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Effect of Glutaraldehyde Treatment on Stability of Permeabilized *Ochrobactrum anthropi* SY509 in Nitrate Removal

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For practical application, the stability of permeabilized *Ochrobactrum anthropi* SY509 needs to be increased, as its half-life of enzymatic denitrification is only 90 days. As the cells become viable after permeabilization treatment, this can cause decreased activity in a long-term operation and induce breakage of the immobilization matrix. However, the organic solvent concentration causing zero cell viability was 50%, which is too high for industrial application. Thus, whole-cell immobilization using glutaraldehyde was performed, and 0.1% (v/v) glutaraldehyde was determined as the optimum concentration to maintain activity and increase the half-life. It was also found that 0.1% (v/v) glutaraldehyde reacted with 41.9% of the total amine residues on the surface of the cells during the treatment. As a result, the half-life of the permeabilized cells was increased from 90 to 210 days by glutaraldehyde treatment after permeabilization, and no cell viability was detected.

Keywords: Denitrification, permeabilization, glutaraldehyde, stability, immobilization

Biological denitrification is a process in which nitrogen (N_2) is formed from nitrate or nitrite *via* nitric oxide (NO) and nitrous oxide (N_2O) as intermediates [11]. Each step of this process is catalyzed by an enzyme system composed of nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase in the periplasmic and/or inner membrane [12, 15]. However, dissimilatory nitrate reduction requires an electron donor, such as NAD(P)H, making carbon sources necessary for its regeneration.

Thus, a novel bioelectro-denitrification process has been suggested using electricity instead of a carbon source as the electron donor [5, 10]. The denitrification reaction was attained using permeabilized *Ochrobactrum anthropi* SY509, which acts as a biocatalyst as it contains the denitrifying enzymes. Electrons were transferred to the enzymes from an electrode *via* mediators. As a result, the permeabilization treatment was able to increase the accessibility of the nitrate and mediators to the enzymes, thereby improving the nitrate removal efficiency [4].

Glutaraldehyde has a bifunctional group, which allows it to be used as a crosslinking reagent and cell-killing agent [13]. A whole-cell biocatalyst, which was mutually bound by several crosslinking agents, has been suggested as a useful tool for enzymatic synthesis [1], as the crosslinking could prevent the biocatalyst from leaching proteins and other cellular components, and provides good mechanical strength to the cells [1]. Immobilized whole cells also showed enhanced enzyme activity due to the permeabilization and enhanced stability resulting from the crosslinking reaction [19]. However, even though glutaraldehyde has been frequently used in the immobilization of enzymes, the main disadvantage of this method is the decreased enzyme activity caused by the crosslinking reaction [14, 20].

Therefore, to apply the bioelectro-denitrification process on an industrial scale, the long-term stability of permeabilized *O. anthropi* SY509 needs to be enhanced. The viability of the cells also needs to be eliminated to prevent breakage of the immobilized matrix, which can inhibit the electron transfer efficiency by increasing the gap between the cells and the electrodes. Moreover, since the newly grown cells do not induce any enzymes under optimum conditions, the enzyme activities of the newly grown cells are also lower compared with those of the initially existing cells. Accordingly, this study combines permeabilization and crosslinking as a new method to increase the stability of whole-cell biocatalysts for denitrification, while retaining their activity and reducing their viability.

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Materials and Methods

Microorganism

A microorganism with high denitrification efficiency was isolated from activated sludge taken from reclaimed land at Kimpo, Korea. The microorganism, *Ochrobactrum anthropi* SY509, showed a higher rate of denitrification than other microorganisms [16].

Permeabilization Procedure

O. anthropi SY509 was used as the model microorganism. The cells were grown and harvested as previously described [16]. The washed cell pellet was resuspended in a potassium phosphate buffer (80 mM, pH 7.0), and an organic solvent was added to the cell suspension (100 g DCW/l). Various concentrations of toluene and chloroform were used for the permeabilization. After incubating the cells in the reaction mixture at 4°C for 15 min, the permeabilized cells were separated by centrifugation at 12,000 rpm for 20 min, washed twice with a potassium phosphate buffer, and then used in the experiments.

Enzyme Activity and Cell Viability Assay

The nitrate and nitrite reductase activities were measured using Chauret and Knowles' method [3]. The assay mixture consisted of the cells (0.29 g dry weight cell/l), potassium phosphate buffer (80 mM, pH 7.0), 1 mM of benzyl viologen as the electron donor, and 10 mM of sodium dithionite. The benzyl viologen was reduced by the sodium dithionate, thereby providing electrons to the denitrifying enzymes. The mixture was stirred and the reaction initiated by the addition of 10 mM of potassium nitrate. After 10 min, the nitrate reduction was stopped by vortexing. The sodium dithionate and benzyl viologen were oxidized to prevent any further donation of electrons. The cells were removed by centrifugation and the nitrate concentrations measured using ion-chromatography (Waters 432) equipped with an IonPak Anion HR column. The mobile phase was composed of a sodium borate/gluconate solution, *n*-butanol, acetonitrile, and distilled water. The cell viability was measured based on the colony forming units (CFUs).

Whole-Cell Immobilization Using Glutaraldehyde

The permeabilized cells were harvested and then reacted with 0.1% (v/v) glutaraldehyde for 12 h. Thereafter, the cells were separated by centrifugation at 12,000 rpm for 20 min and washed twice with an 80 mM potassium phosphate buffer.

Amine Density on Surface of Cells

The *O. anthropi* SY509 was immersed in anhydrous ethanol, containing 4-nitrobenzaldehyde, under an anaerobic atmosphere at 50°C for 12 h [9]. The cells were then separated by centrifugation at 6,000 rpm for 20 min and washed with anhydrous ethanol. Thereafter, the cells were immersed in water, and the aqueous solution was heated at 30°C for 1 h, during which time the 4-nitrobenzaldehyde combined with the amine residue was detached. After centrifugation at 6,000 rpm for 20 min, the absorbance of the supernatant was measured at 267 nm, which represented the amount of amine residues on the surface.

Results and Discussion

Several organic solvents have already been tested as permeabilizing reagents for *O. anthropi* SY509 in previous research, among which chloroform was selected as the most effective [4]. In the cited study, no glucose uptake was observed in cells treated with 1.5% (v/v) or higher concentrations of chloroform. The denitrification activity of the permeabilized cells increased after treatment with 0.5% (v/v) and higher concentrations of chloroform, thus 1.5% (v/v) chloroform was selected as the optimum concentration owing to the high activity of the cells and their low glucose uptake. However, in previous permeabilization experiments, the solubility of the solvent has not been seriously considered [2, 6, 8]. The solubility of organic solvents in water is normally under 1% (w/v). Specifically, the solubility of chloroform in water is 0.5% (w/v), whereas that of toluene is 0.067% (w/v). The phase separation of two solvents can change the degree of cell permeabilization on a batch-to-batch basis. Since ethanol can dissolve both chloroform and toluene, it was added to the reactant to form a 5% (v/v) solution, which was the

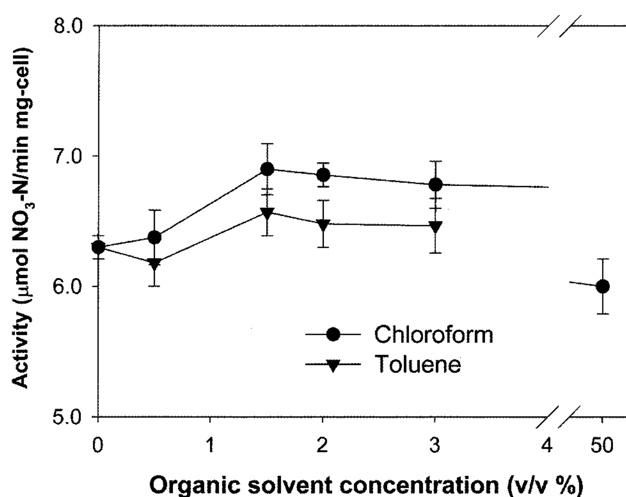

Fig. 1. Effect of chloroform and toluene concentrations on initial activity of *Ochrobactrum anthropi* SY509.

Ethanol [5% (v/v)] was used together with the solvents during the permeabilization. The experiments were performed in triplicate. Error bars (±1 standard deviation) are shown when the size exceeds that of the symbol used. NO_3-N denotes "nitrate-nitrogen", where the value is only the weight of N in NO_3.

Table 1. Effect of chloroform and toluene concentrations on the half-life of *Ochrobactrum anthropi* SY509.

	Half-life (days)	
	Chloroform	Toluene
0.0%	88	88
0.5%	80	80
1.5%	101	104
2.0%	96	116
3.0%	92	110
50%	43	

Temp=30°C pH=7. Ethanol [5% (v/v)] was used together with the solvents during the permeabilization.

minimum concentration making the permeabilization reactant homogeneous.

The effect of the solvent concentration on the cell activity is illustrated in Fig. 1. With the addition of ethanol to the reactant, the optimal concentration shifted from 0.5% to 1.5% for chloroform. The permeabilized cells treated with toluene had a lower initial activity than the permeabilized cells treated with chloroform at the same concentration.

The stability (i.e. the half-life of the cell activity) was also tested (Table 1). Whereas the half-life of the permeabilized cells was similar to the optimum concentration, the 1.5% (v/v) treatment exhibited the longest half-life of 101 days for the cells treated with chloroform. The effect of the toluene concentration on the stability of the permeabilized cells also showed a similar result. However, since the maximum value for toluene was found to be 2.0% (v/v), with a half-life of 116 days, toluene was more effective than chloroform as regards stability.

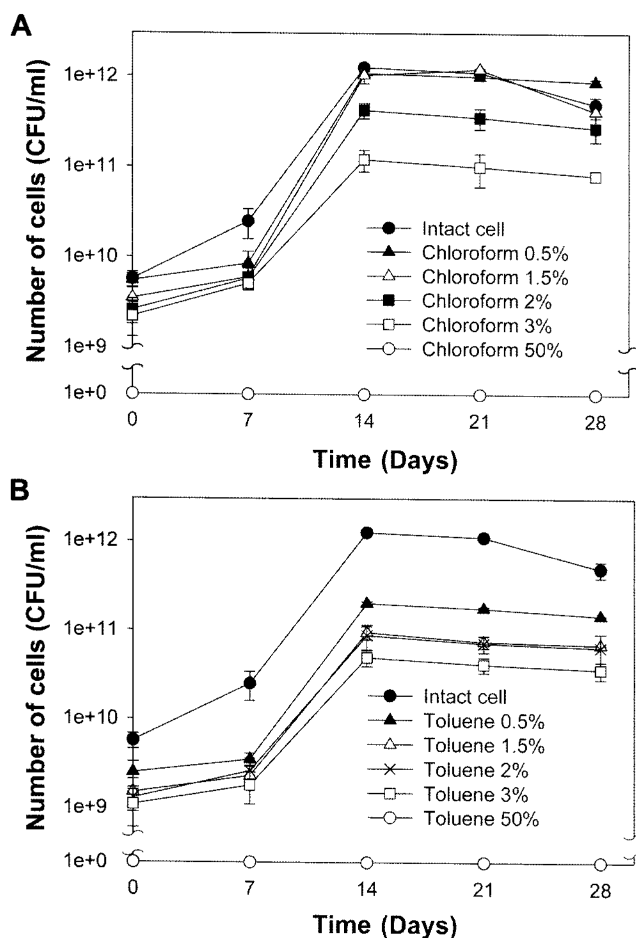

Fig. 2. Effect of chloroform and toluene concentrations on the colony forming units of *Ochrobactrum anthropi* SY509: (**A**) chloroform and (**B**) toluene.

Ethanol [5% (v/v)] was used together with the solvents during the permeabilization. The experiments were performed in triplicate. Error bars (±1 standard deviation) are shown when the size exceeds that of the symbol used.

As the cells appeared to be alive based on microscopic observation, a CFU test was carried out. As shown in Fig. 2, all the tested cells grew on the plates, whether treated by chloroform or not. Since the newly grown cells were unable to induce enzymes under the optimum conditions, their activities were lower than those of the initially existing cells. In addition, the activity of the intact cells was lower than that of the permeabilized cells, which is another problem related to the viability of the cells. Moreover, cell growth can break the immobilizing matrix in a long-term operation, thereby inhibiting the electron transfer efficiency by increasing the gap between the electrodes and the biocatalysts.

The cells grew for 14 days, which was indicated by the increased CFUs. Thereafter, the CFUs decreased, and were reduced as the concentration of chloroform increased, with the permeabilized cells treated with 50% chloroform having zero viability. Twenty and 30% concentrations were also tested, and still revealed viability. Whereas the CFU values were significantly reduced by treatment with high concentrations, the cells continued to grow until 50%. The effect of the toluene concentration on the viability was also similar to that of chloroform. Therefore, according to the results, a high concentration of an organic solvent was needed to lower the viability. However, the reduced activity along with a high concentration of an organic solvent can be problematic under real operating conditions. Thus, a different method that can satisfy the economic and efficiency needs is required.

Although chemical crosslinking has already been utilized to enhance protein stabilization [1, 13], whole-cell biocatalysts or cellular adsorbents require cell fixation to prevent disintegration, stabilization of protein leakage, denaturation or proteolysis, and contamination of the medium and potential product with the cells. Such treatments have also resulted in reduced rates of thermal inactivation [19].

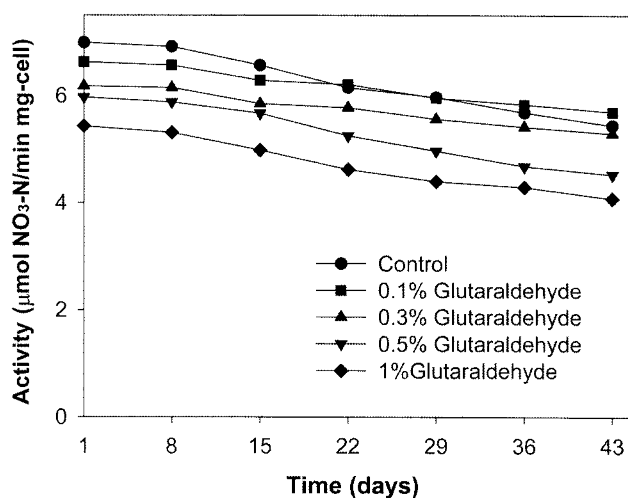

Fig. 3. Effect of glutaraldehyde concentration for crosslinking on the activity and stability of permeabilized *Ochrobactrum anthropi* SY509.

Table 2. Effect of glutaraldehyde on the half-life of *Ochrobactrum anthropi* SY509.

Glutaraldehyde concentration (%)	Half-life (days)
0.0	87
0.1	165
0.3	160
0.5	87
1.0	70

The cells were permeabilized using 1.5% (v/v) chloroform. Ethanol [5% (v/v)] was used together with the solvent during the permeabilization.

O. anthropi SY509 is a Gram-negative bacterium, and its surface contains many types of protein; globular proteins, glycoproteins, protein channels (transport proteins), peripheral proteins, and integral proteins (alpha-helix proteins). Thus, proteins with NH_3^+ residues can react with glutaraldehyde to form inter/intra crosslinking between cells.

The effects of the glutaraldehyde concentration on the activity and stability are shown in Fig. 3 and Table 2. The glutaraldehyde concentration was tested from 0.1% to 1.0%. As expected, the activity decreased as the concentration increased. Interestingly, the half-lives were not proportional to the glutaraldehyde concentration. The cells crosslinked with 0.1% (v/v) glutaraldehyde showed the longest half-life (Table 2). Although interconnected or interbound cells with a high concentration (1.5%) of glutaraldehyde demonstrate an enhanced stability [20], the stability of the current enzyme showed an optimum value at 0.1% (w/v), demonstrating that 0.1% (v/v) glutaraldehyde, in the case of *O. anthropi* SY509 cells, was the most efficient concentration among those tested. Since the location of nitrate reductase is in the periplasmic membrane, it can react directly with the crosslinking agents. Therefore, a high agent concentration can easily have a negative affect on the enzymes. Moreover, the flexibility of the membrane can be reduced by the reaction, which can also negatively affect the cells and enzymes.

To evaluate the degree of crosslinking, the density of the surface amines on the cells was determined. As a chemical tag, 4-nitrobenzaldehyde was utilized to hybridize the surface amine groups, which were detached by water, and their

Table 3. Density of amine groups on the surface of cells.

Types of cell	Density of amine groups (mol/g-cell)
Intact cell	0.005±0.0007
Permeabilized cell (1.5% toluene)	0.0056±0.00019
Crosslinked intact cell	0.0031±0.00033
Crosslinked permeabilized cell (1.5% toluene)	0.0033±0.00016

Ethanol [5% (v/v)] was used together with the solvents during the permeabilization.

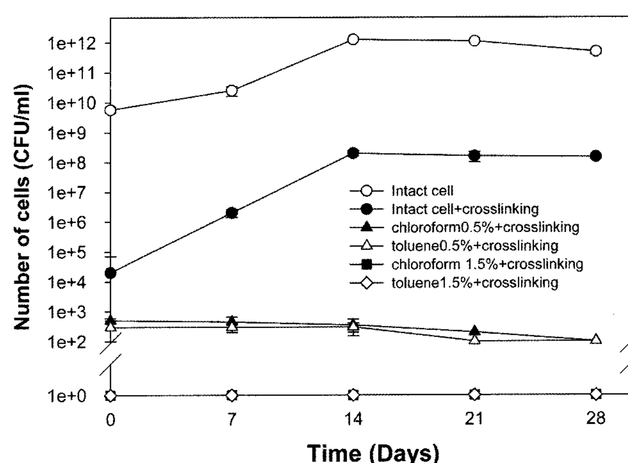

Fig. 4. Effect of crosslinking on colony forming units.
Ethanol [5% (v/v)] was used together with the solvents during the permeabilization. The experiments were performed in triplicate. Error bars (±1 standard deviation) are shown when the size exceeds that of the symbol used.

concentrations were determined by UV spectroscopy (Table 3). The intact and permeabilized cells showed similar values, whereas the crosslinked intact and crosslinked permeabilized cells exhibited fewer amine residues than the uncrosslinked cells. For the intact cells, 38.4% of the amine residues reacted with glutaraldehyde, whereas 41.9% of the amine residues reacted with the permeabilized cells.

The effect of crosslinking on the viability is shown in Fig. 4. With a glutaraldehyde concentration of 0.1% and organic solvents concentrations for the permeabilization of 0.5% and 1.5%, the crosslinked intact cells revealed a 1/10,000 viability compared with the intact cells, with the other cells showing even lower values. Thus, the combined treatment of 1.5% permeabilization and 0.5% glutaraldehyde eliminated the viability of the cells, where glutaraldehyde was effective in reducing the viability when used together with a solvent.

When the permeabilized cells were treated with glutaraldehyde, their outer membranes also gained structural strength. However, this treatment may have caused the decreased initial activity, as shown in Fig. 5. The permeabilized cells were treated with 1.5% chloroform and toluene, respectively. When compared with the untreated cells, the crosslinked cells showed a slightly lower initial activity, yet the degree of change was negligible, demonstrating that the crosslinking treatment did not seriously decrease the activity, which has been considered the main disadvantage of the crosslinking reaction.

The stability of the crosslinked cells is shown in Table 4. The cells were stored at 30°C in a pH 7 phosphate buffer. The stability of the permeabilized cells with 1.5% (v/v) toluene produced the longest half-life of 210 days. The cells crosslinked under other treatment conditions also had longer half-lives when compared with the untreated cells. Crosslinking imparted

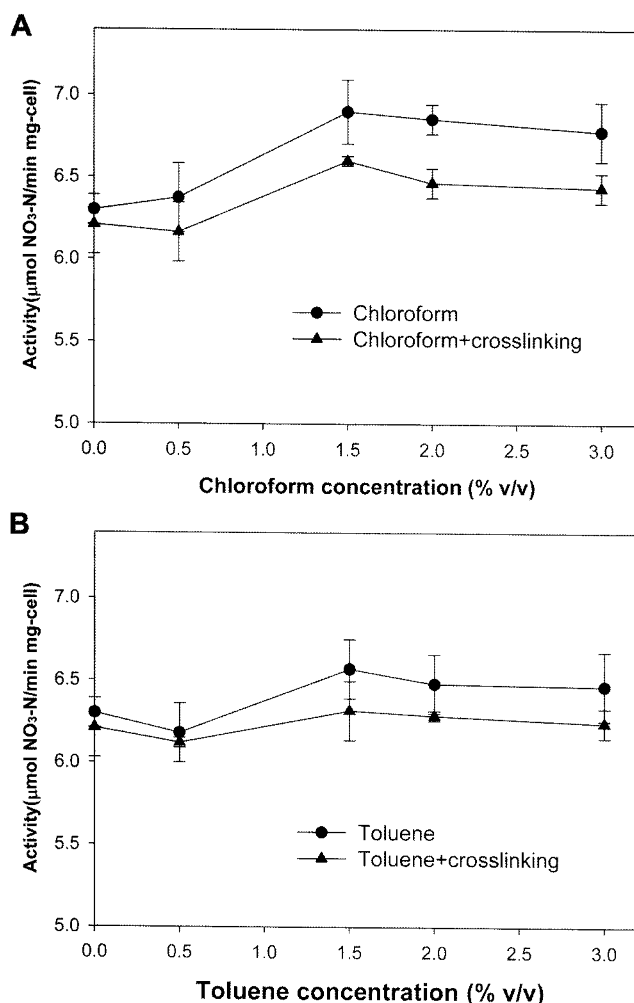

Fig. 5. Effect of crosslinking on initial activity: (**A**) permeabilized with 1.5% (v/v) chloroform and (**B**) permeabilized with 1.5% (v/v) toluene.

Ethanol [5% (v/v)] was used together with the solvents during the permeabilization. The experiments were performed in triplicate. Error bars (±1 standard deviation) are shown when the size exceeds that of the symbol used.

Table 4. Half-life of crosslinked permeabilized cells and untreated permeabilized cells.

Permeabilization conditions	Half-life (days)	
	Crosslinking	Untreated
Intact cell	97	88
Chloroform 0.5%	169	80
Toluene 0.5%	159	80
Chloroform 1.5%	181	101
Toluene 1.5%	210	104
Chloroform 2.0%	159	96
Toluene 2.0%	163	116
Chloroform 3.0%	143	92
Toluene 3.0%	153	110

Ethanol [5% (v/v)] was used together with the solvents during the permeabilization.

the cells with half-lives that were twice as long, and this effect was enhanced with toluene, yet both solvents gave good results at concentrations of 1.5%.

As the induction of enzymes related to nitrate removal can be affected by the nitrate concentration during storage, the nitrate medium was changed once every other day. However, the same stability profile was obtained (data not shown), which confirmed the results.

In conclusion, the stability of *O. anthropi* SY509 was improved when using permeabilization and crosslinking. To reduce the viability and increase the stability, a crosslinking reaction was introduced using glutaraldehyde. When the intact cells were treated with 0.1% (v/v) glutaraldehyde, they became less viable, and nonviable when permeabilized with more than 1.5% of the permeabilizing agent. Moreover, 41.9% of the amine residues on the surface of the cells reacted with glutaraldehyde during the crosslinking reaction, and the half-life increased from 90 to 210 days. In summary, treatment of the cells with glutaraldehyde not only decreased the viability, but also increased the stability.

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