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Reliability and Resilience of Populations and Metabolic Functions within Defined Microbial Communities in Biological Reactors

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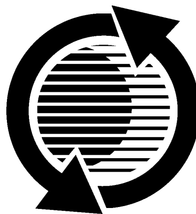

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Reliability and Resilience of Populations and Metabolic Functions within Defined Microbial Communities in Biological Reactors

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ABSTRACT

Bioregenerative components for advanced life support (ALS) systems will need to be reliable and stable for long-duration space travel. To examine the stability and resilience of microbial communities that recover nutrients from inedible wheat residues, we maintained 4 bacterial strains in mixed communities for 7 weeks. After 3 weeks of incubation, aeration was stopped for several days. Although the abundance of each isolate declined during the perturbation, all strains persisted throughout the experiments. However, only 80% of functions lost during perturbation were recovered afterward. Thus, persistence of strains in a community did not guarantee the persistence of metabolic functions which those strains could perform. Niche partitioning of the heterogeneous molecules in the wheat residue apparently contributed to stable coexistence of the 4 strains. A different community, composed of 4 isolates from a hydroponic study grown under either simulated microgravity or 1×*g* control conditions in a comparatively homogeneous 1/2-TSB medium, was less stable. Increased diversity of microbial strains and complexity of the substrates upon which they were maintained seemed to increase the stability of microbial communities in ALS bioreactors.

INTRODUCTION

For long-duration space missions, advanced life support (ALS) systems will be needed to produce food, recycle air and water, and decompose waste [1]. ALS components, including those using microbes, must function reliably [2]. Stability of processes is often included in the concept of component or system reliability because a stable system is less likely to be changed by external variables [2,3]. Stable biological systems maintain a steady-state equilibrium from which there is little deviation [2-4]. Another aspect of system reliability commonly encountered in biological systems is resilience - the ability of an ecological system or community to recover from perturbations [3].

For microbial communities incorporated into ALS components, the most important aspect of reliability is being able to maintain the activities necessary for proper function of that ALS component, whether that function is bioconversion of inedible plant residue to CO_2, recovery of trace nutrients, or decomposition of other wastes [2,5]. Strayer *et al.* [2] have confirmed that CO_2 evolution and total microbial biomass can be reliably maintained for periods of more than a year. Examination of the internal stability of a microbial community as it degrades heterogeneous substrates in inedible plant residue is among the next steps in determining how to control and maintain biological components for ALS, especially since decreases in biological diversity have been observed in microbial communities after closure in bioreactors and mesocosms [6,7]. Since recruitment of new strains into a bioreactor community will be difficult or impossible on a long-duration space flight, the first step in maintaining microbial functions is to maintain the presence of the microbes responsible for particular metabolic activities.

In this paper, we examine the stability of populations and metabolic functions of microbial communities in ALS bioreactors, especially the relationship between bacterial population dynamics within a mixed microbial community and the dynamics of metabolic activities performed by that community. We present results that confirm population stability and resilience within a functionally diverse microbial community assembled to degrade inedible wheat residue. However, maintenance of component strains in the model community did not guarantee the

persistence of metabolic activities performed by those microbes. The stability of the wheat-degrading ALS community is contrasted with the unstable population dynamics of a less functionally diverse community maintained on a simpler medium and subjected to the perturbation of a simulated microgravity environment.

METHODS

BENCHTOP-SCALE REACTOR FOR DEGRADING ALS WHEAT RESIDUE – To maximize the recovery of fixed carbon from higher plants, mixed microbial bioreactors will be used to convert inedible plant matter into CO_2 and microbial biomass [8, 9]. The model community constructed for this project was designed to degrade inedible plant residue under conditions similar to those of the microbial community in the ALS bioreactor studied by Finger and Strayer [9]. Experimental bacterial communities were cultured at 26°C in 3-liter Bellco® spinner flasks with emplaced pH electrodes [Ingold Electrodes] (Figure 1). Magnetized impellers were driven by stir plates at approximately 60 rpm. Air input was filtered through two autoclaved Gelman® bacterial air vent filters, positioned before and after a hydration flask. The outflow air line from the reactor vessel was connected to a trap flask, and then to a final bacterial air vent filter. A flow meter was connected downstream of the final filter.

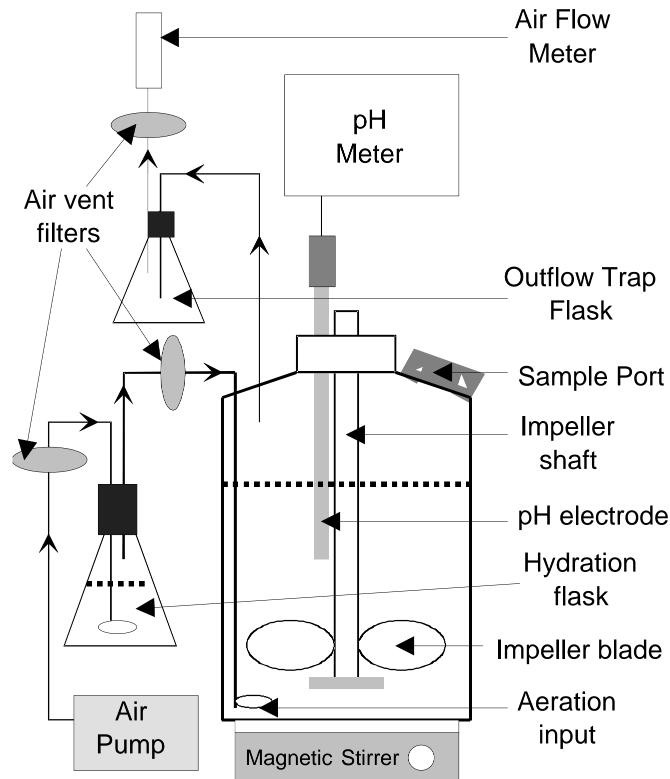


Figure 1. Schematic diagram of 3-L benchtop-scale reactor vessel for model microbial community cultured on inedible (to humans) wheat residue.

Isolation and Characterization of Bacterial Strains – Bacterial strains capable of degrading inedible plant matter were isolated from samples of mixed soil, leaf litter, and rotting wood. Strains were chosen for inclusion in mixed Runs 1 and 2 on the basis of their functional diversity, both in their ability to grow on different components of fibrous plant matter (*e.g.*, cellulose and hemicellulose) and in the presence of non-overlapping active metabolic functions on BIOLOG®-GN plates. The four bacterial strains (6a, 9, 13a, and 19) could be visually differentiated during enumeration on TSA plates (Table 1 and Appendix A) [10].

Table 1. Strains chosen for model community degrading inedible wheat residue.

Strain No.	Traits		
	Cell morphology	BIOLOG®-GN assay profiles[a]	Liquid media which support growth
6a	G^+ rod	25	wheat
9	G^{var} rod,	5	hemicellulose or wheat
13a	G^-, coccus	24	wheat, cellulose, or hemicellulose
19	G^{var}, rod	38	wheat or hemicellulose

[a]Number of compounds on BIOLOG®-GN assay plates degraded at 24 h at 25°C

Combining characterized strains in model communities – Reactor vessels containing sterile medium were inoculated with 15 mL each of 3- to 5-day-old shake-flask starter cultures, which had been undergoing daily 10% removal and replenishment of medium. Inoculation densities were 1:200 of the pure culture density for each strain. The 4 chosen strains were combined in parallel communities in 2 reactor vessels. Fed-batch conditions were maintained for 7 weeks by removing one-tenth (300 mL) of the culture volume every day and replenishing the culture with an equal volume of fresh sterile medium containing 2% (w/v) unleached wheat residue in distilled water (pH 5.65). Inedible wheat residue, supplied by Kennedy Space Center (KSC), consisted of aerial portions of hydroponically grown wheat, dried and milled to <0.635 mm.

The two mixed culture communities, Runs 1 and 2, were maintained in side by side reactor vessels on the benchtop for 50 days (five turnovers of the reactor volume). In Run 1, the baseline aeration rate was 650±150 mL/min. The model community was stressed by ceasing aeration for one week, starting one hour after the Day 22 sampling for functional profile and population densities. Minutes after the Day 29 samples were taken, aeration was returned to the baseline level. Starting at Day 40, aeration became increasingly intermittent and the rate gradually slowed because of blockage in the outflow air line.

Mechanical problems with the air pump resulted in Run 2 having a lower aeration rate than Run 1. For Run 2, the initial aeration rate was 400 ± 200 mL/min. After the Day

24 removal and replenishment of medium, aeration was stopped for 5 days. After the Day 29 samples were taken, aeration was returned to about 400 ±75 mL/min until Day 34, when the aeration rate was reset to 1050±100 mL/min. In summary, Run 2 started with a lower aeration rate than Run 1, but after Day 34, aeration was increased above that of Run 1.

Sampling Cultures in Benchtop Reactors – Bacterial population densities (CFU/mL) were enumerated on TSA plates that were incubated for 4 days at 25°C and counted daily as the colonies for each strain became visible. Population densities were sampled at least three times a week. Profiles of active metabolic functions on BIOLOG®-GN assay plates were obtained from 150-mL samples, the first removed after the first 24 hours of mixed culture, and the remainder removed once weekly during regular aeration. During perturbation without aeration, functional assays were performed 2 days after the onset of perturbation and again immediately before resumption of aeration.

For inoculation of samples onto BIOLOG®-GN assay plates, bacteria were separated from the particulate wheat residue by centrifugation and then resuspended in 0.85% sterile saline [11]. Replicate 1:10 dilutions of the saline suspensions were used to inoculate five BIOLOG®-GN assay plates for each sample. The assay plates were incubated in the dark for 24 h at 25.5 ±1.5°C. The media and conditions used to prepare these microbial samples do not match those required for any of the BIOLOG® databases, so any resulting pure-culture profiles could not provide accurate identifications [12]. The BIOLOG®-GN profiles showed that each strain had at least one substrate which that strain alone metabolized in pure culture (Appendix A).

EFFECTS OF SIMULATED MICROGRAVITY ON A COMMUNITY OF HYDROPONIC ISOLATES – Ground-based simulation of microgravity was attained using 50-mL high aspect-ratio rotating-wall vessel clinostat bioreactors (HARVs), in which slow rotation (20 rpm) of the bioreactor vessel on a horizontal axis randomized the gravity vector under low-shear, fed-batch culture conditions [13].

Microbial communities in HARVs – Three pairs of SMG and Control HARVs were maintained for 10 days each, with daily enumeration of strain densities within the cultures. The members in the constructed community were the yeast *Rhodotorula rubra* and three bacteria: *Pseudomonas azelaica*, a species of *Xanthomonas*, and an unidentified Gram-positive spore-forming bacillus that was in neither the BIOLOG® nor the Vitek® databases (Table 2). All 4 strains were present at detectable population levels in the inoculation cocktail. The dynamics of functional activity could not be examined because profiles based on BIOLOG®-GN assay plates were performed only at termination (T=10) of experimental runs.

Table 2. Strain Identification for HARV communities.

Gram stain characteristics	BIOLOG® ID on TSA or Blood/BUGM®
G- rod	*Pseudomonas azelaica*
G- rod	*Xanthomonas* species
G+ bacillus (spore former)	No ID
	VITEK® ID:
Yeast	*Rhodotorula rubra*

RESULTS AND DISCUSSION

PERSISTENCE OF STRAINS IN ALS-MODEL MICROBIAL COMMUNITIES DEGRADING WHEAT RESIDUE

Persistence of all inoculated strains in the mixed communities – All strains persisted throughout the 50 day mixed culture incubations, even when a contaminant appeared in Run 2 on Day 30 (Figures 2 and 3). Run 1 remained properly gnotobiotic for the entire incubation period (Figure 2). Density of strain 19 dropped at least two orders of magnitude during the perturbation in each run. In Run 1, no strain density dropped below the 2×10^5 CFU/mL detection limit after Day 5, when strain 6a had become detectable. Although strain 6a was undetectable at times in Run 2, its reappearance after the perturbation confirmed that the strain had not become extinct within the reactor. In both communities, the recovery of population densities after perturbation was slower than the drop when the perturbation was begun.

Competition did not result in the extinction of any of the 4 strains inoculated into the two ALS-modeled mixed microbial communities. Reduction of community diversity and species richness has been observed in artificial communities or mesocosms that have been physically separated from their parent communities [6,7,14-16], and is a continuing problem for industrial applications of microbial consortia [17]. Abiotic factors ranging from cycling culture conditions to micro-scale spatial heterogeneity have been identified which can alleviate the effects of competition and allow persistence of multiple strains in a community [7,15,18-22]. The metabolic diversity of the bacterial strains chosen for this ALS-modeled community was intended to maximize the degradation and conversion to CO_2 of the complex plant material [9, 23-25]. When coupled with the heterogeneous nature of the plant substrate, the functional diversity of the strains may have resulted in niche partitioning, which reduced competition for energy and carbon sources and allowed the stable coexistence of all 4 strains.

POPULATION STABILITY IN COMMUNITIES DEGRADING WHEAT RESIDUE – Population stability can be defined in terms of an equilibrium, to which a stable population returns after perturbation, or in terms of

variability about a long-term average, where a population with less variability about that average is considered more stable than a population with more variability [3,4]. According to either definition, the community in Run 1 was more stable than that in Run 2. The rapidity with which the pairwise population density plots for Run 1 converged on an equilibrium point, and returned to that equilibrium after the perturbation satisfy the equilibrium-based definition of stability (Figure 4). The downward deviation only along the axis for strain 19 in Figure 4a indicates that the population density of strain 13a was not significantly affected by the perturbation. Similarly, density of strain 6a was less inhibited by the perturbation than strain 19 (Figure 4b).

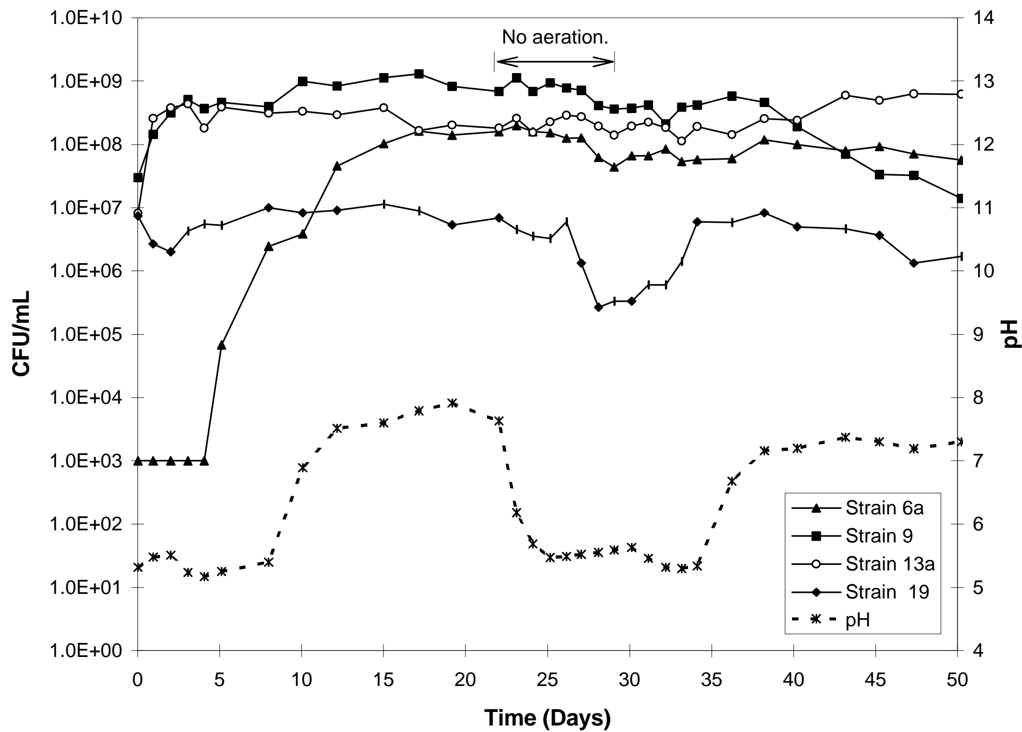


Figure 2. Population Densities of Each of the Four Strains in Run 1 of the Wheat-Degrading Model ALS Community. Density plots are means of triplicate dilution series onto TSA. Error bars are omitted to reduce clutter.

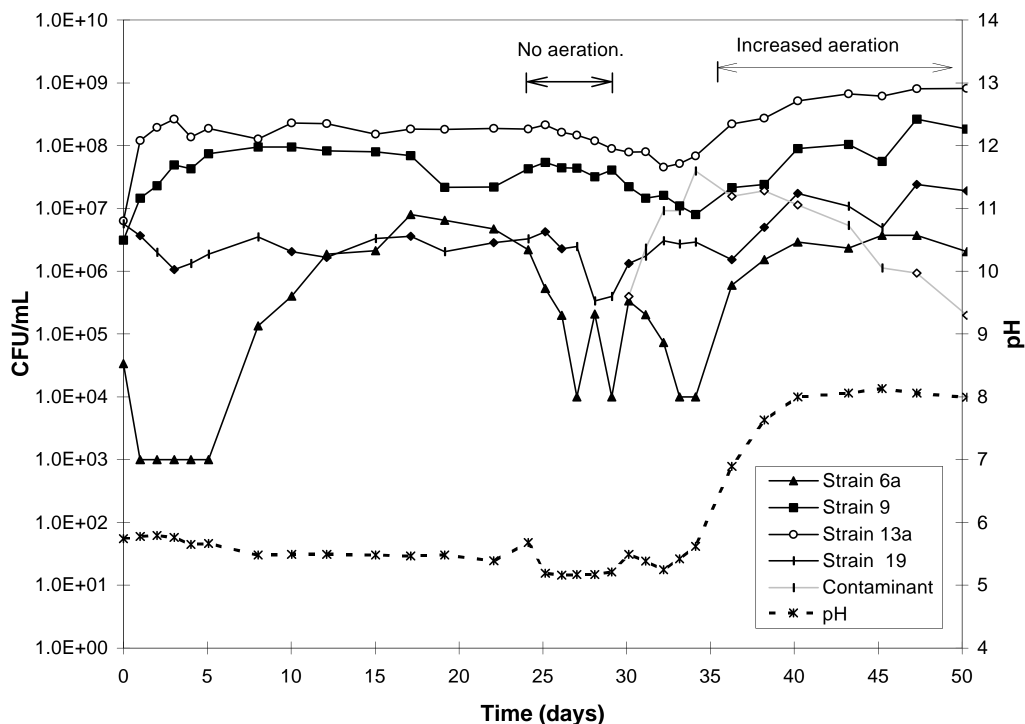
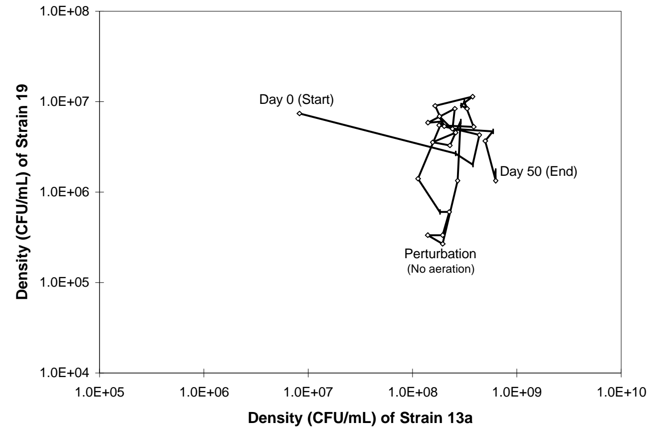


Figure 3. Population Densities of Each of the Strains in Run 2 of the Wheat-Degrading Model ALS Community. Density plots are means of triplicate dilution series onto TSA. Error bars are omitted to reduce clutter.

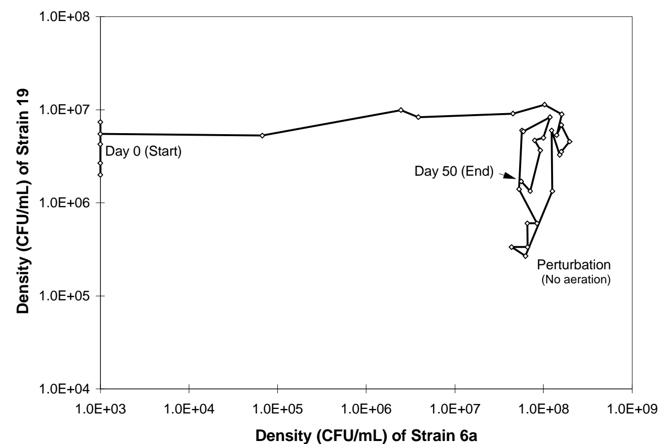
In Run 2, convergence on an equilibrium point was less obvious than in Run 1, especially since the increased aeration after Day 34 was associated with a positive drift away from the local equilibrium point established under the initial aeration rate (Figure 5). The pairwise density plots for Run 2 are not drawn into knots as tight as those for Run 1, indicating less stable population dynamics in Run 2 according to the variability definition of stability. Population densities of strain 13a (Figure 5a) and especially strain 6a (Figure 5b) declined more dramatically in response to the perturbation than they did in Run 1 (Figure 4). However, the obvious perturbation and return to local equilibrium of population densities identify Run 2 as a stable community according to the equilibrium definition.

<u>Factors contributing to population stability of ALS-Model communities</u> – In addition to the niche partitioning mentioned above, cyclic variations in environmental parameters are a well-documented factor which can facilitate coexistence of populations, as long as the amplitude and frequency of the variations are within a tolerance range specific to the particular community [15,20,22,26]. Maintenance of greater diversity has been reported in several microbial communities subjected to fluctuations in temperature [18, 19, 22]. In one study, a fed-batch culture from a natural biofilm maintained structural diversity, while a steady-state continuous culture, inoculated from the same biofilm community, lost diversity and species richness until only three strains could be detected [7]. Temporal fluctuations in pH and population densities accompanying daily removal and replenishment of medium in the fed-batch regimen used for Runs 1 and 2 were apparently within the range that facilitated maintenance of structural diversity in those communities.

STABILITY OF ALS-MODELED COMMUNITY *VS.* INSTABILITY OF A 4-STRAIN COMMUNITY CULTURED IN HARVS – When a hydroponically-based 4-strain microbial community (Table 2) was cultured under simulated microgravity (SMG) conditions, the population dynamics within the community were different from those observed under ~$1 \times g$ control conditions (Figures 6 and 7). The most striking difference between SMG and control communities was the behavior of the yeast *Rhodotorula rubra*. In control mixed cultures, the density of *R. rubra* remained above 1×10^7 CFU/mL at the end of 10 days (Figure 6). A pairwise density plot for *R. rubra* and *P. azelaica* never converges on an equilibrium point, thus reflecting the instability of this community (Figure 8a). The SMG cultures were even less stable (Figures 7 and 8b). In SMG, the density of *R. rubra* peaked at 2.5×10^6 CFU/mL and declined more rapidly than in the control, reflecting the increased destabilization of this community under SMG conditions (Figure 8).



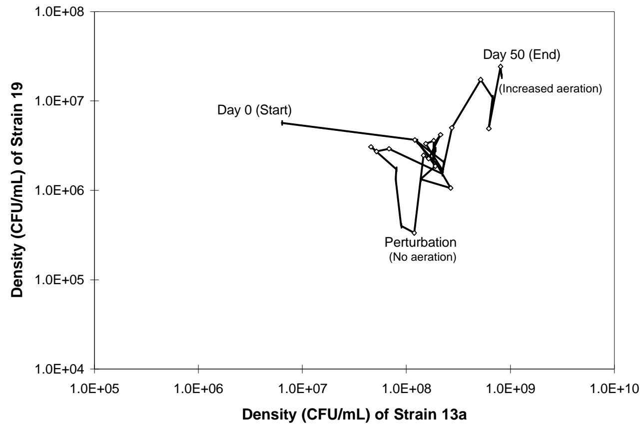
(a) Pairwise density plot for strains 19 and 13a in wheat-degrading model community, Run 1.



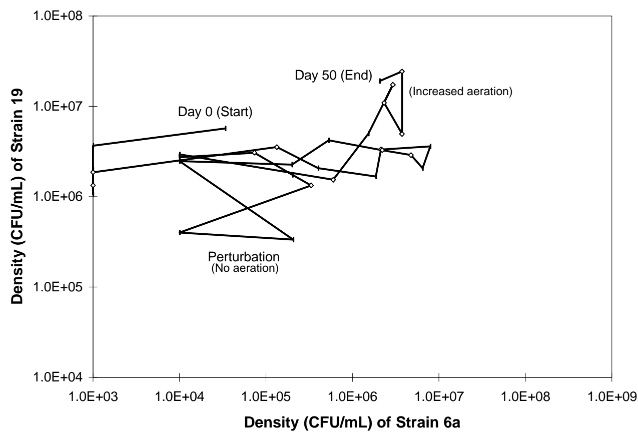
(b) Pairwise density plot for strains 19 and 6a in wheat-degrading model community, Run 1.

Figure 4.

<u>Conditions in HARV communities unfavorable to population stability</u> – Compared to the diverse array of carbon sources (*e.g.*, starches, cellulose and hemicellulose) present as components of unleached wheat medium used for the ALS-modeled community, the standard laboratory medium 1/2-TSB is quite homogeneous and did not provide many niches for partitioning between the component strains in the HARVs. Likewise, the low-shear environment of 50-mL HARV chambers is less diverse than the agitated and aerated 3-L cultures maintained in the ALS studies, providing less physical heterogeneity. Finally, the microbes included in the HARV study were selected for their divergent colony morphologies and *common* ability to grow on TSA and TSB, not for metabolic diversity as had the strains used in the ALS model. Without the potential for niche partitioning or the cyclicity of environmental conditions experienced by the ALS-modeled community, the HARV communities had fewer stabilizing factors.



(a) Pairwise density plot for strains 19 and 13a in wheat-degrading model community, Run 2.



(b) Pairwise density plot for strains 19 and 6a in wheat-degrading model community, Run 2.

Figure 5.

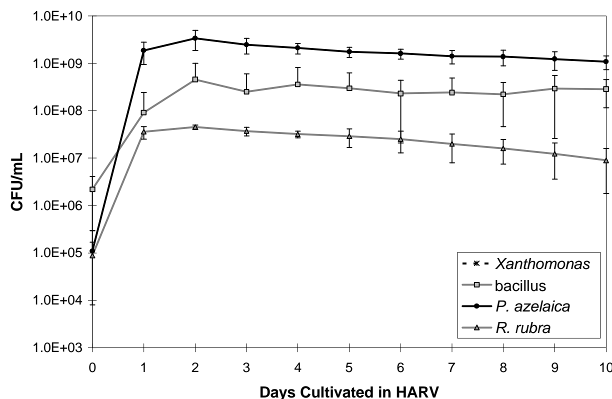


Figure 6. Population Densities of Microbial Community in 1×*g* Control HARV. Mean cell density ±1 SD (n=3 HARV runs) of each strain enumerated on TSA (bacteria) or SAB+cam (*R. rubra* yeast).

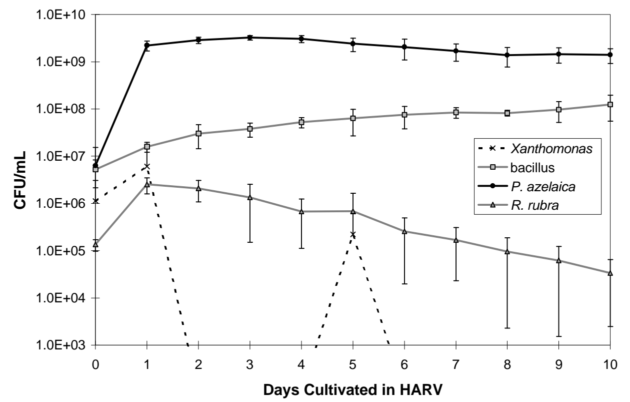
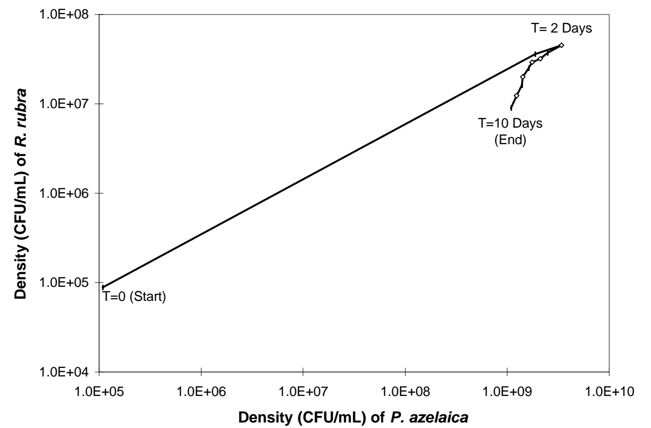
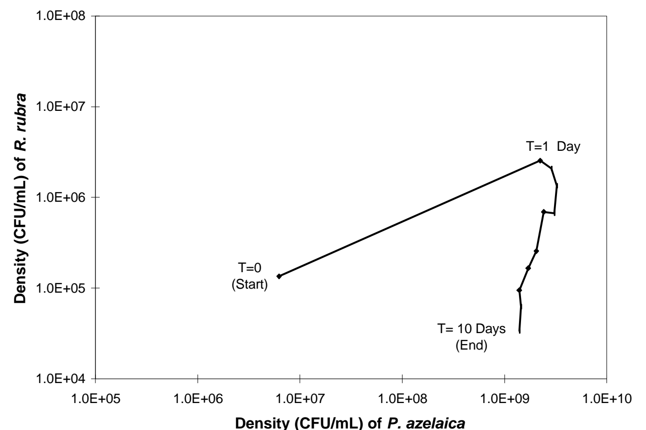


Figure 7. Population Densities of Microbial Community in SMG HARV. Mean cell density ±1 SD (n=3 HARV runs) of each strain enumerated on TSA (bacteria) or SAB+cam (*R. rubra* yeast).



(a) Pairwise plot of average densities (n=3) of *R. rubra* and *P. azelaica* in control HARVs.



(b) Pairwise plot of average densities (n=3) of *R. rubra* and *P. azelaica* in SMG HARVs.

Figure 8.

FUNCTIONAL ACTIVITY IN ALS-MODELED MICROBIAL COMMUNITIES

Presence and persistence of metabolic activities in mixed microbial communities degrading inedible plant residue – Functional dynamics in the model ALS communities were complicated and not determined solely by the population dynamics of the bacterial strains that composed the communities. The number and identity of sole-carbon substrates metabolized by microbial communities changed over time (Appendix A). The number of detectable metabolic functions in Run 1 gradually increased as the community matured, decreased during the perturbation, and increased again after the return of aeration. In Run 2, an initial aeration rate lower than that in Run 1 complicated the functional behavior of the community: the planned period of no aeration occurred in the middle of an "unplanned perturbation" of low aeration which existed for the first 5 weeks of cultivation. Community #2 had fewer active functions than community #1, and the planned perturbation was accompanied by a barely perceptible dip in the functional activity of community #2. Three functions ceased to be detected during the anoxic perturbation in Run 2. All three recovered immediately after the low aeration rate was resumed. After the increase in aeration at Day 34, the community in Run 2 began to degrade 7 substrates which it had never previously metabolized at detectable levels, including the synergistically degraded D-gluconic acid. The activation of functions five weeks after the community was assembled is convincing evidence that prolonged absence of a function from a community does not mean that functional activity cannot be induced from capable organisms present in the community.

Functional Response of Microbial Communities to Perturbation – The ability to degrade carbohydrates and polymers, the major components of the fibrous plant material provided as a carbon and energy source [27, 28], was apparent from the outset and persisted through the perturbation with only a 20% to 30% decrease in the number of degraded substrates. During the perturbation without aeration, both communities degraded only polymers and carbohydrates (Appendix A). This behavior, vital to the survival of most organisms degrading plant material, must be done with enzymes secreted extracellularly because the substrate molecules are too large to cross the cell membrane [29].

In both Runs 1 and 2, functions not observed in any pure cultures, and thus probably resulting from synergistic interaction of the microbes, were especially ephemeral and dependent upon the condition of the community. All synergistic functions were interrupted during perturbation, but some recovered afterward (Appendix A). Thus, the enzymatic alliance necessary for synergistic functions to appear in microbial communities #1 and #2 was fragile and easily disrupted by stress on the bacterial community.

PERSISTENT STRAINS DID NOT GUARANTEE PERSISTENT FUNCTIONS – Unlike the strains themselves, functional activities did not persist throughout either Run 1 or Run 2. Temporal shifts in functional profiles of microbial communities have been noted and linked with succession in some communities [30-33]. In the current study, the appearance and persistence of metabolic functions was affected by the length of time in mixed culture and the aeration rate of the community. The functional profiles of both communities took longer to stabilize than the density profiles.

Environmental conditions, most notably the difference in baseline rate of aeration between the two communities, had a profound influence on the functional profiles exhibited by the same 4 strains in the two communities. Run 1, which had a higher aeration rate, maintained higher densities of bacteria and degraded up to 47 substrates on the functional assay plates. Run 2, which had lower aeration for most of its duration, degraded a total of 37 different substrates, with no more than 30 different substrates being degraded at a time. This difference in the behavior of the two experimental communities supports previous findings which report that bacteria express different properties under different growth conditions, especially with respect to inactivating metabolic activities when conditions are adverse [29,34-36]. The physical presence of bacteria in community #2 did not guarantee detection of metabolic functions of which those bacteria were known to be capable, and that those bacteria were actually expressing in parallel community #1. This finding is consistent with published reports of strains being present in a community but not performing metabolic functions of which they were known to be capable [34,36,37].

SUMMARY AND CONCLUSIONS

The natural heterogeneity of fibrous plant structural molecules and nominal operating cyclicity of bioconversion components of ALS systems seemed to promote niche partitioning that in turn facilitated the long-term maintenance of a 4-strain model microbial community with stable and resilient population densities. However, the relationship between population dynamics of a bacterial community and the dynamics of metabolic activities performed by those bacteria was complex and can be grouped into 3 main categories, as reviewed below.

- Persistence of strains and functions in mixed culture

  (1) Long-term maintenance was achieved for bacterial populations in stable communities that were degrading inedible plant matter. **(2)** Persistence of strains in a microbial community did not ensure the persistence of functional activities performed by those strains. **(3)** Functions obtained by synergistic interactions between strains were especially susceptible to deactivation during adverse conditions.

- Community response to perturbation

  (1) Population densities were more resistant to perturbation and more resilient than functional activities. **(2)** Bacterial population densities recovered fully from perturbation, but only about 80% of lost functions were recovered within 2 weeks after perturbation. **(3)** Strains and functions that degraded polymers and carbohydrates were less affected by perturbation than other strains and functions.

- Relationship between population structure and functional dynamics

  (1) Changes in functional activity were not dictated solely by population dynamics; metabolic activity could be repressed or induced by environmental factors, both biotic and abiotic. **(2)** Since not all possible functions for a strain were detected as soon as a strain was above the threshold density for detection on the BIOLOG®-GN assay plates, it was confirmed that bacterial behavior is different in mixed culture than it is in pure culture.

Since functional activity was not determined solely by bacterial population densities, monitoring the total microbial biomass and gross CO_2 production in ALS bioreactors will not be sufficient indicators of specific degradative processes. Even in the more diverse communities used in operational bioreactors at JSC and KSC, the mere persistence of cellulose- or lignin-degrading microbes in a community will not guarantee that carbon bound into cellulose and lignin is being adequately recovered. The persistence of key degradative processes will need to be monitored.

Fortunately, abiotic factors that affect microbial behavior can be controlled in an artificial community constructed for an ALS. Knowledge that behavior of the microorganisms can be influenced by human operators will be useful in further refining microbial bioreactors. For instance, although functional profiles did not recover from perturbation as quickly as they stabilized after initial establishment of the communities, external manipulation of abiotic factors may be able to accelerate a more complete functional recovery. The delayed appearance of metabolic activities 5-weeks into the cultivation of Run 2 indicates that if the species are present in the community, it is possible to alter culture conditions to induce functional activities.

For the specific ALS application of a mixed microbial community to convert inedible plant residue into microbial biomass and CO_2, the positive correlation between persistence of functions and the polymer and carbohydrate substrates that account for most of the mass of the plant residue to be degraded by these communities is good news for building stable microbial bioreactors. However, trying to integrate additional waste streams, such as human bodily wastes or aquaculture effluent, may require maintaining functions which are not as stable in a mixed community. Again, the persistent success of specific functional activities will need to be confirmed.

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ADDITIONAL SOURCES

Footnotes for Appendix A:

[a] Positive responses in all replicates indicated by: • during aeration, ♦ during periods of no aeration. Clear cells had no indication of metabolism (zero positive responses). Numbers indicate the number of positive responses in cases where not all replicates gave identical responses.

[B] Compounds listed in bold italics were not degraded by any of the strains in pure culture, but were metabolized in one or both mixed cultures.

[C] Strain 6a had 2 replicate BIOLOG®-GN profiles, Day 8 of Run 2 had 4 replicates, all other samples had 5 replicate profiles.

[d] -P indicates no aeration or aeration reduced below baseline, +P indicates aeration increased above baseline.

DEFINITIONS, ACRONYMS, ABBREVIATIONS

ALS: advanced life support
BIOLOG®-GN: assay plate for Gram negative bacteria made by the BIOLOG company
CELSS: controlled ecological life-support system
CFU: colony-forming units
CFU/mL: colony-forming units per milliliter of medium
G^+: Gram positive
G^-: Gram negative
G^{var}: Gram variable
HARV: High Aspect-ratio Rotating-wall Vessel
JSC: Johnson Space Center
KSC: Kennedy Space Center
SAB+cam: Sabouraud-dextrose with chloramphenicol (plates)
SMG: simulated microgravity
TSA: tryptic soy agar (plates)
TSB: tryptic soy broth (liquid)

APPENDIX A. DEGRADATION OF SOLE-CARBON SOURCES ON BIOLOG®-GN ASSAY PLATES 24 H AFTER INOCULATION WITH PURE CULTURES OF STRAINS 6A, 9, 13A, AND 19 OR INOCULATION WITH SAMPLES FROM MIXED CULTURE RUNS 1 AND 2[A].

		Pure Strains[c]				Run 1[c,d]									Run 2[c,d]								
										-P	-P			-P					-P	-P		+P	+P
	Compound[b]	6a	9	13a	19	1	8	15	22	24	29	36	43	50	1	8	15	22	26	29	36	43	50
Polymers	α-cyclodextrin			●	3	●	●	●	●	♦	♦	●	●	♦	●	●	●	●	2		●	●	●
	dextrin	●	●	●	4	●	●	●	●	♦	♦	●	●	♦	●	●	●	●	♦	♦	●	●	●
	glycogen	●		●		●	●	●	●	♦	♦	●	●	♦	●	●	●	●	♦	♦	●	●	●
	tween 40	●	●	●	●	●	●	●	●	♦	♦	●	●	♦	●	●	●	●	♦	♦	4	●	●
	tween 80	●			●		●	●	●	♦		4										●	●
Carbohydrates	***N-acetyl-D-galactosamine***					●			●	1					●	●							
	N-acetyl-D-glucosamine			●	●	●	●	●	●	♦	♦	●	●	♦	●	●	●	●	♦	♦	●	●	●
	L-arabinose			●		●	●	4	●	♦	♦	●	●	♦	●	●	●	●	♦	1	●	●	●
	D-arabitol				●																		
	cellobiose			●		●	●	●	●	♦	♦	●	●	♦	●	●	●	●	♦	♦	●	●	●
	D-fructose			●	●	●	●	●	●	♦	♦	●	●	♦	●	●	●	●	♦	♦	●	●	●
	L-fucose								1	3			3		●								
	D-galactose			●		●	●	●	●	♦	♦	●	●	♦	●	●	●	●	♦	♦	●	●	●
	gentiobiose			●		●	●	●	●	♦	♦	●	●	♦	●	●	●	●	♦	♦	●	●	●
	α-D-glucose	●		●	●	●	●	●	●	♦	♦	●	●	♦	●	●	●	●	♦	♦	●	●	●
	α-D-lactose			●		●	●	●	●	♦	♦	●	●	♦	●	●	●	●	♦	♦	●	●	●
	lactulose			●		●	●	●	●	♦	4	●	●	♦	●	●	●	●	♦	2	●	●	●
	maltose	●		●	4	●	●	●	●	♦	♦	●	●	♦	●	●	●	●	♦	♦	●	●	●
	D-mannitol				●			●	4			●	●									●	●
	D-mannose			●	●	●	●	●	●	♦	♦	●	●	♦	●	●	●	●	♦	♦	●	●	●
	D-melibiose			●		●	●	●	●	♦	♦	●	●	♦	●	●	●	●	♦	♦	4	●	●
	β-methyl D-glucoside			●		●	●	●	●	♦	♦	●	●	♦	●	●	●	●	♦	4	●	●	●
	psicose				●																		
	D-raffinose			●		●	●	●	●	♦	♦	●	●	♦	●	●	●	●	♦	♦	●	●	●
	L-rhamnose					●		4	●	♦		2	●	2	●	3							
	D-sorbitol				●																		
	sucrose	●		●	●	●	●	●	●	♦	♦	●	●	♦	●	●	●	●	♦	♦	●	●	●
	D-trehalose	●		●	●	●	●	●	●	♦	♦	●	●	♦	●	●	●	●	♦	♦	●	●	●
	turanose	●		●		●	●	●	●	♦	♦	●	●	♦	●	●	●	●	♦	4	●	●	●
	xylitol				4																		
Esters	methyl pyruvate	●	●		●		●	●	●	♦		●	●	♦									
	mono-methyl succinate	●		●	●		4	●	●	♦		●	●	♦								●	3
Carboxylic Acids	acetic acid	●			●		4	●	●	♦			●										
	formic acid				●																		
	D-gluconic acid							3					3									1	4
	D-glucosaminic acid				●																		
	D-glucuronic acid					1									●								
	α-hydroxybutyric acid	●			3			2	●	♦			●										
	b-hydroxybutyric acid				●																		
	p-hydroxyphenylacetic acid		3			●	●	●	●	♦		●	●	3	1							2	●
	α-ketobutyric acid	●			●			●	●	♦			●										
	α-ketoglutaric acid				●																		
	α-ketovaleric acid	●																					
	D,L-lactic acid	●						●	●	♦		●	●	2									
	propionic acid				●			1	●	1			2										
	sebacic acid	●			●																		
	succinic acid	●			●				●	1													
Amino Acids	***L-alanylglycine***						1	4	●	♦					●								
	L-asparagine				●																		
	L-aspartic acid				●																		
	L-glutamic acid	●			●				●														
	hydroxy-L-proline														●								
	L-phenylalanine	●																					
	L-proline			3			●	2	●	3				1									2
	L-pyroglutamic acid	●							●	1													
	L-serine	●		2	●		3	●	●	♦			●	1	●								
	L-threonene	●			3		2		●	3			2										
Amides	succinamic acid				●																		
Amines	putrescine		●			●	●	●	●	2		●	●	1								●	●
	2-aminoethanol				●																		
Alcohols	2,3-butanediol				●																		
	glycerol				●		●	●	●	♦		●	●	♦								1	
Brominated Compounds	bromosuccinic acid				●				3														
Aromatic Compounds	urocanic acid	●						1	●	♦			1										
	thymidine	1																					
Phosphorylated Compounds	***glucose-1-phosphate***														2								