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12 Isolation of free-living dinitrogen-fixing bacteria and their activity in compost containing de-
13 inking paper sludge

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1 **Abstract**

2 Knowledge of the microbiology of dinitrogen (N₂)-fixing bacteria in compost rich in de-inking
3 paper sludge (DPS) is limited. Dinitrogen (N₂)-fixing bacteria from DPS composts were isolated
4 and studied for their N₂-fixing activity in vitro and in vivo. Two Gram-negative N₂-fixing
5 isolates were identified as *Pseudomonas*. At 20°C, both isolates revealed N₂-fixing activity
6 higher than three arctic *Pseudomonas* strains. Their N₂-fixing activity was found to occur
7 between 18 and 25°C, a pattern that was similar to the reference isolate *Azotobacter* ATCC 7486.
8 Composts successfully showed N₂-fixing activity after carbohydrate amendments both with and
9 without inoculation of a N₂-fixing isolate. These results suggest that DPS composts support N₂-
10 fixing bacteria and that N₂-fixing activity is dependent on a usable carbohydrate source.

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12
13 Key words: dinitrogen-fixing bacteria, compost, paper sludge, glucose, *Pseudomonas balearica*,
14 *P. putida*

15

1 **1. Introduction**

2 In Quebec, approximately 3.5 Mt of solid waste are produced per year, including 2 Mt
3 produced by the pulp and paper industries. Among these paper wastes, the de-inking process of
4 used paper produces a waste by-product, called de-inking paper sludge (DPS). The
5 characterization of DPS and its compost has indicated that it is relatively safe for the
6 environment and that composting is one promising avenue of recycling (Beauchamp et al. 2002).

7 The high carbon (C) : nitrogen (N) ratio of DPS (250) is the main factor that limits
8 composting. In addition, its high lignocellulose nature increased the time required to compost
9 this material (Brouillette et al. 1996, Charest and Beauchamp 2002). Manure applied at 0.6 to
10 1.0% N decreased the C : N ratio of the composted DPS, but this produced an immature compost
11 having a C : N ratio higher than 25 after 24 weeks of composting (Beauchamp et al. 2002,
12 Charest and Beauchamp 2002). Surprisingly, among the N treatments investigated, the lowest C :
13 N ratio and highest N content were obtained with the 0.6% N treatment, and support the
14 hypothesis that N₂-fixing bacteria contribute to N increases (Charest and Beauchamp 2002).

15 Jain et al. (1987) have reported important differences among free-living N₂-fixing
16 bacteria in their ability to fix atmospheric N₂, and found that these bacteria were adapted to their
17 environment. It is well known that various microorganisms fix atmospheric N₂ such as rhizobia
18 in symbiosis with legumes, and *Azospirillum* in association with plant roots (Steenhoudt and
19 Vanderleyden 2000). In the soil, free-living bacteria, including *Pseudomonas*, also fix N₂ (Chan
20 et al. 1994). In compost, the presence of *Azomonas*, *Enterobacter*, *Klebsiella*, *Clostridium* and
21 *Bacillus* has been reported after the thermophilic phase (de Bertoldi et al. 1983). Inoculation of
22 composts with cellulose decomposing isolates of *Bacillus* sp., *Cephalosporium* sp. and
23 *Streptomyces* sp. increased N₂-fixing activity (Kostov et al. 1991). However, inoculation of
24 compost with N₂-fixing bacteria has received little attention, even though compost rich in

1 lignocellulose has potential N₂-fixing properties (Keeling et al. 1994). In direct line with these
2 observations, attempts were made to select N₂-fixing bacteria from compost to increase the
3 chance to successfully introduce free-living N₂-fixing bacteria, and thus, increase N₂-fixing
4 activity in compost.

5 The source of carbohydrate is important to allow N₂ fixation activity, which requires
6 large amounts of energy and reducing equivalents (Chan et al. 1994). Increases in atmospheric
7 N₂ fixation in soils can readily be shown by the addition of glucose (Azam et al. 1988, Sindhu
8 and Lakshmirayana 1992, Keeling et al. 1996), where the N₂-fixing bacteria have an ecological
9 advantage over other soil microorganisms since they can use the carbohydrates to fix
10 atmospheric N₂ and use the N-derived molecules for their metabolic needs (Keeling et al. 1994).
11 Amending DPS compost with N₂-fixing bacteria and carbohydrate after its thermophilic phase
12 could be an interesting avenue to supply nitrogen to compost rich in lignocellulose and having a
13 C : N ratio higher than 25 after several weeks of composting.

14 The aims of the present study were, (i) to isolate and identify N₂-fixing bacteria from
15 different DPS composts following the thermophilic stage, (ii) to characterize and compare the
16 optimal N₂-fixing temperature and N₂-fixing activity of isolated bacteria to other known N₂-
17 fixing bacteria, and (iii) to evaluate the effect of carbohydrate amendments on N₂-fixing activity
18 of non-sterile compost *in vitro* and on N₂-fixing activity, total C, total N and the C : N ratio of
19 non-sterile compost *in vivo*.

20

21 **2. Materials and methods**

22 *2.1. Isolation of dinitrogen-fixing bacteria*

23 The DPS composts used were obtained from “Les Composts du Québec, Inc.” (Saint-
24 Henri-de-Lévis, QC, Canada). Three composts from the maturation platforms were selected to

1 isolate N₂-fixing bacteria through enrichment cycles (Beauchamp et al. 1991). These composts
2 were 0.5-year-old, one-year-old and three-year-old. Briefly, for each compost, serial dilutions
3 were performed in 100 mM MgSO₄ diluent solution. Five 50-ml flasks containing 20 mL of N-
4 free medium with vitamins (Beauchamp et al. 1991) were subsequently inoculated with 100 µL
5 of the 10⁻² dilution from the compost suspension (cycle 0). The source of carbon was L-malic
6 acid at 5 g L⁻¹. These flasks were subsequently incubated at 25 °C for 7 days, then 100 µL were
7 transferred to fresh medium and incubated again for 7 days. Three enrichment cycles were
8 performed. After each enrichment cycle, serial dilutions were performed and spread on Tryptic
9 Soy Agar (TSA) to recover the putative N₂-fixing bacteria. Putative N₂-fixing bacterial colonies
10 were purified on TSA. These isolates were then evaluated for their ability to fix N₂ on the NFM
11 by the acetylene reduction assay (Beauchamp et al. 1991). Briefly, each flask was closed with a
12 Suba Seal (William Freeman Company Ltd., Barnsley, England) and 10% (v/v) of the air was
13 replaced by acetylene. The flasks were incubated for 30 min at room temperature and ethylene
14 concentration was determined by injecting 0.2 mL of air sample in a gas chromatograph (model
15 8310B; Perkin-Elmer, Montréal, QC, Canada) equipped with a flame ionization detector and a 2
16 m Porapak N column (100 to 120 mesh). The temperatures of the detector, injection port and
17 oven were 150, 100 and 50°C, respectively.

18 The isolates that showed N₂-fixing activity were identified by fatty acid analysis of their
19 cell membrane using the microbial identification system and the similarity index of the computer
20 libraries (MIDI, Rev 4.10) (Bacterial Strain Identification and Mutant Analysis Service, Dept. of
21 Entomology and Plant Pathology, Auburn University, Auburn, AL, USA). In addition, the strains
22 were identified using Biolog (GN-NENT) and using 16S ribosomal DNA (Bacteria Identification
23 services, Dép. de biologie, Université de Sherbrooke, Sherbrooke, QC, Canada), using standard
24 procedures for DNA isolation (Lance et al. 1985, Takeuchi et al. 1996) and comparing 16S

1 rDNA sequences to GenBank.

2

3 2.2 *N₂-fixing activity (Acetylene reduction activity; ARA)*

4 Under industrial composting conditions of eastern Canada, compost reached the
5 maturation phase by the end of the summer (i.e., August) and, therefore, N_2 -fixing activity
6 needed to be quantified at cool temperatures. Initially, the N_2 -fixing isolates from the DPS
7 composts were compared to known N_2 -fixing bacteria. Canadian arctic strains were selected
8 since their N_2 -fixing activities has been demonstrated under cool temperatures (Lifshitz et al.
9 1986). The N_2 -fixing isolates from the DPS composts, isolates 92-3F and 94-3E, and three arctic
10 *Pseudomonas* strains GR3-5, GR17-4 and GR2-2 were grown on TSA for 48 h at 20°C and used
11 for inoculation for the acetylene reduction activity experiments. This was prepared by placing 10
12 mL of $MgSO_4$ (100 mM) solution on TSA, dislodging the bacteria with a hockey stick-shaped
13 glass rod, placing the bacterial suspension into a sterile centrifuge tube and centrifuging the
14 suspension. The bacteria were washed twice by repeating the suspension into buffer and the
15 centrifugation steps. The optical density of the inocula was adjusted to 0.5 at 780 nm, which
16 represented approximately 10^8 colony-forming units (cfu) mL^{-1} , and 0.1 mL of this suspension
17 was inoculated into 7 mL of NFM in 12 ml tubes. These tubes were incubated at 20°C, and after
18 3, 7, 9, 11 and 14 days, N_2 -fixing activity (ARA) was measured on a different tube at each
19 sampling date.

20 Nitrogen-fixing activity of four bacteria was determined at temperatures ranging from 12
21 to 25°C. These temperatures were thought to be representative of compost maturation
22 temperatures by the end of the summer to early fall conditions in eastern Canada. Two N_2 -fixing
23 bacteria isolated from DPS composts, isolates 92-3F and 94-3E, were compared to two reference
24 strains, *Azotobacter chroococum* (ATCC7486) and *Pseudomonas* (GR3-5). These isolates were

1 grown and their inoculum was prepared as described above, except that cultures were incubated
2 at 12, 15, 18, 21 and 25°C, and ARA was measured at day 5, 8, 11 and 15.

3

4 *2.3. Effects of in vitro compost amendments on ARA*

5 For this experiment, isolate 94-3E was selected for its relative constant N₂-fixing activity
6 over time at 21°C and to minimize a time interaction in studying the effects of carbohydrate
7 amendments on ARA. Non sterile compost (2.5 g) was placed in test tubes. These tubes were
8 inoculated with 0.5 mL of isolate 94-3E at 10⁷ to 10⁸ cfu mL⁻¹ or 0.5 mL of MgSO₄ solution. In
9 addition, 0.5 mL of a carbohydrate solution containing glucose, L-malic acid or sucrose added to
10 MgSO₄ solution at 20 g L⁻¹, was added to each tube. A control solution, i.e., without a source of
11 carbohydrate in the MgSO₄ solution, was also tested. At day 7, there was re-addition of 0.5 mL
12 carbohydrate or MgSO₄ solution to the compost. The tubes were incubated at 20°C and the
13 acetylene reduction assay was performed daily from day 1 to 9. At each assay, a new set of tubes
14 was used.

15

16 *2.4. Effects of in vivo compost amendments*

17 For this experiment, isolate 92-3F was selected due to its higher ARA compared to isolate
18 94-3E. The DPS compost for the following experiment was obtained from “Les Composts du
19 Québec, Inc.” (Saint-Henri-de-Lévis, QC, Canada); the DPS compost was ending its
20 thermophilic phase (65 to 55°C) and entering the mesophilic phase (approximately 30°C to
21 ambient temperature). By mid-August, nine compost piles of 3 m³ each were placed on a
22 maturation platform. The first treatment was the control that consisted in the application of 20 L
23 of MgSO₄ solution to compost. The second treatment consisted in the application to compost of
24 1 kg of sucrose diluted in 20 L of MgSO₄ solution. The third treatment consisted in the

1 application to compost of 1 kg of sucrose diluted in 20 L of MgSO₄ solution inoculated with *P.*
2 *putida* isolate 92-3F, prepared as mentioned above. The inoculation yielded a population of 2.28
3 x 10⁶ to 7.80 x 10⁶ cfu g compost⁻¹ (dry matter). Treatments were applied on day 0, 7 and 14.

4 The compost piles were mixed manually at the time of the initial applications and every
5 week thereafter. The compost piles were sampled 2 days later. Therefore, the compost piles were
6 sampled on day 2, 9, 16, 30 and 45 after the first application of treatments. Two liters of compost
7 were sampled at five locations (i.e., sub-samples); three sub-samples from the top, the middle
8 and the bottom of the internal part of the pile, and two sub-samples from the top and bottom of
9 the side part of the pile. These sub-samples were placed on ice and analyzed as soon as possible.
10 At each sampling date, bacterial population, N₂-fixing activity, nitrate, ammonium, total carbon
11 and nitrogen and the C : N ratio were determined on each sub-sample. The population of the
12 introduced isolate 92-3F was determined only for the inoculated treatment by spreading dilutions
13 on TSA + rifampicin (100 µg mL⁻¹) since this isolate was resistant to rifampicin. The water
14 content was determined on each sub-sample by drying 100 g of compost at 105°C until constant
15 weight. Nitrate and ammonium were extracted from 20 g of each sub-sample using 20 ml of 2 M
16 KCl and diluted 10 times before their determination was performed by ion chromatography on a
17 Dionex DX 500 chromatograph (Dionex Corporation, Sunnyvale, CA) equipped with AG5 and
18 CS5 columns. The mobile phase was 35 mM KCl with a flow rate of 1 mL min⁻¹. Nitrate and
19 ammonium were detected by the AD20 absorbance module and quantified using PeakNet
20 Software (Dionex Corporation, Sunnyvale, CA, USA). Finally, each compost sub-sample was
21 dried at 42 °C for a week and ground at ca. 100 µm using a ball mill. The sub-samples (200 mg)
22 were subsequently used to determine the total C and N by dry combustion using a LECO
23 Elemental Analyzer Model CNS-1000 (LECO Corp., St. Joseph, MI, USA.) using wheat flour
24 (prod. No. 502-278, LECO Corp., St. Joseph, MI, USA) to generate the calibration curve (%C =

1 44.36 ± 0.62 ; %N = 2.91 ± 0.03).

2

3 2.5. Statistical Analyses

4 For the experiments on N₂-fixing activity, optimal temperature for N₂ fixation and in
5 vitro carbohydrate amendments to compost, a completely randomized design was used, where
6 treatments were the isolates, temperatures or carbohydrate amendments, respectively, using three
7 replications. For each experiment, Bartlett's test was used to test the homogeneity of the
8 variances (Little and Hills 1978) prior to performing analyses of variance using Statistical
9 Analysis System (SAS) procedures (Steel and Torrie 1980, SAS Institute, Inc. 1990). When the
10 F test was significant, the treatment means were compared using the Least Significant Difference
11 (LSD) test (Little and Hills, 1978).

12 For the experiment on *in vivo* compost amendments, a three-replicate split-plot design
13 was set up. The main treatments were "control (MgSO₄ solution)", "compost amended with
14 sucrose", "compost inoculated with *P. putida* 92-3F and amended with sucrose", whereas the
15 sampling dates were the split factor. Again, Bartlett's test was used to test the homogeneity of
16 the variances (Little and Hills 1978); here, only the N₂-fixing activities were transformed to
17 $\log_{10}(\text{N}_2\text{-fixing activity} + 1)$ and the values presented in the text or tables are their antilog_{10}
18 (Little and Hills 1978). No difference was noticed among the five sub-samples (sampling
19 locations) within each compost pile; thus, their averages were used to perform the analysis of
20 variance (Gomez and Gomez 1984) using the General Linear Models (GLM) procedure of SAS.
21 The analyses of variance included partitioning the treatments into single degree of freedom
22 contrasts. The interpretation of results was mainly based on the means comparison of the
23 treatment "control vs. compost amended with sucrose" and "control vs. compost inoculated with
24 *P. putida* 92-3F and amended with sucrose" when the interaction over time was not significant

1 (i.e. water content, total C, total N, C:N ratio). However, when the interaction was significant
2 between the treatment and the time factors (the linear and quadratic effects of time),
3 subsequently the significant interaction contrast was presented.

4

5 **3. Results**

6 *3.1. Isolation of dinitrogen-fixing bacteria*

7 The N₂-fixing activity increased at each cycle for the 3- and 1-year-old composts, but
8 decreased after two cycles for the 0.5-year-old compost (Table 1). A total of 83 isolates were
9 obtained after three cycles of enrichment; 30 bacteria were isolated from the three-year-old
10 compost, 12 from the one-year-old compost and 41 from the 0.5-year-old compost. Among these
11 isolated bacteria, only four were found to be able to fix atmospheric N₂. To perform the
12 diagnostic tests, the N₂-fixing bacteria were grown on TSA, but the isolates from the 0.5-year-
13 old compost lost their ability to fix atmospheric N₂.

14 According to the fatty acid analyses, isolate 92-3F was identified as *Pseudomonas putida*
15 with a level of match of 0.76, whereas isolate 94-3E was identified as *Pseudomonas balearica*
16 with a level of match of 0.73, but a second choice match of 0.71 as *Pseudomonas stutzeri*. The
17 fatty acid composition of the two isolates appears in Table 2. The Biolog identification system
18 failed to identify the isolates. Finally, the 16S rDNA indicated affiliations with the *Pseudomonas*
19 genus, but failed to confirm the species of the isolates.

20

21 *3.2. N₂-fixing activity (Acetylene reduction activity; ARA)*

22 During the 14 days of incubation at 20° C, *Pseudomonas* isolates 92-3F and 94-3E
23 showed higher N₂-fixing activity than the arctic *Pseudomonas* strains GR3-5, GR17-4 and GR2-
24 11 (P<0.05; Figure 1). The two compost isolates showed N₂-fixing activity 5 to 100-fold higher

1 than these reference strains. Among the GR strains, only GR3-5 was selected as reference strain
2 for the subsequent study. In all cases, the bacterial population increased from day 3
3 (approximately log 7.7) to day 9 (approximately log 8.7), where the maximum were noted, and
4 subsequently decreased until day 14 (approximately log 8.3).

5 The temperature affected significantly the N₂-fixing activity of the two isolates and
6 reference strains tested. *Pseudomonas* isolates 92-3F and 94-3E showed a peak N₂-fixing activity
7 among 18 to 25°C, compared to a peak N₂-fixing activity among 21 to 25°C for *Azotobacter*
8 *chroococum* ATCC7486 and 12 to 15 ° for the arctic strain GR3-5 (Figure 2). The arctic strain
9 GR3-5 exhibited a 10-fold lower activity compared to the others.

10

11 3.3. Effects of *in vitro* compost amendments on ARA

12 No N₂-fixing activity was measured when MgSO₄ solution or MgSO₄ solution plus *P.*
13 *balearica* isolate 94-3E were added to non-sterile compost (Figure 3). In contrast, at 0 and 7 days
14 after glucose or sucrose amendment, and 7 days after L-malic acid amendment, N₂-fixing
15 activity was detected regardless of the compost inoculation with *Pseudomonas* isolate 94-3E
16 (Figure 3). The second carbohydrate amendment 7 days after incubation stimulated the N₂-fixing
17 activity more than the first amendment, and again, regardless of the presence of *Pseudomonas*
18 isolate 94-3E.

19

20 3.4. Effects of *in vivo* compost amendments

21 During the course of this experiment, the population of *Pseudomonas* isolate 92-3F was
22 determined in the inoculated treatment only, and its population showed a decrease over time.
23 Two days after inoculation, its population was log₁₀ 6.6 cfu g⁻¹ compost and decreased to 6.2 to
24 6.0 to 5.8 and to 5.4 log₁₀ cfu g⁻¹ on days 9, 16, 30 and 45 after inoculation, respectively.

1 The temperatures of the compost piles were affected differently by the treatments over
2 time (Treatments*Time $P < 0.05$; Figure 4A). This interaction was mainly due to the different
3 rates of decrease in temperature from day 0 to 2. The amendments of sucrose combined with
4 *Pseudomonas* isolate 92-3F resulted in higher raise in temperature than the control ((Control vs
5 Suc+92-3F)*time quadratic $P < 0.01$). The temperatures increased sharply from day 2 to 16 for
6 the amendments of sucrose combined with *Pseudomonas* isolate 92-3F and decreased thereafter,
7 whereas for the control, the temperatures remained almost similar from day 2 to day 16 and
8 decreased thereafter. The sucrose amendments resulted in slightly higher raise in temperature
9 than the amendments of sucrose combined with *Pseudomonas* isolate 92-3F (Sucrose vs Suc+92-
10 3F)*time quadratic $P < 0.05$). The temperatures of the sucrose amendments alone increased from
11 day 2 to 9 but decreased thereafter.

12 The N_2 -fixing activities were affected differently by the treatments over time, where a
13 treatment by time interaction was again noted. The control differed from the sucrose
14 amendments and the sucrose amendments combined with *Pseudomonas* isolate 92-3F treatments
15 ((Control vs Suc)*time quadratic and (Control vs Suc+92-3F)*time quadratic: both $P < 0.01$;
16 Figure 4B). This effect was mainly attributed to the stimulated N_2 fixation in the DPS compost
17 during the first 10 to 30 days in the sucrose amendments and the sucrose amendments combined
18 with *Pseudomonas* isolate 92-3F, compared to the control treatment. No significant difference in
19 N_2 fixation was noted between sucrose amendments and sucrose amendments combined with
20 *Pseudomonas* isolate 92-3F treatments (Figure 4B).

21 On average and for all treatments, the ammonium content increased from day 2 to 16 and
22 decreased thereafter (figure 4C) and the nitrate content was minimal in the compost pile at 30
23 days after the amendments and increased thereafter (figure 4D). However, the increases in
24 ammonium content were not similar among treatments over time, as well as the decreases and

1 increases in nitrate content that were more pronounced for the sucrose amendments combined
2 with *Pseudomonas* isolate 92-3F than for the control or sucrose treatments ((Control vs Suc+92-
3 3F)*time quadratic and (Sucrose vs Suc+92-3F)*time quadratic: $P < 0.01$ for both nitrate and
4 ammonium; Figures 4C and D).

5 For the water content of the compost piles, the interaction of the main factors was not
6 significant. The water content, averaged over the treatments, increased from the formation of
7 piles to day 16 due to rainfall. However, it decreased thereafter due to dry weather ($P < 0.05$). On
8 the other hand, the water content, averaged over time, indicated that the sucrose amendments
9 combined with *Pseudomonas* isolate 92-3F exhibited lower water content than the control ($P <$
10 0.0001 ; Table 3). However, the sucrose amendments combined with *Pseudomonas* isolate 92-3F
11 had similar water content to the sucrose amendments alone.

12 For the total N and C of the compost piles, the time factor and the interaction of the main
13 factors were not significant. The total N content of compost, averaged over time, was higher in
14 the sucrose amendments combined with *Pseudomonas* isolate 92-3F than control ($P < 0.001$;
15 Table 3), but the total N content of compost was similar to the sucrose amendments alone. The
16 total C content of compost, averaged over time, was lower with the sucrose amendments
17 combined with *Pseudomonas* isolate 92-3F than control ($P < 0.05$; Table 3), but the total C
18 content of compost was higher with the sucrose amendments alone compared to the sucrose
19 amendments combined with *Pseudomonas* isolate 92-3F ($P < 0.001$; Table 3).

20 For the C: N ratio, the interaction of the main factors was not significant. Over time, the
21 C : N ratio, averaged over treatments, decreased linearly with an average over all treatments of
22 about 36.8 on days 2, 9 and 16, to 36.2 on day 30, to 36.1 on day 45. For the C : N ratio,
23 averaged over time, the sucrose amendments combined with *Pseudomonas* isolate 92-3F had the
24 lowest value compared to control and the sucrose amendments ($P < 0.001$ both contrasts; Table

1 3).

2

3 **4. Discussion**

4 In this study, four isolates, out of the 83 originally obtained from compost, i.e. 5%, were
5 able to reduce acetylene, an indirect measure of N₂ fixation. This result indicates that DPS
6 composts are an ecological niche for N₂-fixing bacteria after their thermophilic phase. Even
7 using N-free culture medium, other bacteria that were not N₂-fixing were isolated. The N
8 required for their growth probably came from trace amounts of N in the medium or from the
9 bacterial consortium based on the mutual exchange of fixed carbon and nitrogen (Veal and
10 Lynch 1984). This mutual exchange might explain the loss of N₂-fixing ability observed from the
11 0.5-year old compost isolates when they were in pure culture on TSA medium. TSA is a non-
12 selective medium used here to cultivate isolates that grow easily under laboratory conditions; the
13 possibility of using the N-free culture medium to purify the isolates would more likely not
14 prevent the loss of mutual bacteria, since eventually the mutual exchange would not be possible
15 when the isolates would be in pure culture.

16 Two free-living N₂-fixing bacteria survived the purification process. The present results
17 demonstrate that both isolates belong to the genus *Pseudomonas*. Over the years, *Pseudomonas*
18 isolates reported to fix atmospheric N₂ have been derived from soil (Anderson 1955, Eckford et
19 al. 2002) or the root zone (Paul and Newton 1961, Lifshitz 1986, Zlotnikov et al. 1997) but to
20 our knowledge, this is the first report of free-living N₂-fixing *Pseudomonas* isolated from
21 compost. The fatty acid profile of isolate 92-3F identified it as *P. putida*, that is known to be a
22 N₂-fixing bacterium (Chan et al. 1994); and isolate 94-3E as *P. balearica*, a new specific
23 genomovar of *P. stutzeri* strains (Bennasar et al. 1996). However, Biolog failed to identify these
24 strains, whereas the 16S rDNA only confirmed the *Pseudomonas* genus.

1 For the *in vitro* compost inoculation experiment averaged over both treatments (without
2 and with inoculum), amendments of carbohydrates to non-sterile compost stimulated ARA in the
3 following order of effectiveness; sucrose > glucose > L-malic acid > MgSO₄ solution only; and
4 inoculation of the compost with *Pseudomonas* isolate 94-3E had little effect on this activity. In
5 addition, the results suggested that the first amendment of carbohydrate may have been used for
6 the multiplication and growth of inoculated and indigenous free-living N₂-fixing bacteria
7 (Keeling et al. 1996) whereas the second amendment may have been used for N₂ fixation
8 activity.

9 For the *in vivo* compost experiment, *Pseudomonas* isolate 92-3F was preferred due to its
10 higher ARA compared to *Pseudomonas* isolate 94-3E. In general, the control compost exhibited
11 higher water content and lower temperature than the compost amended with sucrose treatments.
12 These differences in water content support the hypothesis that the sucrose-treated composts had a
13 higher microbial activity resulting in a higher temperature that favored water evaporation
14 compared to the control under similar weather conditions. Without carbohydrate amendments,
15 the compost-C was essentially not available to indigenous free-living N₂-fixing bacteria to
16 support a measurable level of ARA. Nevertheless, when sucrose amendments were applied to
17 compost piles, the N₂-fixing activity increased for up to 10 days, and during that time, the
18 ammonium content of compost increased whereas the nitrate content decreased. In fact, the
19 increase in ammonium content is known to be related to N₂ fixation by free-living N₂-fixing
20 bacteria and excretion of ammonium into the medium (Steenhoudt and Vanderleyden 2000),
21 whereas the nitrate decrease suggested N immobilization by compost microorganisms (Kostov et
22 al. 1991). After 30 days, the ammonium content of compost was possibly oxidized or the cool
23 temperatures were leading to the death of the microorganisms, both processes leading to an
24 increase in nitrate content.

1 Overall, the present results demonstrate the beneficial effect of sucrose amendments to
2 increase the total N content of compost compared to the control. In addition, the sucrose
3 amendments combined with *Pseudomonas* isolate 92-3F decreased the total C content compared
4 to the control and sucrose treatments. A situation similar to other co-inoculation studies may be
5 occurring in the inoculated piles where the N₂-fixing bacteria gave ammonium to other compost
6 microorganisms that degrade lignocellulose and gave a carbon source to N₂-fixing bacteria. Most
7 studies have investigated the impact of inoculation by N₂-fixing bacteria, alone or in co-
8 inoculation, on straw or wood decomposition (Spano et al. 1982, Veal and Lynch 1984, Halsall
9 and Gibson 1986, Kostov et al. 1991, Abd-Alla et al. 1992). For the sucrose amendments
10 combined with *Pseudomonas* isolate 92-3F, the successful increase in total N and decrease in
11 total C gave a lower C : N ratio compared to other treatments and suggests that a lower C : N
12 ratio can be reached for the lignocellulolytic composts in a shorter period of time. The differences
13 in total C and total N were less than 1 %, but gave C:N ratio decreases of 1 to 2 units. In regard
14 to the recalcitrant nature of this lignocellulolytic wastes, this decrease in C and increase in N by
15 sucrose and *P. putida* over 45 days is an important step to decrease the maturation period and
16 increase the microbial quality of compost with regard to sustainable agriculture, especially under
17 the cold and humid conditions of Eastern Canada.

18 In conclusion, this study showed that approximately 5% of the population of DPS
19 composts consisted of free-living N₂-fixing bacteria which belong to the *Pseudomonas* genus.
20 These isolates have the ability to fix atmospheric N₂ when temperatures are appropriate and a
21 source of carbohydrate is available. Under field conditions, when sucrose was amended to non-
22 sterile compost after its thermophilic stage, N₂-fixation was observed. Also, evidence is provided
23 from this study that sucrose amendments combined to compost inoculation with a N₂-fixing
24 isolate resulted in a C decrease, resulting in a compost with a lower C : N ratio. Even though the

1 C : N decrease was slight, this was the first time that we successfully decreased it during the
2 maturation phase; a beneficial decrease since the composting processes are maximal only during
3 the summer months. A more complete understanding of the ecology of free-living N₂-fixing
4 bacteria in compost is required to facilitate the development of new strategies for compost
5 production when wastes rich in lignocellulose are used.

6

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14

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12 activity, stability and the ratio of components in mixed cultures of diazotrophic bacteria.
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- 14

1 Table 1. Isolation of N₂-fixing bacteria from different de-inking sludge composts through
 2 enrichment cycles on nitrogen-free medium containing 5 g L⁻¹ of L-malic acid, and mean N₂-
 3 fixing activity of the isolated N₂-fixing bacteria.

4

Compost Age (year)	Enrichment Cycle	Dinitrogen Fixation (nmol C ₂ H ₄ ml ⁻¹ h ⁻¹)	Number of Isolates	Number of N ₂ - fixing bacteria
3	1	2.9	21	0
	2	4.1	5	0
	3	4.9	4	1
1	1	1.1	9	0
	2	1.3	2	0
	3	2.0	1	1
0.5	1	2.0	32	0
	2	1.8	9	2
	3	1.2	0	0

5

6

1 Table 2. Fatty acid composition of N₂-fixing isolates 92-3F and 94-3E.

2 Numbers in parentheses are standard errors of means.

3

Fatty acid	Isolate	
	92-3F	94-3E
	<i>P. putida</i>	<i>P. balearica</i>
% of total fatty acids		
10 :0	ND -	0.32 (0.02)
10 :0 3-OH	4.13 (0.47)	4.44 (0.08)
12 :0	2.49 (0.16)	7.48 (0.03)
12 :0 2-OH	5.13 (0.06)	ND -
12 :0 3-OH	4.22 (0.06)	2.95 (0.01)
14 :0	0.39 (0.04)	0.45 (0.05)
15 :0	0.30 (0.00)	0.80 (0.27)
16 :0	27.64 (0.06)	16.07 (0.54)
17 :1 ω 8 <i>cis</i>	0.17 (0.00)	0.29 (0.00)
17 :0 cyclo	10.32 (0.66)	0.41 (0.13)
17 :0	0.32 (0.00)	0.52 (0.00)
18 :1 ω 7 <i>cis</i>	21.92 (0.18)	32.55 (0.47)
18 :0	0.44 (0.01)	0.30 (0.02)
19 :0 cyclo ω 8 <i>cis</i>	2.40 (0.23)	0.25 (0.00)
Summed feature 3	20.55 (0.65)	33.20 (0.91)

4 ND, not detected

5 Summed feature 3 = 16 :1 ω 7*cis*/15 iso 20 H, 15 :0 iso 20 H/16 :1 ω 7*cis*

1 Table 3. Overall means of the water content, total nitrogen and carbon, and the C : N ratio *in vivo*
 2 compost amended with sucrose and inoculated with *P. putida* isolate 92-3F, the MgSO₄ solution
 3 (control), or sucrose alone.

4
5

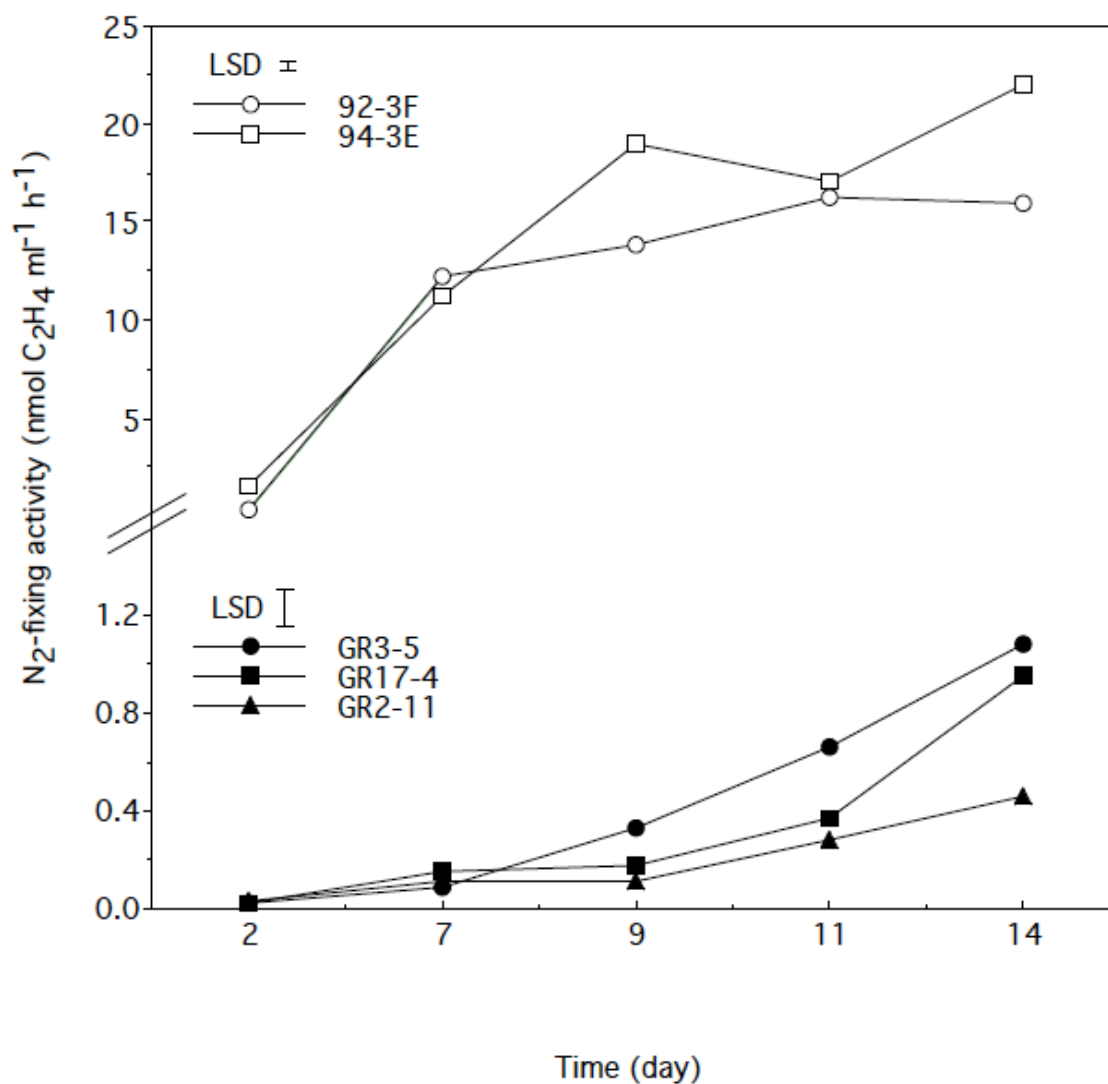
Variable	Treatment			Contrast comparison	
	Sucrose + <i>P. putida</i>	Control	Sucrose	Sucrose + <i>P. putida</i> vs Control	Sucrose + <i>P. putida</i> vs Sucrose
				<i>Level of probability (P)</i>	
Water content (%) ^z	53	57	53	0.0001	NS ^y
Total N (%) ^z	0.74	0.71	0.75	0.001	NS
Total C (%) ^z	26.3	27.0	27.3	0.05	0.001
C : N ^z	35.6	37.8	36.2	0.001	0.001

6 ^z Means of four replicates where each replicate was the mean of five sub-samples.

7 ^y NS = No significant difference between treatments.

8

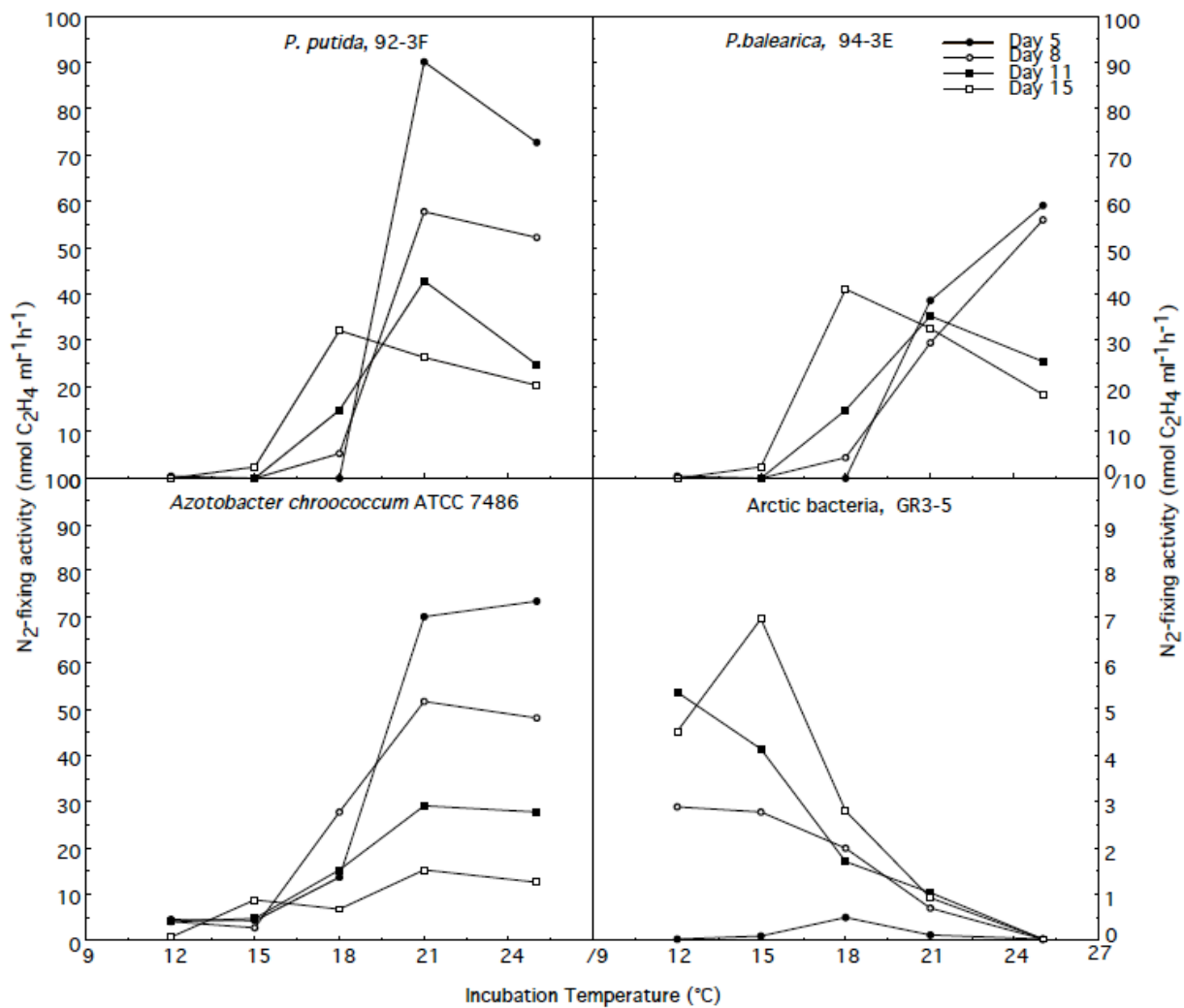
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1
 2 Figure 1. N₂-fixing activity (ethylene production) over time for *Pseudomonas* isolates 92-3F and
 3 94-3E obtained from DPS compost, and arctic *Pseudomonas* strains GR3-5, GR17-4 and GR2-
 4 11 incubated at 20 °C. Data are the means of three replicates. Least significant difference (LSD)
 5 at P < 0.05.

6

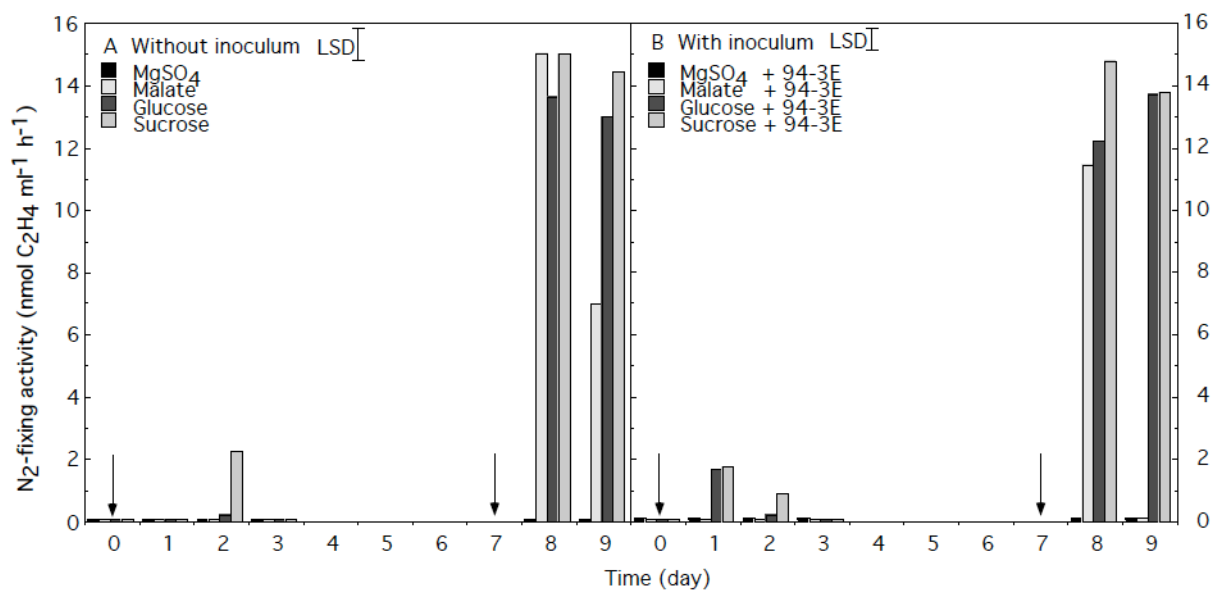
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1
 2 Figure 2. Effect of temperature on the N₂-fixing activity of *P. putida* isolate 92-3F and *P.*
 3 *balearica* isolate 94-3E obtained from composts, *A. chroococcum* strain ATCC7486, and arctic
 4 *Pseudomonas* strain GR3-5 at various incubation temperatures. Data are the means of three
 5 replicates. Least significant difference (LSD) at P < 0.05.

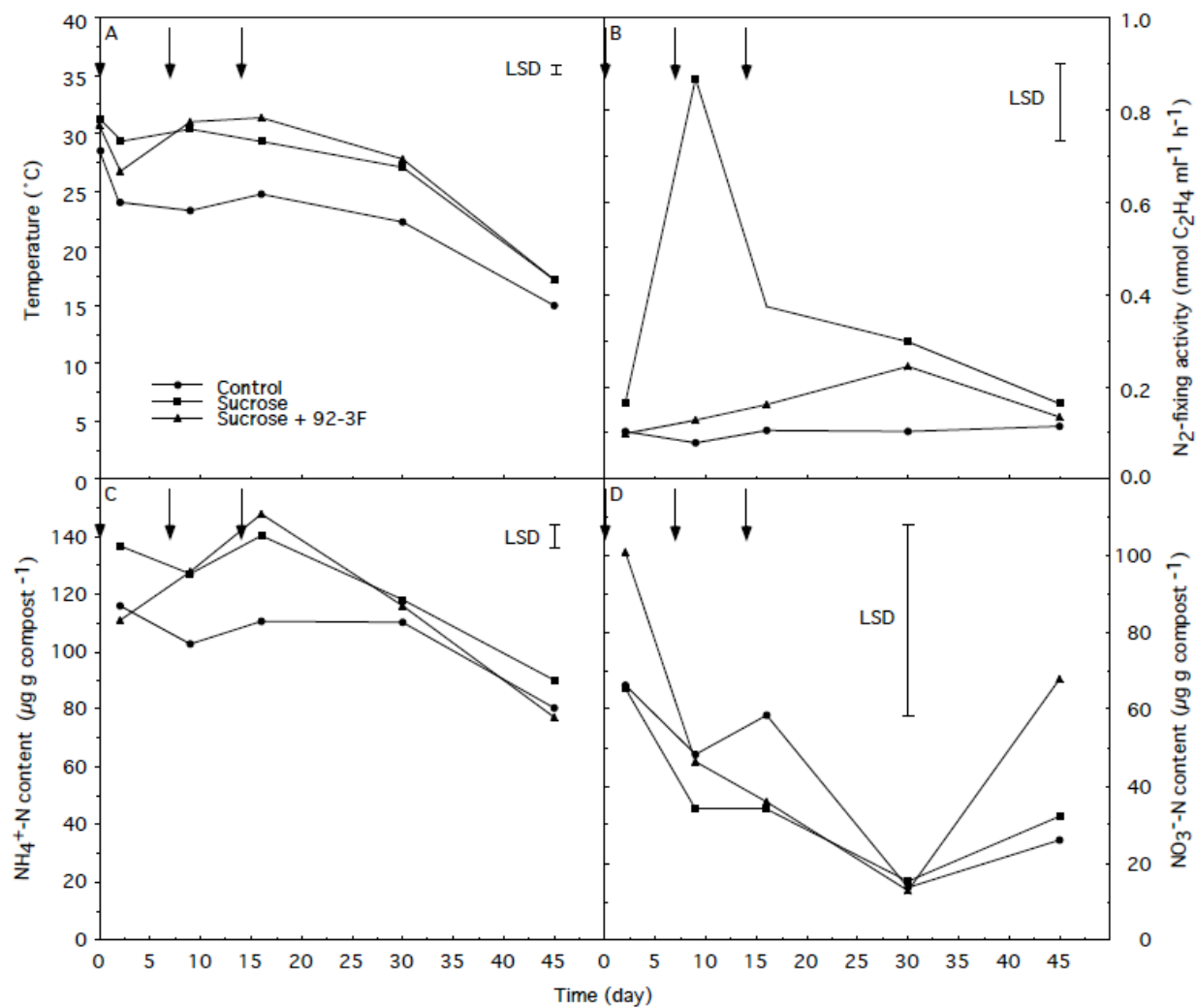
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 2 Figure 3. N₂-fixing activity in compost inoculated without and with *P. balearica* isolate 94-3E,
 3 and amended with different sources of carbohydrates (↓: day of carbohydrate amendment).
 4 Least significant difference (LSD) at P < 0.05.

5



1
 2 Figure 4. Measurement of temperature (A), N₂-fixing activity (B), NH₄⁺-N content (C) and NO₃⁻
 3 -N content (D) of composts over time after their amendments with MgSO₄ solution (control),
 4 sucrose, or sucrose combined with *P. putida* isolate 92-3F (↓: day of sucrose amendment).
 5 Standard Error (SE).
 6