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PNNL-38762

Quantifying Growth of Three Common Bacterium on Phytic Acid:

Investigating Effects of Media Composition

December 2025

Grace Black

U.S. DEPARTMENT of ENERGY

Prepared for the U.S. Department of Energy
under Contract DE-AC05-76RL01830

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ABSTRACT:

Microbes can produce a plethora of metabolites and enzymes that allow mobilization of various elements and nutrients in the soil, including phosphorus. Phosphorus is essential to plant growth; however, only a limited amount of it is in a bio-accessible inorganic form that can be utilized by most plants. Microbes can produce enzymes called phosphatase, which break down phytic acid (organic phosphorus) into bioavailable inorganic phosphate. My work aimed to understand how inorganic phosphate, phytic acid, and calcium in media affect the growth of *Bacillus subtilis168* (*B. subtilis*)*, Escherichia coli K12 BW25113* (*E. coli*)*, Pseudomonas putida KT2440* (*P. putida*)*,* and a strain of *Pseudomonas putida AG5577_MS238* engineered to produce a phytase. Eight media were made to test how these components affect growth, and an additional two to understand the effects of multiple carbon sources versus one. It was observed that *P. putida's* growth increased in the presence of phytic acid and was strongly driven by nitrate availability. Furthermore, it was able to initiate faster growth when multiple carbon sources were available rather than just one. *E. coli's* growth seemed to be limited when inorganic phosphate was unavailable and little to no growth occurred when phytic acid, calcium, and inorganic phosphate were absent. *B. subtilis* was unable to substantially grow within 28 hrs in any of the mediums tested. The results of this research aid in understanding the growth responses of *P. putida, E. coli,* and *B. subtilis* on less accessible and bioavailable forms of phosphorus.

I. INTRODUCTION

The microbial world underfoot plays a vital role in elemental mobilization and cycling. Microbes can be used to remediate polluted soils, collect desired elements, or produce enzymes of interest. Understanding how microbes can accomplish these functions can contribute to securing sustainable agriculture and bioprocesses for a strong bioeconomy.

Modern agriculture heavily relies on optimizing soil conditions to allow for plant growth. Oftentimes, soil conditions are adjusted using fertilizers to ensure that enough nutrients are available to not limit growth. Phosphorus fertilizers have come to play a crucial role in this endeavor, as it is often a rate limiting nutrient, as only about 0.1% of phosphorus in the soil is in a form that can freely be taken up by plants.[1] However, there is only a finite amount of phosphorus available, since the time required for phosphorus cycling ranges from days to decades.[2] The importance of phosphorus in agriculture and its finite supply chain has resulted in it being added to the critical mineral list.[3, 4] Therefore, finding a solution to secure phosphorus is of great relevance.

Microbes have the potential to be part of the solution to this need. They have a vast set of enzymes; some of particular interest are phytases and phosphatases, which allow for the mobilization of organic phosphorus into accessible inorganic phosphorus. Phosphatases break down a broad range of esters and anhydride phosphoric acids to release inorganic phosphate, while phytases are a specific subset that releases inorganic phosphate and *myo*-inositol phosphate derivatives from phytic acid.[5] Phytases are

categorized into two major groups: alkaline phytases and histidine acid phosphatase.[6] Alkaline phytases require metal bound phytate and, as the name suggests, a more basic pH, while histidine acid phosphatases work with free phytate and require a more acidic environment.[6]

Some bacteria that are known to contain these important enzymes are *Bacillus subtilis 168* (*B. subtilis*) and *Escherichia coli K12 BW25113* (*E. coli*). *B. subtilis* is a model gram-positive bacterium and is known to have a β-propeller phytase (BPP), which is a kind of alkaline phytase.[7] *E. coli* is a model gram-negative microbe and has an appA phytase, which is categorized as a histidine acid phosphatase (HAP).[8] Another microbe that is commonly studied is *Pseudomonas putida (P. putida),* which is a soil gram-negative bacterium. Although the wildtype strain (KT2440) of this microbe has no known or previously reported phytase, it can acidify its environment through the production of organic acids such as gluconic acid.[9] Our team engineered a strain (AG5577_MS238) of *P. putida* that contains a β-propeller phytase.[10]

My project aims to understand how various media compositions including the presence or absence of calcium (as calcium nitrate), nitrate (potassium nitrate), inorganic phosphate (monopotassium phosphate), and phytic acid (phytic acid sodium salt hydrate) affect the growth of *B. subtilis 168*, *E. coli K12*, *P. putida KT2440*, and *P. putida AG5577_MS238*. Furthermore, the effects of single versus multiple mixed carbon/nitrogen substrates where we balanced the total carbon and nitrogen, were also studied in *P. putida*. Understanding how these components affect the growth of these bacteria would allow a better understanding of the metabolic capabilities and requirements driving their growth.

II. METHODS

Bacillus subtilis 168, Escherichia coli K12 BW25113, Pseudomonas putida KT2440, and *Pseudomonas putida AG5577_MS238* were grown in LB liquid cultures and passaged every one to three days to fresh LB. The initial liquid cultures were made from either agar stabs or glycerol stocks. The cultures were kept in a shaking incubator set at 30°C.

Standard Modified Strullu-Romand (MSR) medium (M0) was made along with three variations including MSR without calcium (M1), MSR without inorganic phosphorus (M2), and MSR without calcium or inorganic phosphorus (M3). The media without calcium had additional potassium nitrate, since the calcium was being added as a calcium nitrate compound. These four media were then prepared with and without phytic acid, resulting in a total of eight media.

Media Name	Media Unique Components
M0	Standard MSR
M1	MSR without calcium with extra potassium nitrate
M2	MSR without monopotassium phosphate
M3	MSR without monopotassium phosphate and calcium with extra potassium nitrate

Table 1: Media Composition Key. Summary of media names for testing the effects of calcium, nitrate, and inorganic phosphate and what components were changed. Each of the media were also made with/without phytic acid it is denoted in figures by the media name followed by a dot.

Base stock components for the media were made, which included: a macro solution ($MgSO_4 \times 7H_2O$, KNO_3, KCl), an inorganic phosphate stock(KH_2PO_4), a calcium nitrate stock

($Ca(NO_3)_2$ x 4 H_2O), an iron stock (NaFeEDTA), a micro solution ($MnCl_2$ x 4 H_2O, $ZnSO_4$ x 7 H_2O, H_3BO_3, $CuSO_4$ x 5 H_2O, Na_2MoO_4 x 2 H2O, $(NH_4)_6Mo_7O_{24}$ x 4 H_2O), a phytic acid stock, and a glucose stock all in MilliQ. Each of the stocks were filter sterilized. The phytic acid stock was adjusted to pH 6, and the other base components were combined and adjusted to pH 6 before the phytic acid was added. This was done to eliminate precipitation of the phytic acid.

Two additional media were made to test the effects of multiple carbon sources on the growth of the *P. putida* strains. The media were created to balance the carbon and nitrogen sources, one with multiple forms of carbon (glucose, fructose, trehalose, aspartic acid, citrate and malate) and one with a single source of carbon (glucose). The stock solutions mentioned above were used to form a base MSR with solids being added as needed. The base MSR had no calcium nitrate. The final media were adjusted to a pH of 6.

Growth plates were constructed using overnight cultures of the given bacterium. An initial measurement of optical density at wavelength 600 (OD_{600}) was taken. From the OD_{600} read the culture to medium ratio was adjusted to get an OD_{600} of 0.1. The medium with the culture was spun down for 5 min. at 7,000 x g. The supernatants were removed, and fresh medium was used to resuspend the pellets. Replicates of four (250 uL per well) were added into a 96-well plate with triplicates of blanks, except for the mediums testing carbon source in which replicates of 6 were used with 8 blanks per medium. The protocol for the plate reader was set to maintain a constant temperature of 30°C with double orbital shaking for 28 hours, taking an OD_{600} read every hour.

Data processing was done in R[11] using the Tidyverse packages.[12] The average of the replicates was calculated, and the mean of the corresponding media blanks was subtracted. The resulting data was used to graph a mean growth curve. The average of the blanks (per media) was taken and then subtracted from each data point to generate the dataset used to calculate standard deviations.

III. RESULTS:

A. *Pseudomonas putida KT2440*

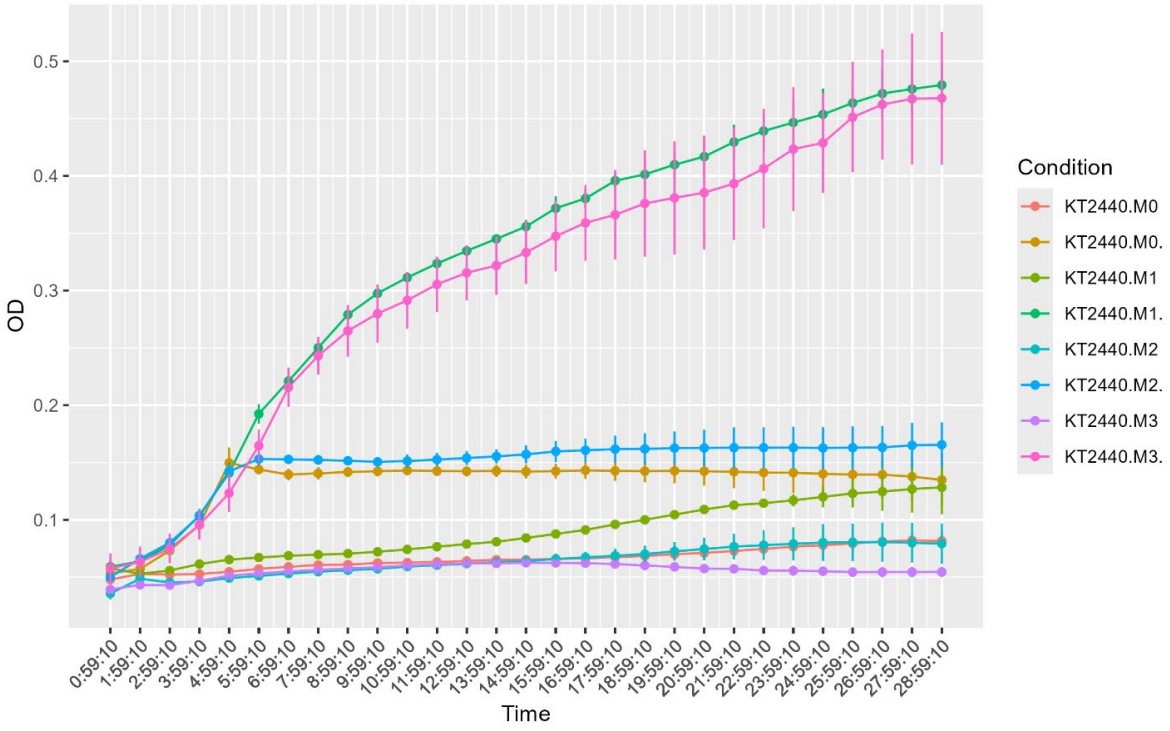


Figure 1: Pseudomonas putida KT2440 wildtype growth curves. Time is on the X-axis and OD_{600} is on the Y-axis. M0 (Red: without phytic acid; Yellow/brown: with phytic acid) is standard media, M1 (moss green: without phytic acid; light green: with phytic acid) is without calcium but has extra nitrate, M2 (turquoise: without phytic acid; blue: with phytic acid) is without inorganic phosphate, M3 (purple: without phytic acid; pink: with phytic acid) is without calcium and inorganic phosphate but has extra nitrate. The dot at the end specifies if there is phytic acid present in the medium.

The data (presented in figure 1) suggest that the wildtype *P. putida* requires phytic acid to grow, since in all the mediums without phytic acid, little to no growth occurred. This

was expected, as very little inorganic phosphate was available to the microbe to utilize. However, since there is no known phytase activity in *P. putida,* this suggests that it can solubilize organic phosphate --- either by producing phytases or acidifying the medium through the release of organic acids. Also, the Na-phytate we used could have some inorganic P in it and this needs to be verified. Moreover, the growth test suggests that nitrate is a key driving factor in the ability for this microbe to grow. When extra potassium nitrate was available a noticeable amount of growth was observed reaching optical densities 2 to 3 times more than what was observed in standard MSR. Removing or adding inorganic phosphorus had little to no effect on its growth. Understanding the effect of calcium on the growth in the wildtype strain is difficult to determine from the data collected as in the absence of calcium extra potassium nitrate was present.

B. *Pseudomonas putida AG_5577 MS238*

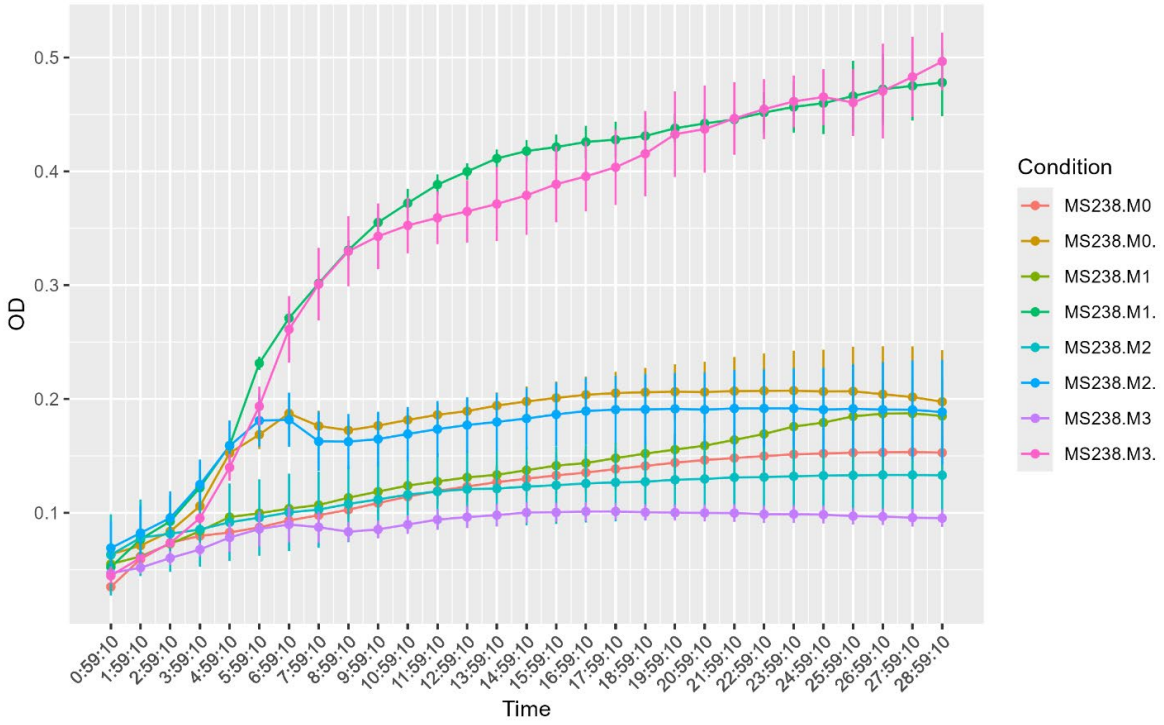


Figure 2: Pseudomonas putida AG5577_MS238 engineered with a BPP growth curves. Time is on the X-axis and OD_{600} is on the Y-axis. M0 (Red: without phytic acid; Yellow/brown: with phytic acid) is standard media, M1 (moss green: without phytic acid; light green: with phytic acid) is without calcium but has extra nitrate, M2 (turquoise: without phytic acid; blue: with phytic acid) is without inorganic phosphate, M3 (purple: without phytic acid; pink: with phytic acid) is without calcium and inorganic phosphate but has extra nitrate. The dot at the end specifies if there is phytic acid present in the medium.

The growth pattern of the engineered *P. putida MS238* was similar to what was observed in the wildtype strain of *P. putida KT2440*. A large increase in growth was seen when additional nitrate was present, and it also grew better in the presence of phytic acid, with inorganic phosphate availability having little to no effect on its growth. Interestingly, this engineered strain seemed to have a small advantage in growth compared to the wildtype *P. putida* in all the media conditions tested. This result suggests that the engineering of the inclusion of its β-propeller phytase allowed for a growth advantage in all conditions whether calcium (the enzyme co-factor) was present or not. However, identifying exactly how calcium affects the growth of this microbe is difficult to determine

from the data collected as extra nitrate was available when calcium was removed from the media.

C. *Bacillus subtilis 163*

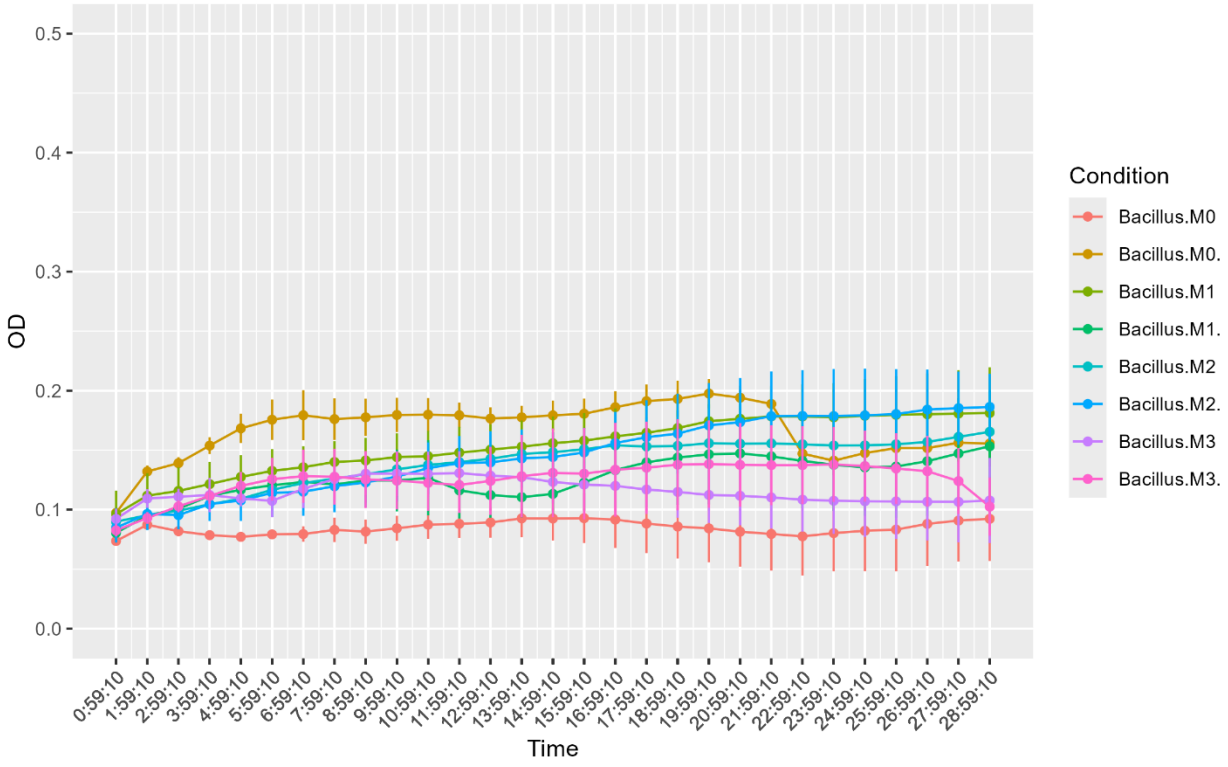


Figure 3: Bacillus subtilis 168 growth curves. Time is on the X-axis and OD_{600} is on the Y-axis. M0 (Red: without phytic acid; Yellow/brown: with phytic acid) is standard media, M1 (moss green: without phytic acid; light green: with phytic acid) is without calcium but has extra nitrate, M2 (turquoise: without phytic acid; blue: with phytic acid) is without inorganic phosphate, M3 (purple: without phytic acid; pink: with phytic acid) is without calcium and inorganic phosphate but has extra nitrate. The dot at the end specifies if there is phytic acid present in the medium.

B. subtilis in all mediums saw slow and limited growth, especially within the 29 hrs when the experimental growth was measured, making it difficult to draw any conclusions. From the data gathered it may suggest that when phytic acid and calcium are present better growth occurs, however, due to the bacterium having such limited growth there is not enough evidence to strongly support this observation. Therefore, to better understand how *B. subtilis'* growth is affected by these components longer growth studies should be

conducted. Additionally, possible changes in the media may be made to better support microbial growth, such as optimizing pH or using an iron compound that does not contain EDTA, as some literature suggests that it inhibits the phytase *Bacillus* produces.[13, 14]

D. *Escherichia coli K12 BW25113*

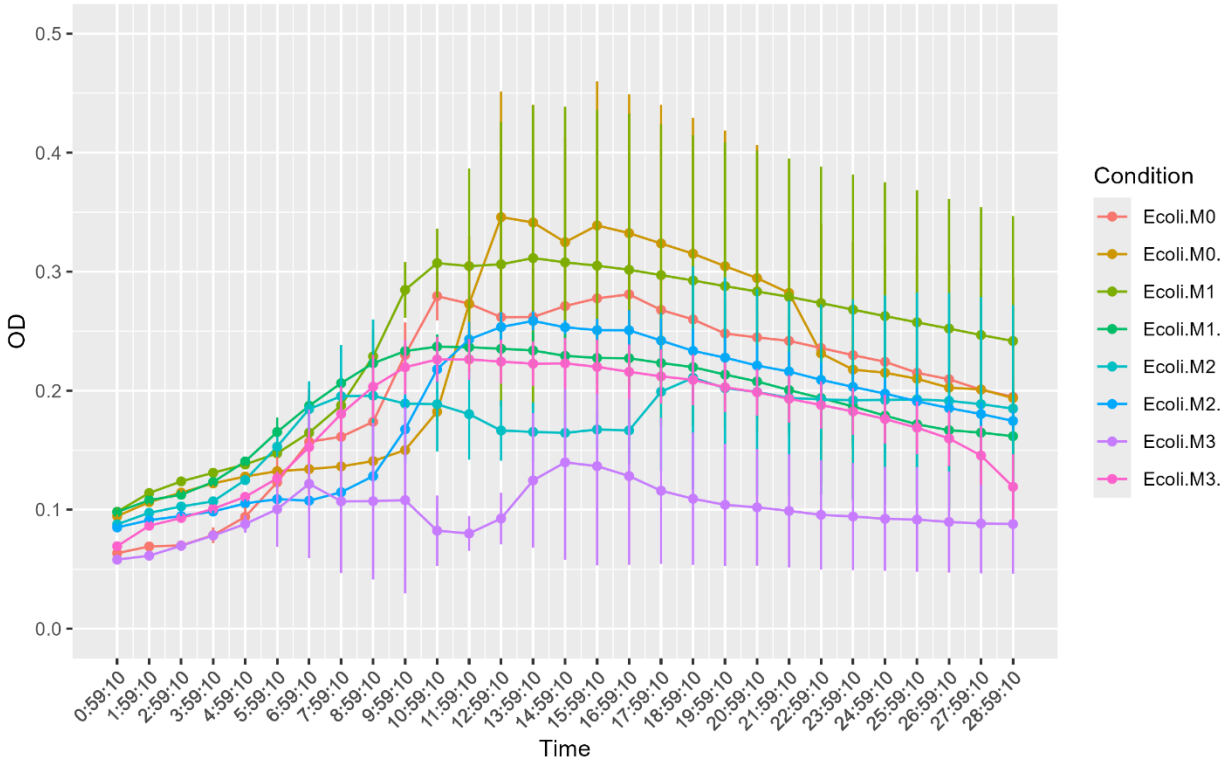
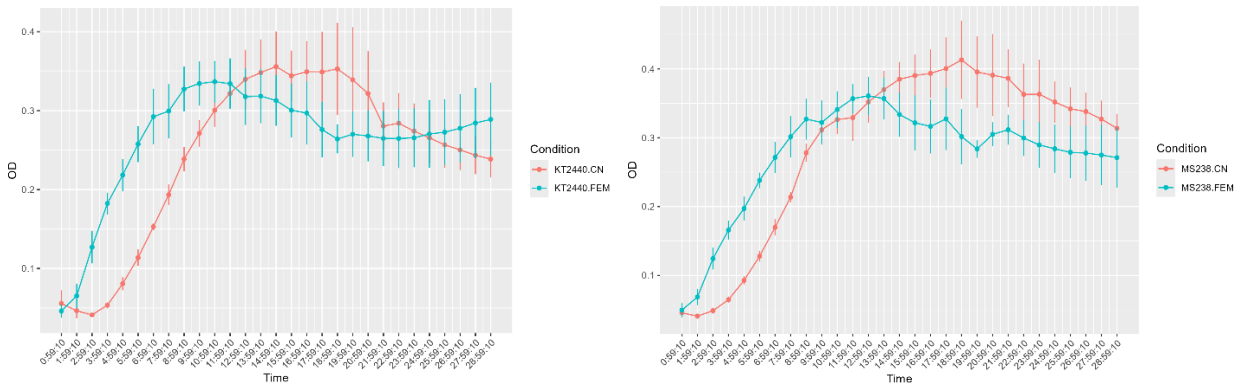


Figure 4: Escherichia coli K12 BW25113 growth curves. Time is on the X-axis and OD_{600} is on the Y-axis. M0 (Red: without phytic acid; Yellow/brown: with phytic acid) is standard media, M1 (moss green: without phytic acid; light green: with phytic acid) is without calcium but has extra nitrate, M2 (turquoise: without phytic acid; blue: with phytic acid) is without inorganic phosphate, M3 (purple: without phytic acid; pink: with phytic acid) is without calcium and inorganic phosphate but has extra nitrate. The dot at the end specifies if there is phytic acid present in the medium.

E. coli saw growth in almost all the conditions except when calcium, phytic acid, and inorganic phosphorus wasn't present. Generally, more growth was observed in the presence of phytic acid. The only exception was when calcium was removed, but inorganic phosphate was still present. However, even without the alternate phosphate source, the bacterium was able to grow. The presence of inorganic phosphate also seemed to allow for

greater growth as the mediums with this component generally saw greater optical densities. The presence or absence of calcium had no clear advantages for growth in *E. coli*. The outcome of this experiment suggests that *E. coli* was able to utilize phytic acid and the presence of inorganic phosphate increased growth. However, phytase enzyme assays should be performed to quantify phytase activity to confirm phytase is what is allowing the growth advantage when phytic acid is present.

E. Comparing microbial strain growth on single versus mixed balanced carbon/nitrogen substrates



A *B*

Figure 5: Growth curves of P. putida (A: Wildtype B: BPP engineered) in a mixed carbon substrate medium (blue) or a single carbon medium (red). The X-axis is time (H:MM:SS) and the Y-axis is OD_{600}.

It was observed that multiple substrates compared to just glucose allowed for faster initial growth in both the wildtype and engineered strains of *P. putida*. Since both media have the same amount of total carbon and nitrogen, the increased growth in the mixed substrate media suggests that *P. putida* has a metabolic mechanism that allows it to utilize carbon quicker when in a mixture. The results of this experiment are intriguing and propose

the need to study bacterial growth in mediums with complex carbon mixtures like what is seen in nature to optimize growth.

IV. CONCLUSION

Phosphorus is required in all living organisms, but only a limited amount is bioavailable. Some microbes have enzymes called phytase, which can break down inaccessible phosphate into accessible forms.

Through the curation of ten media, it was found both *P. putida* strains (wildtype and BPP engineered) showed increased growth in the presence of phytic acid. However, the presence of small amounts of inorganic phosphate had little to no effect on their growth. Interestingly, the driving factor of *P. putida*'s growth in these media seemed to be high nitrate availability, as both strains saw a large increase in growth when calcium was missing but extra nitrate was present. The excess nitrate may have masked the effect of calcium and further experiments are required to clarify this. Furthermore, the data suggest that multiple carbon sources allow for faster initial growth than a single carbon source.

E. coli saw growth advantages when inorganic phosphate was present and was able to utilize phytic acid under all the conditions tested. In contrast, *B. subtilis* exhibited little to no growth in all the media, suggesting longer incubation times or adjusted media may be needed to study its growth and utilization of phytic acid. Additionally, phytase enzyme assays should be conducted to quantify phytase activity to understand if the media are prompting enzyme production or if an alternative mechanism is used. Overall, this research improves our understanding of microbial growth on organic and inorganic

phosphorus, as well as other nutrients. It aids in the mission to utilize microbes to mobilize phosphorus, as we learn what prompts microbes to break down inaccessible phosphate into bioavailable forms.

ACKNOWLEDGEMENTS:

This work was supported in part by the U.S. Department of Energy, Office of Science, Office of Workforce Development for Teachers and Scientists (WDTS) under the Science Undergraduate Laboratory Internship (SULI) Program.

I would also like to especially thank my fantastic mentor Sneha Couvillion who guided me through my project and Natalie Sadler who helped me troubleshoot and calculate all my media recipes. As well as Whitney Garcia who taught me microbial culturing techniques.

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