Denitrification Using Denitrifying Bacterial Cells Immobilized by PVA-Boric Acid Method

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Abstract The relation between pH levels and denitrifying activities was determined using free cells of three bacterial strains; *Pseudomonas* sp. IFO 13302 (PS), *Paracoccus denitrificans* IFO 12442 (PD) and NK-2G, belonging to CDC group Vd-1, isolated from activated sludge. After immobilization by the polyvinyl alcohol (PVA)-boric acid method, these strains were mixed and subjected to a long-term denitrification test. The pH levels of PS, PD and NK-2G at which the maximum denitrifying activities were demonstrated were 6, 8 and 8 respectively. At a pH range of 5-9, the strains maintained satisfactory levels of denitrifying activity. When the three strains which had different denitrifying activities with respect to pH levels were immobilized by the PVA-boric acid method and used in mixture, they retained a 98-100% denitrification efficiency for four months. After that, the denitrification efficiency was slightly decreased and varied at a range of 83-100%. When reactivated, a 100% denitrification efficiency was restored but the efficiency was again decreased and varied at a range of 80-100% after approximately one month.

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Application of immobilized microbes as a bioreactor has recently been introduced in various fields of biotechnology²⁾. However, only a few examples have been described of application to the treatment of waste water, and this method has mostly been tested only on a bench scale^{3,4,9-11,13-15)}. In a previous investigation on carbon sources and optimum temperature conditions for denitrification using 3 denitrifying bacterial strains (NK-2G, NK-69 and NK-18) isolated from activated sludge (swine waste water treatment) and a standard strain (*Pseudomonas* sp. IFO 13302), we have found that the standard strain and NK-2G rapidly

denitrified waste water with a high level of denitrification efficiency and that the temperature range and the variety of carbon sources utilized for denitrification were large⁵⁾. We have then undertaken a short-term (10 days) comparative evaluation of the denitrification efficiency using one more standard strain (*Paracoccus denitrificans* IFO 12442) in addition to the above strains which were all immobilized by the PVA-boric acid method⁶⁾. NK-2G has been found to have the highest efficiency and the two standard strains to have a lower denitrification efficiency compared to the strains isolated from activated sludge⁶⁾.

This has been found attributable to the high susceptibility of the standard strains to boric acid than the isolated strains⁶⁾.

In this study, the relation between specific denitrification rates and pH levels was determined using two standard strains and NK-2G. Then, the three kinds of denitrifying bacterial strains were immobilized by the PVA-boric acid method, and a long-term denitrification test was carried out for the mixture of these immobilized bacterial strains.

Materials and Methods

Denitrifying bacteria: The following strains were used; NK-2G isolated from activated sludge and two standard strains, *Pseudomonas* sp. IFO 13302 (PS) and *Paracoccus denitrificans* IFO 12442 (PD). NK-2G⁵⁾ belongs to VD-1 of the CDC group and is an *Achromobacter* sp. in the former classification system.

Measurement of specific denitrification rate of free cells: The bacteria were cultured in a CGY agar medium¹²⁾ for 24 hours at 30°C. Colonies were inoculated to 125 ml of a mass culture medium¹⁰⁾. After preculture for 24 hours at 35°C, these were inoculated to 125 ml of the same medium for mass culture under the same culture condition. Bacterial cells were concentrated by centrifugation (Kokusan H-103 RLH) at $2,800 \times g$ for 10 minutes at 4°C. The concentrated bacteria were washed twice in 0.05 M Tris-HCl buffer (pH 7.5) and were recovered into test tubes. The broth for analysis (150 mg KH_2PO_4 , 150 mg K_2HPO_4 , 50 mg KNO_3 as nitrogen, 200 mg CH_3COONa as a carbon source and 1,000 ml distilled water) was adjusted to a pH level of 5, 6, 7, 8 and 9 using 0.1 N HCl or 0.1 N NaOH. This medium (20 ml) was added to each bacteria-containing test tube, immediately sealed after replacement of He gas and then allowed to stand at 25°C. Samples were collected at 1, 2, 4, and 8 hours, filtered through a membrane filter (pore size $0.45 \mu\text{m}$) and subjected to analysis. The concentrations of nitrate nitrogen ($\text{NO}_3\text{-N}$) and ni-

trite nitrogen ($\text{NO}_2\text{-N}$) were measured using a GCT·12N·12N-A type analyzer (Sumitomo Chemical Co. and Shimadzu Corp.). GCT is a Shimadzu GC-4C gas chromatograph which used He as carrier gas, two aluminium columns packed with activated carbon and thermal conductivity detector. 12N-A is a GCT attachment required for gasification of NO_2 into N_2 . It consists of a reaction tube filled with an aqueous solution of sodium chloride and sulfamic acid for dissociation of NO_2 into N_2 and an oxidation-reduction tube heated to 55°C for removal of hydrogen and oxygen. NO_3 was reduced to NO_2 with zinc powder and was dissociated into N_2 using this attachment. These N_2 gases were introduced into GCT using carrier gas (He), and $\text{NO}_2\text{-N}$ and NOx-N were measured. $\text{NO}_3\text{-N}$ was obtained by subtracting $\text{NO}_2\text{-N}$ from NOx-N .

The specific denitrification rate (K) for the evaluation of denitrifying activity was obtained by the following equation¹⁶⁾.

$$K = (\text{N}_1 - \text{N}_2) / \text{Sa} (t_2 - t_1)$$

where K denotes the specific denitrification rate (mg N/g cell/hr). Sa is the bacterial concentration (g wet weight/l), t_1 and t_2 are the times, and N_1 and N_2 are the concentrations (mg/l) of NOx-N at t_1 and t_2 respectively.

Immobilization: Immobilization was carried out basically by the method of HASHIMOTO *et al.*¹⁾. PVA used was a completely saponified product (Kuraray Co., Ltd.). Concentrated bacteria for each strain were obtained using 3 l of a mass culture medium by the method previously described⁵⁾. The wet weights of PS, PD and NK-2G were 7.2, 7.5 and 7.9 g respectively. A PVA solution was prepared with 1.3 g of PVA added to 10 ml of distilled water. The solution was sterilized in an autoclave at 121°C for 10 minutes. The PVA solution (30–35°C) and the concentrated bacteria were thoroughly mixed with stirring and dropped into saturated boric acid using a syringe. For complete gelatinization, the beads in boric acid were stirred at 60 rpm for 24 hours in a magnet

stirrer. The gelatinized beads (600 beads, average diameter = 3 mm) were washed twice in 0.05 M Tris-HCl buffer (pH 7.5). In consideration of the possible loss of bacterial cells during the process of immobilization, recultivation was carried out for 48 hours at 35°C using a mass culture medium and the beads were washed twice. After regelatinization by slow stirring for 24 hours in boric acid, the beads were washed and used. Two-hundred beads each were prepared for PS, PD and NK-2G.

Activity assay of immobilized bacterial cells : Six-hundred beads were transferred into a test tube, and 20 ml of the previously described broth for analysis (the carbon/nitrogen weight ratio = 4) were added to the tube. After replacement of He gas, the tube was immediately sealed. The broth for analysis was allowed to react for 24 hours and then collected. This procedure was repeated every day. The temperature used for reaction was 25°C. Using the medium collected daily, the pH level and the concentrations of $\text{NO}_3\text{-N}$ and $\text{NO}_2\text{-N}$ were determined. The denitrification efficiency was calculated by the following equation.

$$\text{Cr} = (1 - N_a/N_o) \times 100$$

where Cr denotes the removal (%) of $\text{NO}_x(\text{NO}_3 + \text{NO}_2)\text{-N}$. N_o is the initial concentration (mg/l) of $\text{NO}_x\text{-N}$ and N_a is the concentration (mg/l) of $\text{NO}_x\text{-N}$ at 24 hours.

Reactivation : When the denitrifying activity of the immobilized bacteria was deteriorated, reactivation was carried out by the following procedure. The beads were placed in 300 ml of a mass culture medium, cultured for 24 hours at 35°C, washed twice in 0.05 M Tris-HCl buffer (pH 7.5) and gelatinized in boric acid for 24 hours. The reactivated beads were then washed twice in Tris-HCl buffer, rewashed three times in a broth for analysis and subjected to a denitrification test.

Results

Relation between pH and specific denitrification rate : As shown in Fig. 1, NK-2G and PD

had the highest denitrification rates at a pH level of 8. The rates were 15.5 and 16.1 mg N/g cell/hr respectively. PS had the highest denitrification rate at a pH level of 6. The rate was 11.7 mg N/g cell/hr. When compared among the three strains, the bacterial strains which achieved the highest denitrification rate at pH 5 and 6, at 7, and at 8 and 9 were PS (8.67 and 11.7 mg N/g cell/hr), NK-2G (8.84) and PD (16.1 and 7.83) respectively.

Changes in denitrification efficiency of immobilized denitrifying bacteria and effluent pH until Day 126 : The denitrification efficiency and effluent pH were gradually increased during the first 6 days. At Day 9, these were increased to 97% and 7.3 respectively. The denitrification efficiency was maintained at a 98-100% level from Day 15 until Day 126. The pH level during this period varied at a range of

Fig. 1. Relation between pH and specific denitrification rates of three denitrifying bacterial strains.

○ : *Pseudomonas* sp. (PS)

● : *Paracoccus denitrificans* (PD)

○ : NK-2G

7.4–8.8 (Fig. 2).

Changes in denitrification efficiency and effluent pH after Day 129 and effect of reactivation: Fig. 3 shows the data obtained after Day 129. The denitrification efficiency decreased to 92% from Day 129. It ranged from 83 to 100% until Day 168, indicating apparent deterioration of the denitrification efficiency. The pH level during this period ranged from 7.2 to 8.9. When the bacteria were reactivated,

the denitrification efficiency was restored to 100% for approximately one month from Day 175 to Day 205. However, it varied at a range of 80–100% from Day 208. The pH level ranged from 7.3 to 8.2.

Discussion

ALEXANDER¹⁾ examined the relation between the denitrifying activities of denitrifying bacteria and pH from the amount of nitrogen gas

Fig. 2. Changes in pH and denitrification efficiency for 126 days of a mixture of three immobilized denitrifying bacteria.

○ : Denitrification efficiency ● : pH

Fig. 3. Changes in pH and denitrification efficiency at 129–262 days of a mixture of three immobilized denitrifying bacteria.

○ : Denitrification efficiency ● : pH ↑ : Reactivation

production and found that the optimum pH of *Pseudomonas denitrificans*, *Micrococcus denitrificans* and *Achromobacter* sp. were 6, 8, and 8 respectively. His results well agree with those obtained in this experiment with PS, PD and NK-2G. In a review of earlier studies, KOKUFUTA *et al.*⁷⁾ found that the optimum pH levels for denitrification varied with the 5 kinds of denitrifying bacterial strains used and ranged from 6 to 7.6. The previous studies^{5,6)} have clearly shown that the kinds of organic carbon source and the levels of optimum temperature for denitrification differed with individual bacterial strains. In immobilization of bacterial cells by the PVA-boric acid method, the bacterial susceptibility to saturated boric acid has been also found to differ with bacterial strains⁶⁾. From these findings it is presumed that stable denitrification efficiency which may be free from the influences of various environmental factors may be achieved, if PS, PD and NK-2G that are different in characteristics are used in mixture after immobilization.

KOKUFUTA *et al.*⁸⁾ carried out a 3-month denitrification test on a continuous process using *Paracoccus denitrificans* immobilized with a polyelectrolyte complex and found that the denitrification efficiency was maintained at more than 80% for 65 days but it was subsequently decreased to 60-70%. In a continuous denitrification test using *Pseudomonas denitrificans* immobilized with sodium alginate, and potassium aspartate as a carbon source, NILSSON *et al.*^{10,11)} described that the denitrification efficiency was gradually decreased from 100% to 15% from the beginning of the test until the 22nd day. The efficiency was, however, restored to more than 95% by reactivation. In an experiment using ethanol as a carbon source, they found that the efficiency was maintained at more than 95% for 53 days but was later rapidly decreased to less than 50%. They further found that reactivation, which was repeated three times, resulted in restoration of the denitrification efficiency to more than 95%. In

this experiment, the denitrification efficiency was maintained at more than 80% for a long period of 8.5 months, although reactivation was needed once. For the duration of 5 months during the above period, the efficiency exceeded 98%. This may indicate that higher denitrification efficiency was achieved in this study, compared to the data reported in the earlier studies.

In the present denitrification test for the triple mixture of immobilized bacterial strains, pH in the broth for analysis, which had been adjusted to 7, was found to vary at a range of 7.2 to 8.9 after 24 hours of reaction, except for the first 9 days. No specific relation was found between this pH change and the denitrification efficiency. Since the triple mixture of these strains was expected to allow denitrification above 7.8mg N/g cell/hr at pH ranging from 7 to 9 (Fig. 1), it seemed that the denitrification efficiency of this mixture was free from the effect of this range of pH change. However, it also seemed that the denitrification efficiency (Cr) could not be used as a sole basis for the evaluation of the effect of pH.

The triple mixture of bacterial strains immobilized by the PVA-boric acid method was thus found to retain its high denitrification efficiency for a long time. The denitrifying activity was also found to recover by reactivation. These results suggested the practical applicability of this method. We intend to carry out a test for actual application of this method to the treatment of animal shed waste water.

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PVA-硼酸法を用いた固定化脱窒細菌による脱窒素

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本試験は *Pseudomonas* sp. IFO 13302 (PS), *Paracoccus denitrificans* IFO 12442 (PD) および活性汚泥由来株である NK-2G の株の pH と脱窒活性の関係を調べるとともに, 各株を PVA-硼酸法により固定化後, 混合し, 長期脱窒試験を試みた. PS, PD および NK-2G 株の最大脱窒活性を示した pH はそれぞれ 6, 8 および 8 であり, pH 5-9 の範囲では 3 株ともに脱窒活性を維持した. そこで, pH 活性の異なる 3 株をそれぞれ固定化し, 脱窒試験を行った. その結果, 4 カ月は 98~100% の脱窒効率を維持したが, その後 83~100% を変動し, 若干の低下を示した. このことから, 再活性化を試みたところ, 100% の効率に復帰したものの, 約 1 カ月後には再び 80~100% を変動した. 以上より, 脱窒素に対する pH 活性が相違する 3 株を, PVA-硼酸法で固定化することにより 4 カ月間は 98% 以上の脱窒効率が維持し, 再活性化を行えば, さらに 1 カ月間の 100% の効率を維持することが明らかとなった.

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