This is an archive post-print (i.e. final draft post-refereeing)

Beauchamp, C.J., Lévesque, G., Prévost, D. and Chalifour, F.-P. 2006. Isolation of free-living dinitrogen-fixing bacteria and their activity in compost containing de-inking paper sludge. Bioresource Technology 97(8): 1002-1011.

DOI: 10.1016/j.biortech.2005.04.041

4/12/2005 1:26:14 PM

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

Chantal J. Beauchamp[1,3], Gabriel Lévesque[2], Danielle Prévost[2] and François-P. Chalifour[1]

[1] Département de phytologie, Université Laval, Sainte-Foy, Québec, G1K 7P4, CANADA

[2] Soils and Crops Research Centre, Agriculture and Agri-Food Canada, Sainte-Foy, Québec, G1V 2J3, CANADA

[3] Corresponding author

Abstract

Knowledge of the microbiology of dinitrogen (N_2)-fixing bacteria in compost rich in de-inking paper sludge (DPS) is limited. Dinitrogen (N_2)-fixing bacteria from DPS composts were isolated and studied for their N_2-fixing activity in vitro and in vivo. Two Gram-negative N_2-fixing isolates were identified as *Pseudomonas*. At 20°C, both isolates revealed N_2-fixing activity higher than three arctic *Pseudomonas* strains. Their N_2-fixing activity was found to occur between 18 and 25°C, a pattern that was similar to the reference isolate *Azotobacter* ATCC 7486. Composts successfully showed N_2-fixing activity after carbohydrate amendments both with and without inoculation of a N_2-fixing isolate. These results suggest that DPS composts support N_2-fixing bacteria and that N_2-fixing activity is dependent on a usable carbohydrate source.

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

1. Introduction

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

The high carbon (C) : nitrogen (N) ratio of DPS (250) is the main factor that limits composting. In addition, its high lignocellulose nature increased the time required to compost this material (Brouillette et al. 1996, Charest and Beauchamp 2002). Manure applied at 0.6 to 1.0% N decreased the C : N ratio of the composted DPS, but this produced an immature compost having a C : N ratio higher than 25 after 24 weeks of composting (Beauchamp et al. 2002, Charest and Beauchamp 2002). Surprisingly, among the N treatments investigated, the lowest C : N ratio and highest N content were obtained with the 0.6% N treatment, and support the hypothesis that N_2-fixing bacteria contribute to N increases (Charest and Beauchamp 2002).

Jain et al. (1987) have reported important differences among free-living N_2-fixing bacteria in their ability to fix atmospheric N_2, and found that these bacteria were adapted to their environment. It is well known that various microorganisms fix atmospheric N_2 such as rhizobia in symbiosis with legumes, and *Azospirillum* in association with plant roots (Steenhoudt and Vanderleyden 2000). In the soil, free-living bacteria, including *Pseudomonas,* also fix N_2 (Chan et al. 1994). In compost, the presence of *Azomonas, Enterobacter, Klebsiella, Clostridium* and *Bacillus* has been reported after the thermophilic phase (de Bertoldi et al. 1983). Inoculation of composts with cellulose decomposing isolates of *Bacillus* sp.*, Cephalosporium* sp. and *Streptomyces* sp. increased N_2-fixing activity (Kostov et al. 1991). However, inoculation of compost with N_2-fixing bacteria has received little attention, even though compost rich in

lignocellulose has potential N_2-fixing properties (Keeling et al. 1994). In direct line with these observations, attempts were made to select N_2-fixing bacteria from compost to increase the chance to successfully introduce free-living N_2-fixing bacteria, and thus, increase N_2-fixing activity in compost.

The source of carbohydrate is important to allow N_2 fixation activity, which requires large amounts of energy and reducing equivalents (Chan et al. 1994). Increases in atmospheric N_2 fixation in soils can readily be shown by the addition of glucose (Azam et al. 1988, Sindhu and Lakshmirayana 1992, Keeling et al. 1996), where the N_2-fixing bacteria have an ecological advantage over other soil microorganisms since they can use the carbohydrates to fix atmospheric N_2 and use the N-derived molecules for their metabolic needs (Keeling et al. 1994). Amending DPS compost with N_2-fixing bacteria and carbohydrate after its thermophilic phase could be an interesting avenue to supply nitrogen to compost rich in lignocellulose and having a C : N ratio higher than 25 after several weeks of composting.

The aims of the present study were, (i) to isolate and identify N_2-fixing bacteria from different DPS composts following the thermophilic stage, (ii) to characterize and compare the optimal N_2-fixing temperature and N_2-fixing activity of isolated bacteria to other known N_2-fixing bacteria, and (iii) to evaluate the effect of carbohydrate amendments on N_2-fixing activity of non-sterile compost in vitro and on N_2-fixing activity, total C, total N and the C : N ratio of non-sterile compost *in vivo*.

2. Materials and methods

2.1. Isolation of dinitrogen-fixing bacteria

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

isolate N_2-fixing bacteria through enrichment cycles (Beauchamp et al. 1991). These composts were 0.5-year-old, one-year-old and three-year-old. Briefly, for each compost, serial dilutions were performed in 100 mM $MgSO_4$ diluent solution. Five 50-ml flasks containing 20 mL of N-free medium with vitamins (Beauchamp et al. 1991) were subsequently inoculated with 100 µL of the 10^{-2} dilution from the compost suspension (cycle 0). The source of carbon was L-malic acid at 5 g L^{-1}. These flasks were subsequently incubated at 25 °C for 7 days, then 100 µL were transferred to fresh medium and incubated again for 7 days. Three enrichment cycles were performed. After each enrichment cycle, serial dilutions were performed and spread on Tryptic Soy Agar (TSA) to recover the putative N_2-fixing bacteria. Putative N_2-fixing bacterial colonies were purified on TSA. These isolates were then evaluated for their ability to fix N_2 on the NFM by the acetylene reduction assay (Beauchamp et al. 1991). Briefly, each flask was closed with a Suba Seal (William Freeman Company Ltd., Barnsley, England) and 10% (v/v) of the air was replaced by acetylene. The flasks were incubated for 30 min at room temperature and ethylene concentration was determined by injecting 0.2 mL of air sample in a gas chromatograph (model 8310B; Perkin-Elmer, Montréal, QC, Canada) equipped with a flame ionization detector and a 2 m Porapak N column (100 to 120 mesh). The temperatures of the detector, injection port and oven were 150, 100 and 50°C, respectively.

The isolates that showed N_2-fixing activity were identified by fatty acid analysis of their cell membrane using the microbial identification system and the similarity index of the computer libraries (MIDI, Rev 4.10) (Bacterial Strain Identification and Mutant Analysis Service, Dept. of Entomology and Plant Pathology, Auburn University, Auburn, AL, USA). In addition, the strains were identified using Biolog (GN-NENT) and using 16S ribosomal DNA (Bacteria Identification services, Dép. de biologie, Université de Sherbrooke, Sherbrooke, QC, Canada), using standard procedures for DNA isolation (Lance et al. 1985, Takeuchi et al. 1996) and comparing 16S

rDNA sequences to GenBank.

2.2 N_2-fixing activity (Acetylene reduction activity; ARA)

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

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

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

2.3. Effects of in vitro compost amendments on ARA

For this experiment, isolate 94-3E was selected for its relative constant N_2-fixing activity over time at 21°C and to minimize a time interaction in studying the effects of carbohydrate amendments on ARA. Non sterile compost (2.5 g) was placed in test tubes. These tubes were inoculated with 0.5 mL of isolate 94-3E at 10^7 to 10^8 cfu mL^{-1} or 0.5 mL of $MgSO_4$ solution. In addition, 0.5 mL of a carbohydrate solution containing glucose, L-malic acid or sucrose added to $MgSO_4$ solution at 20 g L^{-1}, was added to each tube. A control solution, i.e., without a source of carbohydrate in the $MgSO_4$ solution, was also tested. At day 7, there was re-addition of 0.5 mL carbohydrate or $MgSO_4$ solution to the compost. The tubes were incubated at 20°C and the acetylene reduction assay was performed daily from day 1 to 9. At each assay, a new set of tubes was used.

2.4. Effects of in vivo compost amendments

For this experiment, isolate 92-3F was selected due to its higher ARA compared to isolate 94-3E. The DPS compost for the following experiment was obtained from "Les Composts du Québec, Inc." (Saint-Henri-de-Lévis, QC, Canada); the DPS compost was ending its thermophilic phase (65 to 55°C) and entering the mesophilic phase (approximately 30°C to ambient temperature). By mid-August, nine compost piles of 3 m^3 each were placed on a maturation platform. The first treatment was the control that consisted in the application of 20 L of $MgSO_4$ solution to compost. The second treatment consisted in the application to compost of 1 kg of sucrose diluted in 20 L of $MgSO_4$ solution. The third treatment consisted in the

application to compost of 1 kg of sucrose diluted in 20 L of $MgSO_4$ solution inoculated with *P. putida* isolate 92-3F, prepared as mentioned above. The inoculation yielded a population of 2.28 x 10^6 to 7.80 x 10^6 cfu g compost^{-1} (dry matter). Treatments were applied on day 0, 7 and 14.

The compost piles were mixed manually at the time of the initial applications and every week thereafter. The compost piles were sampled 2 days later. Therefore, the compost piles were sampled on day 2, 9, 16, 30 and 45 after the first application of treatments. Two liters of compost were sampled at five locations (i.e., sub-samples); three sub-samples from the top, the middle and the bottom of the internal part of the pile, and two sub-samples from the top and bottom of the side part of the pile. These sub-samples were placed on ice and analyzed as soon as possible. At each sampling date, bacterial population, N_2-fixing activity, nitrate, ammonium, total carbon and nitrogen and the C : N ratio were determined on each sub-sample. The population of the introduced isolate 92-3F was determined only for the inoculated treatment by spreading dilutions on TSA + rifampicin (100 µg mL^{-1}) since this isolate was resistant to rifampicin. The water content was determined on each sub-sample by drying 100 g of compost at 105°C until constant weight. Nitrate and ammonium were extracted from 20 g of each sub-sample using 20 ml of 2 M KCl and diluted 10 times before their determination was performed by ion chromatography on a Dionex DX 500 chromatograph (Dionex Corporation, Sunnyvale, CA) equipped with AG5 and CS5 columns. The mobile phase was 35 mM KCl with a flow rate of 1 mL min^{-1}. Nitrate and ammonium were detected by the AD20 absorbance module and quantified using PeakNet Software (Dionex Corporation, Sunnyvale, CA, USA). Finally, each compost sub-sample was dried at 42 °C for a week and ground at ca. 100 µm using a ball mill. The sub-samples (200 mg) were subsequently used to determine the total C and N by dry combustion using a LECO Elemental Analyzer Model CNS-1000 (LECO Corp., St. Joseph, MI, USA.) using wheat flour (prod. No. 502-278, LECO Corp., St. Joseph, MI, USA) to generate the calibration curve (%C =

44.36 ± 0.62; %N = 2.91 ± 0.03).

2.5. Statistical Analyses

For the experiments on N_2-fixing activity, optimal temperature for N_2 fixation and in vitro carbohydrate amendments to compost, a completely randomized design was used, where treatments were the isolates, temperatures or carbohydrate amendments, respectively, using three replications. For each experiment, Bartlett's test was used to test the homogeneity of the variances (Little and Hills 1978) prior to performing analyses of variance using Statistical Analysis System (SAS) procedures (Steel and Torrie 1980, SAS Institute, Inc. 1990). When the F test was significant, the treatment means were compared using the Least Significant Difference (LSD) test (Little and Hills, 1978).

For the experiment on *in vivo* compost amendments, a three-replicate split-plot design was set up. The main treatments were "control ($MgSO_4$ solution)", "compost amended with sucrose", "compost inoculated with *P. putida* 92-3F and amended with sucrose", whereas the sampling dates were the split factor. Again, Bartlett's test was used to test the homogeneity of the variances (Little and Hills 1978); here, only the N_2-fixing activities were transformed to $\log_{10}(N_2\text{-fixing activity} + 1)$ and the values presented in the text or tables are their antilog_{10} (Little and Hills 1978). No difference was noticed among the five sub-samples (sampling locations) within each compost pile; thus, their averages were used to perform the analysis of variance (Gomez and Gomez 1984) using the General Linear Models (GLM) procedure of SAS. The analyses of variance included partitioning the treatments into single degree of freedom contrasts. The interpretation of results was mainly based on the means comparison of the treatment "control vs. compost amended with sucrose" and "control vs. compost inoculated with *P. putida* 92-3F and amended with sucrose" when the interaction over time was not significant

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

3. Results

3.1. Isolation of dinitrogen-fixing bacteria

The N_2-fixing activity increased at each cycle for the 3- and 1-year-old composts, but decreased after two cycles for the 0.5-year-old compost (Table 1). A total of 83 isolates were obtained after three cycles of enrichment; 30 bacteria were isolated from the three-year-old compost, 12 from the one-year-old compost and 41 from the 0.5-year-old compost. Among these isolated bacteria, only four were found to be able to fix atmospheric N_2. To perform the diagnostic tests, the N_2-fixing bacteria were grown on TSA, but the isolates from the 0.5-year-old compost lost their ability to fix atmospheric N_2.

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

3.2. N_2-fixing activity (Acetylene reduction activity; ARA)

During the 14 days of incubation at 20° C, *Pseudomonas* isolates 92-3F and 94-3E showed higher N_2-fixing activity than the arctic *Pseudomonas* strains GR3-5, GR17-4 and GR2-11 ($P<0.05$; Figure 1). The two compost isolates showed N_2-fixing activity 5 to 100-fold higher

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

The temperature affected significantly the N_2-fixing activity of the two isolates and reference strains tested. *Pseudomonas* isolates 92-3F and 94-3E showed a peak N_2-fixing activity among 18 to 25°C, compared to a peak N_2-fixing activity among 21 to 25°C for *Azotobacter chroococum* ATCC7486 and 12 to 15 ° for the arctic strain GR3-5 (Figure 2). The arctic strain GR3-5 exhibited a 10-fold lower activity compared to the others.

3.3. Effects of in vitro compost amendments on ARA

No N_2-fixing activity was measured when $MgSO_4$ solution or $MgSO_4$ solution plus *P. balearica* isolate 94-3E were added to non-sterile compost (Figure 3). In contrast, at 0 and 7 days after glucose or sucrose amendment, and 7 days after L-malic acid amendment, N_2-fixing activity was detected regardless of the compost inoculation with *Pseudomonas* isolate 94-3E (Figure 3). The second carbohydrate amendment 7 days after incubation stimulated the N_2-fixing activity more than the first amendment, and again, regardless of the presence of *Pseudomonas* isolate 94-3E.

3.4. Effects of in vivo compost amendments

During the course of this experiment, the population of *Pseudomonas* isolate 92-3F was determined in the inoculated treatment only, and its population showed a decrease over time. Two days after inoculation, its population was $\log_{10}$ 6.6 cfu·g^{-1} compost and decreased to 6.2 to 6.0 to 5.8 and to 5.4 $\log_{10}$ cfu g^{-1} on days 9, 16, 30 and 45 after inoculation, respectively.

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

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

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

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

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

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

For the C: N ratio, the interaction of the main factors was not significant. Over time, the C : N ratio, averaged over treatments, decreased linearly with an average over all treatments of 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, averaged over time, the sucrose amendments combined with *Pseudomonas* isolate 92-3F had the lowest value compared to control and the sucrose amendments ($P < 0.001$ both contrasts; Table

3).

4. Discussion

In this study, four isolates, out of the 83 originally obtained from compost, i.e. 5%, were able to reduce acetylene, an indirect measure of N_2 fixation. This result indicates that DPS composts are an ecological niche for N_2-fixing bacteria after their thermophilic phase. Even using N-free culture medium, other bacteria that were not N_2-fixing were isolated. The N required for their growth probably came from trace amounts of N in the medium or from the bacterial consortium based on the mutual exchange of fixed carbon and nitrogen (Veal and Lynch 1984). This mutual exchange might explain the loss of N_2-fixing ability observed from the 0.5-year old compost isolates when they were in pure culture on TSA medium. TSA is a non-selective medium used here to cultivate isolates that grow easily under laboratory conditions; the possibility of using the N-free culture medium to purify the isolates would more likely not prevent the loss of mutual bacteria, since eventually the mutual exchange would not be possible when the isolates would be in pure culture.

Two free-living N_2-fixing bacteria survived the purification process. The present results demonstrate that both isolates belong to the genus *Pseudomonas*. Over the years, *Pseudomonas* isolates reported to fix atmospheric N_2 have been derived from soil (Anderson 1955, Eckford et al. 2002) or the root zone (Paul and Newton 1961, Lifshitz 1986, Zlotnikov et al. 1997) but to our knowledge, this is the first report of free-living N_2-fixing *Pseudomonas* isolated from compost. The fatty acid profile of isolate 92-3F identified it as *P. putida*, that is known to be a N_2-fixing bacterium (Chan et al. 1994); and isolate 94-3E as *P. balearica*, a new specific genomovar of *P. stutzeri* strains (Bennasar et al. 1996). However, Biolog failed to identify these strains, whereas the 16S rDNA only confirmed the *Pseudomonas* genus.

For the *in vitro* compost inoculation experiment averaged over both treatments (without and with inoculum), amendments of carbohydrates to non-sterile compost stimulated ARA in the following order of effectiveness; sucrose > glucose > L-malic acid > $MgSO_4$ solution only; and inoculation of the compost with *Pseudomonas* isolate 94-3E had little effect on this activity. In addition, the results suggested that the first amendment of carbohydrate may have been used for the multiplication and growth of inoculated and indigenous free-living N_2-fixing bacteria (Keeling et al. 1996) whereas the second amendment may have been used for N_2 fixation activity.

For the *in vivo* compost experiment, *Pseudomonas* isolate 92-3F was preferred due to its higher ARA compared to *Pseudomonas* isolate 94-3E. In general, the control compost exhibited higher water content and lower temperature than the compost amended with sucrose treatments. These differences in water content support the hypothesis that the sucrose-treated composts had a higher microbial activity resulting in a higher temperature that favored water evaporation compared to the control under similar weather conditions. Without carbohydrate amendments, the compost-C was essentially not available to indigenous free-living N_2-fixing bacteria to support a measurable level of ARA. Nevertheless, when sucrose amendments were applied to compost piles, the N_2-fixing activity increased for up to 10 days, and during that time, the ammonium content of compost increased whereas the nitrate content decreased. In fact, the increase in ammonium content is known to be related to N_2 fixation by free-living N_2-fixing bacteria and excretion of ammonium into the medium (Steenhoudt and Vanderleyden 2000), whereas the nitrate decrease suggested N immobilization by compost microorganisms (Kostov et al. 1991). After 30 days, the ammonium content of compost was possibly oxidized or the cool temperatures were leading to the death of the microorganisms, both processes leading to an increase in nitrate content.

Overall, the present results demonstrate the beneficial effect of sucrose amendments to increase the total N content of compost compared to the control. In addition, the sucrose amendments combined with *Pseudomonas* isolate 92-3F decreased the total C content compared to the control and sucrose treatments. A situation similar to other co-inoculation studies may be occurring in the inoculated piles where the N_2-fixing bacteria gave ammonium to other compost microorganisms that degrade lignocellulose and gave a carbon source to N_2-fixing bacteria. Most studies have investigated the impact of inoculation by N_2-fixing bacteria, alone or in co-inoculation, on straw or wood decomposition (Spano et al. 1982, Veal and Lynch 1984, Halsall and Gibson 1986, Kostov et al. 1991, Abd-Alla et al. 1992). For the sucrose amendments combined with *Pseudomonas* isolate 92-3F, the successful increase in total N and decrease in total C gave a lower C : N ratio compared to other treatments and suggests that a lower C : N ratio can be reached for the lignocellulolitic composts in a shorter period of time. The differences in total C and total N were less than 1 %, but gave C:N ratio decreases of 1 to 2 units. In regard to the recalcitrant nature of this lignocellulolitic wastes, this decrease in C and increase in N by sucrose and *P. putida* over 45 days is an important step to decrease the maturation period and increase the microbial quality of compost with regard to sustainable agriculture, especially under the cold and humid conditions of Eastern Canada.

In conclusion, this study showed that approximately 5% of the population of DPS composts consisted of free-living N_2-fixing bacteria which belong to the *Pseudomonas* genus. These isolates have the ability to fix atmospheric N_2 when temperatures are appropriate and a source of carbohydrate is available. Under field conditions, when sucrose was amended to non-sterile compost after its thermophilic stage, N_2-fixation was observed. Also, evidence is provided from this study that sucrose amendments combined to compost inoculation with a N_2-fixing isolate resulted in a C decrease, resulting in a compost with a lower C : N ratio. Even though the

C : N decrease was slight, this was the first time that we successfully decreased it during the maturation phase; a beneficial decrease since the composting processes are maximal only during the summer months. A more complete understanding of the ecology of free-living N_2-fixing bacteria in compost is required to facilitate the development of new strategies for compost production when wastes rich in lignocellulose are used.

Acknowledgements

The authors thank "Daishowa, Inc." and Agriculture and Agri-Food Canada for financial support. They are also thanking "Les Composts du Québec, Inc." for providing composts. F.-P. Chalifour acknowledges the support of a Research Grant from the Natural Sciences and Engineering Research Council of Canada (NSERC). Finally, the authors thank Manon Croft, Nadia Goussard and Johanne Tremblay for their technical support. Contribution no XXX of Agriculture and Agri-Food Canada, Sainte-Foy, Québec, G1V 2J3.

References

Abd-Alla, M. H., Omar, S.A., Abdel-Wahab, A. M., 1992. The role of cellulose-decomposing fungi in nitrogenase activity of *Azotobacter chroococcum*. Folia Microbiol. 37 (3), 215-218.

Anderson, G. R., 1955. Nitrogen fixation by *Pseudomonas*-like soil bacteria. J. Bacteriol. 70, 129-133.

Azam, F., Mahmood, T., Malik, A., 1988. Immobilization-remineralisation of NO_3-N and total N balance during the decomposition of glucose, sucrose and cellulose in soil incubated at different moisture regimes. Plant Soil 107 (2), 159-163.

Beauchamp, C. J., Kloepper, J. W., Lifshitz, R., Dion, P., Antoun,, H. 1991. Frequent occurrence

of the ability to utilize octopine in rhizobacteria. Can. J. Microbiol. 37 (2), 158-164.

Beauchamp, C. J., Charest, M.-H., Gosselin, A., 2002. Examination of environmental quality of raw and composting de-inking paper sludge. Chemosphere 46 (6), 887-897.

Bennasar, A., Rossello, M. R., Lalucat, J., Moore, E. R. B., 1996. 16S rRNA gene sequence analysis relative to genomovars of *Pseudomonas stutzeri* and proposal of *Pseudomonas balearica* sp. nov. Int. J. System. Bacteriol. 46 (6), 200-205.

Brouillette, M., Trépanier, L., Gallichand, J., Beauchamp, C., 1996). Composting paper mill deinking sludge with forced aeration. Can. Agr. Eng. 38 (2), 115-122.

Chan, Y.-K., Barraquio, W. L., Knowles, R., 1994. N_2-fixing pseudomonads and related soil bacteria. FEMS Microb. Rev. 13 (1), 95-118.

Charest, M.-H., Beauchamp, C. J., 2002. Composting of de-inking paper sludge with poultry manure at three nitrogen levels using mechanical turning : Behavior of its physico-chemical parameters. Biores. Technol. 81 (1), 7-17.

de Bertoldi, M., Vallini, G., Pera, A., 1983. The biology of composting: a review. Waste Man. Res. 1 (2), 157-176.

Eckford, R., Cook, F. D., Saul, D., Aislabie, J., Foght, J., 2002. Free-living heterotrophic nitrogen-fixing bacteria isolated from fuel-contaminated antartic soils. Appl. Env. Microbiol. 68 (10), 5181-5185.

Gomez, K. A., Gomez, A. A., 1984. Statistical procedures for agricultural research. John Wiley and Sons. Toronto, ON.

Halsall, D. M., Gibson, A. H., 1986. Comparaison of two *Cellulomonas* isolates and their interaction with *Azospirillum brasilense* in degradation of wheat straw and associated nitrogen fixation. Appl. Environ. Microbiol. 51 (4), 855-867.

Jain, D. K., Beyer, D., Rennie, R. J., 1987. Dinitrogen fixation (C_2H_2 reduction) by bacterial

strains at various temperatures. Plant Soil 103 (2), 233-237.

Keeling, A. A., Mullett, J. A. J., Paton, I. K., 1994. Developments in the biology of compost processes. In: A.K.M. Rainbow (Eds.), Why Recycle? Balkema, A.A., Rotterdam, The Netherlands, pp. 1-5

Keeling, A. A., Cater, G. L. F., Cook, J. A., Wilcox, A., 1996. Application of glucose at low concentrations to grass swards in waste-derived compost can significantly increase long-term yields. Plant Soil 184 (1), 117-121.

Kostov, O., Rankov, V., Atanacova, G., Lynch, J. M., 1991. Decomposition of sawdust and bark treated with cellulose-decomposing microorganisms. Biol. Fertil. Soils. 11 (2), 105-110.

Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L., Pace, N. R., 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc. Natl. Acad. Sci. USA. 82 (20), 6955-6959.

Lifshitz, R., Kloepper, J. W., Scher, F. M., Tipping, E. M., Laliberté, M., 1986. Nitrogen-fixing pseudomonads isolated from roots of plants grown in the Canadian high arctic. Appl. Environ. Microbiol. 51 (2), 251-255.

Little, T. M., Hills, F. J., 1978. Agricultural experimentation. Design and analysis. John Wiley and Sons. Toronto, ON.

Paul, E. A., Newton, J. D., 1961. Studies of aerobic non-symbiotic nitrogen-fixing bacteria. Can. J. Microbiol. 7 (1), 7-13.

SAS Institute Inc. 1990. SAS User's Guide: Statistics. Version 6.03, Cary, NC.

Sindhu, S. S., Lakshminarayana, K., 1992. Effect of carbon source amendments on acetylene reduction by *Azotobacter* isolate in sandy loam. Ann. Biol. 8 (1), 81-85.

Spano, S. D., Jurgensen, M. F., Larsen, M. J., Harvey, A. E., 1982. Nitrogen-fixing bacteria in Douglas Fir residue decayed by *Fomitopsis pinicola*. Plant Soil 68 (1), 203-217.

Steel, R.G.D., and Torrie, J.H. 1980. Principles and procedures of statistics: a biometrical approach. McGraw-Hill, New-York.

Steenhoudt, O., Vanderleyden, J., 2000. *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. FEMS Microb. Rev. 24 (4), 487-506.

Takeuchi, T., Sawada, H., Tanaka, F., Matsuda, I., 1996. Phylogenetic analysis of *Streptomyces* spp. causing potato scab based on 16S rRNA sequences. Int. J. Syst. Bacteriol. 46 (2) 476-479.

Veal, D. A., Lynch, J. M., 1984. Associative cellulolysis and dinitrogen fixation by co-cultures of *Trichoderma harzianum* and *Clostridium butyricum*. Nature 310 (5979), 695-697.

Zlotnikov, A. K., Glagoleva, O. B., Umarov, M. M., 1997. Relationships between nitrogenase activity, stability and the ratio of components in mixed cultures of diazotrophic bacteria. Microbiology 66 (6), 676-812.

Table 1. Isolation of N_2-fixing bacteria from different de-inking sludge composts through enrichment cycles on nitrogen-free medium containing 5 g L^{-1} of L-malic acid, and mean N_2-fixing activity of the isolated N_2-fixing bacteria.

Compost Age (year)	Enrichment Cycle	Dinitrogen Fixation (nmol C_2H_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

Table 2. Fatty acid composition of N_2-fixing isolates 92-3F and 94-3E.

Numbers in parentheses are standard errors of means.

Fatty acid	Isolate	
	92-3F	94-3E
	P. putida	*P. balearica*
	% 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*cis*	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*cis*	21.92 (0.18)	32.55 (0.47)
18 :0	0.44 (0.01)	0.30 (0.02)
19 :0 cyclo ω 8*cis*	2.40 (0.23)	0.25 (0.00)
Summed feature 3	20.55 (0.65)	33.20 (0.91)

ND, not detected

Summed feature 3 = 16 :1 ω 7*cis*/15 iso 20 H, 15 :0 iso 20 H/16 :1 ω7*cis*

Table 3. Overall means of the water content, total nitrogen and carbon, and the C : N ratio *in vivo* compost amended with sucrose and inoculated with *P. putida* isolate 92-3F, the $MgS0_4$ solution (control), or sucrose alone.

Variable	Treatment			Contrast comparison	
	Sucrose + *P. putida*	Control	Sucrose	Sucrose + *P. putida* vs Control	Sucrose + *P. putida* vs Sucrose
				Level of probability (P)	
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

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

[y] NS = No significant difference between treatments.

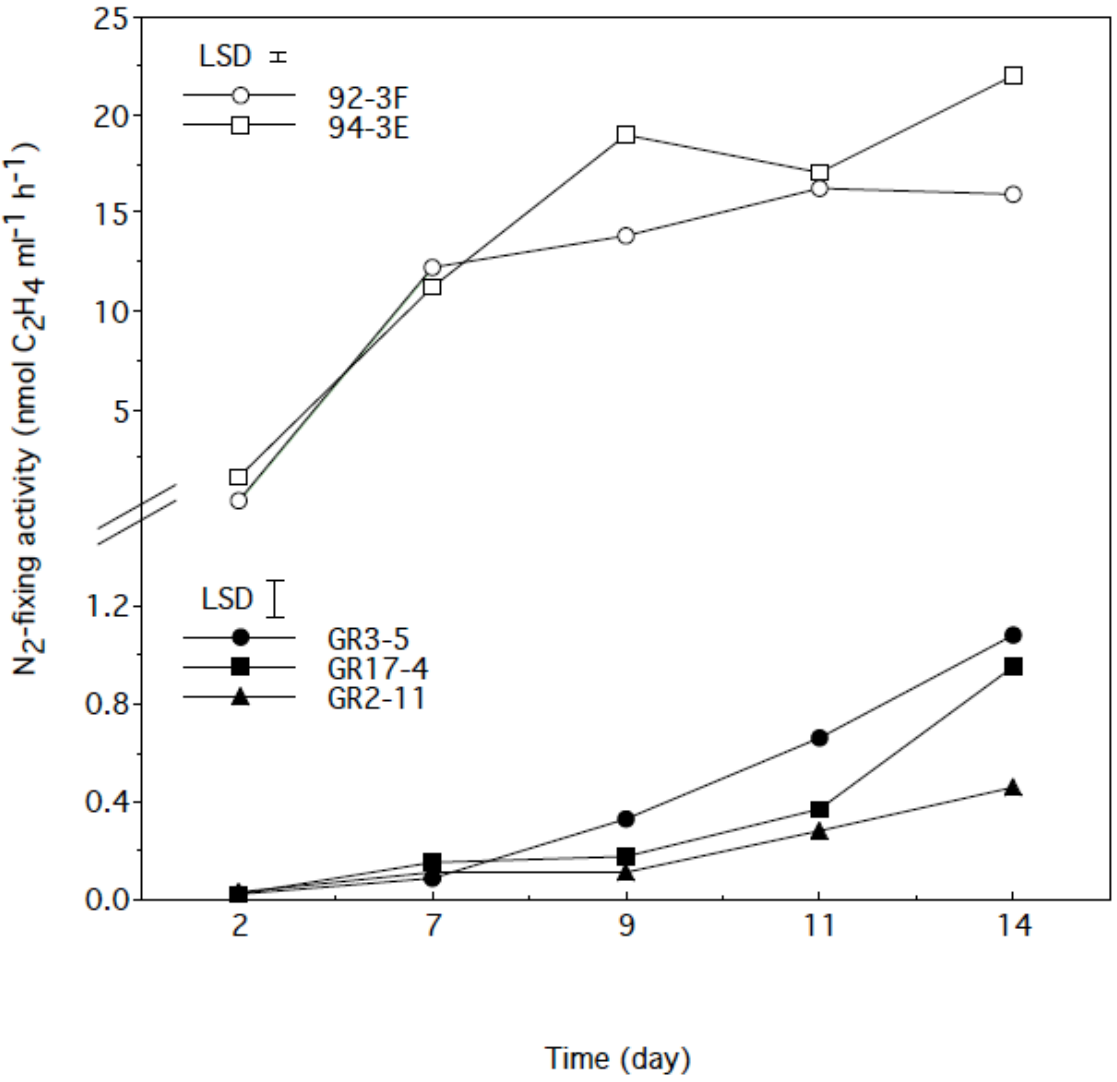

Figure 1. N_2-fixing activity (ethylene production) over time for *Pseudomonas* isolates 92-3F and 94-3E obtained from DPS compost, and arctic *Pseudomonas* strains GR3-5, GR17-4 and GR2-11 incubated at 20 °C. Data are the means of three replicates. Least significant difference (LSD) at $P < 0.05$.

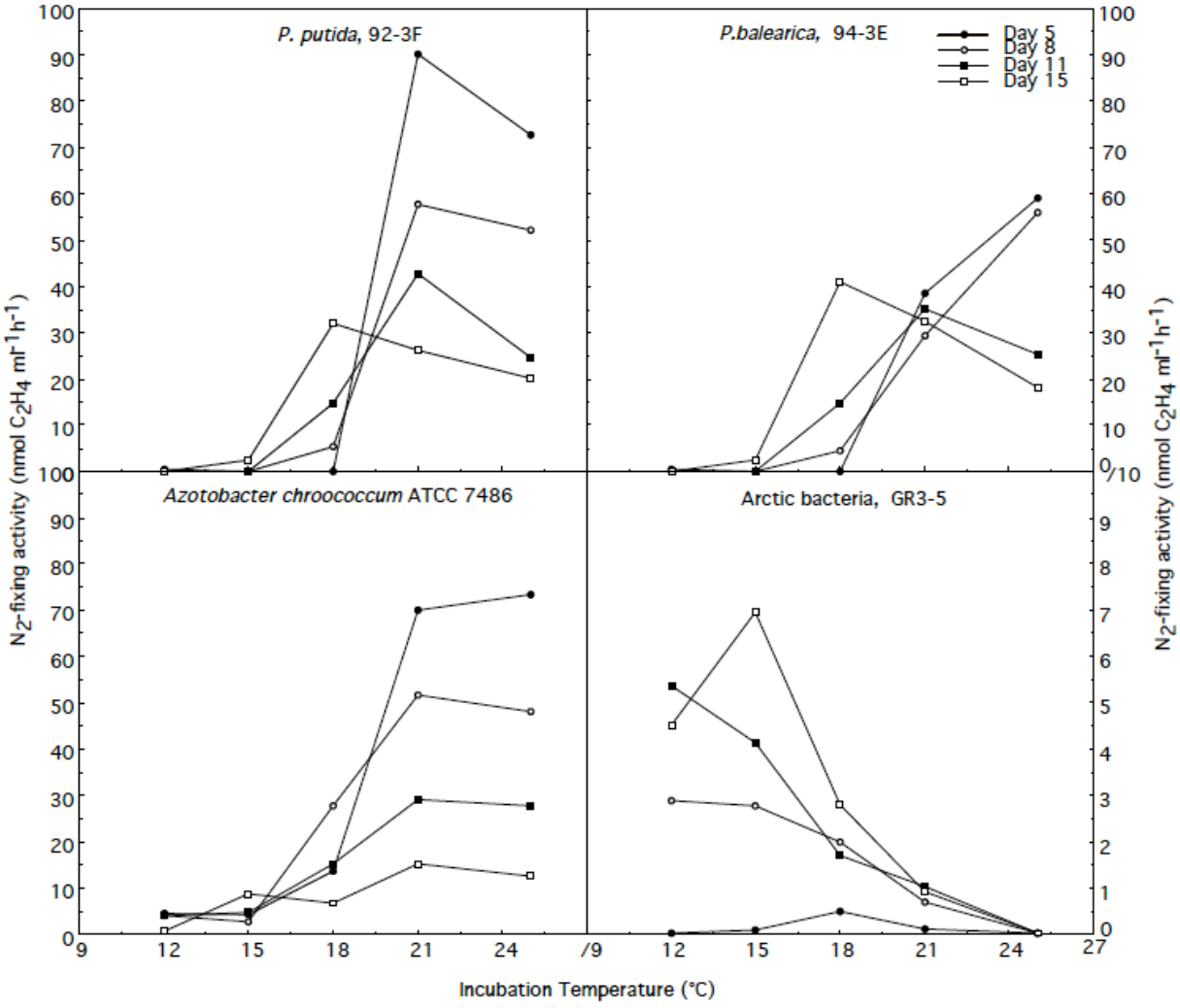

Figure 2. Effect of temperature on the N_2-fixing activity of *P. putida* isolate 92-3F and *P. balearica* isolate 94-3E obtained from composts, *A. chroococcum* strain ATCC7486, and arctic *Pseudomonas* strain GR3-5 at various incubation temperatures. Data are the means of three replicates. Least significant difference (LSD) at $P < 0.05$.

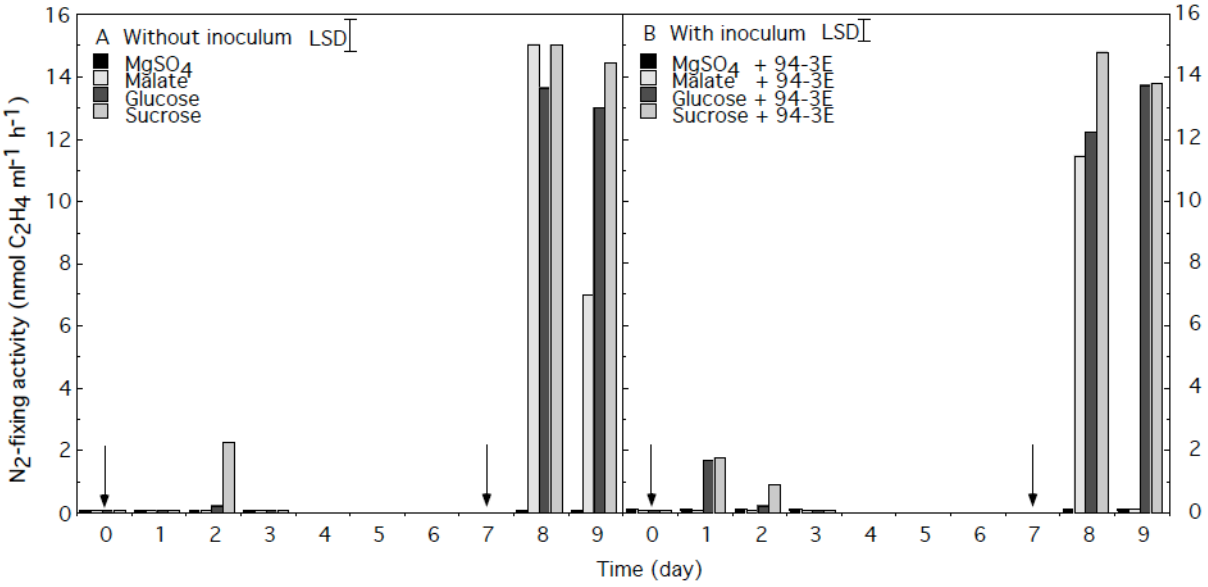

Figure 3. N_2-fixing activity in compost inoculated without and with *P. balearica* isolate 94-3E, and amended with different sources of carbohydrates (↓: day of carbohydrate amendment). Least significant difference (LSD) at $P < 0.05$.

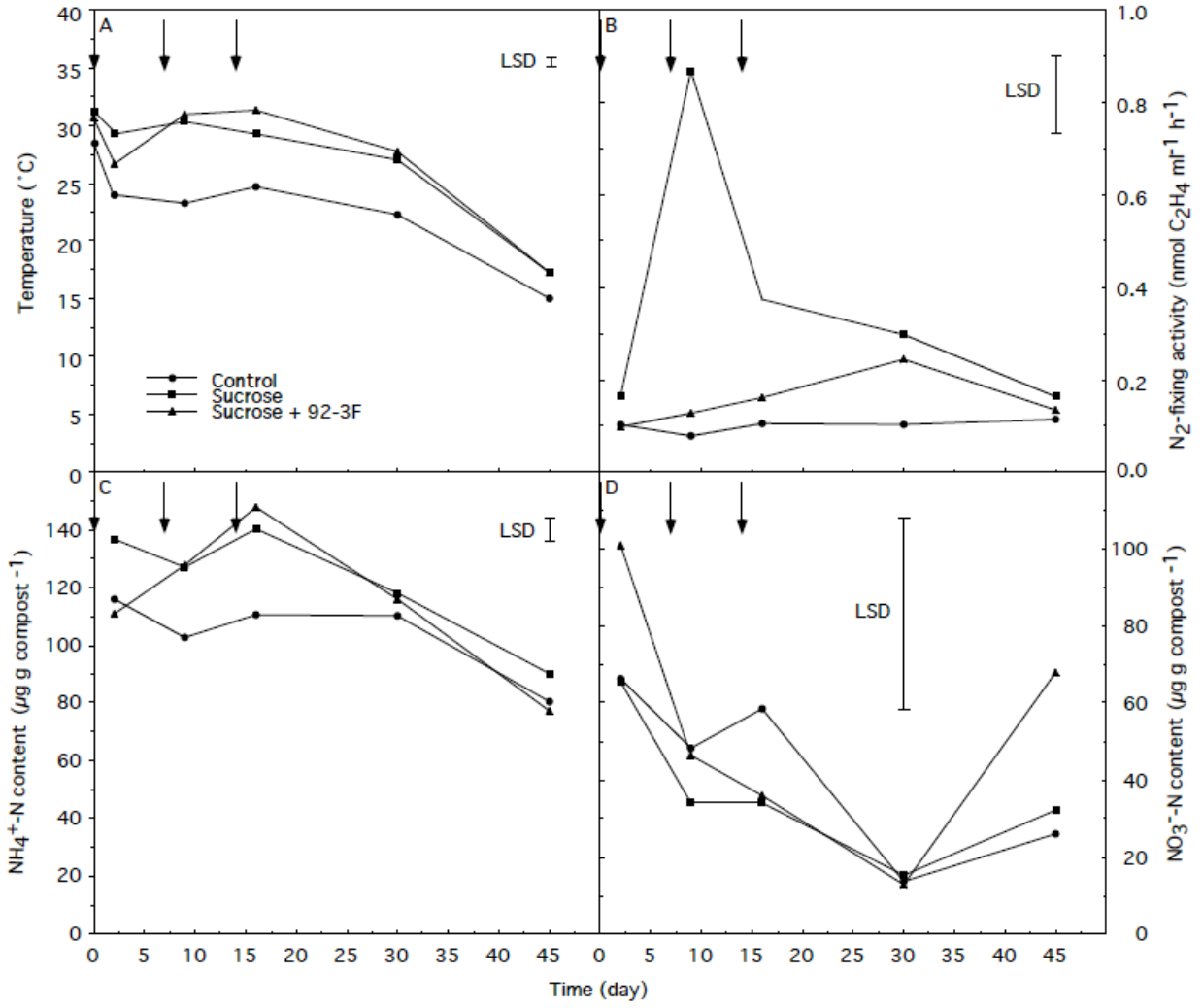

Figure 4. Measurement of temperature (A), N_2-fixing activity (B), NH_4^+-N content (C) and NO_3^--N content (D) of composts over time after their amendments with $MgSO_4$ solution (control), sucrose, or sucrose combined with *P. putida* isolate 92-3F (↓: day of sucrose amendment). Standard Error (SE).