MICROBIAL FERMENTATIONS

LAB MANUAL

MBOI 4470

2006

Lab manual is available as a pdf file on the website.

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INTRODUCTION

This laboratory course is designed to supplement the lectures and to help illustrate various principles of fermentation microbiology. The laboratory format is straight forward. Each student will be given unknowns for the fermentation substrate and end-products, which require accurate concentration determination (10% error). Next the actual fermentation will be performed, with the view of constructing a fermentation balance. The 'unknowns' are in effect practice for the fermentation analysis.

MBOI 4470 Microbial Fermentations SCHEDULE 2006
Lab Location: 204 Buller

TITLE	WEEK #	DATE	
		Lab Experiment	Report Due[b]
Lab 1: Determination of acetaldehyde	1	Sept 11	Sept 18
Lab 2: Determination of CO_2	2	Sept 18	Oct 2
Lab 3: Determination of ethanol and Lab 4: Determination of acetic acid	3	Sept 25	Oct 10
Lab 5: Determination of glucose	4	Oct 2	Oct 16
NO LAB (Thanksgiving Oct 9)	5		
Lab 6: Determination of glycerol	6	Oct 16	Oct 30
Lab 7: Ethanol fermentation group project In-lab calculation Assignment (2:30 pm - 30 min) - individual Part I Media Preparation (3:00 pm)	7	Oct 23	
Lab 7: Ethanol fermentation group project Part II Sample Processing and Analysis (start)	8	Oct 30	
All repeat unknown reports due (it is acceptable to hand in repeat reports any time before this date)	10		Nov 13
Last day for lab clean up. All your solutions must be discarded. Prep Room starts cleaning Nov 20th. If your name is found on anything, marks will be subtracted from your final lab mark.	11	Nov 18	
Lab 7: Ethanol fermentation group project Part II Sample Analysis (continued) Attendance is not compulsory if you have completed your experiments.	11	Nov 6	Nov 20 (includes peer evaluation - one per student)
Lab exam	12		**Nov 27**

[b]All lab reports are due by 4:30 pm on due date.

GENERAL INSTRUCTIONS

Lab Instructor:	Dr. L. Cameron	Office: 414B
Lab Teaching Assistants:	Taweewat Deemargaran	Lab: 410
	Tom Rydzak	Lab: 413

Location: 204 Buller Bldg.and possibly 201 Buller

WEBSITE: www.umanitoba.ca/faculties/science/microbiology/staff/cameron/

OR via University of Manitoba Microbiology Homepage:
http://www.umanitoba.ca/faculties/science/microbiology/labinformation.htm
Information available at the website: changes/corrections, additional information, data, marks

REGULATIONS

1. Lab attendance is compulsory.
2. Students must wear a buttoned lab coat as strong acid or base is used frequently in the lab. There is no smoking, drinking, or eating in the lab. Long hair must be tied back.
3. Bring a permanent marker.
4. When recommended in the lab protocol, disposable gloves and shield glasses should be worn in the lab. The disposable gloves are provided in the lab and glasses are available at the bookstore.
5. Students will work individually unless stated otherwise in a particular experiment pre-lab.
6. Each student or group's bench area must be thoroughly cleaned and wiped with cleaner before leaving the lab. Marks will be subtracted from the final lab mark if this is not performed each lab day.

Evaluation

1. The lab is worth 25%of the final mark:

Six unknowns	6%
Fermentation Project	
Quiz	0.5%
Peer evaluation	0%*
Group report	4.5%**
Lab exam	14%

*No marks are allocated for the peer evaluation but you must hand in your peer evaluation or 0.5% mark will be subtracted.
**If your peer evaluation final mark is below 70%, your group report mark will be reduced dependent on peer evaluation mark. For example, the maximum mark from each student = 30 x 5 (# group members other than yourself) = 150. If group members total evaluation marks for Frederick was 24, 20, 30, 30, 28 = 162 then 132/150 x 100 = 88% resulting in no deduction in project mark. However, Rocky, due to poor participation in lab and assistant in writing report, only received a total of 62 resulting a evaluation mark of 64/150 = 43%. Rocky would only receive a mark 0.43 times the project final mark.

2. Lab clean up: Last day for **lab clean up.** All your solutions must be discarded. See schedule for last clean up day. If your name is found on anything, marks will be subtracted from your final lab mark.

3. Students must pass the lab to complete the course (50%).

4. Lab reports are to be handed in as stated in schedule by 4:30 pm of that day. Hand in your unknown reports and fermentation report through the slotted drawer in room 204 ONLY. Instructor and demonstrators do not accept lab reports. If handing in a lab report late, one mark will be subtracted for each class day late. Marked lab reports will be returned to

students the next week. A late report will not be accepted after that report has been returned to the class.

5. Lab report marks are final unless an obvious error in addition of marks has been made. However, if a student feels they have a legitimate complaint, please direct attention to the lab instructor.

6. Approximately two weeks prior to the lab exam, a brief outline of lab exam format and information content will be will be available on the website. See schedule for lab exam date. Lab exam is in your lab room (unless otherwise announced) and starts at 2:30 pm (1.5 h).

7. You must notify the lab instructor no later than two school days after the missed lab. A Doctor's certificate is required for a missed lab exam. All deferrals will write the lab exam at a scheduled time set by the instructor. Failure to comply will result in a zero on your lab exam.

8. **Plagiarism (copying another student's lab report (present or previous year) or copying published literature without citing is a violation of University regulations. Refer to the STUDENT DISCIPLINE BY-LAW in your student handbook (rule book) for action taken for plagiarism.**

LAB REPORTS PRESENTATION INSTRUCTIONS

Unknown report formats are available online on lab website either as a Word document or Excel Spreadsheet. However, for the final project report you are required to set up some of your own report format including tables.

Before handing in your report review report to ensure that all information is included. When printing Excel spreadsheets make sure you have selected all information before printing. If you are using text boxes, they must be completely within the selected area or they do not print.

1. All reports must have an Honesty Declaration attached at end of report. Available as a pdf file on lab website.

2. Lab reports must be **typed.** Up to 10% of the mark subtracted for reports not typed.

3. Number pages.

4. <u>On the front page of the report state (this does not need to be a separate page):</u>
   - Course name and number
   - Experiment number and Title
   - Group # and section #
   - Individual or Group name(s). If handing in an individual report, also include lab partners name.
   - GROUP report or INDIVIDUAL report
   - Date

5. Lab report information is to be presented exactly as requested in lab manual. Number sections the same as the lab manual.

6. **Always include a sample of each type of calculation in your lab report.**

7. If any of your fermentation project data is not <u>workable</u>, borrow data from another group and reference. Non workable refers to data that cannot be plotted, used for calculations or required analysis. It does not necessarily mean the expected data.

8. Cite reference in text of lab report and record full reference at end of lab report. When should you cite and reference. The following is a good definition of plagiarism that

explains when you should cite a reference. "**The unacknowledged use of another person's work, in the form of original ideas, strategies, and research, as well as another person's writing, in the form of sentences, phases and innovative terminology**." (Spatt[1], 1983, p.438) This is done by using bracketed reference number that you used when listing references at end of lab report or by bracketing first authors name and date. Quote text unless you paraphrase completely in your own words. But remember, quotes should only be a small part of your work. If you are using the name year system, list the references alphabetically. Some examples are as follows (McMillan[2] 1997):

Binder V. Hendriksen C, Kreiner S. 1985. Prognosis in Crohn's disease - - based on results from regional patient group from county of Copenhagen. Gut 26:146-50.
Danforth DN, editor. 1982. Obstetrics and gynecology. 4th ed. Philadelphia: Harper and Row. 1316 p.
Petter JJ. 1965. The lemurs of Madagascar. In: DeVore I, editor. Primate behavior: field studies of monkeys and apes. New York: Holt, Rinehart and Winston. p 292-319.
If available only on the web:
Kingsolver JC, Srygley RB. Experimental analyses of body size, flight and survival in pierid butterflies. Evol. Ecol. Res. [serial online] 2000;2:593-612. Available from: Colgate University online catalog. Accessed 2000 Oct 3.
If journal article assessed on the internet, site as journal. However, if available only on the web, reference as follows:
Kingsolver JC, Srygley RB. Experimental analyses of body size, flight and survival in pierid butterflies. Evol. Ecol. Res. [serial online] 2000;2:593-612. Available from: Colgate University online catalog. Accessed 2000 Oct 3.

9. Personal or Professional Electronic sources[2]:
Cite in-text by putting the following in parentheses, author's last name or file name (if no author's name is available) and publication date or the date of access (if no publication date is available).
At the end of report list: author or organization, publication date or date last revised, title of Web site,URL site in angle brackets, and the date accessed.
Cameron, L. 60.344 Microbial Physiology Lab Information <http://www.umanitoba.ca/faculties/science/microbiology/staff/cameron/60_344.htm>. Accessed 2004 April 12.

Table presentation (if format not available on the website)
- Table number and title (legend) presented above the table body.
- Number tables using arabic numbers, even if only one table in a report.
- Include enough information in title to completely describe table, eliminating the necessity to search elsewhere in the lab report to understand information presented in table. Table title starts with an incomplete sentence. Additional complete sentences may be included to adequately describe the table (this also applies to figures).
- If abbreviations are used in table, indicate what abbreviations mean as a footnote. Other footnotes may be required to clarify material in the table.
- Like information should be in columns making it easier to view the table.

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[1]Spatt, B. (1983). *Writing from Sources*. New York: St. Martin's Press.

[2]McMillan V.E. 1997. Writing Papers in the Biological Sciences. 2nd ed. Boston: Bedford Books: 1997. 197 p. and McMillan, V.E. 2001. Writing Papers in the Biological Sciences. 3rd ed. Boston: Bedford Books. 123 p.

- Data in columns should be listed under the centre of each heading. Align decimal points and dashes. If a number value is less than 1 always include zero before the decimal.
- Column or Row headings should be complete and self explanatory. A heading is a separate entity from the title. It cannot be assumed information given in the title is adequate for a heading. The unit of measurement should only be included in the heading, not in column data.
- Group related column headings under larger headings.
- If information is the same for each column or row do not include but treat as a footnote.
- Make the table as concise as possible but include all necessary information. For example, any constant experimental conditions that would change the data presented.
- Tables should be properly set up with a straight edge. Horizontal lines must be included but it not necessary to always include vertical lines.
- Include somewhere in table dilution used and volume of sample titrated. If applicatAlso include any relevant volumes used to calculation concentration.

Figure presentation (graphs, diagrams, photographs, films)
(all graphs must be computed generated - where applicable, required Excel presentation procedure is given in the lab appendix)

- Figures are to be numbered separate from tables, using arabic numbers. Include figure number even if only one figure.
- Figure number and figure legend should be presented below the graph. The figure legend, like the table, starts with an incomplete sentence describing the graph. For example, do not repeat just the labels of the x- and y-axis but present in a descriptive manner. Additional sentences should be included if additional information is required to completely describe figure, for example, any constant experimental conditions that affect the data presented.
- All diagrams, photographs, and films are figures and should be completely labelled.
- For figures of graphs, there is one dependent variable plotted and one or more independent variables plotted. The dependent variable is a function of the independent variable. It is accepted practise to plot the independent variable on the x-axis and the dependent variable on the y-axis. For example the measurement of absorbance (dependent) with increasing concentration of protein (independent). The size of the graph should fit the plot(s). The axis should not necessarily start at zero. Place graph completely within graph grid, this includes axis labels and legend. The overall size of graph should not be too large but should not be so small that information is obscured. The graph must be completely labelled (always include units). Use different symbols for each plot (not different coloured pens) on a graph. If more than one plot, explain symbols in legend or in a key included in the body of the graph. Graph plots can be drawn in a number of ways (this depends on the plot): (a) best fit straight line, (b) join each point with a straight line, and (c) use a flexible curve ruler or french curve. Do not drawn a free hand line.

Note: When writing your lab reports you are frequently requested to present both a table and a figure for a given set of data, similar to keeping a research journal. This is not the accepted practice for papers published in journals or books. Usually either a table or a figure is presented for a given set of data and depending on nature of data, it may only be summarized in the text. How do you make a choice of data presentation? The aim is to effectively and efficiently demonstrate what you want to show, for example, correlations, comparisons, pattern, trends, etc (McMillan 1997).

REFERENCE

McMillan V.E. 1997. Writing Papers in the Biological Sciences. 2nd ed. Boston: Bedford Books: 1997. 197 p.

LAB STANDARD OPERATIONS PROCEDURE (SOP)
Bench area: Wash bench area before and after use with AIRx109.

Personal safety: You must wear a lab coat. Wear coat only in the lab, transport separately outside of the lab (in a plastic bag). Wash hands with antibacterial soap for 15 sec before leaving the lab. No eating or drinking in the lab. Use aseptic technique for transfer of bacteria. This is to protect yourself as much as to ensure the purity of your culture. Protect hands with gloves and eyes with glasses when needed. The gloves provided in the lab must be disposed of in the petri plate containers not the general garbage cans.

Biohazards: Know biosafety risk groups. Handle all cultures as potential pathogens. Never mouth pipette. Always use a pro-pipette. If you spill a culture, cover the spill with paper towels. Pour AIRx109 over the towels to saturate. Gather up soaked towels and discard. Wipe area to dryness with fresh paper towels. Wash hands with soap and water. Place cultures on discard trolley. All cultures are autoclaved before disposing. Dispose of eppendorf tubes[a] in petri plate containers. Dispose of pipetman tips[a] in clear plastic lined basins along with glass or plastic Pasteur pipets, broken glassware, glass slides, brittle plastic objects, metal objects[a] (not needles or blades). Discard disposable 1 ml and 10 ml pipettes, tip down, in yellow plastic pail. Bacteria dilutions may to be poured down the sink and the tubes rinsed before placing on the discard trolley. Rinse sink with lots of water. Used disposable gloves must be disposed on in the Petri plate containers to decontaminate. When handling level 2 microorganisms you must wear disposable gloves, make sure any cuts on your hands are covered with a bandage, and be aware of the possibility of bacteria aerosol when you flame your loop.
[a] due to the multi-use nature of the teaching lab, all eppendorf tubes, pipetman tips, Pasteur pipets, brittle plastic or metal objects will be treated the same as similar items contaminated with microorganisms.

Glassware (unbroken): Remove tape and pen markings (use alcohol) from glassware before placing on discard trolley. Used glassware should be rinsed and placed on the discard trolley. Rinsed test tubes should be placed in tray provided on the discard trolley. Used glass pipettes should be placed in pipette holders.

Petri plate culture and non-sharps solid culture material disposal: use covered plastic containers lined with clear plastic bags for contaminated petri dishes or any bacteria contaminated solid non-sharps material (eppendorf tubes, API strips, antibiotic strips, microtitration plates, etc)

Hazardous material disposal: Examples: radioactive material, ethidium bromide, sovents, etc. The lab demonstrator will instruct proper disposal methods for labs that contain hazardous materials. These materials must be disposed of in appropriately labelled containers and disposed via the safety office. Use fumehood when recommended. A MSDS binder available in lab gives information on all hazardous materials used in the lab. Use extreme care with flammable solvents. Alcohol used to flame spread rod should never be positioned within 40 cm of flame. Never put a very hot spread rod into a beaker of alcohol. The alcohol may catch fire. Handle caustic (acids and bases) solutions with care. Never discard an acid or base greater than one molar down the sink. Discard in labelled glass containers provided. Use lots of water when discard caustic solutions (< 1M). These materials are disposed of through the university safety office. Never pour solvents down the sink (eg. phenol, ether, chloroform, etc). Discard in labelled containers provided.

Sharps disposal: Dispose of all sharps (needles, syringes, razors, scalpel blades) in specified container. Dispose of syringe with needle attached - do not take apart. Do not replace the needle cap before disposing (high frequency of accidents occur when replacing cap). Sharp's containers are autoclaved before disposing.

Broken glass disposal: Dispose of broken glass in labelled plastic containers lined with clear plastic. Transferred to boxes before discarding.

Know location: Exits, fire extinguisher, eye wash, sink shower, and first aid kit. This information is given in the first pre-lab.
Equipment operation: Know how to operate equipment before use. DO NOT use equipment unless you know exactly how to operate the equipment. The demonstrator is always available to assist. Please follow instructions in appendix for proper clean up of Spectronic 20D. Ensure the spec tubes are thoroughly washed and rinsed with distilled water before replacing in rack upside down as you (hopefully) found the tubes.
Leave your bench area clean All equipment and supplies should be returned to original location.

LABORATORY BIOSAFETY GUIDE
Although, only Level 1 bacteria risk group is used in this lab, level 2 bacteria are used by other labs in this room. Follow standard operation procedures, SOP (see above).

The University of Manitoba Biosafety Guide (Feb 2000) and Health Canada Laboratory Biosafety Guidelines booklets are available in your lab. Biosafety information is also available at Guidelines: http://www.hc-sc.gc.ca/pphb-dgspsp/ols-bsl/lbg-ldmbl/index.html Health Canada http://www.umanitoba.ca/campus/health_and_safety/
MSDS (infectious agents): http://www.hc-sc.gc.ca/pphb-dgspsp/msds-ftss/index.html

There is no listing of level 1 agents in the guidelines or MSDS pamphlets
Risk group 1 bacteria are low individual and community risk and are unlikely to cause disease in healthy workers.
Risk group 2 bacteria are moderate individual risk and limited community risk. Bacteria in this group can cause human or animal disease but are unlikely to infect healthy laboratory workers. Effective treatment is available. Risk of spreading is limited.

CONTAINMENT LEVEL 1 (UM biosafety guide p. 11)
- microbiology lab with washable walls, countertops and hand wash sink
- established safe laboratory practices (hand washing and disinfection of countertops)
- general WHMIS safety training
- UM lab registration

CONTAINMENT LEVEL 2 (UM biosafety guide p.11)
- all of level 1 specifications
- biosafety permit
- biological safety cabinet (not required)
- biohazard signage
- a written standard operations procedure
- MSDS for the infectious agent

Lab Equipment
1. In each lab there are cupboards labelled ‘fermentation cupboard’ that contain basic fermentation equipment. One cupboard is usually shared by 3 or more students. Each student is responsible for cleaning, storing, and reporting any lose or breakage throughout the lab term. The glass biuret is particularly fragile. Please handle the biuret with CARE.
2. At the end of each lab, student equipment, cleaned glassware, etc. should be replaced in cupboard as the lab is used by other groups (biuret, funnel, test tube rack, magnetic stirrer, etc).
3. STORE REUSABLE REAGENTS IN YOUR CUPBOARD FOR FUTURE EXPERIMENTS. The preparations service only supplies reagents for unknowns not the ethanol fermentation lab.

WHMIS

The Workplace Hazardous Materials Information System (WHMIS) is a system for safe management of hazardous materials. WHMIS is legislated by both the federal and provincial governments.

Under WHMIS legislation, laboratories are considered to be a workplace, and students are workers. By law, all workers must be familiar with the basic elements of the WHMIS system.

The WHMIS program includes:
1. Cautionary labels on containers of controlled products. Consumer products, explosives, cosmetics, drugs and foods, radioactive materials, and pest control products are regulated separately, under different legislation.
2. Provision of a Material Safety Data Sheet (MSDS) for each controlled product.
3. A worker education program

1. A. **SUPPLIER LABELS**

Controlled products must have a label of prescribed design which includes the following information:

PRODUCT IDENTIFIER - trade name or chemical name
SUPPLIER IDENTIFIER - supplier's name and address
MSDS REFERENCE - usually, "See MSDS supplied"
HAZARD SYMBOL - (see illustration on next page)
RISK PHRASES - describes nature of hazards
PRECAUTIONARY MEASURES
FIRST AID MEASURES

B. **WORKPLACE LABELS**

All material dispensed in a workplace container must be labelled with the **Product Name, Precautionary Measures** (simplified) and **Reference to Availability of MSDS**.

2. **MSDS**

Individual course MSDS are located in a binder in your lab (Room 201 binder located in 204). The main MSDS binders are located in the Microbiology preparation room, 307/309 Buller. MSDS are also available on the local area computer network (see your demonstrator, if necessary).

The MSDS will provide: relevant technical information on the substance, chemical hazard data, control measures, accident prevention information, handling, storage and disposal procedures, and emergency procedures to follow in the event of an accident.

3. **SAFETY**

The Laboratory Supervisor will provide information on the location and use of safety equipment, and emergency procedures.

University of Manitoba

WHMIS

(Workplace Hazardous Materials Information System)

Hazard Classification / Symbol

Class A - Compressed Gas

Class B - Flammable and Combustible Material
1. Flammable Gas
2. Flammable Liquid
3. Combustible Liquid
4. Flammable Solid
5. Flammable Aerosol
6. Reactive Flammable Material

Class C - Oxidizing Material

Class D - Poisonous and Infectious Material
1. Materials Causing Immediate and Serious Toxic Effects
2. Materials Causing Other Toxic Effects
3. Biohazardous Infectious Material

Class E - Corrosive Material

Class F - Dangerously Reactive Material

Workplace Labels

These labels are applied at the workplace on controlled products or containers of controlled products when
- A. The original supplier label is missing or becomes unreadable,
- B. The material is decanted or transferred from the supplier's container to another container,
- C. The material is produced at the workplace for use within the workplace.

Workplace labels will include the following information
1. **Name/Identity of the Product**
2. **Safe Handling Information** - refers to any precautions that must be followed to minimize the risks of adverse health effect or injury. This also includes any personal protective equipment (PPE) e.g., type of gloves, eye or respiratory protection to be worn or other controls to be used through statements or pictograms (symbols)
3. **Reference to the MSDS.**

Supplier Labels

Supplier labels on a controlled product must be in English and French and include the following information
1. Product Identifier
2. Hazard Symbol(s)
3. Risk Phrase(s)
4. Precautionary Measures
5. First Aid Measures
6. Supplier Identifier
7. Reference to the availability of a Material Safety Data Sheet

This type of border (shown) is the indicator that the label identifies a controlled product.

Material Safety Data Sheet

A Material Safety Data Sheet (valid for three years) will include information relating to each of the following categories
1. Product Identification and Use
2. Hazardous Ingredients
3. Physical Data
4. Fire and Explosion Data
5. Reactivity Data
6. Toxicological Properties
7. Preventive Measures
8. First Aid Measures
9. Date and Source of MSDS

and any other hazard information of which the Supplier is aware or ought reasonably to be aware.

Fire / Medical Emergencies

555 from 474, 789, 975, 977 exchanges or
#555 from a cell phone (MTS or AT&T)
or
911 from other exchanges. If 911 is called you must also call Campus Security at **474-9341**

Chemical / Biohazards Emergencies

474-6633

(8:30 am - 4:30 Mon. to Fri.). If busy or after hours, call **555**

For further information on WHMIS, contact the Environmental Health and Safety Office at 474-6633.

Manitoba Worplace Health Hazard Regulation 53/88 requires that a complete and current chemical inventory is maintained at all times.

The University of Manitoba

WASTE DISPOSAL CHART FOR LABORATORIES

Laboratory Waste

LIQUIDS

- **NON-TOXIC WATER-SOLUBLE LIQUIDS** → Pour down a laboratory drain. Flush with copious amounts of cold water.
- **WASTE MACHINE OIL** → Collect in a suitable container. → Dispose through Safety Office
- **ACIDS, BASES AND ALL OTHER LIQUID CHEMICALS AND LIQUID CHEMICAL WASTE** → Store securely. Send a completed INVENTORY FORM to the Safety Office. (Please set aside a box for packaging at the time of pick-up.)

SOLIDS

- **UNWANTED DRY CHEMICALS AND CHEMICALLY CONTAMINATED WASTE** → Store securely. Send a completed INVENTORY FORM to the Safety Office. (Please set aside a box for packaging at the time of pick-up.)
- **ASBESTOS WASTE** → Seal in plastic. Label as "ASBESTOS WASTE". → Dispose through Safety Office
- **SHARPS** needles, blades, tips *(biomedically involved)* → Pack in a puncture-proof container. → Autoclave if used with any potentially infectious materials. → Dispose through Safety Office
- **GLASS** brittle plastic, tips *(not biomedically involved)* → Pack securely in a box. Label as "GLASS". → Dispose through Physical Plant. (i.e. caretakers)
- **PAPER AND DISPOSABLES** gloves, lab mat → Dispose with regular garbage. → Dispose through Physical Plant. (i.e. caretakers)

Dispose through Safety Office (474-6633) Method of disposal determined by Safety Office → Incinerate / Commercial Hazardous Waste Handler. / Brady Landfill

Dispose through Physical Plant → Brady Landfill

NO CHEMICALS ARE TO BE LEFT FOR THE UNIVERSITY OF MANITOBA CUSTODIAL STAFF. Empty reagent bottles are to be rinsed and have the labels de-faced. All potentially infectious materials (*biomedically involved*) must be autoclaved or de-activated using a chemical sterilizing agent prior to disposal. Animal carcasses are to be incinerated. Radioisotope users should consult the University of Manitoba "Waste Disposal Chart For Radioisotope Users".

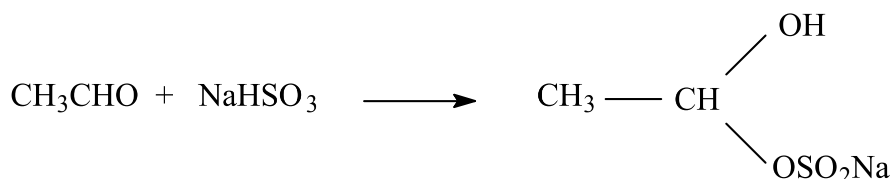
THE ABOVE CHART IS A GUIDE, MORE INFORMATION IS AVAILABLE THROUGH THE OCCUPATIONAL HEALTH & SAFETY OFFICE (474-6633).

October 1993

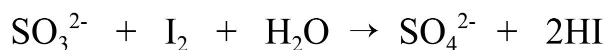
EXPERIMENT 1 DETERMINATION OF ACETALDEHYDE

Principle

Acetaldehyde (aldehydes and ketones) reacts rapidly with aqueous solutions of sodium bisulfite to form the nonvolatile sodium acetaldehyde-bisulfite compound:



This derivative is stable in slightly acid and neutral solutions and is decomposed only when the solution is made distinctly alkaline. These properties permit the direct estimation of acetaldehyde by destroying excess bisulfite with iodine at pH 6 to 7, and subsequently liberating the sulfite combined as sulfurous acid (H_2SO_3) by proper adjustment of the pH. The liberated sulfite is then titrated with iodine, the value being the amount of acetaldehyde.



$$SO_3^{2-} + I_2 + H_2O \rightarrow SO_4^{2-} + 2HI$$

Reagents

Acetaldehyde: M.W. 44.05, d. 0.788. CAUTION: acetaldehyde is extremely dangers in concentrated form. Flammable (boils at 21ºC). Cancer suspect agent. Severe eye irritant. In the lab you work only with acetaldehyde bisulfite in dilute solutions. But still exercise caution - wear gloves when handling acetaldehyde-bisulfite. If you spill it on yourself, rinse immediately. When preparing the student acetaldehyde-bisulfite solutions what precautions must be taken? How would you prepare a 0.61 M solution of acetaldehyde-bisulfite using a 1 M solution of sodium bisulfite? Sodium bisulfite (MW = 104) Acetaldehyde is a liquid.

Sodium Carbonate Buffer: prepare by dissolving 80 g of sodium carbonate in 500 ml distilled water, adding 20 ml glacial acetic acid, and diluting to1 liter. pH ≅10.

Standard Iodine (0.1 Normal): dissolve 12.7 g resublimed iodine and 40 g KI crystals in 800 ml distilled water and stir until completely dissolved. Bring the volume up to 1 liter with distilled water. Store in a brown bottle. Why use distilled water to prepare all solutions unless otherwise state?

Note: The distilled water is tap distilled water (white handle).

Reference

(note: this reference and other references in this lab manual were used in preparation of lab manual, not necessary for student study)

Goldman, F.H. and H. Yagoda. 1943. *Industrial and Engineering Chemistry, Anal.* Edition, 15:377.

Week 1

Procedure (Students work individually.)

Comments
a) Refer to appendix for titration theory and method.
b) The biuret must be cured for each different reagent added. After clamping biuret to stand, rinse first with distilled water and then a small volume of titrant. Discard the titrant used to cure the biuret. Be sure to cure biuret outlet.
c) If the magnetic stirrer does not have a white top, put a piece of white paper or paper towel on the magnetic stirrer. OR if the volume of the solution being titrated is too small or the stirrer is too fast, just shake the solution flask by hand while titrating.

Reminder
Blue Pro-pipettors are for 0.1 ml to 2 ml glass pipettes and Green Propipettors are 5 or 10 ml pipettes. Always hold the glass pipette near the top when attaching propipettor and NEVER USE FORCE when fitting propipettor firmly.

1. The acetaldehyde is available as the bisulfite complex (as it is volatile at room temperature, b.p. 21ºC).
   (i) Each student will receive a standard sample of acetaldehyde-bisulfite (0.61 M) and an unknown sample. Dilute the standard solution 20-fold before titrating.
   (ii) Each student will receive an unknown sample of acetaldehyde-bisulfite. Dilute your sample 1/2 and 1/5. Titrate. Select best dilution and repeat the following procedure in triplicate. The best dilution refers to "best" amount of titrant to add, not too little as incorporates too much error or too much, wastes your time and chemical (possibly not have enough). An approximate gauge is a titrant volume over 20 ml is too much or less than 5 ml is too little.

2. First Titration: Add approximately 10 drops of starch indicator to the 10 ml of diluted sample and titrate with 0.1 N iodine solution to the first end-point (blue/purple). The first titration gives the amount of free SO_3^{2-}. Explain the function of the starch indicator?

3. Second Titration: Add 5 ml of sodium carbonate buffer - the solution will clear. Then titrate until the solution reaches the second end-point (blue/purple). This end point is very unstable. If the color remains for 10-20 seconds then it is the true second end point. The second titration gives the amount of SO_3^{2-} bound to acetaldehyde.

Reminder Keep all leftover re-useable reagents in your cupboards, for example, starch indicator.

Comments
(i) Refer to appendix for definition of normality and relationship to molarity.
(ii)The reason the known standard sample is included in this lab is to familiarize the student with iodine indicator end point, which is difficult to determine correctly. It is advisable to carry out the entire procedure using the known solution. Calculate the concentration (molarity) of known sample from your experimental data and see if it matches expected value of 0.61 M. If it does, you know how to read the iodine endpoint and should proceed with unknown. If inaccurate, repeat using known sample until experimental and theoretical values are close, then proceed to unknown sample.

Lab 1 REPORT (Report format available as a Word document on lab website.)

Date:
Group or Individual Report:
Student Name(s):
If applicable indicate group members name if individual report:

1. Record requested information in the following table. Select triplicate data for only one dilution.

Table 1. Determination of unknown acetaldehyde-bisulfite concentration by titration with 0.1 N iodine in the presence of starch indicator.			
Unknown code: ______________			
Selected dilution of unknown acetaldehyde-bisulfite : _____________ (use only one dilution, the best)			
Trial #	volume of 2nd titration[a] (ml)	unknown acetaldehyde concentration (M)	average unknown acetaldehyde-bisulfite concentration (M)
1			
2			
3			

[a]corresponds to amount of bisulfite released from acetaldehyde in a 10 ml sample.

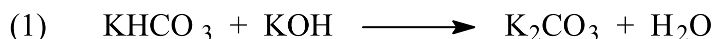
Show calculations.
[Include only one sample calculation for each calculation type. This applies to all reports. For this report you need one sample calculation for acetaldehyde concentration determination and average value.]

Each unknown is marked out of 12. Two marks for data presentation and calculation presentation and accuracy. The remaining 10 marks are assigned to determination of unknown concentration. A mark of 10 will be obtained for 0-10% error in calculation of unknown concentration. 0.1 to 1.0 mark will be deducted for each additional percentage error for each lab. There is variability in the mark subtracted due to differential accuracy of each unknown determination. Mark deducted is determined each year by considering results of all student data for each unknown determination. Students are allowed one additional attempt to get each unknown correct. You are only permitted a second attempt if the first report is handed in on due date. If you repeat an unknown determination, you must obtain a new unknown sample from the teaching assistant that is responsible for that particular unknown. Repeating of unknown determination may be done at anytime, prior to handing in fermentation report, whenever the lab is open. Check lab occupation schedule on lab door.

EXPERIMENT 2 CARBON DIOXIDE DETERMINATION

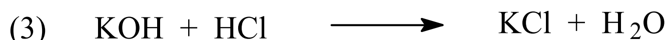
Principle

The 'CO_2 unknown' consists of $KHCO_3$ dissolved in excess KOH. The $KHCO_3$ will be converted to K_2CO_3 in the KOH.

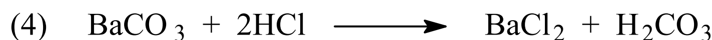

(1) $KHCO_3 + KOH \longrightarrow K_2CO_3 + H_2O$

Excess $BaCl_2$ is added to a sample of the KOH-K_2CO_3, and the carbonate is precipitated.

(2) $BaCl_2 + K_2CO_3 \rightarrow BaCO_3 + 2KCl$


(3) $KOH + HCl \longrightarrow KCl + H_2O$

The $BaCO_3$ precipitate is stable at alkaline pH, so the excess KOH can be titrated with HCl to the phenolphthalein end point (pH 8.3 - 10.0). Does the pH indicator interfere with your titration? Explain why or why not.


(4) $BaCO_3 + 2HCl \longrightarrow BaCl_2 + H_2CO_3$

The titration is continued using a second indicator - bromophenol blue (pH 3.0 - 4.6). Around pH 4.0 $BaCO_3$ is decomposed by HCl to carbonic acid.

NaOH is added in small aliquots (10 µl) to compensate for any over-titration with HCl. This back titration is used to give a precise titration value. The amount of HCl used in the second titration minus the back-titration, measures the carbonate present. Back titration may not be required.

Reagents

Saturated $BaCl_2$: neutral to phenolphthalein

0.1 N HCl (standardized): normality need not be exactly 0.1 N but must be accurately known by titration with standard 0.1 N NaOH

0.04% phenolphthalein in 50% ethanol: low pH (colorless), pH range 8.3 - 10.0 (chalky pale pink), and high pH (pinkish red)

0.04% bromophenol blue in water: The dye is dissolved by addition of excess NaOH, then

adjusted with HCl to its endpoint (turns yellow from blue).

0.04% phenol red

0.1 N NaOH (standardized): The standard solution is prepared by diluting stock 1 N NaOH (commercial) with distilled water - has exact concentration.

Week 2

Procedure (Students work individually.)

1. Prior to start of experimental procedure the 0.1 N HCl has to be standardized. Not all groups need to do this step - details given in prelab. A 0.1 N HCl solution (approximate) is provided by the preparation services. To obtain a standard HCl solution, it has to be titrated against a standard solution of NaOH (this is provided) - refer to appendix for titration protocol (use phenol red as the pH indicator phenol; yellow below pH 6.8 - 8.4[a] and red above pH 6.8 - 8.4). This determines the exact concentration of the HCl solution - defined as a standard HCl solution. Use the exact (standard) concentration when calculating titration value in the subsequent procedure.

2. (i) Each student will receive a standard sample of $KOH=KHCO_3$ (0.5 M). Dilute the standard solution 10-fold. Again like the first lab, the end-points in this lab can be difficult to judge. It is advisable to carry out the complete procedure with known sample and determine if experimental and theoretical concentration of $KOH=KHCO_3$ agree, then proceed to unknown sample concentration determination.
   (ii) Each student will receive an unknown sample of $KOH=KHCO_3$. Dilute your sample 5-fold and repeat the experiment in triplicate.

3. Put 10 ml of dilution into a 150 ml Erlenmeyer flask. Add 20 ml saturated $BaCl_2$ and several drops of phenolphthalein (colorless with a white precipitate below pH 8.3 - 10.0[a] and deep pink/red above pH 8.3 to 10).

4. **First Titration**: Titrate with standard 0.1 N HCl (or whatever the standard concentration is) to a very faint chalky pink or white. This first titration volume gives the amount of excess KOH.

5. **Second Titration:** Add several drops of bromophenol blue (bromophenol blue; yellow below pH 3.0 - 4.6[a] and blue above pH 3.0 to 4.6). Titrate again with standard 0.1 N HCl until the indicator just turns yellow (or yellow/green) and the precipitate has dissolved. The second titration volume used indicates the amount of $BaCO_3$ present, plus a small excess of HCl.

6. **Third Titration:** To eliminate the possibility of over titration with HCl, back-titrate using 10 µl aliquots of standard 0.1 N NaOH to blue-grey (pale green) endpoint of bromophenol blue (pH 4.0).

[a] The transition pH may show an intermediate color.

Lab 2 REPORT (Report format available as a Word document on lab website.)

Date:
Group or Individual Report:
Student Name(s):
If applicable indicate group members name if individual report:

1. a) Record requested information in the following table. Select triplicate data for only one dilution.

Table 1. Determination of unknown carbon dioxide concentration by titration with standardized HCl.					
Unknown code: _______________					
Standardized HCl concentration: _____________________					
Selected dilution of unknown acetaldehyde-bisulfite : ______________ (use only one dilution, the best)					
Trial #	initial volume of bromophenol blue titration[a] (ml)	back titration with 0.1 N NaOH (ml)	final volume of bromophenol blue titration[a] (ml)	unknown CO_2 (M)	average unknown CO_2 concentration (M)
1					
2					
3					

[a]corresponds to amount of carbonic acid released when $BaCO_3$ is decomposed by HCl in a 10 ml sample.

b) Numerical sample calculations.

EXPERIMENT 3 ETHANOL DETERMINATION

Principle

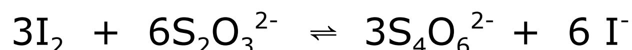
Ethanol and related compounds can be determined with good precision by oxidation with acid dichromate solutions. Ethanol is oxidized stoichiometrically to acetic acid, 4 equivalents of dichromate being consumed per mole of ethanol. Interpret this statement using the following equations.

$$2K_2Cr_2O_7 + 8H_2SO_4 + 3C_2H_5OH \longrightarrow 3CH_3COOH + 2Cr_2(SO_4)_3 + 2K_2SO_4 + 11H_2O$$

What is the oxidative state of chromium in dichromate? There are lots of websites that review oxidative-reduction reactions, for example, http://www.towson.edu/~ladon/oxstate.html and http://chemed.chem.purdue.edu/genchem/topicreview/bp/ch19/1_frame.html. You need to know this to understand why 4 equivalent of dichromate are consumed per mole of ethanol

$$6I^- + Cr_2O_7^{2-} + 14H^+ \longrightarrow 2Cr^{3+} + 3I_2 + 7H_2O$$

For this redox reaction, do you know the substance oxidized, the substance reduced, the oxidizing agent and the reducing agent?



$$3I_2 + 6S_2O_3^{2-} \rightleftharpoons 3S_4O_6^{2-} + 6I^-$$

An accurately measured amount of dichromate is added, and the amount remaining at the end is measured iodometrically. The iodine formed is titrated against a standard thiosulfate solution with starch as an indicator.

Although the estimation of alcohols by dichromate is precise, it is extremely unspecific, the majority of organic compounds of interest to biochemists being attacked. For this reason it is essential to separate the alcohol from the other oxidizable components of the mixture before applying the oxidation.
This can be done by distillation or microdiffusion. In the technique of microdiffusion, the ethanol diffuses from the outer well to the inner well where it is oxidized, allowing the diffusion to go to completion.

Reference Methods in Enzymology, Vol. III, p.255.
Reagents

0.05 N $K_2Cr_2O_7$ in 10 N H_2SO_4: Dissolve 1.226 g of dry $K_2Cr_2O_7$ in 10 N H_2SO_4 to give a final volume of 500 ml. Note: $K_2Cr_2O_7$ MW = 294.2 Express 0.05 N $K_2Cr_2O_7$ in molarity. Hint refer to equations above to determine n knowing that I^- has a n value = 1.

50% Potassium Iodide: Dissolve 25g KI crystals in distilled water to give a final volume of 50 ml.

Standard 0.1 N sodium thiosulfate solution

Note: Prepare 10 N H_2SO_4 on ice in fumehood. Place required amount of distilled water on ice and slowly add measured amount of H_2SO_4. Slowly stir. Leave in fumehood until cool.

Week 3

Procedure
Unless otherwise instructed STUDENTS WORK INDIVIDUALLY.
If class size is greater than 23 students, students work in pairs.

Store supplies required for remainder of week in your cupboard. Store 10 N H_2SO_4 in your cupboard for next's week lab.
Discard the contents of the microdiffusion units in acid discard bottle provided in the fumehood. Rinse the microdiffusion dishes with cold water at first (high concentration of sulfuric acid). Then run the water for at least 1 min afterwards. Make sure the sink and microdiffusion dishes are completely rinsed of sulfuric acid and vasoline wiped off before putting on discard trolley.

DAY 1

1. Set up 4 Conway No. 1 microdiffusion units as follows: pipette **exactly** 1.0 ml of the acid dichromate solution into the centre well of the Conway No. 1 microdiffusion unit (plastic dish with lid containing an inner and outer well). Note: When pipetting dichromate into the centre well place the 1 ml such that it touches the side of the well. Lightly but thoroughly coat the top's bottom rim with vaseline.

2. Add 1 ml diluted unknown solution to the outer well exactly opposite acid dichromate (repeat for all dilutions) and seal the units immediately. Prepare three dilutions of your unknown sample: 1/4, 1/8 and 1/12.

3. Prepare a blank by repeating the above process but add 1 ml distilled water.

4. Let stand overnight (at least 18 hours).

DAY 2

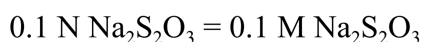
1. Open unit and add 0.5 ml distilled water and approximately 0.5 ml 50% KI to the centre well. This releases I_2.
   Note: Prior to adding any reagents, a good indication that you sample is too concentrated is if it is already a slight blue color (not yellow). This means that it is already past the end

point.

2. Titrate immediately in the microdiffusion plate with 0.1 N sodium thiosulfate using a 0.1 ml or 0.2 ml pipette all the while stirring carefully but thoroughly with the *tip of a modified pasteur pipette. Add 2-4 drops of starch indicator when near the end point (solution is yellow) and titrate until the starch-iodine complex color (faded dark blue - like denim with greenish tinge) just clears (a faint blue color will remain).
   Note: After adding starch indicator, it is a good idea to switch to P20 pipetman and add sodium thiosulfate in 10 µl increments. The end point must be exact for accurate calculation of ethanol concentration.
   * Modified Pasteur pipette: made by heating the tip until it bends and seals.
   Note: if you are having trouble with this assay, starch indicator may be added at the start.

3. Determine which dilution is in the correct concentration range (titrant should be approximately 1/2 of the standard volume titrant) and repeat the procedure in triplicate for the correct dilution and duplicate for the blank.

Notes:



0.1 N $Na_2S_2O_3$ = 0.1 M $Na_2S_2O_3$

Lab 3 REPORT (Report format available as a Word document on lab website.)

Date:
Group or Individual Report:
Student Name(s):
If applicable indicate group members name if individual report:

1. a) Record requested information in the following table. Select triplicate data for only one dilution.

<table>
<tr><td colspan="5">Table 1. Determination of unknown ethanol concentration by titration with 0.1 N Sodium thiosulfate in the presence of KI and starch indicator.</td></tr>
<tr><td colspan="5">Unknown code: _______________</td></tr>
<tr><td colspan="5">Selected dilution of unknown ethanol sample : ______________
(use only one dilution, the best)</td></tr>
<tr><td>sample</td><td>Trial #</td><td>volume of 0.1 N Sodium thiosulfate titration[a] (ml)
TITRE</td><td>average TITRE (ml)</td><td>average unknown ethanol concentration (M)</td></tr>
<tr><td rowspan="3">unknown diluted ethanol</td><td>1</td><td></td><td rowspan="3"></td><td rowspan="3"></td></tr>
<tr><td>2</td><td></td></tr>
<tr><td>3</td><td></td></tr>
<tr><td rowspan="2">blank (no sample added)</td><td>1</td><td></td><td rowspan="2"></td><td rowspan="2">NA</td></tr>
<tr><td>2</td><td></td></tr>
</table>

[a]corresponds to amount of acid dichromate remaining in the Conway diffusion dish, ie., not consumed by 1 ml ethanol sample.

b) Numerical sample calculations.
equivalents dichromate reduced/ml = ((average titre of blank x normality of thiosulfate) - (average titre of diluted ethanol sample x normality of thiosulfate)) x dilution factor

$$\text{moles ethanol/ml} = \frac{\text{equivalents dichromate reduced/ml}}{4}$$

equation units: titre of blank (litre); normality of thiosulfate (equivalent/litre)

EXPERIMENT 4 ACETIC ACID DETERMINATION

Principle

In a clarified solution, salts of a volatile organic acid like acetic acid, if treated with a strong non-volatile acid like sulfuric acid, will result in the weak acid existing in an undissociated state. The weak acid (acetate) may then be distilled from the solution using steam. The acetic acid is then determined by titration against a base.

Reagents

10 g $MgSO_4$ (What is the function of magnesium?)
10 N H_2SO_4
0.05% phenol red: grind 100 mg of phenol red powder with 28.5 ml 0.01 N NaOH and dilute to 200 ml with distilled water. Filter if necessary.

Apparatus for steam distillation (refer to diagram)

2-3 stands	2 tall burners
4 clamps	rubber/glass tubing for connections
1 clamp ring	1 funnel
1 burner stand	1 condenser-rubber stopper-glass tubing
2 wire screen	1 250 ml Erlenmeyer flask

1 1000 ml round bottom flask for main reaction fitted with double glass tubing rubber stopper
1 500 ml Erylenmeyer for boiling water fitted with double glass tubing rubber stopper

Week 3

Procedure

Students work in groups of three students. (Maximum of 10 groups)

1. Collect apparatus as shown in diagram. Because of the complexity of this experiment all groups dilute sample 1/ 2. Add 10 ml diluted unknown acetic acid sample and 25 ml distilled water (just enough to dissolve magnesium sulfate) to the round bottom flask. Add 10 g $MgSO_4$ crystals and swirl to mix. Add 1 ml 10 N H_2SO_4, again swirl to mix.
   Note: Store bottle of 10 N sulfuric acid in cupboard or fumehood as needed for next week's lab.
2. Assemble steam distillation apparatus as shown in diagram.
   Important considerations:
   Keep tubing short between flasks and condenser. You may cut the tubing to fit if necessary.
   The condense should not be higher than the reaction flask.
   The boiling water flask and the reaction flask should be at the same height.
   Use the tall burner. Just remove the hand burner from tubing and attach tubing to the tall burner. Replace tubing on hand burner when you are finished.
   The burner must be close to the water flask as you require a rapid boil for efficient distillation. You may need to use a test tube rack as a stand to adjust height of burner. Use either a burner stand or attach an O-ring clamp to stand to support burner mesh.
   In the water flask both glass tubes should be above the water level.
   Maintain about 250-300 ml in the boiling water flask by adding boiling water. Use heater plate to boil additional water.
   The glass tubing entering the reaction flask **must be submerged in the diluted acetic acid**.

3. Steam distill until 200-250 ml of distillate is collected (until the entire sample is distilled).

Record exact volume of distillate as required for acetic acid concentration calculation. It is important to maintain boiling water level. Add boiling water in small aliquots continuously to boiling water flask.

4. Thoroughly mix total distillate. Put 10 ml distillate in a small beaker. Add three drops phenol red (pH range, 6.8 - 8.4, low pH - yellow, high pH - red). Titrate with 0.1 or 0.05 N standard NaOH to the phenol red end point. Repeat titration twice more.

Lab 4 REPORT (Report format available as a Word document on lab website.)

Date:
Group or Individual Report:
Student or Group Name(s):
If applicable indicate group members name if individual report:

1. a) Record requested information in the following table. Select triplicate data for only one dilution.

<table>
<tr><td colspan="4">Table 1. Determination of unknown acetic acid concentration by titration with 0.1 N NaOH in presence of phenol red pH indicator. Acetic acid sample distilled in presence of $MgSO_4$ and H_2SO_4 prior to titration.</td></tr>
<tr><td colspan="4">Unknown code: _______________</td></tr>
<tr><td colspan="4">Selected dilution of unknown acetic acid sample (10 ml) : _______1/2________</td></tr>
<tr><td colspan="4">Total volume of distilled acetic acid sample: __________</td></tr>
<tr><td>Titration trial number</td><td>volume of 0.1 N NaOH titration[a] (ml)</td><td>acetic acid concentration (M)</td><td>average unknown acetic acid concentration (M)</td></tr>
<tr><td>1</td><td></td><td></td><td rowspan="3"></td></tr>
<tr><td>2</td><td></td><td></td></tr>
<tr><td>3</td><td></td><td></td></tr>
</table>

[a]corresponds to amount of diluted acetic acid in 10 ml sample.

b) Numerical sample calculations.

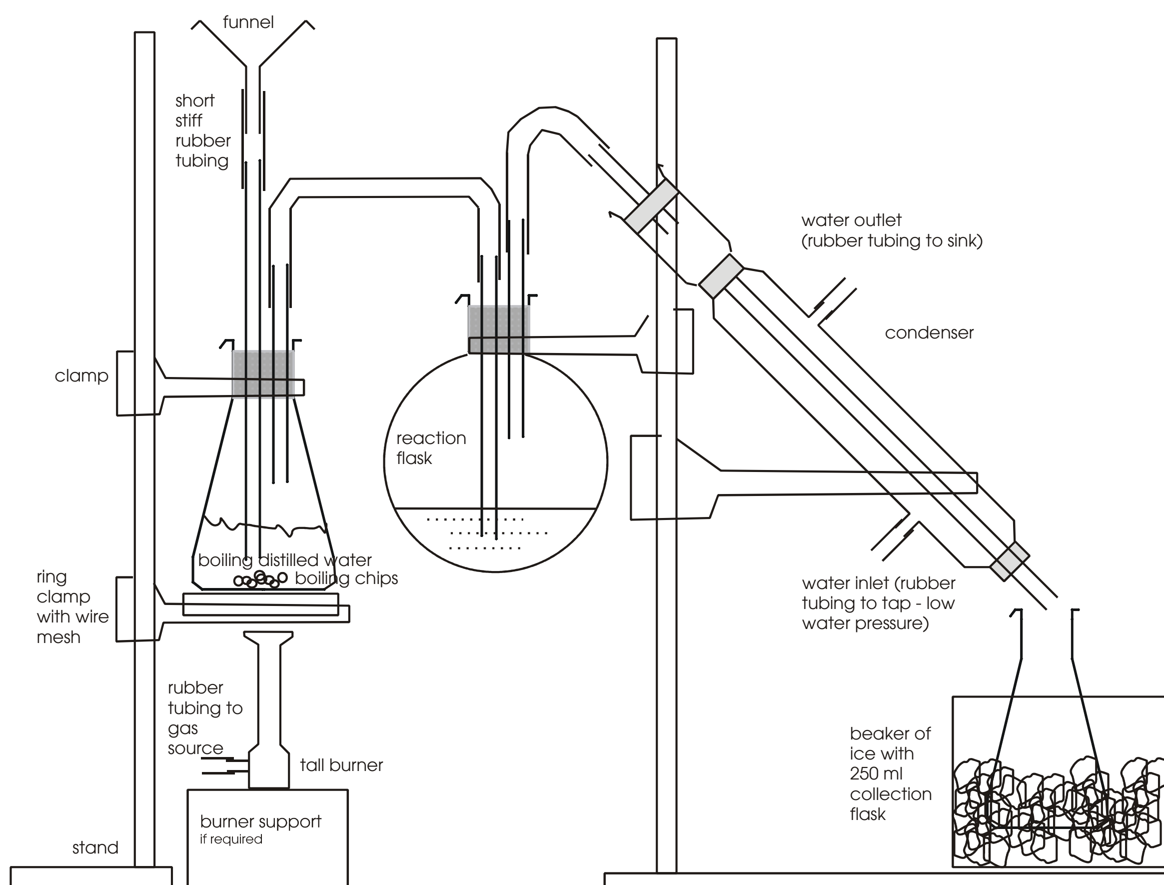
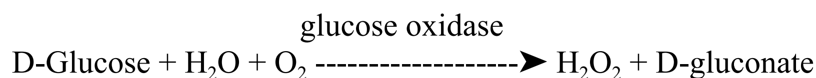


Figure 1. Steam distillation apparatus for acetic acid determination. Keep tygon tubing as short as possible to speed up distillation. Stiff rubber tubing must be used to attach funnel. Make sure water does not enter reaction flask through tubing. Add boiling water via the funnel when required.

EXPERIMENT 5 THE ENZYMATIC DETERMINATION OF GLUCOSE

Principle

Glucose oxidase reagent[3] set is a single reagent kit containing glucose oxidase, peroxidase, aminoantipyrine, N-ethyl-N-phosphate buffer, pH 7.5, sodium ρ-hydroxybenzoate, non-reactive stabilizers and fillers, sodium azide. The hydrogen peroxide formed reacts with 4-amino antipyrine and ρ-hydroxybenzoate (oxidation of the chromagen) in the presence of peroxidase producing a red quinoneimine dye. The intensity of the red color at 500 nm is directly proportional to the glucose concentration and consequently sucrose concentration.



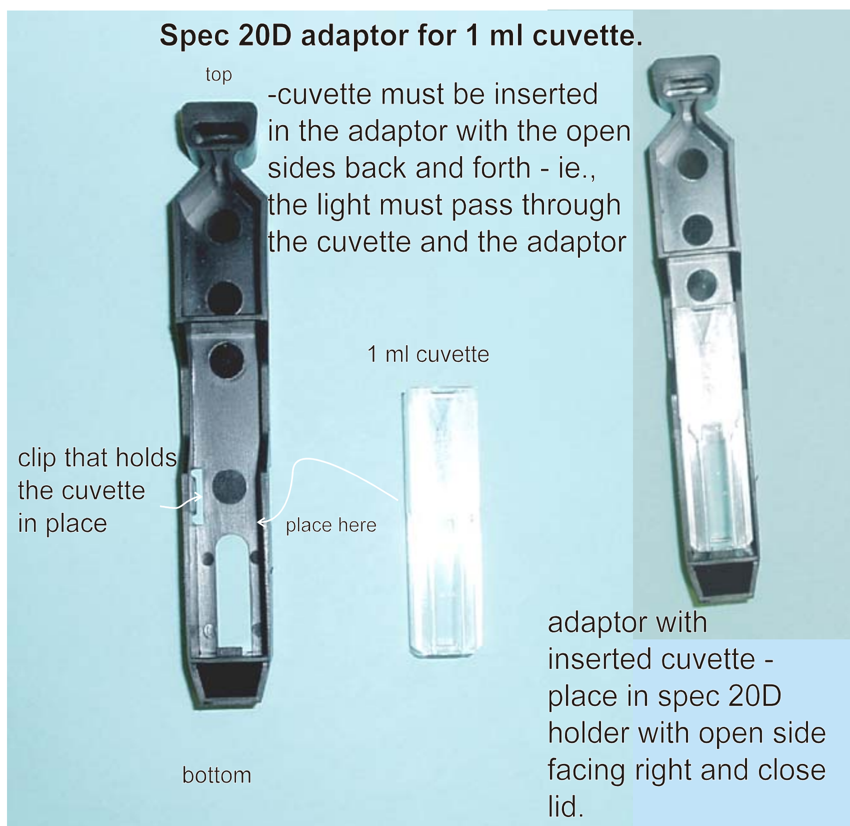
$$\text{D-Glucose} + H_2O + O_2 \xrightarrow{\text{glucose oxidase}} H_2O_2 + \text{D-gluconate}$$

$$H_2O_2 + \text{4-Aminoantipyrine} + \rho\text{-Hydroxybenzoate} + \rho\text{-Hydroxybenzene sulfonate} \xrightarrow{\text{peroxidase}} \text{Quinoneimine dye} + H_2O$$

Reference

Technical Manual. Guocose Oxidase Reagent Set. 2001. Pointe Scientific Inc.

Procedure



Two students share a bottle of glucose oxidase and glucose standard curve, but each student does their own unknown glucose sample.

1. Turn on the spec20D 15 minutes prior to start of experiment. Set at 500 nm. Since the reaction volume is only 1 ml use a spec 20D adaptor to hold the 1 ml cuvet. See diagram for instructions. The cuvet adaptor only fits in the spec 20 sideways, open side facing right. This is the only way the lid closes. Discard in plastic lined bucket when you finish.

2. Completely dissolve glucose oxidase reagent in 100 ml distilled water (white handled tap).

---

[3] Pointe Scientific, Inc. 2001 Glucose oxidase reagent set instructions.

Use a milk dilution bottle. You may shake to dissolve but not too vigorously as bubbles kill enzymes. Glucose oxidase reagent (Pointe Scientific Inc) is located in the brown fridge, room 204.

3. Label a series of disposable plastic cuvets for required samples. Add 1 ml glucose oxidase reagent to each cuvet.

4. To the glucose oxidase reagent add:
   Blank (required to blank spec): 10 µl distilled water
   Glucose standard: add 2, 4, 6, 8 and 10 µl of a 5 mg/ml standard glucose solution to a series of tubes **in triplicate**.

   Experiment samples (each dilution in triplicate): 10 µl glucose undiluted, ½ dilution, 1/4 dilution and 1/8 dilution. Keep in mind that pipetting small volumes may not be accurate. Make dilutions using larger volumes, eg. total volume of 100 µl, then adding 10 µl dilution.

5. Mix by using a small piece of parafilm held in place with your thumb (do not need to wrap) and invert several times. Need only one piece of parafilm if start mixing the lowest concentration first. Incubate at room temperature for 15 min. Read absorbance at 500 nm in spec20D using 1 ml cuvet holder. Final color is stable for 15 min (room temperature).

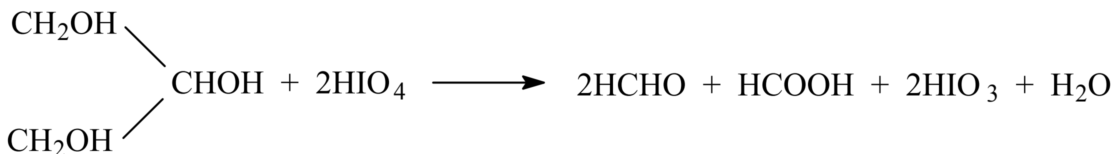
Lab 5 Report
See appendix for excel functions and graph procedure.

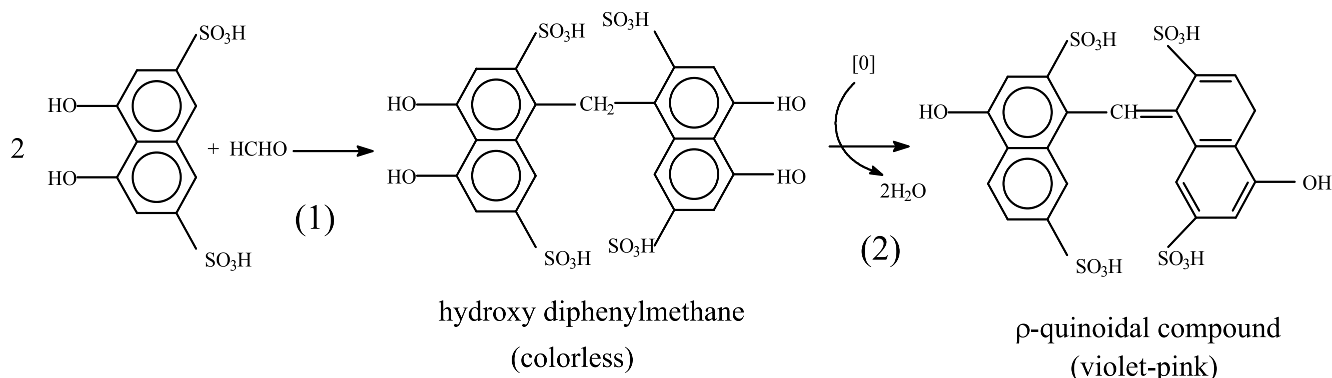
1. Submit only an excel spreadsheet(available at website). Just save spreadsheet then open in Excel. Include all requested information. Complete all columns unless otherwise specified. Insert a linear regression plot for glucose standard curve (absorbance vs mg glucose). Include linear regression equation on graph and R-squared value. Determine the concentration (mg/ml) of unknown glucose sample - only one average value for one selected dilution should appear on spreadsheet. Include requested numerical sample calculations.

EXPERIMENT 6 GLYCEROL DETERMINATION

Principle

Glycerol is oxidized by periodic acid to give 1 mole of formic acid and 2 moles of formaldehyde.





hydroxy diphenylmethane
(colorless)

ρ-quinoidal compound
(violet-pink)

The formaldehyde is measured colorimetrically by the well known chromotrophic acid-formaldehyde reaction.

Sulfuric acid participates in both (1) and (2). In the former it functions as a dehydrant to bring about the condensation, and in (2) it serves as an oxidant and is reduced to sulfurous acid. Periodic acid oxidation of any terminal hydroxyl groups, as in sugar alcohols, will yield formaldehyde and will be determined as glycerol. If the glycerol mixture is contaminated, the glycerol may be quantitatively separated by chromatography and then determined colorimetrically. Since iodate and periodate interfere with the color reaction they are reduced to iodide by excess arsenate in an acid medium before the color is developed.

Reference

Methods in Enzymology, Vol. III, Ed. S.P. Colowich and N.O. Kaplan, Academic Press Inc., p.246

Reagents

Chromotrophic Acid Reagent: Dissolve 1 g of 4,5-dihydroxy-2,7-naphthalene disulfonic acid (sodium salt) in 100 ml distilled water. Add 300 ml concentrated sulfuric acid to 150 ml water (best to do on ice in fumehood), cool, and add diluted sulfuric acid to the sulfonic solution to a final volume of 500 ml. Store in a brown bottle in the dark. Solution is stable for 2 - 3 weeks only. Note: Each group is given excess chromotrophic acid reagent. As the reagent is stable for 2 to 3 weeks, store in your cupboard to use for ethanol fermentation lab.

0.1 M periodic acid

1.0 M sodium arsenite

10 N H_2SO_4: Pour 300 ml of concentrated H_2SO_4 continuously into 600 ml distilled water with stirring (best to do on ice in fumehood). Adjust the volume to 1 liter with distilled water when cool.

Week 6

Procedure

Students work in groups of three students. (Maximum of 10 groups)

1. Repeat the following procedure for each duplicate standard curve sample and triplicate unknown sample dilution. Use three different unknown sample dilutions; 1/5, 1/7 and 1/14.

2. Pipette 2 ml of each sample into a medium size screw capped test tube.
   (i) Blank: distilled water
   (ii) Standard samples (duplicate): 20, 40, 60, 80 and 100 µg/ml using a 100 µg/ml glycerol stock solution. Use distilled water to bring each to 2 ml.
   (iii) Unknown glycerol samples (triplicate): for three different dilutions

   Comment: It is necessary to do all three dilutions (triplicate) at the same time as doing the standard glycerol samples. Why?

3. Add 0.1 ml of 10 N H_2SO_4 to each tube.

4. Pipette 0.5 ml of 0.1 M periodic acid into each tube and incubate 5 minutes.

5. Add 0.5 ml 1 M sodium arsenite to each tube, mix vigorously and let stand at room temperature for 15 minutes. Is arsenite involved in the formation of formic acid?

6. Add 6.9 ml distilled water to each tube.

7. For each reaction tube, aliquot 0.5 ml into a screw capped test tubes (medium tubes that fit into racks of boiling water baths). Add 5 ml chromotrophic acid reagent to each and place in a vigorously boiling water bath for 30 min under DIFFUSE LIGHT (lights out in lab). But not too dark as dangerous. Have some blinds open in the lab. Cool the tube and mix well. Why boil under diffuse light conditions?

8. Measure the absorbance using the spectronic 20D at 570 nm.

9. DO NOT DISCARD REACTION TUBE CONTENTS (concentrated sulfuric acid) down the sink (safety regulations). Labelled **discard concentrated sulfuric acid** glass bottle is located in the fumehood. If you accidentally dump concentrated acid in the sink, slowly add cold water, then continue to flush with cold water for at least one min.

Lab 6 Report

See appendix for excel functions and graph procedure.

1. Submit only an excel spreadsheet(available at website). Just save excel spreadsheet then open in Excel. Include all requested information. Complete all columns unless otherwise specified. Insert a linear regression plot for glycerol standard curve, absorbance vs glycerol (µg/ml). Include linear regression equation on graph and R-squared value. Determine the concentration (µg/ml) of unknown glycerol - only one average value for one selected dilution should appear on spreadsheet. Include numerical sample calculation

EXPERIMENT 7 ETHANOL FERMENTATION PROJECT

Introduction

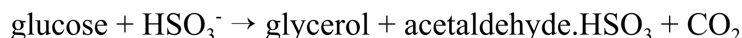
Fermentation is the degradation of an organic substrate, in our case glucose, under anaerobic conditions to produce energy as ATP. In industry the term fermentation is more generic since it means any large-scale microbial process whether it is anaerobic or aerobic.

Microbial fermentation has been important to man for as many as 6000 years, when beer and wine were produced by the alcohol fermentation of sugar by yeasts. In 1815 Guy-Lussac made a quantitative study of the process, and showed that glucose was fermented to ethanol and CO_2. Using the ubiquitous glycolysis pathway, yeast converts glucose to pyruvate. The enzyme pyruvate decarboxylase first converts pyruvate to acetaldehyde and carbon dioxide. In the presence of NADH, the acetaldehyde is reduced to ethanol by alcohol dehydrogenase. NADH is made available from the oxidation and phosphorylation of glyceraldehyde-3-P (glycolysis).

In 1911, Carl Neuberg showed that the products of the fermentation could be varied by changing the conditions of growth. For Neuberg's First Form normal fermentation is carried out with ethanol and CO_2 as products.

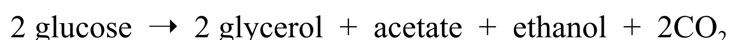
$$C_6H_{12}O_6 \quad 2CH_3CH_2OH + 2CO_2$$

Neuberg's Second Form: When sulfite was added to the medium, the acetaldehyde formed combines with the sulfite to form acetaldehyde-bisulfite. As a result, acetaldehyde is no longer able to act as a hydrogen acceptor for reduced NAD and cannot be reduced to ethanol. However, dihydroxyacetone phosphate acts as an acceptor and is reduced to glycerol, resulting in the products; glycerol, acetaldehyde bisulfite and carbon dioxide.



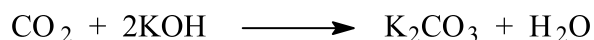
$$\text{glucose} + HSO_3^- \rightarrow \text{glycerol} + \text{acetaldehyde}.HSO_3 + CO_2$$

Neuberg's Third Form: When *Saccharomyces cerevisiae* is grown under alkaline conditions, the dihydroxyacetone -3- phosphate produced during glycolysis tends to become reduced to glycerol instead of being converted to glyceraldehyde phosphate. This results in a deficiency in the amount of reduced NAD available for the reduction of acetaldehyde to ethanol. Acetaldeyde, instead of accumulating, undergoes a dismutation to either acetate or ethanol. Therefore overall:



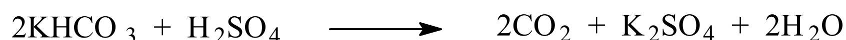
$$2 \text{ glucose} \rightarrow 2 \text{ glycerol} + \text{acetate} + \text{ethanol} + 2CO_2$$

Carbon dioxide trap: The fermentation flask is connected to a carbon dioxide trap (refer to diagram of ethanol fermentation) filled with 60 ml of 4 N carbonate free KOH. The CO_2 produced during the fermentation will be trapped by the KOH as K_2CO_3.



$$CO_2 + 2KOH \longrightarrow K_2CO_3 + H_2O$$

Excess alkali is important to prevent the formation of bicarbonate. What happens if excess bicarbonate is formed? After the fermentation is completed, the CO_2 dissolved in the fermentation



$$2KHCO_3 + H_2SO_4 \longrightarrow 2CO_2 + K_2SO_4 + 2H_2O$$

flask is driven into the KOH by the addition of H_2SO_4.

Clarification principle: Removal of proteins from fermentation solution is necessary to prevent excessive frothing or emulsification in the distillation or extraction procedures for analysis. This can be accomplished by precipitation **with** (not by) zinc hydroxide and centrifugation. What actually causes the precipitation of proteins? The resulting supernatant is clear and can be distilled without difficulty. The equation for the clarification process is as follows:

$$ZnSO_4 + 2NaOH \longrightarrow Zn(OH)_2 + Na_2SO_4 \quad .$$

cotton
Carbon dioxide Trap
Ascarite
20 cm
Clamp
Use Pyrex Tube (height 50 cm x 14 mm (in), 16 mm (out))
Inject 2 ml
Stand
Glass Beads
40 cm
cotton filled glass tube - loosely packed
Autoclave only this part, excluding syringe
~100-200 ml 4 N KOH
Medium

Figure 2. Diagram of carbon dioxide trap attached to incubation flask.

Week 7

Procedure

IN-LAB CALCULATION ASSIGNMENT (2:30 pm - 30 min) - INDIVIDUAL
-open book, bring you lab manual
-bring calculator
-emphasize is on the calculations required to carry out ethanol fermentation project
eg. dilutions required to determine mmole product and/or substrate, determination of mmole product or substrate. Know how to use ethanol fermentation volumes recorded in table for subsequent calculations.
-you are not responsible for constructing a fermentation balance chart at this stage.
-prior to lab ask TAs or instructor for help - THIS IS A DIFFICULT QUIZ.

Work in groups of 6 students (maximum of 7 groups per lab - depends on class size).

PART I MEDIA PREPARATION AND INOCULATION
For all solutions **slowly** add chemical powder to distilled water (smaller volume than required) while stirring. This allows chemical to dissolve and not form insoluble clumps. For the following procedure, this is especially required for the phosphates and glucose. Make sure the additives are completely dissolved before adjusting the pH.

Monday
Label all flasks clearly using white masking tape -include group names to ensure all members of the groups can recognize the flasks. If use just pen to mark the glassware it may wash off in the autocalve.

1. **Prepare medium for Neuberg's First Form** - testing
   a) Dissolve 2.0 g yeast extract, 0.2 g urea, 0.4 g KH_2PO_4 in 175 ml distilled water.
   b) Adjust to pH 7.
   c) Make volume up to 200 ml with distilled water.
   d) Dispense 100 ml in each of two 250 ml flasks.
   e) Loosely fit one flask (First Form INCUBATION) with rubber stopper containing glass tubing-rubber tubing-short piece of glass stuffed with cotton as shown in diagram. Loosely cover stopper and cotton stuffed glass tubing with foil. It is important to loosely fit rubber stopper so they will not seal during autoclaving. Label flask with masking tape, First Form Incubation.
   f) Fit the remaining flask (First Form CONTROL) with a foam stopper.

2. **Prepare medium for Neuberg's Second Form** (no CO_2 trap)
   a) Dissolve 2.0 g yeast extract, 0.2 g urea, 0.4 g KH_2PO_4 and 8 g Na_2SO_3 in 175 ml distilled water.
   b) Adjust to pH 7.
   c) Make volume up to 200 ml with distilled water.
   d) Dispense 100 ml in each of two 250 ml flasks.
   e) Fit each flask with a foam stopper. Label one flask, Second Form CONTROL and Second Form INCUBATION.

3. **Prepare medium for Neuberg's Third Form**

a)) Dissolve 2.0 g yeast extract, 0.2 g urea and 0.4 g K_2 HPO_4, in 175 ml distilled water.
b) Adjust to pH 8.0 with 1 N KOH (supplied with pH meter - do not use 4N KOH for CO_2 trap.
c) Make up volume to 200 ml with distilled water.
d) Dispense 100 ml in each of two 250 ml flasks.
e) Attach rubber stopper with glass tubing outlet to Third Form INCUBATION flask as described above.
f) Fit the remaining flask (Third Form CONTROL) with a foam stopper.

4. Prepare GLUCOSE solution: Dissolve 90 g glucose in a final volume of 300 ml. Dispense 50 ml into each of 6 milk dilution bottles (square 100 ml bottles) with screw caps. Do not tighten the caps tightly as they may seal upon autoclaving. Why must you autoclave the glucose solution separately? State what causes the problem.
Note: the magnesium and calcium stock solutions will be prepared for you and available in the lab Monday.

5. Autoclave all media for 15 minutes.

6. Set up two carbon dioxide traps, one for First Form incubation and the other for Third Form incubation as illustrated in figure 2. Keep tubing as short as possible. Only lightly stuff the class tubing, almost none. If stuffed to thick, no carbon dioxide will enter the trap. Use a 250 ml sidearm flask for carbon dioxide trap. Record total volume of 4 N KOH added to each trap.
Note: the sample size for CO_2 determination must be related to the total volume of KOH which represents the flask volume. This allows you to compare zero time and six day samples also allows you to correlate to samples taken from the flask.
Zero time sample: Take one 100 ml 4 N KOH sample directly from stock bottle, place in a milk dilution bottle, label zero time carbon dioxide trap sample (use for both 1st and 3rd form carbon dioxide determination) and store at 4°C.

DO NOT DISCARD 4 N KOH down the sink, pour into **labelled discard concentrated base** glass bottle located in the fumehood.

Friday

7. After media has cooled, aseptically transfer 50 ml glucose (one flask or bottle) to each medium flask. To each flask also add the following supplied sterile stock solutions to each flask, 0.8 ml 20% $MgSO_4.7H_2O$ and 0.7 ml 0.3% $CaCl_2.2H_2O$.

8. Attach carbon dioxide traps as shown in diagram to only 2 flasks - Neuberg's First and Third Form INCUBATION flasks.

9. Inoculate each INCUBATION flask with one 7.5 ml *Saccharomyces cerevisiae* (Level 1 Biohazard) (5% v/v) culture tube by pouring contents of one culture tube into each flask using aseptic technique. Assume the volume is 7.5 ml. Do not inoculate the CONTROL flasks for each form. There should be a note on the board to indicate the location of the inoculation cultures. Secure all stoppers on the cultures connected to carbon dioxide trap.

10. Immediately take a 30 ml sample (aseptic technique) from each inoculated flask (Zero day

sample). Store samples in milk dilution bottles (masking tape together) at -20ºC until ready to analyse.

11. Incubate all flasks without shaking at 28ºC for 3 days. Use the 28ºC incubator in room 204. Take care when placing cultures in the incubator. Flasks connected to carbon dioxide traps should be place on the bottom of the incubator and remaining flasks place on the upper shelves.

Week 8

PART II SAMPLE PROCESSING AND ANALYSIS

Record all requested volumes in data table provided (also available on website). This information is important for construction of fermentation balances.

1. If any observable change has occurred in the CONTROL flasks this indicates poor media preparation and you have a problem, consult teaching assistants. CONTROL flasks are only included to ensure the purity of the system. Work only with 6 samples, time zero and 3 day samples for the three Neuberg forms of INCUBATION FLASKS and the 2 samples from the carbon dioxide traps (three days) and 1 4 N KOH zero time carbon dioxide.

2. Stop cell growth by adding 2 ml concentrated H_2SO_4 to each flask. The acid must be added very gently (by syringe) so as to allow the CO_2 time to dissolve in the KOH, and not be driven up through the bead tower into the atmosphere. Insert the syringe needle between stopper and top of flask, not through the stopper. A gentle suction (water aspirator) is then applied to the top of the bead tower to swap the remaining CO_2 into the 4 N KOH. This will drive any remaining CO_2 into the CO_2 trap. Why does the addition of acid drive CO_2 into the carbon dioxide trap?

3. Remove a 30 ml sample from each incubation flask. Also remove a 100 ml sample from each carbon dioxide trap. You now have a total of six INCUBATION samples and three CO_2 samples. Milk dilution bottles are available to store samples.

4. Centrifuge INCUBATION samples (sorval-small head) at 10000 rpm for 10 min at 4ºC to remove the *Saccharomyces cerevisiae* cells.

5. Clarification of ALL INCUBATION SAMPLES: Add 1/10 volume of a 25% zinc sulfate ($ZnSO_4$) solution to each sample and mix.
a) Then only to Neuberg's Second Form three day samples add 0.42 g $CaCl_2.2H_2O$ + 0.2 g $Ca(OH)_2$ per 30 ml sample. Adjust samples to pH 7 with 10 N NaOH. If there is too much precipitate, you may need to centrifuge before adjusting the pH. Remove the $CaSO_4$ and $CaPO_4$ precipitates by centrifugation at 10000 rpm for 10 min at 4ºC. Remove the supernatant and record final volume of each sample. Use immediately or store at -20ºC until ready to analyse.
b) For all remaining samples adjust the pH to 7.0. If a precipitate forms, shake and centrifuge at 10000 rpm for 10 min. Decant the supernatant liquid and record final volume of each sample. Use immediately and/or store at -20C until ready to analyse.

Week 8 continued and week 9 (if required)

1. Analyse samples for both control and incubation flasks as follows:

Sample	Analyse
Neuberg's First Form	glucose, CO_2, ethanol
Neuberg's Second Form	glucose, glycerol, acetaldehyde (CO_2 not required - assume equal to the amount of acetaldehyde)
Neuberg's Third Form	glucose, CO_2, ethanol, glycerol, acetate

Notes:
(i)When determining the concentration of carbon dioxide, titrate excess KOH with 1 N HCl (if sample is undiluted), then titrate KOH released from sample with 0.01 or 1 N HCl. This is difficult. It is important that you are using the correct dilution and the correct concentration of HCl when titrating.
(ii) It is best to divide analysis per substrate or product not Neuberg form as this eliminates duplication of standard curves.
(iii) All required solutions not saved from the unknown determinations, must be prepared by each group. Chemicals are available on the shelves in room 201 and room 204. Acids available in the fumehood. Take extreme care when making acid solutions. Remember, add acid to water not vice versa. Prepare in the fumehood on ice.

Hints for sample dilution estimates:
a) You do not use the same dilution as unknowns but must relate to Neuberg's three forms.
b) Each form starts with 15 g glucose per 150 ml medium. Therefore, at zero time you expect all the glucose present. Calculate the theoretical dilution required to fit the standard curve. Then vary 5 to 10 fold above and below for the two other dilutions to consider experimental procedure. This gives you three 'rough' dilutions to find the correct dilution range. Once the correct dilution is found, repeat that dilution in triplicate.
c) Due to limitation of the amount of sample available. Use 5 ml sample for acetic acid distillation once. The do triplicate titrations of distillate. If indicator turns immediate, dilute the titrant, ie NaOH until you have a readable volume.
d) Remember you can always dilute the titrant to get an accurate volume. If necessary, using too much, prepare a more concentrated titrant.
e) It is unlikely that products are present in any quantity at zero time, therefore, use undiluted sample.
f) After three days, it is more difficult to predict the amount of substrate remaining and the amount of product formed. Theoretically follow the equation for each form. As an estimate, assume 5% to 50% glucose remains and determine dilutions as stated for zero time for glucose and products.
g) If it appears that no product is present, assay undiluted sample.

2. As this is the last lab, it is imperative that you clean up all your apparatus, that is, dismantle apparatus completely, rinse and put on discard trolley. **The CO_2 trap must be completely dismantled, ascarite discarded, KOH poured into discard concentrated base glass bottle located in the fumehood, ,trap rinsed thoroughly and put on discard trolley**. Ascarite contains NaOH, handle with care. All saved and new solutions must be discarded, containers rinsed and put on discard trolley.

Ethanol Fermentation Volume Table (available on lab website in Word format)

	Neuberg Form 1		Neuberg Form 2		Neuberg Form 3	
	zero time	day 3	zero time	day 3	zero time	day 3
total volume 4 N KOH in CO_2 trap (ml)			NA	NA		
total volume in each incubation flask (ml)						
initial incubation sample volume (ml)						
final incubation sample volume after processing (ml)						

NA = not applicable

Why do I need these volumes?

- for each substrate and product you need to determine the number of mmoles in order to do a fermentation balance
- if you are not comparing the same total volume for the substrate and each product then the fermentation balance is incorrect
- the best way to do this is to determine the mmoles in the total INCUBATION flask. Make sure you consider the size of the inoculum added and the removal of the time zero sample before the fermentation starts
- you need to know the total volume of the 4 N KOH trap to correlate with the total volume of the attached INCUBATION flask. Determine the mmoles CO_2 in the total trap to correlate to incubation flask.
- you need to normalize (meaning?) the processed INCUBATION sample size. You start with 30 ml but after processing the volume may increase or decrease. For example, you collect a 30 ml sample but after processing you have 32 ml. After determining mmoles in your sample, say 10 ml, determine the mmoles in 32 ml – this is equivalent to mmoles in the original 30 ml sample collected. This must be taken into consideration when determining the mmole substrate or product in the INCUBATION flask.
- also it is important that you compare all samples as you need to subtract zero time data from the three day sample data for each product and vice versa for substrate (glucose consumed).

Project Report (refer to general instructions for additional information)

General Project Comments
Submit only one report per group.
The report must have unity in presentation not just six reports carelessly put together. It is not necessary that one person write the complete report unless that is how your group wants to divide report work. However, the presentation by each person must be of similar format.

0.5 **Include a Title (on title page)**
- The title should identify major finding(s).
- Be concise.
- Avoid abbreviations.
- Include taxonomic names if relevant.

1.0 **Introduction**
- Write in paragraph format.
- Explain why you did the experiment (hypothesis).
- Include information that gives background to your experimental findings.
- Cite and reference all necessary information.
- Write from general to specific.
- End by summarizing results in one sentence.

Data Presentation (Present results exactly as requested)
- Throughout the different experiments, numerous dilution steps are involved. Whenever you have a dilution, state what the dilution is in a data table or figure description.
- When presenting sample calculations, explain all volumes and dilutions involved in calculation.
- Fractional marks will be subtracted each time the same error is made for each product since different group members may or may not make the error.

2.0 1. Determine glucose (mmole) **utilized** per INCUBATION flask for each Neuberg form. Just attach completed excel spread sheet including standard curve, linear regression equation, and table of sample data for each form (table format will need to be modified). There must also be a column of substrate consumed per flask. Include only triplicate data of best dilution. Show all calculations used on spreadsheet to calculate glucose (mmole) utilized per INCUBATION flask. Make sure it is obvious what dilution is used. Remember you need to normalize mmoles glucose due to possible sample volume changes during processing (this applies to all products except CO_2). Make sure you know what **glucose utilized** means.

6.0 2. Determine product (mmole) produced per INCUBATION flask for each Neuberg form. Present one table per product. There must also be a column of mmoles product produced per flask. Include triplicate data of the best dilution for each form. Include dilution in table. Remember you need to consider product produced - must take into consideration the zero time sample (mmole). For glycerol just include excel spreadsheet of data with a modified sample table. For carbon dioxide calculated (Form 1 and 3) remember to calculate the carbon dioxide produced in the entire carbon dioxide trap. Show all calculations used just below table. Make sure you know what **product produced** means.

3.0 3. Construct a fermentation balance for each form and tabulate information as demonstrated below for Neuberg's Third Form. Calculate Carbon recovery, O/R balance, and C_1 balance for each form. Remember mmoles is the amount per flask either produced or utilized. Values presented in table must be the same as calculated values presented in table for each product and the substrate. Fermentation balance tables available on lab website in Word format.

compound	mmoles	mmoles Carbon	oxidation value	oxidized products	reduced products	C_1 observed	C_1 calculated
glucose							
glycerol							
acetate							
ethanol							
CO_2							
Totals							

Carbon recovery = $\frac{\text{matoms Carbon product}}{\text{matoms Carbon substrate}}$ x 100% = expect ~97%

O/R balance = $\frac{\text{oxidized product}}{\text{reduced product}}$ = expect ~1

C_1 balance = $\frac{C_1 \text{ observed}}{C_1 \text{ calculated}}$ = expect ~.96

Refer to appendix for information on how to construct a fermentation balance.

1.5 **Discussion**

- Write from specific to general.
- Write with confidence.
- Be clear and organized.
- Analyze results citing figures or tables and referring to your data.
- Explain what your results mean (if more than one explanation is possible, select the most likely and explain why.
- Do your results support your hypothesis or literature results.
- State experiment limitations and how you would change your experiment to obtain a better fermentation balance?

Appendix

1.0 • Include completed ethanol fermentation volume table

Question

1.0 1. Neuberg's First Form fermentation often produces glycerol even though Neuberg's first form equation does not show any glycerol production. Bideaux et al[4] (2006) attempted to reduce the production of glycerol without sacrificing ethanol production by *Saccharomyces cerevisiae*. Explain why glycerol is produced during Neuberg's first form. State how (no details required) Bideaux et al (2006) reduced glycerol production without much reduction in ethanol production.
Since the reference is recent it is only available via UM NETDOC - ASM journal search. Link available on lab website.

0.0 **References**

- include a list of cited reference as outlined in general introduction (you must have references)
- marks subtracted in introduction and discussion sections

0 **Acknowledgments**

- state what each project member contributed to the experiment for both the report writing and the project presentation (general information only - do not need to be specific). No marks assigned but 0.5 mark subtracted if not completed.

1.0 **Overall Presentation**

- unity
- organization
- grammar
- clarity
- concise
- readable

0 **Peer Evaluation** (submit individually, do not attach to report)

- Each member of the group must hand in a completed ethanol fermentation project peer evaluation form (follows or available as a Word or pdf file on lab website).
See evaluation section in general instructions for possible mark adjustment due to peer evaluation. Marks (2) subtracted if a peer evaluation is not handed in.

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[4]Bideaux, C, Alfenore, S, Cameleyre, X, Molina-Jouve, C, Uribelarrea, J_L, Guillouet, SE. 2006. Minimization of Glycerol Production during the High-Performance Fed-Batch Ethanolic Fermentation Process in Saccharomyces cerevisiae, Using a Metabolic Model as a Prediction Tool. Appl. Env. Micro.72: 2134-2140.

ETHANOL FERMENTATION PROJECT PEER EVALUATION FORM

Date: __________________

Student Name: ______________________ Student Number: _________________

Other group member names	**PEER CRITERIA EVALUATION** See footnotes for criteria[b] and rating scale[c].						Total
	1	2	3	4	5	6	

[b] criteria:

1. Student participated in the organization of the project tasks.
2. Student effectively carried out assigned experiments.
3. Student effectively participated in project report.
4. Student interacted well within the group and respected other group members.
5. Student contribution was essential for the completion of the project with respect to research, talent and thoughtful ideas.
6. Student used time productively and met deadlines.

[c] excellent = 5, good = 4, average = 3, fair = 2, poor = 1

Notes:

i) Do not base evaluation on friendship or personality conflicts.
ii) Evaluations will not be seen by group members.
iii) Your evaluation is a valuable indicator to help assess contributions in a fair manner.

APPENDIX

MS EXCEL PROCEDURES (MAY VARY DEPENDING ON EXCEL VERSION):
Possible ways to use Microsoft Excel if you are not familiar with the software.
HINTS:
(i) Right click on whatever you want to change and select appropriate item from the pull down menu.
(ii) Make use of copy and paste, once you have designed a formula, just copy and paste for like calculations.

CELL FORMULAE NOTATIONS
-start equation with an = sign, do not leave spaces between entries
-use mathematical brackets wherever required
* multiply
/ divide
+ add
- minus
^power

SUM
Put the curvsor in the cell where you want to record SUM . Select AUTOSUM button (∑). The program buttom automatically selects cells, if this differs from what you want use the cursor to select numbers you want to add.. Press ENTER. Or use pull down menu - select Insert, function, statistics, then SUM. Click OK. A pop-up menu appear, use or just move out of the way if necessary. Using your mouse click the first or last1 cell of data set. Hold down button and scroll down to the last or first cell in the data set. Release button. Click OK on pop-up menu or press enter. The SUM appears in your selected cell. If required, repeat for remaining data sets.

AVERAGE
Put the curvsor in the cell where you want to record average. Select paste function button (f_*), then statistics (or defaults to most recently used), then AVERAGE. Or use pull down menu - select Insert, function, statistics, then AVERAGE. Click OK. A pop-up menu appear, use or just move out of the way if necessary. Using your mouse click the first or last1 cell of data set. Hold down button and scroll down to the last or first cell in the data set. Release button. Click OK on pop-up menu or press enter. The average value appears in your selected cell. Repeat for remaining data sets.

STDEV (standard deviation).
Put the cursor in the cell where you want to record standard deviation. Select paste function button (f_*), then statistics (or defaults to most recently used), then STDEV. Or use pull down menu - select Insert, function, statistics, then STDEV. Click OK. A pop-up menu appears, use or move out of the way if necessary. Using your mouse click the first or last cell of data set. Hold down button and scroll down to the last or first cell in the data set. Release button. Click OK on pop-up menu. The standard deviation value appears in your selected cell. Repeat for remaining data sets.

MS EXCEL STANDARD CURVE CHART
Select Insert pull down menu.
Select chart - (standard types), select (XY) Scatter chart type. Defaults to only markers present.
Click next.
Put cursor in data range box, click. Make sure there is no default information in box - delete if present as Excel will often automatically select data. Use the mouse to click and hold down to select y-axis data (absorbance). Column should select - check.
Select series tab. Put the cursor in x values box, click. Again make sure there is no information in this box - delete if present. Use the mouse to click and hold down to select x-axis data (mass or concentration).
Click next.
Chart Options menu appears. Under default titles tab, enter title and axes labels. Unfortunately the title defaults to the top of the graph. After you are finished setting up graph, just select and move below graph. Mostly likely you will need to move the graph up. May require resizing, font size change, etc. Under the legend tab, remove legend check mark - not required as only one plot per standard graph.
Click next. Keep the default setting (graph in data page).
Click finish.
Right click any empty area on graph area. Select format plot area. Change the background color to white. Click OK.
Put the cursor on any one of the plot markers. Right click. Select Add Trendline. (Linear Regression Line). Under the default Type tab the linear regression box should be highlighted. This is what you want.
Select the Options tab. Change the Forecast backward from zero to whatever value is the difference between zero and your first x-axis value (draws the linear regression line to the y-axis). Select display equation on chart. Select display R-squared value on chart. Press OK.
Select cell area you want to print. Under page set up or page set up button, select print to ONE page. The graph, data and calculations must be on ONE page.

R- squared value
The value is between 0 and 1. The R squared value is an indicator of how closely the estimated values used to draw the linear regression trendline correspond to the actual data. The linear regression trendline is most reliable when R value is close to 1.

Spreadsheet Sample Calculation Information
Usually sample calculations are requested on the spreadsheet (must be printed with data and graph). You may insert the calculation directly into the cell. If you want the cell larger, just block and join. Or you may do the calculation in word, copy paste to Text insert box. If the Drawing Taskbar is not alway on the screen (usually bottom) then right click top task bar and select drawing. Then click insert text box. Just copy paste word information to box. Or you may answer directly in the text box. Excel is not user friendly for Greek symbols or formatting. To insert μ need to select text then change font style to symbol (letter m). If you want superscript or subscript again select text to change and right click. Select font, then you can change what you want. The entire text box must be inside the selected area to print or it does not print.

Normality

Reference: Biochemical Calculations; 2nd edition by Irwin H. Segal, ISBN 0-471-77421-9

Normality (N) = number of equivalents of solute per liter of solution

To determine the number of equivalents:

$$\text{equivalents} = wt_g/EW$$

wt_g = weight of dissolved solute
EW = equivalent weight

One equivalent (EW) of an acid or base is the weight that contains 1 g-atom (1 mole) of replaceable hydrogen or replaceable hydroxyl, respectively.

One equivalent (EW) of a compound involved in an oxidation-reduction reaction is the weight that provides or accepts 1 faraday (1 mole) of electrons.

To determine EW:

$$EW = MW/n$$

n = the number of replaceable hydrogen or hydroxyl per molecule (for acids and bases)
OR
n = the number of electrons lost or gained per molecule (for oxidation and reduction respectively)

To determine molarity and normality relationship:

$$N = nM$$

For example, a 0.01 M solution of H_2SO_4 is 0.02 N.

Titration
There are two types of titration that are used in this lab, (1) neutralization reactions to determine the concentration of acids and bases and (2) starch indicator (starch indicator: starch-iodine complex (blue) <-----> starch (clear)). This section deals with the first whereas the second is similar in principle.

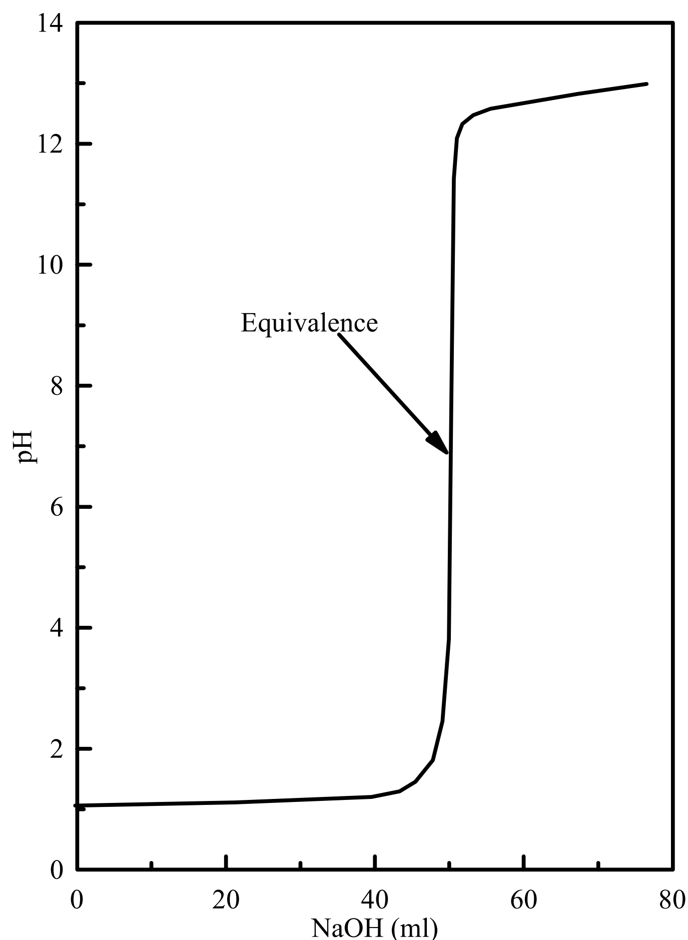
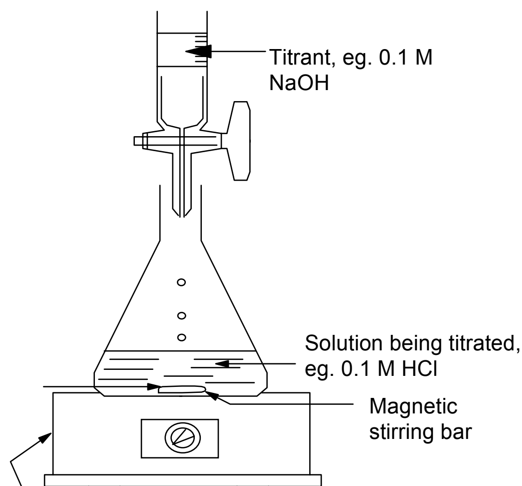


Figure 3. Titration 0.11 N HCl with 0.1 N NaOH.

Titration, involving a neutralization reaction to determine the concentration of acids and bases, is performed by slowly adding a measured volume of basic solution to a known volume of acidic solution until the base is completely neutralized or vice versa (refer to diagram). The point at which neutralization occurs is the equivalence point. A pH indicator (phenol red, pH 7.0) is present in the solution being titrated to show visibly the equivalence point. When the indicator changes color, it is called the END POINT of the titration which is the experimental estimate of the equivalence point. At equivalence point the number of moles of base added is equal to the number of moles of acid initially present. For example, for a HCl-NaOH titration at equivalence point there is just a solution of NaCl with a neutral pH equal to 7.0.

The theory can be experimentally demonstrated by plotting pH of resulting solution as a function of the volume of added solution (the titrant) to obtain a titration curve. The following graph is the curve for the titration of 50 ml of 0.1 M NaOH with 0.1 M HCl. The pH of the solution changes very slowly until the vicinity of the equivalence point. Figure 3 presents a typical curve for the titration of a strong acid with a strong base.

The titration curve of a weak base with a strong acid is somewhat different (not shown). The titration curve for acetic acid titrated with NaOH starts at pH 2.9 (initial pH) and increases slowly until the equivalence point is reached. Around the equivalence point the pH changes from 6 to 11. The equivalence point occurs at pH = 9 not pH = 7. Refer to figure 4 for titration apparatus.



Indicators

Indicators are weak organic acids that change color upon loss of a proton. Because of the intense color of indicators, only a very small concentration is necessary to produce a visible color. The contribution of the indicator acid to the acidity of the solution is negligible. There is a wide range of indicators that change color throughout the pH scale. The indicators that are used in this lab are listed below:

indicator	pH range* (transition pH)	color low pH	color high pH
bromophenol blue	3.0 - 4.6	yellow	blue
phenolphthalein	8.3 - 10.0	colorless (chalky white precipitate)	deep pink/red
phenol red	6.8 - 8.4	yellow	red

* The transition pH may show an intermediate color.

CONCENTRATION OF ACIDS AND BASES - COMMON COMMERCIAL STRENGTHS

Chemical	MW	Moles/liter	Grams/liter	Specific Gravity
Acetic Acid	60.1	17.5	1049	
Hydrochloric acid	36.5	11.6	424	1.18
Sulfuric acid	98.1	18.0	1766	1.84
Potassium hydroxide	56.1	13.5	757	1.52

FERMENTATION BALANCE (chemical balance per mole substrate utilized)

Methods for calculation of Fermentation Balance:

mmoles: total mmoles, remember to keep total mmoles (sample volume) the same for substrate and product
eg. Neuberg's first form 1 mole glucose substrate produces 2 moles ethanol and 2 moles carbon dioxide

Carbon recovery:
mmole Carbon = mmole substrate or product times the number of carbon atoms. The total number of product C atom should equal the C atom for substrate.
eg. glucose 40 mmole = 6 x 40 = 240 C atom

oxidation value: The oxidative value of a compound is equal to the number of oxygen atoms minus ½ the number of hydrogen atoms. Hydrogen is arbitrarily assigned a value of 0.5 and oxygen a value of 1.0 (Sokatch, 1969).
eg. ethanol, CH_3CH_2OH = 1.0 - (6 x 0.5) = -2.0

Oxidized products: mmoles product multiplied by oxidation values
eg. for 80 mmoles carbon dioxide: 80 x 2 = 160

Reduced products: mmoles product multiplied by oxidation value (do not consider minus sign)
eg. for 80 mmoles ethanol: 80 x 2 = 160

C_1 balance =

Observed C_1/Calculated C_1
Observed C_1 = to mmoles $C0_2$ observed
Calculated C_1 = mmoles of product other than CO_2 that have the potential to produce CO_2, ie., C-2 pathway never the C-3 pathway (eg. glycerol).
eg. Neuberg's First Form: C_1 calculated = mmole ethanol since ethanol was produced from pyruvate by decarboxylation.

References:

Sokatch, J.R. 1969 Bacterial Physiology and Metabolism. New York: Academic Press. p. 72-74.
Moat AG, Foster JW, Spector MP, editors. 2002 in Microbial Physiology 4th ed. New York: Wiley-Liss p 412-414

SPECTRONIC 20D OPERATION

The spectronic 20D is a single beam spectrophotometer. The wavelength range is 340 nm to 600 nm with a nominal spectral slit width of 20 nm that is constant over the wavelength range. The wavelength accuracy is 2.5 nm. The spectronic 20D is supplied with 1/2 inch test tubes.

SAMPLE MEASUREMENT: Absorbance

1. Remove dust cover. Turn on **Power Switch** clockwise. Allow the spectrophotometer to warm up for 15 min.

2. Set the required wavelength with the **Wavelength Control Knob**.

3. Set the display mode to TRANSMISSION by pressing the **MODE CONTROL KEY** until the LED beside TRANSMISSION is lit.

4. The sample compartment should be empty and closed. Adjust the display to 0.0%T with the **Zero Control Knob** (same as power switch).

5. Fill a spec 20D 1/2 inch test tube with blank solution. The tube should be at least 1/2 full. Wipe the test tube with tissue to ensure no liquid drops, dust or fingerprints. Place the test tube in **Sample Compartment** and align the guide mark on the test tube with the guide mark at the front of the sample compartment. Press test tube firmly into sample compartment and close lid.

6. Press the **MODE CONTROL KEY** until the LED beside ABSORBANCE is lit. Adjust the display to 0.0A with the **Transmission/Absorbance Control Knob**. Remove the test tube from the sample compartment.

7. Put test tube containing sample(s) in **Sample Compartment** and close lid. Read absorbance directly from display.

8. When all measurements are complete, turn off the spec 20D and replace dust cover. Thoroughly rinse all spec 20D test tubes with distilled water, and place test tubes in spec 20D rack upside down.

COMMENTS

1. Using the same test tube for blank and all samples should minimize error. Although this is not usually required for experiments carried out in undergraduate labs.

2. Keep all solutions free of bubbles.

3. The display must be reset to 100%T or 0.0A every time the wavelength is changed.

OPERATION OF FLOOR MODEL CENTRIFUGES

Note: If procedure varies depending on centrifuge manufacturer a step by step operation procedure is usually located on or nearby the centrifuge or the teaching assistant will help you.

HITACHI HIGH SPEED HIMAC REFRIGERATED CENTRIFUGE

- to select or change settings the CHECK button must first be pressed (light on). The light stays on for 16 sec. When the light is off you can no longer select, change setting or carry out any operation, just press check button again and continue.
- When the centrifuge is turned on and the CHECK button is not pressed. The centrifuge displays real time parameters.

OPERATION

Centrifuge tubes should be balanced by scale by adding or removing appropriate solution from one of the tubes.

1. Turn power switch on. The indicators on the control panel are illuminated. The door lock is released.
2. Open door. If required set the rotor gently in position and close door. Turn the rotor lightly by hand to check that the rotor is correctly set. Remove the rotor lid and place balanced tubes opposite each other in rotor. You cannot run the centrifuge with an odd number of tubes. SCREW ON LID.
3. Call up memory code number or enter parameters.
   <u>Call up pre-programmed memory code number:</u> Press CHECK button, MEMORY button, memory code number, and CALL button. Each memory code number consists of a specified set of operation parameter (see sheet on centrifuge cover). See below for a list of operation parameters and how to set and store operation parameters.
   OR
   <u>Real time operation (enter original parameters)</u>: see setting of operation parameters below.
4. After the parameters are set make sure the check light is still on. If not, press the CHECK button.
5. Press the START button. The rotor starts running. The start lamp begins flashing. The timer starts to count down.
6. The timer counts down to zero or press the STOP button. The rotor begins to decelerate. The stop light begins flashing.
7. The rotor stops. The stop light stops flashing. A buzzer sound occurs. The door lock is released.
8. Unscrew rotor lid and remove tubes. If required, use tweezers to help remove tubes. Wipe out rotor if spills occur. DO NOT SCREW ON THE LID just place on top of the rotor.
9. Close centrifuge lid and turn off power.

PARAMETERS

ROTORS NUMBER: SMALL (maximum volume 40 ml) RPR20-2 = ROTOR #7
LARGE (maximum volume 450 - 500 ml) RPR9-2 = ROTOR #13
TEMPERATURE: 4 to 20 ºC
SPEED: Rotor number 7 (small) - maximum speed 18,000 rpm
Rotor number 13 (large) - maximum speed 8,000 rpm
Example: for 3,520 rpm, press 3 . 5 2
TIME 0 to 99 min 59 sec, FREE
Example: for 5 min and 30 sec, press 5 . 3 0
ACCEL. Higher the value the faster the acceleration - 9 is good for basic centrifugation.
DECEL. Higher the value the faster the deceleration - 7 is good for basic centrifugation.
Example: for loose pellet or phase separation the deceleration number should be decreased to 3.

PIPETMAN OPERATION

In your lab, you have available three different pipetmen depending on the lab. If you look at the top of the plunger it states the size of the pipetman.
P20 measures accurately from 2 µl to 20 µl.
P200 measures accurately from 20 µl to 200 µl.
P1000 measures accurately from 100 µl to 1000 µl.
Never turn the pipetman above the maximum volume; 20 µl for P20, 200 µl for P200, and 1000 µl for P1000 as this breaks the pipetman. The scale on the pipettor is read different for each type - refer to Figure 5 for an example of how to read the scale.

(Excerpted from Gilson pipetman operation manual.)

1. Setting the volume: The required volume is set on the digital volumeter by turning the knurled adjustment ring (Figure 5-2A). The volumeter display is read from top to bottom in µl for P20 and P200 and ml for P1000 (Figure 5-2).

2. Place a disposable tip on the shaft of the Pipetman. Press on firmly with a slight twisting motion to ensure an airtight seal. Depress the push-button to the first positive stop (Fig. 5-3A). While holding the Pipetman vertical, immerse the tip 2-4 mm into the sample liquid. Release the push-button slowly to draw up the sample (Fig. 5-3B). Wait 1 to 2 seconds, then withdraw the tip from the sample.

3. To dispense the sample, place the tip end at a 10-45° angle against the inside wall of the vessel and depress the push-button SMOOTHLY to the first stop (Fig 5-3C). Wait 1 to 2 seconds and then depress the push-button completely to expel any residual liquid (Fig. 5-3D). With the push-button fully depressed, carefully withdraw the Pipetman, sliding the tip along the inside wall of the tube. Release the push-button. Remove the used tip by depressing the tip ejector button (Figure 5-1F).

diagram of pipetman

SAMPLE FINAL LAB EXAM: Microbiology 60.447 FERMENTATIONS

WRITE IN PEN ONLY
ANSWER ALL QUESTIONS in SPACE PROVIDED (abbreviated here to save space).
ANSWERS ACCEPTABLE IN POINT FORM.

1. Briefly answer each of the following questions.

1 a) Explain the relationship between molarity (M) and normality (N). Include an example.
3 b) Explain how the starch indicator functions to determine the concentration of acetaldehyde. Include chemical formulas.
3 c) Explain the following statement: Ethanol is oxidized stoichiometrically to acetic acid with four equivalents of dichromate being consumed per mole of ethanol. Include chemical formulas.

5 2. Explain the function of each of the following components used in your fermentations lab. Include chemical formulas wherever relevant.

a) $ZnSO_4$ in ethanol fermentation lab
b) H_2SO_4 in ethanol fermentation lab
c) sodium arsenate in glycerol determination lab
d) Oxidase reagent in glucose determination
e) steam distillation apparatus in acetic acid determination

3. How do you accurately prepare each of the following solutions or media for use in the fermentations lab?

1 a) 50 ml 0.04% phenolphthalein in 50% ethanol (given phenolphthalein powder and 95% ethanol)
1 b) 100 ml Standard HCl solution (given a 0.1 N HCl solution, prepared by preparation room)
2 c) 100 ml 0.1 N $K_2Cr_2O_7$ in 10 N H_2SO_4 (given bottle of powdered $K_2Cr_2O_7$ and concentrated sulfuric acid)
$K_2Cr_2O_7$ MW = 294.2
Sulfuric acid MW = 98.1, concentrated sulfuric acid contains 1766 g/liter.
3 d) 1liter *Sacchromyces cerevisiae* sterile growth medium, pH 5.5, containing 0.5 g malt extract, 10.0 g KH_2PO_4, 5.0 g $(NH_4)_2SO_4$, and 100 g glucose per liter. Include details of preparation.

SAMPLE LAB EXAM: Microbiology 60.447 FERMENTATIONS
continued

6 4. a) Determine the concentration (M) of carbon dioxide sample.
i) sample dilute 5 fold
ii) 10 ml sample titrated
iii) phenolphthalein end point: 8.2 ml titrant 0.1 N HCl
iv) bromo phenol blue end point: 14.3 ml titrant 0.1 N HCl

b) Determine the concentration (M) of ethanol sample.
i) sample diluted 10 fold
ii) 1 ml 0.05 N acid dichromate solution titrated
iii) starch indicator end point: 0.3 ml titrant 0.1 N sodium thiosulfate
iv) assume no change in blank
v) samples size is 1 ml

c) Determine the concentration (μg/ml) of glycerol sample.
i) sample diluted 15 fold
ii) 2 ml of diluted sample was assayed
ii) regression equation of standard curve (absorbance vs μg/ml glycerol)
$y = 0.007x + 0.02$
iii) average absorbance reading of sample is 0.23 O.D.

4 5. Present a theoretical fermentation balance table for Neuberg's Second Form of ethanol fermentation by *Sacchromyces cerevisiae*. At the start of the experiment 15 g glucose was present in the flask containing 200 ml medium. Include all necessary calculations and explain your data. Glucose MW = 180

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- END -