**Purpald Assay Procedure for Methanol Quantification in Culture Supernatants**

# Procedure Objective

Most typical fermentation substrates are measured by HPLC, using an ion exchange column and refractive index detection (RID). Although methanol can be detected by this approach, there are challenges to its quantification. Specifically, methanol co-elutes with components of tryptone and yeast extract commonly found in rich and minimal media formulations. Additionally, the RID response is poor, leading to small peak areas, and we have observed a shift in the baseline around the time that methanol elutes. As accurate methanol measurements are important to our work, we routinely analyze samples using a colorimetric enzyme-based assay. Briefly, methanol is oxidized to formaldehyde by amethanol oxidase from *Pichia pastoris,* then the formaldehyde is quantified by Purpald assay. This procedure outlines the details of this protocol as used in our lab, modified from previous literature1.

# Health and Safety

Closed toed shoes, gloves, safety glasses should be worn for this procedure.

# Materials List

* Samples collected from the experiment, preferably in microcentrifuge tubes.
* 96-well clear plastic plates
* Purpald
* Alcohol Oxidase
* Stock Solutions:
	+ 100 mM sodium phosphate buffer (pH 7.5 pH),
	+ 0.5 N NaOH
	+ 50 mM methanol in water (for preparation of standard curve)

# Equipment

* Platereader capable of absbornace measurements
* Multichannel pipette

# Pre-lab Steps

Determine the quantities of solutions needed:

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Component** | **Addition (uL) /well** | **# of wells per plate - Approximate** | **Vol per plate (mL) Needed** | **Total mL Needed** | **Notes** |
| Buffer for AO Solution | 100 | 96 | [100\*96\*1/1000] = 9.6 mL/plate | 20 mL at a time, up to 60 mL | Result is 0.1 U in assay well = 100 uL of 0.001 U/uL |
| NaOH for Purpald Solution | 100 | 96 | [100\*96\*1/1000] = 9.6 mL/plate | 20 mL at a time, up to 60 mL | Result is 0.50 mg Purpald per assay well |

**Prepare fresh 1M methanol standard:**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Chemical Name | Chemical Name on Bottle | Lot Number | Amount Planned  | Amount Actual  |
| Methanol |   |   | 3.2 g or 0.405 mL |   |
| DI Water |   |   | 10 mL total (add 9.6 mL RODI) |   |

**Prepare fresh 50 mM methanol in media standard in a tube:**

* 50 uL of 1M methanol stock
* 950 uL of media that matches experimental conditions, or water

**Prepare Solutions**

**Phosphate Buffer,** 7.5 pH, 100 mM (If fresh is needed)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Chemical Name | Chemical Name on Bottle | Lot Number | Amount Planned  | Amount Actual |
| Sodium phosphate monobasic | S0751-500G | BCCB7663 | 0.80 g |   |
| Sodium phosphate dibasic | S9763-500G | SLCD2106 | 1.89 g |   |
| RODI water | -- | -- | 200 mL |   |

Adjust pH to 7.5 using NaOH.

**Alcohol Oxidase –**Add to buffer same day as use, keep cold

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Chemical Name | Chemical Name on Bottle | Lot Number | Ratio for 100 uL addition (current protocol) | Amount Actual  |
| Alcohol Oxidase |   |   | 5.4 uL |  |
| Phosphate Buffer | Lab Stock, 21/06/04, KH | -- | 10 mL |  |

**NaOH Solution, 0.5N**(If fresh is needed)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Chemical Name | Chemical Name on Bottle | Lot Number | Amount Planned  | Amount Actual |
| NaOH | 567530-500GM | 3311947 | * 0.5 N = 0.5 M NaOH
* For 100 mL, need:
* (0.5 mol/L)\*(0.1 L) = 0.05 mol
* 0.05 mol \* 40 g/mol = **2 g**
* (Approx 10 pellets)
 |   |
| RODI water | -- | -- | **100 mL** |   |

**Purpald - MAKE RIGHT BEFORE USING DURING 10 min Heat Step**Concentration needed: 5 mg/mL of Purpald in 0.5 N NaOH (100 uL of Purpald + NaOH per well; 150 uL of Purpald + NaOH + water per well)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Chemical Name | Chemical Name on Bottle | Lot Number | Amount  Planned 1 plate | Amount Actual  |
| Purpald | Alfa Aesar L00982 | 10228117 | 50 mg |   |
| 0.5 N NaOH  | Lab Stock, 21/03/30, KH | -- | 10 mL |   |
| After mixing Purpald with NaOH, add 1/2 the total volume of water for 150% of volume to pipette once | DI water | -- | 5 mL |   |

# Procedure Steps

**4. Prepare 96-well Dilutions and Assay Wells**

**Assay Steps followed (includes updates from Oct 4, 2021 assay improvements):**

a.) Prepare 2x Dilution of 50 mM methanol using Media

b.) Dilute samples and standards into linear assay range:

* Add 20 uL of samples and standards
* Add 320 uL water, pipette mix.
* Helpful to prepare technical replicates for this dilution step.

c.) Prepare Assay wells:

* Add 80 uL water to fresh wells for assay
* Bring 20 uL from Dilution wells into assay wells
* Add 100 uL Alcohol Oxidase in Buffer (prepared above), pipette mix

d.) Incubate plate at 30C for 10 minutes. Now prepare Purpald in NaOH, with water (recipe above).

e.) Add 150 uL of Purpald/NaOH/Water to Assay wells, pipette mix.

f.) Incubate plate at 30C for 30 more minutes.

g.) Read in Platereader at 550 nm absorbance.

# Data Analysis

From the platereader software, export the raw data into an excel file. Use python or excel to prepare a linear regression of the standard curve wells, and then apply the relationship to the absorbances to calculate the methanol concentration.

# References

1. Anthon, G. E. & Barrett, D. M. Comparison of three colorimetric reagents in the determination of methanol with alcohol oxidase. Application to the assay of pectin methylesterase. *J. Agric. Food Chem.* **52**, 3749–3753 (2004).

# Revision Table

|  |  |  |  |
| --- | --- | --- | --- |
| Revision No. | Date of Change | Change Description | Person Responsible |
| 0 | May 23, 2021 | Initial Development | KH |
| 1 | Oct 5, 2023 | Improved assay protocol | KH |
| 2 | April 3, 2024 | Improved for clarity | KH |
| 3 | October 11, 2024 | More clarity improvements | KH |