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Effects of cell entrapment on nucleic acid content and microbial diversity of mixed cultures in biological wastewater treatment

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ABSTRACT

The effects of entrapment on nucleic acid content and microbial diversity of mixed cultures in biological municipal wastewater treatment were investigated. Deoxyribonucleic acid content increased 1.6–5.5 times more in alginate entrapped cells than in free and polyvinyl alcohol (PVA) entrapped cells. PVA entrapment resulted in 1.1- to 5.9-fold more increases in ribonucleic acid content compared to that experienced by free and alginate entrapped cells. Entrapment in carrageenan changed the bacterial community structure more than the alginate and PVA entrapments (35–80% versus 0–35%) as determined by single-strand conformation polymorphism analyses. The change in the bacterial community structure of alginate entrapped cells was less time dependent than that of PVA entrapped cells. This study enhances understandings on the physiology of entrapped cells and their community evolution in wastewater treatment environments.

1. Introduction

In several applications such as wastewater treatment, ethanol production, and α-amylase production, entrapped cell systems performed differently than free cell systems (Konsoula and Kyriakides, 2006; Pramanik and Khan, 2008; Roukas, 1996). Entrapped cells show various modifications in physiology compared to suspended cells such as higher or lower growth rates, metabolic activity and product yields (Doran and Bailey, 1986; Galazzo and Bailey, 1990; Hilge-Rotmann and Rehm, 1990, 1991; Vives et al., 1993; Jamai et al., 2001). When entrapped, the cells experience a change in environment which causes physiological changes (Jamai et al., 2001).

Modifications in the deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) contents of microorganisms have been reported as consequences of entrapment. A significantly higher DNA content but only about one-fourth RNA content in immobilized *Saccharomyces cerevisiae* in crosslinked gelatin compared to those in the free cells was reported (Doran and Bailey, 1986). The DNA content of the macronucleus of alginate entrapped ciliated protozoan *Tetrahymena thermophila* was twice that of the free cells whereas the micronucleus of free and entrapped *T. thermophila* exhibited no difference in DNA content (Kiy and Tiedtke, 1993). The RNA content of *Escherichia coli* immobilized in latex patches was stable after a 16 day incubation period in phosphate-buffered saline while that of the free cells decreased rapidly (Lyngberg et al., 1999).

In the field of wastewater treatment, entrapped cell systems have been widely developed over the last several years and become one of the alternatives for the treatment of municipal as well as industrial wastewater (Canizares et al., 1994; Cao et al., 2004; Chen and Lin, 2007; Chen et al., 1998; Degiorgi et al., 2002; Hsieh et al., 2008; Kuo and Shu, 2004; Muyima and Cloete, 1995; Pramanik and Khan, 2008, 2009; Song et al., 2005; Vilchez et al., 2001; Yang et al., 2003). However, only two studies have been carried out to understand how the entrapment changes the physiological parameters of cells and the possible reasons of such changes. Pramanik and Khan (2008, 2009) showed that the type of entrapment media and cell-to-matrix ratio affected growth rate and metabolic activity of entrapped cells and that these two factors were crucial for the use of entrapped cells for wastewater treatment.

The objective of this research was to investigate the effects of entrapment on the nucleic acid content and microbial diversity of mixed cultures in biological wastewater treatment to enhance the understanding of the physiological characteristics of entrapped cells in wastewater treatment environments. To the best of knowledge, this is the first research that examined the microbial diversity of entrapped mixed cultures used for municipal wastewater treatment. The type of polymeric matrix and the source of mixed

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cultures were the variables studied. Free and entrapped cells were studied in parallel in sequential aerobic batch reactors fed with real municipal wastewater. The effect of entrapment on nucleic acid content quantity was investigated using total DNA and RNA measurements which were performed spectrophotometrically. The microbial diversity or the change of bacterial community was examined using polymerase chain reaction (PCR) and single-strand conformation polymorphism (SSCP) analyses. Dissolved organic carbon (DOC) removal was monitored to examine the wastewater treatment performance of free and entrapped cells.

2. Methods

2.1. Materials

Sodium alginate, κ-carrageenan, polyvinyl alcohol (PVA), calcium chloride, potassium chloride, boric acid, sodium orthophosphate, sodium citrate and citric acid were purchased from VWR International Inc. (West Chester, PA, USA). The first three chemicals were the cell entrapment media while the rest of them were used in the cell immobilization and deimmobilization procedures. Primary treated wastewater was collected from the Moorhead Wastewater Treatment Facility, Moorhead, MN, USA. Mixed-liquor suspended solids (MLSS) were collected from the aeration tanks of the Moorhead Wastewater Treatment Facility, Fergus Falls Wastewater Treatment Facility in Fergus Falls, MN, USA and Grand Forks Wastewater Treatment Plant in Grand Forks, ND, USA to provide three sources of mixed cultures. The MLSS were centrifuged (2546g for 15 min) and the settled solids were used for entrapment. The MLSS were aerated until centrifuged or used to represent free cells.

2.2. Cell entrapment

Each mixed culture was entrapped within three different entrapment media at a cell-to-matrix ratio of 2.5%. The cell-to-matrix ratio (2.5%) was selected because it provided the highest growth rate and metabolic activity according to a previous study (Pramanik and Khan, 2008). The cell-to-matrix ratio of 2.5% (g of centrifuged MLSS/volume of media) was prepared by mixing 2.5 g of the centrifuged MLSS with 100 ml of entrapment media. The entrapment procedures to produce spherical beads (5 mm in diameter) containing cells were modified from the methods of Konsoula and Kyriakides (2006) for sodium alginate, Lau et al. (1998) and Sankalia et al. (2006) for κ-carrageenan, and Chen and Lin (1994) for PVA. The modifications were as follows. For sodium alginate, the beads were allowed to harden overnight. For κ-carrageenan, the concentration of potassium chloride solution used for hardening the beads was 0.85 M. For PVA, the beads were hardened in a saturated boric acid solution for 2 h followed by another 3 h of hardening in a 1.0 M sodium orthophosphate solution.

2.3. Experimental setup

Polypropylene bottles (500 ml volume) were used as reactors for both entrapped and free cells. Air was provided through a stone diffuser at the bottom of each reactor to maintain a dissolved oxygen (DO) level of 5.0–5.5 mg/l. Each entrapped cell reactor contained beads prepared from 33.3 ml of the mixture of MLSS and entrapment media. The mass of MLSS in the 33.3 ml mixture was 0.83 g. In the free cell reactors, the same MLSS mass as in the entrapped cell reactors was used. The primary treated wastewater from the Moorhead Wastewater Treatment Facility was used as a feed.

2.4. Experimental design and procedure

2.4.1. Reactor operation and experimental design

To determine the effects of entrapment on nucleic acid content quantity and microbial diversity, the experiment was conducted for 4 weeks in a sequencing batch reactor mode since it was reported that 2–3 weeks may be required for changes in nucleic acid content to occur (Doran and Bailey, 1986). For each mixed culture source, one free cell reactor and three entrapped cell reactors (three types of entrapment media) were operated in parallel. The feeding, decanting, and aeration times were 15 min, 105 min, and 22 h, respectively (24-h cycle). For each feeding, the reactors were filled with 250 ml of autoclaved (at 121 °C and 15 psi for 15 min) primary treated wastewater. Since primary treated wastewater used in all experiments was from the Moorhead Wastewater Treatment Facility while MLSS were from the Moorhead, Fergus Falls and Grand Forks Treatment Plants (three sources of mixed culture), the substrate (primary treated wastewater) and the culture were from different sources for the experiments using MLSS from the last two treatment plants. All 12 experiments [3 cultures × (3 entrapment matrices + 1 free cell)] were triplicated. For the mixed culture from the Grand Forks Treatment Plant, two additional free cell reactors with cell concentrations equivalent to cell-to-matrix ratios of 5% and 15% were operated in parallel in order to investigate the effect of high cell concentrations on DOC removal efficiency and nucleic acid content quantity.

2.4.2. DOC removal monitoring

Influent and effluent substrate DOC concentrations were measured every other day. The DOC removal efficiency was used to represent the performance of the systems. For both free and entrapped cell reactors, 10 ml of the fluid was collected and filtered through a GF/F Whatman glass fiber filter (0.7 μm pore size). The filtrate was analyzed for DOC.

2.4.3. Quantification of nucleic acid content

The nucleic acid content of free and entrapped cells was monitored once per week. For the free cell reactors, at each sampling time, 11.25 ml of fluid was collected for DNA/RNA extraction. The collected liquid samples were centrifuged at 14,000 *g* for 2 min and the settled pellets were used for DNA/RNA extraction. For the entrapped cell reactors, 15 beads were collected. The 11.25 ml fluid and 15 beads had the same initial cell mass. The initial cell mass in 15 beads was calculated by dividing the total cell mass (determined from known volume and concentration of MLSS) used in the entrapment procedure by the total number of beads produced to obtain the initial cell mass per bead and then multiplying it by 15. The initial cell mass in 15 beads was later divided by the known initial concentration of MLSS to determine the sampling fluid volume from the free cell reactor (11.25 ml) that would provide the same initial cell mass. It should be noted that it was not possible to accurately determine real-time entrapped cell mass. As a result, the equivalency in the initial cell mass was used as a basis for the samplings from the free and entrapped cell reactors.

After the collection, entrapped cells were released by immersing the beads in appropriate solvents. The alginate beads were dissolved in 10 ml of a 0.3 M sodium citrate solution, adjusted to pH 5.0 with 1 M citric acid with continuous shaking (Roukas, 1996). The κ-carrageenan beads were dissolved in 15 ml of a 1.0% sodium citrate solution with continuous shaking at 37 °C (Baudet et al., 1983). The PVA beads were cut and mixed thoroughly in 20 ml of 20 mM phosphate buffer (pH 6.8) using a vortex mixer (Fisher Scientific, Pittsburgh, PA, USA) at 3200 rpm for 5 min to release entrapped cells from the media. After releasing the entrapped cells, the liquid samples were centrifuged (14,000 *g* for 2 min) and the

settled pellets were used for extracting DNA/RNA. The genomic DNA extraction procedure followed the instruction from the DNA extraction kit (Wizard Genomic DNA Purification Kit, Promega, USA). The total RNA extraction procedure was according to the instruction from the RNA extraction kit (SV Total RNA Isolation System, Promega, USA). The ratio of spectrophotometric absorbance of the extracted DNA and RNA samples at the wavelengths of 260 nm to that of 280 nm (A_{260}/A_{280}) were 1.7–2.0 and 1.8–2.1, respectively.

2.5. Analytical methods

DOC was measured by using a total organic carbon analyzer (Phoenix 8000, Emerson Process Management: Tekmar-Dohrmann Division, Mason, OH, USA). The pH was measured by using a 250Aplus pH meter (Thermo Orion, Beverly, MA, USA). DO was measured by using a 850Aplus DO meter (Orion Research Inc., Beverly, MA, USA). The extracted DNA and RNA were quantified by using a nanodrop spectrophotometer (ND-1000, NanoDrop Technologies Inc., Wilmington, DE, USA).

2.6. Detection of microbial diversity using PCR-SSCP technique

2.6.1. DNA amplification

The DNA amplification procedure by PCR was modified from Lin et al. (2007). The V3 region of the 16S rDNA (nucleotide positions 334–514 of *E. coli*) was amplified with primers EUB1 (5′-CAG ACT CCT ACG GGA GGC AGC AG 3′) and UNV2 (5′-GTA TTA CCG CGG CTG CTG GCA C 3′). A 25-μl PCR reaction contained 1.5 mM of $MgCl_2$, 200 μM of dNTP, 5.0 μl of Taq Polymerase buffer 5× (Promega, CA, USA), 50 μM of each primer, 1.25 U of Taq Polymerase (Promega, CA, USA), and 2 μl of DNA template. Dnase-free water was used for making up the volume of samples. The 0.2 ml PCR tubes were then placed into a thermocycler (Applied Biosystems 2720, Applied Biosystems Inc., Foster City, CA, USA). The PCR conditions consisted of an initial denaturation at 94 °C for 5 min, 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. The presence of PCR products (approximately 200 base pair) was confirmed by 1.5% agarose gel electrophoresis.

2.6.2. SSCP gel electrophoresis

The SSCP was carried out in a horizontal electrophoresis setup (Origins, Elchrom Scientific, Switzerland). The SSCP procedure followed the instructions from the manufacturer. Three microliters of PCR products were mixed with 7 μl of a denaturing solution (1 ml of formamide, 10 μl of 1 M NaOH, and 20 μl of 0.02% (w/v) bromphenol blue). The mixture was heated at 95 °C for 5 min and immediately placed in ice until loading to the SSCP gel. The 10 μl denatured PCR products were loaded into a pre-cast polyacrylamide gel (GMATM, Elchrom Scientific, Switzerland). The gel was run at a constant voltage of 72 V for 10 h. The gel temperature was maintained at 9 °C by circulating TAE buffer. The gel was visualized by using a SYBR® Gold-stain method (Molecular probes, OR, USA).

2.6.3. SSCP gel data analysis

The relative positions of the DNA bands in the SSCP gels were analyzed using Bionumerics version 5 (Applied Maths, TX, USA). The pair-wise similarity among the samples was calculated using the Dice index and an unweighted pair-group method with arithmetic average.

2.7. Statistical analysis

The DOC removal efficiency and nucleic acid content quantity data of free and entrapped mixed cultures were analyzed by analysis of variance (ANOVA) and *t*-test at 5% significance level using the Microsoft Excel 2007. ANOVA was used to examine the relationships among free cells, alginate entrapped cells and PVA entrapped cells while *t*-test was used to analyze the relationship between any two of them.

3. Results and discussion

3.1. Effect of entrapment on DOC removal efficiency

The MLSS collected from the Moorhead, Fergus Falls and Grand Forks treatment plants are referred to as mixed cultures 1, 2 and 3, respectively. Fig. 1a–c shows the DOC removal efficiency of the free and entrapped cells for mixed cultures 1, 2, and 3, respectively. Note that the data were highly reproducible that the error bars were within the data symbols. The statistical analysis results of the data are shown in Table 1. The *p*-value indicates whether there was a significant difference in DOC removal efficiencies of free cells, alginate and PVA entrapped cells. The range of initial DOC was from 70 to 90 mg/l. There was no experimental data of carrageenan entrapped cells since the carrageenan beads of all three mixed cultures disintegrated within 4–6 days.

For all three mixed cultures, the DOC removal efficiency of free cells was 77–83% at the beginning and gradually reached 86–90% after 3 weeks. For alginate entrapped cells, the DOC removal efficiency increased up to 80–86% after 3 weeks from its initial value of 62–65%. The alginate matrix dissolved after 3 weeks. The DOC removal efficiency of PVA entrapped cells initially was 73% and finally reached 88% after 3 weeks. The free cells had slightly higher DOC removal efficiency at the beginning but became quite comparable to the alginate and PVA entrapped cells after 2 weeks for mixed culture 2 and to the PVA entrapped cells after 3 weeks for mixed cultures 1 and 3 (Table 1). The higher DOC removal by free cells at the beginning compared to entrapped cells was likely because of faster acclimation by free cells as found in previous studies (Pramanik and Khan (2008, 2009)). Although the mixed culture 1 was acclimated to the substrate, its physiology changed after entrapment.

Based on less or similar DOC removal by the entrapped cells compared to the free cells, it is unlikely that DOC adsorption on PVA and alginate (if there was any) was substantial. Even if DOC adsorbed on the matrices, it should be biodegraded sooner or later by the entrapped cells that were in close proximity. It should be noted that wastewater derived DOC adsorption on PVA and alginate has not been reported. When the beads dissolved/disintegrated, they contributed very high DOC, much higher than that of the adsorbed DOC because they are made up of organic compound(s). Consequently, the DOC data showed substantial fluctuations and was not meaningful, and the experiments with carrageenan beads were discontinued at an early stage.

The PVA beads were durable against abrasion and no recognizable destruction of the beads was observed during the experimental period of 4 weeks whereas the alginate and carrageenan beads disintegrated after 3–4 weeks and 4–6 days, respectively, as was also reported in previous studies (Bonilla and Rand, 1991; Chen et al., 2003; Hill and Khan, 2008; Konsoula and Kyriakides, 2006; Leenen et al., 1996; Siripattanakul et al., 2008). Higher initial cell concentrations (free cell concentrations equivalent to 5% and 15% cell-to-matrix ratios) did not improve the DOC removal efficiency (Fig. 1c and Table 1). As reported in a previous study (Pramanik and Khan, 2008) on mixed cultures utilizing wastewater as a

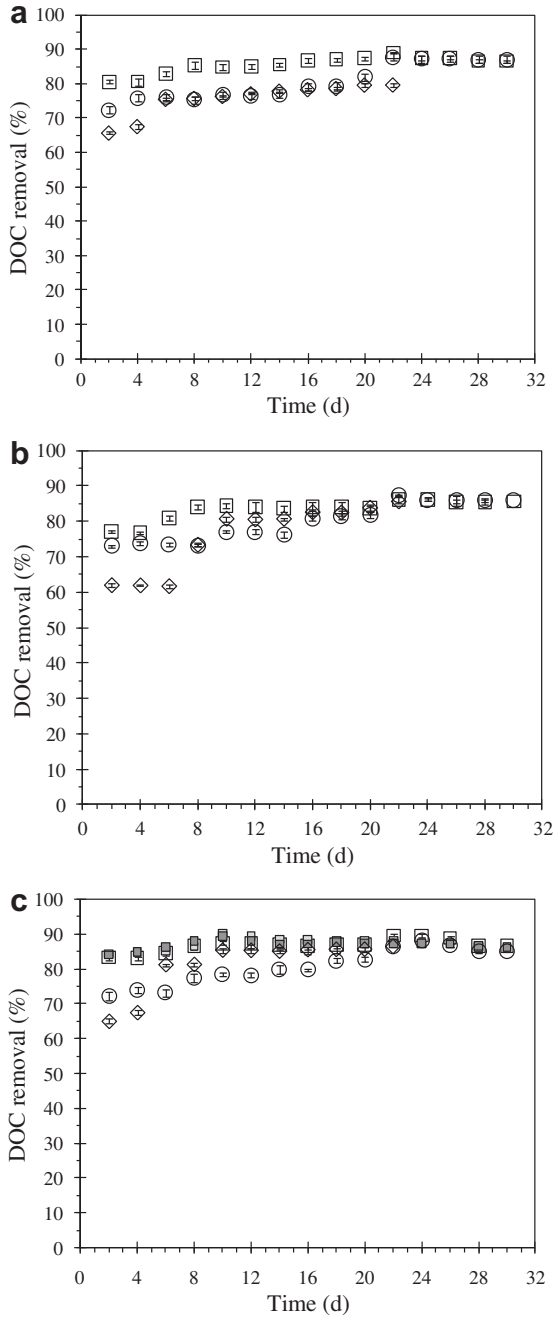

Fig. 1. DOC removal efficiency of (a) mixed culture 1, (b) mixed culture 2, and (c) mixed culture 3. □ Free cells (cell concentration equivalent to 2.5% cell-to-matrix ratio), ◇ alginate entrapped cells (2.5% cell-to-matrix ratio), ○ PVA entrapped cells (2.5% cell-to-matrix ratio), [□] free cells (cell concentration equivalent to 5% cell-to-matrix ratio), and [■] free cells (cell concentration equivalent to 15% cell-to-matrix ratio). Data shown is mean and error bar represents standard deviation based on the triplicate experiments.

substrate, higher cell-to-matrix ratios and equivalent free cell concentrations did not increase the specific growth and substrate utilization rates.

3.2. Effect of entrapment on nucleic acid content

Figs. 2 and 3 show the percent increase of DNA and RNA of the free and entrapped cells for mixed cultures 1, 2, and 3. The statistical analysis results of the data are presented in Table 2. The *p*-value indicates whether there was a significant difference

Table 1
Statistical analysis results of DOC removal efficiency data.

Mixed culture	Week	DOC removal efficiency	*p*-Value
1	1	Significant difference among F, A and P	0.0143
	2	Significant difference among F, A and P	<0.0001
	3	Significant difference among F, A and P	0.0025
	4	No difference between F and P	0.7742
2	1	Significant difference among F, A and P	<0.0001
	2	Significant difference among F, A and P	0.0025
	3	No difference among F, A and P	0.5225
	4	No difference between F and P	0.1436
3	1	Significant difference among F, A and P	0.0480
	2	Significant difference among F, A and P	<0.0001
	3	Significant difference among F, A and P	0.0176
	4	No difference between F and P	0.1903
	1–4	No difference among F, F (5%) and F (15%)	0.7243

F = free cells (cell concentration equivalent to 2.5% cell-to-matrix ratio), A = alginate entrapped cells (2.5% cell-to-matrix ratio), P = PVA entrapped cells (2.5% cell-to-matrix ratio), F (5%) = free cells (cell concentration equivalent to 5% cell-to-matrix ratio), and F (15%) = free cells (cell concentration equivalent to 15% cell-to-matrix ratio).

in percent increase of DNA or RNA of free cells, and alginate and PVA entrapped cells. The percent increase of DNA of the alginate entrapped cells was significantly higher than (1.6–5.5 times) that of free cells and PVA entrapped cells for all three mixed cultures. The free cells had similar percent increases of DNA to the PVA entrapped cells (Fig. 2 and Table 2). The percent increase of RNA was the highest for the PVA entrapped cells (1.1–5.9 times higher than the free and alginate entrapped cells) for all three mixed cultures although the statistical analysis showed that for mixed culture 2, the PVA entrapped cells had a similar percent increase of RNA as the free cells. The percent increase of RNA of the alginate entrapped cells was comparable to that of the free cells (Fig. 3 and Table 2). The percent increase of DNA decreased as initial free cell concentration increased but there was no statistical difference in the percent increase of RNA among different initial free cell concentrations (Figs. 2c and 3c & Table 2). Similar to DOC removal, the DNA and RNA contents tended to level off between weeks 3 and 4, which might indicate a steady state condition.

Compared to free cells, higher DNA contents in calcium alginate entrapped ciliated protozoan and gelatin attached yeast cells have been reported. Disturbed cell division but continuous synthesis of DNA components was suggested as a possible reason for higher DNA content of entrapped cells (Doran and Bailey, 1986; Kiy and Tiedtke, 1993). The higher RNA content in PVA entrapped cells might be responsible for its higher metabolic activity even though the cell concentration was much less compared to the free and alginate entrapped cells as discussed in a previous study (Pramanik and Khan, 2008). According to previous studies, entrapment is responsible for stabilizing the protein synthesis capacity of bacteria or decreasing the total RNA degradation rate as compared to free cells (Junter et al., 2002; Lyngberg et al., 1999). Entrapped cells have been shown previously to have different protein content and specific physiological behavior than free cells (Perrot et al., 2000; Vilain et al., 2004).

Entrapment affected the nucleic acid content of the cells depending on the entrapment media. The DNA content of entrapped cells increased when alginate was used as an entrapment media whereas the RNA content increased when PVA was used as an entrapment media. Alteration in the environment of cells by the media might be responsible for such diversified nucleic acid content of entrapped cells as compared to free cells (Lyngberg et al., 1999). At higher initial cell concentrations, cells might have less space to grow and more competition for substrate and in turn lesser increases in DNA and RNA were observed.

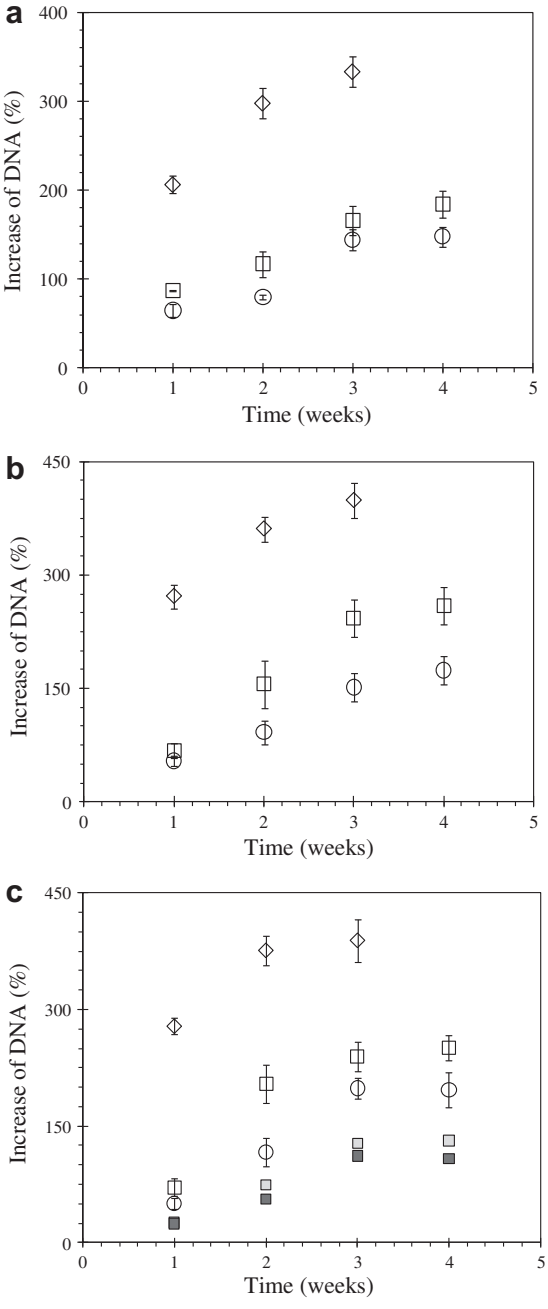

Fig. 2. Percent increase of DNA: (a) mixed culture 1, (b) mixed culture 2, and (c) mixed culture 3. □ Free cells (cell concentration equivalent to 2.5% cell-to-matrix ratio), ◇ alginate entrapped cells (2.5% cell-to-matrix ratio), ○ PVA entrapped cells (2.5% cell-to-matrix ratio), [□]free cells (cell concentration equivalent to 5% cell-to-matrix ratio), and [■] free cells (cell concentration equivalent to 15% cell-to-matrix ratio). Data shown is mean and error bar represents standard deviation based on the triplicate experiments.

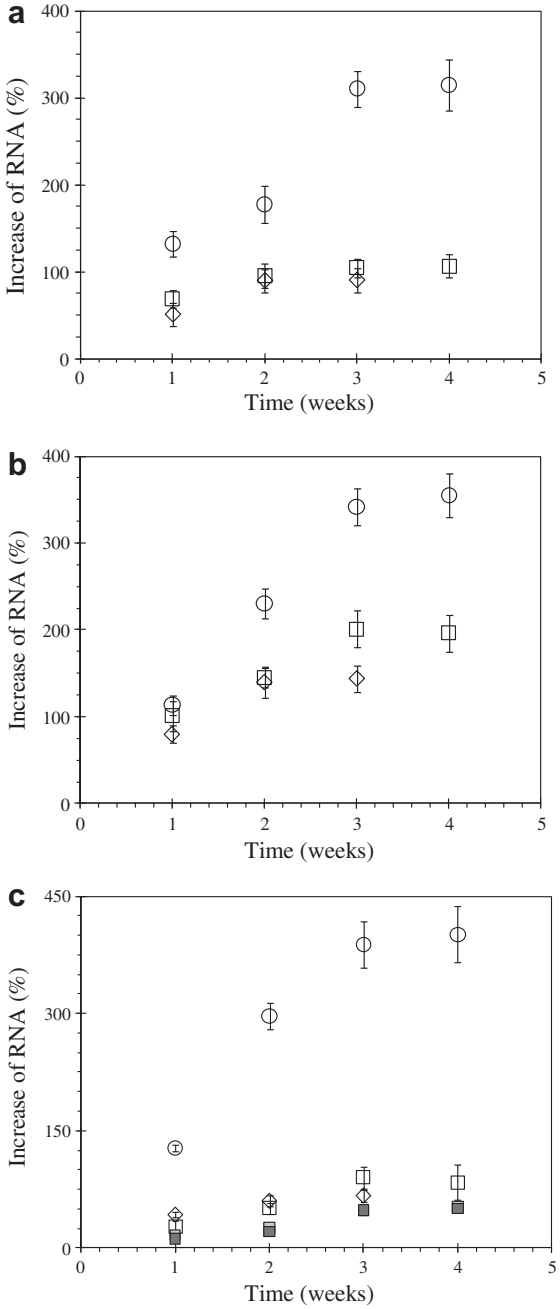

Fig. 3. Percent increase of RNA: (a) mixed culture 1, (b) mixed culture 2, and (c) mixed culture 3. □ Free cells (cell concentration equivalent to 2.5% cell-to-matrix ratio), ◇ alginate entrapped cells (2.5% cell-to-matrix ratio), ○ PVA entrapped cells (2.5% cell-to-matrix ratio), [□]free cells (cell concentration equivalent to 5% cell-to-matrix ratio), and [■] free cells (cell concentration equivalent to 15% cell-to-matrix ratio). Data shown is mean and error bar represents standard deviation based on the triplicate experiments.

3.3. Effect of entrapment on microbial diversity of mixed cultures

The SSCP profiles and the results of the cluster analysis of mixed cultures 1, 2, and 3 are presented in Figs. 4–6, respectively. Free cells, alginate entrapped cells, PVA entrapped cells and carrageenan entrapped cells are represented by notations F, A, P and C, respectively. The numbers 0, 1, 3, and 4 after the notations represent the sample collection times of 0, 1, 3, and 4 weeks. For mixed culture 1, both entrapment and time affected the bacterial community structure since the results were not clustered by either entrapment media or time (Fig. 4). In the free cell system, the initial bacterial community structure ($t = 0$) was only 50% similar to the community structure at week 1 or after, which changed very slightly ($\geqslant$90% similarity). The cluster analysis showed that the entrapment caused the change in the bacterial community structure of about 30% for alginate and 35% for carrageenan and PVA compared to free cells at $t = 0$. The community structure of the alginate entrapped cells changed 30% and 40% compared to free cells after 1 and 3 weeks, respectively. For the PVA entrapped cells, the community structure was 55%, 20%, and 10% different from that of the free cells at the 1st, 3rd, and 4th weeks, respectively.

Table 2
Statistical analysis results of nucleic acid content data.

Mixed culture	Percent increase of DNA	*p*-Value	Percent increase of RNA	*p*-Value
1	A > F	0.0243	F ≈ A	0.3298
	F ≈ P	0.3788	P > F	0.0298
	A > P	0.0148	P > A	0.0237
2	A > F	0.0190	F ≈ A	0.2621
	F ≈ P	0.2753	P ≈ F	0.0895
	A > P	0.0042	P > A	0.0407
3	A > F	0.0170	F ≈ A	0.7109
	F ≈ P	0.3855	P > F	0.0169
	A > P	0.0044	P > A	0.0150
	Significant difference among F, F (5%) and F (15%)	0.0478	No difference among F, F (5%) and F (15%)	0.1681

F = free cells (cell concentration equivalent to 2.5% cell-to-matrix ratio), A = alginate entrapped cells (2.5% cell-to-matrix ratio), P = PVA entrapped cells (2.5% cell-to-matrix ratio), F (5%) = free cells (cell concentration equivalent to 5% cell-to-matrix ratio), and F (15%) = free cells (cell concentration equivalent to 15% cell-to-matrix ratio).

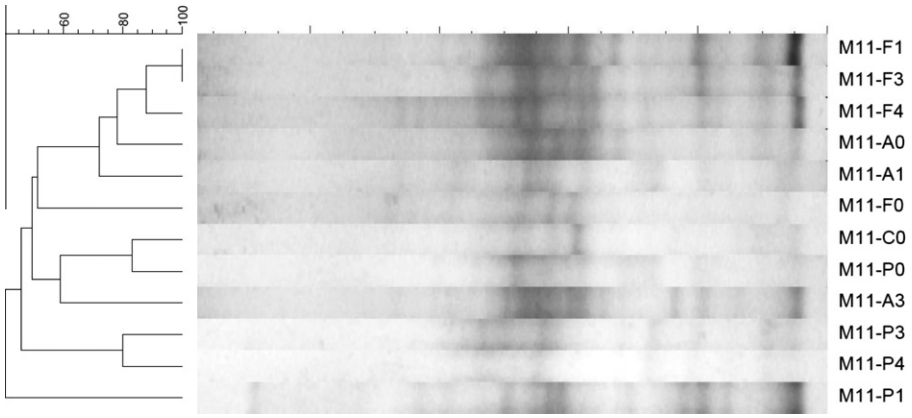

Fig. 4. SSCP profiles and cluster analysis of PCR-amplified 16S rDNA fragments of mixed culture 1. F, A, P and C represent free cells, alginate entrapped cells, PVA entrapped cells and carrageenan entrapped cells. The numbers 0, 1, 3, and 4 represent the sample collection times of 0, 1, 3, and 4 weeks.

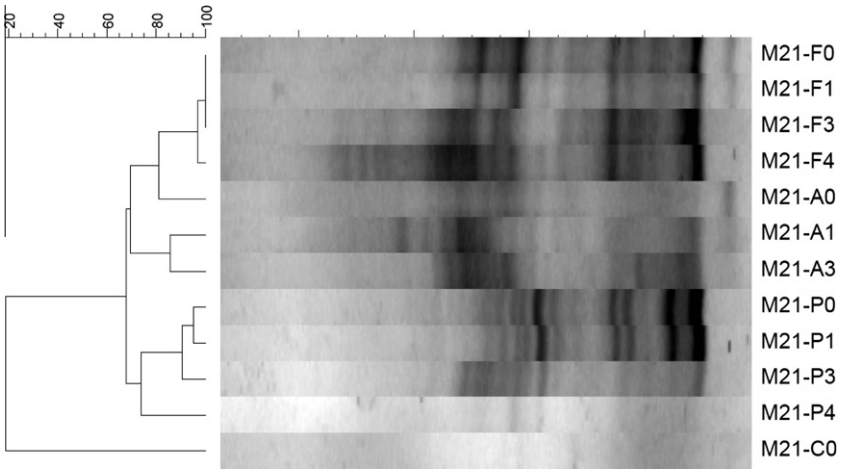

Fig. 5. SSCP profiles and cluster analysis of PCR-amplified 16S rDNA fragments of mixed culture 2. F, A, P and C represent free cells, alginate entrapped cells, PVA entrapped cells and carrageenan entrapped cells. The numbers 0, 1, 3, and 4 represent the sample collection times of 0, 1, 3, and 4 weeks.

The bacterial community structure of mixed culture 2 was obviously affected by the entrapment since the results were clustered by the entrapment media (Fig. 5). The community structure of the free cells did not change much throughout the experiment, a slight difference of less than 5% was observed among the free cell samples. Immediately after the entrapment (at $t = 0$), the community structure of alginate, carrageenan, and PVA entrapped cells changed 20%, 80%, and 5%, respectively, compared to the free cells. The community structure of the alginate entrapped cells was 15% different from that of the free cells at the 1st and 3rd weeks. The PVA entrapped cells exhibited 5%, 10%, and 22% difference in bacterial community structure compared to the free cells at the 1st, 3rd, and 4th weeks, respectively.

As shown in Fig. 6, for mixed culture 3, the free cells at $t = 0$ and 1 week were 8% different in the community structure while 5% difference in the community structure was observed between the free

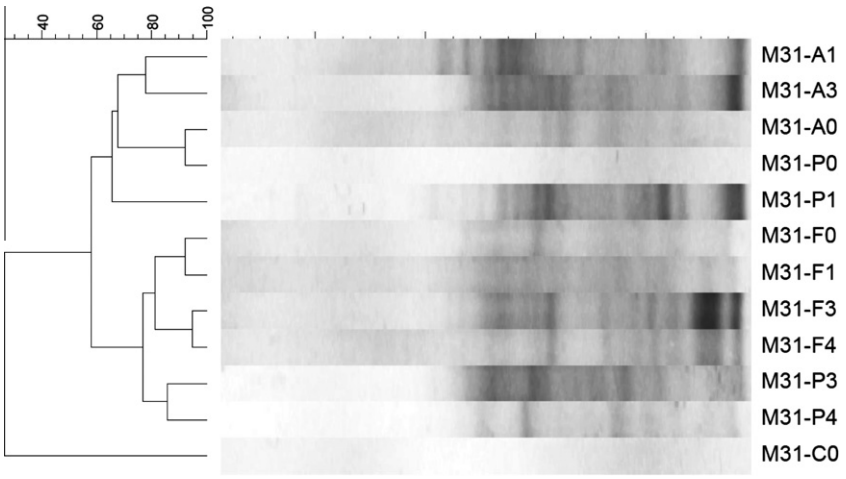

Fig. 6. SSCP profiles and cluster analysis of PCR-amplified 16S rDNA fragments of mixed culture 3. F, A, P and C represent free cells, alginate entrapped cells, PVA entrapped cells and carrageenan entrapped cells. The numbers 0, 1, 3, and 4 represent the sample collection times of 0, 1, 3, and 4 weeks.

cells after 3 and 4 weeks. The community structure difference between the beginning period (t = 0 and 1 week) and the 3rd and 4th weeks was about 15%. Immediately after the entrapment, no substantial difference in the bacterial community structure was observed among the free, alginate, and PVA entrapped cells whereas the carrageenan entrapped cells showed almost 67% change in bacterial community structure compared to the free cells. At the 1st and 3rd weeks, the community structure of the alginate entrapped cells was 86% and 83% similar to that of the free cells. For the PVA entrapped cells, the community structure deviated 27%, 10%, and 10% from the free cells at the 1st, 3rd, and 4th weeks, respectively.

The results suggest that the entrapment procedure affected the bacterial community structure depending on the types of entrapment media, sources of bacterial cultures, and time. Stresses generated by chemicals such as potassium chloride in the carragenan entrapment, boric acid and orthophosphate in the PVA entrapment, and calcium chloride in the alginate entrapment might be responsible for the initial changes observed between free cells and cells entrapped by different media. Physical factors such as high temperatures (40–50 °C) in the carragenan entrapment procedure and mixing in all three types of entrapment might contribute to the changes as well. For all three mixed cultures studied, the collected MLSS were aerated until used. During the aeration period, the cells experienced a different environment which might cause an initial change in the bacterial community structure. Although during the experiment the free cells of mixed culture 1 experienced the same substrate that it was acclimated to previously, a sudden and substantial change in the community structure of mixed culture 1 (50% change between t = 0 and the 1st week) was likely because of a re-adaption to the substrate by the cells after the aeration maintenance period prior to the entrapment. The free cells of mixed culture 1 adapted the substrate very quickly within the 1st week and no significant change in its community structure was observed after that. On the contrary, the substrate was new for the free cells of mixed cultures 2 and 3 and the cells adapted to it slowly along with gradual changes in their community structures. The carageenan entrapment immediately changed the bacterial community structure more than the alginate and PVA entrapments. High temperatures (40–50 °C) associated with the carrageenan entrapment procedure compared to the room temperature involved in the alginate and PVA entrapment procedures could be a possible reason for the more drastic and immediate change in the bacterial community entrapped in carrageenan. The changes in the bacterial community structure of the alginate entrapped cells were less time dependent for all three mixed cultures.

The bacterial community structure of the PVA entrapped cells changed in an inconsistent manner with time for all three mixed cultures as different levels of community structure similarity were observed between the PVA entrapped cells and free cells at 1st, 3rd, and 4th weeks. Moreover, the community structure similarity between the PVA entrapped cells and free cells increased with time for mixed cultures 1 and 3 but decreased with time for mixed culture 2. The substantial changes with time of the community structure of the PVA entrapped cells could be a result of continuous selection by substrate and environment. The substrate (DOC) removal efficiency of the PVA entrapped cells was stable or better with all these community changes. Less difference between the bacterial community structures of free and entrapped (alginate and PVA) cells was observed when the MLSS and substrate were from different sources (mixed cultures 2 and 3). This could be because the communities of the free and entrapped cells were adapting in a similar manner to acclimate to the new substrates. Entrapment changed the bacterial community structure but did not affect DOC removal likely because the mixed cultures were acclimated to municipal wastewater and the changes in their community structures were not substantial enough to affect overall DOC removal efficiency.

4. Conclusions

This study investigated the effects of entrapment and type of entrapment media on nucleic acid content quantity and community of mixed cultures in biological wastewater treatment. Entrapment affected the nucleic acid content in a media-dependent manner and changed the bacterial community structure depending on the media type, culture source, and time. Since the study dealt with mixed cultures, it was difficult to examine whether the entrapment affects cell morphology, cell surface properties and stress. These characteristics could provide more information related to the altered metabolic behavior of entrapped cells and therefore are recommended for future work.

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