# Vitazyme *Enzyme Activity*

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***Experimental design:*** The goal of Phase 1 of this two-phase study plan was to screen for the presence of enzyme activity in Vitazyme by analyzing for broad activities identified through a review of the literature. The literature review identified the enzyme activities listed below as commonly present in substrates such as soils, composted solids, and fermented products.

| Enzyme Activity | Role |
|---|---|
| β-glucosidase | Catalyzes the breakdown of cellobiose into glucose.<br>A predominant enzyme in soils.<br>Commonly found in fermented and composted samples. |
| Chitinase | Catalyzes the breakdown of chitin, a major component of fungal cell walls. |
| Sulfatase | Catalyzes the breakdown of sulfur containing compounds.<br>Had been linked to suppression of root rot (Leon, 2006). |
| Phosphatase | Catalyzes the removal of phosphate groups from molecules.<br>Is thought to influence the ability of plants to cope with P-stress conditions.<br>(Karthikeyan, 2002. Mudge, 2002. Versaw, 2002) |
| α-glucosidase | Catalyzes the breakdown of starch. |
| Cellulase | Catalyzes the breakdown of cellulose into smaller polysaccharides. |

The analysis was performed using a modified version of ISO method 22939, which details the use of fluorogenic substrates to measure enzyme activity in soils. The fluorogenic reporting molecule used for this analysis is 4-methylumbelliferone (MUF) and associated MUF-linked substrates are available for each of the enzyme activities listed above.

### *Analysis of enzymes in Vitazyme:*

MUF labeled substrates were received in powdered form. Many of the compounds had limited solubility in water and all were diluted in dimethyl sulfoxide (DMSO). Stock solutions were prepared at 25 mg/ml. Due to the limited stability of some of these compounds, stock solutions were prepared fresh each day. MUF labeled substrates were diluted 1:10 into the matrix with a final concentration of 2.5 mg/ml. Reactions were incubated at 37°C and stopped with the addition of 3 mL MUB to the 1 mL reaction mixture. Reactions run with heat treated Vitazyme served as a negative control and were used as blanks for activity calculations.

A summary of the results is shown in the table to the right. There was significant time dependent breakdown of 4-MUF-phosphate that was both consistent and reproducible, providing strong evidence for acid phosphatase activity. Most of the activity was complete by four hours, with the reaction continuing at a slower pace between 4 and 15 hours.

Breakdown of 4-MUF-N-acetyl-β-D-glucosaminide, while not as intense, followed the same time dependant pattern as the breakdown of 4-MUF-phosphate. This combined with the consistent and reproducible nature of result points to possible chitinase activity.

**Summary of Enzyme Activity Results**

| Substrate | MUF Produced (uM) | | |
|---|---|---|---|
| | 2 hrs | 4 hrs | 15 hrs |
| 4-MUF-N-acetyl-β-D-glucosaminide | 1.8 | 3.6 | 4.4 |
| 4-MUF-phosphate | 5.5 | 18.6 | 19.8 |
| 4-MUF-α-D-gulcopyranoside | none | NA | none |
| 4-MUF-β-D-gulcopyranoside | none | NR | NR |
| 4-MUF-sulfate | none* | NA | none |
| 4-MUF-β-D-cellobioside | none | NA | none |

Samples incubated with 2.5 mg/ml substate for 2 hrs at 37°C.
*4-MUF-sulfate concentration is 5.0 mg/ml for sample incubated for 2 hrs.
None = a value of zero or below background.
NA = Data was not collected for the 4 hr time point since no enzyme activity was exhibited at the 15 hr time point.
NR = not reported, values were obtained but the data was not consistent or reproducible.

Low levels of 4-MUF-β-D-gulcopyranoside breakdown occurs with overnight incubations. However, this activity is not consistently reproducible. There is not convincing evidence of the presence of significant levels of β-glucosidase activity.

There is no breakdown of 4-MUF-α-D-gulcopyranoside, 4 MUF-sulfate, or 4-MUF-β-D-cellobioside or evidence of corresponding α-glucosidase, sulfatase, or cellulase enzyme activity by this method. Incubation of the reaction mixtures for 15 hours gave no indication of the presence of these enzyme activities.

### *Conclusions:*

There is strong evidence of the presence of **acid phosphatase** in the product. However, it is recommended that additional studies be done using commercially available acid phosphatase as a control to confirm that the breakdown of 4-MUF-phosphate is enzymatically driven, and to quantify acid phosphatase activity in the product relative to microgram amounts of the enzyme. Acid phosphatases are active across a wide range of temperatures and pH. This, combined with inhibition of phosphatase activity by a variety of compounds including phosphate, the product of the reaction, may require some additional refinement of the assay in order to clearly quantify the phosphatase activity. It is also recommended that due to the strong signal associated with acid phosphatase that future investigations and assay refinements be done with this enzyme first.

There is convincing evidence of **chitanase** activity in the product. Additional study using a commercially available chitinase as a control is recommended as a way to both confirm the activity and to quantify chitinase activity in terms relative to microgram amounts of the enzyme.

There is not convincing evidence of the presence of significant levels of β-glucosidase activity. Any signal produced by the breakdown of 4-MUF-β-gulcopyranoside was weak at best.

There is no evidence of α-glucosidase, sulfatase, or cellulase enzyme activity by this method. Even overnight incubation of the reaction mixtures gave no indication of the presence of these enzyme activities. It is recommended that there should be no further investigation of β-glucosidase, α-glucosidase, sulfatase, or cellulase enzyme activities at this time.