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Chapter 12

A Novel Bioremediation Method for Shallow Layers of Soil Polluted by Pesticides

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Additional information is available at the end of the chapter

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1. Introduction

Excess amounts of pesticide have often been used to enhance the yield of agricultural products, and simultanously causing serious problems by polluting the soil and groundwater all over the world. For example, in the United States, large amounts of atrazine were used as a weed killer. The excessive atrazine sprayed by planes polluted rivers and soil near the farms, and this eventually was transferred to urban areas. The soil pollution in China is even more serious. Many agricultural fields and groundwater supplies are no longer used because of their contamination with pesticides and chemical fertilizers. An investigation by the FAO/UNEP/WHO suggested that 1-5 million patients develop pesticide poisoning every year, and several thousand patients die [1].

As a result, many countries have passed laws about the remediation and conservation of soil. In the United States, the remediation and monitoring of polluted soil must be done based on the federal Superfund Act. In Germany, a law about the preservation of water and soil was passed in 1997. The remediation of soil is carried out according to these laws in these countries.

Among the various processes used for the remediation of soil, bioremediation has been remarkable because it is high safety and inexpensive running cost compared to the physical and chemical methods, such as burning and adsorption. Bioremediation methods can be classified into two broad classes (*in situ* and *ex situ* bioremediation), and *in situ* bioremediation processes employs the treatment method without moving the polluted soil. Some pilot studies have applied *in situ* bioremediation methods, such as bioventing and oxygen release compounds (ORC) methods, to remediate soil polluted by pesticides [2]. Such projects have succeeded in decreasing the herbicide concentration to a safe level, but large scale equipment and high expenses were major drawback associated with these methods. As most of the agriculture-related soil pollution occurs over a large area, bioremediation processes with high

costs cannot be applied for this pollution. Therefore, the development of a new bioremediation method, which is applicable to huge areas and is low cost, has been desired.

In this chapter, the author proposed a novel bioremediation method called "Bioremediation with the self-immobilized system (BSIS)". Using this method, degrading microorganism can be self-immobilized by the help of *Bacillus subtilis*, and the immobilized cells rapidly degrade the pollutant in the shallow layer of the soil. The equipment and running cost associated with BSIS are low, and therefore, I think that this method is a superior method for remediating soil polluted by pesticides.

2. Necessity of remediation of soil and groundwater polluted by pesticides

2.1. Harmful influences of pesticides on living organisms

Typical pesticides employed in the agriculture sector are shown in Figure 1. Until the 1980s, organochlorine insecticides, such as DDT and BHC, were the most commonly used agents. However, Rachel L. Carson warned in her book "Silent Spring," which was published in 1962, that these insecticides were hard to degrade and subject to accumulation in the environment. In fact, many deformed birds and fish that had accumulated the organochlorine compounds were found. Based on this book and the findings of numerous other scientists, the production and utilization of DDT is now forbidden in many countries. Moreover, the discontinuation of persistent organic pollutants (POPs) such as DDT and BHC was decided to be a worldwide goal at the Stockholm Convention on Persistent Organic Pollutants in 2001. As a result, DDT cannot be used except for eliminating mosquitoes carrying malaria.

Following the banning of POPs, organophosphorous insecticides, such as parathion, dichlorvos, penitrothione and diazinon, were gradually applied to patties and fields instead of DDT and BHC. The persistence of organophosphorous compounds is much lower than that of DDT or BHC. These compounds, however, strongly inhibit the activity of acethylcholine esterase in nerve cells, and some of them showed strong toxicity to humans [3]. The use of many organophosphorous compounds was prohibited by the European Union (EU) in the 2000s, but they are still used in other countries. The derivatives of pyrethroid, such as chrysanthemic acid and pyrethrolone, and the derivatives of nicotine, such as imidacloprid, were similarly developed to decrease the toxicity of these herbicides. Imidacloprid is currently speculated to be the cause of colony collapse disorder, but remains widely use all over the world.

Another compound, 2,4-dichlorophenoxyacetic acid (2,4-D), was first developed in 1944 as a weed killer for treating wheat and corn fields. The compound kills dicotyledons, but functions as a phytohormone for monocotyledons. It is still used today. Triazine herbicides, such as atrazine and simazine, were developed as weed killers in the 1970s. These compounds kill weeds after absorption from the leaves and roots. They were used in Europe and the United States with great success, but atrazine was subsequently found to act as an endocrine disruptor [4,5]. According to Hayes *et al*. [4], when 40 male frogs (*Xenopus laevis*) were exposed to water containing 2.5 ppm of atrazine for three years, 30 frogs became infertile and four frogs became

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females. The four female frogs could be fertilized by a male frog. These results strongly suggested that atrazine was harmful. The EU decided to prohibit the use of atrazine after noting that concentrations often exceeded the upper limit in groundwater. The US Environmental Protection Agency (EPA) set the upper limit of atrazine as 3 ppm in the drinking water, which was much higher than that in the EU. The EPA does not currently prohibit the utilization of atrazine, because the proof of adverse effects was not considered to be reliable. Therefore, atrazine is still used in the United States. Some specialists also warn that the sharp decrease of frogs in all over the world might be related to the excess use of triazine herbicides.

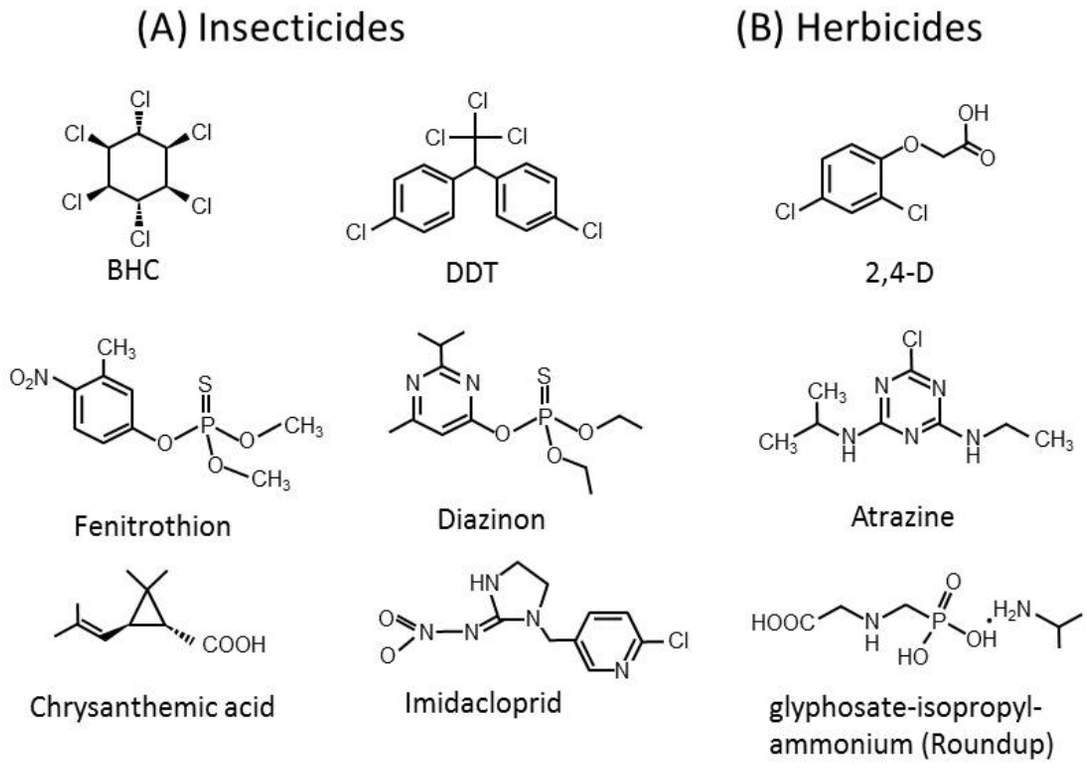

Figure 1. Pesticides commonly used for agriculture.

In the 1980s, Roundup was developed by Monsant Company. Glyphosate-isopropyl ammonium is a main component of the herbicide. It inhibits the synthesis of an amino acid, and works against all kinds of plants. The company also developed genetically modified organisms (GMO) which were resistant to Roundup. Based on these successes, Roundup is widely used to grow GMO in the United States. However, excess utilization of the non-selective herbicides (2, 4-D and Roundup) causes a decrease in soil insects and bacteria, which supply the nitrogen, carbon and phosphate sources to plants.

As described above, the use of many harmful herbicides has been prohibited or regulated since the 2000s, and the pesticides showing low toxicity and a short retention time are now being used. However, dangerous pesticides continue to be used in many countries, especially

developing countries, and the acreage where serious damage has been inflicted have been increasing all over the world.

2.2. Soil and groundwater pollution by pesticides

Atrazine and simazine have been used extensively in the United States and Europe. In the United States, 36,000 tons/year of atrazine are still being used in the patties and fields. The fields and rivers in the northwest part of the United States have been monitored for several years, and many kinds of pesticides were detected there [6-9]. In fact, 75% and 40% of samples collected in rivers and groundwater contained atrazine and other harmful pesticides. The concentrations of harmful pesticides were gradually decreased, but the concentrations of diazinon, chlorpyrifos and malathion often exceeded the benchmark value [6].

Atrazine sprayed from the airplanes is often carried by wind to other locations at a distance of over 1000 km from the spraying place, and these fall with rain. As noted above, the EU forbade the use of triazine herbicides, because their concentrations in groundwater exceeded the limit they set for safety standards. However, because the yield of crops can be enhanced by the use of atrazine, the farmers in the EU do not necessarily obey this decision, and harmful pesticides are still used and detected from the river water and groundwater in some countries in Europe [10-12].

Soil pollution is much more serious in the countries in Asia. Although the current amount of pesticide consumption in developing countries is only 25% of the total amount produced, 99% of the people killed by pesticide poisoning are from developing countries. Soil pollution also induces groundwater pollution. As groundwater is used for drinking water without any treatment in many countries in Asia due to the water shortage, this may lead to many human cases of poisoning. Additionally, many kinds of POPs which were produced before 2001 are still used in some countries, even though their use and production is prohibited by the Stockholm Convention. For example, in Vietnam, the soil is polluted by 4 tons of harmful pesticides that were buried in soil to discard them, and 108 tons of toxic POPs are still being stored in a warehouse. Such POPs can be easily obtained at markets in Nepal and India. BHC is still used for the production of crops and cotton cultivation in those countries, because it is cheap and effective [13].

In China, the pollution of soil and groundwater are extremely serious [14-16]. Approximately one-third of the wastewater from industries, and 90% of the drainage from households is directly discharged to the river. It has been estimated that 40% of river water (95% in urban areas) is already impossible to utilize as drinking water due to pollution, and thus, it has the potential to harm the 160 million people who are known to use this water.

The amount of pesticide consumption in China has increased ten times compared to that in the 1990s. Because of excess use (13.4 kg/ha) for agricultural fields, 60% of the pesticides used remain in the soil without degradation. Therefore one-sixth of the agricultural fields need to be remediated. Chemical fertilizers are also excessively used, and approximately 60% of the fertilizer also remains in the soil. Excrement (3 billion tons) discarded in pastures is also present in the soil. These contaminants cause pollution due to nitrate nitrogen. The use of plastic

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sheeting buried in soil, which help to maintain nutrients and moisture, exacerbates these problems. In addition, discarded plastic sheeting (500,000 tons/year) spoils the soil by interrupting the exchange of air and water.

Soil pollution in China is caused by not only pesticides, but also wastewater that is insufficiently treated by industry. For example, melamine, which is used for the production of melamine resin, contains a very high level of nitrogen. In 2004-2007, melamine was intentionally added to dairy products by some food manufacturers in China to increase the apparent content of protein. Consequently, serious food-related toxicity occurred in the people, dogs and cats that consumed the dairy products. Wastewater containing melamine was discarded into the environment without any treatment until the incidents occurred. Therefore, the soil, groundwater and crops near the manufacturing facilities were polluted with melamine [17]. A similar incident occurred related to the production of rice in China. Methamidophos, an organophosphorous compound, was mistakenly included in rice, although the precise reason for this accident was not clarified. One million tons/year of food is affected by soil pollution in China according to an investigation by a public organization.

The shortage of drinking water caused by groundwater pollution is also serious. To avoid groundwater polluted by toxic compounds, wells were dug to much deeper layers in the aquifer [18-19], but the groundwater at these layers contains arsenic and fluoride. Around 25 million people who drank this water showed arsenic toxicity and dental fluorosis. Since the 1980s, poisoning by arsenic has been gradually increased in many countries, such as India, Thailand and Bangladesh [20].

3. A novel bioremediation process targeting the shallow layers of soil

3.1. Problems with conventional bioremediation processes

As described in section 2, excess use of pesticides in agricultural fields causes serious pollution of the soil and groundwater. The characteristics of soil pollution by pesticides are that the area of pollution is huge, and the pollution reaches to deep layers. *Ex situ* bioremediation, which is a process performed after movement of the polluted soil, is not adequate to control such widespread pollution. Therefore, *in situ* bioremediation, which involves treating the soil in place, had been applied for pollution with pesticides.

Several *in situ* bioremediation processes have been proposed [2], and the ORC method developed by the REGENESIS Corp and bioventing are often used. Figure 2 shows a schematic diagram of these processes. In the bioventing method, air is supplied from the upstream side of the pollutant at deeper layers of soil by using high-pressure pumps. In the ORC method, oxygen release compounds (ORC) are injected into the deeper layer by using high-pressure pumps. The supply of oxygen activates the microorganisms present in the polluted soil, enhancing the degradation rate.

Bioventing is suitable for the remediation of contaminants at deeper layer and over wide areas of soil, and some projects using bioventing have been performed. For example, bioremediation

of organochloride compounds was examined using a pilot plant [21]. Anaerobic bacteria could be activated by injecting vegetable oil as a carbon source, and the concentration of tetrachloroethylene was decreased until the level was lower than the limit of the benchmark. The ORC method also succeeded in the remediation of oil pollution in practical cases.

However, there are several conditions that must be overcome in cases of soil pollution by pesticides: (1) The polluted area is huge and requires the use of large-scale equipment; (2) the cost for the remediation should be low; (3) the operation for remediation must be continued permanently, because pesticides are sprayed periodically every year; (4) the polluted area cannot be dug up, because grass, crops and other plants are already present there. The bioventing and ORC methods don't conform to these conditions and are therefore not applicable. A novel bioremediation method for soil pollution due to pesticides is strongly needed.

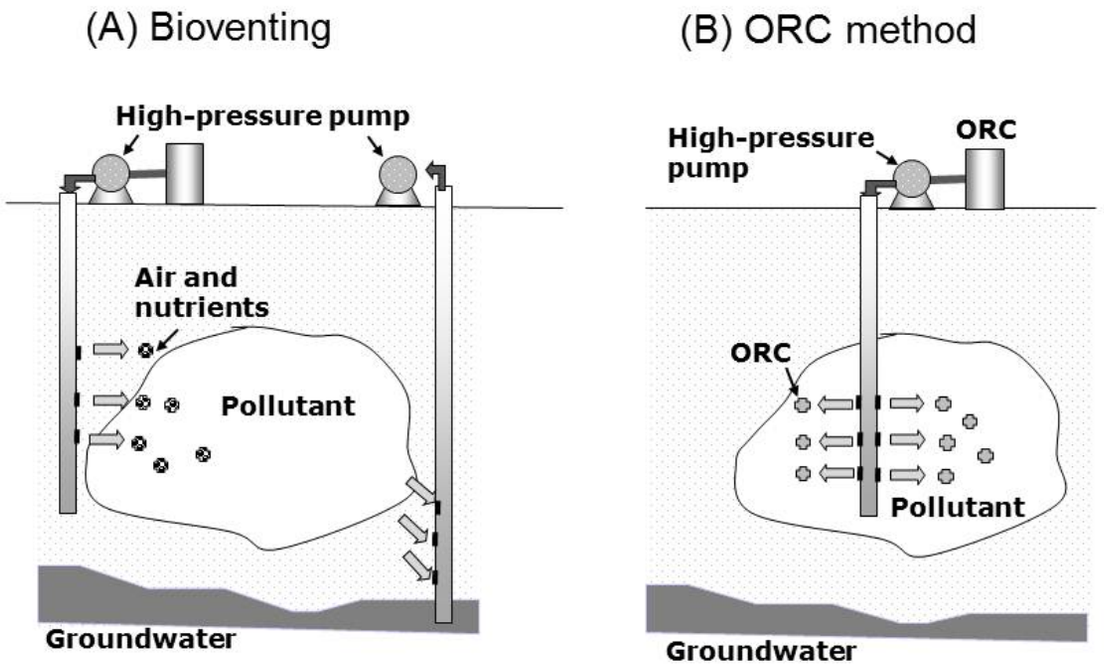

Figure 2. *In situ* bioremediation methods: Bioventing (A) and the ORC method (B).

3.2. Advantages of targeting the shallow layer of soil as the degrading zone

The author previously proposed a new concept for bioremediation [22]: the shallow layer of soil should be targeted for the bioremediation of pesticides. Targeting the shallow layer (A0 and A1 horizons) of soil has many advantages compared to the deeper layers (B and C horizons). Figure 3 shows a schematic diagram of the advantages of targeting the shallow layer, and can be explained as follows:

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The first advantage is that the degradation rate of the pollutant at shallow layers is much higher than that in the deeper layers. Pesticide is sprayed in the air, and drops on the soil surface with rain or sprayed water. Because the pesticide diffuses from the soil surface to the deeper layer, the concentration is the highest at the soil surface. The degradation rate of a pollutant is generally proportional to the concentration, and thus, the highest degradation rate can be obtained at the shallow layer.

The second advantage is that oxygen is sufficiently present at the shallow layer. Many microorganisms require oxygen for their activation. In the shallow layer, water in soil contains sufficient oxygen, because air passes through the cavities in soil. Therefore, the exogenous supply of oxygen is not necessary at the shallow layer, making large-scale equipment unnecessary.

The third advantage is that it is easy to supply a degrading bacterium and nutrients to the shallow layer. Pollution is partially caused by the shortage of degradation by microorganisms. Therefore, microorganisms showing high degradation potential should be supplied to compensate for the shortage. In the deeper layer of the soil, it is very hard to supply microorganisms, and huge amounts of cells must be supplied. In the shallow layer, however, microorganisms can be easily supplied by spraying them with a sprayer, and the amount of cells required is much lower than that required for the deeper layer.

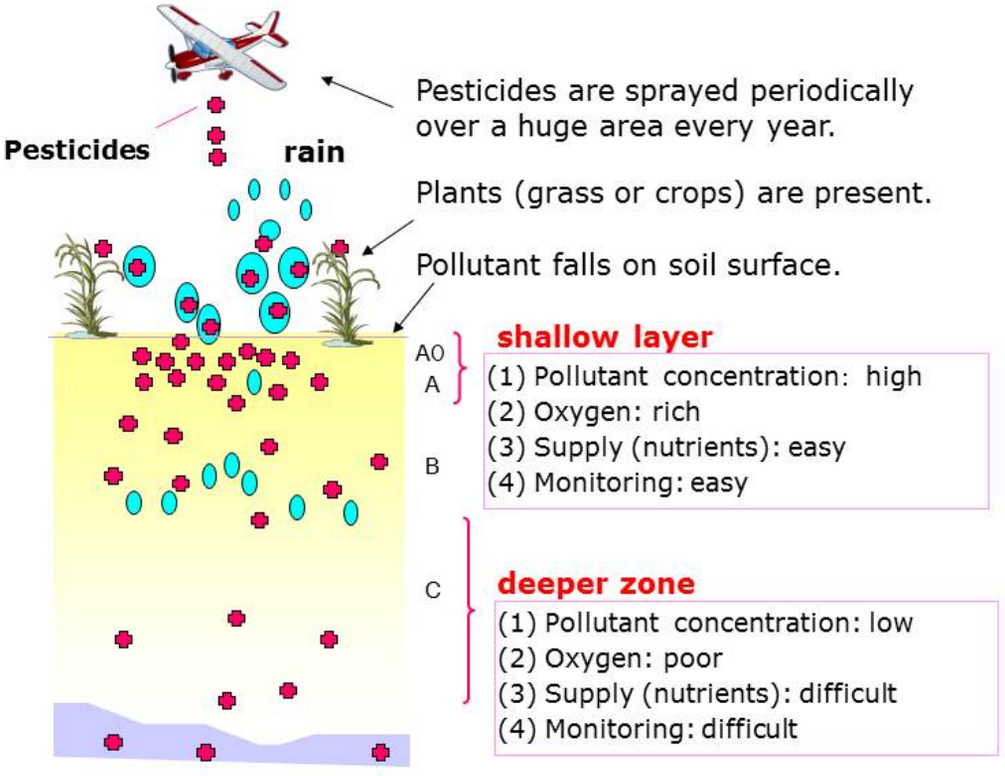

Figure 3. A schematic diagram of the advantages of targeting the shallow layer of soil for bioremediation.

The fourth advantage is that the maintenance and monitoring of the degradation are very easy. A pesticide is periodically sprayed every year, and there is always some contamination. Therefore, the ability to degrade the compounds must be permanently maintained. It is important to provide a supply of nutrients for the microorganisms to maintain the degradation activity. In the bioventing method, nutrients are often unable to reach the polluted areas. In contrast, the supply of nutrients in the shallow layers can be easily provided and assessed. Furthermore, in the agricultural fields in China or other countries in Asia, it is unnecessary to provide carbon and nitrogen sources or phosphate because chemical fertilizers are excessively used, and acid rain containing SOx and NOx often falls.

3.3. Bioremediation in the shallow layer using cells immobilized by a carrier

Although there are many advantages to bioremediation targeting the shallow layer, the passage time of pesticides in the shallow layer is short, and the degradation must be completed during this short period so that it does not reach the deeper layers. This can be realized by covering the soil surface or the shallow layer with immobilized cells. By using immobilized cells, the degradation rate and activity can be maximized.

Many studies have reported the immobilization of microorganisms [23] and its application for the treatment of waste water [24,25]. To use immobilized cells for bioremediation, it is necessary to ensure that the techniques are inexpensive and that the pore size is large enough to provide a high flow rate. Immobilization by biomass-supported particles (BSPs) [26], as shown in Figure 4A, may be the best applicable method. Yeast and bacteria showing cohesiveness can be immobilized by BSPs [27]. A high flux and low cost can be obtained because of large pore size for the immobilization, as shown in Figure 4B. The excessive growth of cells in BSPs can be easily remedied by their flowing out from the carrier, and the high degrading activity can be maintained by these released cells.

Charcoal also may be suitable for this purpose. It is inexpensive and can be used in the immobilization of bacteria. Immobilization with charcoal has been examined previously in the treatment of waste water. For example, Takagi *et al.* [28] examined the degradation of simazine in an experimental plot of a golf course. The *Arthrobactor sp.* cells that had the capacity to degrader simazine were immobilized with charcoal particles (1 cm^3 average size), and covered the soil surface. Simazine (25 g) was sprayed twice every 10 days. Consequently, around 90% of the simazine was degraded, and the degradation capacity was maintained for at least two months.

These results suggest that the degradation in the shallow layer is very useful for the remediation of the pollution by pesticides. However, two problems must be solved. One is the cost. When the immobilization method must be applied to a large area, the immobilization operation will likely be costly, even though the running cost is low. The other issue is digging. Most fields cannot be dug to cover them with the immobilization particles, because grass or crops are present. If the cover by these particles is inadequate, an insufficient degradation rate will be obtained.

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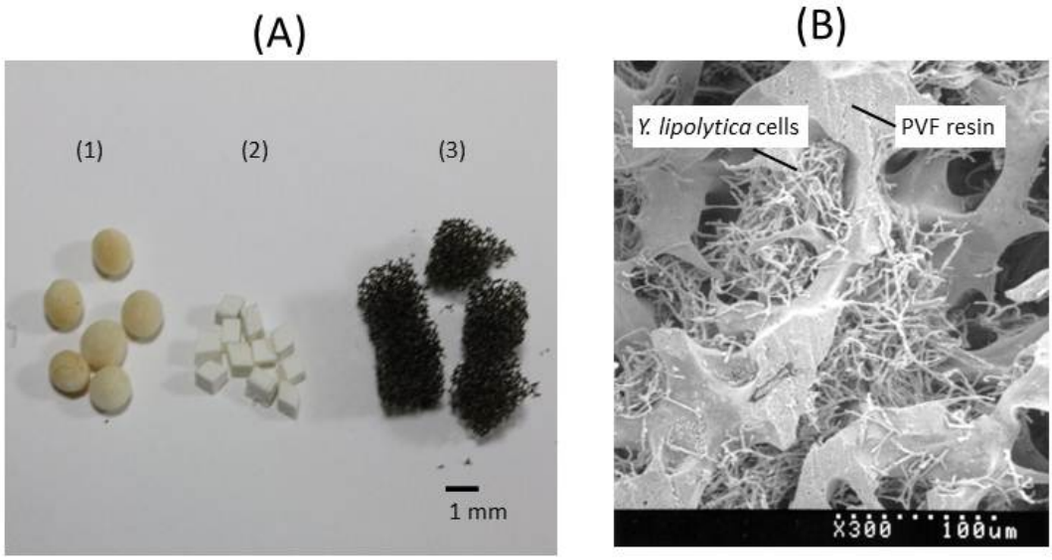

Figure 4. Immobilization with BSPs. Various kinds of BSPs (A) and a photograph of *Yarrowia lipolytica* cells immobilized by polyvinyl formal (PVF) resin (B). (1) The cellulose form; (2) the polyvinyl formal resin; (3) the urethane form

3.4. Bioremediation with the Self-Immobilized System (BSIS)

To compensate for the defects of the immobilized method, the author proposed a novel bioremediation method, which the author named "bioremediation with a self-immobilized system (BSIS)". BSIS is based on the idea that a polymer secreted by a microorganism can be utilized instead of an immobilization carrier, and the microorganism can immobilize itself in the shallow layer of soil without any other immobilization carrier (BSPs or charcoal). Below, BSIS is described in detail.

Figure 5 shows a schematic diagram of the protocol for BSIS. The *Bacillus natto* (*Bacillus subtilis*) strain, which is used for the production of "Natto" (a traditional fermented food in Japan), secretes a poly-glutamate (PGA) polymer. The PGA polymer shows high viscosity and can be used as a humectant, and was used in an attempted greening of a desert. The author used the PGA polymer as an immobilization carrier. As the first and second steps in Figure 5, a cell suspension of *B. natto* (*Bacillus subtilis*) is sprinkled on the soil surface, and medium is supplied for several hours. The *Bacillus* cells grow and form cell aggregates by secreting the PGA polymer in a shallow layer. As the third and fourth steps, the cells showing the ability to degrade pollutant (degrading cells) are sprinkled on the soil surface, and the medium is supplied. The degrading cells are trapped by the PGA polymer previously secreted by *B. subtilis* and grow. The medium is supplied for another several hours until cell aggregation at high-density constructs in the shallow layer of soil is achieved. By these steps, *B. subtilis* and degrading cells are co-immobilized by attaching to the PGA polymer, and the zone for degradation is constructed in the shallow layer of soil.

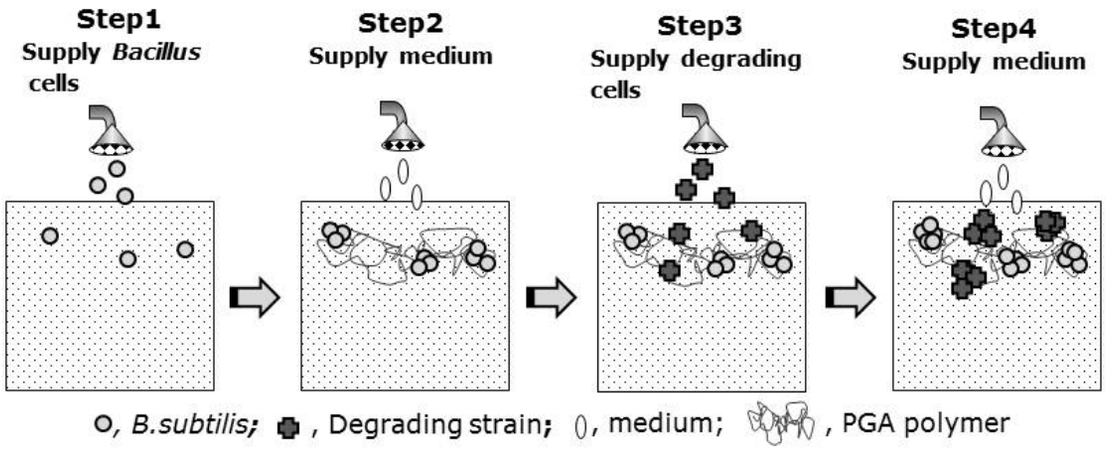

Figure 5. The protocol for the BSIS. Step 1, Supply *B. subtilis*; Step 2; Supply the medium; Step 3, Supply the degrading strain; Step 4, Supply the medium.

BSIS has many advantages compared to the other bioremediation methods. For example, BSIS does not require a supply of exogenous oxygen or nutrients with a high pressure pump, because BSIS treats pollutants at a shallow layer in the soil, where oxygen is rich and nutrients are easily supplied by an inexpensive sprinkler. BSIS therefore does not require expensive equipment. The cost for the equipment and operation of BSIS are much lower than those of the other methods. BSIS can be applied even in cases where many crops and grass are present, because it can be performed without having to dig up the soil. These advantages suggest that BSIS can be applied for the bioremediation of huge areas, and that continuous operation can be easily performed for many years. Thus, the BSIS method is an optimal remediation technique for soil polluted by pesticides.

3.5. Bioremediation of acid rain and pesticides by using BSIS

Finally, this section precisely explains how the idea of BSIS can be realized by introducing the author's studies. In a previous investigation [22], the author selected the *B. subtilis* NBRC3335 strain, which showed the highest cohesion among the stock bacteria at Kobe College. Figure 6 shows the cohesion of the strain. The *B. subtilis* NBRC3335 or *Eshcerichia coli* (control) bacteria were poured and penetrated into soil or sand packed in a column (5 cm at height), and 160 ml of water was poured into the column. A large amount of *E. coli* cells were passed through the column, especially in the case of the column packed in sand, which had a larger particle diameter (average diameter 400 μm) than that of the soil (average diameter 40 μm). On the contrary, *B. subtilis* cells were retained even in the column packed with sand. Therefore, *B. subtilis* cells have ability to attach to the shallow layer of various types of soil, and can be used as the first step of BSIS, as shown in Figure 5 (although *B. natto* which is used in the actual production of natto is superior to the NBRC3335 strain).

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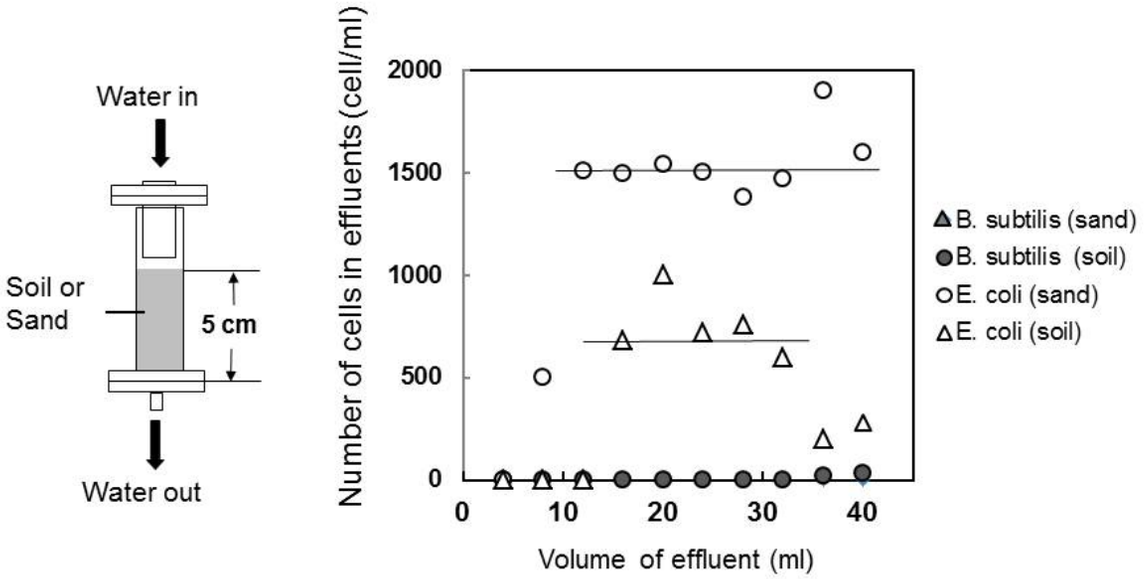

Figure 6. The self-immobilization capacity of *B. subtilus* NBRC3335 in the shallow layer. Four milliliters of *B. subtilis* or *E.coli* cell suspensions (0.01 OD) were supplied to the soil or sand in a column (5 cm height), and water was added. The numbers of cells in effluent samples (4 ml) were respectively counted.

Moreover, the author examined whether BSIS can be applied for the remediation of soil acidified by acid rain [29,30]. In recent years, acid rain has damaged many kinds of plants through the leaching of calcium and magnesium ions from the soil, and it has become a serious problem worldwide [31]. Preservation of the pH value of soils is important to avoid this problem. To protect the soil from acidification by acid rain, calcium carbonate powder or stone (marble) has often been used for neutralization of acids in Europe.

The author tried to use microorganisms showing a high neutralization potential instead of using calcium carbonate powder. The *Aureobasidium pullulans* NPH6 strain was isolated [32], and showed the high neutralization by degrading nitrate and secreting ammonia, as shown in Figures 7A and B. A neutralization test for BSIS was performed using the NPH6 strain and *B. subtilis* NBRC3335. Figure 7C shows the apparatus used for the experiment. An acrylic column (5 cm in inside diameter and 57 cm in height) was packed with sterilized silica sand (600-800 μm of particle sizes), which was used as a model soil. A mixture of *A. pullulans* NPH6 and *B. subtilis* IFO3335 cells (1.0 OD) was applied to the top of the column at a flow rate of 16.6 ml/h, then the modified Luria-Bertani medium (LB medium; 10 g/L bactopeptone, 10 g/L yeast extract, 5 g/L NaCl, 1g/L glucose, pH7.2) was supplied at a rate of 5.6 ml/h for 18 h to grow and co-immobilize these cells (mixed culture: BSIS). For comparison, immobilization was carried out by application of only NPH6 cells (pure culture: control). Figure 8A shows the distribution of the cell number of the NPH6 strain, which is expressed as the total cell number in each section of the column, after supplying the LB medium for 18 h. By supplying of LB medium, the cell number in the column increased about 17 times the number initially supplied in the mixed culture (BSIS). This increase in the cell number is almost the same as that in the batch culture. Furthermore, in the mixed culture, over 80% of the total cells existed in the layer

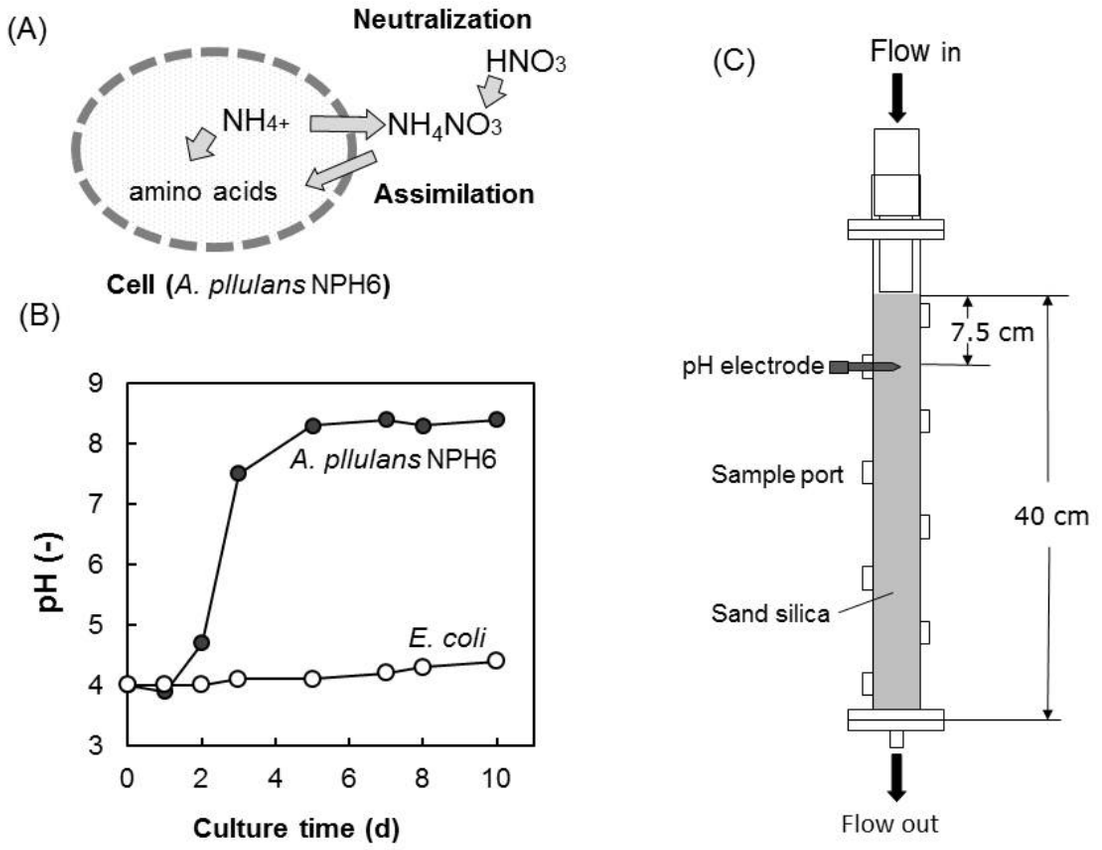

Figure 7. The strain and apparatus used for the BSIS experiment. A schematic diagram of the neutralization of nitric acid by *A. pllulans* NPH6 (A) and the time courses of the pH changes (B) when the *A. pllulans* NPH6 or *E. coli* cells were cultured in the LB medium. (C), A schematic diagram of the column used for the experiment. Sand silica was packed in the column, and the cells and medium were supplied at the top of the column with a micro-pump.

0-5 cm from the surface of the packed silica. The DO value measured by a DO electrode inserted to a depth of 7.5 cm was about 50% of air saturation after supplying the LB medium for 18 h.

Figure 8B shows the neutralization characteristics of the sulfuric acid solution (pH 4.9) by BSIS after the cells were immobilized (as shown in Figure 8A), when the sulfuric acid solution was supplied at the top of the column at a flow rate of 24.9 ml/ h. The pH value in the mixed culture was kept above 6.5 for 96 hours, while it decreased below 5 in the pure culture. The total amount of solution corresponded to a rain fall of 1200 mm, which is the average value of rain fall during a one year period in Japan. This means that BSIS is applicable for the neutralization of a large amount of acid rain.

The author also tried to apply the BSIS for the remediation of soil polluted by pesticides [33]. Figure 9 shows the metabolic pathway of atrazine. In this pathway, cyanuric acid is a key intermediate, and the cleavage of its ring is the rate limiting step in its degradation [34]. Thus, the author examined the bioremediation of cyanuric acid instead of atrazine by using the *Pseudomonas sp*. NRRL B-2227, which showed a high degradation capacity for cyanuric acid. The same apparatus and model soil (Figure. 8A) were used for the experiment. To co-immo-

bilize the *Pseudomonas sp.* NRRLB-12227 with *B. subtilis*, 20 ml of a cell suspension of *B. subtilis* NBRC3335 at 1.0 OD was supplied to the top of the column at a flow rate of 16.6 ml/h. Next, LB medium was supplied at a rate of 5.6 ml/h for 24 h to grow *B. subtilis* cells. A 20 mL suspension of *Pseudomonas sp.* at 1.0 OD was supplied at a flow rate of 16.6 ml/h. The R medium was supplied at a rate of 10 ml/h for 18 h to co-immobilize the *Pseudomonas* cells with *Bacillus* cells (mixed culture: BSIS). For comparison with the mixed culture, *Pseudomonas sp.* cells were immobilized without *Bacillus* cells (pure culture: control).

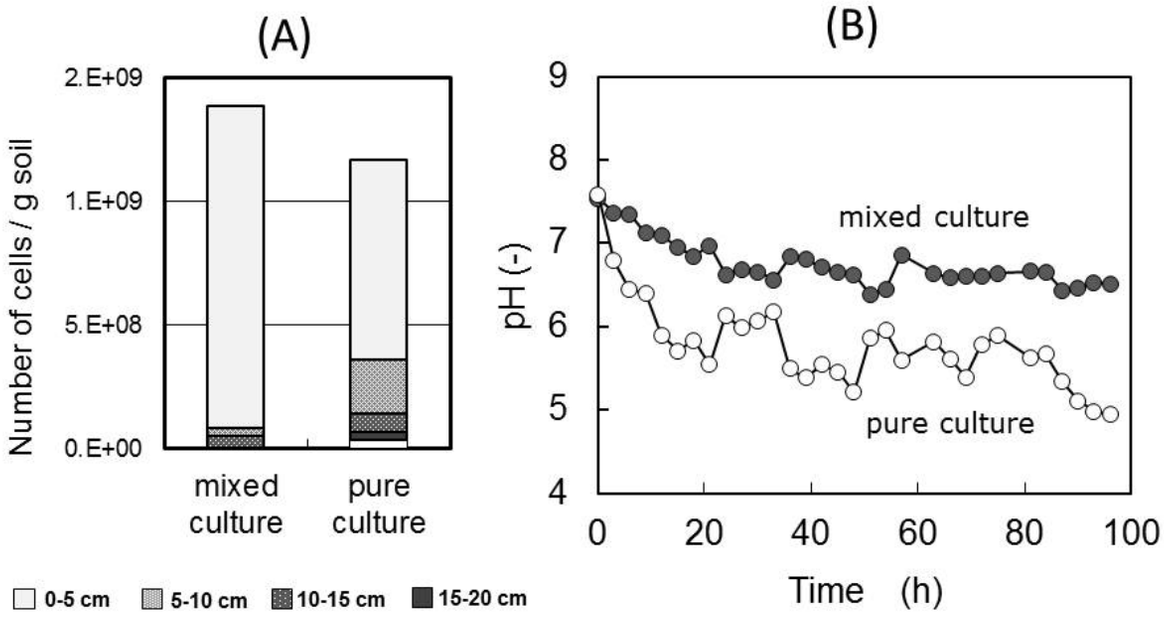

Figure 8. Neutralization of sulfuric acid with *A. pllulans* NPH6 in a mixed culture (BSIS) and a pure culture (control). (A), The distribution of the *A. pllulans* NPH6 cells along the column axis after the cells were respectively immobilized by mixed culture (BSIS) and pure culture (Control). (B), the pH value was measured 7.5 cm from the top, when sulfuric acid solution (pH 4.7) was supplied at a flow rate of 24.9 ml/h for 96 h.

After immobilization on the packed soil, the R medium containing 1 mM cyanuric acid was supplied to the column at a flow rate of 24.9 ml/h. Figure 10A shows the viable cell number in each layer of soil in the column after supplying the R medium containing 1 mM cyanuric acid. In both cases, 60-80% of the cells were immobilized in a shallow layer (0-7.5 cm) of packed soil, and the cell numbers in the mixed culture were much higher than those in the pure culture. Figure 10B shows the time course for the cyanuric acid concentrations for liquid flowing 7.5 cm from the surface of the packed bed and in the effluent solution (35 cm), when the medium containing 1 mM cyanuric acid was supplied for 72 h to the mixed culture. The rate of degradation of cyanuric acid in the BSIS gradually increased after 24 h. This increase may have been caused by acclimation of the cells to cyanuric acid and an increase in cell activity during cultivation in the medium containing 1 mM cyanuric acid. The rate of degradation in the BSIS was higher than that in the control, and the cyanuric acid concentration was decreased to 0.1-0.2 mM at the column exit. The degradation rate could be maintained for a long period of time

(two weeks) by supplying suitable nutrients in another test. Therefore, we concluded that BSIS is an excellent method for the bioremediation of soil polluted by pesticides.

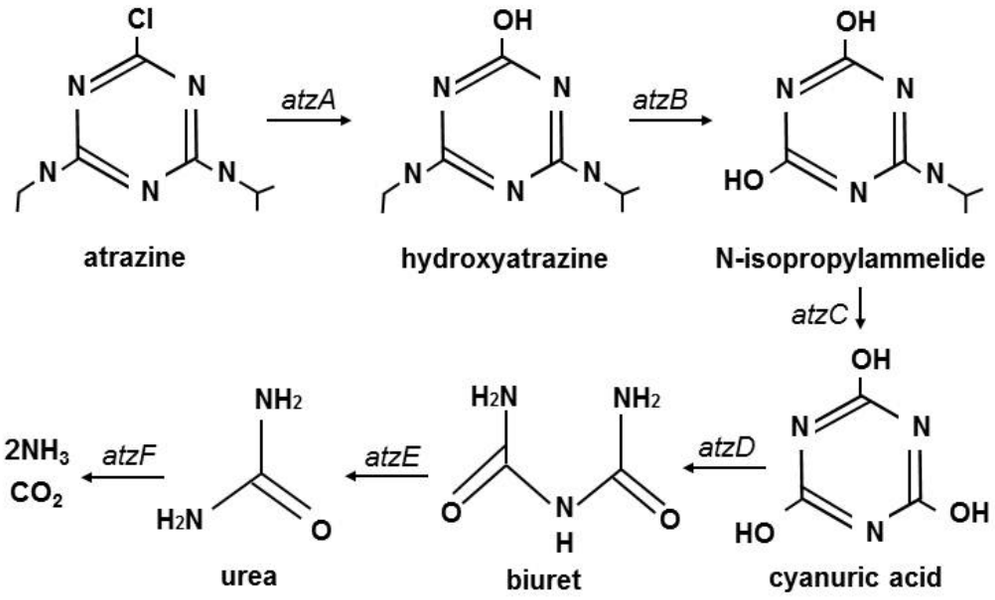

Figure 9. The biological degradation pathway of atrazine, and the genes corresponding to the enzymes.

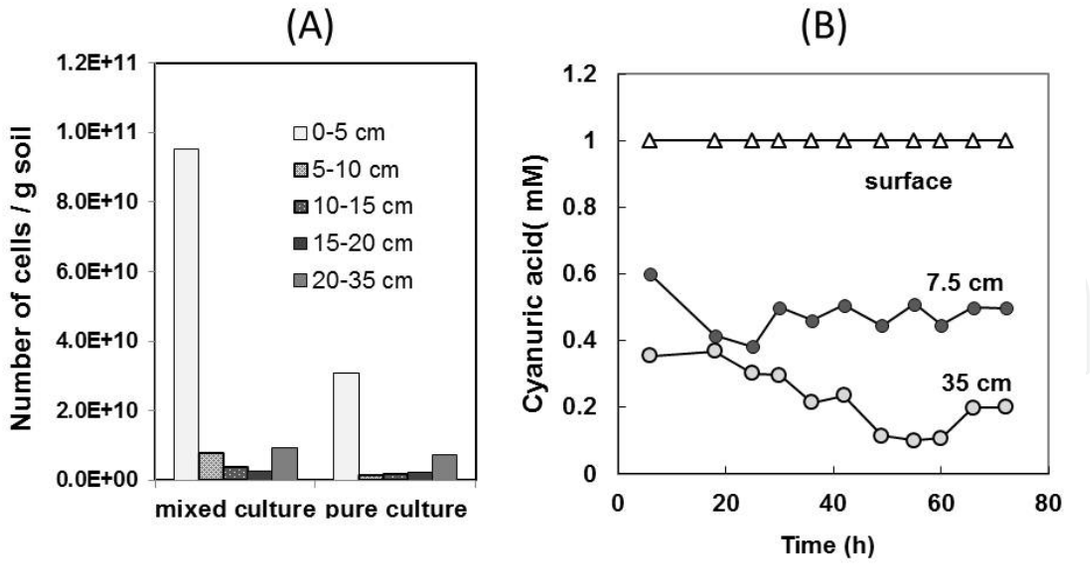

Figure 10. Bioremediation of soil polluted with cyanuric acid by BSIS. (A), The distribution of the *Pseudomonas sp*. cells along the column axis after the cells were respectively immobilized by a mixed culture (BSIS) and mono culture (Control). (B), the cyanuric acid concentration was measured 0, 7.5 and 35 cm from the top, when 1 mM cyanuric acid was supplied at a flow rate of 25 ml/h for 72 h.

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3.6. Enhancement the performance of degradation and stability using the optimal microorganisms in the BSIS

Despite these successful experiments, there were some points that need to be improved in further studies. Enhancement of the performance in the BSIS is necessary for its practical use, and the following two improvements in the degrading microorganisms are important.

One is the activity for degrading pesticides. A very high degradation rate is necessary to complete the degradation in the shallow layer. A higher degradation rate can be obtained by a higher density of degrading cells, but there is an upper limit for the rate. Additionally, the extremely high-density operation can cause a shortage of nutrients and oxygen, and it decreases the levels of other important natural soil microorganisms. Therefore, during the operation of BSIS, the increase of degrading activity per cell is more effective and important than providing a higher density operation.

Gene manipulation is one of the most effective methods to enhance the activity of the microorganisms. Many microorganisms showing the ability to degrade triazine and organophosphorous herbicides have been isolated [35-39], and the metabolic pathways and their genes are known. For example, Figure 9 shows the genes encoding the enzymes involved in the metabolism of atrazine. The atzA-atzD genes shown in Figure 9 were cloned from bacteria, and their activity has been examined [36]. The performance of BSIS will be increase if recombinant bacteria showing a higher degrading capacity can be used.

Other interesting studies were also reported on other aspects that increased the activity. The rate limiting step of degradation is the permeability of the cell membrane. To decrease the time required for the agent to cross the membrane, recombinant strains that can express the organophosphorous hydrolase on the surface of the cells [40,41] and secrete it into the periplasmic space [42] were constructed. As another method, a bacterium carrying a high functionality catabolic plasmid was also constructed, which showed the ability to perform conjugal transfers [43]. When this bacterium is present in the soil, the degradation ability can be increased by conjugal transfer. A recombinant strain carrying a special sensor to search for pollution has also been sought [44]. These results suggest that the performance of BSIS will be able to approach to an ideal value by using these recombinant strains if they are successfully constructed and their use is permitted.

The other point that could use improvement is the synergy between microorganisms. Many microorganisms play important roles in maintaining the healthy condition of the soil. For example, a nitrogen-fixing bacterium provides nitrogen sources to plants, and photo-chemical bacteria help degrade chemical compounds. Degradation of pollutants by multiple strains is much more effective than that by a single strain. Some studies have reported on the optimal combinations that can be used to enhance the synergy between strains [45-49]. As many different strains can be simultaneously immobilized in the BSIS, the optimal combination will be help to increase the performance and stability of the operation. Recently, microorganisms producing electricity have been reported. Those strains are present even in the agricultural field, and can produce electricity in the field [50]. If the electrical power generated by these

bacteria can reach the level of a solar battery, a permanent operation in the BSIS using the battery may be possible, further minimizing the cost of operation.

4. Conclusion

The pollution of soil and groundwater by the use of pesticides has become a serious condition all over the world. The soil pollution is not only present in huge areas, but also is repeated continuously, leading to permanent contamination, because the pesticide is used many times each year. Therefore, the conventional methods require high budgets, making their set-up and maintenance are impractical. The author proposed the use of a novel bioremediation method, namely BSIS. Using BSIS, degrading microorganisms can be self-immobilized at high density in the shallow layer with the help of a PGA polymer secreted by the *Bacillus* strain, and can rapidly degrade a pollutant *in situ*. The author applied the BSIS for acid rain and triazine herbicides by using model soil packed in a column. BSIS was effective to remediate soil acidified by the acid rain and polluted by the pesticide. Moreover, this BSIS has advantages in that it can be applied without any expensive apparatus and can operate easily and permanently. These results suggest that BSIS may be the best method for the bioremediation of soil pollution by pesticides, although further improvements in the microorganisms might be necessary for the practical application of the technique.

Everyone can freely study and use our BSIS method without any restrictions by a patent. Therefore, the author expects that many researchers will recognize the advantages of BSIS, and by further improvements, it can applied for the pollution by pesticides, which is a serious worldwide problem.

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