Quantitative Analysis of Exo-Enzymatic Activity in *Bacillus subtilis* using Commercial Assay Procedures

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Abstract

Probiotics are health-benefiting organisms that are useful supplements in the maintenance of homeostasis within a host's body. The *Bacillus* genus is currently considered the best alternative for use as probiotics because of their endospores naturally high thermal stability, exo-enzymes high specificity, neutral optimum pH, and proteolysis resistance. Currently in the laboratory of Dr. Patricia Rayas-Duarte, there is an on-going project to evaluate strains of *Bacillus spp.* that may prove to be beneficial in the use of probiotics. Experiments have already been done to identify selected strains of *Bacillus spp.* via 16S rDNA that have phytase, α-amylase, cellulase, and protease activities. From those experiments, strains have been selected to undergo quantitative analysis of enzyme activities that will later provide evidence on strains that can be prepared singularly, or in blends, for *in vivo* testing of their efficacy in a monogastric model (poultry) via growth and feed efficiency tests. The objective of this study was to evaluate quantitatively the protease, phytase, and cellulase enzymatic activities of eight selected OSU strains.

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Introduction

The gastrointestinal (GI) track harbours a rich flora of more than 500 different bacterial species that help to stimulate the immune system, provide protection from invading bacteria and viruses, and aid in digestion. Antibiotics, immunosuppressive therapy, etc. may disrupt flora homeostasis and can have potential negative effects. Probiotics are defined as "live micro-organisms," which, when administered in adequate amounts confers a health benefit on the host.[1] Bacillus genus is currently considered the best alternative for use as probiotics because of their endospores naturally high thermal stability, exo-enzymes high specificity, neutral optimum pH, and proteolysis resistance. Bacillus spores can survive in the acid gastric environment, and reach the intestinal tract where they germinate to vegetative forms. The spores can also survive pelleting processing used during the preparation of feed and thus have competitive advantage compared to live organisms and enzymes presently used as supplements in farm animals rations.

Probiotics also work by selective enzyme activities that help maintain homeostasis within the body. Proteases are involved in digesting long protein chains into shorter fragments by splitting the peptide bonds that link amino acid residues.[2] Phytase breaks down phytate, a major form of phosphorus, and phytic acid, a strong chelator of cations that has been shown to cause deficiencies in minerals such as calcium, iron, zinc and magnesium.[3] Cellulase breaks down the cellulose molecule into monosaccharides ("simple sugars") such as beta-glucose, or shorter polysaccharides and oligosaccharides.[4]

Currently in the laboratory of Dr. Patricia Rayas-Duarte, there is an on-going project to evaluate strains of *Bacillus spp.* that may prove to be beneficial in the use of probiotics. Experiments have already

been done to identify selected strains of *Bacillus spp.* via 16S rDNA that have phytase, α-amylase, cellulase, and protease activities. From those experiments, strains have been selected to undergo quantitative analysis of enzyme activities that will later provide evidence on strains that can be prepared singularly, or in blends, for *in vivo* testing of their efficacy in a monogastric model (poultry) via growth and feed efficiency tests. The objective of this study was to evaluate quantitatively the protease, phytase, and cellulase enzymatic activities of eight selected OSU strains.

Experimental Section

Selection of Bacteria Strains

Eight strains of *Bacillus spp.* were selected from a library of isolated strains from previous experiments. The strains were qualitatively analysed and ranked to determine potential strain candidates for *in vivo* testing in chickens as probiotics. The strains, OSU1015-08 and OSU1015-13, were selected to act as negative-comparative controls. These strains had qualitatively low enzymatic activity in all areas of interest. Strains, OSU1015-03, OSU1015-19, and OSU1015-24, were selected due to their high qualitative activity and their current trial use as probiotics in chickens. These strains were isolated from grain products. The last set of strains, OSU1015-09, OSU1015-12, and OSU1015-21, were selected based on their high qualitative activity and were isolated from chicken GI tract.

Inoculation and Incubation of Bacteria

There were six treatment conditions of the bacteria to evaluate optimal growth conditions and whether the desired enzymatic activity had to be induced. These treatments would later undergo three different commercial assays to evaluate enzymatic activity. Four treatments were used in all three enzymatic assays. Treatment One, “Activity Broth + Plate in Capped Tube – Tilted,” was conducted by first streaking each individual strain on an enzyme-inducing plate media and then incubated for 24 h.[5,8] After incubation, a single loop of bacteria was used to inoculate 20 mL of enzyme-inducing broth in capped tubes.[5,8] The tubes were incubated at a tilted angle in a shaker at 500 rpm and at 39° C for 24 h. Well-vortexed mixtures of bacteria and broth were used to conduct the assays 3 days following final incubation.

Treatment Two, “LB Broth + Activity Plate in Capped Tube – Flat” was performed by streaking the desired strains on an enzyme-inducing media plate and incubated for 24 h, similar to Treatment One, and then a single loop of bacteria was used to inoculated 20 mL of Lysogeny broth (LB) in a capped tube. The tubes were incubated on a flat surface in a shaker at 500 rpm and 39° C for 24 h. Well-vortexed mixtures of bacteria and broth were used to conduct the assays 2 days following final incubation.

Treatment Three, “’New’ LB Broth in Capped Tube – Tilted,” was performed by streaking the desired strains on LB agar plates and incubating them for 24 h. Single colonies were transferred into 20 mL of LB in a capped tube via inoculating loop and incubated at a tilted angle within a shaker, at 500 rpm and 39° C. The assays were conducted 5 days following final incubation using well-vortexed mixtures of bacteria and broth. The LB and LB agar plates were made as directed by the manufacturer.

Treatment Four, “’Old’ LB Broth in Flask,” was performed by streaking the desired strains on LB agar plates and incubated for 24 h. A loop full of bacteria was transferred into 100 ml of LB broth in a 250 Erlenmeyer flask sealed with aluminium foil and tape. They were incubated in a shaker at 500 rpm and at 39° C. The assays were conducted 20 days following final incubation using well-vortexed mixtures of bacteria and broth.

Two additional inoculation/incubation combinations were conducted for the protease assay. Treatment Five, “LB Broth + Protease Activity Plate in Capped Tube – Tilted,” was performed by streaking the desired strains on to the protease-inducing plate[5] and incubating for 24 h. Single loops of bacteria were then inoculated into LB broth in capped tubes at a tilted angle in a shaker at 500 rpm and 39° C for 24 h. The protease assay was conducted 2 days following final incubation using well-vortexed mixtures of bacteria and broth.

Treatment Six, “LB Broth + Protease Activity Plate in Flask,” was performed by streaking the desired strains on to the protease-inducing plate[5] and incubating for 24 h. Single loops of bacteria were inoculated in LB broth in a flask for 24 hours at 39° C at 500 rpm in a shaker. The protease assay was conducted 2 days following final incubation using well-vortexed mixtures of bacteria and broth.

Protease Assay Development

The protease-inducing broth was made in accordance with Gomaa (2013) method[5], containing 1.0 g glucose, 0.5 g yeast extract, 0.1 g $CaCl_2$, 0.5 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_20$, 10.0 g skim milk, and 10.0 g casein. The mixture was diluted to 1 L and stirred until a mostly homogenous mixture had formed. The solution was autoclaved to prevent contamination of bacteria samples for 45 minutes. Protease-inducing plates were made following the same recipe as above; however, 20 g of agar was added to the solution, and the mixture was slightly heated and then later poured into plates.

The protease assay was conducted with slight modification of Anson (1938) method and available on the Sigma Aldrich website. Instead of filtration, samples were centrifuged at 10,000 rpm for 2.5 minutes and the supernatant was collected to be used for analysis. Enzyme solution was 200 µL of well-mixed broth and diluted to 1 mL using buffer.

Table 1. Description of *Bacillus* Strains and Growing Conditions used for Protease Activity Analysis

Sample ID	Strain ID	Conditions
1	OSU1015-08	Protease Activity Broth + Plate in Capped Tube - Tilted
2	OSU1015-13	Protease Activity Broth + Plate in Capped Tube - Tilted
3	OSU1015-03	Protease Activity Broth + Plate in Capped Tube - Tilted
4	OSU1015-19	Protease Activity Broth + Plate in Capped Tube - Tilted
5	OSU1015-24	Protease Activity Broth + Plate in Capped Tube - Tilted
6	OSU1015-09	Protease Activity Broth + Plate in Capped Tube - Tilted
7	OSU1015-12	Protease Activity Broth + Plate in Capped Tube - Tilted
8	OSU1015-21	Protease Activity Broth + Plate in Capped Tube - Tilted
9	OSU1015-08	LB Broth + Protease Activity Plate in Capped Tube - Flat
10	OSU1015-13	LB Broth + Protease Activity Plate in Capped Tube - Flat
11	OSU1015-03	LB Broth + Protease Activity Plate in Capped Tube - Flat
12	OSU1015-19	LB Broth + Protease Activity Plate in Capped Tube - Flat
13	OSU1015-24	LB Broth + Protease Activity Plate in Capped Tube - Flat
14	OSU1015-09	LB Broth + Protease Activity Plate in Capped Tube - Flat
15	OSU1015-12	LB Broth + Protease Activity Plate in Capped Tube - Flat
16	OSU1015-21	LB Broth + Protease Activity Plate in Capped Tube - Flat
17	OSU1015-08	"New" LB Broth in Capped Tube - Tilted
18	OSU1015-13	"New" LB Broth in Capped Tube - Tilted
19	OSU1015-03	"New" LB Broth in Capped Tube - Tilted
20	OSU1015-19	"New" LB Broth in Capped Tube - Tilted
21	OSU1015-24	"New" LB Broth in Capped Tube - Tilted
22	OSU1015-09	"New" LB Broth in Capped Tube - Tilted

Sample Number	Strain ID	Conditions
23	OSU1015-12	"New" LB Broth in Capped Tube - Tilted
24	OSU1015-21	"New" LB Broth in Capped Tube - Tilted
25	OSU1015-08	"Old" LB Broth in Flask
26	OSU1015-13	"Old" LB Broth in Flask
27	OSU1015-03	"Old" LB Broth in Flask
28	OSU1015-19	"Old" LB Broth in Flask
29	OSU1015-24	"Old" LB Broth in Flask
30	OSU1015-09	"Old" LB Broth in Flask
31	OSU1015-12	"Old" LB Broth in Flask
32	OSU1015-21	"Old" LB Broth in Flask
33	OSU1015-8	LB Broth + Protease Activity Plate in Capped Tube - Tilted
34	OSU1015-13	LB Broth + Protease Activity Plate in Capped Tube - Tilted
35	OSU1015-03	LB Broth + Protease Activity Plate in Capped Tube - Tilted
36	OSU1015-19	LB Broth + Protease Activity Plate in Capped Tube - Tilted
37	OSU1015-24	LB Broth + Protease Activity Plate in Capped Tube - Tilted
38	OSU1015-09	LB Broth + Protease Activity Plate in Capped Tube - Tilted
39	OSU1015-12	LB Broth + Protease Activity Plate in Capped Tube - Tilted
40	OSU1015-21	LB Broth + Protease Activity Plate in Capped Tube - Tilted
41	OSU1015-21	LB Broth + Protease Activity Plate in Flask
42	Protease Standard 1	Standard
43	Protease Standard 2	Standard
44	Protease Standard 3	Standard
45	Protease Standard 4	Standard
46	Protease Standard 5	Standard
47	Protease Enzyme Blank	Blank
48	Protease Standard Blank	Blank

Phytase Assay Development

Phytase-inducing media were made following the recipe described in Bae, et al. method in 1999.[8] The method describes making LB medium as described on the package and adding 0.5% phytic acid. The phytase assay was conducted in accordance with Heinonen, and Lahti 1981 method and available on the Sigma Aldrich website.[9] Enzyme solution was 600 µL of well-mixed broth and diluted to 1 mL using buffer.

Table 2. Description of *Bacillus* Strains and Growing Conditions used for Phytase Activity Analysis

Sample Number	Strain ID	Conditions
49	OSU1015-08	Phytase Activity Broth + Plate in Capped Tube - Tilted
50	OSU1015-13	Phytase Activity Broth + Plate in Capped Tube - Tilted

51	OSU1015-03	Phytase Activity Broth + Plate in Capped Tube - Tilted
52	OSU1015-19	Phytase Activity Broth + Plate in Capped Tube - Tilted
53	OSU1015-24	Phytase Activity Broth + Plate in Capped Tube - Tilted
54	OSU1015-09	Phytase Activity Broth + Plate in Capped Tube - Tilted
55	OSU1015-12	Phytase Activity Broth + Plate in Capped Tube - Tilted
56	OSU1015-21	Phytase Activity Broth + Plate in Capped Tube - Tilted
57	OSU1015-08	LB Broth + Phytase Activity Plate in Capped Tube Flat
58	OSU1015-13	LB Broth + Phytase Activity Plate in Capped Tube Flat
59	OSU1015-03	LB Broth + Phytase Activity Plate in Capped Tube Flat
60	OSU1015-19	LB Broth + Phytase Activity Plate in Capped Tube Flat
61	OSU1015-24	LB Broth + Phytase Activity Plate in Capped Tube Flat
62	OSU1015-09	LB Broth + Phytase Activity Plate in Capped Tube Flat
63	OSU1015-12	LB Broth + Phytase Activity Plate in Capped Tube Flat
64	OSU1015-21	LB Broth + Phytase Activity Plate in Capped Tube Flat
65	OSU1015-08	"New" LB Broth in Capped Tube - Tilted
66	OSU1015-13	"New" LB Broth in Capped Tube - Tilted
67	OSU1015-03	"New" LB Broth in Capped Tube - Tilted
68	OSU1015-19	"New" LB Broth in Capped Tube - Tilted
69	OSU1015-24	"New" LB Broth in Capped Tube - Tilted
70	OSU1015-09	"New" LB Broth in Capped Tube - Tilted
71	OSU1015-12	"New" LB Broth in Capped Tube - Tilted
72	OSU1015-21	"New" LB Broth in Capped Tube - Tilted
73	OSU1015-08	"Old" LB Broth in Flask
74	OSU1015-13	"Old" LB Broth in Flask
75	OSU1015-03	"Old" LB Broth in Flask
76	OSU1015-19	"Old" LB Broth in Flask
77	OSU1015-24	"Old" LB Broth in Flask
78	OSU1015-09	"Old" LB Broth in Flask
79	OSU1015-12	"Old" LB Broth in Flask
80	OSU1015-21	"Old" LB Broth in Flask
81	Phytase Standard 1	Standard
82	Phytase Standard 2	Standard
83	Phytase Standard 3	Standard
84	Phytase Standard 4	Standard
85	Phytase Standard 5	Standard
86	Phytase Enzyme Blank	Blank
87	Phytase Standard Blank	Blank

Cellulase Assay Development

The cellulase-inducing broth was made in accordance with Gomaa (2013) methods containing 1.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_20$, 0.5 g NaCl, 0.01 g $FeSO_4 \cdot 7H_20$, 0.01 g $MnSO_4 \cdot 7H_20$, 0.3 NH_4NO_3, 10.0 g

CMC.[5] The mixture was diluted to 1 L and stirred and slightly heated a clear solution formed. The solution was autoclaved to prevent contamination of bacteria samples for 45 minutes. Cellulase-inducing plates were made following the same recipe as above; however, 20 g of agar was added to the solution, and the mixture was slightly heated and then later poured into plates.

The cellulase assay was conducted with slight modification of the 1988 Worthington Enzyme Manual and is available on the Sigma Aldrich website.[10] Instead of using SigmaCell micro-crystalline cellulose, methylcellulose was used. The resulted in a very thick, gel-like substance that made measurements difficult. Enzyme solution was 800 μL of well-mixed broth and diluted to 1 ml using buffer.

Table 3. Description of *Bacillus* Strains and Growing Conditions used for Cellulase Activity Analysis

Sample ID	Strain ID	Conditions
88	OSU1015-08	Cellulase Activity Broth + Plate in Capped Tube - Tilted
89	OSU1015-13	Cellulase Activity Broth + Plate in Capped Tube - Tilted
90	OSU1015-03	Cellulase Activity Broth + Plate in Capped Tube - Tilted
91	OSU1015-19	Cellulase Activity Broth + Plate in Capped Tube - Tilted
92	OSU1015-24	Cellulase Activity Broth + Plate in Capped Tube - Tilted
93	OSU1015-09	Cellulase Activity Broth + Plate in Capped Tube - Tilted
94	OSU1015-12	Cellulase Activity Broth + Plate in Capped Tube - Tilted
95	OSU1015-21	Cellulase Activity Broth + Plate in Capped Tube - Tilted
96	OSU1015-08	LB Broth + Cellulase Activity Plate in Capped Tube - Flat
97	OSU1015-13	LB Broth + Cellulase Activity Plate in Capped Tube - Flat
98	OSU1015-03	LB Broth + Cellulase Activity Plate in Capped Tube - Flat
99	OSU1015-19	LB Broth + Cellulase Activity Plate in Capped Tube - Flat
100	OSU1015-24	LB Broth + Cellulase Activity Plate in Capped Tube - Flat
101	OSU1015-09	LB Broth + Cellulase Activity Plate in Capped Tube - Flat
102	OSU1015-12	LB Broth + Cellulase Activity Plate in Capped Tube - Flat
103	OSU1015-21	LB Broth + Cellulase Activity Plate in Capped Tube - Flat
104	OSU1015-08	"New" LB Broth in Capped Tube - Tilted
105	OSU1015-13	"New" LB Broth in Capped Tube - Tilted
106	OSU1015-03	"New" LB Broth in Capped Tube - Tilted
107	OSU1015-19	"New" LB Broth in Capped Tube - Tilted
108	OSU1015-24	"New" LB Broth in Capped Tube - Tilted
109	OSU1015-09	"New" LB Broth in Capped Tube - Tilted
110	OSU1015-12	"New" LB Broth in Capped Tube - Tilted
111	OSU1015-21	"New" LB Broth in Capped Tube - Tilted
112	OSU1015-08	"Old" LB Broth in Flask

113	OSU1015-13	"Old" LB Broth in Flask
114	OSU1015-03	"Old" LB Broth in Flask
115	OSU1015-19	"Old" LB Broth in Flask
116	OSU1015-24	"Old" LB Broth in Flask
117	OSU1015-09	"Old" LB Broth in Flask
118	OSU1015-12	"Old" LB Broth in Flask
119	OSU1015-21	"Old" LB Broth in Flask
120	Cellulase Enzyme Blank	Blank

Results

Protease Activity

Table 4. Standard Curve Data for Protease Activity

Concentration (µmoles of Tyrosine)	Absorbance (660 nm)
0.000	0.000
0.055	0.112
0.110	0.242
0.220	0.463
0.440	0.939
0.550	1.043

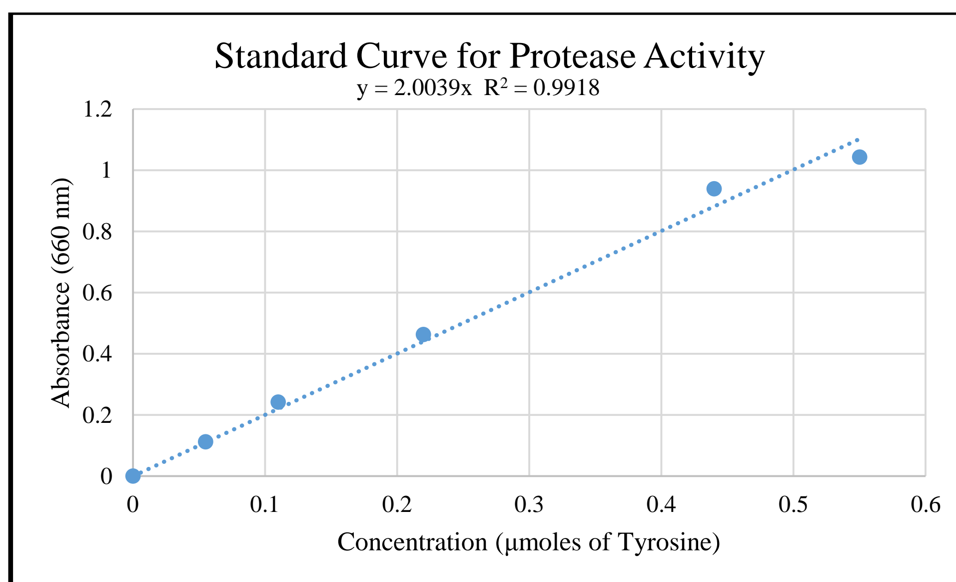

Figure 1. Standard curve of Protease Activity

Table 5. Concentration and Protease Activity of OSU Strains Identified in **Table 1.**

Sample ID	Absorbance (400 nm)	Concentration (µmoles of Tyrosine)	Units/ml enzyme
1	0.094	0.0469	0.129
2	0.059	0.0294	0.0809
3	0.099	0.0494	0.136

4	0.011	0.00549	0.0151
5	0.06	0.0299	0.0822
6	0.061	0.0304	0.0836
7	0.12	0.0599	0.165
8	0.002	0.000998	0.00274
9	0.004	0.002	0.0055
10	0.001	0.000499	0.00137
11	0.036	0.018	0.0495
12	0.052	0.0259	0.0712
13	0.057	0.0284	0.0781
14	0.072	0.0359	0.0987
15	0.091	0.0454	0.125
16	0.067	0.0334	0.0919
17	0.098	0.0489	0.134
18	0.113	0.0564	0.155
19	0.145	0.0724	0.199
20	0.068	0.0339	0.0932
21	0.127	0.0634	0.174
22	0.099	0.0494	0.136
23	0.115	0.0574	0.158
24	0.093	0.0464	0.128
25	0.254	0.127	0.349
26	0.450	0.225	0.619
27	0.716	0.357	0.982
28	0.302	0.151	0.415
29	0.480	0.240	0.660
30	0.269	0.134	0.369
31	0.267	0.133	0.366
32	0.103	0.0514	0.141
33	0.085	0.0424	0.117
34	0.108	0.0539	0.148
35	0.106	0.0529	0.145
36	0.056	0.0279	0.0767
37	0.127	0.0634	0.174
38	0.088	0.0439	0.121
39	0.094	0.0469	0.129
40	0.089	0.0444	0.122
41	0.081	0.0404	0.111

*Highlighted: largest activity value per section

- Calculation of units per millilitres of enzyme:

$$\frac{units}{ml\ enzyme} = \frac{(\mu mole\ Tyrosine) \times 11}{10 \times 2 \times Df}$$

Where:

- *11 = total volume of assay in ml*
- *10 = time in minutes of assay*
- *2 = volume of colorimetric determination in ml*
- *Df = dilution factor*

Phytase Activity

Table 6. Standard Curve Data for Phytase Activity

Concentration (µmoles of Phosphate)	Absorbance (400 nm)
0.0	0.000
0.5	0.184
1.0	0.365
1.4	0.562
2.0	0.589
2.5	0.863

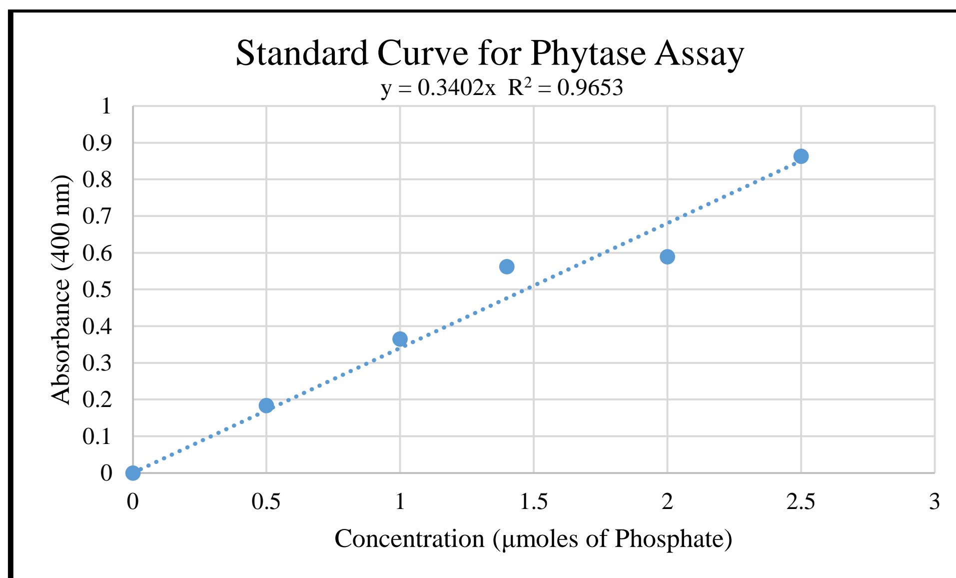

Figure 2. Standard curve of Phytase Activity

Table 7. Concentration and Phytase Activity of OSU Strains Identified in **Table 2.**

Sample ID	Absorbance (400 nm)	Concentration (µmoles of Phosphate)	Units/mL enzyme
49	0.12	0.353	0.188
50	0.121	0.356	0.195
51	0.149	0.438	0.234
52	0.051	0.150	0.08

53	0.079	0.232	0.124
54	0.061	0.179	0.0955
55	0.097	0.285	0.152
56	0.118	0.347	0.185
57	0.002	0.00588	0.00314
58	0.037	0.109	0.0581
59	0.059	0.173	0.0923
60	0.006	0.0176	0.00939
61	0.044	0.129	0.0688
62	0.083	0.244	0.130
63	0.017	0.050	0.0267
64	0.055	0.162	0.0864
65	0.087	0.256	0.137
66	0.057	0.168	0.0896
67	0.095	0.279	0.149
68	0.107	0.315	0.168
69	0.122	0.359	0.191
70	0.086	0.253	0.135
71	0.076	0.223	0.119
72	0.08	0.235	0.125
73	0.089	0.262	0.140
74	0.055	0.162	0.0864
75	0.079	0.232	0.124
76	0.088	0.259	0.138
77	0.04	0.118	0.0629
78	0.082	0.241	0.129
79	0.043	0.126	0.0672
80	0.058	0.170	0.0907

*Highlighted: largest activity value per section

- Calculation of units per millilitres of enzyme:

$$\frac{units}{ml\ enzyme} = \frac{(\mu mole\ Phosphate) \times Df}{45 \times 0.025}$$

Where:

- *Df = dilution factor*
- *45 = time in minutes of assay per the unit definition*
- *0.025 = volume of enzyme used in ml*

Cellulase Activity

Table 8. Cellulase Activity of OSU Strains Identified in **Table 3.**

Sample ID	Absorbance (340 nm)
88	0.002

89	0.001
90	0.002
91	0.000
92	0.002
93	0.003
94	0.005
95	0.006
112	0.015
113	0.016
114	0.025
115	0.011
116	0.011
117	0.016
118	0.025
119	0.024

*Highlighted: largest activity value per section

Analysis of cellulase activity could not be completed. When attempting to make the cellulose solution, the solution became a very thick gel with clumps of cellulose that would not dissolve. This prevented accurate allocation of cellulose to the samples and thus the absorbance reading is not reliable. Also, less samples were ran in the cellulase assay due to insufficient glucose reagent available for the experiment. The "Activity Broth + Plate" and "Flask" samples were selected to use since the flask samples were the most successful to grow and the high, activity-inducing samples were chosen with consideration that the enzymatic activity may require induction.

Overall Results of Enzyme Activities in OSU Strains

Table 9. Total Enzyme Activities of OSU Strains

Rank ID	Strain ID	Protease*	Phytase*	Cellulase*
1	OSU1015-08	0.147	0.117	0.009
2	OSU1015-13	0.201	0.107	0.009
3	OSU1015-03	0.302	0.150	0.014
4	OSU1015-19	0.134	0.099	0.006
5	OSU1015-24	0.234	0.112	0.007
6	OSU1015-09	0.162	0.122	0.010
7	OSU1015-12	0.187	0.091	0.015
8	OSU1015-21	0.099	0.122	0.015

Values are determined by the average of units/ml enzyme over all conditions
*Activity given in units/ml enzyme

Rankings of Enzyme Activites

- Protease: 3 >> 5 > 2 > 7 > 6 > 1 > 4 >> 8
- Phytase: 3 >> 6 > 8 > 1 > 5 > 2 > 4 > 7
- Cellulase: 8 = 7 > 3 > 6 > 2 = 1 > 5 >

Effects of Growing Conditions

Table 10. Effect of Enzyme Activity per Growing Condition

Condition	Protease Activity*	Phytase Activity*	Cellulase Activity*
Enzyme activity inducing broth + plate in capped tube – tilted (3 days)	0.087	0.157	0.003
LB broth + enzyme activity inducing plate in capped tube – flat (2 days)	0.065	0.059	N/A
LB broth + protease activity plate in capped tube – tilted (2 days)	0.129	N/A	N/A
LB broth + protease activity plate in flask (2 days)	0.111	N/A	N/A
"New" LB broth in capped tube – tilted (6 days)	0.147	0.139	N/A
"Old" LB broth in flask (20 days)	0.488	0.105	0.017

Values are determined by the average of units/ml enzyme of all samples per condition
*Activity given in units/ml enzyme

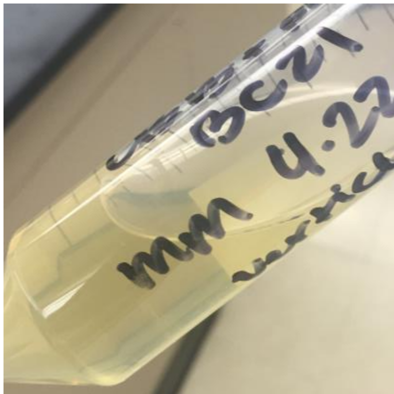

Figure 3. – Flat Tube

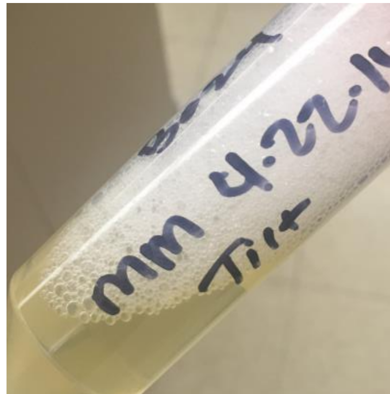

Figure 4. – Tilted Tube

Figure 5. – Flask

The three images above display the growth of bacteria in various conditions. The flask visibly had a significant growth of bacteria whereas the tubed samples were still translucent. Tilted tubes had a significant amount of foam after incubation in the shaker, and many tubes had a noticeable, suction-type lock when one tried to open up the cap.

Conclusions and Future Work

The strain, OSU1015-03 has proved to be the most active strain in the categories of protease, phytase, and cellulose activity. No other strain had significant activity in the multiple areas as in OSU1015-03. Growth conditions in flasks seemed to exceed other conditions, however, more studies need to be done to evaluate the effectiveness of the type of media used.

Problems that arose during experimentation came from replacing SigmaCell microcrystalline cellulose with methylcellulose which resulted in a “jello” like substance. This may have to do with the low solubility of methylcellulose in the broth. There was also a limited availability of Glucose HK Assay Reagent for the cellulase activity assay which resulted in a small sample size. Due to time constraints, only 1 trail of each sample was achieved. Lastly, during the protease assay, there was a visible fizzing when each solution was placed in a water-wetted cuvette. The assay solution may have had a reaction with the water, or a possible impurity that may have skewed results.

Quantitative analysis assay for phytase determined there was similar/noticeable phytase activity in all OSU strains. This result is counter intuitive in comparison with the qualitative analysis performed in an earlier experiment. This may signify that there was an error within the assay, particularly, in designing a “blank” sample. The phytase assay is conducted by determining the amount of free phosphorous in a sample. Free phosphorous is abundant and prosperous in all media, and also may be difficult to filter out or eliminate by taking the supernatant of a centrifuged sample. The free phosphorous that was detected in the samples during the assay may have been a result of free phosphorous within the media rather than the phytase activity of the samples. The selected blanks did not account for the different phosphorous levels present within the different media thus the recorded phytase activities above may just be a result of the free phosphorous present within the media.

Future work that needs to be completed are optimizing growing conditions for the strains, repeat Cellulase Activity Assay using SigmaCell microcrystalline cellulose, changing concentration of cellulose, and/or alter pH, test reactivity of protease assay with water, design new blanks for the phytase activity that account for the different media types, and to repeat all assays to ensure reliability of results.

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