{2+} between the external and internal cell fractions also increased with duration of exposure indicating that the process of adsorption and absorption occurred simultaneously. The amount of Pb^{2+} associated with the external cell fractions was higher than the internal at each time interval. While the extracellular adsorption was found gradually increased, intracellular uptake appeared fluctuating at different time intervals although the tendency of Pb^{2+} association was of increasing order. Of the total Pb accumulated (3.41 µmol per 10^9 cells) at 48 hours, 75% was externally bound to the cell surface whereas only 25% was found inside the cells.

Table-3
Total Pb^{2+} accumulation and distribution between external and internal cell fractions of the WT at different time intervals; mean (standard deviation; n = 3)

Culture	Time Interval (hr.)	Pb^{2+} association (µmol / 10^9 cells)		
		External	Internal	Total Pb^{2+} accumulated
WT	0.25	1.11 (0.10)	0.15 (0.02)	1.26
	0.5	1.70 (0.00)	0.15 (0.04)	1.85
	1.0	1.70 (0.00)	0.41 (0.09)	2.11
	2.0	1.70 (0.00)	0.67 (0.00)	2.37
	4.0	2.04 (0.00)	0.59 (0.10)	2.63
	8.0	2.13 (0.10)	0.93 (0.10)	3.06
	12.0	2.39 (0.25)	0.84 (0.04)	3.23
	24.0	2.56 (0.00)	0.76 (0.09)	3.32
	48.0	2.56 (0.00)	0.85 (0.01)	3.41

The PbR-101 cell line demonstrated a higher accumulation of Pb^{2+} per 10^9 cells compared to that of the WT (table 4). Besides, the distribution of Pb^{2+} between the external and internal cell

fractions of the cell line was also higher to the WT. The cell line accumulated almost all of the metal supplemented within the few hours showing a saturation point at early treatment hours. The lead in external and internal cell fractions increased with duration of exposure to the metal solution. But they were found to be saturated with the metal during the first few hours showing that the strain promptly responded to the lead toxicity. This further indicates that the cell line demonstrated more resistance to the toxic metal ion. Of the total metal accumulated (4.85 µmol /10^9 cells) by the cell line at 48 hours, 68% and 32% of Pb^{2+} were externally bound and inside the cells respectively.

Table-4
Total Pb^{2+} accumulation and distribution between external and internal cell fractions of the PbR-101 at different time intervals; mean (standard deviation; n = 3)

Culture	Time Interval (hr.)	Pb^{2+} association (µmol / 10^9 cells)		
		External	Internal	Total Pb^{2+} accumulated
PbR-101	0.25	2.50 (0.01)	0.87 (0.01)	3.37
	0.5	2.75 (0.01)	1.16 (0.04)	3.91
	1.0	2.80 (0.01)	1.32 (0.01)	4.12
	2.0	2.81 (0.07)	1.32 (0.01)	4.12
	4.0	3.10 (0.00)	1.45 (0.05)	4.55
	8.0	3.30 (0.10)	1.48 (0.00)	4.78
	12.0	3.32 (0.25)	1.48 (0.10)	4.80
	24.0	3.34 (0.12)	1.49 (0.00)	4.83
	48.0	3.30 (0.06)	1.55 (0.10)	4.85

Active intracellular uptake occurs once metal ions are bound by the cell wall. The membrane potential, which is negative on the inside of the plasma membrane, provides a strong driving force for the uptake of metal ions through secondary transporters[22]. In the present study, the intracellular Pb^{2+} uptake in the tested algal cells was significantly less compared to the extracellular adsorption (tables 3 and 4). It is due to the reason that when the binding sites of the algal cells became exhausted or nearly saturated, the cells began taking up the metal ion by active physiological mechanisms[19]. Inside the cell, metals are chelated and excess metal is sequestered by transport into the vacuole exhibiting intracellular detoxification mechanisms[23]. A common response of organisms to metal toxicity is the synthesis of metallothioneins[24] and phytochelatins[25], which may play a role in the intracellular detoxification of metal ions. But the present study could not investigate the fate of Pb^{2+} inside the cells. However, the total Pb^{2+} accumulation and distribution between the external and internal cell fractions show that both the processes occur simultaneously in response to the metal toxicity. Indeed, the PbR-101 cell line showed significant accumulation and distribution of the metal ions compared to that of the WT indicating its better resistance capacity. It was also noted that Pb^{2+} bound to the external cell surface was significantly high (t-test, $p>0.05$) compared to that of the metal inside the cells in both the tested cells (tables 3and4). However, the presence of other metal ions in the growth medium, light, temperature, time of exposure to metal ions and pH are some of the dependent and sensitive parameters of the processes[21,26,27].

Conclusion

It can be concluded from the study that the PbR-101 cell line possesses comparatively higher Pb^{2+} accumulating potential than the WT exhibiting a certain degree of resistance to the metal toxicity. The rapid removal of the metal followed by the simultaneous extracellular adsorption suggests that the cell line plays important role in reducing the level of metal concentration from the medium. Extracellular Pb^{2+} adsorption was found significantly higher than intracellular uptake in both the tested algal cells. Total Pb^{2+} accumulation and distribution between the external and internal cell fractions of the PbR-101 were significantly higher to that of the WT. This indicates that this resistant cell line is likely to be used as a biological tool for remediation of metal contaminated sites. However, a comprehensive understanding of physiological, biochemical and molecular mechanisms conferring Pb^{2+} resistance in *Chlorella* sp. would enable the engineering of metal accumulating organisms such that they could serve as a tool in water treatment, wastewater treatment and controlling the environment from toxic metal pollution.

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