INDUSTRIAL MICROBIOLOGY
MBIO 4510
LAB MANUAL

2007

Lab manual available as a pdf file on website.

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MBIO 4510 INDUSTRIAL MICROBIOLOGY LAB SCHEDULE 2007

Date	Week #	Description
Jan 12	1	Lab 1: Exponential growth analysis of *Escherichia coli* Lab 2: Microbial industrial fermentation: Citric Acid Production by *Aspergillus niger* Part I: Experiment design
Jan 19	2	Lab 2: Microbial industrial fermentation: Citric Acid Production by *Aspergillus niger* Part II: Media preparation and inoculation
Jan 26	3	Lab 2: Microbial industrial fermentation: Citric Acid Production by *Aspergillus niger* Part III: Sample collection and biomass determination. Part IV: Citric Acid determination
Feb 2	4	Lab 3: Investigation of batch culture specific production and consumption rates in *Aspergillus niger* Part I: Culture inoculation, sampling and biomass determination
Feb 9	5	Lab 3: Investigation of batch culture specific production and consumption rates in *Aspergillus niger* Part II: Sucrose determination
Feb 16	6	MID-TERM BREAK
Feb 23	7	Lab 3: Investigation of batch culture specific production and consumption rates in *Aspergillus niger* Part III: Citric acid determination
Mar 2	8	Tissue Culture lab tour
Mar 9 or 16	9	Beer Tour
Mar 30	12	Lab Exam

Lab Report Due dates

Due Date	Report or Data
Jan 19	Lab 2: Microbial industrial fermentation: Citric Acid Production by *Aspergillus niger* Part I: Experiment design procedure checked in lab
Jan 26	Lab 1: Batch culture growth analysis of *Escherichia coli*
Feb 9	Lab 2: Microbial industrial fermentation: Citric Acid Production by *Aspergillus niger*
Mar 9	Lab 3: Investigation of batch culture specific production and consumption rates in *Aspergillus niger*
Mar 9 or Mark 16	Students not attending beer tour, Virtual Beer Tour assignment due day of tour

GENERAL INSTRUCTIONS

Lab instructor:	Dr. L. Cameron	Office: 414B
Demonstrators:	Brad Pickering	Lab: 105-107
	Vikash Rha	Lab: 410

Lab room: 201 (204) Buller Bldg. All students come to room 201for the first lab.

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 lab coat. Food or beverages are not permitted in the lab. Require a fine permanent marker.
3. When recommended in the lab protocol, disposable gloves and shield glasses should be worn. The disposable gloves are provided in the lab.
4. Students are required to supply their own marker for tubes.

EVALUATION

General

1. Lab Mark (Total = 20%):

   Lab exam: 12%

   Lab reports 8%

   Lab report mark includes beer tour attendance or virtual beer tour assignment (no mark is given but up to 1% may be subtracted).

   Marks will be subtracted from your final lab mark if lab is not cleaned up when working on your project.
2. Students must pass the lab to complete the course (50%).
3. The lab exam date is included in your lab manual schedule.

   Place: lab (unless student is large)

   Time: 2:30 - 4:00 p.m.
4. Lab reports are to be handed in as stated in schedule by 4:30 pm of that day. Hand in lab reports through slotted drawer in room 204 Buller ONLY. Instructor and demonstrators do not accept lab reports. If handing in lab late, 1 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 in 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 available on the website.

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 REPORT PRESENTATION INSTRUCTIONS

[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. Include a title page. Lab reports **MUST BE TYPED**. Up to 10% of the mark subtracted for reports not typed. Number each page. If a Word document or Excel spreadsheet is available on lab website, they must be used to write report.

3. Lab reports may be done as an individual effort <u>or</u> a group effort by the two students that carried out the experiment. One report or more reports may be handed in per group. The decision on the number of reports per group is totally dependent on members of the group. This decision may be changed any time during the term. Therefore for each lab report the group has the option to hand in one or two reports exclusive of what has been done before or after that particular report. Indicate on the cover page of the report if the report is a group report or an individual report. If handing in an individual report also include lab partner's name. Do not put a student's name on the report who has not assisted with the report. Marks only given to students whose names are listed on the report.

4. If your group's data (or part of data) is 'not workable' borrow data from another group and reference. The definition of 'not workable' is data that cannot be used for required calculations. It does not necessarily mean the correct data as long as the calculations can be performed and data presented as requested.

5. Always include one sample of each type of calculation used when analyzing data. When presenting sample calculations, explain all volumes and dilutions involved in calculation.

6. 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 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.

7. 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)

1. Table number and title (legend) presented above the table body.
2. Number tables using arabic numbers, even if only one table in a report.
3. 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).
4. If abbreviations are used in table, indicate what abbreviations mean as a footnote. Other footnotes may be required to clarify material in the table.
5. Like information should be in columns making it easier to view the table.
6. 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.

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

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

7. 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.
8. Group related column headings under larger headings.
9. If information is the same for each column or row do not include but treat as a footnote.
10. Make the table as concise as possible but include all necessary information. For example, any constant experimental conditions that would change the data presented.
11. Tables should be properly set up with a straight edge. Horizontal lines must be included but it not necessary to always include vertical lines.

Figure presentation (graphs, diagrams, photographs, films)

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

1. Figures are to be numbered separate from tables, using arabic numbers. Include figure number even if only one figure.
2. 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.
3. All diagrams, photographs, and films are figures and should be completely labelled.
4. 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)[3]

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

References available in the reference binder on reserve in the Science and Technology Library.

**Reference**

Lab 2 & 3: Microbial industrial fermentation: Citric Acid Production by *Aspergillus niger* and Investigation of batch culture specific production and consumption rates in *Aspergillus niger*

1 Hilton, MD. 1999. Small-scale liquid fermentations. In: Demain, AL, Davies, JE., editors. Manual of Industrial Microbiology and Biotechnology, 2nd edition. Washington: ASM Press. p 49-60.

2 Strobel, RJ., Sullivan, GR. 1999. Experimental design for improvement of fermentations. In: Demain, AL, Davies, JE., editors. Manual of Industrial Microbiology and Biotechnology, 2nd edition. Washington: ASM Press. p 80-93

3 Dahod, SK. 1999. Raw materials selection and medium development for industrial fermentation processes. In: Demain, AL, Davies, JE., editors. Manual of Industrial Microbiology and Biotechnology, 2nd edition. Washington: ASM Press. p 213-220.

Miscellaneous

4 McMillan V.E. 2001 Writing Papers in the Biological Sciences. 3rd ed. Boston: Bedford Books. p 55-59 (available in reference binder)

*Many scientific journal articles are available on the internet. Search using Highwire Library of Sciences and Medicine Search and Browse Tools at http://highwire.stanford.edu/cgi/search/ © 2001 - 2002 by the Board of Trustees of the Leland Stanford Junior University. (accessed Dec 2003) or ejournals available at UM libraries. http://www.umanitoba.ca/libraries/online/ejournals/

or UM NetDoc http://www.umanitoba.ca/libraries/online/netdoc/

LAB STANDARD OPERATIONS PROCEDURE (SOP)

Bench area: Wash bench area before and after use with AIRx109 or Savlon.

Personal safety:

•You must wear a buttoned lab coat. . Wear coat only in the lab, transport separately outside of the lab (in a plastic bag). If you forget your lab coat, dark blue lab coats are available in the lab. Sign the IN/OUT sheet.

Laundry instructions: wash separately from other clothes with detergent and bleach. When taking lab coat home for washing, carry separate from all other personal effects, ie. not in your back pack.

•No personal effects (this includes outer clothing and back packs)are permitted in the lab, only lab notebook, pen and permanent ink marker.

•Long hair must be tied back. Keep your hands away from your hair.

•Wash hands with antibacterial soap (SWISH contains sodium lauryl sulfate (SDS) a detergent, coco diethanolamide, coco amido betaine, and copolymer of acrylamide) before leaving the lab - require 15 - 30 seconds of hand washing. Use the sinks in the corners as they are foot petal operated. Do not put your hands on your face or anything in your mouth (eg. pen) while in the lab. Protect hands with gloves (available in lab) and eyes with glasses when handling level II Biohazard microorganisms and when needed.

•Dispose of gloves in Petri plate containers.

•No eating or drinking in the lab.

•Use aseptic technique for transfer of bacteria and fungi. This is to protect yourself as much as to ensure the purity of your culture.

•Never mouth pipette. Always use a pro-pipette.

•When handling level II microorganisms you must wear disposable gloves.

•Cover any cuts with a bandage (if necessary, available in the first aid kit).

•Be aware of the possibility of bacteria aerosol when you flame your loop. It only takes one tiny droplet of Salmonella (~1000 organisms) to make you very sick if ingested. Be aware what other students are doing around you. You could be getting their loop aerosol on you.

•Students are advised to wear closed toed shoes.

LAB ENVIRONMENT:

•Signage in the lab listing level II biohazard that may be used. Signage in the lab outlining requirements for level II biohazard. Hard copy of all MSDS information for both chemicals and biologicals used in the lab.

•Only students taking the assigned lab and trained workers are permitted in the lab.

•Wash bench area before and after with BDD (Backdown Detergent Disinfectant containing nonyl phenoxy polyethoxy ethanol, alkyl-aryl ammonium chloride and ethyl benzyl ammonium chlorides). This helps prevent contamination of your notebook and pens. Even if you wash your hands when you leave, your pen or book could be contaminated.

•First aid kit present in lab.

•Know location of exits, fire extinguisher, eye wash, full body shower, and first aid kit.

• 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.

•Leave your bench area clean. All equipment and supplies should be returned to original location.

DISPOSAL:

•All biohazard disposable containers must be labelled with a biohazard label. After autoclaving the biohazard label is removed.

•All biohazards must be autoclaved. Biohazards include any surface that has come in contact with bacteria. The autoclave is monitored weekly to ensure all organisms are destroyed. After autoclaving the Petri plate container the plastic bag is removed, tied and placed in a second black plastic bag before disposing. After autoclaving the plastic lined basin, the plastic bag is carefully removed, tied and placed in a corrugated cardboard box, taped shut and labelled broken glass before disposing.

•**Bacteria and fungal cultures :** All bacteria cultures must be autoclave. Place broth cultures on discard trolley after removing markings and masking tape.

•**Petri plate containers**: All cultures agar plates must be autoclave. Discard culture plates in plastic lined Petri plate containers. Discard all non-sharp biologically contaminated items in the Petri plate container, this includes microtubes, API strips, antibiotic strips, microtitration plates, etc.

•**Plastic lined basin**[4]: Any 'pointy' item must be disposed in plastic lined bucket (not Petri Plate container), this includes disposable plastic 1 ml cuvets, pipetman tips, sticks, toothpicks, slides, Pasteur pipettes, broken glassware, brittle plastic objects, metal objects[a] (not needles or blades), etc.

•**Yellow plastic bucket:** Place all disposable 1 ml and 10 ml pipettes, tip down in yellow plastic pails. Pipettes are autoclaved and boxed before disposing.

•**Biological Spills**: Put on glove. Immediately wash container or test tube rack with BDD disinfectant. Put stack of paper towels on top of spill, pour disinfectant around and over. Do not press down. Collect soaked paper towels in Petri plate container. If spill includes broken glass or any sharp item put in plastic lined basin.

•**Glassware (unbroken)**: Remove tape and pen markings (use alcohol) from glassware before placing on discard trolley. Used glassware that has not contained bacteria should be rinsed and placed on the discard trolley. Rinsed test tubes (no biological contact) should be placed in tray provided on the discard trolley.

•**Chemical hazardous material**: Read the MSDS information available in lab or online at http://ccinfoweb.ccohs.ca/msds/search.html . Organic solvents must be disposed of in organic solvent container. The lab demonstrator will instruct proper disposal methods for labs that contain hazardous materials. These containers are disposed of through the university safety office. Never pour solvents down the sink. 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.

Some supplied chemicals may be preserved with 0.1% Na azide, handle with gloved hands.

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 discarding caustic solutions (< 1M). These materials are disposed of through the university safety office.

When handling stains or reagents, wear disposable gloves as the majority of stains or reagents contain hazardous material.

•**Biohazard sharps disposal**: Dispose of all sharps (needles, syringe tops, razors, scalpel blades) in specified container. Dispose of syringe with needle attached - do not take apart. Do not replace the

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[4] due to the multi-use nature of the teaching lab, all 'pointy' items will be treated the same as similar items contaminated with microorganisms.

needle cap before disposing (high frequency of accidents occur when replacing cap). Sharp's containers are autoclaved before disposing. You must dispose of the syringe top in the biohazard sharps container even if not used for biologicals as it is a perceived hazard by the general public.

Wear gloves when handling level 2 microorganisms (*Aspergillus niger* is level 2). All cuts should be covered with bandages (available in first aid in the lab). Be aware that whenever you flame your loop aerosols are created.

LABORATORY BIOSAFETY GUIDE

Aspergillus niger is a level 2 microorganism. It is important that you follow standard operation procedures, SOP (see above). When handling environmental samples you must assume they contain level 2 microorganisms.

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 the Health Canada websites:

Guidelines: http://www.hc-sc.gc.ca/hpb/lcdc/biosafty/docs/index.html

MSDS (infectious agents): http://www.hc-sc.gc.ca/hpb/lcdc/biosafty/msds/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

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

UM

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. (i.e. caretakers) → 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

LAB 1: EXPONENTIAL GROWTH ANALYSIS OF *Escherichia coli*

Batch Culture

It would be preferential to use an 'Industrial' organism to study log phase cell growth[5]. However, due to lab time constraints *E. coli* will be used as it grows rapidly. Optimum growth conditions for *E. coli* are selected to ensure log phase growth is maintained, 37°C, rotation (aeration), rich medium (T-soy broth) and subculture 1/10 with a log phase culture. There should be no or little lag phase as the cells are subcultured at a 'critical' concentration ensuring required growth factors are present. Under optimum growth conditions *E. coli* doubling time is ~30 min.

During the cell growth phase, cell number increases exponentially.

$$N = N_o 2^x \text{ or } \log_{10} N = \log_{10} N_o + x \log 2$$

Where x is the number of generations of cell growth (in some texts n = number of generations), N is the final cell concentration, and N_o is the initial cell concentration.

The following formula is obtained when solving for the number of generations (x).

$$x = \frac{\log_{10} N - \log_{10} N_o}{\log 2}$$

As time passes the rate of increase in cell number increases dramatically. Graphically log phase growth is best observed on a semi-log plot of cell concentration (log y-axis) vs time (linear x-axis). When plotted in this manner log phase growth is linear. Once you know the number of generations the doubling time(t_D) is easily determined using the following formula.

$$t_D = T/x$$

Where t_D = doubling time, T = total elapsed time and x = number of generations

Another parameter used to describe cell growth is specific cell growth rate (μ). Specific cell growth rate (μ) is a measure of the rate of cell number increase at a particular cell concentration.

$$\ln N = \ln N_o + \mu t$$

Where N is cell concentration at time t, N_o is initial cell concentration

[5]Butler, M. 2004. Animal Cell Culture & Technology. Londaon: BIOS Scientific Publishers. p 49-50.

and μ is the specific growth rate. The following formula is obtained when solving for specific growth rate (μ).

$$\mu = \frac{\ln N - \ln N_o}{t}$$

If the doubling time is already known the specific growth may also be calculated using the following formula.

$$\mu = \ln 2/t_D$$

Specific glucose consumption rate (Q_s) for a log phase culture is calculated using the following formula when the initial and final cell concentration and substrate concentration are known during an elapsed time. To calculate the specific glucose consumption rate for a chemostat the formula needs to be modified using the steady state cell concentration and μ (D).

$$Q_s = \frac{\Delta S}{T} \cdot \frac{(\ln N - \ln N_0)}{(N - N_0)}$$

Chemostat

In a chemostat culture, nutrients are continuously added while cells are continuously removed. The growth of the culture is dependent on entry rate of fresh nutrient. This is expressed as

$$D = F/V$$

where D is the dilution rate the culture, F is medium flow rate and V is the volume of the culture. To adequate sustain a chemostat culture the dilution rate should be 0.2-1.0 volume/day. At steady state the dilution rate equals the growth rate of the culture. The growth rate of the culture is maintained slightly below maximum by limiting one of the nutrients in the in-flow medium. If the limiting nutrient concentration (increased rate of adding nutrients, ie diluting the culture) is increase the growth rate increases until it's maximum rate. After the maximum rate has been obtained increased dilution rate will start to dilute out the culture until growth ceases.

Oxygen Supply

In your lab you have little difficulty supplying enough oxygen to the culture to maintain logarithmic growth. However, once you scale up the culture oxygen supply becomes a real problem. To ensure that the culture does not die, the oxygen transfer rate (OTR) must be equal the oxygen utilization rate by the culture (OUR).

$$ORT = OUR$$

Numerous methods are used to increase the supply of oxygen to a large culture, changing the headspace to pure oxygen instead of air (only 21% oxygen), direct aeration by sparging (bubbling air through the culture or medium before it enters the chemostat), and diffusions via tubing.

PROCEDURE

Cultures should remain on rotator as much as possible. Incubator should be opened and closed as quickly as possible. Quickly remove and return culture to rotator for additions and sampling.

1. *E. coli* cells were grown overnight with rotation at 37ºC in T-soy broth.

2. Day of lab, the *E. coli* cells were subcultured (1/50) into 5 ml fresh T-soy broth 1½ hours prior to start of lab.

Before starting experiment prepare zero time saline dilutions and get a bucket of ice.

3. **Student lab starts**. Take two log phase 5 ml *E. coli* culture - located on 37ºC culture rotator. Select the two cultures such the rotator remains balanced, opposite each other in position of the turntable. Always keep your cultures balanced on the rotator when removing or adding. Clearly label top with your group number only. This allows quick identification of your cultures and balancing of tubes. Immediately remove 0.1 ml culture from each culture tube to a labelled 5" metal capped test tube containing 0.9 ml saline. Place on ice. Growth stops upon dilution and the cold ensures growth stops. Immediately return culture tubes to rotator. It is important that the cultures are on the rotator as much as possible during the experiment.

4. At 30, 60 and 90 min remove cultures from rotator, transfer 0.1 ml of each culture to a labelled sterile 5" metal capped test tube containing 0.9 ml saline (10^{-1} dilution for each time point) and place on ice. Continue to prepare 10-fold serial dilutions (10^{-2} to 10^{-7}) in saline (total volume of 1 ml) for each sample and each time point. Spread plate 0.1 ml of dilutions 10^{-4} to 10^{-7} for each culture on T-soy agar plates.

5. Incubate plates at 25ºC over the weekend.

6. Monday record plate count data. Colony counters are available in the lab (see appendix for instructions). Plates must be counted Monday as colonies will be already be quite large.

LAB 1: EXPONENTIAL GROWTH ANALYSIS OF *Escherichia coli* DATA SHEET
(available on website as Excel document)

Table 1. T-soy agar plate count data of exponentially growing *Escherichia coli*.								
Time (h)	Duplicate plate count data for 0.1 ml of dilution plated.							
	10^{-4}		10^{-5}		10^{-6}		10^{-7}	
	plate 1	plate 2	plate 1	plate 2	plate 1	plate 2	plate 1	plate 2
0								
0.5								
1.0								
1.5								

LAB REPORT

Data Presentation and Analysis

Reminder: Report must be typed including calculations.

4 1. a) Attach completed Excel spreadsheet of *Escherichia coli* plate count data and bacteria/ml for each time point for your group data. Record all requested information including all requested numerical sample calculations. Insert figure of bacteria/ml (left y-axis log format) vs time. See appendix for Excel information and figure presentation. Include all important information in figure title. (Available on website as a Excel spreadsheet, save and open in Excel to enter requested information.). Use requested information to determine b), c) and d) - may be included on spreadsheet or done separately.

Notes:

(1) For 60.344 labs, significant *E. coli* plate count numbers are between 30 and as high as bacteria can be counted accurately (colonies not overlapping). If no *E. coli* plate counts (for all dilutions plated) are above 30, use numbers below 30 to calculate the number of bacteria per ml - state below significance but only data available.

(2) For each sample (eg. time point) there should only be one calculated bacteria/ml value, that is, average duplicate plates, then average dilutions if more than one dilution in significant plate count range.

(3) Make sure you footnote the numbers used to calculate the titre in your table.

(4) See appendix for sample bacteria titre calculation.

1 b) Determine the number of generations of E. coli cell growth. Include calculation.

1 c) Determine the doubling time during cell growth. Include calculation.

1 d) Determine the specific growth rate. Include calculation.

Questions

1 1. How much oxygen (μmole) needs to be supplied by sparging to a scaled up mammalian cell fermenter to support cell growth. The fermenter culture volume is 200 liters with a head space area of 3000 cm The maximum cell density is1.5 x 10^6 cells/ml. The head space area is 2500 cm^2. The rate of head space aeration was measured at 0.6 μmol oxygen/ cm^2 per h. The cellular utilization rate was measured at 0.06 μmol oxygen/ 10^6 cells per h.

2. A 2.7 liter continuous (chemostat) culture of mammalian cells is established at a steady-state equilibrium. The rate of medium inflow is 1.8 ml/ min. After 4 days the cell concentration attains a steady state of 1.8 x 10^6 cells/ ml. The chemostat input glucose concentration is 22 mM and the output spent medium has a concentration of 5 mM glucose.

1 a) What is the specific growth rate?

0.5 (b) Does the dilution rate fit expected range of 0.2 to 1.0 volume per day? Answer YES or NO and show calculation.

1.5 c) What is the specific consumption rate of glucose (μmol/10^6 cells/h)?

—
11

EXCEL OPERATIONS AND STANDARD CURVE PLOT METHODS.

Procedures may vary depending on excel version. There are numerous ways to use Excel, only one described below. HINT: Right click on whatever you want to change and select appropriate item from the pull down menu.

Formula and Function calculations

Put the curvsor in the cell where you want to enter formula or function. Type in formula or select function button. In the spreadsheet just set up formula for one cell then copy and paste for similar calculations.

Cell Formulae Notations:

Remember to use correct **mathematical brackets** in your formulae.

start equation with an = sign, do not leave spaces between entries

* multiply

/ divide

+ add

- minus

^power or use the POWER function

FUNCTIONS

EXP (e raised to the power of a given number)

You do not actually use the function alone but incorporate it into the exponential equation required to calculate N_o and N.

Example:

for $y = ce^{bx}$ the exponential regression equation

$y = 2E+08e^{1.45x}$ for bacteria/ml (y-axis) vs time (h)

for time (x) 0 h (N_o) : =(2E+08)*(EXP(1.45*0)) = 2 x 10^8 bacteria/ml

for time (x) 1.5 h (N) : =(2E+08)*(EXP(1.45*1.5)) = 1.76 x 10^9 bacteria/ml

MS EXCEL Exponential Growth CHART

Notes:

(i) Bacteria/ml values are scientific number category which permits growth rate constant calculation and keeps all data consistent.

Select Insert pull down menu.

Select chart - (standard types), select (XY) Scatter chart type. Select chart with data points not connected by lines. 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 data (x-axis time (h) and y-axis bacteria/ml). Row should be selected - just check. This is the method to select all data at one time. Should work. If not select series tab and add data for x-axis and y-axis one at a time. Click next.

Chart Options menu appears. Under default titles tab, enter figure 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 (8 is best), etc. When changing font select the entire graph box.. Click next. Keep the default setting (graph in data page).

Click finish.

Right click any empty area on graph area. Select clear. Removes lines and background color.

Select legend. Delete (may delete earlier when chart options menu is open).

Right click on y-axis. Select Format Axis. Select scale tab. Check logarithmic scale. Adjust the y-axis scale range (minimum and maximum) to fit your data.

Add Trendline by putting the cursor on any one of the plot markers. Right click. Select Add Trendline. Select the exponential regression box.

Select the Options tab. Select display equation on chart and R-squared value. Press OK. If required, move the equation so completely visible. You require this regression equation to calculate N_o and N_t for growth rate constant calculation.

Make sure you select print all information on one page, under page setup.

Sample calculations may be done directly on spreadsheet or as set up, Textbox. Either work directly in Excel or copy and paste from Word. Remember, data, calculations and graph must be printed on one page.

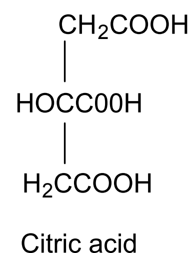
LAB 2 MICROBIAL INDUSTRIAL LIQUID FERMENTATION: Citric Acid Production by *Aspergillus niger*

OBJECT

The object of this experiment is to (i) optimize citric acid production by *Aspergillus niger* by investigating environmental parameter one-factor-at-a-time, (ii) design the experiment (iii) media preparation and (iv) reporting of findings.

INTRODUCTION

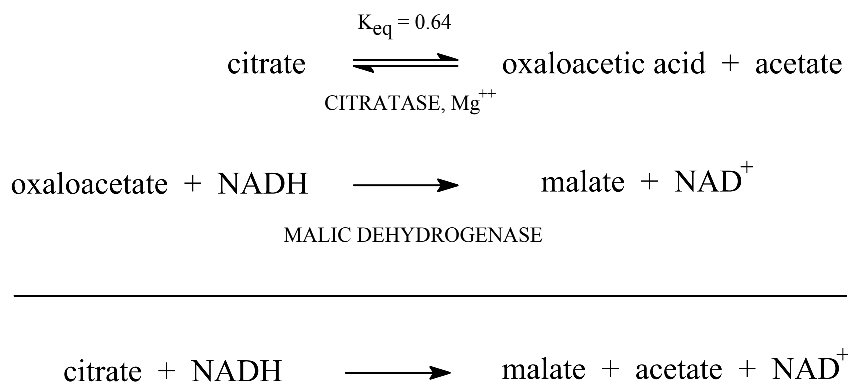
In your lab you will carry out small-scale liquid fermentation for the production of citric acid. Small-scale liquid fermentation allows testing of experimental parameters that lead to increased product production essential before step-up to large scale fermentation. In any fermentation it is important to understand what limits growth of the organism and subsequently what limits product (1). In your lab you will use a shaking waterbath to control oxygen transfer and temperature. In small fermentation, a sample should only be removed once (1). If time points are required, a series of identical tubes should be set up and sample taken only once from each tube.



Citric acid production by *Aspergillus niger* is a world wide industry, 400,000 tons produced annually (2). Citric acid, sold as sodium citrate, calcium citrate, or potassium citrate, is an safe edible acidifier. There are many reasons why citric acid is so widely used, for example, it is soluble, has low toxicity, chelates cations, pH adjustor, environmentally safe and has a good taste. It is used as a food preservative, anticoagulant, found in many medicines, ore leaching, detergent ingredient, chemical industry, etc.

Citrate acid excretion in large amounts by *A. niger* only occurs when certain aspects of cell growth is not optimal. Roehr et al Legisa and Gradisnik-Grapulin showed that high levels of citric acid accumulated in the absence of manganese, high initial sucrose concentration and aeration (3). The absence of zinc also seems to be important for accumulation of citric acid (4). Low pH is important for the accumulation. Ruijter et al demonstrated the an *A. niger* mutant (did not produce oxalic acid), lacking glucose oxidase and oxaloacetate acetylhydrolase, grew at pH 5 in the presence of manganese was able to accumulate citric acid (5). It is apparent, the ability of *A. niger* to excrete citrate depends on many variables, both genetic and environmental. Some strains are good citrate accumulators, while other strains do not accumulate at all. Citrate accumulation property may be induced by mutation.

Citric Acid Determination (6): Citric acid concentration may be determined accurately and



conveniently by coupling two reactions catalysed by the enzymes citratase (citrate lyase) and malic dehydrogenase. As the products of the citratase reaction inhibit it, a large excess of malic

dehydrogenase must be used. This will force the citratase reaction to the right and bring it rapidly to completion (the equilibrium of the malic dehydrogenase reaction lies far to the right). The reaction is followed on spectrophotometer by observing the decrease in absorbance at 340 nm (due to NADH, NAD^+ does not absorb). When there is no further decrease in absorbance, it is assumed that all the citrate has reacted. This is equivalent to the amount of citrate originally present in the cuvet.

In your lab, you will design, carry out and report on the effect of various environmental factors on citrate acid production.

References used to write the introduction not necessarily required reading.

(1) Hilton, MD. 1999. Small-scale liquid fermentations. In: Demain, AL, Davies, JE., editors. Manual of Industrial Microbiology and Biotechnology, 2nd edition. Washington: ASM Press. p 49-60.

(2) Legisa, M., Gradisnik-Grapulin, M. 1995. Sudden substrate dilution induces a higher rate of citric acid production by Aspergillus niger. Appl. Env. Micro. 61: 2732-2737.

(3) Roehr, M, Kubicek, CP, Kominek, J. 1992. Industrial acids and other small moleculs. In: Bennett, JW, Klich, MA, editors. Aspergillus-biology and industrial applications. Stoneham: Butterworth-Heinemann. p 91-131.(not in reference binder)

(4) Wold, WSM, Suzuki, I. The citric acid fermentation by Aspergillus niger: regulation by zinc of growth and acidogenesis. Can. J. Microbiol. 22: 1083-1092.

(5) Ruijter, GJG., van de Vondervoort, PJI., Visser, J. 1999. Oxalic acid production by Aspergillus niger: an oxalate-non-producing mutant produces citric acid at pH 5 and in the presence of manganese. Microbiology. 145: 2569-2576.

(6) Seiffer, S, S Seymour, B Novie, and E Munteoyler, Archives of Biochemistry vol. 25 (1950), p.191.

(8) Strobel, RJ., Sullivan, GR. 1999. Experimental design for improvement of fermentations. In: Demain, AL, Davies, JE., editors. Manual of Industrial Microbiology and Biotechnology, 2nd edition. Washington: ASM Press. p 80-93

Week 1

Part I: Experiment Design

See Lab 2 appendix for some references. You are not limited to these references but may prove useful for your group's project.

Experiment Group Assignment (maximum of 12 groups)

group #	Object: To optimize citric acid production by *A. niger* by varying designated environmental parameter:
1	dilution effect on high initial
2	media composition: sucrose concentration
3	media composition: nitrogen concentration
4	media composition: manganese concentration
5	media composition: carbon source(s)
6	media composition: trace metals such as Zn^{++}, Fe^{++}, etc (do not include Mn^{++}) [Note: Magnesium is an essential mineral not a trace metal.]
7	media composition: vitamins
8	oxygen transfer rate (removal of aeration at varying times)
9	pH
10	media composition: methanol concentration or boost
11	spore inoculum density (must measure spore density using a hemocytometer -see appendix and instructor to get hemocytometer and location of microscope.
12	media composition: citric acid boost
13	media composition: ammonia boost
14	media composition: glycerol

1. Before coming to lab next week you must have completed your experiment procedure (detailed step by step protocol). Please see instructor during the week if you have any difficulties. Experiment procedure will be checked **in lab** next week. Marks will be subtracted from final report if procedure found to be lacking. You do not need to include any procedures that are detailed in 60.451 lab manual.

2. Follow guidelines and materials available to write procedure (see below). Come next week ready to prepare your medium.

Materials available for media preparation and culture incubation:

(unless a special request you must limit your supplies used to the following list)

supplies	number/group
6" metal capped test tubes	12
pipetmen: P200 and P1000/tips	1
digital scale	
pH meter	
28ºC shaking water bath (200 rpm)	
20 ml sterile 0.5% Tween 80 (prepared in double distilled water) in MDB[a]	1
sporulated culture of *Aspergillus niger*	1
sterile large screw capped tube to transfer *A. niger* spore suspension	1
all supplies necessary for preparation of BASIC medium	
if other media components, supplies or conditions are not available - request of instructor (eg. hemocytometer)	

Experiment Design Guidelines:

- STUDENTS WORK IN PAIRS (same group as beer or wine making)
- experiment must demonstrate objective
- do not rewrite any procedure found in lab manual
- read list of available supplies - work within supplies available unless absolutely necessary
- All groups measure biomass and citric acid production regardless of parameter varied.
- Even though most groups are doing a different experiment, all experiments must be carried out as scheduled in lab manual.
- each flask should only be sampled once
- no zero time sample required, negative control should be sampled at the same time as the inoculated flasks but not inoculated with *A. niger* (only one flask required)
- all groups use BASIC *Aspergillus niger* medium (given in lab manual) and vary specified environmental requirement
- duplicate tubes of each condition must be inoculated
- make sure parameter range includes expected optimum - reference material may be helpful to determine optimum conditions for citric acid production

Week 2

Part II: Media Preparation and Inoculation

Friday

Each group should use the basic medium and incubation conditions other than the environmental parameter varied. Remember to measure and record all constant parameters.

Standard environmental parameters:
temperature: 28-30°C
sampling time: ~96 h (4 days)
pH: 2.0
rate of aeration: rotation

BASIC Aspergillus niger Medium[6]:

1. **Prepare salts solution.** Add ~200 ml double distilled water to a 250 or 500 ml beaker. Place on a stirrer and put a stirring bar in the water. Start stirring at a good rate but water should not be splashing out of the beaker. Slowly add 0.8 g NH_4NO_3. Stir until dissolved. Slowly add 0.3 g KH_2PO_4. Stir until dissolved. Adjust pH to 2.0 with 1 N HCl. Bring volume to 250 ml with double distilled water. Dispense 2.5 ml into each 6" metal capped test (number varies depending on your experiment).

2. Prepare **magnesium stock solution**. Your group may work jointly with other groups to prepare the magnesium stock solution but make sure you know how it is labelled and where to find it when it comes time to set up your media and inoculate your cultures. Put ~40 ml double distilled water in a beaker on a stirrer. Add stirrer and start stirring. Slowly add 2.5 g $MgSO_4.7H_2O$. Stir until dissolved. Bring volume to 50 ml. Pour into a milk dilution bottle. A separate magnesium stock solution is prepare to prevent precipitation as phosphates and divalent cations precipitate upon autoclaving.

3. Prepare **sucrose solution**. The sucrose solution must be prepared separately. Put ~30 ml double distilled water in a beaker on a stirrer. Add stirrer and start stirring. Slowly add 15 g sucrose - make sure no clumps form. Stir until dissolved. Bring volume to 50 ml. Pour into a milk dilution bottle. If any carbohydrate is added to salt solution (phosphates present) and subsequently autoclaved, the carbohydrate will caramelize. The carbohydrate that has caramelized (phosphates interact with the carbohydrate upon heating) is no longer available as a carbon source for the microorganism.

4. Clearly label all containers with group # , group names (no initials) and solution name. Use masking tape for label or thick permanent marker, if writing on glass. Autoclave[b] all media solutions for 10 min.

5. When solutions have cooled to below 50°C, **add 2.5 ml sucrose solution and 250 µl**

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[6]Legisa, M., Gradisnik-Grapulin, M. 1995. Sudden substrate dilution induces a higher rate of citric acid production by Aspergillus niger. Appl. Env. Micro. 61: 2732-2737.

magnesium stock solution to each flask. This is done MONDAY when setting up cultures. Use ONE sterile 10 ml pipette to add sucrose solution.

[a] high yield of citric acid production is obtained with 14%-22% sucrose

[b]do not over autoclave

[c] When **weighing chemicals always use a CLEAN spatula**. Rinse spatula with distilled water before using even if it is sitting on the shelf and appears clean. Cross contamination of chemicals can ruin your experiment.

[d] **Add ONLY if experiment requires added trace elements**: at final concentration of 1.0 mg/l manganese chloride citric acid should be inhibited. Prepare a 0.1 mg/ml stock solution by dissolving 10 mg $MnCl_2.4H_2O$ in a final volume 100 ml double distilled water. Dispense in MDB. Autoclave. To add a final concentration of 1 mg/L add 50 µl to each cooled test tube.

Monday

1. All autoclaved media will be in the lab Monday. Remember to add appropriate components to you salts solution before inoculation.

2. *Aspergillus niger* slants are located in the student's cold box (room 202 off room 201) - 1 slant/gr. Prepare a spore suspension by adding10 ml sterile 0.5% sterile Tween 80 per slant. Tween 80 is a non-ionic detergent which serves as a wetting agent to produce a homogeneous suspension of highly hydrophobic spores. Vortex slant to loosen slopes. Use a P1000 to loosen tenacious spores but be careful not to breakup the agar.

3. Unless you are varying the spore inoculum, use a P1000 to add 0.2 ml Tween 80 spore suspension to each flask (10^4 to 10^5 spores/ml) except control. Be careful not to puncture the agar.

4. Incubate tubes (properly labelled) on culture rotator 28ºC incubator. Make sure tubes are balanced, the rotor is turned on and the incubator door is closed. Remember to record all constants.

5. No samples are collected until Friday (4 days, ~96h).

Week 3

Part III: Sample collection and biomass determination

You do not need to centrifuge the negative CONTROL flask - assume zero mass.

1. Pour duplicate inoculated cultures (all liquid and fungal growth) into one sterile centrifuge tube (total of 10 ml). Centrifuge at 10000 rpm for 10 min. Pour clear supernatant into a labelled culture tube (not sterile). Put on ice. Often the fungal pellet is loose. Stop collecting the supernatant if the pellet is going to be transferred. This is okay as you have plenty of supernatant for the citrate assay. Any extra liquid will be evaporated upon drying and contribute little to cell mass.

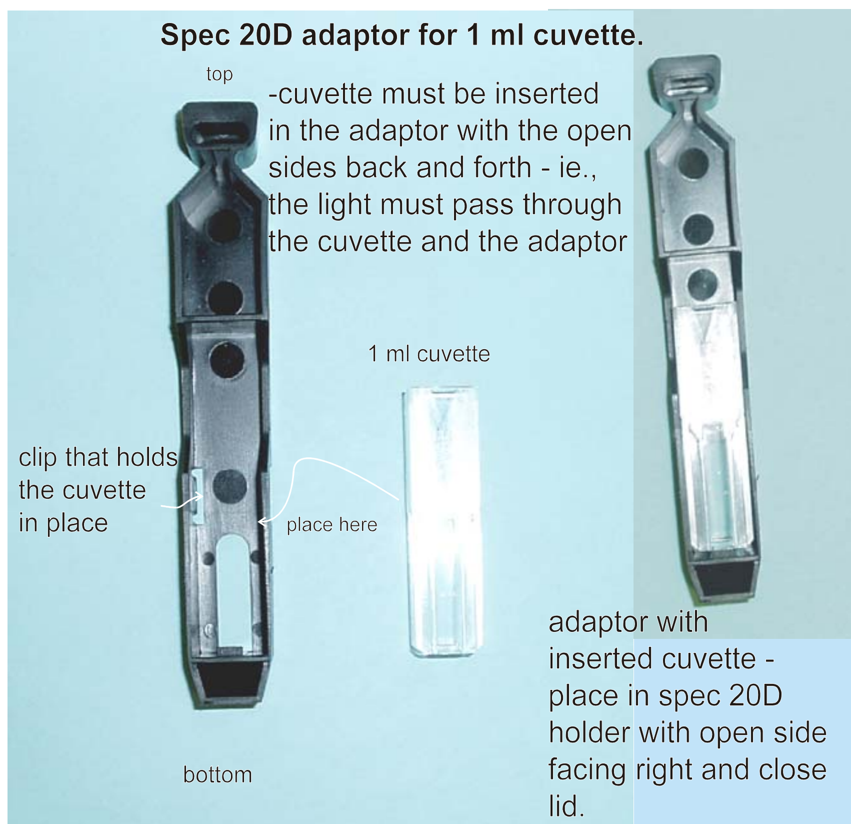
2. Weight and record weight of aluminum weigh dish. Use a spatula to scrap pellet(s) into labelled aluminum weigh dish. Make sure you add complete fungal pellet collected for each experimental condition. Put on tray for drying. The samples will be dried for 22 h at 100ºC.

   Monday: The tray containing the samples will be available in the lab. As soon as possible weigh and record the weight. Discard trays in Petri plate container after weighing.

Part IV: Citric Acid Determination

1. Turn on the spec20D 15 minutes prior to start of experiment. Set at 350 nm. Ideally the wavelength should be 340 nm but that is the lower limit of the spec 20D and for some specs it just doesn't work. 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. Each group has available 50 disposable cuvets (1/2 box). If you need more, rinse and reuse. Discard in plastic lined bucket when you finish.

2. **Blank the spec 20** with 1 ml 0.1 M phosphate buffer, pH 7.5, not the reaction mixture. Use disposable 1ml cuvet and cuvet holder. Remember NADH aborbs at 340 nm and must not be added when blanking the spec20.



3. **Reaction Mixture**: Keep all solutions on ice, especially the enzymes. Once you remove the NADH solution from the fridge, cover with foil as NADH is light sensitive.

   For each reaction use a separate1 ml disposable cuvet. Carefully pipette the following:

20 mM magnesium chloride	0.1 ml
malic dehydrogenase (1.5 mg/ml)	0.1 ml
citratase (1.7 U/ml)	0.1 ml
3 mM NADH	0.1 ml
0.1 M phosphate buffer, pH 7.5	to 1.0 ml (including sample volume)

4. **Change in absorbance measurement**: Add all components of reaction mixture except sample (either your experiment sample or known citrate standard). Mix the cuvet well by inverting several times (capped with parafilm).

   For each measurement your need **two spec 20D readings**, before and after you add the sample (subtract fo △absorbance value).

   Absorbance reading 1: First take a spec 20D absorbance reading of reaction mixture at 340 nm.

   Absorbance reading 2: Next, add 0.1 ml sample(or required volume of stock citrate), mix, wait 1 min, then take spec 20D absorbance. The 1 min wait allows the enzyme reaction to go to completion.

   Enter absorbance values in citric acid raw data tables provided.

5. **Standard Curve (change in absorbance at 350 nm vs mM citrate)**: Prepare samples for standard curve in duplicate. The standard curve for citrate is made by following the total change in absorbance at 340 nm using 200, 150, 100, 50 and 20 µl of 0.5 mM citrate standard solution. Maintain the total volume in the cuvet at 1.0 ml by varying the amount of phosphate buffer added each time.

6. **Experiment samples**:

   Undiluted sample: Prepare reaction mixtures for each sample and negative control in triplicate. Measure the change in absorbance for each.

   1/10 diluted sample excluding negative control: Dilute10-fold by adding 100 µl to 900 µg 0.1 M phosphate buffer, pH 7.5. Prepare reaction mixtures for each 1/10 sample in triplicate. Measure the change in absorbance for each.

Citrate standard curve RAW DATA TABLE							
0.5 mM citrate added* (μl)	citrate (mM)	absorbance at 350 nm					
		trial 1			trial 2		
		initial	final	change	initial	final	change
20	0.10						
50	0.25						
100	0.50						
150	0.75						
200	1.00						

-check that your standard curve data is acceptable, the change should increase proportional to concentration.

Citric Acid Sample RAW DATA TABLE			
Parameter	**Sample dilution**	**Absorbance at 350 nm -triplicate samples**	
		Initial	**Final**
	undiluted		
	diluted 1/10		
	undiluted		
	diluted 1/10		
	undiluted		
	diluted 1/10		
	undiluted		
	diluted 1/10		
Negative Control (uninoculated)			

This is your raw data. For the lab report you need to select either the undiluted results or the 1/10 diluted results. If there is too much citrate produced then the assay system becomes maxed out, all data very similar with a large change (almost maximum) for all parameter variables. Then it is best to select the 1/10 data for your lab report as this should show variation in the amount of citrate produced dependent upon parameter variable.

LAB REPORT

One single spaced TYPED report per group must be submitted no later than 4:30 pm Friday Feb 9.

Submit an abstract of your report **by email** le_cameron@umanitoba.ca by 4:30 pm Friday Feb 9. The abstract must be either a Word or Wordperfect document. Abstracts will be posted on the website.

2 **ABSTRACT**[7]

The abstract is marked as part of your report.

Include title, group # and student names

- 250 words or less - be concise, do not repeat
- it is best to write the abstract after writing the report
- write in complete sentences
- summarize main points of the report: objective, methods, results, and conclusion
- the abstract is a “stand alone” document that must be understandable and informative. Do not refer to any part of the report that is not accessible to the reader.
- do not use abbreviations
- do not mention other relevant literature
- see examples in McMillan V.E. 2001 Writing Papers in the Biological Sciences. 3rd ed. Boston: Bedford Books. p 55-59 (available in reference binder)

REPORT

0.5 **Include a Title**

- The title should identify major finding(s).
- Be concise.
- Avoid abbreviations.
- Include taxonomic names if relevant.

1.0 **Introduction**

- Write in paragraph format, maximum one page.
- Write from general to specific.
- State the relevance of citrate production by *Aspergillus niger*.
- State your experiment purpose relating to research in this area. Cite and reference work.
- End by summarizing your results in one sentence.

1.0 **Materials and Methods**

- Present in paragraph form.
- Organize information.
- Be concise, do not include unnecessary details. If materials or methods are found in lab manual, do not include, just cite.
- Must include details that refer to your group’s particular experiment.
- Do not include results.
- Use materials and methods section of Applied and Environmental Microbiology journal as an

[7]Be concise, do not include unnecessary details. McMillan V.E. 2001 Abstract. In: Writing Papers in the Biological Sciences. 3rd ed. Boston: Bedford Books. p 55-59.

example.

Data Presentation

Present results as requested using Excel spreadsheet (worksheet 1 (standard curve), worksheet 2 (sample data) and worksheet 3 (chart) available on lab website. See lab 2 appendix for Excel spreadsheet functions and chart preparation.

2.0 1. Standard Curve (Excel worksheet 1): Record all requested information. Include a standard curve, average absorbance change vs mM citrate. Also include R-squared value and linear regression equation on standard curve.

2.0 2. Experiment Data (Excel worksheet 2): Record all requested information in worksheet 2. Include requested sample calculations. Remember you select the best dilution (see raw data sheet) either undiluted or 1/10 diluted.

3.0 3. Chart (Excel worksheet 3): Record requested information and calculations. Insert requested figure of average mM citrate produced and biomass (g/L) against measured parameter (varies for each group) using Microsoft excel. The figure must have two y-axes. For each mM citrate point include a standard deviation bar.

Also insert a chart plotting mM citrate/biomass (g/L) ratio (y-axis) vs measured parameter (x-axis). Follow basic Excel instructions for previous graph omitting second y-axis and standard deviation bars.

1.0 **Discussion**

- Write from specific to general. Maximum one page.
- 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 agree with literature results.
- State why the knowledge of the ratio mM citrate/biomass ratio is important for industrial production of citrate .
- State experiment limitations.

Questions

1.0 1. In your lab each group carried out a one-factor-at-a-time experiment. Ideally a 'fractional factorial design' should be carried out. Explain why fractional factorial design is ideal for understanding multi-factor process.

0.5 **Acknowledgments**

State what each project member contributed to the experiment and report.

References

Include a list of cited reference as outlined in general introduction.

(no assigned mark, included with introduction, materials and methods, discussion and question)

Lab 2 APPENDIX

Some Reference Suggestions

Links to reference available on lab website.

Haq, I, Ali, S, Qadeer, MA, Iqbal, J. 2003. Stimulatory effect of alcohols (methanol and ethanol) on citric acid productivity by a 2-deoxy D-glucose resistant culture of *Aspergillus niger* GCB-47. Bioresource Technology 86: 227-233. [UM NetDoc - Science Direct or ejournals.]

Hesse, SJA, Ruijter, GJG, Dijkema, C, Visser, J. 2002. Intracellular pH homeostatis in the filamentous fungus Aspergillus niger. Eur. J. Biochem. 269: 3485-3494.

Karaffa, L, Kubicek, CP. 2003. *Aspergillus niger* citric acid accumulation: do we understand this well working black box? Appl. Microbiol. Biotechnol. 61: 189-196. [UM NetDoc Springer-Link]

Kubicek, CP and M. Rohr 1977. Influence of Manganese on Enzyme Synthesis and Citric Acid Accumulation in *Aspergillus niger*. Appl. Microbiol. Biotechnol. 4: 167-175. [UM NetDoc Springer-Link] -journal previously called European J. Appl. Microbiol.

Kubicek,C.P. Zehentgrubcr, O.E1-Kalak, Housam and M. Rohr 1980. Regulation of Citric Acid Production by Oxygen:Effect of Dissolved Oxygen Tension on Adenylate Levels and Respiration in *Aspergillus niger*. Appl. Microbiol. Biotechnol. 9: 101-115. [UM NetDoc Springer-Link] -journal previously called European J. Appl. Microbiol.

Legisa, M., Gradisnik-Grapulin, M. 1995. Sudden substrate dilution induces a higher rate of citric acid production by Aspergillus niger. Appl. Env. Micro. 61: 2732-2737. [UM NetDoc ASM Journals]

Roukas, T. 2000. Citric and gluconic acid production from fig by Aspergillus niger using solid-state fermentation. J. Ind. Micro. & Biotech. 25:298-304. [available on-line UM NETDOC via Goggle Scholar or Springer-Link]

Ruijter, GJG., van de Vondervoort, PJI., Visser, J. 1999. Oxalic acid production by Aspergillus niger: an oxalate-non-producing mutant produces citric acid at pH 5 and in the presence of manganese. Microbiology. 145: 2569-2576. [available on-line UM NETDOC via Goggle Scholar, search for article]

Xu, D-B, Madrid, CP, Rohr, M, Kubicek, CP. 1989. The influence of type and concentration of the carbon source on production of citric acid by Aspergillus niger. Appl Microbiol Biotechnol 30: 553-558 (available on-line UM NETDOC via Goggle Scholar, search for article)

Xu, D-B, Kubicek, CP 1, and M Riih. 1989. A comparison of factors influencing citric acid production by *Asperyillus niger* grown in submerged culture and on filter paper. Appl Microbiol Biotechnol. 30:444-449 [UM NetDoc Springer-Link]

Yigitoglu, M., McNeil, B. 1992. Ammonium ion & citric acid supplementation in batch cultures of *Aspergillus niger.* Biotechnology Letters. 14:831-836. [UM NetDoc Springer-Link]

MS Excel calculation procedures (may vary depending on Excel version):

Procedures may vary depending on excel version. There are numerous ways to use Excel, only one described below. HINT: Right click on whatever you want to change and select appropriate item from the pull down menu.

Formula and Function calculations

Put the cursor in the cell where you want to enter formula or function. Type in formula or select function button. In the spreadsheet just set up formula for one cell then copy and paste for similar calculations.

Cell Formulae Notations:

Remember to use correct mathematical brackets in your formulae.

start equation with an = sign, do not leave spaces between entries

* multiply

/ divide

+ add

- minus

^power or use the POWER function

Functions

AVERAGE Select the cell where you want to record average. Select paste function button, then statistics (or defaults to most recently used), then AVERAGE. Or use pull down menu - select Insert, function, statistics, then AVERAGE. A pop-up menu appear. Using your mouse right click the first or last[1] 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 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, then statistics (or defaults to most recently used), then STDEV. Or use pull down menu - select Insert, function, statistics, then STDEV. A pop-up menu appear. Using your mouse right 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 or hit return. The standard deviation value appears in your selected cell. Repeat for remaining data sets. Or copy and paste.

MS EXCEL CHART PROCEDURES

Changes to graph may be made at any time by right clicking or double clicking the appropriate area of the chart.

Standard Curve

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 (citrate mM).

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 8 is good, 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.

Right click on any horizontal grid line. Select clear.

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.

If required, move the equation so completely visible.

Move the graph to specified area on data sheet.

Make sure you select print all information (both chart and table) on one page under page setup.

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.

Two y-axes chart

Excel sometimes is not 'Science Friendly', this is one of those cases where two y-axes are required on a scatter plot. Sigma Plot is an excellent Excel based graph program but is not available on the University computers (expensive) so we need to make do.

Select Insert pull down menu.

Select chart - (standard types), select (XY) Scatter chart type for numerical parameter variables, eg pH, media concentration. Select chart with data points connected by lines. Click next.

OR select Column (bar graph) for non-numerical parameter variables (x-axis), eg. C-source, vitamin, etc. For bar graph majority of instructions similar, just need to adjust bars at the end, see comment at end of instructions.

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 the first y-axis data (average mM citrate produced minus negative control). Column should be selected - just check.

Select series tab. Series 1 plot is highlighted. 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 (varied parameter - differs depending on group). Put the cursor in Name box. Select appropriate heading (average mM citrate produced minus negative control). Click ADD under series box. Repeat above for remaining y-axes data (biomass g/L). All data will default to one axis - okay for now. Click next.

Chart Options menu appears. Under default titles tab, enter figure title and axes labels (enter right y-axis name). 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 (8 is best), etc. When changing font select the entire graph box. May need to redo after adding second y-axis. 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.

Right click on any horizontal grid line. Select clear.

Right click on biomass plot. Select Format Data series. Select Axis tab. Change plot series on - from primary to secondary axis. Click OK. Now you need to add a right y-axis name. Right click anywhere in the plot area. Select Chart Options. Select Title tab. Enter second value (y) axis. Just leave second value (x) axis blank.

Fit data plot to y-axis range if not done by default. Right click on appropriate y axis and change the range to fit your data. This allows the plot to fill the graph area.

On average mM citrate produced minus negative control plot right click. Select Y error bars. Under display, select both. Under error amount, select custom. Put the cursor in the + box, click. Use the mouse to click and drag to select all deviation data. Put the cursor in the - box, click. Use the mouse to click and drag to select all deviation data. Press OK.

Move the legend to empty space on graph if not already positioned under chart options. A good location is the bottom of the figure. Resize graph if required.

Move the graph to specified area on data sheet.

Make sure you select print all information (both chart and table) on one page under page setup.

Bar Graph Additional Information.

Right click on any bar. Select Format Data Series from menu. Click Format Data Series. Select Options tab. Adjust overlap and gap width by scrolling up or down until bars do not overlap. Press OK.

Lab 3: Investigation of batch culture specific production and consumption rates in *Aspergillus niger*.

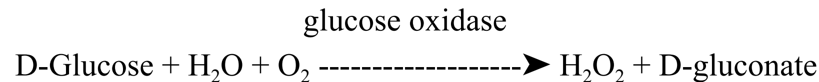
OBJECT:

The object of this experiment is to determine the specific production rate of citric acid and the specific consumption rate of sucrose by *Aspergillus niger* in a batch culture.

INTRODUCTION

In this experiment you will use *Aspergillus niger* to follow production of citric acid with time at optimum growth conditions. In addition, *Aspergillus niger* biomass will be measured as gram wet weight. Due to the size of filamentous fungi, biomass will be used instead of cell number. Sucrose consumption will be determined enzymatically. First sucrose will be hydrolysed using yeast acid invertase yielding glucose and fructose. One unit of invertase hydrolyzes 1.0 umol/min sucrose at pH 4.5 and 37°C. Then glucose oxidase reagent will be used to measure glucose which is equivalent to the amount of sucrose.

Glucose oxidase reagent[8] set is a single reagent kit containing glucose oxidase, peroxidase, aminoantipyrine, phosphate buffer, pH 7.5, sodium ρ-hydroxybenzoate, non-reactive stabilizers and fillers, sodium azide. The intensity of the red color produced by the oxidation of the chromagen quinoneimine dye at 500 nm is directly proportional to the glucose concentration and consequently sucrose concentration.



The specific consumption rate of sucrose and production rate of citric acid is calculated taking into consideration ∫N.dt since growth rate varies during sampling time (not always logarithmic). Cell mass (g wet-weight/l) is plotted against time. ∫N.dt (area under curve) is determined for each sampling time. The area is determined for each day. Each day is calculated by taking the sum of all previous time points (days). See Excel lab 3 spreadsheet for details. To determine consumption and production rates sucrose (mM) and citric acid (mM) are plotted against ∫N.dt. and all possible straight lines drawn. The slope of each linear part of the plot is the specific rate (Q) expressed as mM/g/day. Most likely each plot will have two linear regression slopes. The metabolic coefficient (C_o) of citric acid/sucrose is the ratio of Q product/Q substrate using only the slopes that correspond to rapid substrate utilization.

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

PROCEDURE

Week 4

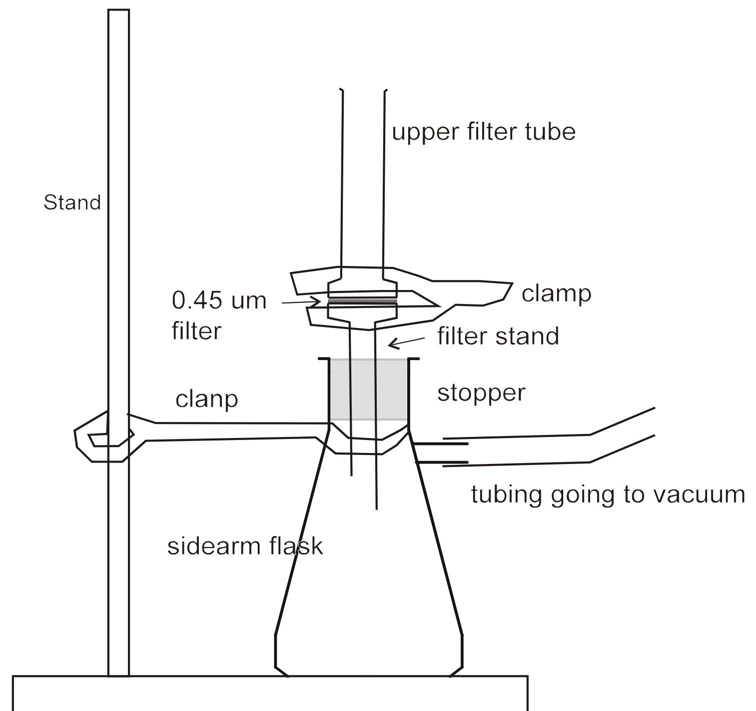
Part I: Culture inoculation, sampling and biomass determination

1. Using freshly prepared 0.5% Tween 80 *Aspergillus niger* spore suspension, add 60 µl spore suspension to twelve 5 ml *Aspergillus niger* basic broth tubes. Incubate on 28ºC rotator. Label clearly with group # and names (no initials). Basic broth is identical to Lab 2. You have two extra tubes in case something goes wrong.

2. Take a tube of basic broth and transfer ½ to two screw capped plastic vials. Label group #, full names and time zero. Store at -20ºC, freezer in room 201. For time zero, assume the cell biomass is zero.

3. Starting Monday process duplicate cultures as follows.

Each day (MONDAY through FRIDAY): Set up a small vacuum filter apparatus using a 0.45 µm nitrocellulose filter (see diagram). Make sure the filter stand tube covers the vacuum outlet. Turn on vacuum. Pour one culture in top funnel. When the filter is dry remove clamp and upper filter tube. Turn off vacuum. Use forceps to remove filter. Place filter containing sample on digital scale that has been zeroed using a dry filter. Record biomass (gram wet weight) of sample. Record weight to 2 decimal places. Ideally the scale should measure to 3 decimal places but not available. Pour filtrate into a small screw capped plastic vial available on shelves. Clearly label with group #, names, and day. Repeat with duplicate sample. Store vials in -20ºC freezer (use a labelled plastic container (box on floor along windows in each lab) to hold your tubes - if necessary, use crunched up paper towel to support vials upright. **Rinse filter apparatus** by setting up filter apparatus without a filter and running about 50 ml tap distilled water (white handled tap) through the filter. Turn off vacuum, discard filtrate and return apparatus to supply bench. Do not discard on trolley until Friday. If the filter apparatus is not rinsed, the sucrose will clog the filter stand and you will not be able to filter your samples later in the week.



make sure the long side of the filter stand tube faces vacuum outlet to prevent liquid from entering vacuum system

Week 5

Part II: Sucrose Determination

1. Remove one vial of each sample (0, 3, 4, 5, 6, and 7 day) from the freezer. Leave the remaining vial in the freezer for citrate determination. Thaw.

2. Conversion of sucrose to glucose:

Set up the following reaction in an eppendorf tube for each sample time point.

6 µl	1.0 M Na acetate, pH 4.8
60 µl	sample
2 µl	invertase (yeast) (10 mg/ml or 7 U/2 µl)

Incubate at 40ºC for 10 min.

Notes
(a) assume 11 µl of invertase reaction is equivalent to 10 µl of culture sample.
(b) 1 unit of invertase hydrolzes 1.0 µmole sucrose/min.

3. Determination of glucose:

(i) Completely dissolve oxidase reagent in 100 ml distilled water (white handled tap). Use a milk dilution bottle. You may shake to dissolve but not too vigorously as bubbles kill enzymes. Oxidase reagent powder may be found in student cold box or brown fridge. ONLY one oxidase reagent bottle available per group - this gives you double the amount you need. Keep oxidase reagent at room temperature once prepared. Do not put on ice.

(ii) Label required plastic disposable cuvets (upper non-transmission side). Add 1 ml glucose oxidase reagent to each cuvet.

(iii) 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 duplicate**.
Experiment samples (0, 3, 4, 5, 6, and 7 day): use 11 µl invertase treated sample for undiluted **in triplicate** for days 3, 4, 5, 6 and 7.
Also do 1/20 dilution in triplicate. Add 10 µl 1/20 diluted sample for days: 0, 3, 4, 5 and 6.
Prepare 1/20 dilution by adding 11 µl invertase treated sample to sample to 209 µl distilled water.

(iv) Add sample, 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 at 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).

Week 7

Part III: Citric Acid Determination

Use 1 ml disposable cuvets and cuvet holder for spec20 D (350 nm).

1. Remove remaining sample vials from freezer.

2. **Blank the spec 20D** with 1 ml 0.1 M phosphate buffer, pH 7.5, not the reaction mixture. Remember NADH aborbs at 340 nm and must not be added when blanking the spec20.

3. **Reaction Mixture**: Keep all solutions on ice, especially the enzymes. Once you remove the NADH solution from the fridge, cover with foil.

   In a spec 20D cuvet, carefully pipette the following:

20 mM magnesium chloride	0.1 ml
malic dehydrogenase (1.5 mg/ml)	0.1 ml
citratase (1.7 U/ml)	0.1 ml
3 mM NADH	0.1 ml
0.1 M phosphate buffer, pH 7.5	to 1.0 ml (including sample volume)

4. **Change in absorbance measurement**: Add all components of <u>reaction mixture</u> except sample (either your experiment sample or known citrate standard). Mix the cuvet well by inverting several times (capped with parafilm).

   For each measurement your need **two spec 20D readings**, before and after you add the sample (subtract fo △absorbance value).

   <u>Absorbance reading 1</u>: First take a spec 20D absorbance reading of reaction mixture at 350 nm.

   <u>Absorbance reading 2</u>: Next, add 0.1 ml sample, mix, wait 1 min, then take spec 20D absorbance. The 1 min wait allows the enzyme reaction to go to completion.

5. **Experiment samples**: Prepare reaction mixtures for each time sample in triplicate. Use undiluted for day 0. Prepare dilution in total volume of 1 ml, for1/10 mix 100 µl sample + 900 µl 0.1 M phosphate buffer (3 and 4 day), pH 7.5, for 1/20 mix 50 µl sample + 950 µl 0.1 M phosphate buffer (5 and 6 day) and for 1/50 mix 20 µl sample + 980 µl 0.1 M phosphate buffer (7 day). Measure the change in absorbance for each. An absorbance worksheet is included to record data.

6. To save time and reduce repetition you are given the slope of the citric acid standard curve on spreadsheet.

Sucrose Worksheet for recording raw data.

Table 1. Glucose standard curve colorimetrically determined by absorbance at 500 nm using oxidase reagent.

glucose (5 mg/ml) added* (µl)	glucose (mM)	absorbance at 500 nm		
		trial 1	trial 2	average
2				
4				
6				
8				
10				

*cuvet volume = 1 ml

Sucrose worksheet for recording raw data.

Table 2. Glucose determination in *A. niger* batch culture samples.

time (day)	dilution	absorbance at 500 nm in triplicate
0	1/20	
3	undiluted	
	1/20	
4	undiluted	
	1/20	
5	undiluted	
	1/ 20	
6	undiluted	
	1/20	
7	undiluted	

Citrate worksheet for record raw data.

Table 3. Citrate determination in *A. niger* batch culture.

time (day)	dilution	absorbance at 350 nm - triplicate samples		change in absorbance at 350 nm
		initial	final	
0	undiluted			
3	1/10			
4	1/10			
5	1/20			
6	1/20			
7	1/50			

LAB REPORT

Data Presentation and Analysis

Excel spreadsheet is available on lab website. The spreadsheet consists of four worksheets, glucose standard curve, glucose data, citrate data, and chart analysis. See Lab 2 appendix for Excel figure presentation information, the basics only as you may need to change axis labels, etc.

2.5 1. Include a completed glucose standard curve worksheet. Record all requested information and calculations. Standard curve plot: absorbance at 500 nm (y-axis) vs glucose mM (x-axis). Include linear regression equation and R-squared value in figure.

2.5 2. Include a completed glucose data worksheet. Record all requested information and include requested spreadsheet cell calculation.

2.0 3. Include a completed citrate data worksheet. Record all requested information and include requested spreadsheet cell calculation. Linear regression equation for citrate standard curve supplied on spreadsheet.

7.0 4. Include a completed chart analysis worksheet. Record all requested information and include requested spreadsheet cell calculations.

Include two figures on spreadsheet:

Figure 1. *Aspergillus niger* growth curve for 'viability index' calculation (plot biomass as g/L against time (day)

Figure 2. *Aspergillus niger* consumption and production rate (plot glucose mM and citrate mM against $\int N.dt$). For both plots include standard deviation bars.

For Table 6 of your chart spreadsheet you need to calculate specific production (Q_p) and specific consumption (Q_s) rates for each slope of each plot of figure 2. If you have more than one slope this is a problem for Excel. Excel only calculates one slope per plot. Either calculate the slope by hand and enter data or manipulate the data (ie removing points for the other slope(s) to get the linear regression equation (ie. slope).

Print all to fit one page.

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APPENDIX

Beer Making

The process starts with barley. Barley is the grain of choice since there are few technical problems when compared to other grains. Barley has a high starch and a low protein content. International Malting Company of Canada (previously Dominion Malting) in Winnipeg is a world renown source of malt for breweries. Much of the barley is grown here in Manitoba. Fort Garry brewery, which you will possibly tour later in term, uses two varieties (light and dark) of Dominion Malt barley which they blend to develop different flavoured brews. Students should be able to relate brewery vessels to following processes.

MALT: The first step in the process of making malt is steeping of the barley seeds. The barley grains are re-hydrated by cyclic soaking in water and air. Next the moist seeds are spread out and allowed to germinate (1). Germination activates enzymes, among them amylases that breakdown (15ºC) starch (amylose and amylopectin) to polyglucose molecules. The germination time depends on the degree of modification (breakdown) of the barley kernels required. Next, the germinate barley is kilned. This is a drying process with air circulation to halt germination while preserving the integrity of the amylases. The grain is initially dried at 38ºC until it has reached 5-8% moisture. At this moisture content, the amylases are stable at high temperatures (80-100ºC)(1). This temperature is used to achieved desired grain color (caramelisation) and moisture content.

GRIST: The malt is crushed (milled) to produce a “gritty flour” or grist (1).

MASHING (Mashing vessel): Water is added and mashing of malt takes place. Enzymes, especially amylases degrade the mash to soluble carbohydrates and other components until only the husks and small particles of the grain remain.

WORT (Brew Kettle): The sweet liquid (wort) is separated from the spent grain (used as cattle feed). The wort is further processed by boiling in the presence of hops. Boiling serves many purposes, (i) stop enzyme activity, (ii) sterilization, (iii) coagulation of proteins and tannins, (iv) lowers pH, (v) distillation of volatile substances, (vi) wort concentration and (v) color (caramelisation of sugars) production and (vi) flavor production (nutty, burnt, toffee) (2) . The hops add a bitter taste to the beer. After boiling the wort, it is clarified, separated from the hops and cooled.

FERMENTATION: The wort is transferred to Fermentation Vessels where the yeast is added. After fermentation the yeast settles out and the beer is transferred to the filtration system.

FILTRATION AND CARBONATION: Filtration system may be a series of meshes, diatomaceous earth, etc. Just before bottling the beer is carbonated often using the recovered carbon dioxide given off during yeast fermentation.

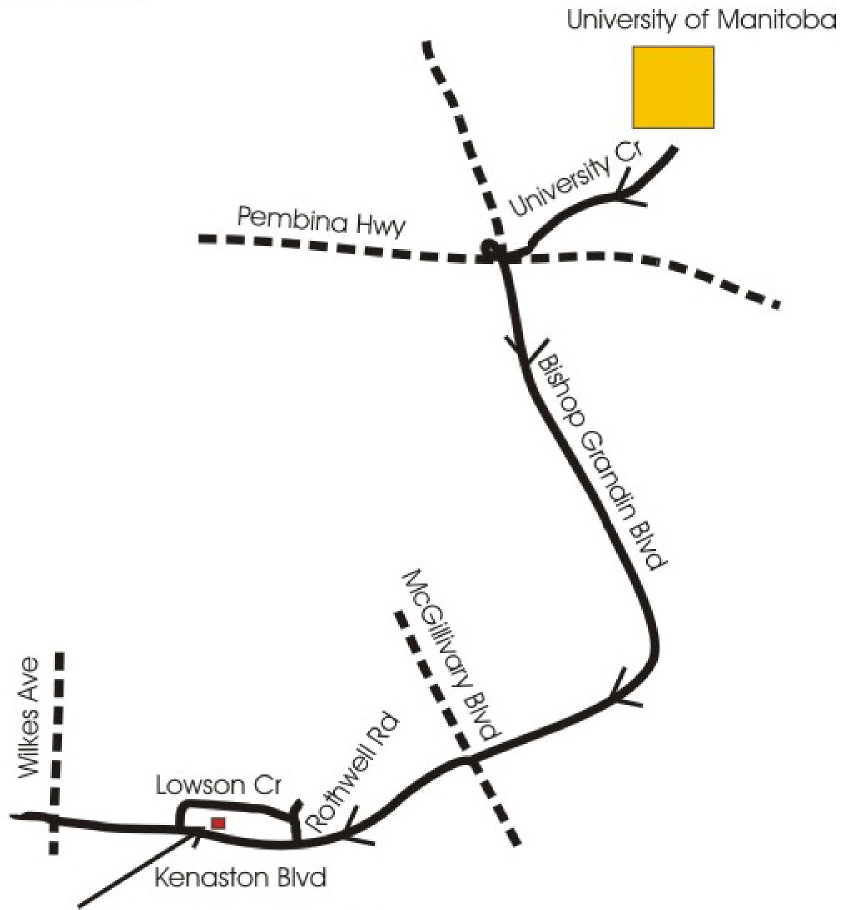
BOTTLING MACHINERY: Bottles are rinsed, filled, capped, labeled, inspected (may or may not be pasteurized). The bottles are capped when there are still bubbles therefore the air space is only carbon dioxide.

References

(1) Coopers Brewing Kits http://www.coopers.com.au/homebrew/d.htm (accessed, July 04/02)

(2) Hough, J.S. 1995. The Biotechnology of Malting and Brewing. Cambridge University Press. p 1-159.

ROUTE TO FORT GARY BREWERY



SAMPLE CALCULATION of the number of bacteria per ml

Data for example calculations using the following sample data

Dilution plated	Number of colonies	
	Plate 1	Plate 2
10^{-2}	TNTC	TNTC
10^{-3}	320	316
10^{-4}	34	27
10^{-5}	2	3

TNTC = too numerous to count

<u>Terms</u>

Plating factor = reciprocal of volume plated

Dilution factor = reciprocal of dilution for significant counts

Significant plate counts = the sum of the plate counts at significant dilution divided by number of plates. Often more than one dilution has significant plate counts. It is important to use all significant plate count data. There are several ways to deal with data that has more than one significant plate count dilution.

Number of plates = number of significant plates

<u>Calculation</u>

Do not average an average value as it incorporates error in your calculation (not statistically accurate). Use one of the following methods to calculate bacteria titer.

<u>Bring all significant counts to the same dilution</u>:

$$\text{Bacteria/ ml} = \frac{\text{significant plate counts}}{\text{number of plates}} \times \text{dilution factor} \times \text{plating factor}$$

$(320 + 316 + 340)/3 \times 1/10^{-3} \times 1/10^{-1} = 3.25 \times 10^{6}$ bacteria/ml, since the smallest number of significant figures for plate counts is two, the answer is 3.3×10^{6} bacteria/ml

<u>Or calculate the titer for each significant plate count and average.</u>

Bacteria/ml = significant plate count x dilution factor x plating factor

$320 \times 1/10^{-3} \times 1/10^{-1} = 3.20 \times 10^{6}$ bacteria/ml

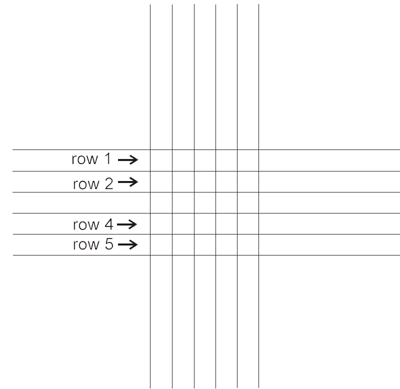
$316 \times 1/10^{-3} \times 1/10^{-1} = 3.16 \times 10^{6}$ bacteria/ml

$34 \times 1/10^{-4} \times 1/10^{-1} = 3.4 \times 10^{6}$ bacteria/ml

Average all values: $(3.20 \times 10^{6} + 3.16 \times 10^{6} + 3.4 \times 10^{6})/3 = 3.25 \times 10^{6}$ bacteria/ml, since the smallest number of significant figures for plate counts is two, the answer is 3.3×10^{6} bacteria/ml

HEMOCYTOMETER INSTRUCTIONS (estimation of spore count)

1. Each hemocytometer holds two samples, inlet either side. Just before using rinse slide and cover in EtOH. Remove and wipe off the hemocytometer and coverslip with a kimwipe.
2. Transfer ~10 µl of solution to V-shaped grove of the hemocytometer*, cover lengthwise with cover slip and allow sample to settle for 1 minute then count.
3. Place on the microscope. Microscopically count microorganisms (rods to cocci ie regular shaped) using 40x objective.
4. See figure below for diagram of center part of ruled slide consisting of a 5 by 5 **double lined** grid. There is a smaller single line grid inside each double lined square but is used only to help you count. Each row consists of 5 doubled lined squares. Count the number of sporess in rows 1, 2, 4 and 5 (omit row 3) - total of 20 squares. If microorganisms overlap rows, make sure you do not record them twice.
5. After using hemocytometer, return slide and cover slip to ethanol container. Replace cover.



Row 1, magnification of row 1 above. Example of microorganism distribution (22 microorganisms)

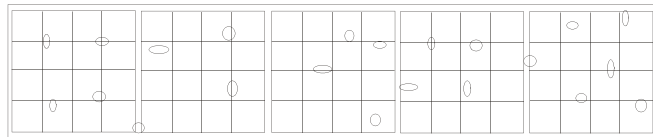


Figure . Hemocytometer counting area.

Hemocytometer Titer Calculation:

Total the number of microorganism in rows 1, 2, 4 and 5. Total of 20 squares.

Divide total number of microorganisms by 20 to get the number of microorganisms in one square.

The volume of each square is 0.02 cm x 0.02 cm x 0.01 cm depth. This is equivalent to 4.0 x 10^{-6} ml since 1 cubic cm is equivalent to 1 ml.

For example:

Hemocytometer spore counts: row 1 (5 squares) contains 19 sporess , row 2 contains 18 spores, row 4 contains 16 spores and row 5 contains 17 spores.

$$\frac{(19 + 18 + 16 + 17)}{20} = 3.5 \text{ spores/square or per } 4 \times 10^{-6} \text{ ml.}$$

$$3.5 \times \frac{1}{4.0 \times 10^{-6} \text{ ml}} = 8.8 \times 10^{5} \text{ spores/ml}$$

AUTOMATIC COLONY COUNTER

There are several makes of automatic colony counters in this department. All automatic colony counters work on the same principle. The counter registers a count every time you touch the colony with the counter probe as long as the L-shaped probe in inserted into the agar at the edge of the plate. This completes th electrical circuit through the agar from the L-shaped probe to the counter probe (needle shaped probe) touching the colony.

Operation

1. Push or flip the power switch to turn on counter.
2. Press the button on the counter that resets the counter to zero.
3. Place agar culture plate on counter and remove cover.
4. Insert L-shaped probe into the agar at the edge of the plate.
5. Count colonies by touching each colony with the counter probe tip (needle shaped probe).
6. Remove plate, replace lid.
7. Remember to turn off power switch when you are finished counting.

Notes:

(i) Use a marker to divide the plate into sections or use the grid on the automatic colony counter to facilitate counting.

(ii) The counter also comes with a magnifying glass but it is not required unless you are counting very small closely spaced colonies.

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 <u>scale on the pipettor is read different for each type</u> - 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 6-2A). The volumeter display is read from top to bottom in µl for P20 and P200 and ml for P1000 (Figure 6-2).
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. 6-3A). 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. 6-3B). 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 6-3C). Wait 1 to 2 seconds and then depress the push-button completely to expel any residual liquid (Fig. 6-3D). 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 6-1F).

pipetman operation diagram

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 with blank solution. 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. DO NOT PUT SPEC 20D TEST TUBES ON DISCARD TROLLEY.

COMMENTS

1. Keep all solutions free of bubbles.
2. 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

8. 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.
9. 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.

   Call up pre-programmed memory code number: 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

   Real time operation (enter original parameters): 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.

FINAL LAB EXAM: Industrial Microbiology 60.451

DATE: SAMPLE PAGE: 1 of 2 TIME: 1.5 h

INSTRUCTOR: Dr. L. Cameron

Student Name: __________________ Student Number: ________________________

Briefly all questions on exam paper in space provided. (Spaces reduced for sample exam.)

WRITE EXAM IN PEN ONLY. The sample exam contains more questions than would be on the final to expose you to a wide range of question type.

1. State what happens in the

1 (a) mashing vessel

0.5 (b) brew kettle

2. A 1 liter batch culture of *E. coli* was inoculated with an actively growing log phase culture (1/50). After 20 min the culture was titered, 1.5×10^7 bacteria/ml. The culture continued to be sampled over the next two hours at regular intervals and the titer of the culture 2 hours after inoculation was 2.4×10^8 bacteria/ml.

1 a) Determine the number of generations of cell growth.

2 b) Determine the specific growth rate.

1 c) Present a schematic graph and indicate on graph how N_o and N are determined.

3. A continuous (chemostat) culture of mammalian cells is established in suspension with a working volume of 2.4 liter. Medium enters the chemostat at a rate of 1.4 ml/ min. After 3 days the cell concentration attains a steady state of 1.4×10^6 cells/ ml.

1 a) What is the specific growth rate ?

2 b) The chemostat input glucose concentration is 22 mM. It is determined that the output spent medium has a concentration of 5 mM glucose. What is the specific consumption rate of glucose (μmol/10^6 cells/h) ?

1 4. How is the *Aspergillus niger* spore suspension prepared? Explain the function of each major step or solution.

2 5. The following components are in the medium for citric acid fermentation by *Aspergillus niger* (per liter):

2.6 g NH_4NO_3

2.6 g KH_2PO_4

0.26 g $MgSO_4.7H_2O$

8.8 g sucrose

final pH of 3.5

Outline in detail how to prepare 100 ml of this medium for inoculation with *Aspergillus niger*. You have available a microbiology lab with all necessary supplies and equipment required.

2 6. When assaying for citric acid in a sample, 5 components are present in the cuvet in addition to the citrate sample. List the 5 components and function of each with reference to sample citrate determination.

FINAL LAB EXAM: Industrial Microbiology 60.451

DATE: example PAGE: 2 of 2 TIME: 1.5 h

INSTRUCTOR: Dr. L. Cameron

1 7. Use the following citric acid determination data to calculate citric acid concentration. Analyze data results.

sample time (hour)	Absorbance values at 340 nm of citric acid assay sample.							
	sample (zinc added to media)				sample (no zinc added to media)			
	sample 1		sample 2		sample 1		sample 2	
	initial	final	initial	final	initial	final	initial	final
72	0.58	0.19	0.66	0.23	0.64	0.03	0.67	0.03

Linear regression equation (straight line formula) $y = 0.15x + 0.01$ for absorbance vs mM citrate.

1 8. Explain why a sample should only be removed only once from a small fermentation flask.

1 9. What environmental factors influence citrate production by *Aspergillus niger*? State the optimum condition for each.

1 10. Outline spectronic 20D operation prior to putting your blank solution in the spec.

1 11. a) Calculate the concentration (mM) citrate for the following data. 150 µl 5 mM citrate added to a final volume of 1 ml.

b) Calculate the concentrate (mM) glucose for the following data. 6 µl 5mg/ml glucose added to a final volume of 1 ml. MW glucose = 180.

0.5 12. Why is a standard deviation bar included in 60.451 citrate production graphs?

0.5 13. Why type of experiment did you design to study the effect of environmental conditions of citric acid production? What type of experiment(s) would give you more information?

1 14. Present a schematic graph that allows you to determine the viability index. Give the formula for calculation of $\int N.dt$.

1.5 15. Calculate the specific production rates of citrate (mM citrate/g/day), specific consumption rates of sucrose (mM sucrose/g/day) given the linear regression equations for each plot. Calculate the metabolic coefficient of citric acid relative to sucrose.

	day range for ∫N.dt	linear regression equation for citrate plot	day range for ∫N.dt	linear regression equation for sucrose plot
slope 1	3-6	y = 133.6x + 24.2	3-5	y = -400.2x + 619.2
slope 2	6-7	y = -15.6x + 327.6	5-7	y = -56.9x + 262.6

-END-