Polymer Bulletin 56, 275–284 (2006)
DOI 10.1007/s00289-005-0478-y

Polymer Bulletin

Core-shell microbioreactor microencapsulated denitrifying bacteria for nitrate-nitrogen treatment

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Received: 7 September 2005 / Revised version: 14 November 2005 / Accepted: 22 November 2005
Published online: 5 December 2005 –

Summary

Droplets of water-in-oil-in-water (w/o/w) emulsion were prepared using sodium alginate solution with denitrifying bacteria (*Paracoccus denitrificans* IFO13301) and dichloromethane (DCM) with polymethylmethacrylate (PMMA). By phase-separation and solvent-evaporation these droplets could be formed into core-shell PMMA microencapsulated denitrifying bacteria (PMMA-MC) possessing a large single core and a highly porous wall. The average thickness of the PMMA shell was 30 μm and the denitrifying bacteria were incorporated in the inner core at a high density. The PMMA-MC completely reduced 20 mg/L nitrate-nitrogen (NO_3-N) and the intermediate product, nitrite-nitrogen (NO_2-N), to N_2 in the presence of H_2 using a batchwise method. Thus, incorporated denitrifying bacteria can be used to treat water polluted with NO_3-N. The PMMA-MC can be used repeatedly, and the third denitrification experiment directly denitrified (NO_3-N → N_2) without the intermediate step (NO_3-N → NO_2-N). In addition, the PMMA-MC with decreased activity could be reactivated by incubating in a culture medium.

Introduction

Recently, chemical pollution of soil and groundwater pollution has been of interest. In particular, potable groundwater contaminated with NO_3-N has become a serious global problem [1-3]. NO_3-N contamination can cause methemoglobinemia and carcinoma in humans and animals [3-5]. The increase in the NO_3-N concentration is attributed to the increase in nitrogen components from applied fertilizer and farm animal excrement, which are oxidized to NO_3-N by nitrifying bacteria in the soil, resulting in contaminated groundwater. Since current purification plants still have some problems in treating NO_3-N, efficient treatment for NO_3-N has been investigated from various angles.

Techniques for treating NO_3-N are largely classified into physicochemical and biochemical methods [3, 6]. The physicochemical methods, largely ion-exchange and

reverse-osmosis-membrane methods, are widely known, but only provide separation, with after-treatment of NO_3-N then required, giving a relatively high running cost. The biochemical methods, which are classified into autotrophic and heterotrophic denitrifications, can reduce NO_3-N to N_2 under anaerobic conditions. Although, these methods are regarded as powerful removal techniques for NO_3-N, heterotrophic denitrification requires feeding with organic carbon sources, the excess addition of which may cause groundwater contamination. On the other hand, autotrophic denitrification requires an inorganic material such as H_2 as an energy source (hydrogen donor) [6-12]. Since H_2 provides clean energy, we focused on the autotrophic-denitrification method.

Some of the drawbacks in utilizing free denitrifying bacteria for denitrification include the difficulty of maintenance and secondary environmental pollution by leakage to the external environment. To overcome some of these drawbacks, various techniques of microorganism immobilization have been developed such as bioreactors with biofilm or microorganism encapsulation. Biofilm-bioreactors utilizing hollow-fiber are able to eliminate NO_3-N effectively and possess a long-term stability [13, 14]. However, the peeling of the biofilm composed of denitrifying bacteria would be worried. An efficient technique is the encapsulation of microorganisms in a polymeric carrier. In this technique, hydrogel microcapsules such as polyvinyl alcohol, calcium alginate, κ–carrageenan, and chitosan are widely used as the immobilization carrier due to their simple preparation procedure, good properties such as biocompatibility, and high diffusion ability of substrates and products [7, 15-21]. In application of immobilized microorganisms in a long-term reaction, however, the hydrogel microcapsules have only limited stability, in which problems such as breakdown of the carrier and leakage of the microorganisms are well known. Therefore, in previous reports, we proposed a core-shell solid polymeric microcapsule with denitrifying bacteria or baker's yeast using polystyrene (PSt), which possesses good mechanical and chemical strength [12, 22, 23]. A two-step preparation procedure was used to obtain the desired microcapsules. In the first step, the microorganism was immobilized into a calcium alginate bead to prevent the microorganism from directly contacting the organic solvent. In the second step, the calcium alginate bead with the immobilized microorganism was incorporated into the inner core of a PSt microcapsule. 2,2,4-trimethylpentane (isooctane) was used as the phase-separation material.

However, in the present study, PMMA was used instead of PSt in order to form a stronger microcapsule wall. Then, to produce the microcapsule in one step, sodium alginate solution was used for the protective material for the denitrifying bacteria and for the phase-separation material. Herein, we describe the development of a novel core-shell microcapsule for the efficient incorporation of denitrifying bacteria in the PMMA carrier and the examination of denitrification properties of the PMMA-MC in simulated water containing NO_3-N.

Experimental

Reagents

Polypeptone, $NaNO_3$, sodium alginate (100-150cP), polymethylmethacrylate (PMMA), polyethylene glycol 4,000 (PEG, Mw: 3,000), sorbitan monooleate, polyvinyl alcohol (PVA, n= 400-600, completely hydrolyzed), dichloromethane (DCM) and hydrochloric acid were purchased from Wako Pure Chemical Industries,

Ltd. Yeast extract was purchased from Difco Laboratories. $MgSO_4 \cdot 7H_2O$ and KH_2PO_4 were purchased from Nacalai Tesque, Inc. Calcium triphosphate (TCP-10U) was purchased from Taihei Chemical Industrial Co., Ltd. Other reagents were of special commercially available grades.

Culture methods

Paracoccus denitrificans IFO 13301 was cultivated in 5 g of pre-culture medium containing 1.0 % (w/w) polypeptone, 0.2 % (w/w) yeast extract and 0.1 % (w/w) $MgSO_4 \cdot 7H_2O$ in distilled water. A pre-incubation was carried out in a reciprocal shaker (150 rpm) at 30 °C for 10 h. A 2 mL aliquot of the pre-culture medium was transferred to 100 g of main-culture medium containing 4.0 % (w/w) polypeptone, 0.8 % (w/w) yeast extract and 0.4 % (w/w) $MgSO_4 \cdot 7H_2O$ in distilled water, and incubated in a rotary shaker incubator (150 rpm) at 30 °C for 18 h. After the incubation, the denitrifying bacteria were collected by centrifuging at 8,000 rpm for 5 min.

Preparation of the PMMA-MC

We prepared the PMMA-MC by the following one-step process, and the differences from two-step processes have been described in previous reports [12, 22-24]. A conceptual diagram of the PMMA-MC and the schematic diagram of the PMMA-MC preparation scheme are shown in Figures 1 and 2, respectively.

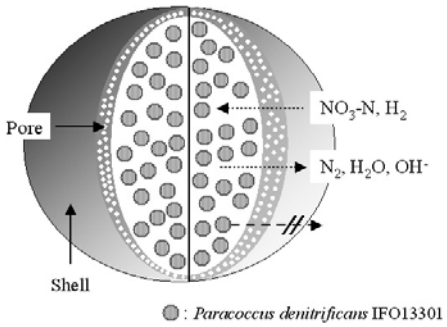

Figure 1. Conceptual diagram of the PMMA-MC

At first, an organic solution containing 20 g dichloromethane, 2 g PMMA, 0.6 g PEG, 0.6 g sorbitan monooleate was mixed with 6 g of internal aqueous solution containing 2g (wet mass) of denitrifying bacteria and 3 % (w/w) sodium alginate solution with some nutrients to support the growth of denitrifying bacteria. The nutrients added to the internal aqueous solution based on the sodium alginate solution were: 4 % (w/w) polypeptone, 0.8 % (w/w) yeast extract and 0.4 % (w/w) $MgSO_4 \cdot 7H_2O$. The mixed solution was then dispersed in a 755 g of external aqueous solution containing 5 g PVA and 250 g TCP-10U with stirring at 200 rpm and 5-10 °C (pre-temperature) for 1 h to form a w/o/w emulsion. DCM was evaporated at 30 °C and 300 hPa for 3 h while maintaining a constant stirring rate. The microcapsules prepared were washed with 0.5 mol/L hydrochloric acid to remove TCP-10U. Then, to grow the denitrifying bacteria in the core, the microcapsules obtained were incubated in the main-culture medium with a rotary shaker incubator (24 h, 30 °C, 150 rpm). The incubation was carried out two more times under the same conditions.

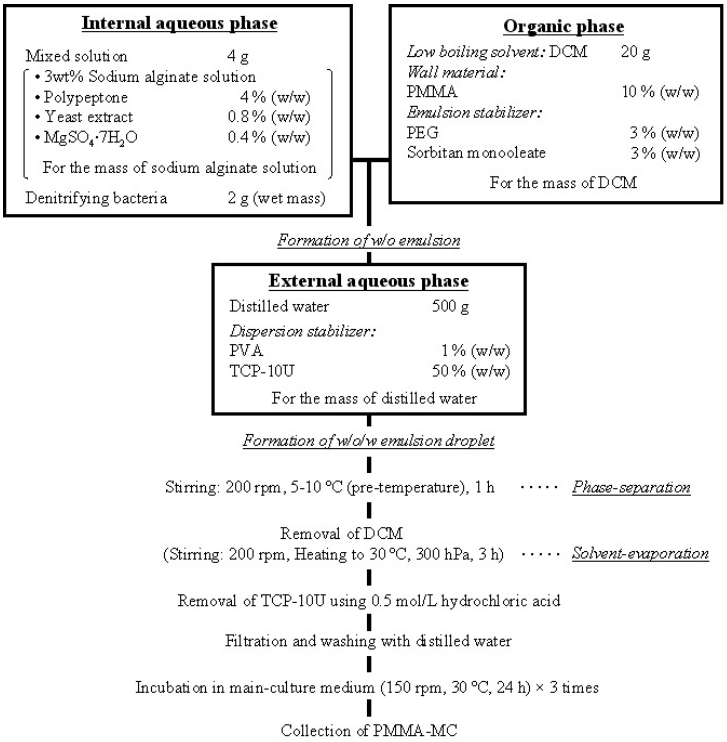

Figure 2. Schematic diagram of PMMA-MC preparation

A schematic diagram of the PMMA-MC formation mechanism is shown in Figure 3. Firstly, the mixed solution of the internal aqueous solution and the organic solution, which mainly consists of sodium alginate solution with denitrifying bacteria and DCM with PMMA, is dispersed in the external aqueous solution to form the w/o/w emulsion droplets (Figure 3 (a)). Phase-separation then occurs, and the internal aqueous solution

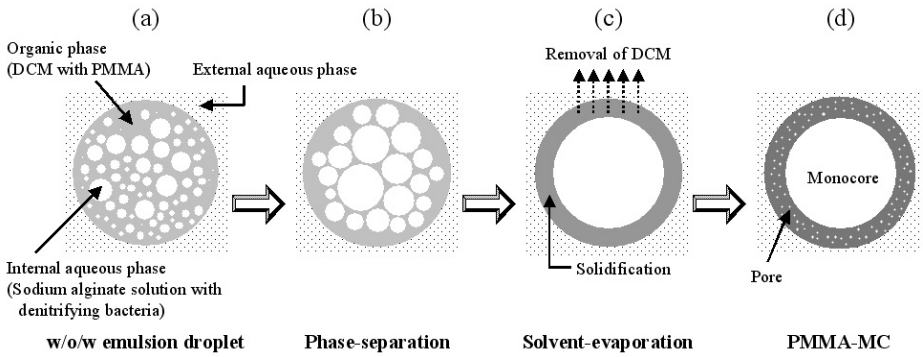

Figure 3. Schematic diagram of PMMA-MC formation mechanism: (a) w/o/w emulsion droplet, (b) phase-separation, (c) solvent-evaporation, (d) PMMA-MC

components combine with each other in the droplet center to cause core formation (Figure 3 (b)). Next, the solvent-evaporation of DCM at a low temperature with a decompressor leads to the solidification of PMMA within the droplet to form a microcapsule shell with a porous structure (Figure 3 (c)). In this way, a core-shell microcapsule with denitrifying bacteria can be formed, as shown in Figure 3 (d).

Denitrification ability on the batch experiment

Denitrification on simulated water containing NO_3-N was evaluated using a batchwise method. One hundred milliliters of simulated water was prepared with 0.121 g/L $NaNO_3$ and 0.2 mg/L KH_2PO_4. The initial concentration of NO_3-N was adjusted to 20 mg/L. Six grams (wet mass) PMMA-MC was shaken at 100 rpm and 30 °C. During the denitrification reaction, H_2 was bubbled into the simulated water at 30 mL/min. The experiment was carried out until the denitrification reaction stopped, after which the PMMA-MC was collected and washed with distilled water. The same PMMA-MC was used twice more under the same conditions. After the third use, the PMMA-MC was intentionally kept at about 5 °C for 276 h in the refrigerator to decrease denitrification activity for the reuse test. Denitrification ability (4-5th repetitions) was continuously investigated under the same conditions as described above. The PMMA-MC in which the activity deteriorated was then incubated in the main-culture medium adding 0.5 g $NaNO_3$ at 150 rpm and 30 °C for 24 h. Then, to evaluate reusability, denitrification (6th repetition) using the PMMA-MC was performed under the same conditions. NO_3-N and NO_2-N were analyzed using an ionic chromatograph (SM-8020, Tosoh Co, Ltd.). The chromatography column, mobile phase and detector were TSK-GEL IC-ANION-PW (4.6 mm × 5 cm, Tosoh Co, Ltd.), 2 mmol/L sodium benzoate and electric conductivity detector (CM-8020, Tosoh Co, Ltd.), respectively. The pH of sample solution was measured using a pH meter (HM-30G, Toa Electronics, Ltd.).

Morphology observation

The morphology (surface and internal structures) of the PMMA-MC was characterized using a scanning electron microscope (SEM, HITACHI S-2500, HITACHI Co., Ltd.). The internal and cross-sectional structures were observed by cutting the PMMA-MC with a diamond knife.

Results and discussion

Morphology observation

Figure 4 shows SEM photographs of the PMMA-MC. The diameters of the PMMA-MC were generally 200-700 μm, and the PMMA-MC was spherical (Figure 4 (a)). The PMMA-MC shell had a porous structure with an average thickness of 30 μm, and the denitrifying bacteria were incorporated into the inner core at a high density (Figure 4 (b) and (c)). As shown in the Figure 4 (b), the PMMA-MC with a large single core could be prepared by this method, and the large volume of the core is useful for high-density incorporation and free movement of denitrifying bacteria.

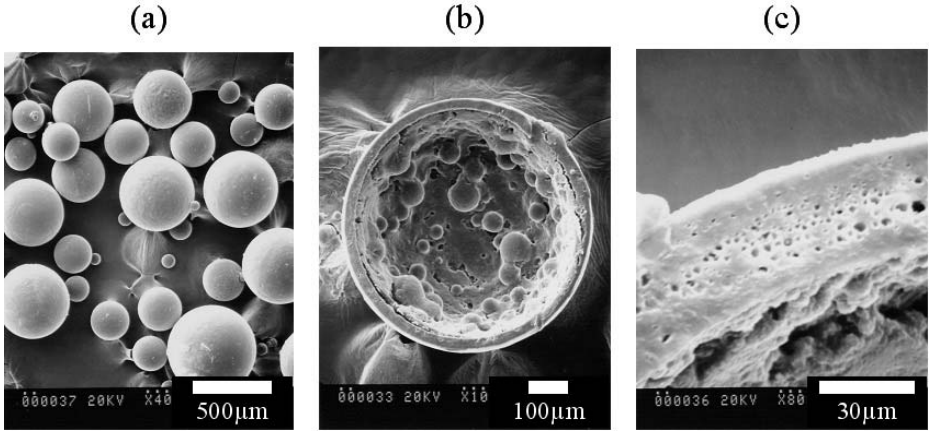

Figure 4. SEM photographs of PMMA-MC: (a) outline of PMMA-MC, (b) cross-section of PMMA-MC, (c) shell of PMMA-MC

Denitrification experiment

In the future field scale, we consider to treat water polluted with NO_3-N under the constant temperature (30 °C), after the groundwater is pumped. Hence, we carried out the reaction by the temperature condition (30 °C) obtained the high denitrification activity. The results of denitrification activities on NO_3-N, NO_2-N and change of pH during the course of experiments (1-3rd repetitions) are shown in Figures 5, 6 and 7, respectively. At present, the acceptable concentration of NO_3-N in drinking water by the World Health Organization (WHO) is less than 11.3 mg/L. In the first treatment, NO_3-N and NO_2-N were completely eliminated after 9 h and 50 h, respectively (Figures 5 and 6). In the second treatment, both NO_3-N and NO_2-N were completely eliminated after 21 h (Figures 5 and 6) and formation of the intermediate product, NO_2-N, was suppressed. In the third treatment, NO_3-N was completely eliminated after 18 h (Figure 5), and the NO_2-N was not completely formed, as shown in Figure 6. Generally, if H_2 is used as the energy source, the denitrifying bacteria sequentially reduced NO_3-N and NO_2-N to N_2 as shown in the following stoichiometric reactions.

$$2\ NO_3^- + 2\ H_2 \rightarrow 2\ NO_2^- + 2\ H_2O \quad (\mathbf{1})$$

$$2\ NO_2^- + 3\ H_2 \rightarrow N_2 + 2\ H_2O + 2\ OH^- \quad (\mathbf{2})$$

The total reaction is:

$$2\ NO_3^- + 5\ H_2 \rightarrow N_2 + 4\ H_2O + 2\ OH^- \quad (\mathbf{3})$$

According to the above denitrification process, NO_2-N is formed. However, in our procedure, we found that the formation of NO_2-N decreased with repetition of denitrification from 1-3 times. These results clearly demonstrate that the PMMA-MC can suppress formation of toxic NO_2-N as well as the previous research papers [7, 25]. We think that the NO_2-N reductase in the denitrifying bacteria is up-regulated by long-term use, and therefore the NO_2-N reductase reaction (2) rate was increased with

repetition, resulting rapid elimination of NO_2-N. In the above denitrification process, a hydroxide ion is formed as an end product, and the pH of denitrification solution increased. In the repetition experiment (1-3rd repetitions), the pH of the sample solution increased with progression of denitrification and stabilized below pH 9 (Figure 7). We also found that pH can rapidly achieve equilibrium with increasing repetitions of denitrification (Figure 7), therefore, the total denitrification ability can be improved by particular long-term use of the PMMA-MC. This phenomenon may also occur with continuous treatment (long-term use) with a bioreactor.

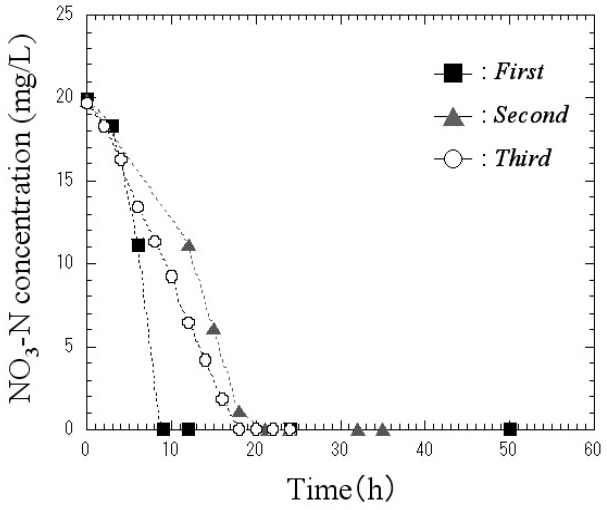

Figure 5. Denitrification results of NO_3-N (1-3rd repetitions)

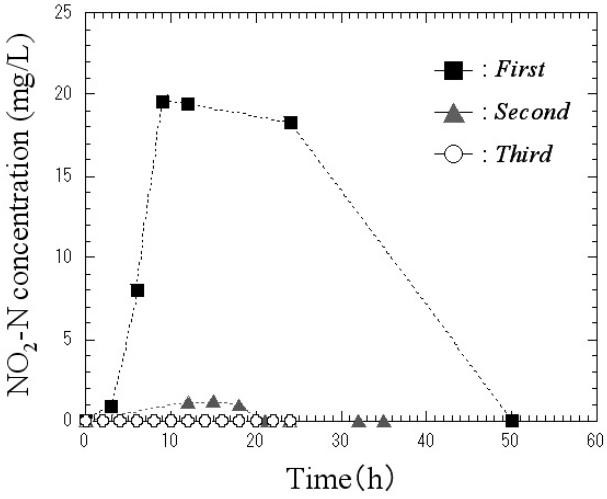

Figure 6. Denitrification results of NO_2-N (1-3rd repetitions)

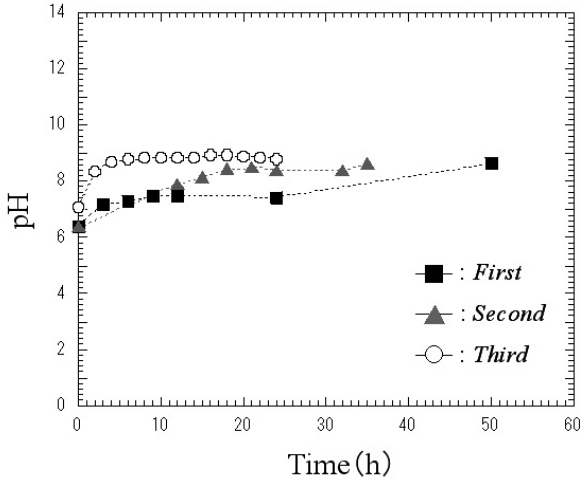

Figure 7. Change of pH (1-3rd repetitions)

Figure 8 shows the results of denitrification activities during the course of the 4-6th repetitions of the experiment with the same PMMA-MC. For the 4-5th repetitions, NO_2-N was also not formed as well with the 3rd repetition, however NO_3-N only decreased from 20 mg/L to 8.5 mg/L in 24 h. For the 6th repetition, NO_3-N and NO_2-N were completely eliminated after 18 h and 22 h, respectively (Figure 8). From this result, we confirmed that the denitrification activity of PMMA-MC, when its activity had decreased, could be recovered by incubation with the culture medium. In the first and sixth denitrifications after the incubation, a large quantity of NO_2-N was formed. The behavior of NO_2-N in the 6th repetition differs from results for the 4-5th repetitions, demonstrating that the denitrification characteristics changed after

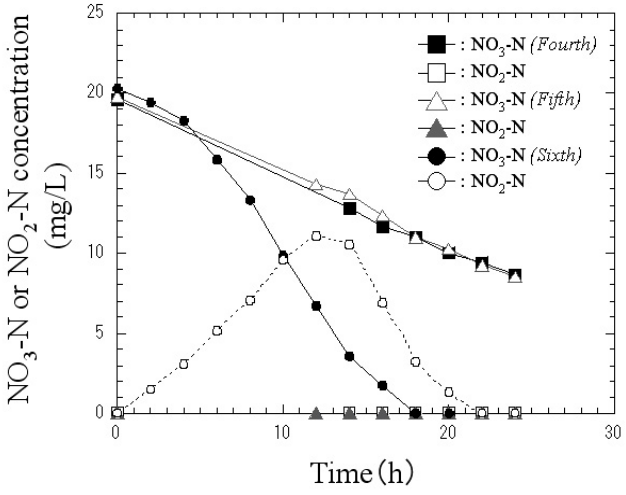

Figure 8. Denitrification results (4-6th repetitions)

incubating in the culture medium. We consider that the reductase in the denitrifying bacteria was oriented toward the environment of the incubation. The 6th denitrification was able to suppress half of the formation of NO_2-N compared to the first. This may be due to the addition of $NaNO_3$ to the main-culture. From the above results, the reductase in the denitrifying bacteria can orient toward each environment. Thus, the denitrification activity can be increased by repetitive use, that is, the PMMA-MC can completely eliminate NO_3-N and NO_2-N, and the formation of NO_2-N can be suppressed by repetitive use. In addition, the microcapsules could be reused, and the breakdown of the microcapsules was not observed.

Conclusions

PMMA-MC, possessing a large single core and a highly porous wall, was obtained by a novel preparation method based on the combination of simple phase-separation and solvent-evaporation techniques. The PMMA-MC could completely eliminate NO_3-N and NO_2-N, and the formation of poisonous NO_2-N could be suppressed by a certain number of repetitive uses. Moreover, the denitrification activity of the PMMA-MC could be recovered by the use of an incubation process when the activity of the denitrifying bacteria in the core had decreased, and the PMMA-MC did not break down during all denitrification experiments. As can be seen from the above results, we obtained a NO_3-N treatment microbioreactor with the targeted functions. We expect that PMMA-MC will be very useful for the development of an economical groundwater purification system.

Acknowledgements.
This research was supported by the New Energy and Industrial Technology Development Organization (NEDO) Grant (Project 2D: 04A27008).

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