

Butanol Dehydrogenase Enzyme Assay

Method 1.

1. Reference paper

Enzymatic investigation on butanol dehydrogenase and butyraldehyde dehydrogenase in extracts of *Clostridium acetobutylicum*. P Durre, A Kuhn, M. Gottwald, and G. Gottschalk. Applied Microbiology and Biotechnology, 1987. 26:268-272.

Level of Enzymes Involved in Acetate, Butyrate, Acetone and Butanol Formation by *Clostridium acetobutylicum*. W. Andersch, H Bahl, and G. Gottschalk. Applied Microbiology and Biotechnology, 1983. 18:327-332.

2. Gasses

- 5% H₂/95% N gas
- 99% H₂ gas
- 99.99% Carbon Monoxide gas

3. Glassware

- 1ml narrow Cuvettes
- butyl stoppers
- sealers/crimpers
- anaerobic chamber
- serum bottles

4. Growth medium

1. Phosphate buffered solution
2. Reducing agent Na₂S
3. Phosphate-buffered medium (includes Wolfe's Mineral Solution and Wolfe's vitamin solution)

5. Equipment

Spectrophotometer
Anaerobic chamber
Vortex
Refrigerated centrifuge (15000 rpm)
Autopipettes

Rubber stoppers
 Serum bottles
 Serum stoppers
 Aluminum seals
 Needles (size 20 gauge 1½ in length)
 1 mL syringes
 Glass beads for vortexing

6. Growth medium

Phosphate-buffered medium

Composition per 985 ml of distilled water:

CaCl ₂	1 g
NH ₄ Cl	1 g
MgCl ₂ .6H ₂ O	0.2 g
Wolfe's mineral solution	10 ml (see recipe below)
Wolfe's vitamin solution	5 ml (see recipe below)
Yeast extract	0.5 g
Reazurin	1 ml

Preparation of phosphate-buffered medium Add components except for the carbon source (i.e. glucose) and bring the volume to 985 ml. Adjust the pH to 7.2-7.4 with NaOH. Gently heat and boil. Cool medium and distribute anaerobically in test tubes in 10 ml volumes using 100% N₂ until the reazurin indicator. Stopper the tubes anaerobically. Autoclave for 15 min at 15 psi pressure, 121°C. Aseptically add 0.2 ml of NaS.9H₂O solution and 0.2 ml of phosphate buffer solution to each tube. Mix thoroughly. **Note:** These two solutions and other additions to the medium should be added in syringes. All solutions added after medium sterilization must be autoclaved or filter sterilized separately in sealed serum vials.

Wolfe's vitamin solution

Composition per liter:

Pyrodoxine-HCL	10 mg
Thiamine-HCL	5 mg
Riboflavin	5 mg
Nicotinic acid	5 mg
Calcium pantothenate	5 mg
<i>p</i> -Aminobenzoic acid	5 mg
Thioctic acid	5 mg
Biotin	2 mg
Folic acid	2 mg
Cyanocobalamin	100 µg

Preparation of Wolfe's vitamin solution

Add above components to distilled/deionized water and bring the volume to 1L. Mix thoroughly and **filter sterilize**.

Wolfe's mineral solution

Composition per 1 liter:

MgSO ₄ ·7H ₂ O	3 g
Nitroloacetic acid	1.5 g
NaCl	1 g
MnSO ₄ ·H ₂ O	0.5 g
FeSO ₄ ·7H ₂ O	0.1 g
CoCl ₂ ·6H ₂ O	0.1 g
CaCl ₂	0.1 g
ZnSO ₄ ·7H ₂ O	0.1 g
CuSO ₄ ·5H ₂ O	0.01 g
AlK(SO ₄) ₂ ·2H ₂ O	0.01 g
H ₃ BO ₃	0.01 g
Na ₂ MoO ₄ ·2H ₂ O	0.01 g

Preparation of Wolfe's mineral solution

Add nitroloacetic acid to 500 ml distilled/deionized water. Dissolve by adjusting pH to 6.0 with KOH. Add remaining components. Add distilled/deionized water to 1.0 L.

Reducing agent:

Composition per 20 ml (2.5%)

NaS·9H ₂ O	0.5 g
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Preparation of reducing agent

Add NaS·9H₂O to distilled/deionized water and bring volume to 20 ml. Mix thoroughly. Gas with 100% N₂ for 20 min. Capped with rubber stopper. Autoclave for 15 min at 15 psi pressure, 121°C. Use fresh prepared solution. Cool medium to room temperature.

Phosphate buffer solution

Composition per 100 ml:

KH ₂ PO ₄	15%
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Na₂HPO₄ 29%

2. Preparation of buffer solution

Gas with 100% N₂ for 20 min and cap with a rubber stopper. Autoclave for 15 min at 15 psi pressure, 121°C.

Buffers for adjusting pH

- 0.1N KOH for adjusting pH
- NaOH for adjusting pH

G. Carbon monoxide medium

When carbon monoxide is used as a carbon source, gas a tube containing 10ml of culture medium (containing 0.2ml Na₂S, 0.2ml phosphate buffer solution, 8.5ml phosphate-buffered medium) with CO gas for a duration of 2 minutes. The CO gas apparatus should have a line that runs from the CO gas tank to a 0.22 micron air filter. The air filter then passes the gas through a 22G x 4" injecting needle. The anaerobic culture tube should have an exhaust system consisting of a 20G x 1" needle in the rubber stopper cap. Bacteria can then be injected into the CO media at a volume of 1ml.

Lysis buffer

Tris pH 8.0	100mM
NaCl	150m
Lysozyme	1mg/ml
antiprotease DNase I	1mg/ml
MgCl ₂	2mM

Prepare 25ml of Lysis buffer for our 20 isolates. (1.25ml lysis buffer *20)

Tris pH 8.0	0.0025ml
NaCl	0.21915g
Lysozyme	25mg/ml
antiprotease DNase I	25mg/ml
MgCl ₂	0.0101665g

Store the lysis buffer at 4°C

Standard Reaction mixture

Preparation of Standard Reaction Mixture for Assay

Composition per 0.75 ml

- Tris pH 8.5 50mM
- Methyl viologen 1 mM

Note: Amount of solution that should be prepared is (0.75mL) x (# of isolates to be assayed)

For a 60ml standard reaction

Take a 100ml beaker and add 0.015g of MV with around 10-20ml of Tris pH 8.5. Swirl to dissolve and once it is in solution, pour into a graduated cylinder and bring it up to 60ml with Tris pH 8.5. Transfer to a serum bottle, gas with N₂ and seal and crimp. **Do not autoclave.**

Preparation of the anaerobic chamber

1.) Place the following items in anaerobic chamber (Don Whitley model DG250):

- sterile 1.5ml eppendorf tubes
- 70% ethanol
- Spectrophotometer
- vortexer with eppendorf attachment
- thermometer
- 3ml syringe
- 20G x 1" needle
- prepared lysis buffer
- standard reaction mixture
- test tube rack
- glass beads (Bio 101 systems)
- oxygen indicator strips should be used to ensure that the conditions within the chamber are anaerobic (BBL dry anaerobic indicator strips)
- conical vial (3-4 ml, Fischer Science)

2.) Ensure that the chamber shell is flushed with the base and no leaks are present.

3.) Ensure that the condenser is properly connected to the unit.

NOTE – The condenser socket is extremely sensitive and can be disconnected easily.

4.) Ensure that the inside and outside doors are properly sealed.

5.) Check O-rings and ensure that the sleeves are not leaking or torn.

6.) Connect the 90% N₂, 5% CO₂, 5% H₂ gas tank to the anaerobic chamber.

7.) Open the main valve entirely.

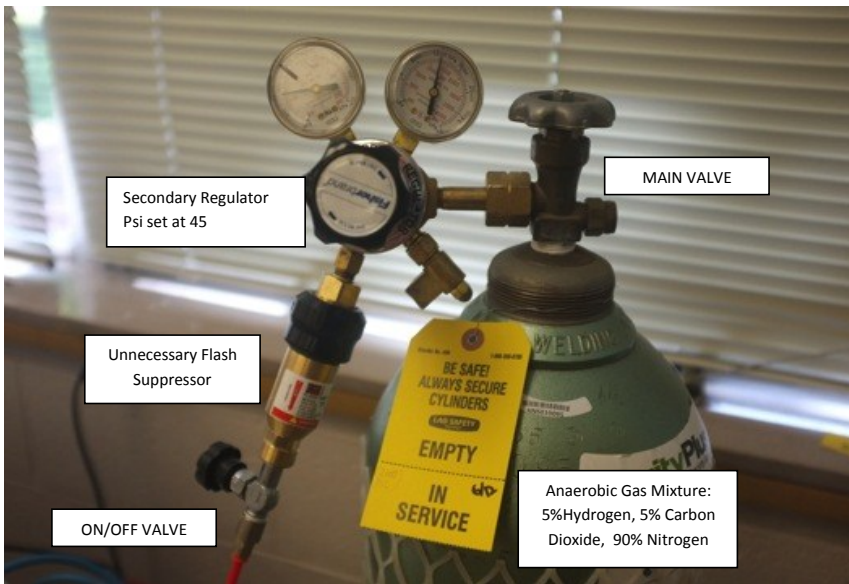
8.) Set the secondary regulator at 45 psi. The secondary regulator controls the pressure of gas

being expelled from the tank which ranges from 0-200psi. The middle protruding lever is the secondary regulator.

9.) Plug in and turn on the anaerobic chamber. Both the gas and flow fault lights should light up.

10.) There should be a buzzing sound to signal that the fault alarm is on. (Toggle the buzzer switch until the check alarm is heard.)

11.) Turn the ON/OFF valve to gas to flow through and the alarm should stop.



12.) Run the commission cycle by pressing and holding the commission buzzer until 2 distinct beeps are heard (Gas should be sucked in approximately every 20 seconds for 20 minutes, if the chamber continues sucking in gas after this time period, then there may be a leak in the system and the operation should be aborted.)

13.) Allow the chamber to stabilize for 24 hours.

Protocol

A. Preparation of cells

Background information: Mesophilic bacteria are characterized as being able to grow at moderate temperatures (between a 20-45°C) and are labeled “M” on the culture tube and thermophilic bacteria are characterized as being able to survive at higher temperatures (between 45-80°C) and are labeled “T” on the culture tube.

1. Prior to adding media for inoculation, label anaerobic culture tubes by copying down the name provided on the frozen culture stock. **Note:** prepare two extra culture tubes and label one M-blank and the other T-blank. These uninoculated tubes will serve as blanks when measuring absorbance values.
2. Set the spectrophotometer to 660_{nm} and allow it to warm up for 30 minutes.
3. Obtain 1 ml of cells from frozen stock cultures and grow in 9 ml phosphate medium containing 8 ml phosphate-buffered medium with 0.2ml phosphate buffer, 0.2ml Na₂S₉H₂O, and 0.5 ml glucose (400 mM) as carbon source. The cells should be incubated at 37°C (mesophiles), 55°C (thermophiles) for 20-24 hours.
4. After inoculation, place the M-blank in the spectrophotometer and adjust the absorbance is set to 0. Record the absorbance of the mesophilic isolates. Place the T-blank in the spectrophotometer and adjust the absorbance is set to 0. Record the absorbance of the thermophilic isolates. Incubate the culture tubes at 37°C (for mesophiles) and 55°C (for thermophiles).
5. Monitor and record absorbance hourly for the first 8 hours and again around 15-20 hours. Plot the growth curve and determine the growth rate. Note: Do not store the growth cultures in the refrigerator; store them room temperature in a cool dry place away from light.
6. Repeat steps 3 to 5 by inoculating a second batch of fresh culture medium. Incubate the culture tubes at 37°C (for mesophiles) and 55°C (for thermophiles). Monitor and record absorbance hourly for the first 8 hours and again around 15-20 hours. Plot the growth curve and determine the growth rate. The absorbance value of the cells should be at least 0.1 absorbance at 660 nm.
7. Repeat the process a third time by inoculating a third batch of fresh phosphate-buffered medium with 1 ml of cells from the second batch. Monitor and record absorbance hourly for the first 8 hours and again around 15-20 hours. Plot the growth curve and determine the growth rate. After the third batch of growth cycle, the cells should be successfully revived. The absorbance value of the cells should be at least 0.1 absorbance at 660nm.
8. Transfer 1 ml of cells from Step 7 in a fresh phosphate-buffered medium (4 ml phosphate medium with 0.1ml phosphate buffer, 0.1ml Na₂S, [final volume should be 5.2 ml]. Incubate cells at 55°C (thermophiles) and 37°C (mesophiles) for 20-24 hours. Record the absorbance hourly for the first 8 hours and around 15-20 hours at 660 nm. The target absorbance should be 0.1 at 660nm.
9. After cells have grown, place the 5ml of media -into serum bottles filled with 51 ml of fresh phosphate-buffered medium (45 ml phosphate-buffered medium, 1 ml phosphate buffer, 1ml Na₂S), [final volume should be around 50 ml].

10. Incubate cells in serum bottles at 55°C (for thermophiles) and 37°C (for mesophiles). Measure the initial absorbance at 660 nm by drawing 1 ml of the culture. Grow for 20-24 hours and check for signs of growth periodically (increase in turbidity of the solution). Cells should be incubated until absorbance of 0.1 at 660nm is reached. (withdraw 1ml of the culture from bottle and place in cuvette and measure with spectrophotometer).
11. Dispense entire contents of serum bottle into a 50 ml Falcon tube centrifuge at 4000 g for 5 minutes.
12. Repeat for the rest of the isolates. The target goal is 200 mg of cells.
13. Add 1.25 ml of lysis buffer (see recipe below) into the culture tube containing the pellet and resuspend the cells by pipetting the cell suspension up and down.

Lysis buffer

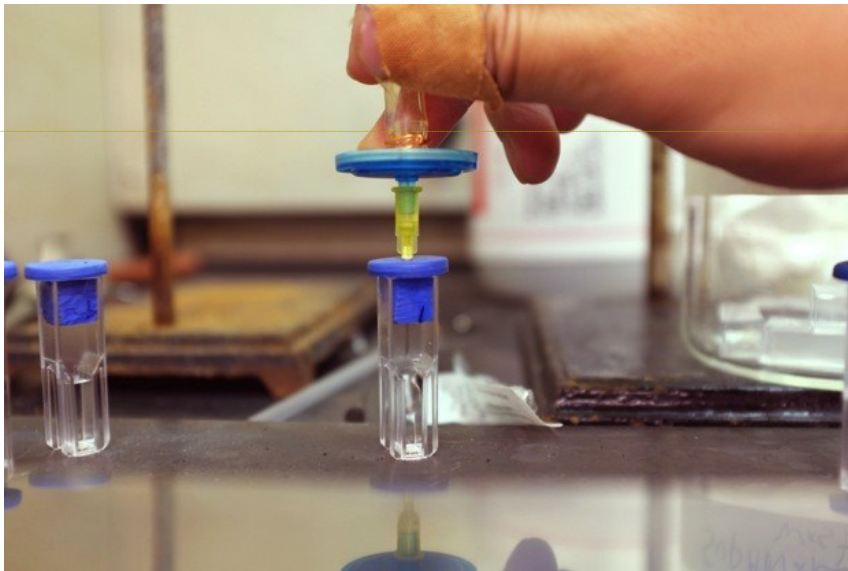
Tris pH 8.0	100mM
NaCl	150mM
Lysozyme	1mg/ml
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MgCl ₂	2mM

Prepare 25ml of Lysis buffer for our 20 isolates. (1.25ml lysis buffer *20)

Tris pH 8.0	0.0025ml
NaCl	0.21915g
Lysozyme	25mg/ml
antiprotease DNase I	25mg/ml
MgCl ₂	0.0101665g

Store the lysis buffer at 4°C

14. Transfer the cell suspension into a sterile 1.5ml eppendorf tube using the 3ml syringe with a 20G x 1" syringe.
15. Incubate cells at 55°C for 10 minutes in a dry heating block (Labnet Int. Accublock Digital Dry bath). Vortex the cells every 2 minutes.
16. Add 0.1mg of glass beads (Bio 101 systems) and place on a vortexer (Daigger vortex genie 2 -22220A) with a modified microcentrifuge attachment and vortex for 10 minutes at maximum speed. Note: repeat for the rest of the isolates.
17. Place centrifuge tubes containing crude cell extracts on a rack and prepare for hydrogenase assay.
18. Place the eppendorf tubes in a tabletop microcentrifuge (Eppendorf centrifuge 5145D) and centrifuge at 16,000 g for 5 minutes to pellet the cells



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Example of modified butyl stopper for air tight cuvette.

Enzyme Assay

Preparation of Standard Reaction Mixture

Composition per 0.83 ml Composition per 0.83 ml

- | | |
|-------------------------------|--------|
| • Butanol | 19.6mM |
| • NAD ⁺ | 0.39mM |
| • Semicarbazide hydrochloride | 73.5mM |
| • Tris-HCl pH7.8 | 68.8mM |

Note: Amount of solution that should be prepared is $(0.75\text{mL}) \times (\# \text{ of isolates to be assayed})$

Prepare the following stocks

50ml of 500mM butanol

10ml of 100mM NAD⁺

100ml of 500mM Semicarbazide hydrochloride

100ml of 500mM Tris HCl pH 7.8

To prepare 60ml of standard reaction mixture:

Add 2.35ml of 500mM butanol, 0.2ml of 100mM NAD⁺, 8.82ml of 500mM semicarbazide hydrochloride, 8.25ml of Tris HCl pH 7.8 in 20ml of distilled water inside a 250ml beaker.

Adjust pH to 7.8 and bring volume up to 60ml with distilled water and store in a labeled serum bottle inside of a refrigerator.

Protocol

1. Set spectrophotometer to 365_{nm} and allow it to warm up for 30 minutes.
2. Pipette 0.75 ml of standard reaction mixture (19.6 mM butanol,) into four 1 ml cuvettes sealed with a modified blue stopper (labeled 1, 2,3, and B). 1=replicate 1; 2 replicate 2; 3=replicate 3; B=blank.
3. Add 0.25ml of the crude cell extract (lyzed cells) into conical vials labeled 1,2, and 3, do not add cell extract to B (blank).
4. Blank the spectrophotometer at 578_{nm} using the mixture in cuvette B (without cell extract).
5. Remove the stopper from conical vial 1 and empty the entire contents of conical vial 1 into cuvette 1 (replicate 1). Record the absorbance every minute for 5 minutes.
6. Repeat steps 2-7 for the rest of the isolates.
7. Rates of methyl viologen reduction are calculated using an absorption coefficient of $13.6 \text{ mM}^{-1} \text{ cm}^{-1}$. One unit (U) of hydrogenase activity was defined as the amount of enzyme, which catalyzes the oxidation of $1 \mu\text{mol H}_2$ per min.