



Evaluation of petroleum hydrocarbons adsorption and biodegradation by *Pseudomonas aeruginosa* cells entrapped into silica-alginate beads

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ABSTRACT

The aim of the work was to elaborate new method of wool entrapping into silica alginate beads and checking of their adsorption properties. The purpose of the present study was also to determine water bioremediation efficiency using *Pseudomonas aeruginosa* cells immobilized into wool entrapped into silica-alginate beads. The results of study showed that obtained capsules are stable in saline water environment. Additionally neither silica adsorbers nor wool impacted negatively on viability of bacterial strains that were entrapped into these capsules. Moreover, it should be underline that 83,01% of adsorbed engine oil has been degraded by immobilized microorganisms after 3 months of biodegradation process. The results proved that properties and non-toxic character of these beads are suitable enough to use them in purification of water from petroleum hydrocarbons.

Keywords: bioimmobilization, biodegradation, encapsulation, petroleum hydrocarbons, *Pseudomonas aeruginosa*

1. INTRODUCTION

Nowadays, the purification of contaminated water from petroleum hydrocarbons is one of the most emerging problems. The main challenge is caused by the fact, that the standard treatment used to decontamination of water is limited. First, this process is selectively expensive, and on the other hand it can be only partially effective. One from various scientific method that is useful in pollutant degradation, is bioremediation (Hua 2006, Liste and Felgentreu 2006, Powell *et al.* 2007). During bioremediation in saltwater environment, nutrients, surfactants and microorganisms are used to purification of contaminated water from crude oil. However, potential dilution of these materials can cause decrease of efficiency especially in open-water systems. A suitable solution of this problem might be seeding with immobilized microorganisms. Hence immobilization of cells onto macroalgae (Suzuki *et al.* 1997), chitin and chitosan flakes (Gentili *et al.* 2006), inside of polyurethane foam (Oh *et al.* 2000, Quek *et al.* 2006, Mizielińska *et al.* 2017) or polystyrene surfaces (Ionata *et al.* 2005) could offer a higher surface area to facilitate growth of biomass and degradation rate. Unfortunately polyurethane and polystyrene foams are not biodegradable materials. This is why immobilization of specific bacteria on suitable and biodegradable carriers to achieve higher cell density and activity could be an alternative method (Kuyukina *et al.* 2009, Hrenovic *et al.* 2010, Ivankovic *et al.* 2010).

Various mineral and organic materials are used as carriers for the immobilization of growing cells. It is very important that such carriers are also cheap materials, nontoxic for the microorganisms, they have also the ability to adsorb hydrocarbons and bacterial cells, and are resistant to chemical and mechanical damage. Cellulose-containing materials, such sawdust, shavings, wood chips meet these requirements. Moreover, these materials have a porous structure, and they provide enough surface area for cells to bind (Kuyukina *et al.* 2009). Perlite is a glassy volcanic rock that can be used as mineral carrier of oil-degrading bacteria. The immobilization of microorganisms on perlite is more effective and stable during the degradation of oil in comparison to non-immobilized (Ivankovic *et al.* 2010). Sepiolite is commercially obtainable of low cost and non-toxic. This carrier does not exhibit swelling properties that can provide difficulties. Sepiolite is commonly used in the wastewater treatment as very good adsorber. Peanut hull powder may also be chosen as the carrier for immobilizing microorganisms because of its porous structure, large surface area and strong adsorption capability (Xu and Lu 2010). Other types of carrier, polymeric cryogels and hydrogels are also used as immobilization matrices for hydrocarbon-oxidising bacteria (Bogusławska-Wąs *et al.*, Patil *et al.* 2006, Kuyukina *et al.* 2009, Sarma and Pakshirajan 2011). The encapsulation of cells in sodium alginate may facilitate and increase degradation rate. The main problem of this process is that adsorption of hydrocarbons by alginate beads may be difficult. This is because of hydrophilic character of beads. Therefore the solution of this problem can be addition of hydrocarbons adsorbers into alginate beads. Silica carriers and wool have good hydrocarbons adsorption properties besides they are natural – they are also resistant enough for application even in higher salinity of the water, they are not toxic for bacteria and they are also biodegradable materials.

The first aim of the work was to elaborate new method of wool entrapping into silica alginate beads and checking of their adsorption properties. The purpose of the present study was also to determine bioremediation efficiency using bacterial cells immobilized into wool entrapped into silica-alginate beads.

2. MATERIAL AND METHODS

2. 1 Materials

One hydrocarbon-degrading *Pseudomonas aeruginosa* strain (non pathogenic) used in this study belongs to CBIMO collection. This strain was chosen among 25 isolates that had been previously characterized by classic and PCR methods (Mizielińska and Bartkowiak 2009). To check viability of bacteria strain, used during research works *Pseudomonas* P agar and TSB medium were used (Merck, Germany). Both of mediums were prepared according to the Merck protocol. Sheep wool obtained from producer X was used as hydrocarbons adsorber. Silica carriers: Syloid Al-1FP, and Syloid 244 FP (Grace Davison, UK) were used as petroleum hydrocarbons adsorbers and to increase strength and adhesion of bacterial cells entrapped into capsules. The sea salt Sera (Sera, GmbH) was used to prepare 11‰ and 35‰ solutions. The solutions were prepared according to Sera protocol. Sodium alginate – Manugel HVCR (ISP, USA) was used for alginate/Ca beads preparation. Diesel oil (Shell) and engine classic oil SJ/CF 15W/40 (ORLEN) were used as petroleum hydrocarbon source.

2. 2 Methods

2. 2. 1. Trials *P. aeruginosa* immobilization in wool/calcium alginate beads

The beads containing bacterial cells and wool have been prepared. As first step, the bacterial inoculum was prepared. One strain of *P. aeruginosa* was plated into TSB medium. After 24 hours of incubation at 37 °C 10 mL of bacterial inoculum was mixed with 30 mL of 2% sodium alginate. After it 40 mL of 1,5% sodium alginate containing 6.8×10^6 CFU/mL of bacterial cells were obtained. As next step 1g of wool have been cut into 50 small pieces. Each piece was formed into the small ball by rolling. All formed mini-balls have been immersed separately into 1,5% aqueous solution of sodium alginate with bacteria cells. Then, impregnated globules have been introduced sterile one by one into 0,155M solution of CaCl₂. After 20 minutes of reactions all beads have been washed with sterile water and direct applied in further tests. To determine the effectiveness of oils adsorption by beads formed using both methods each time 1g of capsules was introduced for 24 hours into the same amount of diesel and into engine oils. After that beads were isolated and weighted.

The mechanical quality of beads was determined after 2 weeks of immersing in following liquids: engine oil, diesel oil and also salty water (11‰ and 35‰). The bursting force was determined for each set of beads using Zwick/Roell Z 2.5 tensile machine. As reference (base value) the same set of measurements was conducted for new prepared capsules (without contact with water and oils). To determine viability of bacterial cells 1g of beads was placed separately into 100 mL of water (distilled water, 11‰ and 35‰ water solutions) and incubated 14 days at room temperature. After incubation the beads were suspended in 10 mL of 2% sodium citrate solution. Viability of *P. aeruginosa* (CFU) strain was determined as colony-forming units (CFU) per mL and determined by making serial decimal dilutions of capsules suspended in sodium citrate and plating on *Pseudomonas* P agar. CFU were counted after 24 hours of incubation at 37 °C.

2. 2. 2. Preparation of capsules with silica adsorbers

Before preparation of capsules containing Syloid Al-1FP and Syloid 244-FP effectiveness of oils adsorbtion by these silica adsorbers was measured. Next 4,44g; 2,22g;

1,11g of Syloid A1-1FP and the same amount of Syloid 244FP have been immersed separately into 1,5% aqueous solution of sodium alginate (in total 40 g of solution) with bacteria culture (6.8×10^6 CFU/mL) and mixed in mild condition using magnetic stirrer (with 500 rpm). The preparation of solutions containing more than 10% of adsorbers was not possible, because of too high viscosity of solutions (there was no possibility to mix such solutions). 10%, 5%, 2,5% of Syloid A1-1FP and 10%, 5%, 2,5% of Syloid 244FP solutions have been extruded into 0,155M solution of CaCl_2 . After 20 minutes of reactions all beads have been washed with distilled water and tested directly in further investigations.

To determine the effectiveness of oils adsorption by capsules contained both adsorbers, 1g of capsules was introduced for 24 hours into the same amount of diesel and into engine oils. After that beads were isolated and weighted.

The mechanical quality of beads containing silica adsorbers and viability of encapsulated *P. aeruginosa* cells was tested before (Mizielińska and Bartkowiak 2012).

2. 2. 3. Immobilization of *P. aeruginosa* cells into Syloid 244FP/wool beads

As first step 40 mL of 1,5% sodium alginate solution containing 6.8×10^6 CFU/mL of bacteria cells was obtained. 4,44g of Syloid 244FP have been immersed into that solution (in total 40 g of solution) and mixed using magnetic stirrer (with 500 rpm). Thus 1g of wool have been cut into 50 small fragments. Each piece was formed into the small ball by rolling. All formed mini-balls have been immersed separately into 1,5% aqueous solution of sodium alginate containing Syloid 244FP and bacterial cells; then impregnated globules have been introduced one by one into 0,155M solution of CaCl_2 . After 20 minutes of reaction all beads have been washed with water and used directly in further tests.

To determine the effectiveness of oils adsorption by beads, 1g of capsules was introduced for 24 hours into engine oils. After 24 hours of incubation the beads were isolated and weighted.

The mechanical stability of beads containing wool, Syloid 244FP and bacterial cells was determined after 14 days of immersing in following liquids: engine oil, diesel oil and also water (distilled, 11‰ and 35‰). As reference the same measurements were conducted for freshly obtained capsules (without contact with water and oils).

2. 2. 4. Engine oil biodegradation test

The criteria chosen for selection of the most suitable bead type for engine oil biodegradation were viability of immobilized bacterial cells, mechanical stability of the beads and adsorption effectiveness. The beads containing bacterial cells entrapped into Syloid 244FP-alginate beads with wool with and without bacteria were obtained. After, 200 mL of water and 50 mL of engine oil were introduced to either 4 conical flasks. Thus 45g of beads without bacterial cells were introduced separately to 2 conical flasks containing water and engine oil, to test effectiveness of oil adsorption by capsules containing both adsorbers. As next step 45g of beads with bacteria were also introduced separately to 2 conical flasks, to test effectiveness of engine oil biodegradation. The flasks were placed in incubator with shaker for 24 hours (2 flasks containing beads with bacterial cells and 2 flasks containing beads without bacterial cells) and also for 3 months at room temperature. Samples after 24h of incubation (1 flask containing beads with bacterial cells and 1 flask containing beads without bacterial cells) and after 3 months of incubation (1 flask containing beads with bacterial cells and 1 flask

containing beads without bacterial cells) were pull out, purified, weighted, and introduced to 2% sodium citrate (to dissolve calcium alginate). As next step, content of engine oil in solution was determined using Blight-Dyer extraction method. The arithmetic average of results for 3 samples repeats was converted to 1g of the beads, and tabulated.

3. RESULTS

Trials of encapsulation of wool showed positive results. Shape of alginate beads was globular approximately 10 mm in diameter. Mass of each capsule was 0,444g, thus the content of wool was calculated and was equal to 4,5%. The effectiveness of oils adsorption by capsules was evaluated. It was noticed, that 21,2g of capsules (21,2g of capsules contained 1g of wool) was able to adsorb about 5,11g of engine oil.

The analysis of mechanical properties showed, that bursting force of capsules has decreased slightly from 14.97 N to 12.95 N (about -13,5%) after 24h of contact with tested oil. After 14 days of contact with distilled water bursting force also decreased (about -7.75%). It should be underline that bursting force of capsules, which were incubated in salty water increased, but also narrowly (Table 1).

Table 1. The changes of bursting force of wool/calcium alginate beads after 2 weeks of incubation in salty water (SD* - standard deviation)

Samples	F_{\max} [N]	SD*
The beads incubated in:		
Engine oil	12.95	3.55
35‰ water	15.48	3.28
11‰ water	16.09	5.00
Distilled water	13.81	3.27
Non incubated beads	14.97	2.98

Viability of *P. aeruginosa* strain into beads was evaluated after 14 days of incubation at room temperature. The results of the tests showed, that number of bacterial cells that were incubated in distilled water decreased from 6.8×10^6 to 2.7×10^5 CFU/mL. The tests demonstrated that incubation of beads in 11‰ water caused decrease of number of *P. aeruginosa* cells from 6.8×10^6 to 2.6×10^5 CFU/mL. It was also observed, that amount of bacterial cells decreased from 6.8×10^6 to 2.2×10^5 CFU/mL after incubation in 35‰ water. The results of tests showed, that concentration of bacterial cells was comparable in all samples after first 14 days of incubation.

The effectiveness of oil adsorption by silica adsorbers was evaluated as well. The results showed that 1g of Syloid Al-1FP adsorbed 0,132g of engine oil and only 0,044g of diesel oil. Syloid 244-FP was more effective because 1g of this substance adsorbed 0,330g of diesel oil

and even 0,976g of engine oil. The effectiveness of oil adsorption by capsules containing Syloid Al-1FP and Syloid 244 FP was also evaluated (Table 2)

Table 2. The amount of engine/diesel oil adsorbed by alginate-silica capsules after 24 hours of incubation at room temperature

Adsorber	The amount of adsorber in capsules [%]	The amount of oil adsorbed by 1g of capsules [g/g]		The amount of oil adsorbed by 1g adsorber [g/g]	
		OS	ON	OS	ON
Syloid 244-FP	2.5	0.103	0	4.15	0
	5	0.102	0	7.26	0
	10	0.037	0	0.37	0
Syloid Al-1FP	2.5	0.073	0	2.94	0
	5	0.107	0	2.15	0
	10	0.0027	0	0.027	0

Viability of *P. aeruginosa* cells entrapped into silica-alginate beads was tested as well. The results demonstrated that number of bacterial cells after 28 days of incubation of capsules at room temperature, in distilled water and in 11‰ and 35‰ decreased, but narrowly. The tests showed that concentration of *P. aeruginosa* was comparable in all samples what proved that silica adsorbers did not influence on bacterial viability.

P. aeruginosa strain was immobilized in wool entrapped in silica/alginate capsules. The results demonstrated, that the beads containing wool and Syloid 244 FP adsorbed more engine oil than the capsules, which contained only wool as hydrocarbons adsorber. It was proved that capsules containing 1g of wool could adsorb about 5.11g of engine oil, while the beads containing both adsorbers could adsorb even 5.8g of oil.

Analyzing the bursting force of beads containing both adsorbers was considered, that values of this parameter were different than in the case of beads containing only wool. This phenomenon was caused by the fact that after 2 weeks of contact with engine oil and with distilled and also salty water the bursting force of all capsules increased more than 20%. As the comparison the bursting force for samples with beads containing only wool after 14 days of contact with distilled water to the beads containing wool and Syloid 244-FP increased significantly (23.56%). Similar effect was observed in the case of beads that were incubated in engine oil. There was noticed the increase (14.8%) of bursting force of beads containing both of adsorbers and decrease (-13.5%) of bursting force of beads containing only wool. It should be underline, that bursting force of two types of capsules, which were incubated in 11‰ and 35‰ water increased, but slightly (Table 3).

Table 3. The changes of bursting force (F) of beads containing wool and Syloid 244-FP after 2 weeks of incubation at room temperature in engine oil, and in distilled and salty water conditions

Reference samples	The beads	
	F _{max} [N]	SD
	12.31	1.27
The beads incubated in:	F _{max} [N]	SD
Engine oil	14.13	2.41
35‰ Salty water	14.98	2.17
11‰ Salty water	13.09	3.50
Distilled water	15.21	3.14

The results of research showed that alginate - Syloid 244-FP capsules containing wool could adsorb the higher amount of the oil after the first day of incubation (Table 4). Over the next three months of incubation in flasks with water contaminated with oil, the capsules adsorbed only 25% of the initial amount of adsorbed oil (which was adsorbed the first day). It was important that the adsorbed oil did not flow back from the capsules into the water. The experiments also demonstrated, that after 3 months of incubation the beads containing microorganisms contained 0.0080 grams of engine oil in one gram of capsules while the capsules with microorganisms incubated 24h adsorbed 0.0471g of oil per 1 gram of capsules. It could be a proof that 83.01% of adsorbed engine oil was degraded by microorganisms (Table 4).

Table 4. The content of engine oil in the beads after bioremediation process at room temperature.

Number of sample	Time of incubation [day]	Average content of engine oil [g] /1g of beads
1	1	0.0471
2		0.0404
3	90	0.0080
4		0.0505

4. DISCUSSION

During bioremediation in marine environment, the capsules should be resistant to oils influences and also to presence of salty water. This is the reason, why bursting force of beads after 2 weeks contact with oils and salty water was also evaluated. As was emphasis above the bursting force of all prepared capsules decreased slightly after 24h of contact with tested oil. The bursting force of beads incubated in salty water increased. Viability of *P. aeruginosa* strain into beads was evaluated after 14 days storage in the same conditions. The results of the tests showed that salinity of environment had no significant impact for bacterial viability. It was very important to check the oils adsorption properties by silica-alginate beads. The results demonstrated that the beads containing Syloid 244-FP can adsorb higher amount of engine oil in the comparison to the capsules containing Syloid Al-1FP as adsorber.

The results also showed that beads have not adsorbed diesel oil at all. Higher viscosity of engine oil in the comparison to diesel oil can cause limited adsorption of diesel oil by capsules. Higher engine oil adsorption caused that capsules with Syloid 244 FP were tested in biodegradation process (because of their higher absorption level). Syloid 244FP and Syloid Al-1FP are good adsorbers of petroleum hydrocarbons what is very important in biodegradation process especially with application of hydrophilic alginate capsules that can not adsorb the hydrocarbons. The presence of silica carriers is necessary to enable of oil adsorption by the beads. The earlier experiments (Mizielińska and Bartkowiak 2012) have indicated that 35‰ water and 11‰ water did not negatively affect mechanical strength of microcapsules containing Syloid 244-FP and Syloid Al-1FP what was proved by increase of bursting force after 28 days of incubation. Viability of *P. aeruginosa* cells entrapped into silica-alginate beads was also evaluated (Mizielińska and Bartkowiak 2012).

Lack of negative influence of wool and silica adsorbers on viability of bacteria, improvement of mechanical strength of beads after their incubation in salty water caused that *P. aeruginosa* strain was immobilized in wool entrapped in silica/alginate capsules. Because of better adsorption of engine oil by capsules containing Syloid 244-FP than by capsules containing Syloid Al-1FP the first adsorber was used to prepare beads with wool. Then effectiveness of engine oil adsorption by the capsules was evaluated. The results demonstrated, that the beads containing wool and Syloid 244 FP adsorb more engine oil than the capsules, which contains only wool as hydrocarbons adsorber. Summarizing, incubation of beads containing Syloid 244-FP and wool in engine oil and in salty water has no negative impact on mechanical properties of the beads. It is also important, that addition of Syloid 244-FP even improves the properties. The obtained results are tempting to suggest that Syloid 244-FP can be used in water bioremediation.

The success of the application of microbial inoculants for water decontamination depends on how favorable to its survival the target environments is or can be made (Gentili *et al.* 2006). In general, the ultimate fate of spilled oil depends primarily on the ability of microorganisms to use hydrocarbons as sources of carbon and energy. In this study *P. aeruginosa* strain was used to decontaminate water from engine oil. The high level of efficiency of this species in removing crude oil and its polluted derivatives from water is known in literature (Cybulski *et al.* 2003, Kiraye *et al.* 2016, Nwinyi *et al.* 2016, Sakkos *et al.* 2016, Varjani and Upasani 2016). It was proved that this strain can use petroleum hydrocarbons as source of nourishment (Mizielińska and Bartkowiak 2009). It is important that the cells of this strain can growth in the enough wide temperature range (10-50 °C) to be

used in bioremediation process. During bioremediation in marine environment, dilution of microorganisms may be a problem especially in open-water systems. Immobilization of microorganisms can be very good solution to solve this problem. Adhesion of cells in polyurethane foam (Oh *et al.* 2000, Patil *et al.* 2006, Quek *et al.* 2006, Mizelińska *et al.* 2017) offers several advantages including high mechanical strength, resistance to organic solvents or cost effective, but also disadvantage for example: polyurethane foams are not biodegradable. Immobilization of bacteria on different mineral carriers is becoming more interesting especially in the contest of bioremediation of contaminated water. Immobilization process offers higher density of bacterial cells, and enhanced metabolic activity in bioreactors (Hrenovic *et al.* 2008, Hrenovic *et al.* 2009). The solution of this problem can be also encapsulation. Kuyukina *et al.* (2009) used different types of hydrophobized carriers for *Rhodococcus* cells immobilization. The carriers included sawdust, poly(vinyl alcohol) cryogel (cryoPVA) and poly(acrylamide) cryogel (cryoPAAG).

Carrier hydrophobization was performed to increase the contact surface between immobilization matrices and *Rhodococcus* cells. Results showed that biotreatment of petroleum – contaminated water resulted in 77% decrease in hydrocarbons concentration after 3 weeks. Their results suggested also that the sawdust-immobilized bacterial cells have a better potential for use in bioremediation of hydrocarbon-contaminated water than bacteria immobilized in cryoPVA and cryoPAAG beads. Barreto *et al.* (2010) presented different solution. Spores of *Bacillus subtilis* LAM1008 were entrapped in 3-mm chitosan beads and cross-linked with 0,3% glutaraldehyde for n-hexadecane biodegradation. When exposed to nutrients spores generated vegetative cells. The entrapped cells degraded almost 100% of 1% of n-hexadecane in medium supplemented with 1% glucose. The overall date of their work showed the potential to develop products based on beads that can preserve the cells for long storage period. The main goal of present research work was to obtain very stable beads containing bacterial cells. Due to the non-sporing character of *P. aeruginosa* strain and because of antimicrobial properties of chitosan sodium alginate was used in immobilization process. It was also important, that there was used so easy degradable product such as alginate. Besides sodium alginate is immobilizing carrier most frequently applied in natural systems (Bogusławska-Wąs *et al.* 2005, Bayat *et al.* 2015, Tirumale *et al.* 2016).

Entrapping of microorganisms into alginate capsules is effective process. The problem is, that alginate capsules do not adsorb hydrocarbons. Sarma and Pakshirajan (2011) used *Mycobacterium frederiksbergense* entrapped in alginate beads to degrade pyrene. Biodegradation of this hydrocarbon in presence of non-ionic surfacant Tween 80 was performed. Assumptions for their experiments were similar to the assumptions of this work. In present work Tween 80 was not used to allow hydrocarbons degradation but wool and Syloid 244-FP as engine oil adsorbers. It is important that Sarma and Pakshirajan (2011) prepared different types of beads. They extruded sodium alginate into calcium chloride, sodium alginate with chitosan into CaCl₂ with NaOH, sodium alginate with PVA into NaNO₃ with CaCl₂, chitosan with acetic acid into TPP etc. Their results showed that calcium alginate capsules were highly stable than the other bead types. On the other hand, bead types, prepared using a mixture of sodium alginate and PVA as the immobilizing matrix, showed very poor mechanical stability. Beads prepared using chitosan-TPP also showed remarkable mechanical stability, but earlier revealed poor viability of the immobilized cells in it. Hence, based on mechanical stability and viability of the immobilized cells inside the beads, calcium alginate was selected as the most suitable beads. In this work Tween 80 was not used to allow

hydrocarbons degradation. Wool and Syloid 244-FP were used as hydrocarbons adsorbers that can be effective from the viewpoint of its biodegradability and good adsorption properties, it may be effective also because of stability of capsules in marine environment. Moreover the bursting force of silica-alginate capsules with wool increases after contact with saline water and neither silica adsorbers nor wool impact negatively on viability of bacterial strains, which were entrapped into the capsules. The results of Sarma and Pakshirajan (2011) demonstrated that complete degradation of pyrene was achieved. Results obtained by Kuyukina *et al.* (2009) demonstrated decrease (77%) in petroleum hydrocarbons concentration after 3 weeks of biodegradation. Results of experiments presented in this work showed 83.01% decrease of hydrocarbons concentration after 3 months. But some results (Sarma and Pakshirajan 2011, Nwinyi *et al.* 2016) proved that biodegradation of petroleum hydrocarbons can be complete.

5. CONCLUSIONS

Pseudomonas aeruginosa strain entrapped in Syloid 244-FP – alginate beads containing wool can be used to complete biodegradation of petroleum hydrocarbons after more than 3 months of degradation process. It is very important that these beads are stable during storage, non-toxic and also biodegradable (environmental friendly). Moreover, selecting of such very cheap immobilization method allows industrial production of immobilized bacterial cells, able to hydrocarbons degradation for environmental application

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