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ACTIVITY AND STRUCTURE OF STORED AEROBIC GRANULES

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ABSTRACT

This study investigates how storage temperatures and periods under extended idle conditions affected the stability and activity of granules that have a high phenol-degrading capability. The granule activity increased as storage temperature decreased, and declined as storage time increased. Granules stored with pure water at room temperature (20.8±5.6° C) lost stability after three months storage. Particularly, granules stored with 500 mg l^{-1} phenol solution demonstrated high phenol degradation capability following storage. Further, storage at subfreezing temperatures (-20° C) is an ideal way of preserving stability and activity of phenol-fed granules. Anaerobic degradation of proteins in granule core by obligate anaerobic strain such as *Bacteroides* sp. corresponded to the stability loss of stored granules.

Keywords: Granule structure, confocal laser scanning microscope, phenol degradation

INTRODUCTION

Aerobic granules lost stability and activity after storage under anaerobic conditions. Tay *et al.* [1] observed that after 4 months of storage at 4° C in tap water, glucose-fed and acetate-fed granules lost 60% and 90% of their initial metabolic activity, respectively. Liu *et al.* [2] demonstrated that after 8 weeks of storage at 4° C in tap water and in a physiological solution, the color of glucose-fed and acetate-fed granules changed from brownish-yellowish to dark black. Moreover, granules stored in 4°C phosphate-buffered saline (PBS) had the smallest color change after being stored for 4 months. Zhu and Wilderer [3] determined that their glucose-fed aerobic granules did not significantly change in size, color, and settleability after storage for 7 weeks at room temperatures. Liu *et al.* [4] recovered microbial activity of aerobic granules stored for 4 months after 2 days of operation in a pilot-scale aerobic granular sludge reactor. Zhang *et al.* [5] re-activated their granular sludge after storage at room temperature for two months. Zhu [6] claimed that granules remained stable even after storage for 2 years in tap water at an ambient temperature (16–26° C). This case is not easily attained in practice as cell hydrolysis of granules always occurs in extended idle conditions.

Structural integrity of aerobic granules gradually deteriorates under long-term operation [7]. Cultivation of phenol-fed granules commonly takes 3 to12 weeks of incubation [8, 9], rendering the on-site cultivation of new granules an impractical option for replacing the old, deteriorated ones. Fresh granules can be produced in full-scale reactors and stored for further use as seed sludge to easily and quickly initiate another aerobic reactor.

This study investigates the effects of storage temperatures and periods under extended idle conditions on the stability and activity of granules that have a high phenol-degrading capability. The recovery of granule activity on phenol degradation following storage was demonstrated in this study. Most bacterial cultures could retain their bioactivity at temperatures below -20ºC [10-12]. The structure of microbial aggregates deteriorated when cells were disinfected via freezing and thawing [13, 14]. Preservation of aerobic granules at subfreezing temperatures is considered unfeasible. This study, however, indicated that storage at subfreezing temperatures (-20° C) is an ideal way of preserving stability and activity of phenol-fed granules.

MATERIALS AND METHODS

Granule Cultivation

Aerobic granules were cultivated in a column-type sequential aerobic sludge reactor. The reactor, seeded with

activated sludge collected from a local municipal wastewater treatment plant, was fed with phenol as the sole carbon source through synthetic wastewater (1000 mg $l^{-1}$$(NH_4)_2SO_4$; 200 mg l^{-1} $MgCl_2$; 100 mg l^{-1} NaCl; 20 mg l^{-1} $FeCl_3$; 10 mg l^{-1} $CaCl_2$; phosphate buffer (1350 mg l^{-1} KH_2PO_4, 1650 mg l^{-1} K_2HPO_4) and micronutrients ((mg l^{-1}) H_3BO_3, 50; $ZnCl_2$, 50; $CuCl_2$, 30; $MnSO_4.H_2O$, 50; $(NH_4)Mo_7O_{24}.4H_2O$, 50; $AlCl_3$, 50; $CoCl_2.6H_2O$, 50; and $NiCl_2$, 50). The medium was sterilized by autoclaving for 15 min at 121°C. The 400 mg l^{-1} phenol solution was filtered, sterilized, and added to the autoclaved medium. The reactor was operated in 12 h cycles. Aeration bubbles were supplied through the reactor bottom.

Storage Test

Six parallel test batches were utilized and in each test batch, 12 plastic tubes containing equal quantities of granules (1.4±0.1 g VSS l^{-1}) were stored at room temperature (20-22ºC, group A), at 4° C (group B), and at -20° C (group C). The following storage liquids were added to the granules separately: pure water, 500 mg l^{-1} phenol, basal salt medium, basal salt medium with 500 mg l^{-1} phenol, synthetic medium, and synthetic medium with 500 mgl^{-1} phenol. The composition of basal salt medium (mg l^{-1}) was as follows: NH_4Cl, 200; $MgSO_4$, 130; K_2HPO_4, 1650; KH_2PO_4, 1350; 10 ml micronutrient solution; and, 10 ml vitamin solution ((mg l^{-1}) biotin, 2; folic acid, 2; pyridoxine hydrochloride, 10; thiamine hydrochloride, 5; riboflavin, 5; nicotinic acid, 5; Ca-pantothenate, 5; vitamin B_{12}, 0.1; p-aminobenzoic acid, 5; and lipoic acid, 5). The synthetic medium consisted of: (mg l^{-1}) peptone, 400; beef extract, 250; NH_4Cl, 200; K_2HPO_4, 45; $CaCl_2$, 30; $MgSO_4$, 25; 10 ml micronutrient solution; and, 10 ml vitamin solution. The plastic tubes were sealed firmly and stored in the dark for 90–180 d at the prescribed temperatures.

Phenol Degradation Test

Following storage, the granules were removed from the storage box or freezer and warmed to room temperature. The granules were then added to sequential batch reactors containing 500 mg l^{-1} phenol solution. Fine air bubbles were supplied through the reactor bottom. All reactors were equipped with condensers that eliminated possible phenol loss due to air stripping. The time course of phenol degradation was determined.

Some stored granules were placed in the sequential batch rector and fed with 500 mg l^{-1} phenol for 48 h for reactivation. The "reactivated" granules were then tested for phenol degradation capability in batch tests.

Analytical Methods

The dry weight and volatile suspended solids (VSS) of granules were determined according to Standard Methods [15]. Phenol concentration in the reactor was determined by high-performance liquid chromatography (HPLC) equipped with C18 column (Varian, Inc., CA, USA), measured spectrographically at 280 nm. The mobile phase was 70% methanol.

For identifying the metabolites from stored granules, fresh granules and granules stored at 4º C in 500 mg l^{-1} phenol for 90 d were washed several times using Milli-Q water. Then the washed samples were sonicated (20 amplitude, 10 sec pulse) for 5 min on ice, centrifuged at 6000 x g for 10 min and the supernatant was analyzed by high-performance liquid chromatography (HPLC) equipped with C18 column (Varian Inc, CA, USA). The mobile phase was 25 mM potassium phosphate (pH 2.5).

The ammonia nitrogen was determined by salicylate method using "High Range Ammonia Test 'N Tube" kit (Hach, GmbH, Germany) following the manufacturer's instruction. Briefly, the sample was mixed with diluted reagent (supplied with kit) followed by addition of ammonia salicylate and then ammonium Cyanurate. A blank was prepared with ammonia free water and without sample. The mixtures were incubated for 20 min at ambient temperature and the ammonia nitrogen was measured at 655 nm by using Hach spectrophotometer (DR/2500, Hach, GmbH, Germany).

Granule Staining and CLSM Imaging

The collected granules were maintained fully hydrated during staining. During staining, SYTO 63 (Molecular Probes, Carlsbad, CA, USA) solution (20 μM, 100 ml) was first added to the sample and the sample was placed on a shaker for 30 min. Next, 0.1 M sodium bicarbonate buffer (100 ml) was added to retain the amine group in non-protonated form, which was followed by the addition of fluorescein isothiocyanate (FITC) (Molecular Probes, Carlsbad, CA, USA) solution (10 mg ml^{-1}, 100 ml) and the mixture was stirred at room temperature for 1 h. Subsequently, the fluorescently labeled lectin Concanavalin A (Con A) (Molecular Probes, Carlsbad, CA, USA) was conjugated with tetramethylrhodamine solution (250 mg l^{-1}, 100 ml) and incubated with the sample for an additional 30 min followed successively by the addition of calcofluor white (fluorescent brightener 28, SIGMA, USA) solution (300 mg l^{-1}, 100 ml) for 30 min. Following each of these five staining stages, the stained sample was washed twice with phosphate-buffered saline (pH 7.2) to remove excess stain and the stained sample was stored at 4° C. Prior to observations, the sample was incubated in SYTOX Blue (Molecular Probes, Carlsbad, CA, USA) solution (2.5 mM, 100 ml) for 5 min. The stained granules were then embedded for cryosectioning and frozen at -20°C, after which 60 μm sections were cut on a cryomicrotome and mounted onto microscopic slides for analysis.

Confocal laser scanning microscopy (CLSM) (Leica TCS SP2 Confocal Spectral Microscope Imaging System, Gmbh, Germany) was employed to visualize distributions of cells and extracellular polymeric substances in the aerobic granules. The granules were imaged with a 10x or 20x

objective lens and analyzed using Leica confocal software.

Anaerobic Strain Identification and FISH

The DNA from the stored granules was extracted via enzymatic lysis as described previously [8]. Polymerase chain reaction (PCR) amplification of the 16S ribosomal DNA (16S rDNA) was performed using the extracted DNA with forward primer P1 (5'-CCT ACG GGA GGC AGC AG-3') and reverse primer P2 (5'-ATT ACC GCG GCT GCT GG-3') as described by Muyzer *et al.* [16]. The GC rich sequence of 40 nucleotide (GC clamp) was attached at 5′ end of primer P1. Homology retrieval of the sequence obtained was performed by NCBI blast, which had 96% sequence similarity with *bacteroides* sp.

The stored granules were prepared for fluorescence *in situ* hybridization (FISH) by washing them with PBS (130 mM NaCl, 10 mM sodium phosphate buffer pH 7.2) and fixing for 1 h in 4% paraformaldehyde. Following fixation, the granules were washed again with PBS and hybridized using hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl at pH 7.4, 0.01% sodium dodecyl sulfate) containing 5 ng μl^{-1} of probe (5′-CTC CCA GTC GAT ATC CAC G-3′ labeled at the 5′-end with Fluorescein phosphoramidite (FAM dye) for 2 h at 48°C. This step was followed by a 20-min washing step at 48°C in wash buffer prior to a final wash with Milli-Q water

RESULTS

Phenol-degrading Granules

The granule had a compact surface. Close examination demonstrated that numerous rods, cocci, and filaments were packed onto the granule surface (photo not shown).

The phenol degradation tests for fresh granules with an initial phenol concentration of 500 mg l^{-1} exhibited zero order kinetics without a time lag, producing a degradation rate for phenol of 42.4 mg phenol g VSS^{-1} h^{-1}.

Figure 1 presents fluorescent staining results probed at 360 mm from the granule outer surface. According to fluorescent intensity, protein formed the core, and the cells (total and dead) and α-polysaccharide accumulated at the outer layer, whereas β-polysaccharide formed the outer layer and skeleton inside the granule.

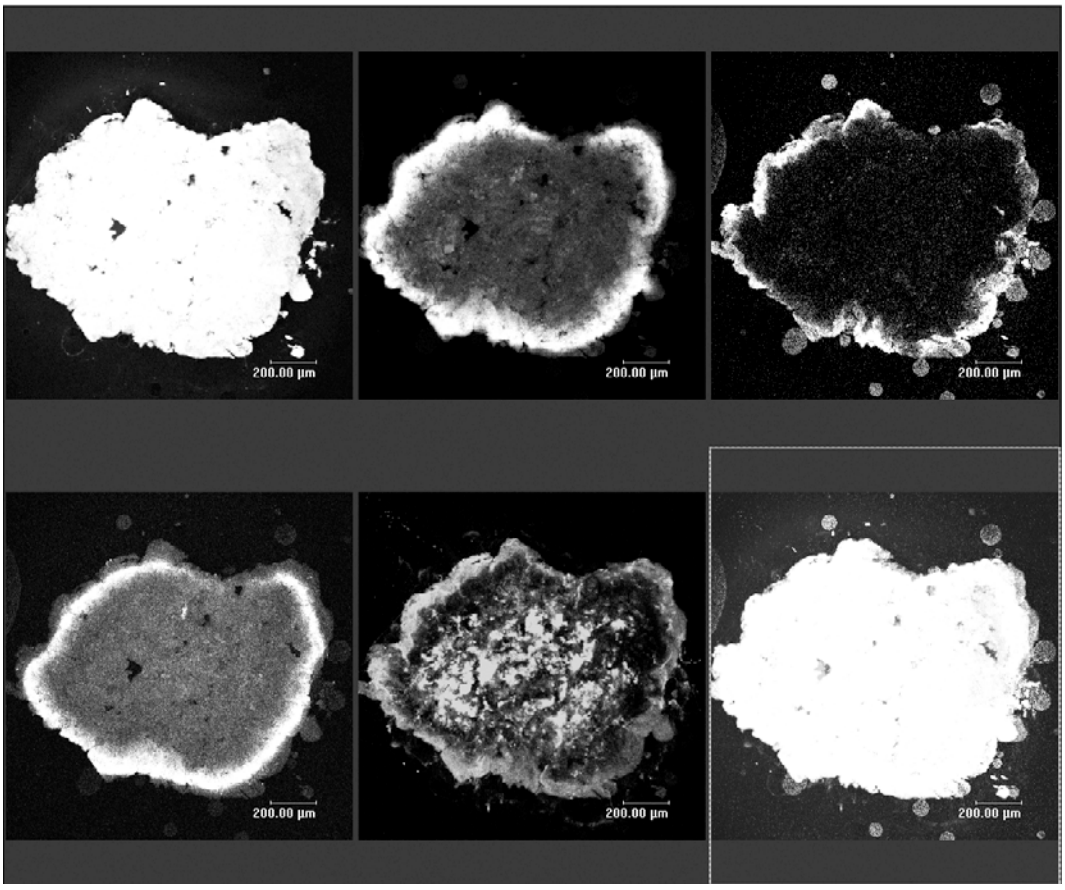

Figure 1. The CLSM image of fresh granule, cross-sectioned at 360 mm from top surface. Green (FITC): proteins; red (SYTO 63): nucleic acids; light blue (Con A): α-polysaccharide; pink: (SYTOX blue): dead cells; blue (calcofluor white): β-polysaccharides; Right-bottom image: combined image of five colors.

Phenol Degradation by Stored Granules

Figure 2 presents the appearance of granules stored for 180 d. Granules stored at 4°C or -20°C for 180 d generated darker interiors (Figures 2b and 2c). Conversely, storing granules at room temperature for 180 d generated dark granule fragments. Granules began losing stability at 90 d of storage at room temperature. Storage at 4°C or at -20°C preserves granule integrity up to 180 d.

The stored aerobic granules after 2 h recovery at room temperature were immediately tested for their phenol degradation capabilities in batch reactors fed with 500 mg l^{-1} of phenol. Table 1 presents their average phenol degradation rates. All stored granules had lower phenol degradation rates than fresh granules. Additionally, granules stored for 180 d at 4° C exhibited lower phenol degradation rates than those stored for 90 d or at -20°C. Effects of storage medium were not significant when stored at room temperature (12–18 mg phenol g^{-1} VSS h^{-1}). When stored at -20°C, the bioactivity of granules in different mediums followed the order: 500 mg l^{-1} phenol > Milli-Q water, synthetic medium+500 mg l^{-1} phenol, basal salt medium+500 mgl^{-1} phenol > basal medium, synthetic medium. When stored at 4°C, granules in Milli-Q water showed lower bioactivity than the other four media.

Phenol Degradation Activity of Reactivated Granules

Stored aerobic granules after 48 h reactivation were tested for their phenol degradation capabilities in batch reactors fed with 500 mg l^{-1} phenol. Following reactivation, all granules recovered their original color.

Figure 3 presents the comparison of phenol degradation rates of stored and reactivated granules. Reactivation improved the phenol degradation capability of stored

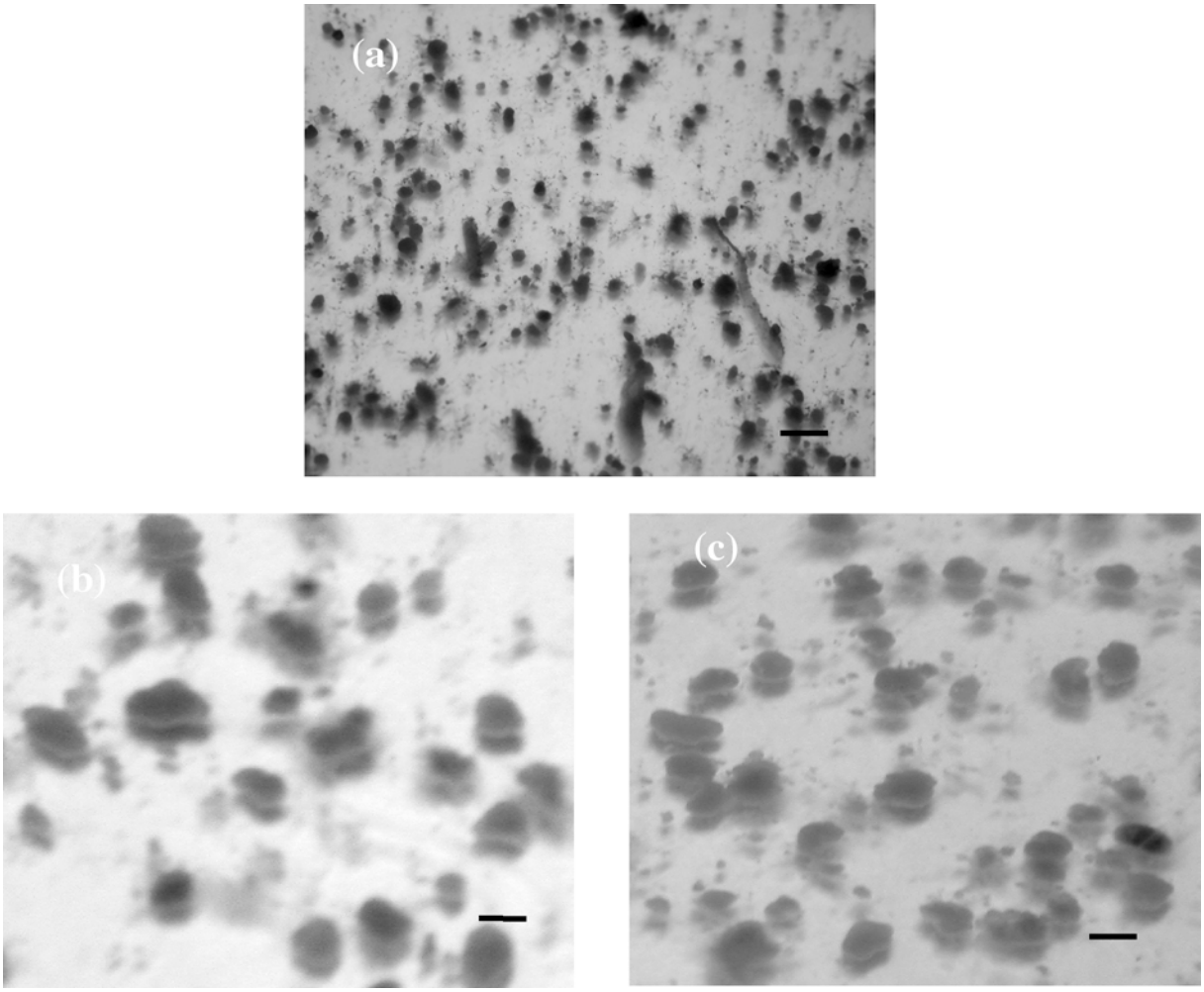

Figure 2. Image of stored granules after 2 h recovery to room temperature (bar =1mm). (a) Granules stored at room temperature for 180 d; (b) granules stored at 4°C for 180 d; (c) granules stored at -20°C for 180 d.

Table 1. Phenol degradation rates for stored granules recovered to room temperature for 2 h. Degradation rate for fresh granules: 42.4 mg phenol g VSS^{-1} h^{-1}.

Storage Medium	Phenol degradation rate of granules stored at room temperature (mg phenol g^{-1} VSS h^{-1})	Phenol degradation rates of granules stored at 4ºC (mg phenol g^{-1} VSS h^{-1})		Phenol degradation rates of granules stored at -20ºC (mg phenol g^{-1} VSS h^{-1})	
	Stored for 90 d	Stored for 90 d	Stored for 180 d	Stored for 90 d	Stored for 180 d
Pure water	12.1 ± 2.9	16.2 ± 2.7	11.4 ± 1.9	28.0 ± 1.7	24.5 ±1.9
500 mg l^{-1} phenol	15.6 ± 2.1	21.8 ± 4.1	18.3 ± 3.3	33.6 ± 0.3	28.0 ±0.6
Basal salt medium	17.4 ± 1.7	23.2 ± 1.6	19.8 ± 1.4	22.6 ± 0.6	22.6 ±0.6
Basal salt medium with 500 mg l^{-1} phenol	17.7 ± 3.1	24.2 ± 3.0	20.6 ± 2.5	25.0 ± 0.8	22.5 ±0.7
Synthetic medium	13.1 ± 3.9	24.8 ± 3.7	16.4 ± 4.0	22.5 ± 1.2	21.2 ±0.2
Synthetic medium with 500 mg l^{-1} phenol	17.4 ± 3.2	20.3 ± 2.7	19.1 ± 2.0	25.7 ± 1.0	25.7 ±1.0

granules from 24–40% to 38–62% for granules stored at room temperature, from 29–55% to 50–97% for granules stored at 4°C, and from 51–81% to 52–99% for granules stored at -20°C. Particularly, granules stored in 500 mg l^{-1} phenol solution had the highest phenol degradation capability among all storing conditions. Additionally, all granules stored at -20°C, except those stored in Milli-Q water, retained 82–99% of phenol degradation capacity of fresh granules following 48 h reactivation.

DISCUSSION

Figure 4 presents fluorescently stained images of granules stored in 500 mg l^{-1} phenol solution at 4°C and at -20°C for 180 d. These granules were intact at their outer surfaces, but had large vacuoles in their interiors when compared with the CLSM images for fresh granules (Figure 1). The distribution of anaerobic strain in stored granules was demonstrated by using *Bacteroides* sp. specific probe with the sequence 5′-GCACTTAAGCCGACACCT-3 labeled with the Fluorescein phosphoramidite (FAM). The probed anaerobic strain spread over the entire interior of the granules stored at -20ºC for 180 d (Figure 5).

Experimental results suggested that storage of granules in Milli-Q water at room temperature for 90 d had reduced stability and activity. Granules were preserved better when storage temperatures were reduced; however, prolonged storage time typically degraded granules further. The activity of granules stored at -20°C for up to 180 d retained 80–99% of their initial activity, after 48 h of reactivation. The presence of phenol significantly preserves the bioactivity of granules at all storage temperatures.

Fluorescently stained images of granules stored in 500 mg l^{-1} phenol solution at 4°C and at -20°C for 180 d were intact at their outer surfaces, but had large vacuoles in their interiors. Compared with the CLSM images for fresh granules (Figure 1), certain mechanisms "consume" proteins and likely some β-polysaccharides as well, in the granule cores. Additionally, granules stored at 4°C had more vacuoles than those stored at -20°C. Restated, freezing the suspension did not significantly degrade granule integrity, significantly dissimilar to cases for wastewater sludge flocs [17]. McSwain *et al.* [18] suggested that a non-cellular protein core stabilizes granule structure. Wang *et al.* [19] claimed that the polysaccharide outer shell generated structural strength for granules. The present result (Figure 4) revealed that both proposals may be true for the investigated granules.

Tay *et al.* [20] investigated anaerobic bacteria at the core regime of aerobic granules. Low storage temperature and phenol toxicity likely delay hydrolysis or biotransformation of proteins at the core regime, thereby retaining granule stability following storage. The DNA from the stored granules was extracted and the PCR-amplified 16S rDNA with forward primer P1 and reverse primer P2 was identified as an anaerobic strain *Bacteroides* sp. The *Bacteroides* sp. is gram-negative and an obligate anaerobic strain that can produce fatty acids from peptone. Tay *et al.* [21] noted the presence of this obligate anaerobic strain in the core regime of large, active aerobic granules.

The FISH-CLSM image using bacterial probe with the sequence 5′-GCACTTAAGCCGACACCT-3 labeled with FAM detects the *Bacteroides* sp. [22] in stored granules. The probed anaerobic strain spread over the entire interior of the granules stored at -20ºC for 180 d. In other words, the anaerobic strain did exist in the present stored granules that had the potential to consume gradually the proteins in the core regime. Also, gas bubbles were noticeable in the studied granule. Junge *et al.* [12] also reported actively respiring bacteria activity in

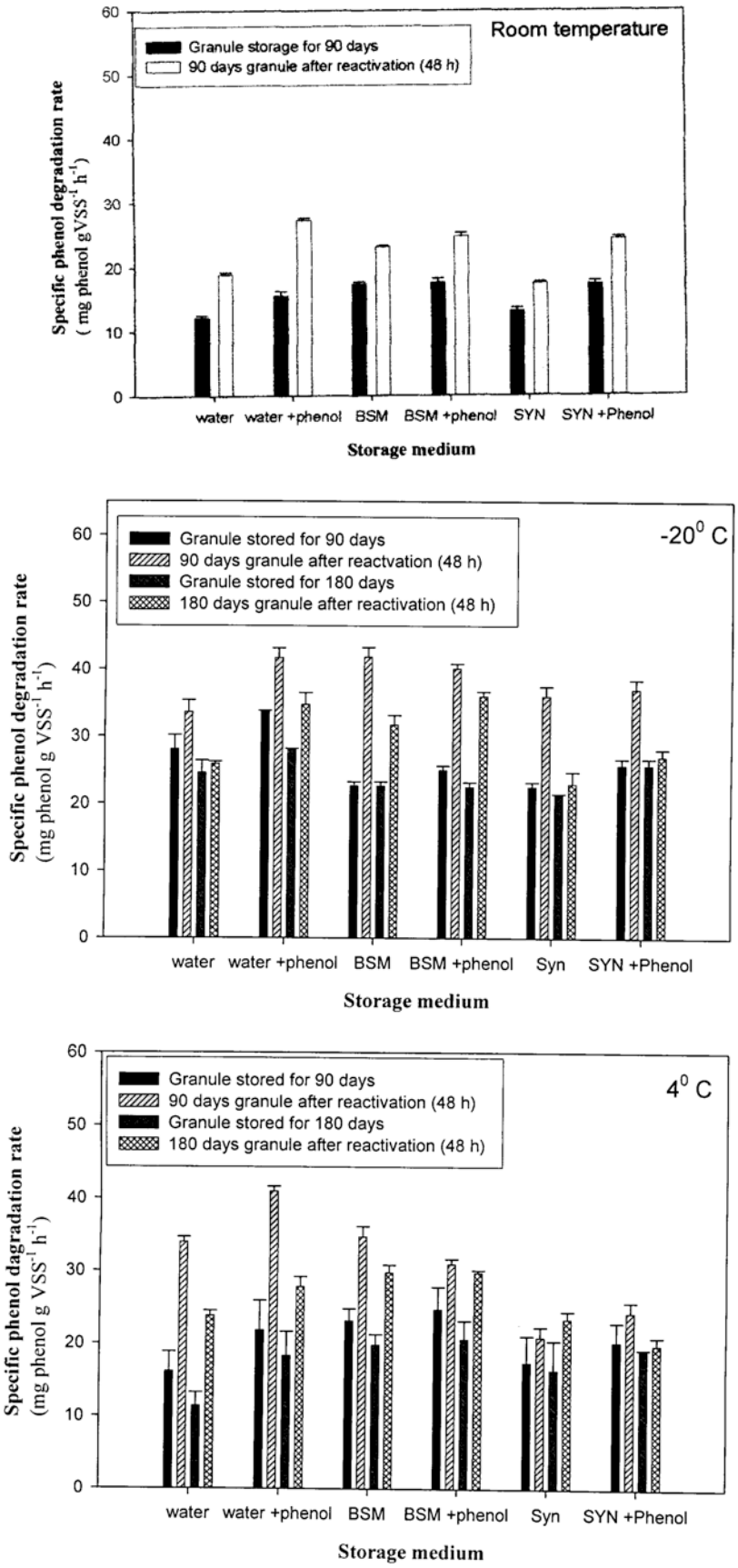

Figure 3. Comparison of batch phenol degradation tests of stored and reactivated granules.

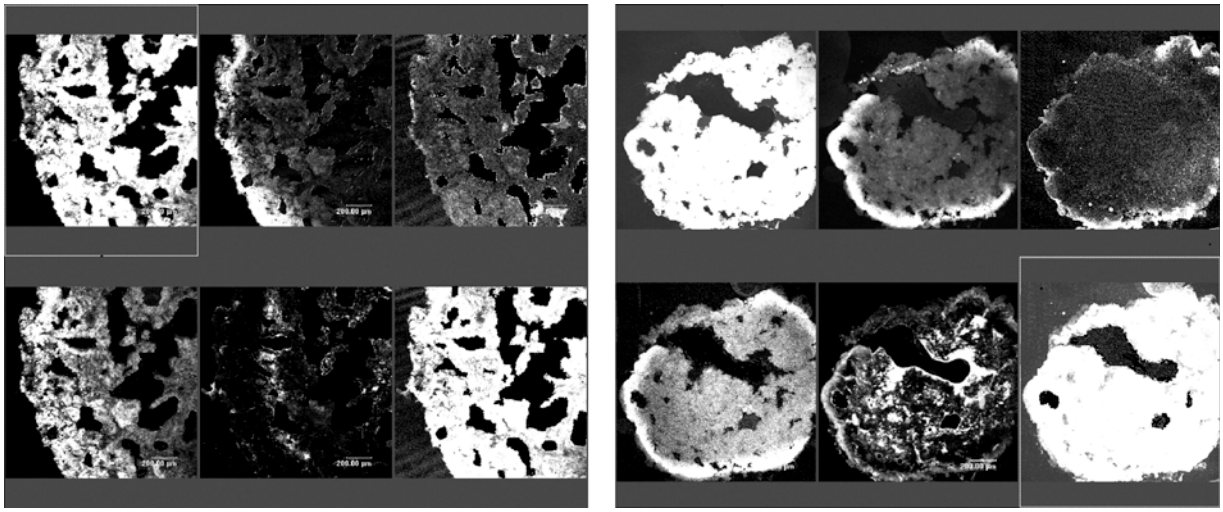

Figure 4. The CLSM image of stained granules, cross-sectioned at 360 mm from top surface. Green (FITC): proteins; red (SYTO 63): nucleic acids; light blue (Con A): α-polysaccharide; pink: (SYTOX blue): dead cells; blue (calcofluor white): β-polysaccharides; Right-bottom image: combined image of five colors. (Left) 180 d of storage at 4°C (Right) 180 d of storage at -20°C.

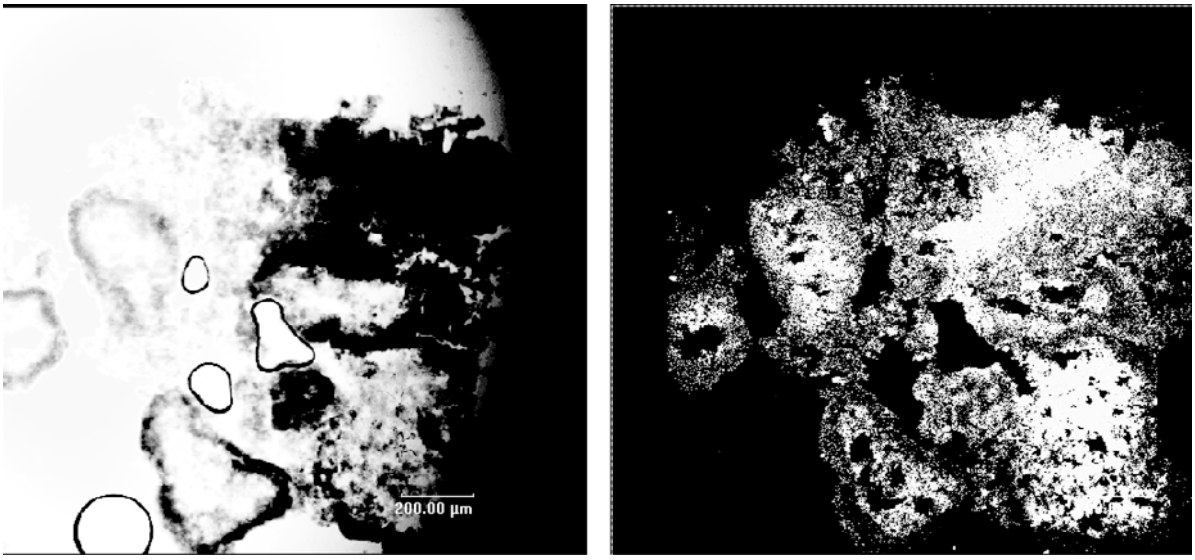

Figure 5. FISH-CLSM image at the outer layer for the *Bacteroides* sp. strained with probe 5′- CTGGTTGATCCTGCCAGT -3′ labeled at the 5′-end with fluorescein phosphoramidite (FAM) in granule stored at -20° C for 180 d.

in Arctic wintertime sea ice cores at temperature gradient of -2 to -20°C and detected *cytophaga-flavobacteria-bacteroides* by applying FISH technique.

The metabolite analysis revealed that the supernatant of sonicated, fresh granules contained few volatile fatty acids and low NH_3-N (<0.15 mg l^{-1}). The applied ultrasound level was not sufficient to deteriorate cell membranes to release intra-cell materials. On the other hand, certain anaerobic metabolites were detected from the sonicated, stored granules. For instance, the acetate and NH_3-N concentrations in supernatant of granules stored at -20° C for 180 d were 7.7 mg acetate l^{-1} supernatant, and 6.0 mg-N l^{-1} supernatant, respectively. Anaerobic degradation occurred in the stored granules (although at a very low rate), probably via the detected *Bacteroides* sp. strain. The low storing temperature and the high phenol concentration inhibited the activity of

anaerobes, hence prolonged the stability of the stored granules. Compositions of storing medium were not significant to granule stability following storage.

CONCLUSIONS

The stability and activity of phenol-fed, aerobic granules following 90 and 180 days of storage in six different media were studied at room temperature, 4° C, and -20° C. Cultivated, fresh granules had compact surfaces and a dense core filled with proteins and β-polysaccharides, and had a degradation rate for phenol of 42.4 mg phenol g VSS^{-1} h^{-1} at 500 mg l^{-1} phenol. The storing mediums were Milli-Q water, 500 mg l^{-1} phenol solution, basal salt medium, basal salt medium with 500 mg l^{-1} phenol, a synthetic medium, and the synthetic medium with 500 mg l^{-1} phenol. Granules lost stability and activity after a prolonged period of storage under anaerobic conditions; particularly, the granules in Mlli-Q water completely disintegrated after 180 days of storage at room temperature.

The stored aerobic granules had lower phenol degradation rates than fresh granules. However, the granules reactivated for 48 h in 500 mg l^{-1} phenol regained some activity. Overall, granule activity increased as storage temperature decreased, and declined as storage time increased. Particularly, granules stored with 500 mg l^{-1} phenol solution demonstrated high phenol degradation capability following storage. All reactivated granules stored at -20° C, except for those in Milli-Q water, had 82–99% of the phenol degradation capacity of fresh granules. Obligate anaerobic strain *Bacteroides* sp. was noted to exist over the entire stored granule. Moreover, acetate and NH_3-N were noted to be produced by stored granules. Anaerobic degradation of proteins in granule core corresponded to the stability loss of stored granules. The fluorescently stained images of stored granules suggested that low storage temperature and the toxicity of phenol likely delay hydrolysis or biotransformation of proteins in the cores of granules, thereby enhancing granule stability following storage.

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