# Rhizosphere Augmentation Using RhizoPlates for Strain Screening and Candidate Selection with Nitrogen Fixing and Phosphate Solubilizing Bacteria

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## Introduction

The rhizosphere, a dynamic microhabitat of soil surrounding plant roots, is teeming with bacteria and fungi that play essential roles in nutrient cycling and crop growth promotion. Nitrogen and phosphorus, two critical nutrients for robust plant development, are often limited in agricultural systems. Enhancing the rhizobial community by introducing bacteria capable of nitrogen fixation and phosphate solubilization offers a promising and sustainable strategy for improving crop yields. The ability to screen and select suitable microbial candidates for these functions is key.

Biolog's RhizoPlates™ in combination with the Odin™ system offer a high-throughput, reliable platform for screening microbial strains or communities for nitrogen fixation (RhizoPlate N) and phosphate solubilization (RhizoPlate P). These assays provide significant advantages over traditional methods such as acetylene reduction assays (ARA) and Pikovskaya's (PVK) agar. In this study, we evaluated a variety of bacterial strains known for their nitrogen-fixing and phosphate-solubilizing abilities. Strain performance was assessed using two approaches: (1) the Average Well Growth Development (AWGD) metric, which allowed us to rank strains based on their overall growth and nutrient-processing capabilities, and (2) an analysis of the impact of individual carbon sources on growth when reliant on atmospheric nitrogen or insoluble phosphate. This comprehensive evaluation enabled the selection of the optimal candidate strains for rhizosphere augmentation, paving the way for sustainable agricultural practices through microbial supplementation.

## Methods

RhizoPlates from Biolog, when used in conjunction with the Odin instrument and software, enable rapid screening of purified strains and rhizobial communities for the ability to fix nitrogen or solubilize phosphate in the presence of 30 different carbon sources, with significantly less hands-on time than traditional methods.


- Side-by-side strain comparisons
- Traditional OD measurements for biomass/growth
- Reads plates every 2-20 minutes for kinetic determinations
- Automatically incubates at a set temperature and reads up to 8 plates (Odin VIII) or 50 plates (Odin L, pictured) at a time
- Automated software analysis and data reporting

**Characterization of candidates for rhizosphere augmentation:** Four strains were selected based on their predicted ability to fix atmospheric nitrogen or solubilize inorganic phosphate. Positive and negative control strains were selected for established nitrogen fixation and phosphate solubilization ability (Table 1). Test strains included C1, C2, C3, and C4 whose phosphate solubilizing and nitrogen fixing phenotypes were predicted using genome analysis (Table 1). Cell suspensions were created for each strain in IF-P (for RhizoPlate P) and IF-N (for RhizoPlate N) to a density of 80% transmittance as measured by a turbidimeter. Suspensions were inoculated into the corresponding RhizoPlates in triplicate at 100 µL per well. Plates were transferred to Odin for incubation at 30 °C and read automatically every 20 minutes for 120 hours.

**Table 1: Strain information**

| Species name or Strain ID | Predicted Phenotypes |
|---|---|
| *Escherichia coli* | Negative control for both $N_2$ fixation and $PO_4$ solubilization |
| *Acinetobacter pittii* | Positive control for $PO_4$ solubilization |
| *Azospirillum brasilense* | Positive control for $N_2$ fixation |
| *Pseudomonas putida* | Positive control for $PO_4$ solubilization |
| *Staphylococcus epidermidis* | Negative control for both $N_2$ fixation and $PO_4$ solubilization |
| *Derxia gummosa* | Positive control for $N_2$ fixation |
| Candidate 1 (C1) | Predicted both $N_2$ fixation and $PO_4$ solubilization |
| Candidate 2 (C2) | Predicted $PO_4$ solubilization only |
| Candidate 3 (C3) | Predicted both $N_2$ fixation and $PO_4$ solubilization |
| Candidate 4 (C4) | Predicted both $N_2$ fixation and $PO_4$ solubilization |

## Methods Continued

**Data Analysis:** RhizoPlate data was analyzed using Odin software v3.2. Kinetic data from each isolate were used to automatically calculate Average Well Growth Development (AWGD), which was plotted for each plate type. To control for intracellular reserves of organic phosphate and nitrogen, each graph was investigated to determine the latest inflection point indicative of when the samples switched from utilizing stockpiled nutrients to fixed nitrogen or solubilized inorganic phosphate. This time point was then used to normalize data across all samples for that plate type to give a corrected AWGD representative of only growth post-transition. Samples were ranked according to the area under the curve (AUC) of the normalized AWGD curves.

## Results: $N_2$ Fixation and $Ca_3(PO_4)_2$ Solubilization

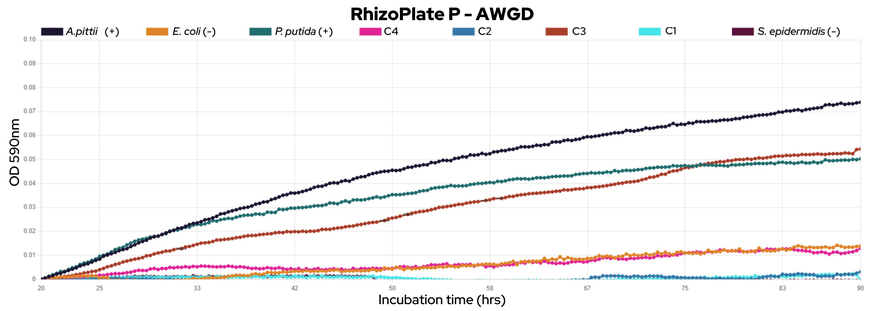


**Figure 1: AWGD for RhizoPlate P shows clear differences in growth between strains when the only source of phosphate was insoluble $Ca_3(PO_4)_2$.** Positive control strains, *A. pittii* and *P. putida* showed the most significant growth followed by candidate C3 whereas the remaining candidates, C1, C2, and C4 failed to surpass baseline growth yielded by the negative controls, *E. coli* and *S. epidemidis,* after 90 hours of incubation.

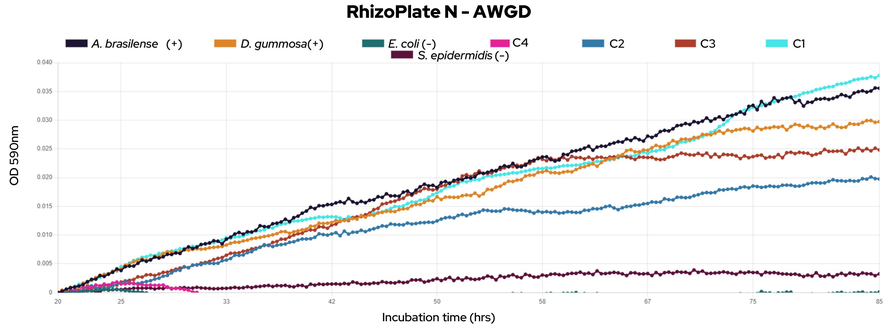


**Figure 1: AWGD for RhizoPlate N shows clear differences in growth between strains when the only source of nitrogen was atmospheric $N_2$.** Positive control strain *A. brasilense* and candidate C1 showed the most significant growth followed by *D. gummosa,* candidate C3, and candidate C2 whereas the remaining candidate, C4, failed to surpass baseline growth yielded by the negative controls, *E. coli* and *S. epidermidis,* after 85 hours of incubation.

| RhizoPlate P Rank | Candidate ID | AWGD AUC |
|---|---|---|
| 1 | *A. pittii* | 17.1 |
| 2 | *P. putida* | 12.16 |
| 3 | C3 | 9.11 |
| 4 | *E. coli* | 2.83 |
| 5 | C4 | 1.74 |
| 6 | C1 | 0.40 |
| 7 | *S. epidermidis* | 0 |
| 8 | C2 | 0 |

**Tables 2 and 3: Ranked AWGD Area Under the Curve (AUC) show C3 is the top performing strain overall.** AUC was calculated for each normalized AWGD curve to quantitatively rank all 8 candidates for RhizoPlate P (Table 2: Left) and RhizoPlate N (Table 3: Right)

| RhizoPlate N Rank | Candidate ID | AWGD AUC |
|---|---|---|
| 1 | *A. brasilense* | 8.35 |
| 2 | C1 | 8.20 |
| 3 | *D. gummosa* | 7.04 |
| 4 | C3 | 5.66 |
| 5 | C2 | 4.57 |
| 6 | *S. epidermidis* | 0.84 |
| 7 | *E. coli* | 0.02 |
| 8 | C4 | 0 |

## Results: Differential Carbon Usage

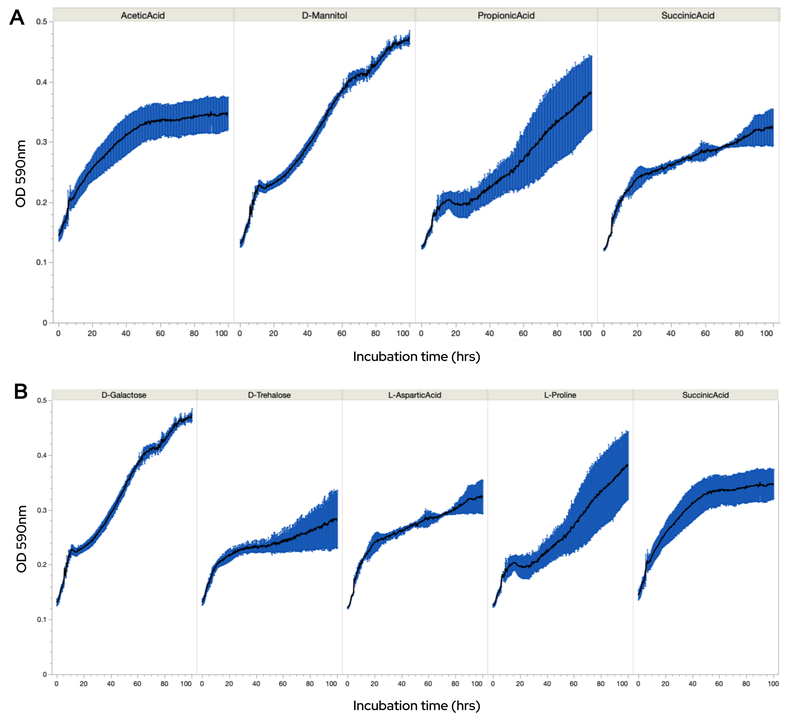


**Figure 3: Carbon source utilization of overall top performing candidate, C3.** Several carbon substrates from RhizoPlates N and P for Candidate C3 show significant growth.
**Panel A** shows significant growth from RhizoPlate N in wells containing acetic acid, D-mannitol, propionic acid, and succinic acid.
**Panel B** shows significant growth from RhizoPlate P in wells containing D-galactose, D-trehalose, L-aspartic acid, L-proline, and succinic acid
These results demonstrate metabolic flexibility in addition to the ability to fix nitrogen and solubilize inorganic phosphate, further solidifying C3 as the ideal augmentation candidate.

## Conclusions

- Significant substrate utilization differences were identified, resulting in sample stratification on AWGD plots.
- Kinetic monitoring using Odin was essential for the rank based on AWGD AUC calculated in Odin software.
- Candidate strain C3 demonstrated both nitrogen fixation and phosphate solubilization, presenting as the optimal candidate for rhizosphere augmentation.
- Communities of strains with differing substrate utilization preferences should be explored as a strategy to develop improved bio-stimulants.
- The combination of Odin and RhizoPlates significantly reduces hands-on time and provides relative quantitation of nitrogen fixing and phosphate solubilizing ability for many strains at the same time.