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BIOCHEMICAL ENGINEERING LABORATORY MANUAL

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by

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LABORATORY MANUAL
ENCH485

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Prerequisite

Satisfactory performance of this laboratory course work in biochemical engineering requires that the student has had experience in freshman chemistry or physics laboratories. In addition, familiarity in biochemical engineering at the introductory level is assumed. The student should have taken or is concurrently enrolled in an introductory undergraduate course in biochemical engineering. Because this is essentially a senior elective course offered by the chemical engineering department, the student should be able to apply meaningfully what one has learned from the previous fundamental courses in transport, kinetics, thermodynamics, and engineering mathematics.

Course Format

Immediately preceeding each experiment, there will be a short lecture ranging between 10 to 45 minutes, depending on the subject. This introductory lecture will briefly cover the background principles, caution avoidable pitfalls, answer any questions that may rise, and demonstrate techniques that have not been explicitly discussed in the instruction manual. Any questions may be directed at any time to the instructor or Mr. Cheng-han Liu, our Teaching Assistant for this semester. The laboratory itself will be closely supervised by the T.A. It is the T.A.'s responsibility to perform the entire experimental procedures at least once before the class to ensure that all the necessary equipment and supplies are available for the smooth operation of each experiment. The T.A. should be thoroughly familiar with the instructions. Conducting a trial run is a key to the success of a laboratory course because there are always many components that can be overlooked.

Minimum Class Performance

As with any other laboratory course, it is imperative that the student prepares for each experiment beforehand. I can promise that the amount of time and ef-

fort spent on preparation shall reflect on the student's work and, ultimately, my evaluation of the student's performance. The student is to read not only the instructions but also comprehend all the procedures and concepts *prior* to coming to the class. This is to be achieved by requiring that the objectives and procedures for that experiment be clearly stated in the student's *bound*, *paged* notebook in his own words *before* coming to the class. The student is encouraged to raise questions during the introductory lecture if additional explanation or clarification of laboratory procedures is required. Otherwise, the student is assumed to understand the material thoroughly. Because various chemicals and equipment can be potentially dangerous if not used correctly, laboratory admission will not be granted to those who are unsure of what needs to be carried out. Furthermore, because most of the critical biochemicals and enzymes required in this course are relatively expensive and unstable, only a minimal amount will be provided to each student. Very likely, there will not be enough supplies to repeat the experiment if it fails, nor will there be sufficient time for the class is tightly scheduled.

Because this course is offered in a chemical engineering program, analytical aspects of the experiment as well as the conceptual aspect will be emphasized. The thorough analytical treatment of the data will ensure that the engineering aspect will be preserved, whereas the questions at the end of each experiment are designed to enhance the conceptual understanding of the underlying principles of the subject. The student must master the laboratory techniques to yield good results. One must apply the correct analytical methods to reduce the raw laboratory data to a set of more meaningful parameters. Above all, one must understand, not merely memorize, the guiding principles. In summary, this course will not at all emphasize rote memorization, but will encourage creativity in each student. If better procedures can be found, let them be followed.

Grading

There will be no formal examinations, except for 2-3 short quizzes on laboratory techniques. The University's final examination requirement can be satisfied in lieu by the individual report. Both the laboratory notebook and the experimental reports will be considered in issuing the final grade. Grading will be partially based on the write-ups before and after performing an experiment. The questions at the end of the instruction manual will count heavily, in proportion to the degree of difficulty or effort required for an acceptable answer. Thus, the grade for the regular experiments will be based on two components: the handwritten laboratory notebook and the *typewritten* report. Combined, they represent the work of this course.

A text editor or word processor is highly recommended for the preparation of the report. An introduction to the use of Disk Operating System (DOS) and text editors on Chemical and Nuclear Engineering Department's Zenith microcomputers will be hosted outside the regular laboratory period to help those students who have not yet mastered the techniques of computer-assisted document preparation.

The grades for the individual project will be based on both the quality of the proposal that is to be submitted before the experiments are conducted and the experimental findings presented in the formal report due at the end of the semester. The relative weight given to each part is as follows:

Regular Experiments		70%
Experiment Write-up	50%	
Notebook	10%	
Quizzes (3)	10%	
Individual Project		30%

Since this is a 3-credit course, one is expected to spend 3 hours in class and an additional 6 hours outside of class in order to pass this course at the minimum level;

thus, the student can expect to pass with a "C" grade if he spends the minimum required 9 hours for a 3-credit course. An effort beyond this minimum amount should result in a proportionately better grade. The grade will be issued based on an absolute scale, not on a curve. A healthy collaborative effort among fellow classmates, which is clearly distinguished from cheating or academic dishonesty, is highly encouraged. However, every student must ultimately do his own work.

Types of Experiments

A wide range of experiments will be covered in this course, reflecting the wide range of applications of biotechnology. Quite many experiments are original and are being offered to the students for the first time. Some will cover the fundamental laboratory techniques involved in modern biotechnology research. It is hoped that the student will find these experiments exciting and challenging. Above all, this course will provide the student a chance to learn something that cannot be conveyed well in a traditional lecture setting and hand-on experience that is so vital to future practicing biochemical engineers. The following is a tentative list of the representative experiments for which I plan to write this year. Because we have only allocated about twelve three-hour sessions to these experiments at the rate of one session per week, we can, unfortunately, perform only a small fraction of them.

Section 1. Enzymes

Examples of Enzymatic Reactions

Cheese Production with Rennet

Protein Hydrolysis with Protease

Detergent Action with Bacterial Protease

Starch Degradation with α-Amylase

Silver Recovery

Fruit Juice Extraction with Pectinase

On-Line Data Acquisition

A/D, D/A

Low-Level Bioprocess Control – Temperature, pH, DO

Advanced On-Line Bioprocess Control

Section 3. Fermentation

Types of Fermentors

Wine Fermentation with Yeast

Beer Brewing with Yeast

Single Cell Protein (SCP) Fermentation

Ethanol from Biomass

Yogurt Production with *Lactobacillus* Strains

Penicillin/Antibiotics Biosynthesis with Fungi

Plant Cell Culture

Mammalian Tissue Culture

Section 4. Molecular Biology

Amino Acid Sequencing – Sanger's Method

Cell Fractionation with Centrifugation

Plasmid Stability, Copy Number, Control Strategy

Application of Selection Pressure

Section 5. Individual Projects

Possible Projects

Standard Research Report

The following is a tentative schedule to be followed during this semester. It is by no means final. The student is highly encouraged to discuss with the instructor the types of experiments he wishes the class to perform so that the class's interest

may be better reflected in the choice of the experiments.

Week 1.	Cheese and Detergent
Week 2.	Protein Digestion
Week 3.	Cellulose Degradation
Week 4.	Starch Degradation
Week 5.	Enzyme/Protein Purification
Week 6.	Enzyme Immobilization
Week 7.	Yogurt Production and Aseptic Culture Techniques
Week 8.	Batch Submerged Fermentation of Yeast in Shaker Flasks
Week 9.	Continuous Fermentation of Phenol with Cell Recycle
Week 10.	Immobilized Cell Bioreactor
Week 11.	Pilot-Scale Fed-Batch Ethanol Fermentation
Week 12.	Individual Project
Week 13.	Individual Project
Week 14.	Individual Project
Week 15.	Presentation and Party!

Individual Project

The last 3 weeks will be devoted to special individual projects where each student is to propose and execute one's own experimental plan. The student should consult with the instructor in planning the experiment to ensure the availability of the necessary reagents and instruments. A typewritten proposal is due at the middle of the semester; this will allow the instructor and the T.A. enough time to order the requested reagents or to arrange the required equipment. Further revisions will be requested from the student if the proposed experiment is deemed unfeasible. At the minimum, the proposal should be similar in format to the instruction manual, with well defined objectives, clearly stated procedures, relevant background information,

and suitable references. At the least, the proposed experiment itself should be similar in difficulty to those performed in class but with a much more ambitious goal. Again, because the student has only two weeks to execute the experimental part of the individual project, the choice of suitable objectives and carefully planned steps are highly critical. The semester will be concluded with the submission of the final report for this project. The schedule is outlined below:

Week 8.	(3/21)	Initial project proposal due
Week 9.	(3/28)	Initial project proposal returned for modification
Week 10.	(4/04)	Final project proposal due
Week 11.	(4/11)	Final project proposal returned with comments)
Week 12.	(4/18)	Laboratory phase, equipment setup
Week 13.	(4/25)	Laboratory phase, data acquisition
Week 14.	(5/02)	Laboratory phase, data analysis
Week 15.	(5/09)	Final project report due; presentation, and PARTY!
Week 16.	(5/16)	Final grades assigned

The student should start planning the individual project to be performed at the end of the semester as soon as possible. One should not wait until the night before the day the initial proposal is due. Start by defining the objectives. The project should be completed within the semester. So do not be overly ambitious, nor should it be trivial. However, the experiment should be slightly more thorough than thoese performed in the class because there will be three weeks, instead of one week, to gather the data. After the objectives are defined, specify the steps needed to reach those objectives. Identify the instruments and reagents required for each step. This is important in order to ensure that what one's wish is realizable.

It will be helpful to discuss one's special needs with the T.A. or the instructor to see if they can be arranged. The student may have to modify his objectives and

iterate the planning process until it is apparent that his plan is feasible. The student may save much time in planning if he keeps in mind that there is no working budget for this course, and that some equipment is limited in supply. Other than the somewhat ancient shaker and one of the spectrophotometers, all the equipment is diverted from the instructor's own research. This is also the case with regards to most non-capital items such as glassware and consumables. Besides, it is impossible to have ten students carrying out fermentation experiments all requiring the prolonged use of the same fermentor. However, at the same time, be aware of the wide range of possibilities that exist. One can do quite a bit with just what he has if an experiment is designed well. The request for the needed equipment and chemicals for the individual project must be submitted as early as possible, not only because even simple chemicals may take more than one month between the placement of an order and the delivery of the item on one's desk, also because there may be considerable competition for the same pieces of equipment from fellow students. The final proposal will be due during the tenth week of class.

The types of experiments that are suitable for the project are the ones with originality. One of the best places to identify ideas is in journals called Biotechnology Letters and Biotechnology Techniques, which contain short articles on various recently performed experiments. The following is a partial list of some possible projects:

Enzyme purification/isolation: various protocols

Membrane ultrafiltration

Thin layer chromatography

Enzyme production: induction and repression, control/feeding strategies

Enzyme deactivation: shear sensitivity

Enzyme probes

Enzyme immobilization: various protocols

Cofactor immobilization/regeneration

Kinetics of gel (alginate) formation, prediction of pore size

Cell immobilization: various protocols

Controlled release of cells

Cell flocculation and sedimentation

Solid substrate fermentation on agar plate

  Estimation of biomass/substrate concentrations

Use of yeast in dough preparation

Cell disruption: cell homogenizer, ultrasonic devices

Cell disruption: shear sensitivity

Wine/beer/sake/yeast fermentation

Plant cell culture

Microbial waste treatment

Mixed culture: competition, commensalism

Effects of water activity on cell growth rate

Binding of magnetites to enzymes to effect separation

Incorporation of magnetites in immobilized enzyme/cell matrices

Aqueous-organic extractive fermentation

Aqueous two-phase fermentation and extraction

  Water activity effect

  Polymer recovery, recycle

Solubilization of enzymes in organic solvents with PEG

Oxygen transfer enhancement with fluorocarbons, water activity study

Alternative oxygen sources other than air

Waste treatment: adsorption of poisonous metals on microbial cells

Micro-encapsulation of enzymes/cells

Fluorescence sensor

HPLC and GC work

On-line monitoring/analysis of fermentation variables

Computer applications: A/D and D/A interfaces, on-line monitoring

Computer controlled actuators: motors, setpoint input to controllers

Optimal control of an enzyme reactor

  Packed/fluidized bed

  Temperature programming

Optimal control of a transient bioreactor

  Feed, withdrawal, and concentration manipulations

Optimal control of a mixed substrate fermentation

Programs to analyze chromatograph; integrator

Deconvolution of spectrum signals to estimate multiple components

Computer modeling

Laboratory Notebook

Use a *bound* (not the spiral type) laboratory notebook with pages numbered consecutively at the upper corners. A permanent-ink pen should be used in entering information in the laboratory notebook; no pencils or water-soluble felt-tip pens will be allowed. The student is forewarned that this rule will be enforced by placing his notebooks under running water or by wiping the surface with a wet sponge in class periodically and unannounced. After this deliberate destructive procedure is carried out, the student will be held responsible for the permanent loss of data as a result of using pencils or water-soluble ink; i.e., he may be assigned an "F" for the experiment in which there is a significant loss of data, as if the data were never taken.

In general the laboratory notebook should serve as the quantitative aspect of the course. Procedures, instrumentation, and data should be recorded in such a way that any person could pick up the notebook and conduct the experiment with reproducible results. The typewritten report, on the other hand, should incorporate qualitative analysis as well as insightful interpretations and observations of one's experimental results. This is why one may sometimes find himself doing library research in order to answer fully the questions in the handouts. Remember, good lab techniques go hand in hand with the ability to analyze data meaningfully – they are equally important.

As mentioned previously, enter the experiment title, the objective, and the procedure in your own words before coming to the class. The notebook will be collected and graded periodically.

Title of the Experiment

This part is self explanatory, simply copy from the instruction manual.

Objectives

The objectives should be concise and explicitly stated, usually in one sentence.

Procedures

Write down the steps of the experiment. A graphical representation of the steps outlined in the instruction manual may greatly help the student visualize the experiment. Also note the purpose of the step if it is not totally obvious. It is to be emphasized that the student is not asked to copy the laboratory manual, nor will a mere copying be accepted under any circumstances, for it is universally considered as a form of plagiarism, a serious academic offense punishable by an immediate and automatic dismissal from this class and a possible expulsion from the University of Maryland. The amount of information entered in this section is considered adequate if one can perform the experiment in class without referring back to the original laboratory manual.

Data

The very first entry on the day of the class should be the date. All data taken in the laboratory must be entered directly in the notebook. Data recorded on a loose sheet of paper will be discarded whenever it is found. Once entered, data are never erased from the notebook. Strike out erroneous entries with a continuous, straight line. Also remember to record other pertinent qualitative observations, as well. Convert all data into physical units whenever possible. For example, the content of a colored compound in a solution may be measured in absorbance or transmittance units, but it is the absolute concentration units such as g/l, mole/l, and weight percent that are desired. Graphic results should be affixed permanently in the notebook. In addition, calculations and data analysis needed to reduce the raw data into meaningful results are carried out and recorded directly in the notebook.

Format for the Laboratory Report

A typewritten report is to be submitted promptly a week after each experiment. No handwritten reports or illegible photocopies are accepted. The equations, figures, and tables must also be professionally done. Number all pages of the report; number all equations consecutively. There is absolutely no excuse for misspelled words. Consult a dictionary if there is the slightest doubt about spelling. It is straight forward to run the report through a spelling check program if it is composed on a computer. Furthermore, there is also no excuse for simple grammatical errors such as subject-verb agreement, whether English is one's native tongue or not. The report, as in other aspects of one's work here at the University of Maryland, is expected to be carried out to the best of one's ability. Hurried, sloppy work will not be tolerated. The submitted work should be the repeatedly revised version of the preliminary draft. The regular use of a text editor or a word processor in composing the report on a computer should make the process of revision much less painful.

A major shortcoming in student report writing is that of being too wordy. The goal of any report is to present to the reader the necessary information so that he will understand what was done, why it was done, how it was done, and what was learned, but no more. To do this efficiently is difficult and requires practice. Keep in mind that communication skills, both writing and oral, are just as important as the technical contents; one will not advance very far in his career if no one else is informed of his great discoveries.

All written material must be the student's own work; use one's own words, not someone else's, especially in describing the background or literature survey. Plagiarism will not be tolerated, not even for half a sentence. Plagiarism will be dealt with according to University Regulations. Give proper credit to other people's

work if used.

The scope and length of each section are governed by the nature of the subject material. Depending upon the purpose of the report, some of the sections may be emphasized, combined, or even deleted, while others may be added. Use subheadings to divide the report into smaller logical units if necessary. In general, the following format, somewhat complementing that of the instruction manual, is suggested for the laboratory report.

The report shall simply start with the title and the objectives of the experiment, followed by a summary of the results obtained. Discuss the results in, of course, the **Discussion** section. Finally, answer the questions to the best of one's ability.

Title Page

- ⋆ Title of the experiment. It may be copied from the instruction manual.
- ⋆ Author's name and affiliation (i.e., our department/school).
- ⋆ Date of submission of report.
- ⋆ The course number (i.e., ENCH485 Biochemical Engineering).

Objectives

Simply copy from one's notebook.

Introduction

This should be a very brief and incisive summary that covers the following:

- ⋆ Purpose of the investigation
- ⋆ Underlying fundamental chemical and physical principles
- ⋆ Method or principles used in the solution
- ⋆ Quantitative/qualitative statement of the principal results

Materials and Methods

This section is required only for the individual project. However, the student may include this section if the procedures he followed differed significantly from those specified in the instruction manual.

- ⋆ Usually, the construction of the equipment, the source of the chemicals, etc. are described here, if the paper contains any experimental aspects.
- ⋆ Give a succinct description of the apparatus with the aid of a neatly drawn diagram where appropriate. Avoid triviality. Describe the methods of measurement and indicate their reproducibility. List briefly each series of experiments run and the reason for running it.
- ⋆ Describe the procedure used. Present the essential equations. This section is considered incomplete if another person who is scientifically trained but is not at all familiar with the student's investigation cannot reproduce exactly what he has done.

Results

Summarize the data and present any significant experimental findings. Whenever possible, the results should be presented in graphical or tabular forms because they are much easier to comprehend and interpret. The graphs and tables should be accompanied by a verbal description of the results that draws attention to their most noteworthy characteristics. The data should be in the reduced form (e.g. actual physical units rather than voltage readings) in order to interpret them in the most general terms. All of the results of the experiment or theoretical calculation that are to be referred to in the **Discussion** and **Conclusions** sections should be presented in this section. The significance of the numbers, the general trends, or the lack of them should be pointed out. Explain any significant or unexpected deviations. Although there should be no surprises in these experiments, the student must be trained to make a careful note of any abnormalities habitually. This is es-

pecially important in a research environment, for many great discoveries have been made accidentally. (If the phenomenon is normal and commonplace, someone else must have already observed it.)

Discussion

Interpret the experimental results in terms of physical and chemical principles and the particular circumstances of the experiment. Indicate the reliability of the reduced experimental data and the sensitivities and uncertainties in the theories. Compare the results with those expected on the basis of theory or empirical correlations, and discuss any discrepancies. State any assumptions explicitly and verify their validity rigorously. Compare the results to what the student expects and to other classmates' whenever possible. State how one's finding differ from others, or how the result depend on the various assumptions.

Mention any nonstandard steps taken to calculate the results and indicate one's confidence level in them. Interpret the results in terms of the underlying physical and chemical principles. State any assumptions *explicitly* and verify their validity rigorously.

Discuss any alternatives that should be considered. Propose what is to be done next. Since this is the first time that the course is offered in a well organized manner, the student's constructive comments on all aspects of the laboratory will be wholeheartedly welcomed to improve this course for the next year. How can the procedures be changed to achieve better and clearer results? Should the objective be modified? What parts were interesting, and what were boring? What was actually learned, or what did the student wish he could have learned instead? Within the limits of time, budget, availability of equipment, and overall course description, what sort of experiments would the student really like to perform? (Remember that this is a biochemical engineering laboratory; there will not be experiments

dealing with bombs made from nitroglycerin.) Although a tentative schedule has been made, by issuing constructive comments, the student can greatly influence the type of experiment he and his classmates will perform and enjoy in the coming weeks.

Conclusion

Briefly summarize the findings supported by the data and the subsequent calculations of this experiment. Describe how the phenomenon investigated depends upon the variables that have been investigated.

Answers to Questions

Attempt to answer all the questions to the best of one's ability before coming to the laboratory class. Direct questions to the instructor or the teaching assistant if help is needed. Hints and pointers will be given liberally; however, the student should not expect them to carry out his work. Note that the answers to some of the questions result from the subsequent analysis of the experimental data obtained in class. Therefore, it is emphasized that the student work them out first as he is writing down the procedures. The student should be fully aware of the additional steps other than the ones explicitly stated in the procedure that one must perform to obtain the required data in the laboratory. Make a note of these hidden steps in the **Procedures,** section because successes in any experiment depend heavily on careful planning. Otherwise, one may later find that he cannot produce a satisfactory report because he has failed to perform certain steps or neglected to record some essential data.

Tables and Figures

Tables and figures must have descriptive titles and must include important details. All tables and figures must be referred to in the text, otherwise there is no need for them in the report. However, the contents of the tables and figures must

be clear without having to refer to the report's text. For a figure, for example, a poor title is "Heat-Transfer Coefficient versus Flow Rate." A better title is "Effect of Water Flow Rate on Overall Heat-Transfer Coefficient in Methanol Condenser at 1 Atm." Use words as well as letters for labelling axes. Thus, write "Pressure" as well as "P," "Friction Factor" as well as "f," etc. The units should be included in all figures and tables. The same data should only be represented either as a table or as a figure but not both.

References

One may follow the alphabetical format (sorted according to last names) or numerical format (sorted according to the order of citation in the main text). Use the same format consistently throughout the entire report. See any standard journal articles if the student is not familiar with the standard formats.

Appendices

Those materials that do not quite fit in the main text should be included in the **Appendices** section. Each appendix should be a stand alone unit.

Overall Hints

Here are listed some general hints on clear writing styles.

⋆ Hyphenate compound adjectives. Examples:

a three-inch pipe, a copper-constantan thermocouple,
a heat-transfer coefficient, a stirred-tank reactor

⋆ Effective writing usually places the verb near the beginning of the sentence, close to the subject. Example:

Poor: The mass-transfer coefficient, shown in Figure 1 as a function of temperature and compared with theory in Table 2, was calculated from Equation (3).

Better: The mass-transfer coefficient was calculated from Equation (3). Figure 1 shows the effect of temperature on that coefficient, and Table 2 gives a comparison with theory.

⋆ Use tenses carefully. Use the past tense for acts that are now history or for results that are no longer true. Use the present tense for results that are contemporary. Avoid the future tense; it is rarely needed. Example:

Poor: The pressure of the evaporator was constant at 3 psia, but it is difficult to say exactly because the pressure meter was flopping back and forth.

Better: Although the pressure meter fluctuated, the evaporator pressure was nearly constant at 3 psia.

⋆ Use relative pronouns properly. In defining clauses (i.e., those necessary to identify the subject of the clause) the pronoun *that* is appropriate. Example:

The particle volume *that* was measured by displacement was greater than the calculated volume.

In non-defining clauses (i.e., those providing additional information about the subject of the clause) the pronoun *which* is appropriate, and the clause should be set off by comas. Example:

The measured particle volume, *which* was greater by 3% than the calculated volume, was used in the porosity determination.

⋆ Avoid unattached participles. Active participles require a subject except in rare circumstances where, by usage, they have acquired the power of adverbs (e.g., roughly speaking).

Incorrect: Substituting for x from Equation (6), the expression

for velocity becomes:

Correct: Substituting for x from Equation (6), we find the expression for velocity to be:

Correct: With substitution for x from Equation (6), the expression for velocity becomes:

★ Avoid extra words. Consistent with clarity, use only the minimum number of words necessary to say what one wants to say. After writing a paragraph, go back over it and remove all words that are not essential.

★ Use simple, direct words and sentences and avoid jargon or false elegance. Here is an oft-quoted example of a passage written by a sociologist on three characteristics of teenage culture:

> Compulsive independence of and antagonism to adult expectations and authority. This involves recalcitrance to adult standards of responsibility.
>
> Compulsive conformity within the peer groups of age mates. It is intolerable to be *different.*
>
> Romanticism: an unrealistic idealization of emotionally significant objects.

This wordy passage can be expressed by one simple sentence:

> Teenagers are disobedient, group-minded, and unrealistic.

★ Avoid essentially meaningless phrases such as:

> It may be stated that ...
> You will find it interesting to know ...

For your information ...

In this connection the statement may be make that ...

At this point in time it may be appropriate to ...

It is a fact that ...

★ Avoid generalities; be as specific as possible. Also, whenever possible, be quantitative rather than qualitative. Examples:

Poor: The temperature measurement was not accurate because the thermometer was no good.

Better: Temperature accuracy can be improved by better thermometer calibration.

Poor: Figure 1 shows that the data are in bad agreement with the results calculated from Equation (3).

Better: Figure 1 shows that the experimental heat-transfer coefficients are about 60 percent larger than those given by Equation (3).

★ It takes much more effort to write a short report than a long report.

Future Plans

What hampers the understanding of biochemical engineering concepts is the lack of feel for a real system. For example, somehow there exists a strong mental block for enzymes because the student thinks he/she has never seen the substance, no matter how strongly an instructor emphasizes the contrary. But the entire concept is suddenly understood once he has seen the enzyme. It is no surprise that the smell of protease is similar to that of rotten eggs or decomposing meat because the protein degradation in nature is often accomplished by the presence of protease. Discovering this fact, the student suddenly feels at ease with the subject because he has just realized that he has always known the material and it is no longer so alien and threatening. There is a conscious effort throughout this manual to provide experiments that the student can relate to his/her daily experience. The transformation of an abstract idea to a concrete experience is the key to a true understanding and lifelong appreciation of the subject in any field of study, and biochemical engineering is certainly no exception. Because biochemical engineering deals with materials that are really significantly closer to a student's everyday experience than, say, nuclear physics or electronics, which is difficult to perceive using our five senses, a well designed laboratory encounter can ensure that the student retains what he/she has learned year after year.

Partly because only in recent years have we witnessed the rapid expansion of the field of biochemical engineering, only an extremely limited number of departments in this country offer an introductory laboratory course in biochemical engineering. Thus, there is currently no established textbook or laboratory manual at all that we can use off the shelf. It is my intention that the manual given to this class be further refined in the coming years to eliminate unworkable experiments and to expand those that are of more interest to the students. There are also plans

to add many more different experiments so that various aspects of biochemical engineering, from the food/nutritional science to the highly delicate recombinant DNA technology.

Presently, due to years of neglect, we have a rather devastated biochemical engineering laboratory with practically no instrument/equipment, minimal essential glassware, and no budget. Within the limited resources, I am working very hard to plan inexpensive experiments that are both interesting and educational. The experiments are planned in an interactive mode; so I will need quite a bit of feedback from the class to make them work. I will be writing the instructions as the course proceeds. The instruction of each experiment will have the following format:

Title

Introduction

Objective

Procedures

List of Reagents and Instruments

Discussion

Questions

Extra Credit Problems for Advanced Students

Reference Readings

Extra Handout from Last Year

The following message was issued to the students last year. It is reproduced verbatim here in the hope that some of this year's students may find part of the message helpful. I do not intend to issue one this year.

There had been many complaints that no quantitative conclusion could be drawn from the experiments and that the experiments are too long. Having done most of the experiments myself, I somewhat disagree with that statement. The poor

quantitative observation may be due to the general lack of instinct as to what types of data must be taken and how they are to be taken. For example, in the detergent experiment, one should have realized that if no dye is released to the washing liquid at the beginning of the run, the solution should be clear and colorless, meaning that the absorbance should be 0.000. If the solution is not in that ideal condition, he should have the flexibility to employ a filter. Having a rough feeling of what kinds of result to expect and understanding the objectives will be of great help in facilitating the course work.

Or, perhaps, a lack of preparation is sometimes to blame. Think through the entire procedure, step by step, before coming to the class. Picture oneself going through the entire experiment, as if he is actually conducting it. Unless noted otherwise, an efficient student should be able to complete an experiment within the lab period. Be warned that the experiments will become longer as microorganisms are introduced because the rate of reaction will be controlled by the nature of the microorganism utilized. Long time constants are one of the main features that distinguish a biological system from a purely chemical one. For example, a typical microbial fermentation run may last for the entire week, 24 hours a day. In that case, the student needs to coordinate the various tasks and share responsibilities and results with his lab mates. Because he will partly depend on his lab mates's data, his confidence in the peer's techniques will be revealed in the error analysis of the data. Effectively, part of the grade for this course will be assigned by the fellow students.

Because all senior chemical engineering students should have had prior exposure to chemistry and physics laboratories, out of respect for the student, familiarities with certain fundamental experimental tools are already assumed. For example, it is not the objective of this course to teach the proper use of a pipet or a spec-

trophotometer, although it must be mastered if the reminder of the course is to be fruitful. The student is advised to review them to refresh his memory. However, do not hesitate to ask for assistance from the T.A. or myself as needed. As indicated in the first syllabus, it is the student's responsibility to ask questions if certain points need be clarified. Otherwise, it is only reasonable for me to assume that everything is understood.

I regret the shortage of some instruments; however, I believe that the shortage is made much worse by the unfamiliarity with their proper use. For example, some students felt that there were not enough test tubes for the spectrophotometer. The fact is that all the test tubes one needs is one, provided that there is another blank test tube next to the instrument; more test tubes will only make the calibration much more tedious, for it must be done for each test tube. The markings on a test tube can be easily cleaned off with ordinary solvents. The time it takes to use various instruments could be shortened significantly, leaving enough time for everyone else. For example, the entire procedure for reading the absorbance consisting of inserting a blank test tube, turing the zero knob, withdrawing the blank test tube, inserting the sample test tube, reading the absorbance, and withdrawing the sample test tube, may be shortened to less than ten seconds, even less than the time needed to read this sentence.

An intelligent person must improvise and learn to do with what he already has. For example, the limitation posed by the number of available sinks can be easily overcome. In most of the experiments, one needs to use the sink only occasionally, if a large beaker is placed on his bench and acts as a temporary holder of waste liquid, which is to be emptied and cleaned only at the end of the lab period. A small quantity of water can be squeezed from a plastic bottle to wash small pieces of glassware. One need not crowd around the heated bath, hold a hot test tube

with his bare hand, and wait for the color to develop. That time can be wisely spent preparing the next sample or conducting other parts of the experiment if the student only uses a small piece of wire to secure the test tube in the water bath.

Many parts of the experiments can be carried out concurrently instead of sequentially; this is especially true when more than one substrate is used in different conditions. For example, hot, warm, and cold water washes can all be carried out simultaneously. Some of the smaller repetitive details can also be taken care of in one step. For example, one need not make a separate trip to the reagent bench each and every time he takes a sample. He may decide that he will be taking, say, 10 samples. He can repeatedly pipet the required reagent to each of the 10 test tubes all within the same trip. Thus, 10 trips are cut to only 1. Even the pipet needs to be cleaned only one time instead of ten times. Although the procedure may state that the reagent is to be added to the sample, occasionally there is absolutely no difference if the sample is add to the reagent. In addition, some procedures can even be combined. For instance, could the addition of DNS reagent, which is alkaline, reverse the pH of the samples in the acid cellulose hydrolysis experiment? Thus, the efficiency with which one works is the direct result of knowing what he is doing.

Why not specify everything *exactly* in the Procedure, down to the exact size of pipets or beakers to be used? Why not stop torturing the poor students who stay past midnight and come back day after day for more of the same torture? I do notice the enthusiasm and am surprised by the degree of motivation. However, I personally feel that too much has already been specified. Many parts of the experiment are intrinsically quite flexible, and over-specification tends to stymie creativity. What must be avoided is the degeneration of potentially exciting experiments to the mere lifeless following of cookbook recipes, even that should be pursued with discretion according to taste. Given the objectives of each experiment, the student should

use the stated procedures as a guide to reach those objectives, not blindly following them like zombies. Frustration is an inescapable part of the learning process. The student must continually stay sharp. If an experiment does not work, find the causes, identify the sources, and rectify the mistakes so as to make it work. One should discuss what he has done in his laboratory report.

Part of the overall objective of this class, aside from introducing students to various aspects of biochemical engineering fundamentals through experimentation, is to gain the ability to follow the type of procedures specified in scientific literature. If the procedures and the purpose behind each step are truly understood, adaptation of the procedure to meet one's needs is straight forward. For example, in the colorimetric method of concentration determination, the ratio of the sample and reagent can be varied, depending on the concentration of the original sample. As a matter of fact, this variation allows the same method to be effectively employed for an extremely wide range of concentrations. Nothing is carved in the stone; a little flexibility will carry a long way.

Finally, some forceful measure will be taken unless the student voluntarily clean after himself before leaving the room each day. This includes all the equipment and glassware used, his work bench, and garbage on the floor.

EXPERIMENT NO. 1

CHEESE PRODUCTION FROM MILK

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ENCH 485, Spring 1988

Objective

To demonstrate the use of rennet in casein coagulation under different pH values.

Introduction

Cow's milk is rich in a wide range of chemical compounds that can be processed into various dairy products such as cheese, butter, and yogurt. Specifically the milk component involved in cheese production is a soluble protein called casein. The enzyme rennet can be used to catalyze the conversion of casein in milk to para-casein by removing a glycopeptide from the soluble casein. Para-casein further clots, i.e. coagulates, in the presence of calcium ions to form white, creamy lumps called the *curd*, leaving behind the supernatant called the *whey*.

$$\text{casein} \xrightarrow{\text{rennet}} \text{para-casein (aq.)} \xrightarrow{Ca^{++}} \text{para-casein (ppt)}$$

The precipitate is soft at this point and can be separated from the whey by the use of cheese cloth or other filtration devices.

There is no standard method of cheese making; limitless variations exist for all stages of the process: pre-ripening, curdling, addition of artificial ingredients and salt for flavor, and aging. This variation in processing accounts for the wide

range of cheeses commercially available, differing in texture and flavor. The curd can also be processed with other techniques to make a variety of desserts. However, all processes have one thing in common: the separation of the curd from the whey.

Three ways of preparing milk for curdling will be introduced in this experiment. Industrially, the lactic acid level in the milk is increased by adding a starter culture of *Streptococci* or *Lactobacilli* to the milk and fermenting at 32°C for 10 to 75 minutes. In addition to biologically converting the lactose present in the milk to lactic acid, these strains of microorganisms also greatly affect the flavor of the final product. Thus, the selection of a suitable strain, the amount of starter culture, and the length of pre-ripening, is of the utmost importance in creating the subtle differences in the final color and aroma that distinguishes an expensive cheese from a cheap one.

If one has not yet acquired a keen palate for cheeses, the second approach should suffice. In this approach, one takes advantage of the existing *Lactobacillus* culture in buttermilk and uses it as the starting culture. One ml of buttermilk is added per 100 ml of milk, and the mixture is then fermented at room temperature for 4-12 hours. At the end of fermentation, the temperature of the mixture is raised to 32°C, and artificial coloring is added to the mixture prior to curdling.

The third way to prepare the milk in a short time frame is to add acid (HCl) and to heat to 32°C. Of course, there leaves much to be desired in this method if you are a cheese connoisseur.

After rennet is added to the pre-cured milk, the coagulation process is started. In cheese making, as coagulation comes to completion, the temperature is gradually raised to about 38°C. This slightly elevated temperature facilitates the separation of the curd from the whey. A higher temperature also hardens the curd. The curd may be hardened further by cooking it for a longer period of time, either with or

without the whey.

After the curd is separated from the whey, salt, seasoning, and other curing and flavoring ingredients are added. The curd is wrapped in cheese cloth and pressed for 12 to 18 hours to remove the additional whey soaked in the curd. The curd hardens and forms a cheese block in the shape of the press as the whey is squeezed out. Finally, the cheese block is dried for 6 hours.

It is now ready for consumption, or it may be left to age in a controlled cool environment (2-13°C). Although a higher temperature promotes faster curing, there is also a higher chance of spoilage due to undesirable microbial activities at elevated temperatures. Prior to aging, the cheese block is usually wrapped tightly to exclude air and microbial contaminants from entering and spoiling the cheese. One way to accomplish this is to dip the cheese block in a pot of melted wax. During the aging process, many complicated microbial and chemical actions continue to take place in the cheese block. Thousands of techniques exist to develop various distinctive flavors. These reactions are not well characterized; thus, cheese making is still an art rather than a science. Depending on the technique employed, this final aging process takes any where from 2 weeks to 6 months.

Precuring at a Warm Temperature (Acidify the Milk)
⇓
Coloring
⇓
Coagulation by Rennet
⇓
Separation of the Whey
⇓
Flavor addition
⇓
Compression of the Curd
⇓
Aging
⇓
Finished Cheese

Figure 1. Steps in cheese making.

List of Reagents and Instruments

A. Equipment

2 beakers, 400 ml

Graduated cylinder

Pipet or medicine dropper

Temperature bath or heat source

Thermometer

Stirring rod

Cheese cloth

Balance

B. Reagents

Pasteurized milk, (to be supplied by the student)

Butter milk or lactic acid producing strains, (to be supplied by the student)

Rennet enzyme

Procedures

1. The T.A. will prepare several batches of cultured milk before the class by mixing 2.5ml of buttermilk (starter culture) to 250ml of normal pasteurized milk in a beaker. The content in the first beaker is to be fermented at room temperature for 12 hours (G); the second for 10 hours (F); the third for 8 hours (E); etc. Do not add buttermilk to the batch labelled A, which is kept free of a starter culture. The number of mixtures with differing pre-curing times depends on the number of students enrolled. Each student will be responsible for analyzing one of these beakers.

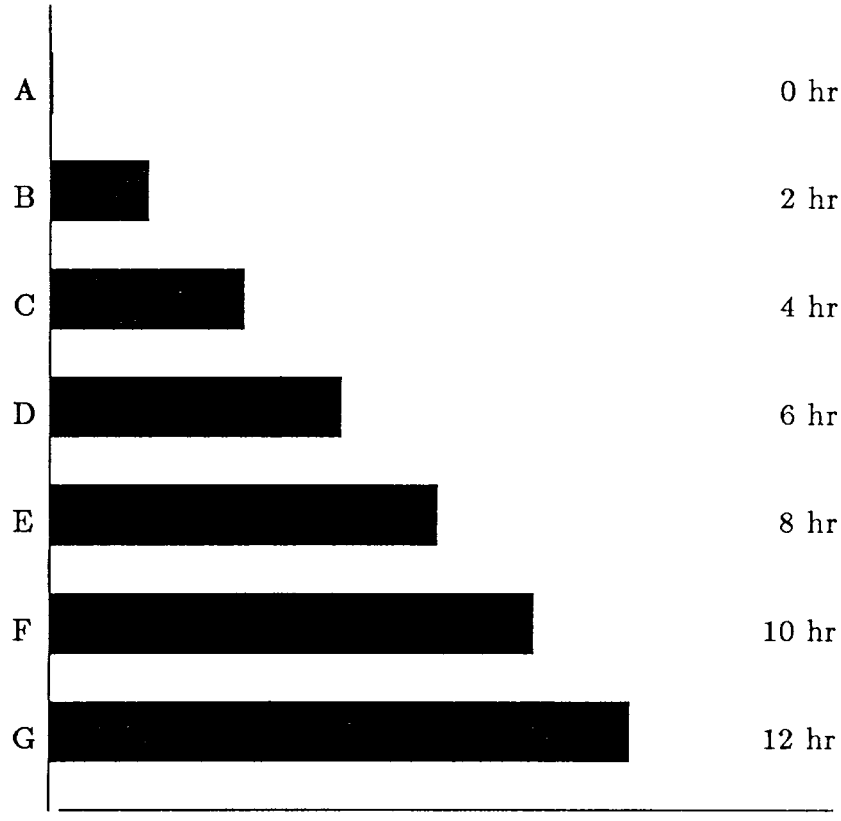

Figure 2. Different beakers contain mixtures incubated for different periods of time.

2. Analogous to Step 1., prepare several synthetic "cultured" milk by adding 0-1 ml of 1N HCl to 250 ml of milk in a beaker. Each student will be responsible

for one of these beakers.

3. Record the pH of each batch.
4. Gently heat the authentically and synthetically cultured milk to 32°C in a temperature bath.
5. Stir and add 0.5 ml (approx. 5 drops) of rennet to each beaker.
6. After the enzyme addition, let the milk stand on the bench top for 30 minutes to allow coagulation to proceed. Do not disturb the coagulation process in the beakers; otherwise, the curds will be broken into pieces too small to filter efficiently with cheese cloth.
7. After curd formation, break up the curds with a stirring rod and drain the whey through layers of cheese cloth. Filtration through a medium pored filter paper may give better results.
8. Weigh the amount of curd obtained in its wet state and approximate the yield.
9. Taste your cheese. (Yum-m-my, I ♡ cheese!) Is there anything missing?

Discussion

It is generally quite straight forward to locate the sources of enzymes; the natural sources of enzymes are where nature needs them. It comes as no surprise that rennet had traditionally been isolated from the fourth stomach of young calves because digestion by suckling calves is nature's primary way of processing cow milk. However, the ways of nature are not always the most economical from man's perspective when a process is adapted to different uses to benefit mankind in ways not originally intended by the nature. Thus enters the study of biochemical engineering.

Chemical engineering is the study of how to make a large quantity of chemicals in an economical fashion. For example, frequently a chemical engineer must devise a process to mass-produce a polymer that is totally different from the one

originally used by an organic chemist working with small test tubes and beakers in a laboratory. Biochemical engineering, being a sub-field of chemical engineering, also deals with the same kinds of problems facing chemical engineering, except that the chemicals are not synthetic (manmade) but biological (naturally existing) in nature. Although rennet is naturally excreted from a calf's stomach lining, extracting it from its natural source is not economical. Other proteases can also convert casein to para-casein, but their action does not stop there. They further degrade the curd to soluble subunits. Fortunately, large quantities of rennet of consistent quality can now be produced easier and cheaper in a well controlled environment by microbial fermentation.

A word of caution is in order here. Enzymes as a class of chemicals are not generally considered as dangerous, toxic, nor poisonous; they do not cause skin irritations or burns as acids or bases. Some exceptions are proteases that catalyze the breakdown of protein molecules to amino acids components. Because meat is mainly protein, protease can digest the soft moist sections of the skin. We can all imagine how that is going to feel. Thus, you should exercise the same caution with enzymes as you do with any other chemicals.

Questions

1. Report your observation of this experiment and discuss the effect of pH on the yield of the curd. Try out other types of processed milk such as nonfat milk and powdered milk. Are they suited for cheese making?
2. Taste the cheese you have made. How does it taste compared to cottage cheese? (The product should be edible if the glassware is clean, which is exactly what it should be.)
3. In Step 1, why is buttermilk not added to the batch labelled "A"?
4. Because its action has long been discovered, rennet does not follow the rules

of enzyme nomenclature discussed in class. Its conventional name has already been deep-rooted in the craft of cheese making. Considering the action of rennet, how would you rename the enzyme to conform to the general rules of nomenclature?

5. What microorganisms are currently used to manufacture rennet? How is rennet isolated?

6. Why is the coloring added before coagulation instead of afterwards?

7. Give a list of the chemical components found in milk. In this experiment, you used an enzyme to react selectively with a protein called casein; what other carbohydrates, proteins, and fats are found in milk? List the nutritional information for some popular types of milk and break down each category into individual chemical components. (Example: Nonfat milk has x g/liter of carbohydrates, y% of which is lactose, z% of which is sucrose, etc.)

8. What are some of the products other than cheese that are derived mainly from milk? Identify the principle component of milk utilized in each product and point out the use of enzymes if applicable. Choose a specific dairy product and give a description of the processes involved in the production.

9. Milk spoilage can be detected visually by the presence of coagulated curd. Identify the chemical mechanisms responsible for coagulation in this case. Are these chemicals/enzymes produced by contaminant microbial actions? Is the curd resulting from spoilage the same as that you have obtained in class? If they are the same, why would you consume cheese but not spoiled milk?

10. Comment on ways to improve the experiment.

Reference Readings

1. Prins, J., Microbial rennet, *Process Biochem.*, May, 1970.

2. Robinson, R.K., *Dairy Microbiology*, Vol. 2, Applied Science Publishers, New Jersey, 1981.

3. Richmond, H.D., *Dairy Chemistry, A Practical Handbook*, Charles Griffin & Co., London, 1930.

4. Constituents of milk: Jenness, R. and Patton, S., *Principles of Dairy Chemistry*, John Wiley, 1959, p3.

EXPERIMENT NO. 2

ENZYMES IN LAUNDRY DETERGENTS

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ENCH 485, Spring 1988

Objective

To hydrolyze protein-based stains in fabrics into soluble amino acids.

Introduction

In today's laundry detergents, enzymes such as proteases and amylases are some of the active ingredients. In the U.S., about 50% of liquid detergents, 25% of powder detergents, and almost all powdered bleach additives now contain enzymes to help break down stains that are otherwise hard to remove with conventional surfactants alone. For example, amylase catalyzes the breakdown of starch-based stains to smaller segments that make up the larger starch molecule. Oligosaccharides and dextrins released from the enzyme's hydrolytic action are soluble; thus, the stain is physically cut off from the surface of the fabric piece by piece, with the enzyme acting as scissors. The action of proteases, as implied by the name itself, is similar to that of amylase, except that a large protein molecule is hydrolyzed. During the process of hydrolysis, the peptide bonds that hold various amino acids together to form a protein molecule are broken down, releasing smaller polypeptides and individual amino acid units. Generally, polymers made of less than approximately one hundred amino acid monomer units are called polypeptides and larger ones are called proteins.

In this experiment, the same milk protein casein that we have experienced in the previous experiment on cheese making is bound with a blue dye. A small piece of fabric soiled by the dyed casein will be provided to each student. The purpose of this experiment is to observe the hydrolytic action of bacterial protease in removing protein-based stains. Because detergents, especially bath soaps, are generally formulated to degrade mainly oil and grease, protein-based stains have traditionally been among the hardest to remove. Proteins can act as strong natural bonding agents that make all sorts of dirt adhere stubbornly to textile fibers. Anyone trying to wash away blood stains can testify to this effect. Other proteinaceous dirt includes perspiration, grass, and slime stains. This exercise demonstrates that it takes protein to get out protein, as some television commercials claim.

List of Reagents and Instruments

A. Equipment

Flasks, 250 ml

Graduated cylinder

Household clothing iron or drying oven

Spectrophotometer

Balance

B. Reagents

Household detergent, (to be supplied by the student)

Bacterial protease

Dyed casein cloth

Procedures

1. Dissolve 1 g of household laundry detergent in 200 ml of hot (60°C) water in a 250 ml flask. Colored detergent obviously interferes with the quantitative

determination of the rate of protein degradation by the colorimetric method; thus, it should not be used if quantitative information is desired. Because the commercial detergent is not completely soluble in water, filter the detergent solution before use.

2. Similarly, mix 0.1 g of powder protease in 200 ml of hot water in a second flask. Remove the insoluble solids by filtration or centrifugation.

3. Place a small piece of the dyed casein or gelatin cloth into each washing liquid.

4. Seal the top of each flask with paraffin or with a rubber stopper. Swirl both flasks gently to simulate the agitating washing motion. A thermostated flask shaker may be used for this purpose.

5. Periodically take out a small (5-10 ml) portion of the washing liquid and measure the absorbance with a spectrophotometer until the changes in the color intensity level off. The stain should be removed in approximately 15-20 minutes. Because of the small particles of protein released during the wash, one may need to filter the sample again with a 25mm syringe filtration unit fitted with a 10 ml plastic syringe. This should be done immediately before measuring the absorbance.

6. After briefly rinsing the cloth under running tap water, dry it with a clothing iron.

7. Compare the effectiveness of the protease with your detergent. Also compare the cloth washed with your detergent with the other students' to see how effective your detergent is in cutting the stain.

8. Simulate a "warm" (40°C) and a "cold" (20°C) wash cycle by repeating the above experiment at appropriate temperatures. (Note that all the temperatures can be run simultaneously.)

Discussion

An enzyme was first used to improve the effectiveness of a laundry detergent in 1913 by a German named Otto Röhm, the founder of the giant chemical company Rohm and Hass. The proteolytic enzyme he used, derived from milled animal pancreases, was quite crude and contained many impurities which, in turn, sometimes stained the very textile it was supposed to clean. Neither was the process of enzyme extraction economical enough to include it routinely in household detergents. Currently, these enzymes are manufactured commercially in large quantities through fermentation by common soil bacteria *Bacillus subtilis* or *Bacillus licheniformis.* This was made possible in the last two decades by the rapid advances in enzymology and fermentation technology. Although numerous other microorganisms produce proteases and amylases, the types secreted by the above strains have the advantage that they work best at the warm alkaline conditions prevailing in washing liquids. They also must not lose their activity in an environment which contains a multitude of potentially inhibitory chemicals routinely formulated into laundry detergents such as surface active agents, magnesium or calcium ions, builders (sodium tripolyphosphate), perfumes, and other additives.

Questions

1. Plot the color intensity as a function of time for both washing liquids. Which one has stronger cleaning power, your detergent or protease? Explain the temperature effect.
2. From the above curve, comment on the possible rate expression for the enzymatic reaction.
3. How would you determine the amount of protein removed if no dye was used?
4. How does the temperature affect the rate of stain removal?

5. List some of the biological functions of the pancreas. What type of digestive fluid does it secrete? Is it surprising that one may obtain protease from milled dehydrated and defatted animal pancreas?

6. List ways in which enzymes can be utilized to break down toxic wastes, thus controlling pollution. Are there any commercial processes at the present that utilize the unique selective catalytic capability of an enzyme in waste treatment?

7. There is a great concern over the phosphate content in a detergent because of the problems of eutrophication, the almost complete depletion of dissolved oxygen in a body of water resulting from the explosive growth of marine microorganisms in the presence of excess nutrient. Do you consider the enzymes contained in the laundry liquid as water pollutants? What about the possibility of the denatured protein as a source of scarce amino acids? Justify your answer.

8. All enzymes are proteins, and protease is no exception. Does protease attack each other canabalistiaclly? If not, what prevents protease from digesting each other? (Hint: does protease digest all proteins, or can it recognize only some and exerts its action on only specific ones?)

9. Comment on ways to improve the experiment.

Reference Readings

1. Duffy, J.I., *Chemicals by Enzymatic and Microbial Processes,* Noyes Data Corp., New Jersey, 1980, p368-373.

EXPERIMENT NO. 3

DIGESTION OF PROTEIN INTO AMINO ACID

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ENCH 485, Spring 1988

Objective

To analyze amino acid concentrations by the ninhydrin colorimetric method during the enzymatic hydrolysis of a protein.

Introduction

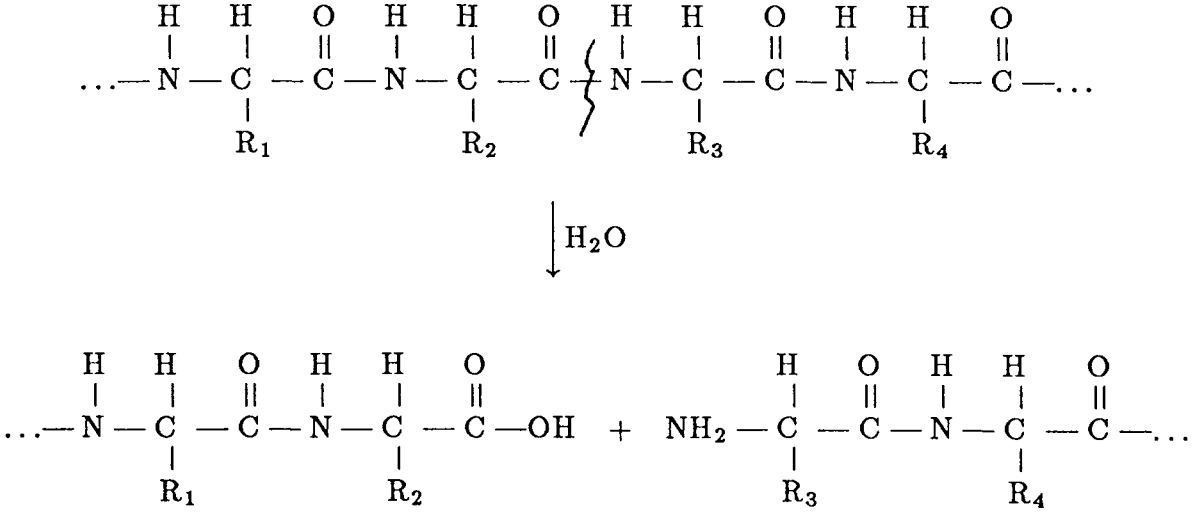

Figure 1. The breakdown of protein through the hydrolysis of peptide bonds

Various species of organisms cannot synthesize or are not efficient in generating all of the twenty amino acids needed to construct the proteins and enzymes essential for their survival. To sustain growth and to maintain metabolic functions, these

amino acids must be provided from outside sources. This can be accomplished by the intake of proteins. Humans are a good example of living organisms that ingest proteins as part of their nutritional requirements. However, protein molecules are generally quite large, and these large molecules cannot be transported across cell membranes for the same reason that you cannot bring an elephant through the door into your dorm room. Some organisms secrete proteolytic enzymes extracellular to break down the protein to its component monomeric amino acid units by hydrolyzing peptide bonds at the end of the polymer chain. A series of shorter polypeptides of different lengths are also formed if the broken peptide bonds are not at the end of the polymer chain. Thus, depending on the location of the attack, proteases can be further classified into exopeptidases (attack on the terminal group) and endopeptidases (attack on internal linkages).

In the previous experiment, the hydrolysis of a protein was monitored with the release of a dye that was bound to the protein. In this experiment, another more accurate and generally accepted color method is introduced. In this method, an organic compound called ninhydrin is reacted with the amino acids released during the hydrolysis of the protein. The original unreacted ninhydrin is yellowish in color, but the reacted product of ninhydrin has a deep purple-blue color. For example, the procedure given at the end of this section yields an absorbance of 0.27 for $1. \times 10^{-4}$M of glutamic acid. Since ninhydrin does not react with the undegraded protein, one can measure the amino acid concentration by following the development of the purple color by measuring the absorbance of the solution with a spectrophotometer. Because the color intensity is a measure of the amino acid present, the color should intensify as more protein is degraded to amino acid over time. The upper limit in color intensity is reached when all the ninhydrin originally present in the solution has been consumed. Thus, the amount of ninhydrin originally present in the reaction mixture determines the maximum amino acid concentration

that can be detected.

List of Reagents and Instruments

A. Equipment

2 beakers, 100 ml

Beaker, 400 ml

Graduated cylinder

Pipets, 1ml, 10ml

Temperature bath (or heat source – Bunsen burner or hot plate)

Thermometer

Stirring rod

Funnel and filter paper (or centrifuge)

Test tubes

Balance

Blender

Spectrophotometer

B. Reagents

Amino acid standard (for calibrating spectrophotometer)

Protein source (cottage cheese or curds from Experiment No. 1)

Protease

Ninhydrin solution

Procedures

1. Make a 10g/liter protein mixture by dissolving casein (Sigma) in water. (See Notes 1. and 3.)

2. Make a saturated protease solution by adding 0.5 g of powder protease to 1

liter of water. Spin the solution in a centrifuge to separate the undissolved powder. Keep the supernatant. (See Note 2.)

3. Mix equal volumes of protein solution and protease solution obtained in Step 1 and Step 2. Note the time at the start of the hydrolysis reaction.
4. Withdraw 5 ml of the solution and measure the amino acid concentration of the solution as a function of time by using the ninhydrin colorimetric method. Suggested sampling time interval: 10 minutes for at least one hour.
5. If time permits, repeat the same procedures for hair.

Notes

1. Gelatin and albumin may be used in lieu of casein. The hydrolysis rate for different substrates may be studied.
2. Alternatively, add 0.05 g protease directly to 50 ml of the protein mixture. However, one must perform the ninhydrin test on the supernatant obtained either through centrifugation or filtration for each sample.
3. If reagent grade casein is not available, a 10g/l mixture can be made by the following steps:
    a Add 1 g of protein (cheese from the previous experiment or from a to 70 ml of water; using a blender, "liquefy" the mixture; pour into a 100 ml graduated cylinder or a 100 ml volumetric flask.
    b. Rinse the blender with about 20 ml of water from a squeezable plastic bottle; pour the rinse into the measurement device in Step 3a; add water to 100 ml.

Discussion

This experiment will also introduce students to the fundamentals of quantita-

tive assays. First, when a spectrophotometer is used to quantitatively measure the absorbance of a colored solution, an absorbance spectrum should be obtained to determine the best wavelength to use. Thereafter, the same wavelength is used for all the subsequent determinations. Secondly, when there is a gradual development of color due to the limited reaction rate of the color reaction, the time needed for the completion of the reaction should be determined from the plot of absorbance versus time. Obviously, this time is when the change in the absorbance as a function of time is no longer significant. Thirdly, a calibration curve must be obtained by subjecting standard solutions of known concentrations to the same procedure so that the absorbance measurement can be correlated to actual physical units, in this case, the amino acid concentration. Finally, one should always remember to compare his sample to some reference, in this case, the reference being the protein mixture with no protease added. To obtain a blank reading, add ninhydrin reagent to the protein solution in the absence of protease and find the absorbance from the ninhydrin reaction. Protein solution in the absence of protease may not always give absolutely negative results due to other contaminants present in the solution, or the protein itself may contribute to the development of purple color to a certain degree.

Questions

1. Report the absorption spectrum of the ninhydrin – amino acid mixture. Which wavelength did you choose to measure the amino acid concentration? Why?
2. How long was needed to develop the color fully? Justify your answer with experimental data. (It should be 4-7 minutes.)
3. Report your findings on protein digestion as a function of time. Explain what you have observed. Also calculate the enzyme activity and the rate of hydrolysis.
4. What amino acids can human cells manufacture? What amino acids must be

provided from outside sources? Can a well balanced amino acid diet replace the human intake of all the proteins?

5. What are the amino acid contents of a typical protein? Express your answer in weight percent and in relative number of occurrences.

6. From suitable references, find how to determine experimentally the amino acid sequence of a protein? (Hint: one of the most widely used and the simplest is Sanger's method. Since its introduction, many modified and improved versions are available today. Edman's is one such method.)

7. Assuming that all the peptide bonds are equally susceptible to protease attacks and given that the starting protein has a well defined number of amino acid sub-units, say 1000, find the distribution of chain length as a function of reaction time. What is the free amino acid concentration as a function of time. Assume a 0th order reaction mechanism, say 100 bonds per minute per unit of enzyme. (Consult references on probability theories if you have difficulties in answering this question.)

8. Repeat the above calculation for a system in which the starting protein has a given size (chain length) distribution, say a normal (Gaussian) distribution with an average of 1000 and a standard deviation of 100. Quantitatively how would your answer be affected if the bonds near the end of the chain were more susceptible to enzyme attacks than those away from the end? For this last part, assume an enzyme activity of 100 bonds per minute per unit of enzyme for the last bond, exponentially decreasing to half of that original value at 30th bonds away from the end. In the following figure, each AA represents an amino acid monomer unit.

9. Do proteolytic enzymes attack themselves, given that the enzyme themselves are protein? If no, what make them special? If yes, what can be done to

nth bond	1	2	3	4	...	26	27	28	29	30		
	AA —	AA —	AA —	AA —	...	— AA —	AA —	AA —	AA —	AA —	AA —	AA —
enzyme activity	100					50						

prolong their activities?

10. Comment on ways to improve the experiment.

Reference Readings

1. James E. Bailey and David F. Ollis, *Biochemical Engineering Fundamentals*, 2nd Ed., p172-174, McGraw-Hill, 1986.

2. Albert L. Lehninger, *Biochemistry*, p99, Worth Pub., 1975.

EXPERIMENT NO. 3 – SUPPLEMENT A

AMINO ACID ASSAY
BY NINHYDRIN COLORIMETRIC METHOD

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ENCH 485, Spring 1988

Method

The reaction between α-amino acid and ninhydrin involved in the development of color are described by the following five mechanistic steps:

(1) $\alpha-$amino acid + ninhydrin $\longrightarrow$ reduced ninhydrin + $\alpha-$imino acid + H_2O

(2) $\alpha-$imino acid + $H_2O \longrightarrow \alpha-$keto acid + NH_3

(3) $\alpha-$keto acid + $NH_3 \longrightarrow$ aldehyde + CO_2

(4)

reduced ninhydrin + residual ninhydrin + $NH_3 \longrightarrow$ intermediate complex + $3H_2O$

(5) intermediate complex + $NH_3 \longrightarrow$ final complex(BLUE)

Step (1) is an oxidative deamination reaction that removes two hydrogen from the α-amino acid to yield an α-imino acid. Simultaneously, the original ninhydrin is reduced and loses an oxygen atom with the formation of a water molecule. In

Step (2), the NH group in the α-imino acid is rapidly hydrolyzed to form an α-keto acid with the production of an ammonia molecule. This α-keto acid further undergoes decarboxylation reaction of Step (3) under a heated condition to form an aldehyde that has one less carbon atom than the original amino acid. A carbon dioxide molecule is produced here. These first three steps produce the reduced ninhydrin and ammonia that are required for the production of color in the last two Steps (4) and (5). The overall reaction for the above reactions is simply (slightly inaccurately) expressed in Reaction (6) as follows:

(6)

$$\alpha\text{–amino acid} + 2\,\textit{ninhydrin} \longrightarrow CO_2 + \text{aldehyde} + \text{final complex(BLUE)} + 3H_2O$$

In summary, ninhydrin, which is originally yellow, reacts with amino acid and turns deep purple. It is this purple color that is detected in this method.

Ninhydrin will react with a free α amino group, NH_2-C-COOH. This group is contained in all amino acids, peptides, or proteins. Whereas, the decarboxylation reaction will proceed for a free amino acid, it will not happen for peptides and proteins. Thus, theoretically only amino acids will lead to the color development. However, one should always check out the possible interference from peptides and proteins by performing blank tests especially when such solutions are readily available. For example, one can simply add the ninhydrin reagent to a solution of only proteins and see if there is any color development. There is no excuse for failing to perform such a vital test when the sample mixture contains both proteins and amino acids. There are also reports that chemical compounds other than amino acids also yield positive results.

This test can be used routinely for the detection of glycine in the absence of other interfering species. Although this is a fast and sensitive test for the presence of α-amino acids, because of the nonselectivity, it cannot be used to analyze the

relative individual contents of a mixture of different amino acids. Furthermore, the color intensity developed is dependent on the type of amino acid. Finally, it does not react with tertiary or aromatic amines.

Note that since ninhydrin is a strong oxidizing agent, proper caution should be exercised in handling this compound. It is especially potent at the elevated temperature under which the reaction is carried out. The ninhydrin reagent will stain the skin blue and cannot be immediately washed off completely if it comes in contact with the skin. However, as in any other stain on the skin, the color will gradually rub off after about a day.

A. Equipment

Test tubes

Pipets

Spectrophotometer

B. Reagents

Ninhydrin Reagent Solution

Ninhydrin: 0.35 g

Add ethanol to: 100 ml (See Note 1.)

Procedures

1. Add 1 ml of the ninhydrin solution to 5 ml of sample. Cover the test tube with a piece of paraffin film to avoid the loss of solvent due to evaporation. A capped test tube can also be used instead.

2. With gentle stirring, react at 80-100°C for 4-7 minutes. (How would one find out the amount of time needed to ensure a complete reaction?) If a large heated water bath is used for the entire class and if there is no good provision

for holding the test tube in the hot water bath, the test tube may be held with a piece of wire and hang on the side of the water container. A clamp usually does not work too well.

3. After cooling to room temperature in a cold water bath, record the absorbance with a spectrophotometer. (How would you find out the best wavelength to use for this purpose? Hint: the final purple colored complex of ninhydrin absorbs the most amount of light at the wavelength of 570 nm.)

Notes

1. Iso-propanol or a 1:1 mixture of acetone/butanol may be used in lieu of ethanol in preparing the ninhydrin reagent.

Questions

1. Why was the sample cooled to room temperature before measuring the absorbance?
2. What properties of the ninhydrin-amino acid reaction other than color can be used to detect the presence of amino acids?
3. List and describe briefly a representative method used to measure a *specific* amino acid in a mixture. Choose three amino acids and find a selective analytical method for each of the three.

Reference Readings

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EXPERIMENT NO. 4

CELLULOSE DEGRADATION

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ENCH 485, Spring 1988

Objective

To compare the enzymatic and acid hydrolysis of cellulose.

Introduction

Currently, there are two major ways of converting cellulose to glucose: chemical versus enzymatic. The research on both methods has for decades occupied the attention of many investigators world wide. Because each cellulose molecule is an unbranched polymer of 1000 to 1 million D-glucose units, linked together with β-1,4 glycosidic bonds, cellulose from various sources are all the same at the molecular level. However, they differ in the crystalline structures and bindings by other biochemicals. It is this difference that make possible a persistent research on cellulose. The model chemical compounds most commonly used in today's research are carboxymethyl cellulose (CMC), which has a generally amorphous structure, and Avicel, which has a highly crystalline structure. In this experiment, cellulose from a variety of sources will be subjected to depolymerization conditions.

There are two types of hydrogen bonds in cellulose molecules: those that form between the C_3 OH group and the oxygen in the pyranose ring within the same molecule and those that form between the C_6 OH group of one molecule and the

oxygen of the glucosidic bond of another molecule. Ordinarily, the β-1,4 glycosidic bonds themselves are not too difficult to break. However, because of these hydrogen bonds, cellulose can form very tightly packed crystallites. These crystals are sometimes so tight that neither water nor enzyme can penetrate them; only *exogluconase*, a subgroup of cellulase that attacks the terminal glucosidic bond, is effective in degrading it. The inability of water to penetrate cellulose also explains why crystalline cellulose is insoluble. On the other hand, amorphous cellulose allows the penetration of *endogluconase*, another subgroup of cellulase that catalyzes the hydrolysis of internal bonds. The natural consequence of this difference in the crystalline structure is that the hydrolysis rate is much faster for amorphous cellulose than crystalline cellulose. The process of breaking the glucosidic bonds that hold the glucose basic units together to form a large cellulose molecule is called *hydrolysis* because a water molecule must be supplied to render each broken bond inactive. In addition to crystallinity, the chemical compounds surrounding the cellulose in plants, e.g. lignin, also limit the diffusion of the enzyme into the reaction sites and play an important role in determining the rate of hydrolysis. Sometimes, wood chips are pretreated with acid at approximately 160°C to strip hemicellulose and lignin before they are In general, 20 to 70 % yield of glucose can be expected after 24 hours.

The conversion of cellulose into glucose is now known to consist of two steps in the enzyme system of *Trichoderma viride*. In the first step, β-1,4 glucanase breaks the glucosidic linkage to *cellobiose*, which is a glucose dimer with a β-1,4 bond as opposed to maltose, a counterpart with an α-1,4 bond. Subsequently, this β-1,4 glucosidic linkage is broken by β-glucosidase:

$$\text{Cellulose} \xrightarrow{\beta\text{-1,4 glucanase}} \text{Cellobiose} \xrightarrow{\beta\text{-glucosidase}} \text{Glucose}$$

The kinetics of cellulose hydrolysis has been widely studied, and Michaelis-Menten

types of rate expressions with substrate or product inhibition terms have been proposed to describe the observed reaction kinetics.

A wide variety of fungal and bacterial species produce cellulase and transport the enzyme across the cell membrane to the outside environment. Although it is common to refer to a mixture of compounds that can degrade cellulose as cellulase, it is really composed of more than one distinctive enzymes. Recent research has shown that one of the components is relatively inert with the ability of recognizing and attaching itself to the surface of the cellulose mass, in addition to the ability of recognizing and holding onto another protein component that exhibits enzymatic activities. Thus, the chance of reaction is significantly enhanced by a proximity effect, because the active enzyme is held onto the surface of a solid substrate by an inert protein which acts as a glue.

The species most often used to study the production of cellulase are white-rot fungal cultures of *Trichoderma ressei* and *Trichoderma viride.* We all have seen a piece of rotting wood. And perhaps without knowing it, we are actually quite accustomed to the appearance and action of this fungi. As in Experiment No. 1, it is only natural that the most promising place to search for cellulase is in a piece of rotting wood. The microorganisms responsible for this enzyme can easily be isolated from a piece of rotting wood, or from a termite's gut if bacterial species are desired. Other fungal species often used are *Fusarium solani*, *Aspergillus niger*, *Penicillium funicolsum,* and *Cellulomonas* sp. The bacterial species *Clostridium thermocellum* and *Clostridium thermosaccharolyticum* also represent promising candidates for cellulase production because they are thermophilic (less contamination problem and faster rate at a high temperature), anaerobic (no oxygen transfer limitation), and ethanologenic (conversion of cellulose to ethanol via glucose with a single culture). In general, different species of microorganisms produce different

cellulolytic enzymes.

List of Reagents and Instruments

A. Equipment

Erlenmeyer flasks

Graduated cylinder

Pipets, 1ml, 10ml

Test tubes

Incubator or thermostated shaker

Temperature bath (or heat source – Bunsen burner or hot plate)

Thermometer

Balance

Syringe

Filter holder

Filter paper

Spectrophotometer

B. Reagents

Cellulose source (filter paper, wood chips, carboxymethyl cellulose, cotton)

Cellulase, buffered at pH=5.00±0.01, 10g/l solution

HCl, 5% solution

H_2SO_4, 5% solution

KOH

Reagents for sugar analysis

Procedures

1. *Enzymatic Hydrolysis:* Repeat the same procedures for shredded wood chips (a complex and impure mixture of cellulose, lignin, and a variety of others),

carboxymethyl cellulose (a model amorphours-structured cellulose), and cotton (90 % cellulose, mostly crystalline-structured). If time permits and if there is extra enzyme solution, try other sources of biomass and waste materials such as newsprint, grass, straw, and corn stalk. See Note 1.

a. Shred a 10 cm^2 piece of cellulose filter paper and weigh 0.1 g. (As opposed to other type of papers with binding materials, a piece of cellulose filter paper without wetting agents has minimum impurities and is almost pure in cellulose. The result of a quantitative analysis using a filter paper would have been very unreliable had impurities leached out into the filtrate.

b. Submerge the shredded paper in 10 ml of the buffered cellulase solution in a test tube. Note the starting time.

c. Incubate the mixture at 40°C. (The enzyme is most active at a temperature of 40°C and a pH of approx. 4.5.)

d. This reaction should last for approximately 24 hours. Take 1 ml samples at some predetermined appropriate intervals. Note that one does not have much to waste because the starting sample is small. (A volume of 1 ml is actually considered as a huge sample when working with biochemicals.)

e. Stop the hydrolysis reaction in the sample. The first method of stopping the reaction is to deprive the mixture of substrate. This can be easily achieved by filtering out the residual solid material from the solution. The individual samples may be stored frozen for later analysis. The samples are thawed and brought to room temperature before they are subjected to measurements. However, this first method is not applicable to soluble cellulose, e.g., CMC. Alternatively, the enzymatically catalyzed reactions can be halted either by adding a strong enzyme inhibitor or by raising the temperature of the mixture to 90°C for 5-10 minutes in a heated bath to

inactivate the enzyme.

f. Measure the glucose concentrations of the samples with the dinitrosalicylate colorimetric method. (Reference: Gail Lorenz Miller, Use of dinitrosalicylic acid reagent for determination of reducing sugar, *Analytical Chemistry*, **31**, 427, 1959.) See Note 2.

2. *Acid Hydrolysis (Sulfuric Acid):* Use the same cellulose sources as in enzymatic hydrolysis.

   a. Add 0.2g cellulose to 10ml of 5% H_2SO_4 solution in a lightly capped test tube. See Note 3. One may choose to carry out the reaction at 90°C instead of at room temperature.

   b. This reaction should last for 2 hours. Take 1 ml samples at some predetermined appropriate intervals.

   c. Stop the hydrolysis reaction in the sample by neutralizing the acid and slightly reversing the pH with the addition of a small volume of a concentrated potassium hydroxide solution. Make a quick calculation to see how much KOH is needed for this purpose. Note that one has to keep a close track of the volume of KOH solution added because this information will be needed to calculate the glucose concentration in the original undiluted sample.

   d. Measure the glucose concentration of the alkaline sample.

3. *Acid Hydrolysis (Hydrochloric Acid):* Substitute 5% sulfuric acid with 5% hydrochloric acid and repeat the same procedures as in sulfuric acid.

Discussion

The most abundant organic compound on earth is cellulose, which provides the primary structural component for plants. (Chitin, present in insects, crustacean,

and bones, is the second most abundant organic compound.) Like starch, cellulose is a polymer of glucose monomer units, linked together at the β-1,4 locations as opposed to the α-1,4 locations for amylose (insoluble starch). Enzymes are generally extremely specific in their catalytic actions. They can recognize even the subtlest difference in the substrate structure and often exhibit no measurable catalytic behavior toward other similarly structured substrates. The difference in the glucose linkage between starch and cellulose makes it impossible for the starch digesting enzymes, e.g. α-amylase, to break down cellulose. The direct consequence of this specificity is that various organisms, including humans, cannot use cellulose to satisfy their nutritional requirement for carbohydrates. However, some animals and insects, such as cattle, sheep, horses, termites, and caterpillars, can subsist on wood and grass, although they themselves do not produce cellulolytic enzymes. This is due to the synergistic effect of the bacteria present in their digestive tracts. These gut bacteria flora secret the necessary cellulolytic enzymes to digest cellulose, and the hosts, in turn, provide them with a shelter as well as nutrient. The inability of most organisms in attacking cellulose is not necessarily undesirable. For example, wood, which is mostly cellulose bound together by lignin, has traditionally been used as building materials due to its relatively stable microscopic structures. Wouldn't it be terrible if your home could be digested by bugs too easily? Perhaps, that is why no one uses bread (starch material) or candy (easily digestible saccharides) to build a durable house except in fairy tales.

There has been a large amount of research work done on the digestion of cellulose into glucose. The generated glucose can be used to produce single cell protein as food for livestock or even for humans. Glucose can also be used as the starting raw material in the production of a wide variety of chemicals and fuels. This is usually carried out with the help of microorganisms. For example, glucose can be easily fermented to ethanol by *Saccharomyces cerevisiae* (yeast) or *Pseudomonas*

mobilis (bacterium). Ethanol can be used as gasoline or processed further to make other common petrochemicals. Another example is the conversion of glucose into solvents such as acetone and butanol by *Clostridium acetobutylicum.* Because the volume of cellulose is so overwhelming and because the resource is renewable, the world will likely to depend on it more heavily for food, fuel, chemical supplies, and raw materials in the future. It has the great potential of alleviating the need for petroleum, whose supply is fast dwindling.

Thus, the ability to manipulate this organic chemical has extremely important implications. A breakthrough in the investigation of cellulose digestion processes will not only have an enormous impact on the world food supply, economy, and geopolitical balance of power, it will also greatly influence the various types and ways products are produced by the chemical industry and enjoyed by the end users. This experiment introduces a student in biochemical engineering to one of tomorrow's technologies with the most far-reaching impacts.

As demonstrated in this experiment, the breaking down some of the cellulose is really not very difficult. However, translating a process from a laboratory scale to a commercial scale is not so trivial. First of all, the entire operation has to be *both* technically sound and economically feasible. In order for a process to be actually adapted, it, of course, has to be technically possible first. In addition, it must offer some clear advantage over all other competing processes. This advantage is almost always measured in the form of a larger profit margin, irrespective of the political system in which the process is to be employed. Note that in calculating the profit, one must duly include various costs that are sometimes not obvious nor easy to estimate, e.g. the public images, institutional responsibilities, and environmental impacts. Unprofitable processes are a waste of natural and human resources and must not survive. As a chemical engineers, whether conducting basic research or

designing a plant, one is continually reminded of the economical impact.

Two typical approaches to effect a similar end result are studied in this experiment. However, one should keep in mind that there are numerous other competing approaches, and one is constantly faced with multiple choices. For example, acetic acid can be produced by fermentation means or chemical synthesis. So are a wide range of pharmaceuticals. As a matter of fact, life is rarely simple and straight forward enough that there is only one choice.

Notes

1. As in any other experiments, remember to include simultaneously a control experiment or a blank solution. An enzyme solution without any solid substrate can be used as one of the controls which will yield the level of glucose entrainment, if any, originally present in the enzyme preparation. This is especially important when one is unsure of the content of a complex solution. Furthermore, the result of enzymatic actions should be compared to another control experiment in which only water is added to the solid substrate. This second control will give the background leaching out of glucose from the substrate, if there is any at all. One should always guard against these possibilities.

2. One needs to outline how to measure the glucose concentration with the dinitrosalicylate colorimetric method. The extent of disclosure of the procedures associated with a particular analytical method in science communication is commonly comparable to, if not less than, what is said in this manual. It is critical that the student learns how to read scientific literature and obtain the necessary information from it.

3. Concentrated hydrochloric and sulfuric acids are among the most corrosive, dangerous chemicals. They are more so when heated. We all know how a splash of these acids can ruin one's clothing (cotton) and permanently and

severely disfigure one's face. In an actual process, much more concentrated acids are used at even higher temperatures (180 °C). Be sure to wear a pair of safety glasses to protect your eyes. Put on a lab coat or an apron to protect your expensive clothing. Thoroughly wipe up any chemical spill immediately before someone else puts his elbow over it.

4. Other assay methods may be used to measure the reducing sugar concentration. When the substrate is soluble, viscosity measurements in lieu of sugar measurements may be used to indicate enzyme activities. When the substrate is insoluble, turbidity and weight loss are sometimes used as indicators of enzyme activities.

5. The solution of commercially available cellulase can easily support the growth of molds and cannot be kept long at room temperature.

Questions

1. Based on sound engineering economics principles, you, as an engineer, now must make the choice between the two hydrolysis methods studied in this experiment. Justify your choice, preferably backed by a rough estimate for the unit cost associated with the glucose production. Compare this to the current market price of a related compound, say, sucrose (table sugar) or ethanol. (Make sure that your comparison is fair.) Is the proposed process profitable? What items or processing steps contribute significantly to the final cost? How can these costs be drastically reduced to make the process more attractive?

2. If you had the time and resources, what other experiments would you perform to reach better conclusions on the merits of acid hydrolysis versus an enzymatic one? percent acid used, source of enzyme, etc.

3. Is it possible to introduce a suitable bacterial flora into our intestinal tract so that we can digest grass as cattle do? If yes, why has it not been done to solve

the problem of food shortage in some of the developing countries? If no, what makes it possible for cattle but not for humans?

4. With the help of better screening techniques and recombinant DNA technology, many scientists are actively engaged in the isolation/creation of a super bug that can digest lignocellulose at an extremely high rate. What are the potential damages if this organism is released to the outside environment? What safety features can a scientist deploy to minimize the impact of such an event that is certainly unavoidable if the organism is put to use in a large scale process?

5. Comment on ways to improve the experiment.

Reference Readings

1. Bailey, J.E. and Ollis, D.F., *Biochemical Engineering Fundamentals*, 2nd Ed., p163-172, McGraw-Hill, 1986.

2. Bertran, M.S. and Dale, B.E., Enzymatic hydrolysis and recrystallization behavior of initially amorphous cellulose, *Biotech. Bioeng.*, **27**, 177, 1985.

3. Linko, M., An evaluation of enzymatic hydrolysis of cellulosic materials, in *Advances in Biochemical Engineering*, **5**, 39, 1977.

4. Ghose, T.K., Cellulase biosynthesis and hydrolysis of cellulosic substances, in *Advances in Biochemical Engineering*, **6**, 25, 1977.

5. Grethlein, H.E., Comparison of the economics of acid and enzymatic hydrolysis of newsprint, *Biotech. Bioeng.*, **20**, 503, 1978.

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EXPERIMENT NO. 4 – SUPPLEMENT A

GLUCOSE ASSAY
BY DINITROSALICYLIC COLORIMETRIC METHOD

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ENCH 485, Spring 1988

Method

This method tests for the presence of free carbonyl group (C=O), the so-called reducing sugars. This involves the oxidation of the aldehyde functional group present in, for example, glucose and the ketone functional group in fructose. Simultaneously, 3,5-dinitrosalicylic acid (DNS) is reduced to 3-amino,5-nitrosalicylic acid under alkaline conditions:

(1) $$\text{aldehyde group} \xrightarrow{\text{reduction}} \text{carboxyl group}$$

(2) $$\text{3,5-dinitrosalicylic acid} \xrightarrow{\text{oxidation}} \text{3-amino,5-nitrosalicylic acid}$$

Because dissolved oxygen can interfere with glucose oxidation, sulfite, which itself is not necessary for the color reaction, is added in the reagent to absorb the dissolved oxygen.

The above reaction scheme shows that one mole of sugar will react with one mole of 3,5-dinitrosalicylic acid. However, it is suspected that there are many side reactions, and the actual reaction stoichiometry is more complicated than that previously described. The type of side reaction depends on the exact nature of the

reducing sugars. Different reducing sugars generally yield different color intensities; thus, it is necessary to calibrate for each sugar. In addition to the oxidation of the carbonyl groups in the sugar, other side reactions such as the decomposition of sugar also competes for the availability of 3,5-dinitrosalicylic acid. As a consequence, carboxymethyl cellulose can affect the calibration curve by enhancing the intensity of the developed color.

Although this is a convenient and relatively inexpensive method, due to the relatively low specificity, one must run blanks diligently if the colorimetric results are to be interpreted correctly and accurately. One can determine the background absorption on the original cellulose substrate solution by adding cellulase, immediately stopping the reaction, and measuring the absorbance, i.e. following exactly the same procedures for the actual samples. When the effects of extraneous compounds are not known, one can effectively include a so-called internal standard by first fully developing the color for the unknown sample; then, a known amount of sugar is added to this sample. The increase in the absorbance upon the second color development is equivalent to the incremental amount of sugar added.

List of Reagents and Instruments

A. Equipment

Test tubes

Pipets

Spectrophotometer

B. Reagents

Dinitrosalicylic Acid Reagent Solution, 1%

Dinitrosalicylic acid: 10 g

Phenol: 2 g (optional, see Note 1)

Sodium sulfite: 0.5 g

Sodium hydroxide: 10 g

Add water to: 1 liter

Potassium sodium tartrate solution, 40%

Procedures

1. Add 3 ml of DNS reagent to 3 ml of glucose sample in a lightly capped test tube. (To avoid the loss of liquid due to evaporation, cover the test tube with a piece of paraffin film if a plain test tube is used.)
2. Heat the mixture at 90°C for 5-15 minutes to develop the red-brown color.
3. Add 1 ml of a 40% potassium sodium tartrate (Rochelle salt) solution to stabilize the color.
4. After cooling to room temperature in a cold water bath, record the absorbance with a spectrophotometer at 575 nm.

Notes

1. Phenol, up to 2g/l, intensifies the color density. It changes the slope of the calibration curve of absorbance versus glucose concentration but does not affect the linearity. The above procedure yields an absorbance of 1 for 1 g/l of glucose in the original sample in the absence of phenol in the reagent, as opposed to an absorbance of 2.5 for 1 g/l of glucose in 2 g/l of phenol. This property can be exploited to achieve the maximum sensitivity for dilute samples.

Questions

1. How much time was needed for the complete color development? Justify your answer with a plot of color intensity as a function of time.
2. Obtain an absorption spectrum over wavelengths in the visible range (i,e. 400-700 nm). Justify the use of 575 nm chosen in the Procedure.

3. Find the procedures for at least two other methods commonly employed to measure sugar concentrations. List the advantages and disadvantages of these methods.

Reference Readings

1. Miller, G.L., Use of dinitrosalicylic acid reagent for determination of reducing sugar, *Anal. Chem.*, **31**, 426, 1959.

EXPERIMENT NO. 5

STARCH HYDROLYSIS BY AMYLASE

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ENCH 485, Spring 1988

Objective

To study the various parameters that affect the kinetics of α-amylase catalyzed hydrolysis of starch.

Introduction

Starchy substances constitute the major part of the human diet for most of the people in the world, as well as many other animals. They are synthesized naturally in a variety of plants. Some plant examples with high starch content are corn, potato, rice, sorghum, wheat, and cassava. It is no surprise that all of these are part of what we consume to derive carbohydrates. Similar to cellulose, starch molecules are glucose polymers linked together by the α-1,4 and α-1,6 glucosidic bonds, as opposed to the β-1,4 glucosidic bonds for cellulose. In order to make use of the carbon and energy stored in starch, the human digestive system, with the help of the enzyme amylases, must first break down the polymer to smaller assimilable sugars, which is eventually converted to the individual basic glucose units.

Because of the existence of two types of linkages, the α-1,4 and the α-1,6, different structures are possible for starch molecules. An unbranched, single chain polymer of 500 to 2000 glucose subunits with only the α-1,4 glucosidic bonds is

called *amylose*. On the other hand, the presence of α-1,6 glucosidic linkages results in a branched glucose polymer called *amylopectin*. The degree of branching in amylopectin is approximately one per twenty-five glucose units in the unbranched segments. Another closely related compound functioning as the glucose storage in animal cells is called *glycogen*, which has one branching per 12 glucose units. The degree of branching and the side chain length vary from source to source, but in general the more the chains are branched, the more the starch is soluble.

Starch is generally insoluble in water at room temperature. Because of this, starch in nature is stored in cells as small granules which can be seen under a microscope. Starch granules are quite resistant to penetration by both water and hydrolytic enzymes due to the formation of hydrogen bonds within the same molecule and with other neighboring molecules. However, these inter- and intra-hydrogen bonds can become weak as the temperature of the suspension is raised. When an aqueous suspension of starch is heated, the hydrogen bonds weaken, water is absorbed, and the starch granules swell. This process is commonly called *gelatinization* because the solution formed has a gelatinous, highly viscous consistency. The same process has long been employed to thicken broth in food preparation.

Depending on the relative location of the bond under attack as counted from the end of the chain, the products of this digestive process are dextrin, maltotriose, maltose, and glucose, etc. Dextrins are shorter, broken starch segments that form as the result of the random hydrolysis of internal glucosidic bonds. A molecule of maltotriose is formed if the third bond from the end of a starch molecule is cleaved; a molecule of maltose is formed if the point of attack is the second bond; a molecule of glucose results if the bond being cleaved is the terminal one; and so on. As can be seen from the exercises in Experiment No. 3, the initial step in random depolymerization is the splitting of large chains into various smaller sized segments.

The breakdown of large particles drastically reduces the viscosity of gelatinized starch solution, resulting in a process called *liquefaction* because of the thinning of the solution. The final stages of depolymerization are mainly the formation of mono-, di-, and tri-saccharides. This process is called *saccharification*, due to the formation of saccharides.

Since a wide variety of organisms, including humans, can digest starch, α-amylase is obviously widely synthesized in nature, as opposed to cellulase. For example, human saliva and pancreatic secretion contain a large amount of α-amylase for starch digestion. The specificity of the bond attacked by α-amylases depends on the sources of the enzymes. Currently, two major classes of α-amylases are commercially produced through microbial fermentation. Based on the points of attack in the glucose polymer chain, they can be classified into two categories, liquefying and saccharifying.

Because the bacterial α-amylase to be used in this experiment randomly attacks only the α-1,4 bonds, it belongs to the liquefying category. The hydrolysis reaction catalyzed by this class of enzymes is usually carried out only to the extent that, for example, the starch is rendered soluble enough to allow easy removal from starch-sized fabrics in the textile industry. The paper industry also uses liquefying amylases on the starch used in paper coating where breakage into the smallest glucose subunits is actually undesirable. (One cannot bind cellulose fibers together with sugar!)

On the other hand, the fungal α-amylase belongs to the saccharifying category and attacks the second linkage from the nonreducing terminals (i.e. C4 end) of the straight segment, resulting in the splitting off of two glucose units at a time. Of course, the product is a disaccharide called maltose. The bond breakage is thus more extensive in saccharifying enzymes than in liquefying enzymes. The starch chains

are literally chopped into small bits and pieces. Finally, the amyloglucosidase (also called glucoamylase) component of an amylase preparation selectively attacks the last bond on the nonreducing terminals. The type to be used in this experiment can act on both the α-1,4 and the α-1,6 glucosidic linkages at a relative rate of 1:20, resulting in the splitting off of simple glucose units into the solution. Fungal amylase and amyloglucosidase may be used together to convert starch to simple sugars. The practical applications of this type of enzyme mixture include the production of corn syrup and the conversion of cereal mashes to sugars in brewing.

Thus, it is important to specify the source of enzymes when the actions and kinetics of the enzymes are compared. Four types of α-amylases from different sources will be employed in this experiment: three of microbial origin and one of human origin. The effects of temperature, pH, substrate concentration, and inhibitor concentration on the kinetics of amylase catalyzed reactions will be studied. Finally, the action of the amylase preparations isolated from microbial sources will be compared to that from human saliva.

List of Reagents and Instruments

A. Equipment

Erlenmeyer flasks

Beakers

Graduated cylinder

Pipets, 1ml, 10ml

Test tubes

Temperature bath

Thermometer

Balance

Syringe

Filter holder and filter paper

Spectrophotometer

Brookfield viscometer

B. Reagents

Enzymes

Bacterial amylase solution, 3000 SKB units/ml

Fungal amylase powder, 40,000 SKB units/g. (Concentration of the fungal amylase solution to be used in class: 75g/l)

Amyloglucosidase solution, 75 AG units/ml

Human salivary amylase

Corn starch

HCl Stopping Solution, 0.1N HCl

Iodine Reagent Stock Solution (in aqueous solution) See Note 1.

Iodine 5 g/l

KI 50 g/l

Potassium Phosphate Buffers

KH_2PO_4 (monobasic phosphate) (FW=136.1)

$K_2HPO_4 \cdot 3H_2O$ (dibasic phosphate) (FW=228.23)

$CaCl_2 \cdot 2H_2O$, 0.1M solution

Reagents for the analysis of reducing sugars

Procedures

Because there is a variety of kinetic studies in this experiment, work will be divided among the entire class. Each student will be assigned responsibilities for different sections.

1. Prepare a 20g/l starch solution.

a. Mix 20 g of soluble potato starch in approx. 50 ml of cold water.

b. While stirring, add the slurry to approx. 900 ml of gently boiling water in a large beaker.

c. Mix well and cool the gelatinized starch solution to room temperature.

d. Add more water to bring the total volume to 1 liter.

e. Put a few drops of the starch solution on a glass plate. Add 1 drop of the iodine reagent and see that a deep blue color is developed. The blue color indicates the presence of starch in the solution.

2. *Effect of the pH:*

a. Prepare 0.1M pH buffer solutions ranging from pH=4.5 to pH=9 in increments of one pH unit. (Note that phosphate buffer is only good for ph=4.5–9 due to the dissociation constant.) Before coming to the lab, review how to make a pH buffer solution in a freshman chemistry textbook and calculate the relative amounts of KH_2PO_4 (monobasic phosphate) and $K_2HPO_4 \cdot 3H_2O$ (dibasic phosphate) needed to make these phosphate buffer solutions.

b. Add an equal volume of one of the above buffer solutions to 5.0ml of the 20g/l starch solution prepared in Step 1. The resulting solution should contain 10g/l of starch in a buffered environment.

c. Start the enzymatic digestion process by adding 0.5 ml of the bacterial amylase solution; shake and mix.

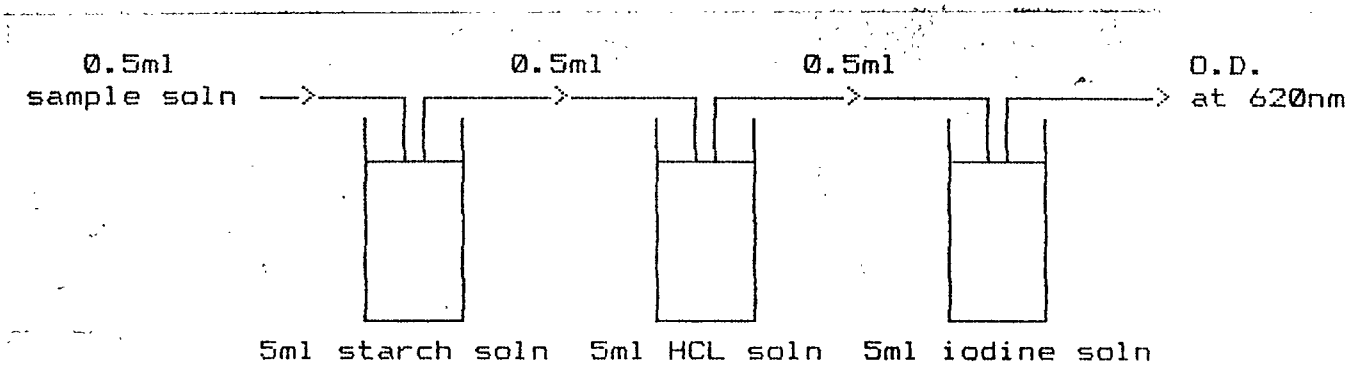
d. Let the hydrolysis reaction proceed for exactly 10 minutes at 25°C.

e. Add 0.5 ml of the reacted starch solution to 5ml of the HCl stopping solution (0.1N)

f. Add 0.5 ml of the above mixture to 5ml iodine solution to develop color. Shake and mix. The solution should turn deep blue if there is any residual, unconverted starch present in the solution. The solution is brown-red colored for partially degraded starch, while it is clear for totally degraded starch.

g. Measure the absorbance with a spectrophotometer at 620nm. See Note 2.

h. Carry out the same procedure for the other starch solutions buffered at different pH's. (Use your time wisely; all the solutions can be handled simultaneously if you are familiar with the procedure. Slightly stagger the sequential sample withdrawal so that there is enough time for sample preparation and handling.)



3. *Effect of Temperature:*

a. Obtain hot water from either a faucet or a hot temperature bath. Adjust the temperatures of the temporary water baths in 500 ml beakers so that they range from 30°C to 90°C in increments of 10°C.

b. Prepare the starch substrate by diluting the 20g/l starch solution prepared in Step 1 with an equal volume of pH=7.0 phosphate buffer solution. This results in a working starch concentration of 10 g/l. Add 5 ml of the starch solution to each of the test tubes.

c. Allow the temperature of each of the starch solutions to come to equilibrium with that of the water bath.

d. Add 0.5 ml of the bacterial amylase solution to each of the thermostated test tubes to start the reaction.

e. Stop the reaction after exactly 10 minutes and analyze the starch content by following the procedures outlined in Step 2.

4. *Effect of Heat Treatment:*

a. Place 0.5 ml of the bacterial amylase solution each of eleven test tubes.

b. Heat-treat the enzyme solution by placing all the test tubes, except one, in a hot (90°C) water bath. The untreated enzyme is used as the control. Take out the first test tube from the heat after one minute and quickly bring it to room temperature by immersing it in a cool water bath. Remove the second test tube after 2 minutes, the third after 3 minutes, and so on.

c. Add 5 ml of the 10 g/l buffered (pH=7.0) starch solution to each of the test tubes containing the enzymes.

d. Carry out the hydrolysis reaction at room temperature and analyze the sample after exactly 10 minutes by following the procedures outlined in Step 2.

e. Mix an equal volume of the $CaCl_2$ solution to the enzymes and repeat the same procedures to investigate the heat stabilization of the enzymes in the presence of Ca^{2+} ions.

f. This set of studies can be done quickly if the procedures are synchronized. If time permits, try 0.5 ml samples of the amyloglucosidase and 0.5 ml samples of the fungal amylase solution. Compare the sensitivity to heat for these related enzymes. Hint: The liquefaction step in the production

of high-fructose corn syrup is carried out at about 105°C.

5. *Activity of Human Salivary Amylase:* Obtain enough saliva to repeat the pH effect study as in Step 2.

6. *Enzyme Specificity:* Use 0.5 ml of the cellulase left over from the previous experiment. Follow a similar procedure to determine the decrease in the starch concentration as outlined in Step 2. Measure the rate with buffered starch solution at pH=4.0 and 7.0.

7. *Effect of Substrate Concentration:*

   a. Add 0.5 ml of the bacterial amylase solution to 50 ml of a 10g/l starch solution buffered at pH=7.0. Note that less enzyme per ml of substrate is used in this part of the experiment than the previous parts. The objective here is to slow down the reaction so that multiple sampling is possible with reasonable accuracy before all the starch is consumed.

   b. Take samples periodically to monitor both the decrease in the starch concentration and the increase in the reducing sugars until most of the starch is hydrolyzed. The starch concentration is measured with the same steps outlined above and the sugar concentration with the dinitrosalicylic colorimetric method used in the previous experiment.

   c. Continuously monitor the viscosity of the substrate-enzyme mixture with a viscometer. Generate a calibration curve for the viscosity as a function of the starch concentration. Note that this part of the study is fruitful only when the starch solution is extremely thick.

8. *Effect of Enzyme Sources:*

   a. Repeat Procedure 7 with 0.5 ml of the fungal amylase solution.

   b. Repeat Procedure 7 with 0.5 ml of the amyloglucosidase.

c. Repeat Procedure 7 to study the joint action of a mixture of 0.167 ml of bacterial amylase, 0.167 ml of fungal amylase solution, and 0.167 ml of amyloglucosidase.

d. This entire Procedure 8 can be concurrently carried out along with Procedure 7.

9. *Sequential Enzymatic Treatment (Corn Syrup Production):* In making industrial sugars, e.g. corn syrup, large gelatinized starch molecules are first chopped into smaller dextrins with the help of bacterial amylase. The liquefaction step is followed by saccharification with either fungal amylase or amyloglucosidase, depending on the end use of the sugar. These sequential enzymatic treatment steps will be simulated in this part of the experiment

   a. Add 0.5 ml of the bacterial amylase solution to 50 ml of the 20g/l non-buffered starch solution prepared in Step 1. Periodically place a few drops of the reaction mixture on a glass plate and add one drop of the iodine reagent. The color should finally turn red, indicating the total conversion of starch to dextrin. This liquefaction step should last for approximately 10 minutes.

   b. When the process of liquefaction is complete, adjust the pH of the starch solution to 4.7 with 1N HCl.

   c. Filter the starch solution if it is turbid. Separate the solution into two equal parts.

   d. To the first starch solution, add 0.5 ml of amyloglucosidase; to the second solution add 0.5 ml of fungal amylase solution.

   e. Measure the sugar concentrations periodically. Note that you need to use the appropriate calibration curves because one is maltose and the other

is glucose. Also do not forget to reference your observation to the initial absorbance at the start of the saccharification process so that the increase in the sugar concentration can be correctly measured. This saccharification step should last for about 30-60 minutes.

f. Taste the two sugar solutions and compare the sweetness. See Note 3.

10. *Inhibition:* Follow Procedure 7, except that the buffered starch solution at pH-7.0 also contains hydrogen peroxide at a level of 0.5 g/l. If time permits, try hydrogen peroxide at a level of 1.0 g/l.

11. *Enzyme Activity versus Enzyme Concentration:* Mix 0.5, 1.0, 1.5, 2.0, and 2.5 ml of enzyme solutions with 5 ml of 10g/l starch solution. Measure the starch concentration after 10 minutes as in Step 2.

12. *For Curious Students:* Study the simple cleavage of the α-1,4 glucosidic bonds by using maltose as the substrate and amyloglucosidase as the enzyme. Plan ahead to see how one can perform this. For example, you need to be able to distinguish between maltose and glucose. Note that in order to use a colorimetric method, maltose and glucose must have different extinction coefficients. Can material balance be used to back out the individual concentrations?

Notes

1. Dilute the stock solution 1:100 to obtain a working solution. Other dilutions may be used, depending on the enzyme activity.

2. Remember to take care of the background absorbance caused by the colored iodine solution. The true absorbance should be roughly proportional to the starch concentration. The enzyme solution may have to be diluted first if all the starch present in the sample is digested and all the color disappears in 10 minutes. The most reliable results are obtained when the decrease in the

absorbance is approximately 20-70% of the absorbance of the original, undigested starch solution. To measure the amount of starch digested, you need to know the absorbance corresponding to the initial undigested starch solution by following the same procedure with a sample in which plain water in lieu of the enzyme solution is added to the starch solution.

3. Be sure you do not contaminate your sugar solution during the various stages of the reaction. Do not taste the sugar solutions and risk your health if you are not confident about your lab techniques. However, do not expect others to trust your results if you cannot even convince yourself. All glassware used in this biochemical engineering laboratory should always be much cleaner than the eating utensils on your dinning table. The reagent or analytical grade chemicals we use are also much purer than the food grade ones. Thus, as long as they are not poisonous or toxic and as long as you do not contaminate them when weighing, the intake of a small amount of them should not cause you any harm. Furthermore, as sterility will be stressed in the later part of the course when microorganisms are introduced, the glassware used then should be thoroughly aseptic, certainly cleaner than your finger. If you have hesitation in eating and drinking from any of the glassware or spatula that you use, I suggest that you get into the habit of *really* cleaning them before using them in the experiment. Although I am not encouraging you to go around and lick everything in sight, you should develop a good, aseptic laboratory habits so that you know you will not hesitate to do so if needed. The small amount of HCl added to adjust the pH to 4.7 should not affect you at all; many carbonated drinks are much more acidic than this.

4. Do as many experiments as you wish or as time and supplies/materials permit. You can effectively cover all the procedures by teaming up with a few other

classmates and exchanging data at the end of the lab period. (Be sure to give the proper credit, or blame for that matter, to your lab partners. Also be sure you know what your lab partners have done.) However, you must prepare your own lab report.

Questions

1. Plot the enzyme activity versus pH. From this curve, what is the optimal pH? Explain why enzyme activities depend on the pH. Similarly plot the enzyme activity versus temperature. Report the optimal temperature.
2. To what extent did the heat treatment affect the enzyme activities? What happens to an enzyme when it is subjected to heat?
3. What is the amylase activity in your saliva? How does it compare to those preparations isolated from microbial sources? Do they all share the same optimal pH? How does the optimal pH for the salivary amylase compare with the pH of the stomach? If the pH of the stomach is not at all favorable for amylase, has the nature made a mistake? Considering the relatively short period of time food stays in the mouse and considering the amylase activities of human saliva, there is really not much degradation of starch molecules in the mouse. Why does the nature seemingly endow amylase activities in saliva so inefficiently?
4. Did cellulase exhibit any amylo-saccharifying activities?
5. Combine the data from this experiment with those from the previous experiment and derive the rough ratio of the cellulase activity to the amylase activity. For the purpose of this comparison, you may define the activity to be the number of glucosidic bonds broken per gram of enzyme per minute. Which type of bond was easier to break, α-1,4 or β-1,4? One should base his comparison on the same conditions, e.g., the same acid concentrations and temperature.

6. From the experimental data for Procedures 7 and 8, plot the starch concentration, reducing sugar concentration, and viscosity as functions of time. Do you get a closed material balance between the starch converted and the sugar generated? If not, explain this discrepancy. Is it possible to detect no starch at all and at the same time only a negligible amount of sugar during the course of the hydrolysis reaction? Make a plot of the reaction rate versus the substrate concentration. Are Michaelis-Menten kinetics applicable to this enzyme system? If so, what are the values for the model parameters? (You may need to use a Lineweaver-Burk plot or other closely related plots to derive these parameters.) If not, what model best describes what you have observed?

7. Did the presence of hydrogen peroxide affect the enzyme activities? If so, is it a competitive, a non-competitive, or uncompetitive inhibitor?

8. Is the enzyme activity directly proportional to the enzyme concentration? If not, which quantity better describes the amount of enzymes present?

9. How would you make an acetate pH buffer solution? List the required chemicals and the composition needed to make one liter of acetate buffer as a function of the pH. Repeat for a citrate buffer. (Phosphate, acetate, and citrate buffers are the most commonly encountered ones.)

10. The more accurate name for α-amylase is 1,4-α-D-glucan-glucanohydrolase (EC 3.2.1.1), it is 1,4-α-D-glucan maltohydrolase (EC 3.2.1.2) for β-amylase, and exo-1,4-α-glucosidase or 1,4-α-D-glucan glucohydrolase (EC 3.2.1.3) for amyloglucosidase. What reaction does β-amylase catalyze?

11. Comment on ways to improve the experiment.

Reference Readings

1. Bailey, J.E. and Ollis, D.F., *Biochemical Engineering Fundamentals*, 2nd Ed.,

Chapter 3, McGraw-Hill, 1986.

2. Standard SKB method to determine enzyme activity: *Cereal Chem.*, **16**, 712, 1939.

PH OPTIMUM

Steps #2 #5 #6 (2 Students)

pH	*Enzyme Sources*				
	Bacterial Amylase	Fungal Amylase	Amylo-Glucosidase	Saliva	Cellulase
5.0				—	—
6.0				—	—
7.0					
8.0				—	—
9.0				—	—

TEMPERATURE OPTIMUM

Step #3 (1 Student)

Temperature (°C)	Bacterial Amylase
30	
45	
60	
75	
90	

HEAT STABILITY

Step #4 (2 Students)

Time of Heat Treatment at 90°C (min)	*Enzyme Sources*					
	Bacerial Amylase		Fungal Amylase		Amylo-Glucosidase	
	no Ca^{++}	/w Ca^{++}	no Ca^{++}	/w Ca^{++}	no Ca^{++}	/w Ca^{++}
0						
4						
8						
12						
16						
20						

REACTION KINETICS

Steps #7 #8 #10 (2 Students)

Reaction Time (min)	*Enzyme Sources*							
	Bacterial Amylase				Fungal Amylase		Amylo-Glucosidase	
	no H^2O^2		/w H^2O^2					
	Starch conc. (g/l)	Glucose conc. (g/l)	Starch conc. (g/l)	Glucose conc. (g/l)	Starch conc. (g/l)	Glucose conc. (g/l)	Starch conc. (g/l)	Glucose conc. (g/l)
0								
5								
10								
15								
20								
25								
30								

ENZYME CONCENTRATION

Step #11 (1 Student)

Enzyme Volume (ml)	*Enzyme Sources*		
	Bacerial Amylase	Fungal Amylase	Amylo-Glucosidase
0.5			
1.0			
1.5			
2.0			
2.5			

EXPERIMENT NO. 6A

ENZYME PURIFICATION BY SALT (AMMONIUM SULFATE) PRECIPITATION

Prepared by

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ENCH 485, Spring 1988

Objective

To recover proteins/enzymes from a solution by salting-out.

Introduction

The solubility of protein depends on, among other things, the salt concentration in the solution. At low concentrations, the presence of salt stabilizes the various charged groups on a protein molecule, thus attracting protein into the solution and enhancing the solubility of protein. This is commonly known as *salting-in*. However, as the salt concentration is increased, a point of maximum protein solubility is usually reached. Further increase in the salt concentration implies that there is less and less water available to solubilize protein. Finally, protein starts to precipitate when there are not sufficient water molecules to interact with protein molecules. This phenomenon of protein precipitation in the presence of excess salt is known as *salting-out*.

Many types of salts have been employed to effect protein separation and purification through salting-out. Of these salts, ammonium sulfate has been the most widely used chemical because it has high solubility and is relatively inexpensive.

Because enzymes are proteins, enzyme purification can be carried out by following the same set of procedures as those for protein, except that some attention must be paid to the consideration of permanent loss of activity due to denaturation under adverse conditions.

There are two major salting-out procedures. In the first procedure, either a saturated salt solution or powdered salt crystals are slowly added to the protein mixture to bring up the salt concentration of the mixture. For example, the salt concentration reaches 25% saturation when 1 ml of the saturated salt solution is added to 3 ml of the salt-free protein solution; 50% for 3 ml added; 75% for 9 ml added; and so on. The precipitated protein is collected and categorized according to the concentration of the salt solution at which it is formed. This partial collection of the separated product is called *fractionation*. For example, the fraction of the precipitated protein collected between 20 and 21% of salt saturation is commonly referred to as the 20-21% fraction. The protein fractions collected during the earlier stages of salt addition are less soluble in the salt solution than the fractions collected later.

Whereas the first method just described uses *increasing* salt concentrations, the following alternative method uses *decreasing* salt concentrations. In this alternative method, as much protein as possible is first precipitated with a concentrated salt solution. Then a series of cold (near 0°C) ammonium sulfate solutions of decreasing concentrations are employed to extract selectively the protein components that are the most soluble at higher ammonium sulfate concentrations. The extracted protein is recrystallized and thus recovered by gradually warming the the cold solution to room temperature. This method has the added advantages that the extraction media may be buffered or stabilizing agents be added to retain the maximum enzyme activity. The efficiency of recovery typically ranges from 30 to 90% , depending

on the protein. The recrystallization of protein upon transferring the extract to room temperature may occur immediately or may sometimes take many hours. Nevertheless, very rarely does recrystallization fail to occur. The presence of fine crystals in a solution can be visually detected from the turbidity.

List of Reagents and Instruments

A. Equipment

Test tubes

Graduated cylinder

Pipets

Balance

Centrifuge

Filtration devices

B. Reagents

Protein solution, 1.0 g/l hemoglobin, 200 ml

(May need centrifugation to obtain a clear solution.)

Fungal α-amylase, 10 g/l

Protease, 10 g/l

Saturated $(NH_4)_2SO_4$ solution. See Note 1.

Procedures

1. *Isolation of Hemoglobin:*

   a. Record the absorbance of the hemoglobin solution. Suggested wavelength: 577nm. This measurement is to be used in the calculation of the recovery of the protein.

   b. Pipet 4 ml of the hemoglobin solution into a test tube.

c. While stirring, add the saturated ammonium sulfate solution drop-wise to the protein solution until precipitates start to form. In order to record accurately the amount of ammonium sulfate solution added, the salt solution should be dispensed from a graduated pipet or a buret. It is critical to avoid the spatial nonuniformity in the salt concentration during the addition of the salt solution. Localized concentration hot spots will prematurely initiate the precipitation of other proteins and inadvertently affect the purify of the protein crystals. Record the volume of the saturated ammonium sulfate solution needed to cause precipitation. Also note that protein precipitation is not instantaneous; it may require 15–20 minutes to equilibrate.

d. Centrifuge the mixture at 10,000 g for 15 minutes. Collect the precipitate by carefully discarding as much supernatant as possible.

e. Reconstitute the original hemoglobin solution by resuspending the precipitate in 4 ml of water. This can be done by first adding approximately 2 ml of water from a water bottle to the centrifuge tube, shaking the test tube to redissolve the precipitate, and transferring as much as possible the hemoglobin solution in the centrifuge tube into a test tube with a pipet while noting the volume. Rinse the centrifuge tube with another ml of water, pipetting this rinse in the test tube as well, again, while noting the volume transferred. Finally, add the residual water to bring the total volume in the test tube to 4 ml.

f. Measure the absorbance of the reconstituted hemoglobin solution with a spectrophotometer.

2. *Isolation of Fungal α-Amylase:* Instead of hemoglobin solution, use 4ml of 20 g/l of fungal α-amylase.

tions.

Notes

1. Add 750 g of ammonium sulfate to 1000 ml of water in a beaker or flask. Simply stir the solution at room temperature with a magnetic stirrer for 15 minutes or until saturation. Gently decant the clear supernatant solution after the undissolved solids settle on the bottom of the flask. (Filtration is not really necessary.)

Discussion

To assure the maximum yield and to avoid unnecessary denaturation of the enzymes, most of the protein purification work is usually carried out at low temperatures, i.e. between 0 and 4°C. However, it is simply far more convenient to work in a regular laboratory room as opposed to a cold room. Since the purpose of this experiment is to demonstrate the use of common purification techniques, unless noted otherwise when it is truly critical, the procedures will be carried out at room temperature without any significant loss of educational values.

The recovery of protein can have very significant economical implications. Because a fixed fraction of the original protein stays soluble in the solution, the recovery of protein is often not near 100% . Of course, a yield of over 100% indicates that there may be problems associated with the assay method.

In a typical protein preparation or purification step carried out in a laboratory where the aim is to isolate a small quantity of a product for structural or kinetic studies, a saturated ammonium sulfate solution is routinely used. It is also the procedure taken in this experiment. However, in an actual large scale commercial process, it is better to add ammonium sulfate directly into the protein mixture as powdered solids so that the effect of dilution by the salt solution is minimized.

Questions

1. From the volume of the saturated ammonium sulfate added to the protein solution at the onset of precipitation, calculate the salt concentration in terms of percent saturation. Note that this is the same as a dilution calculation. Did the supernatant have the characteristic red/brown color of hemoglobin? (Oxygenated hemoglobin is red, and the deoxygenated form is brown.) What does the color in the supernatant, if any, indicate? From the absorbance readings before and after the protein separation, how much many percent of the original protein is recovered in the ammonium sulfate salting-out procedure?

2. At what salt concentration did α-amylase precipitate? Was there any enzyme denaturation? If yes, suggest an alternate procedure of isolation. Answer the same set of questions for protease.

3. Did you think α-amylase and protease could be separated from each other and from hemoglobin? Why? If enzyme separation from a mixture was attempted, were you successful in separating the mixture into respective components? If yes, what fraction was α-amylase? What fraction was protease? Was there any shift in the incipient precipitation concentrations of the salt solution? Was there any decrease in the enzyme activities?

4. Comment on ways to improve the experiment.

Reference Readings

1. Jakoby, W.B., Crystallization as a purification technique, *Enzyme Purification and Related Techniques*, in *Methods in Enzymology*, Vol. 22, Jakoby, W.B., Ed., Academic Press, 1971.

EXPERIMENT NO. 6B

ENZYME PURIFICATION BY ACETONE PRECIPITATION

Prepared by

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ENCH 485, Spring 1988

Objective

To recover proteins/enzymes from a solution by adding acetone.

Introduction

The solubility of protein depends on, among other things, the *dielectric constant* of the solution. In general, solvent molecules with large dielectric constants, e.g. water and dimethylsulphoxide, can stabilize the interaction between themselves and protein molecules and favor the dissolution of protein. On the other hand, organic solvents with small dielectric constants, e.g. acetone and methanol, discourage the dispersion of protein molecules in the media. Thus, the solubility of proteins can be lowered and precipitation can be induced by lowering the effective dielectric constant of the media. This is commonly achieved by adding a water-soluble solvent such as acetone to an aqueous solution of protein. Acetone had the advantage that it is relatively inexpensive and is available in in a pure form with few contaminants that may inhibit or poison the enzyme. It is also frequently used in sterol extraction.

List of Reagents and Instruments

A. Equipment

Test tubes

Graduated cylinder

Pipets

Balance

Centrifuge

Filtration devices

B. Reagents

Protein solution, 5.0 g/l (albumin, gelatine, casein)

Enzymes solution, 10 g/l (α-amylase, protease)

Acetone

Procedures

1. *Precipitation of Protein from Individual Protein Solution:*
   a. Pipet 4 ml of the protein or enzyme solution into a test tube.
   b. While stirring, add acetone drop-wise to the protein solution from a graduated pipet or a buret until precipitates start to form. Vigorous stirring and slow acetone addition rate will avoid the localized high concentration of acetone.
2. *Effect of Temperature on Enzyme Isolation:* Perform Step for enzyme solutions 1 at 0°C and compare the enzymatic activities.
3. *Isolation of Protein Components from a Mixture:* Repeat the same procedures as in ammonium sulfate precipitation.

Discussion

Most of the enzyme/protein separation and purification work is conducted at low temperatures, and acetone precipitation is no exception. In practice, it is routinely carried out at a temperature below 0°C.

Questions

1. Calculate the volume fraction of acetone at the onset of precipitation for each protein solution. Calculate the protein yield for each case by following the same procedure as in ammonium sulfate precipitation.
2. How did the temperature affect the enzyme activities? How would you extend this conclusion to the other proteins tried in this experiment?
3. Answer the same set of questions as in ammonium sulfate precipitation.
4. Comment on ways to improve the experiment.

EXPERIMENT NO. 6C

ENZYME PURIFICATION BY ISOELECTRIC PRECIPITATION

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ENCH 485, Spring 1988

Objective

To recover proteins/enzymes from a solution by changing the pH of the solution.

Introduction

The solubility of protein depends on, among other things, the *pH* of the solution. Similar to the amino acids that comprise protein, protein itself can be either positively or negatively charged overall due to the terminal amine (-NH_2) and carboxyl (-COOH) groups and the groups on the side chain. It is positively charged at low pH and negatively charged at high pH. The intermediate pH at which a protein molecule has a net charge of zero is called the *isoelectric point* of that protein. In general, the net charge on the protein, either positive or negative, can interact with water molecules, meaning that it is more likely for a protein molecule to dissociate itself from other protein molecules, thus, more soluble. As a result, protein is the least soluble when the pH of the solution is at its isoelectric point.

When microorganisms grow in milk, they often produce acids and lowers the pH of the milk. The phenomenon of precipitation or coagulation of milk protein (casein) at low pH as milk becomes spoiled is one of the common examples of protein

isolation due to changes in the pH.

List of Reagents and Instruments

A. Equipment

Test tubes

Graduated cylinder

Pipets

Balance

Centrifuge

Filtration devices

B. Reagents

Protein solution, 5.0 g/l (albumin, gelatine, casein)

Enzymes solution, 10 g/l (α-amylase, protease)

NaOH or KOH solution, 1N

Acetic Acid solution, 0.1N

Procedures

1. *Precipitation of Protein in Acidified Solution:*

   a. Add 5.0 g of casein to 200 ml of 1N NaOH solution.

   b. Pipet 4 ml of the protein solution into a test tube.

   c. While stirring, add the acid solution drop-wise to the alkaline protein solution from a graduated pipet or a buret until precipitates start to form. Stir thoroughly to avoid the localization of low pH spots in the solution. Note the volume of the acid solution added at the incipient of precipitation. Since precipitation is not an instantaneous process, let the test tube stand undisturbed for 30 minutes.

d. Repeat the same process for a series of test tubes, each containing 4 ml of the alkaline protein solution. To each test tube, add slightly less acid solution than the previous one so that a series of pH values can be established. Let each test tube stand for 30 minutes. Measure the pH of each solution and note the pH region around which the amount of precipitate is the maximum.

2. Try out other proteins or enzymes available in the laboratory.

3. *Isolation of Protein Components from a Mixture:* If the student conclude that the individual protein component can be fractionally isolated, repeat the same procedures as in ammonium sulfate precipitation.

Questions

1. Report the amount of precipitate as a function of the pH for each of the proteins tried.

2. Justify why pH fractionation can/cannot be used to isolate different proteins.

3. Comment on ways to improve the experiment.

EXPERIMENT NO. 7

ENZYME IMMOBILIZATION BY GEL ENTRAPMENT

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ENCH 485, Spring 1988

Objective

To compare the effectiveness of three methods of enzyme immobilization by gel entrapment.

Introduction

Three different commonly used entrapment media will be introduced in this experiment: polyacrylamide, calcium alginate, and gelatin. All these gels can be formed with a simple set of equipment and share similar procedures. In all the protocols, enzymes are well mixed with monomers/polymers and cross-linking agents in a solution. The solution is then exposed to polymerization promoters to start the process of gel formation. The solution is poured into a mold to achieve the desired shapes. A gel block may be cut into smaller cubes to increase the surface area. Commercially, it is common to force the unpolymerized solution through a set of nozzles to form spherical beads, whose size can be controlled by adjusting the back pressure. The resulting beads may be further hardened to enhance structural integrity.

Of the three gels, polyacrylamide is the most widely used matrix for entrapping

enzymes. It has the advantage that it is non-ionic. The consequence is that the properties of the enzymes are only minimally modified in the presence of the gel matrix. At the same time, the diffusion of the charged substrate and products is not affected, neither. However, dimethylaminopropionitrile, the polymerization initiator, is highly toxic and must be handled with great care. The requirement to purge the monomer solution with nitrogen is also troublesome, although not totally crippling.

Calcium alginate is just as widely used as polyacrylamide. Unlike polyacrylamide gels, gelation of calcium alginate does not depend on the formation of more permanent covalent bonds between polymer chains. Rather, polymer molecules are cross-linked by calcium ions. Because of this, calcium alginate beads can be formed in extremely mild conditions, which ensure that enzyme activity yields of over 80% can be routinely achieved. However, just as easily as calcium ions can be exchanged for sodium ions, they can also be displaced by other ions. This property can both be advantageous and disadvantageous. If needed, enzymes or microbial cells can be easily recovered by dissolving the gel in a sodium solution. On the other hand, proper caution must be exercised to ensure that the substrate solution does not contain high concentrations of those ions that can disintegrate the gel.

The main attraction of using gelatin as the immobilization media is that the gel formation process requires only simple equipment and that the reagents are relatively inexpensive and nontoxic. The retention of enzymatic activities for immobilization with a gelatin gel is typically 25-50% of the original free enzyme. Gelatin gel has the advantage that the mass transfer resistance is relatively low compared to other entrapment methods, but the rate of enzyme loss due to leakage is high.

In summary, the efficiency of an immobilization process can be measured by the following criteria. Most important of all, a high percentage of the enzymes

must be initially retained in gel matrices. Secondly, the enzyme activity must be preserved. And thirdly, the enzymes must be physically restrained from diffusing back into the substrate solution at a later time. It is quite difficult to create a fine and uniform mesh so as to prevent the entrapped enzymes from leaking out of the matrices. On the other hand, highly cross-linked matrices can result in higher mass transfer resistances for both the substrate and the product.

Bacterial α-amylase will be used in this experiment to demonstrate and compare the effectiveness of various entrapment techniques. The class will be divided into groups of three. Before attempting the experiment, identify the major steps and devise a work plan among the group members. Each member should be explicitly assigned an equal portion of the responsibilities. For example, one member may be responsible for making all three types of gels, and other members may be assigned the tasks of measuring the enzyme leakage and immobilized enzyme activities. Alternatively, each member may be responsible for remain responsible throughout all phases of the study associated only one type of gel. It is the responsibility of the entire group to make sure that each member is carrying out the work according to the plan, whatever the plan is. Work closely together and help each other if needed to coordinate the smooth execution of the plan. Share the data at the end.

List of Reagents and Instruments

A. Equipment

Erlenmeyer flasks

Beakers

Graduated cylinder

Pipets

Test tubes

Temperature bath

Thermometer

Balance

Syringe

Spectrophotometer

See Supplement A, B, and C for the equipment required in each immobilization protocols.

B. Reagents

Bacterial α-amylase

HCl solution, 1N

KOH solution, 1N

See Supplement A, B, and C for the reagents required in each immobilization protocols.

Procedures

1. *Prepare immobilized enzyme beads:* Immobilize α-amylase by entrapping it inside gel matrices according to the immobilization protocols accompanying this write-up. See Note 1.

2. *Immobilized enzyme activities:* Follow a similar procedure as in the previous amylase experiment to measure the activities of the immobilized enzymes. Instead of the enzyme solution, immerse about 1 g of the gel beads prepared in the above step in 10 ml of the buffered starch solution at pH=7.0. Constantly shake the mixture to make sure that the solution is not stagnant. As before, add 0.5 ml of the reacted starch solution to 5ml of the 0.1N HCl stopping solution. Mix with the iodine solution to detect the presence of residual starch. Rinse the gel beads with water and reuse the same beads twice more, each time measuring the enzyme activities.

3. *Shift in the optimal enzymatic condition – pH effect:* Study the effect of pH on the activities of the immobilized enzymes. Refer to the previous experiment on α-amylase. Because of the time limitation, perform this part for only one of the gels of your choice.

4. *Recovery from adverse pH condition:* Immerse about 1g of the gel bead in 5 ml of the 1N HCl solution. After shaking for 15 minutes, discard the HCl solution and thoroughly wash the gel beads with water. Measure the enzyme activity with buffered (pH=7.0) starch solution. Repeat for a 1N KOH solution.

5. *Recovery of enzymes:* Dissolve the gels and measure the enzyme activities afterward.

6. *Enzyme leakage and inactivation:* Immerse 5 ml of the fresh gel which has not been exposed to adverse pH or temperature conditions in 5 ml of water in a test tube for over 24 hours. Record the duration allowed for enzyme leakage. Measure and report the amylase activity in the surrounding water by following the same procedure as in the previous experiments. In addition measure the activities of the immobilized enzyme gel beads.

7. *For Curious Students:* Follow the same procedure as in the previous amylase experiment to study the effect of temperature on the activities of the immobilized enzymes. Is there any shift in the optimal temperature?

Notes

1. Note that each protocol produces approximately 10 ml of gels, barely enough for the explicitly stated steps of this experiment. However, if the student plans to perform additional investigation on his own, or if he feels that he is prone to repeated mistakes, double or triple the amount stated in the recipes so that there is a small reserve.

Discussion

Because enzymes are biological catalysts that promote the rate of reactions but are not themselves consumed in the reactions in which they participate, they may be used repeatedly for as long as they remain active. However, in most of the industrial, analytical, and clinical processes, enzymes are mixed in a solution with substrates and cannot be economically recovered after the exhaustion of the substrates. This single use is obviously quite wasteful when the cost of enzymes is considered. Thus, there is an incentive to use enzymes in an immobilized or insolubilized form so that they may be retained in a biochemical reactor to catalyze further the subsequent feed. The use of an immobilized enzyme makes it economically feasible to operate an enzymatic process in a continuous mode.

Numerous methods exist for enzyme immobilization, sometimes referred to as enzyme insolubilization. The overwhelming majority of the methods can be classified into four main categories: matrix entrapment, microencapsulation, adsorption, and covalent binding. Of these methods, matrix entrapment is the focus of this experiment.

Many entrapment methods are used today, and all are based on the physical occlusion of enzyme molecules within a "caged" gel structure such that the diffusion of enzyme molecules to the surrounding medium is severely limited, if not rendered totally impossible. What creates the "wires" of the cage is the cross-linking of polymers. A highly cross-linked gel has a fine "wire mesh" structure and can more effectively hold smaller enzymes in its cages. The degree of cross-linking depends on the condition at which polymerization is carried out. Because there is a statistical variation in the mesh size, some of the enzyme molecules gradually diffuse toward the outer shell of the gel and eventually leak into the surrounding medium. Thus, even in the absence of loss in the intrinsic enzyme activity, there is a need to re-

plenish continually the lost enzymes to compensate for the loss of apparent activity. In addition, because an immobilized enzyme preparation is used for a prolonged period of operation, there is also a gradual, but noticeable, decline in the intrinsic enzyme activity even for the best method. Eventually, the entire immobilized enzyme packing must be replaced.

Besides the leakage of enzymes, another problem associated with the entrapment method of immobilization is the mass transfer resistance to substrates, products, and inhibitors. Because the average diameter of a typical bead of enzyme impregnated gel is much larger compared to the average diffusion length, substrate cannot diffuse deep into the gel matrix, as in any other conventional non-biological immobilized catalysts. At the same time, the diffusional resistance encountered by the product molecules can sometimes cause the product to accumulate near the center of the gel to an undesirable high level, leading to product inhibition for some enzymes. Thus, ideally the network of cross-linking should be coarse enough so that the passage of substrate and product molecules in and out of a gel bead is as unhindered as possible. For this reason, entrapment is not suitable for special cases where the substrate has a large molecular weight such that it cannot easily move freely in the gel matrix.

Unlike the adsorption and covalent bonding methods, most polymerization reactions that cause cross-linking and gel formation in entrapment methods do not directly involve the formation of bonds between the support material and the enzyme molecules. There are reports that these bonds change the conformation of the enzyme protein and modify the enzyme properties. Since the enzyme molecules do not themselves participate in the polymerization reaction in the entrapment methods, the same entrapment techniques can be successfully applied to a wide range of enzymes with only minor modifications between different enzymes.

Questions

1. Compare the activities of the immobilized beads of different methods. Be sure that the comparison is based on the same standard and is fair.
2. How would you measure the mass transfer resistance in a gel matrix?
3. How would you estimate the relative contribution of the following factors in the overall loss of enzyme activities: enzyme deactivation, enzyme leakage, mass transfer?
4. How fast did the activity decline over a period of one or more days, if at all? What fraction of that decline can be attributed to the leakage of the enzyme from the gels?
5. Was there any shift in the optimal pH or temperature due to immobilization? Do you expect any?
6. What industrial enzymatic processes are routinely carried out with immobilized enzymes instead of free enzymes? What methods of immobilization are actually being used?
7. List some of the advantages and disadvantages of an immobilized enzymatic conversion process versus a free one.
8. Comment on ways to improve the experiment.

EXPERIMENT NO. 7 – SUPPLEMENT A

ENZYME IMMOBILIZATION PROTOCOL ENTRAPMENT IN POLYACRYLAMIDE GEL

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ENCH 485, Spring 1988

Method

This technique is based on the polymerization of acrylamide with N,N'-methylene-bis-acrylamide (Bis) as the cross-linking agent. The degree of cross-linking, thus, can be partly controlled by adjusting the ratio of acrylamide to Bis used.

List of Reagents and Instruments

A. Equipment

Beakers

Pipets

Balance

Graduated cylinder

Syringe and needle

B. Reagents

Buffered Monomer Solution

0.1 mM EDTA

0.1 M Tris-HCl

11 g/l N,N'-Methylene-bis-acrylamide $(CH_2{=}CHCONH)_2CH_2$

1 g/l Acrylamide $CH_2{=}CHCONH_2$

Adjust the pH to 7.0 (See Note 1)

Washing Solution

0.5 M NaCl

0.1 mM EDTA

0.1 M Tris-HCl

Adjust the pH to 7.0

Dimethylaminopropionitrile (polymerization catalyst)

Potassium persulphate solution, 10g/l (polymerization initiator)

Nitrogen gas cylinder

Enzyme

Procedures

1. *Buffered Monomer Solution:* Add 1.1 g of Bis and 0.1 g of acrylamide to a 100 ml of buffered solution (pH 7.0) of 0.1mM EDTA and 0.1M Tris-HCl in a beaker. See Note 1.
2. To 10 ml of the buffered monomer solution of the above step, add enzyme powders (approximately 0.015g) or an equivalent concentrated enzyme solution; mix.
3. Purge the dissolved oxygen in the solution that can interfere with the polymerization process with nitrogen for 20 minutes. This step is *critical* in achieving a high degree of cross-linking.
4. Add 2.0 ml of dimethylaminopropionitrile; mix.
5. Add 5.0 ml of freshly prepared 10g/l potassium persulphate solution to initiate

polymerization.

6. Now is the time to pour the solution into a mold if one does not desire the gel to form in the original beaker. Leave the solution undisturbed; gel will form in approximately 30 minutes.

7. Cut the resulting gel into small cubes of approximately 3mm per side. Alternatively, if smaller pieces are desired, the gel can be forced through a syringe fitted with a fine needle.

8. Gently wash the free enzyme off the gel surface in 10 ml of the Washing Solution. Repeat the washing process two additional times.

Notes

1. The pH of the buffer should be adjusted to match the optimum value of the enzyme to be entrapped.

Discussion

The above methods of enzyme immobilization by gel entrapment can be directly applied to live cells with minor modifications. For example, dimethylaminopropionitrile used in forming the polyacrylamide gel may not be employed because of its toxicity to viable cells. The monomers of acrylamide are also somewhat toxic to cells. On the other hand, cells can be immobilized with much less degree of cross-linking due to its much larger size.

Reference Readings

1. M.D. Trevan and S. Grover, *Trans. Biochem. Soc.*, **7**, 28, 1979.

EXPERIMENT NO. 7 – SUPPLEMENT B

ENZYME IMMOBILIZATION PROTOCOL ENTRAPMENT IN ALGINATE GEL

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Method

Alginate, commercially available as alginic acid, sodium salt, commonly called sodium alginate, is a linear polysaccharide normally isolated from many strains of marine brown seaweed and algae, thus the name *algi*nate. The copolymer consists of two uronic acids: D-mannuronic acid (M) and L-guluronic acid (G). Because it is the skeletal component of the algae it has the nice property of being strong and yet flexible.

Alginic acid can be either water soluble or insoluble depending on the type of the associated salt. The salts of sodium, other alkali metals, and ammonia are soluble, whereas the salts of polyvalent cations, e.g., calcium, are water insoluble, with the exception of magnesium. Polyvalent cations bind to the polymer whenever there are two neighboring guluronic acid residues. Thus, polyvalent cations are responsible for the cross-linking of both different polymer molecules and different parts of the same polymer chain. The process of gelation, simply the exchange of calcium ions for sodium ions, is carried out under relatively mild conditions. Because the method is based on the availability of guluronic acid residues, which will not vary once given a batch of the alginate, the molecular permeability does

not depend on the immobilization conditions. Rather, the pore size is controlled by the choice of the starting material.

$$2\ \text{Na(Alginate)} + \text{Ca}^{++} \longrightarrow \text{Ca(Alginate)}_2 + 2\ \text{Na}^{+}$$

The ionically linked gel structure is thermostable over the range of 0-100°C; therefore heating will not liquefy the gel. However, the gel can be easily redissolved by immersing the alginate gel in a solution containing a high concentration of sodium, potassium, or magnesium. Maintaining sodium:calcium$\leq$25:1 will help avoid gel destabilization. In fact, it is recommended by alginate vendors to include 3mM calcium ions in the substrate medium. On the other hand, citrate or phosphate pH buffers cannot be effectively used without destabilizing the alginate gel.

Alginate is currently widely used in food, pharmaceutical, textile, and paper products. The properties of alginate utilized in these products are thickening, stabilizing, gel-forming, and film-forming. Alginate polymers isolated from different alginate sources vary in properties. Different algae, or for that matter different part of the same algae, yield alginate of different monomer composition and arrangement. There may be sections of homopolymeric blocks of only one type of monomer (-M-M-M-) (-G-G-G-), or there may be sections of alternating monomers (-M-G-M-G-M-). Different types of alginate are selected for each application on the basis of the molecular weight and the relative composition of mannuronic and guluronic acids. For example, the thickening function (viscosity property) depends mainly on the molecular weight of the polymer; whereas, gelation (affinity for cation) is closely related to the guluronic acid content. Thus, high guluronic acid content results in a stronger gel.

List of Reagents and Instruments

A. Equipment

Beakers

Graduated cylinder

Balance

Pipets

Syringe

B. Reagents

Alginic acid, sodium salt

$CaCl_2$

Enzyme

Procedures

1. Dissolve 30g of sodium alginate in 1 liter to make a 3% solution. See Note 1.
2. Mix approximately 0.015 g of enzyme with 10 ml of 3% (wt.) sodium alginate solution. The concentration of sodium alginate can be varied between 6-12 % depending on the desired hardness. See Note 2.
3. The beads are formed by dripping the polymer solution from a height of approximately 20 cm into an excess (100 ml) of stirred 0.2M $CaCl_2$ solution with a syringe and a needle at room temperature. The bead size can be controlled by pump pressure and the needle gauge. A typical hypodermic needle produces beads of 0.5-2 mm in diameter. Other shapes can be obtained by using a mold whose wall is permeable to calcium ions. Leave the beads in the calcium solution to cure for 0.5-3 hours.

Notes

1. Sodium alginate solution is best prepared by adding the powder to agitated water, rather than vice versa, to avoid the formation of clumps. Prolonged stirring

may be necessary to achieve the complete dissolution of sodium alginate. After sodium alginate is completely dissolved, leave the solution undisturbed for 30 minutes to eliminate the air bubbles that can later be entrapped and cause the beads to float.

2. Although not necessary, the beads may be hardened by mixing some amines in the sodium alginate solution and cross-linking with glutaraldehyde.

Discussion

Because of the mild conditions needed for gelation, calcium alginate is also widely used for cell immobilization.

Reference Readings

1. S. Ohlson, P.-O. Larsson, and K. Mosbach, Steroid transformation by living cells immobilized in calcium alginate, *European J. Appl. Microbiol. Biotechnol.*, **7**, 103, 1979.

2. J. Vaija, et al., *Appl. Biochem. Biotechnol.*, **7**, 51, 1982.

3. J.M. Lee and J. Woodward, *Biotech. Bioeng.*, **25**, 2441, 1983.

EXPERIMENT NO. 7 – SUPPLEMENT C

ENZYME IMMOBILIZATION PROTOCOL
ENTRAPMENT IN GELATIN GEL

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Method

Since this process is quite familiar to all of us who have ever made Jell-O, there is really no need to elaborate. The only difference is that the gel is hardened to provide structural strength.

List of Reagents and Instruments

A. Equipment

Beakers

Graduated cylinder

Pipets

Constant temperature bath

Freezer

B. Reagents

Gelatin Solution, 10 wt %

Hardening Solution

20 vol% Formaldehyde

50 vol% Ethanol

30 vol% Water

Enzyme

Procedures

1. Dissolve 10 g gelatin in 100 ml of water to prepare a 10% (wt.) aqueous solution. Heating the solution gently to facilitate the dissolution process.
2. Adjust the temperature of the gelatin solution to 35-40°C. The temperature is kept relatively high so that the gelatin solution is not too viscous, but not so high as to cause enzyme denaturation.
3. Add approximately 0.015 g of the enzyme powder (or an equivalent of concentrated enzyme solution) to 10 ml of the gelatin solution.
4. Add 2 ml of the Hardening Solution to the above enzyme solution.
5. Pour the solution into a mold or a small beaker. Freeze at -28°C for 4 hours to facilitate the gel formation. If this temperature is not readily available, a regular freezer will also suffice for the purpose of demonstrating the technique.
6. When the gel is set, warm the gel to room temperature simply by leaving it on a lab bench.
7. Cut the gel into small cubes of approximately 3mm per side.
8. Gently wash the gel liberally with deionized water.

Discussion

The gel may be liquefied by raising the temperature; however, this may also inactivate many heat labile enzymes.

Reference Readings

1. E. de Alteriis, P. Parascandola, S. Salvadore, and V. Sardi, Enzyme immo-

bilization within insolubilized gelatin, *J. Chem. Tech. Biotechnol.*, **35B**, 60, 1985.

EXPERIMENT NO. 8

YOGURT FERMENTATION WITH LACTOBACILLUS CULTURES

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Objective

To demonstrate the use of microorganisms in food processing by using yogurt as an example.

Introduction

Actually this experiment has already been performed. One may have noticed in Experiment No. 1 that mushy substance formed during the prolonged precuring process in cheese manufacturing in which the natural action of lactose fermenting culture originally resident in butter milk was utilized to acidify milk. Of course, this custard-textured substance was none other than yogurt, sometimes spelled yoghurt or yoghourt.

Other than cheese, buttermilk, and yogurt, lactic starter cultures are also used to help prepare or manufacture a wide variety of food products such as sour dough bread, pickles, and sausages. As implied by the name "lactic cultures," they belong to a category of microorganisms that can digest the milk sugar lactose and convert it into lactic acid. For the cells to utilize lactose, deriving carbon and energy from it, they must also possess the enzymes needed to break lactose into two compo-

nents sugars: glucose and galactose. Some representative strains are *Streptococcus lactis, S. cremoris, S. thermophilus, Lactobacillus bulgaricus, L. acidophilus,* and *L. plantarum.* These cultures can be purchased directly from local health food and drug stores in tablet form. These tablets, taken orally during the intake of dairy products, help those people who have digestive tract disorder and cannot tolerate lactose. The major steps involved in a large scale production of lactic starter cultures are the following: media preparation (constitution, mixing, straining, sterilization), inoculum preparation, fermentation, cell concentration by centrifugation, liquid nitrogen freezing, and packaging.

In summary, commercial yogurt production is composed of the following steps: pretreatment of milk (standardization, fortification, lactose hydrolysis), homogenization, heat treatment, cooling to incubation temperature, inoculation with starter, fermentation, cooling, post-fermentation treatment (flavoring, fruit addition, pasteurization), refrigeration/freezing, and packaging. For set yogurt, the packaging into individual containers is carried out before fermentation. In addition to the above steps, the starter culture is propagated in parallel. Although a batch process is followed in this illustrative experiment, the commercial production of yogurt is carried out in an automated continuous fermentation process. A good strain of starter culture not only affects the flavor and aroma, it can also speed up the process and thus reduces the effective equipment cost.

List of Reagents and Instruments

A. Equipment

Beakers

Heat source

Incubator, 43°C

Thermometer

B. Reagents

Milk

Starter culture or plain yogurt from local stores

Procedures

1. Heat 1 liter (approximately 1 quart) of milk in a beaker slowly to 85°C and maintain at that temperature for 2 minutes. This step kills undesirable contaminant microorganisms. It also denaturizes inhibitory enzymes that retard the subsequent yogurt fermentation. If you are attempting this procedure at home with a sauce pan, use caution so as not to allow the milk to boil over and make a mess on your kitchen stove. See Note 1.

2. Cool milk in a cold water bath to 42-44°C. The cooling process should take about 15 minutes.

3. Add 5 g of starter culture to the cooled milk and mix with a glass rod. See Note 2.

4. Cover the container to minimize the possibility of contamination. Incubate at 42°C for 3 to 6 hours undisturbed until the desired custard consistency is reached. Yogurt is set when the mixture stops flowing as the container is tipped slowly. Fluid yogurt results if the mixture is stirred as the coagulum is being formed. See Note 3.

5. The fresh made yogurt is ready for consumption when it is set. However, you may want to refrigerate it first if you are not accustomed to warm yogurt. Refrigeration also stops the growth of the lactic acid culture, which is thermophilic. (Thermophilic cultures grow best at high temperatures.) See Note 4.

6. *Use of Lactobacillus acidophilus:* Grind 4 yogurt tablets (about 1 g) into fine

powder. Repeat Steps 3-5.

7. *For entrepreneurs or simply hungry/thrifty students:* You can recycle a small part of the finished product as the starter culture for the next batch. Theoretically, you can multiply or maintain your supply of yogurt indefinitely. However, in actuality, extended recycling is not recommended because the composition of the mixed culture will gradually deviate from the ideal one, and hence the flavor.

Notes

1. Any type of milk may be used. Use nonfat or lowfat milk you are watching your fat intake. For example, one cup of nonfat dry milk powder dissolved in one liter of hot water may be convenient. The consistency and the flavor of the final product depend on the type of milk used. You may experiment at home to find your favorite recipe.

2. The yogurt in a local market usually contains an active culture. Thus, if a starter culture is not readily available, it can be easily derived from plain store-bought yogurt. In this case, a few teaspoonfuls of the store-bought yogurt will adequately act as as the starter culture. (Make sure the label on the package indicates that it indeed contains an active culture.) The culture in fresh yogurt is healthier and more active than that in an outdated one. A stale one is also more likely to be contaminated with undesirable microorganisms, so check the expiration date. If possible, choose the "All-Natural" variety, because stabilizers and additives, included to suppress microbial activities, are generally harmful to the culture. If one is making yogurt at home, it is more convenient to pour the mixture into smaller containers before incubation; drinking glasses are just about the right serving size. Seal the glasses with a lid or plastic food wrap. Place all the glasses in a baking pan for easy handling.

3. At home, a household electric or gas oven is an ideal substitute for the incubator. The middle shelf, slightly away from the direct heat, usually gives the most even temperature. The temperature can be controlled better if a pan of warm water is placed on the bottom rack.

4. You may add your favorite fruits, fruit preserves, puree, jam, or sweeteners to enhance the taste, or you may add equal part of water to make a yogurt drink. Many types of yogurt differ mainly in the post-incubation processing. For example, the yogurt may be frozen, spray-dried or freeze-dried, carbonated, or concentrated.

Discussion

Yogurt originated in the Balkans and the Middle East; it is now quite popular in Europe and America, as well. The microorganisms used in the production of yogurt accomplish two tasks: production of lactic acid and flavor components. The secret to tasty yogurt is in the proper control of the temperature at various stages. If the temperature is too low, the culture grows too slowly to adequately acidify milk and to achieve a good texture. The commercial starter is a mixed culture of S. thermophilus and *L. bulgaricus*. The culture is killed if the temperature is too high. In addition, there is a subtle difference in the taste because the formation and secretion of metabolites which contribute to the overall taste are dependent on the growth rate. The window of proper fermentation is quite small, i.e. from 42°C to 44°C. In general, as the temperature is raised up to 44°C, the rate of culture metabolism is higher, and the yogurt is sweeter. Faster growth also prompts the yogurt to set faster. When the desired acidity is reached, yogurt is quickly cooled to halt further fermentation and metabolic activity. This cooling step is quite critical in industrial yogurt production; it must be done quickly to control tightly the acidity of the yogurt, which has a profound effect on the taste.

Questions

1. Compare the texture and taste of yogurt made from different sources of starter cultures. Also compare your "homemade" yogurt to commercial brands.
2. What was the cost of "homemade" yogurt? Compare this to the market price of a comparable item. Did you make a profit?
3. The yogurt starter culture is a mixed population of *S. thermophilus* and *L. bulgaricus*, both competing for the common substrate lactose. How does the principle of competitive exclusion apply here?
4. Can the human intestinal tract be infested with lactic acid cultures? If yes, why has this method not been employed to to treat lactose-intolerant consumers who cannot intake dairy products without the usual gastro-intestinal discomfort? If no, what make these strains different from, for example, *Escherichia coli*, normal flora of digestive tract?
5. Why is the shelf life for unpasteurized yogurt longer than that for pasteurized milk?
6. Comment on ways to improve the experiment.

Reference Readings

1. Tamime, A. Y. and Deeth, H. C. Yogurt: technology and biochemistry, *J. Food Protection*, **43**, 939, 1980.
2. Driessen, F. M., Ubbels, J., and Stadhouders, J., Continuous manufacture of yogurt. I. Optimal conditions and kinetics of the prefermentation process, *Biotech. Bioeng.*, **19**, 821, 1977.

EXPERIMENT NO. 8 – SUPPLEMENT A

ASEPTIC CULTURE TECHNIQUES — USE OF A STEAM AUTOCLAVE

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Introduction

Practice the procedures, especially the closing and opening of the autoclave door, without introducing any steam into the autoclave chamber until the entire sequence can be performed without any difficulty. The student is authorized to use the autoclave only after he has demonstrated to the instructor his ability without referring back to this write-up. Do not use the autoclave if the student is not *absolutely* sure of the proper operating procedures. The pressurized steam is more likely to cause burns than boiling water. First, it is hotter than boiling water. The temperature and pressure relationship is shown in the following table. More importantly, the heat of vaporization released upon the condensation of steam causes much severer damage than does the same quantity of boiling water. Always watch out for the hot metal parts on the autoclave. Wear heat insulating (asbestos) gloves when handling hot autoclaved items.

Temperature and Pressure Relationship

Steam Pressure (psi∿)	Temperature (°C)				
	AIR DISCHARGE				
	100%	66%	50%	33%	0%
0	100.0				
1	101.9				
2	103.6				
3	105.3				
4	106.9				
5	108.4	100	94	90	72
6	109.8				
7	111.3				
8	112.6				
9	113.9				
10	115.2	109	105	100	90
11	116.4				
12	117.6				
13	118.8				
14	119.9				
15	121.0	115	112	109	100
16	122.0				
17	123.0				
18	124.1				
19	125.0				
20	126.0	121	118	115	109
21	126.9				
22	127.8				
23	128.7				
24	129.6				
25	130.4	126	124	121	115
26	131.3				
27	132.1				
28	132.9				
29	133.7				
30	134.5	130	128	126	121

⋆ Around the working range of 15 psi, each psi raises the autoclave temperature by approximately 1 °C.

Operating Procedures

1. Check the chamber pressure (GAUGE # 2 in the Figure) if the autoclave door is already closed and locked, because a locked door usually indicates that autoclaving is in progress. Do not attempt to open the door unless the chamber pressure is at ambient. Opening a chamber filled with pressurized steam can be suicidal. Although some newer models of autoclaves have built-in safety features to guard against this possibility, never subject one's own well being to such a shaky assumption. Check the jacket pressure (GAUGE # 1). If the gauge indicates a pressure of between 15-17 psig, proceed to Step 4; otherwise, continue onto Step 2.

2. Open the condensate valve (VALVE # 1 in the Figure).

3. Slowly open the steam supply valve (VALVE # 2). Allow condensate to drain. When the condensate is completely drained, as evidenced by the flow of steam from the outlet of VALVE # 1, close the condensate valve (VALVE # 1). (If pipes start to "bang" hard during condensate drainage, close VALVE # 2 slightly.) Wait for the jacket pressure to build up to the required 15-17 psig.

4. Open the door on the autoclave slowly and cautiously. Take out all the items from the previous autoclaving runs if they are found inside the chamber. Place items to be autoclaved inside the chamber. Make sure that all the components of each item can withstand the heat of autoclaving; many plastic materials will melt or deform. Leaving a few drops of water inside a closed container where the steam in the chamber cannot easily penetrate will enhance the sterilization effect. If liquid is to be autoclaved, place all such containers in a stainless steel pan so that any spill can be readily caught. Many liquids will boil over in the autoclave. The boiling is especially violent when the pressure is released at the end of the autoclave cycle. The use of a pan will also facilitate handling if more

than one container is to be autoclaved. Vent all containers to avoid explosion. If bottles are capped, make sure that the caps are screwed on lightly so that any excess pressure can escape. The caps can be screwed tightly later as they are taken out from the autoclave. At the same time, make sure that everything is closed so that contaminants cannot enter after autoclaving. Note that the need for pressure relief and prevention of contaminant entry are not contradictory. For example, shaker flasks can be vented with cotton seals; fermentor jars, and nutrient jars can be vented with in-line filter units. On the other hand, some items to be autoclaved cannot be conveniently vented; they must be placed in a protective steel casing. The glass pH electrodes are such examples.

5. Near the center of the door locking wheel, there is a pin that, when in the inserted position, prevents the door locking wheel from turning freely. The pin is released from its hole by pushing the small (approx. 2 inches) lever parallel to the face of the door locking wheel. To engage the pin, set the lever perpendicular to the face of the door locking wheel, then turn the wheel slightly to allow the pin to drop into one of the holes intended for it. Close the autoclave door. Engage the pin, and turn the wheel clockwise by about 1/8 of a turn to insert *all* the locking bars in the respective slots on the rim of the autoclave. These bars do not always all slide into the slots at the first attempt. If so, wiggle the bars as the wheel is turned. After make sure that all the the locking bars extend securely through the slots, release the pin so that the door locking wheel is now free to spin. Rotate the wheel clockwise until it is hand-tight; this applies pressure to the door to seal it air tight so that steam pressure can be built up inside the chamber. Do not over tighten the wheel. Engage the pin to lock the wheel and prevent it from turning back. The door is now securely locked.

6. Turn the four-position selection valve (VALVE # 3) on top of the autoclave to the sterilize position (STER) by lining up the STER mark with the arrow on the valve. This position allows the steam to enter the autoclave chamber from the jacket.

7. Wait for the chamber pressure, as indicated by GAUGE # 2, to reach the maximum, where the pressure is to remain stationary during the entire autoclave cycle. The pressurization of the chamber will take approximately 5 minutes. This marks the start of the autoclave. The temperature in the chamber can be read with GAUGE # 3 at the lower front of the autoclave.

8. After autoclaving for 20-30 minutes, terminate the autoclaving cycle by shutting off steam to the chamber and venting it slowly to the ambient pressure. This can be accomplished by turning VALVE # 3 from the sterilization position (STER) to the exhaust position (EXH). It takes about 3-5 minutes to vent the excess steam. It is safe to open the door only when the needle of the chamber pressure indicator, GAUGE # 2, falls within the narrow white area marked around 0 psig. When the chamber reaches ambient pressure, release the pin that locks the door locking wheel. Slowly turn the wheel counterclockwise to relieve pressure on the door. When the door locking wheel is fully released, engage the pin and turn the wheel 1/8 turn further to disengage the locking bars from the slots. Open the door slowly. Watch out for a small puff of residual hot steam being released as the door is first opened. Note that a slight vacuum may be created in the chamber as the hot air is cooled further while the door is left locked. In this case, a screwdriver may be used to pry open the autoclave door with care; however this is not recommended for beginning students. Alternatively, introduce steam into the chamber for a few seconds after securely locking and pressurizing the door will bring back a positive pressure

in the chamber.

9. Again, remember to wear gloves. Watch out for the boiling liquid and handle the hot autoclaved items with great care so as not to burn oneself. Do not touch the hot chamber wall. Now is the time to make sure that the sterile side of all the sterilized items are not open to invasion by contaminants. For example, plug the mouth of shaker flasks if the plugs have come off during autoclaving. Check all connections on a fermentor jar to make sure that they all remain sealed and that no tubings have been broken. Reconnect them quickly whenever possible. Remove all autoclaved items from the chamber.

10. Clean up any spills and close the autoclave door when done. Do not pressurize the door.

Summary of Procedures

1. Check the jacket pressure.

4. Place items in the chamber.

5. Close and lock the autoclave door.

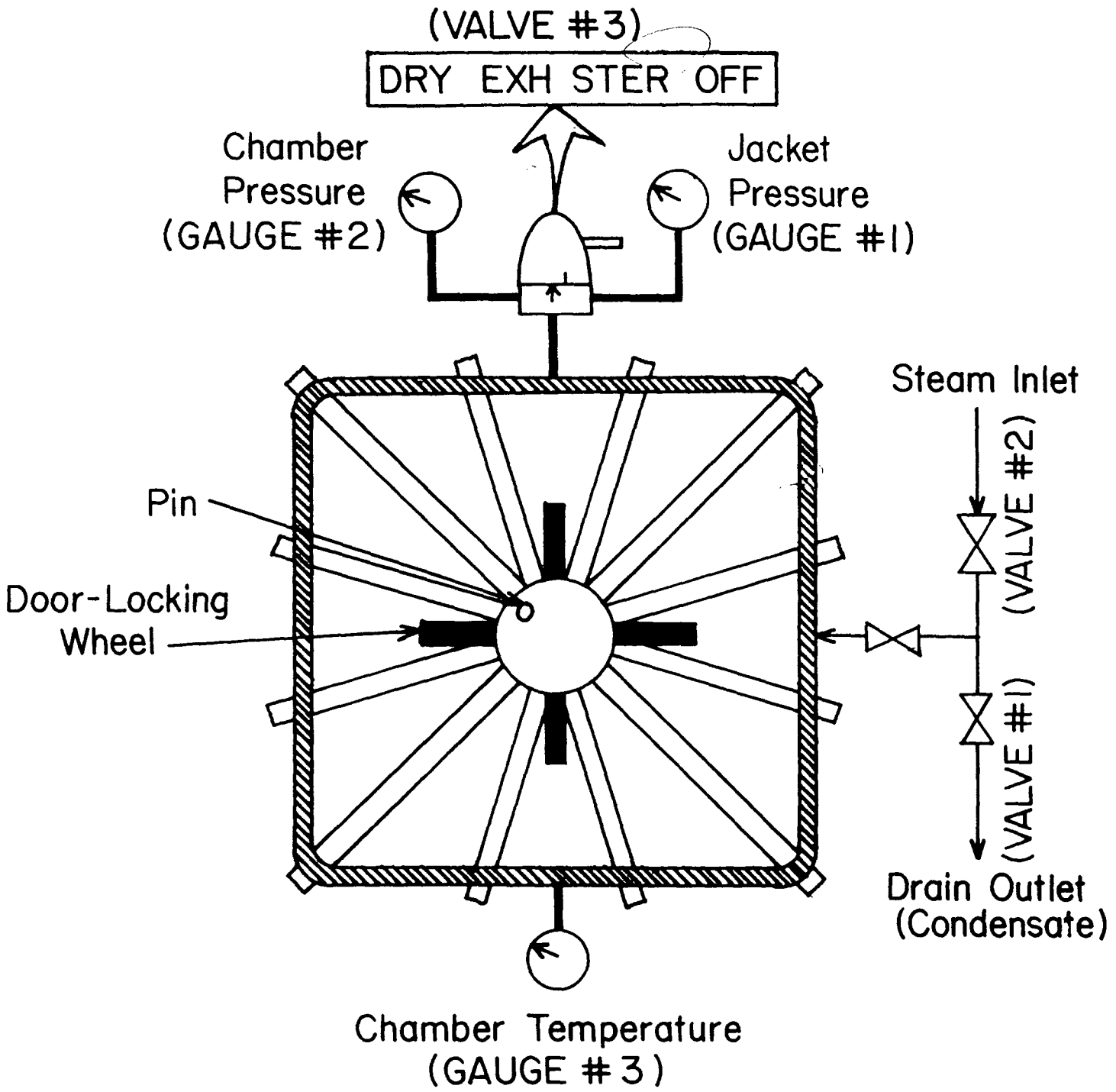
6. Introduce steam into the chamber.

7. Autoclave for 20-30 minutes.

8. Unlock and open the autoclave door.

9. Remove autoclaved items from the chamber.

10. Clean up.



STEAM AUTOCLAVE

EXPERIMENT NO. 8 – SUPPLEMENT B

ASEPTIC CULTURE TECHNIQUES — PETRI DISH PREPARATION

Prepared by

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ENCH 485, Spring 1988

Objective

To demonstrate pure culture techniques with Petri dishes.

Introduction

In microbiological and biochemical engineering studies, one almost always deals with a pure culture or a mixture of known cultures, except perhaps in wastewater treatment studies. Unless aseptic culture techniques are followed strictly, an originally pure culture will definitely become contaminated with other unwanted species. For illustrative purposes, an extraterrestrial who has never seen a human being simply cannot accurately study human behavior if the group of subjects to be observed includes, in addition to humans, monkeys, dogs, cats, and many more other "contaminants." The results of such a study will certainly be unreliable. Similarly, the use of a contaminated culture with unknown microorganisms will only lead to incredible results that are of little value. Thus, the isolation and maintenance of a pure culture is of utmost importance in many microbiological studies.

It is especially important to work with a well characterized strain if the microorganism is used for food preparation, as well as in antibiotic production where

the product is to be taken internally. For the same reason that a student will not lick an incubated plate that was exposed to the air, the presence of a large number of contaminants may present a potential health hazard, especially when the exact natures of the contaminants are unknown. Licking that agar plate probably will not kill the student; however, the probability of fatality certainly increases with higher numbers of organisms. Conversely, the probability of a wasted fermentation run due to contamination is directly proportional to the number of microorganisms in the environment, which in turn is directly related to the cleanliness of the laboratory.

The need for a clean working environment in biochemical engineering studies must be stressed again, for cleanliness is the prerequisite to any meaningful work. (This obsession is not the result of the instructor having had bad toilet training as Sigmund Freud may have concluded.) As demonstrated by the plate transfer process, many parts of the aseptic procedures require occasional exposure to the surrounding environment. Since our laboratory cannot be made totally sterile economically, it is imperative that the room be kept clean. The use of a laminar hood, which creates an air curtain to reduce the chance of contaminants drifting into the working space enclosed by the hood, is highly recommended if it is available. However, a laminar hood itself must still be maintained in a clean condition.

List of Reagents and Instruments

A. Equipment

Erlenmeyer flasks

Culture tubes

Petri dishes, 100×15mm

Bunsen burner

Inoculation loop

Autoclave

Balance

Magnetic Stirrer or stirring rod

Incubator, 37°C

Thermometer

Refrigerator

B. Reagents

Nutrient media:

- Yeast extract
- Peptone
- Agar
- Glucose
- Lactose
- Sucrose
- Dipotassium phosphate
- Eosin
- Methylene blue

HCl, 0.1 N solution

KOH, 0.1 N solution

Procedures

1. Petri dish preparation:

   a. Mix the following nutrient ingredients in proportion to the amount required. Note that a Petri dish pour requires about 12 ml and a slant needs 6 ml. Adjust the pH to the desired value (pH=5.0) with 0.1N HCl or 0.1N KOH. If a large number (more than 5) of plates are to be poured, mix the nutrient in 1-2 liter flasks. It is difficult to handle flasks larger

than 2 liters with one hand. If only a few (less than 5) are needed, the nutrient can be divided and poured into test tubes, each holding enough media for one Petri dish. The advantage of using test tubes is that they can be autoclaved separately and may later be heated to melt the agar in a beaker of boiling water. The liquefied agar may then be poured into a Petri dish directly from the test tube. However, the use of test tubes is not practical when making large quantities of agar plates.

YPG (Yeast extract-Peptone-Glucose) Agar

Yeast extract	5g
Peptone	10g
Glucose	5g
Agar	15g

Add water to make 1 liter

EMB (Eosin-Methylene Blue) Agar

Peptone	10g
Lactose *	5g
Sucrose *	5g
Dipotassium phosphate	2g
Agar	13.5g
Eosin	0.4g
Methylene blue	0.065g

Add water to make 1 liter

*5 g/l of glucose may be substituted for lactose and sucrose.

b. Cover the flask with a beaker or a piece of aluminum foil, and cover the culture tubes with caps, cotton plugs, or gauge plugs. Autoclave the media

for 20 minutes. The heat of sterilization will dissolve the agar. Place a watch glass over the flask.

c. Set the media on a bench top and cool until the flask can be handled with your bare hands. Wrap paper towel around the neck of the flask when pouring agar into Petri dishes just in case the media reheats the glassware as the hot liquid passes through the neck in the process of pouring. Of course, one cannot pour out a block of solidified agar if the flask is cooled excessively. The agar will solidify at approximately 42°C; thus, the temperature window for handling the liquefied agar is quite narrow. If the agar starts to solidify at the bottom as a result of not pouring fast enough, the flask can be gently heated with a flame to raise the temperature and melt the agar.

d. Stack sterile Petri dishes in a row, three or four per stack, on a disinfected lab bench. The number os dishes per stack can be adjusted according to the size of the student's hand. With the right hand holding the neck of the flask, open the cover of the lowest Petri dish with the left hand just wide enough to pour the media. (The top cover is the larger dish of the two; the actual dish is the smaller one.) Pour about 12 ml into each Petri dish. If too little agar is poured, there may not be enough to cover the dish or the agar plate will dry up easily. If too much is poured, the cover dish will come in contact with the nutrient agar, leaving no room for microbial growth. The plates are rendered useless either way. After pouring the bottom dish, pour the second one from the bottom, and so on until the entire stack is poured. Proceed to the next stack until all dishes are poured.

e. Immediately clean the flask with hot water before the residual agar solid-

ifies, or have fun painstakingly scraping out the solidified agar from the flask.

f. Leave the plates undisturbed until the agar solidifies. The plates may now be streaked.

2. Slant tube preparation:

   a. Mix and prepare the YPG nutrient agar as in the preparation of Petri dishes. Pour 6 ml of the nutrient into a test tube. Plug the test tube with a cotton ball or a cap. Culture tubes with screw caps may also be used.

   b. After autoclaving the media for 20 minutes, the tubes are placed in a slanted position to allow the agar to solidify. These tubes are called *slants.*

3. Plate streaking with inoculum from a liquid suspension: This entire sequence must be repeatedly practiced with an empty Petri dish and plain water until it can be carried out smoothly without referring back to this write-up.

   a. Gently shake the flask or tap the bottom of the culture tube with your finger to suspend microbial cells in the broth that are to be transferred. Watch out for the broth level and do not wet the cotton plug when shaking.

   b. Light a Bunsen burner and flame an inoculation loop until the wire turns red hot. This will kill all the bugs on the loop surface. Slightly flame the handle as well to minimize the chance of contamination.

   c. Cool the loop for about 5 seconds.

   d. While the loop is cooling, with the thumb and index finger of the right hand holding the inoculation loop and with the left hand holding the flask or culture tube, take out and hold onto the plug with the baby finger and palm of the right hand. Quickly pass both the neck and rim of the flask through the flame.

e. Carefully dip the loop into the broth that contains the microorganism to be streaked. Note that only the small loop at the tip needs be dipped, not the entire extension of the wire leading to the loop and definitely not the handle. Since the handle is not really well sterilized, do not touch the side of the flask or culture tube with the handle.

f. Flame the neck and rim of the flask or tube as before. After lightly flaming the bottom and side of the plug, place the plug back into the mouth of the flask. Note that the side and bottom of the plug never comes in contact with anything but air; the plug is never placed on a table.

g. Put down the flask and pick up a Petri dish with the left hand. Flip open the cover plate with the middle finger and the thumb. *Gently* stroke 3-5 parallel lines at one corner of the plate with the loop to spread the cells. (Lines marked A in the following Figure.) Re-close the Petri dish; the best results are achieved when the dish is recovered immediately following the streaking. Do not gouge the agar with the loop; a light touch is all that is necessary to deposit the culture onto the plate.

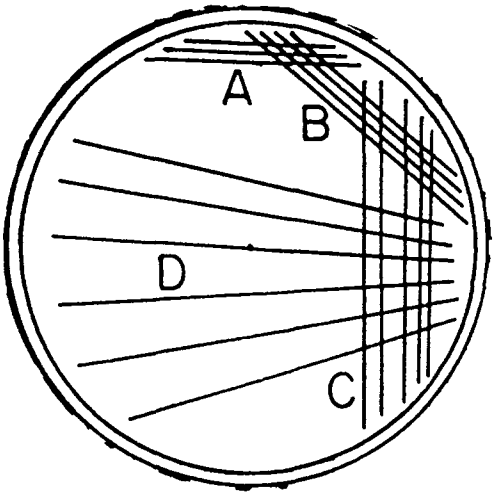


Figure 1. Streak patterns on an agar plate.

h. Flame the loop as before and cool it for about 5 seconds. Gently touch the free surface of the agar plate to cool it further to avoid killing all the organisms upon the initial contact. Spread the cells further with 5-6 strokes (Lines B in the Figure); start each stroke by crossing the original set of lines.

i. Repeat the above step twice more to create Lines C and D, flaming the loop each time before streaking.

k. When done streaking, flame the loop for the last time before returning it to the bench. This is to prevent the spread of microorganisms on the bench. This last step is especially crucial when working with pathogenic strains.

l. Incubate the inoculated plate upside-down at 37 °C for 48 hours. Store the plate, also upside-down, in a refrigerator thereafter.

3. Plate streaking with inoculum from another plate: Repeat the above steps of plate streaking, except that the inoculum is taken from another Petri dish.

a. Find a single colony on the original agar plate that is located nearby Line D and is physically isolated from all other colonies on the same plate. This colony is homogeneous in the sense that all the cells degenerate from the same parent. Thus, this can be considered as a pure culture.

b. Flame the loop and lift a minute amount of culture from the original plate. Remember that one need not see the colony of organisms on his loop to prove that that the microorganisms are indeed there. There is already too much if one can see the culture on the inoculation loop.

c. Transfer the cells in the loop to a new agar plate by following the same set of steps involved in streaking a plate as before. Incubate the inoculated

plate.

Discussion

A wide variety of dehydrated medium formulations are available from Baltimore Biological Laboratory (BBL) Microbiology Systems (a division of Becton, Dickson & Co, Cockeysville, Maryland) or Difco Laboratories (Madison, Wisconsin).

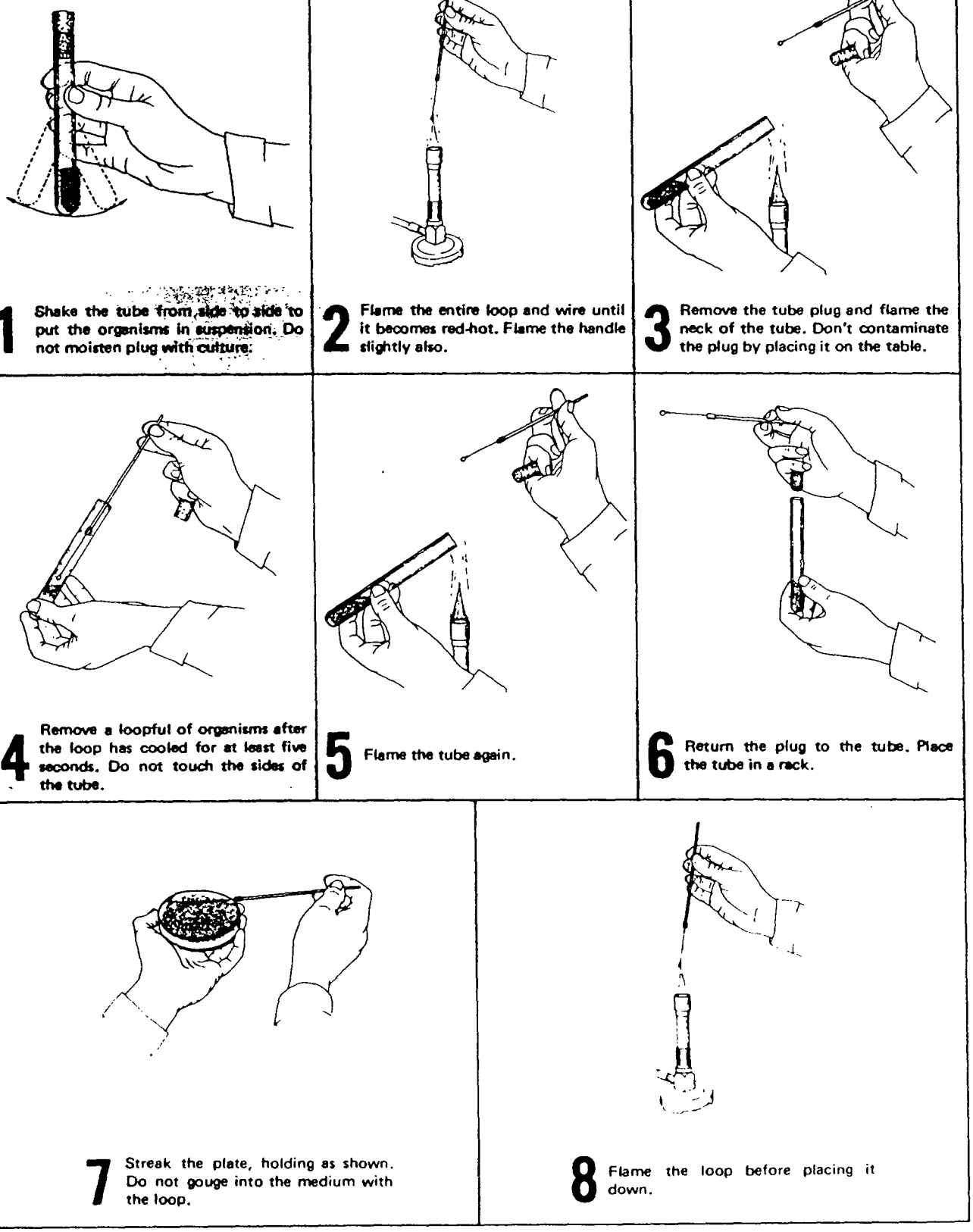
Uninoculated agar plates may stacked and stored *upside down* in a refrigerator for about 3 months if not used immediately. The inverted position ensures that water does not condense on the bottom of the cover plate and retards the loss of moisture. An inoculated agar plate is also incubated and stored upside down to retain moisture. In addition, the water condensate can act as a medium for transport of cells from one location on an agar plate to another. The slants may be stored in a refrigerator for over a year if culture tubes with screw caps are used.

Incubation of the inoculated plates should last for about 48 hours. They are stored in a refrigerator, and the culture can stay viable for over one month in agar plates or for one year in sealed slants. Prolonged storage of the plates in the incubator dries up the agar and kills the culture. The severe loss of moisture can be visually detected by the appearance of macroscopic cracks that develop in the agar plate.

The used agar plates and other biological wastes should be autoclaved in a pan to destroy all biological activities before being disposed.

Questions

1. Why is the agar in test tubes allowed to solidify in a slanted position?
2. During the preparation of an streak plate, why flame the loop between different sets of strokes?



1 Shake the tube from side to side to put the organisms in suspension. Do not moisten plug with culture.

2 Flame the entire loop and wire until it becomes red-hot. Flame the handle slightly also.

3 Remove the tube plug and flame the neck of the tube. Don't contaminate the plug by placing it on the table.

4 Remove a loopful of organisms after the loop has cooled for at least five seconds. Do not touch the sides of the tube.

5 Flame the tube again.

6 Return the plug to the tube. Place the tube in a rack.

7 Streak the plate, holding as shown. Do not gouge into the medium with the loop.

8 Flame the loop before placing it down.

Figure 21-3 Inoculation routine

EXPERIMENT NO. 8 – SUPPLEMENT C

ASEPTIC CULTURE TECHNIQUES — BUGS ARE EVERYWHERE!!

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ENCH 485, Spring 1988

Objective

To demonstrate the necessity of aseptic techniques.

Introduction

The fact that microorganisms are everywhere must be realized and firmly believed by all students if nothing else is to be learned in this course. This fact will be demonstrated by briefly exposing Petri dishes to the ordinary working environment in a laboratory. However, keep in mind that the presence of potential contaminants need not hinder biochemical engineering experiments so long as the investigator makes a conscious effort to maintain a tidy, clean lab and to practice aseptic techniques.

List of Reagents and Instruments

A. Equipment

Incubator, 37°C

B. Reagents

YPG Petri dishes

Procedures

1. Obtain the prepared Petri dishes from the instructor and expose them to the following:
    a. Expose to the air for 30 minutes
    b. Touch with unwashed fingers
    c. Touch with fingers washed with water and soap
    d. Touch with fingers dipped in ethanol
    e. Kiss with moist lips
    f. Press lightly against the lab door knob
    g. Press lightly against the restroom door knob
    h. Smear a little yogurt
    i. Smear 1 ml of milk
    j. Press coins
    j. Press against the bottom of a flask or beaker
    k. Cough vigorously from 4-6 inches away
2. These plates should be labeled appropriately and incubated upside down at 37°C for 48 hours.

Notes

1. If culture tubes are used instead of petri dishes, cover the tubes with finger. Shake, then incubate.

Discussion

There are actually many more numbers and varieties of microorganisms in our immediate environment than those appearing on the incubated plates. Some of

these will not grow in aerobic conditions or on glucose, but will flourish in different media.

When working with microorganisms, it is important that the working environment stays as clean as possible. For example, a minuscule puff of mold spores can drift in air for an extended period of time and contaminate many subsequent experiments. Some of the spores can withstand even the temperature existing inside an autoclave and emerge from the sterilization process in dormant but viable forms. In general, the chance of contamination is proportional to the number of microorganisms in the environment when the same sets of aseptic procedures are taken. Of course, contamination means nothing but the waste of both the materials and the time spent. It can be an extremely maddening experience even for the calmest researcher. It is one's prerogative to be dirty or clean in one's own living quarters, but it is plainly irresponsible not to practice good aseptic techniques and keep the working environment clean, for not only himself but for everyone else as well. All the autoclaving will not help much in reducing the chances of contamination if the rest of the working environment is full of bugs as a result of improper attention to cleanliness. Although the presence of microorganisms cannot be totally eliminated in our lab economically, the numbers can certainly be reduced drastically by *orders of magnitude.* Uncleanliness simply does not pay in biochemical engineering!

Questions

1. Describe the morphology of the colonies growing on the plates (color, texture, size, shape, elevation, number of colonies, etc.)
2. You breath the air and lick your finger without much hesitation. Will you lick the plate full of microorganisms? Why?

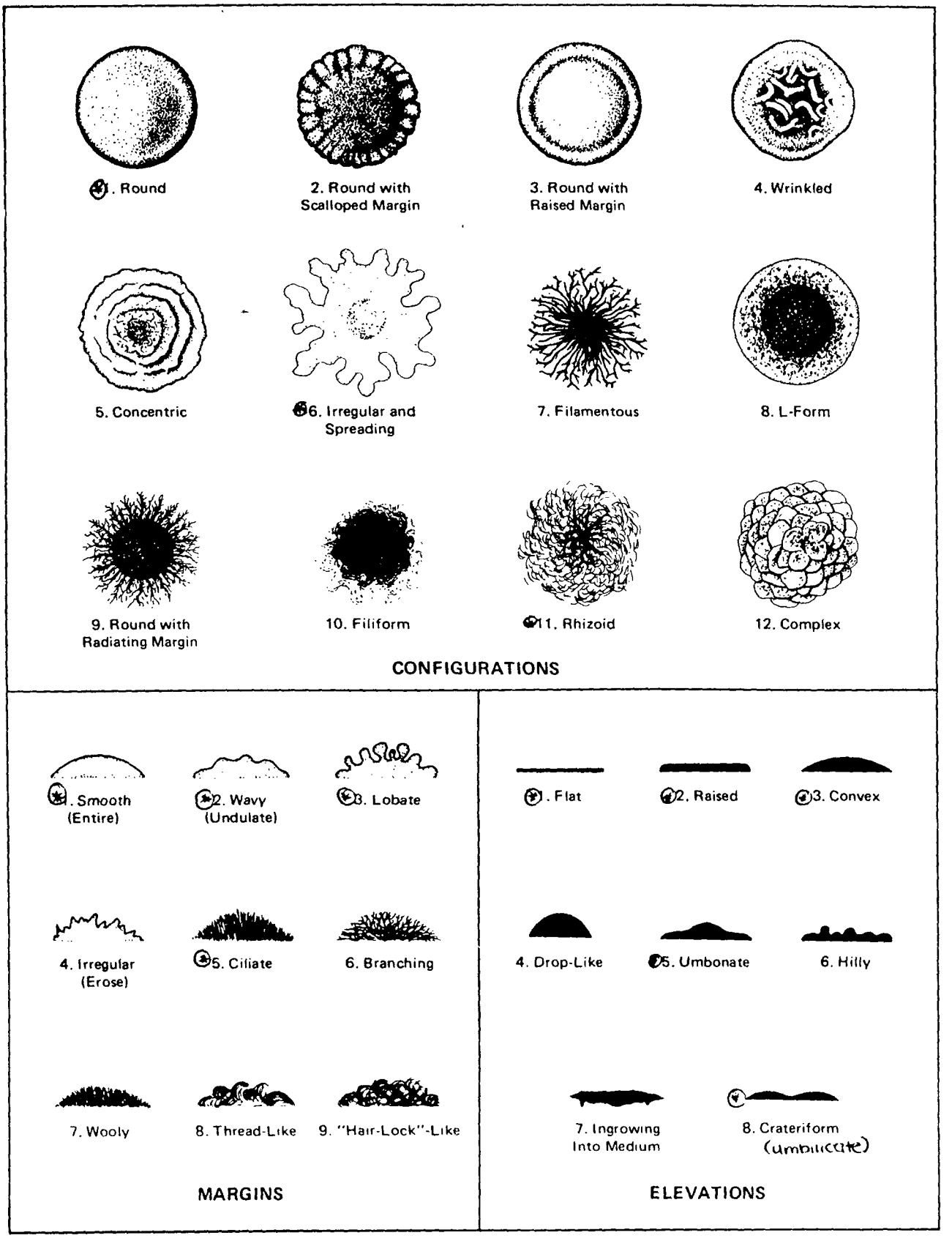

Figure 34-5 Colony characteristics

morphotypes = structural characteristics

Resolving Power

It might seem that infinite magnification could be achieved simply by increasing the power of the ocular and objective, but lenses are limited by a phenomenon known as resolving power, or resolution. As two small objects are moved closer to each other, a point is reached where the lens is unable to distinguish the objects as separate entities, and only a single object is observed. The smallest distance at which two points can be seen separately is called the **resolving power** of the lens. The resolving power of the human eye at ten inches is 0.1 mm. (100 μm).

The resolving power is determined by the wavelength of light and the numerical aperture. The relationship of these two factors can be expressed as follows:

$$\text{Resolving power} = \frac{\text{wavelength}}{\text{numerical aperture}}$$

If you keep in mind that a high resolving power represents a low numerical value, it is

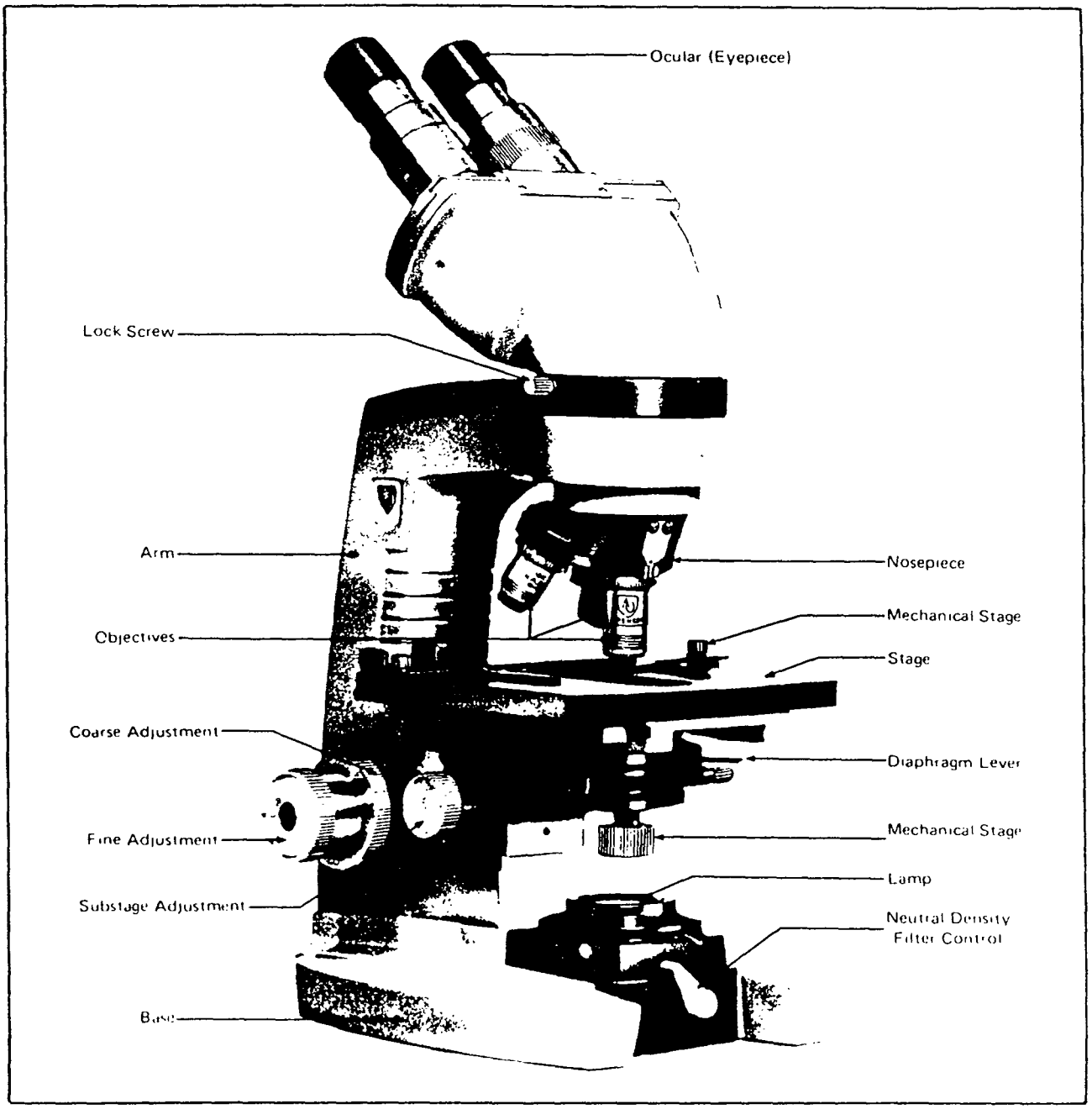

Figure 1-2 Parts of a microscope

EXPERIMENT NO. 9

BATCH SUBMERGED FERMENTATION OF BAKER'S YEAST IN A SHAKER FLASK

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ENCH 485, Spring 1988

Objective

To study the batch growth kinetics of a submerged culture.

Introduction

The proper procedure for a batch fermentation is first to inoculate a small flask of nutrient broth with a pure culture from a Petri dish, a culture tube (containing liquid nutrient), or a slant tube (containing solid gel). The inoculated flask is constantly agitated in a temperature controlled flask shaker. A small amount of the culture in the original flask is pipetted out during the *exponential growth phase*, or *log phase*, and is used to inoculate the next flask. This process is repeated a few times to ensure that the culture is acclimated before it is employed to study the fermentation kinetics. A similar process of repeated inoculation is carried out in the fermentation industry to build up enough inoculum needed to seed a larger fermentor. To reduce the shock resulting from a drastic change in the growth environment, the composition of the media used in preparing the inoculum should optimally be identical as that used in the main process.

When working with a pure culture, one must operate under the assumption that

contaminating microorganisms are present everywhere in the open environment, a fact demonstrated in our previous experiment. It is important to know intuitively when sterile tools or glassware must be used and when sterilization is not necessary. This requires the ability to distinguish clearly the sterile side from the nonsterile side. In this experiment, the interior of the shaker flask is the sterile portion of the system. Anything that is that part of the system and anything that ever comes in direct contact with that part of the system must be sterile. Thus, the nutrient in the shaker flask before inoculation must be sterile, which in turn requires that the reservoir storing the filtered nutrient is sterile and that the entire process of dispensing the nutrient from the medium jar to the shaker flask is carried out aseptically. In addition, items that enter the shaker flask such as the cotton plug, inoculation loop, sampling pipets, and even air must all be sterile.

Most practical industrial fermentation processes are based on complex media because of the cost and the choice of the nutrients and the ease of nutrient preparation. For example, complex media for yeast fermentation can be easily prepared in a lab by following the same recipe as that used in the YPG agar, minus the agar: 5g/l yeast extract, 10g/l Peptone, and 5g/l glucose. However, the use of complex media is discouraged in the fundamental studies of fermentation kinetics because of the possibility of variations in the nutrient composition from run to run. For example, the exact content of a yeast extract preparation is not known, and its nutritional quality may vary from batch to batch. On the other hand, a defined medium can be reproduced time after time to ensure the reproducibility of biochemical experiments. The disadvantage of a defined medium is that there is always the possibility of missing some important growth factors. The formulation of a defined medium is often a tedious process of trial and error. However, a well formulated defined medium can support the healthy growth and maintenance of cells as effectively as, or sometimes superior to, a complex one. A defined medium will be used in this

experiment.

List of Reagents and Instruments

A. Equipment

Erlenmeyer flasks, with cotton plugs

Graduated cylinders

Nutrient jar, with rubber stoppers

In-line filters, 0.2μm, for nutrient and vent

Temperature controlled flask shaker, thermometer

Bunsen burner

Inoculation loop

Autoclave

Balance

Magnetic stirrer

Sterile Pipets, 1ml, 10ml

Sampling vials, 8cc

Centrifuge or filtration setup for dry cell mass measurement

In-line filter holder, 25mm, 0.45μm, for sample clarification

Syringe

B. Reagents

Baker's yeast

Nutrient. See Table. 1.

Sucrose

Glucose

Ethanol, 100%

Procedures

1. *Nutrient Preparation (Defined Medium):* The overall nutrient composition is shown in Table 1. To facilitate nutrient preparation and to minimize the chance of making the fatal mistake of omitting one or two trace components, two concentrated (100 fold) stock solutions can be made according to the formula given in Table 2 for minerals and Table 3 for vitamins.

    a. Mix the concentrated mineral stock solution according to Table 2. Adjust the quantity according to the need. Bottled solutions can be stored on a shelf for more than six months. A yellowish color may develop upon prolonged storage; however, yellowing of the solution seems to impart no harmful effect.

    b. Mix the concentrated vitamin stock solution according to Table 3. Keep the stock solution in the dark at 4°C. Discard the solution if mold growth can be visually detected. The storage life of the stock solution is approximately 2-3 months.

    c. To obtain a working nutrient solution, add to 750 ml of stirred deionized water the components of Table 4 in the order listed. Precipitates may form if the order is not strictly followed. KOH pellets are used to neutralize the phosphoric acid and bring the pH close to the desired value. Add water to 1 liter. Finally, adjust the pH to the desired value by dropwise adding 1N KOH solution. (A pH value of 5.00 will be used in this experiment.)

2. *Nutrient Sterilization:* Next, the prepared nutrient must be sterilized. Usually, this is done by autoclaving. However, autoclaving is not a practical sterilization method for the formulation used in this experiment. First, the heat of autoclaving will caramel the sugar and darken the nutrient to a brown color. Secondly, vitamins will be destroyed by the heat. Furthermore, the loss of liquid due to

boiling during the autoclaving process will change the concentration of various nutrient components, including the rate limiting carbon source. Evaporation loss is especially severe when ethanol is the designated carbon source.

Instead, membrane filtration will be used to sterilize the nutrient in this experiment. This can be accomplished by drawing the nutrient from a mixing jar and forcing it through an in-line filter (0.2 μm pore size) either by gravity or with a peristaltic pump. See Figure 1. The sterilized medium is fed into an autoclaved nutrient jar with a rubber stopper fitted with a filtered vent and a hooded sampling port. Do not overfill the nutrient jar, for the nutrient will be forced out of the venting filter and wet it. A wet venting filter must be aseptically replaced with another dry sterile one. Otherwise, the wet filter will support the unwarranted proliferation of a wide range of microorganisms which will soon destroy the filter membrane and enter into the nutrient jar and contaminate the broth. For the same reason, soon after the filling process is completed, clamp the tubing at position "A" as indicated in Figure 1, disconnect the in-line nutrient filtration unit, and wash the residual nutrient from the newly exposed part of the tubing. If the nutrient filter is to be reused, wash and autoclave it before it is destroyed by the microbial growth either due to clogging or breaking of the membrane.

The hooded sampling port consists of a tubing reaching into the bottom of the nutrient jar and a rubber bulb on the side. Normally, a sampling bottle is attached to the sampling port to keep its tip airtight and sterile. When the rubber bulb is squeezed, the air in the sampling bottle is forced into the nutrient jar. When the bulb is released, nutrient equal to the volume of the displaced air is sucked up the sampling tube and is collected in the sampling bottle. A sterile medium bottle with the same cap thread size may be attached

to the sampling port in place of a sampling bottle if fermentation is to be conducted directly in the medium bottle or if it is more convenient to store the media in smaller bottles, each holding enough for one or two shaker flasks. The content from the media bottles may later be poured into shaker flasks as needed. Alternatively, a sterilized flask may be placed under the exposed sampling tube. Applying pressure to the vent will force liquid out from the jar into the flask. Flame both the sampling port and the mouth of the sterile sampling bottle before screwing on the sampling bottle.

Although the turbidity in the nutrient jar may be due to the precipitation of some of the nutrient components, it is almost always due to the presence of contaminants. At the first sign of contamination, either totally kill the contaminants by autoclaving or reduce the viability by adding bleach solutions. Discard the contents only after sanitization.

The setup in Figure 1 is useful for preparing a relatively large volume of sterile nutrient. A simpler setup consisting of a vented filtration flask as shown in Figure 2 may be desired if the quantity needed is not too large.

3. *Shaker Flask Preparation:*

   a. Make cotton-gauze plugs to fit the mouth of 250ml shaker flasks.

   b. Plug the flask and cover the plug with a piece of aluminum foil before autoclaving. The aluminum foil will prevent dust from directly settling on the cotton plug while standing on the shelf waiting to be used. This is generally the case where many flasks are simultaneously autoclaved for later use.

   c. After autoclaving the flasks, cool them to room temperature.

   d. Pour sterile nutrient into the flasks aseptically.

4. *Inoculum Preparation:*

   a. Find a single isolated colony of yeast on the Petri dish from which the culture is to be transferred.

   b. Following the aseptic plate streaking techniques introduced in the previous weeks, lift a small loopful of the creamy culture off the agar plate. Dip the loop into a 250 ml flask containing 100 ml of 5.0g/l glucose. Swirl the loop in the nutrient solution to dislodge the selected culture from the loop.

   c. Flame the neck of the flask and the cotton plug before inserting the plug back on the flask. Also, flame the loop to kill the residual microorganisms.

   d. Place the flask in a temperature controlled shaker at 37°C. The exponential growth phase will last from 2 to 24 hours after inoculation. The exact time and duration depend on the physiological condition of the inoculum. The instructor will provide an exponentially growing culture.

5. *Shaker Flask Inoculation:*

   a. Follow the same procedure as Step 1 to prepare nutrient solutions with the following carbon sources:

Run	Carbon Source	Weight
A	Ethanol	5.0 g/l
B	Glucose	5.0 g/l
C	Sucrose	5.0 g/l
D	Glucose	2.5 g/l
	Sucrose	25 g/l

   b. Follow the same procedure as Step 2 to sterilize the nutrient through filtration.

c. Follow the same procedure as Step 3 to autoclave 1000 ml flasks and transfer 500 ml of the nutrient into each of them.

d. Inoculate the flask with 10 ml of suspended actively growing yeast culture obtained at the end of Step 4 with a sterile 10ml pipet.

6. *Batch Fermentation Monitoring:* Remove the flask from the shaker and draw a sample at 90-120 minute intervals. The fermentation should last for approximately 24-36 hours before the culture enters the stationary phase. See Notes 1-2.

   a. Although a 10 ml sample is more than adequate to analyze for optical density, glucose/sucrose concentration, and ethanol concentration, an extra 10 ml will ensure the availability of a sufficient quantity of sample broth, should the quantitative analysis fail repeatedly. Immediately after taking a 20 ml aliquot with a sterile pipet, flame the neck of the flask and place the plug back in the mouth.

   b. Record the pH of the sample just taken.

   c. Save a drop of the sample on a slide for microscopic examination later.

   d. Initially, when the cell density is still low, the optical density of the sample can be measured without dilution with water. Perform this step quickly with a spectrophotometer at 550 nm. Then, filter out the cells from the sample. After the optical density is over 0.5, save 1 ml of the sample by pipetting it into a test tube for optical density measurement. Force the remaining sample through a filter.

   e. The clear filtrate is collected in a tightly capped sampling vial for later analysis. Freezing the filtrate will better preserve the existing condition.

   f. If a 1 ml sample is saved for the optical density measurement, dilute the

sample with 5 ml of water. Record the optical density.

g. Clean the filter unit, test tubes, and pipet. Return the flask back to the shaker and get ready for the next sampling.

7. Dry Cell Weight Measurement: Terminate the experiment when the stationary phase is reached. Obtain a calibration curve for the cell concentration in g/l as a function of the optical density.

   a. Measure the volume of the remaining culture.

   b. Weight an empty aluminum weighing pan or a sheet of dried filter paper stored in a desiccator.

   c. Separate the cells from the broth either by centrifugation or by filtration.

   d. After drying the cell paste in an oven set at 100 °C for 24 hours, measure the weight of the weighing pan or the filter paper.

8. Quantitative Analysis: After the experiment is concluded, for each sample, measure the glucose concentration with the DNS reagent, the sucrose concentration according to the accompanying Supplement D, and the ethanol concentration according to the accompanying Supplement E or a gas chromatograph.

Notes

1. Because of the limited rate of microbial growth, the experiment will continue day and night. Each student will be assigned a period of time during which he is responsible for overseeing the experiment, including sample taking. Thus, more than ever, it is necessary that the group cooperate closely to complete the experiment. All the runs will be performed side by side.

2. Because the entire sampling sequence must be carried out quickly, make sure that everything is in order before taking a sample.

Discussion

Yeast has been in use since the beginning of human civilization. It is still a very versatile microorganism widely used in a wide range of fermentation industries. For example, the carbon dioxide released as a result of carbohydrate metabolism is used to raise dough in baking; the ethanol produced supports a multi-billion dollar alcoholic beverage industry; single cell protein is used to supplement animal feed. It is also currently the most widely used microorganism for ethanol production as the alternative energy source, *Zymomonas mobilis* being the other potentially dominant one. Although fermentation was practiced even before recorded history, the fact that microorganisms were responsible for the leavening and brewing actions was not realized until the last century.

Questions

1. For each run, calculate and plot the cell biomass concentration, glucose concentration, ethanol concentration, and pH as a function of time. Identify the major phases in a batch fermentation: lag, exponential, stationary, and death. How many growth phases are there? How many lag phases are there?
2. Speculate on the existence of lag phases.
3. Microorganisms are "picky eaters." Which carbon source does the yeast prefer the most? Which the least? Comment on the biological reason of the observed preference on the carbon source as related to the survival of the microorganism.
4. What kinds of plots are needed to find the parameters of the Monod cell growth model, i.e. the maximum specific growth rate and the Michaelis-Menten constant? Report the parameters observed in this experiment for baker's yeast.
5. Did the pH change during the course of the fermentation? If so, what was the cause of the change in the pH? Did the change in the pH coincide with the

different batch growth phases?

6. Identify the major elements of life, i.e. nutrient requirement, in a typical yeast cell. List the amount of each element in a typical yeast cell. Of these essential elements, which are represented in our synthetic medium formulation? What chemicals are used to provide each of these elements in our experiment? From the relative ratios of the elements present in the media, identify the limiting substrate. (Is the carbon source indeed the limiting substrate as we have implicitly assumed in applying the Monod model? Is every thing else in excess? If so, by how much?)

7. Which nutrient components are vitamins, and why are they provided in the media? (Obviously they are not included to derive trace elements.)

8. What are the roles of EDTA and phthalic acid?

9. Comment on ways to improve the experiment.

Reference Readings

1. Oura, Erkki, Effect of aeration intensity on the biochemical composition of baker's yeast. I. Factors Affecting the type of metabolism, *Biotech. Bioeng.* **16**, 1197, 1974.

Table 9-1.

The Composition of Defined Medium for Baker's Yeast

Compound	Concentration	
$MgCl_2 \cdot 6H_2O$	0.52	g/l
$(NH_4)_2SO_4$	12.0	g/l
H_3PO_4 (85%)	1.6	ml/l
KCl	0.12	g/l
$CaCl_2 \cdot 2H_2O$	0.2	g/l
NaCl	0.06	g/l
$MnSO_4 \cdot H_2O$	0.024	g/l
$CaSO_4 \cdot 5H_2O$	0.0005	g/l
H_3BO_3	0.0005	g/l
$Na_2MoO_4 \cdot 2H_2O$	0.002	g/l
NiCl	0.0025	mg/l
$ZnSO_4 \cdot 7H_2O$	0.012	g/l
$CoSO_4 \cdot 7H_2O$	0.0023	mg/l
KI	0.0001	g/l
$FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$	0.035	g/l
myo-Inositol	0.125	g/l
Pyridoxine-HCl (Vitamin B_6)	0.00625	g/l
Ca-n-Pantothenate	0.00625	g/l
Thiamine-HCl (Vitamin B_1)	0.005	g/l
Nicotinic Acid	0.005	g/l
D-Biotin (Vitamin H)	0.000125	g/l
Carbon Source (e.g. Glucose)	0-50	g/l
EDTA	0.1	g/l

Table 9-2.

Mineral Stock Solution (100X)

Compound	Weight-Volume	
H_3PO_4 (85%)	160.	ml
KCl	12.00	g
$CaCl_2 \cdot 2H_2O$	20.00	g
NaCl	6.00	g
$MnSO_4 \cdot H_2O$	2.40	g
$CaSO_4 \cdot 5H_2O$	0.05	g
H_3BO_3	0.05	g
$Na_2MoO_4 \cdot 2H_2O$	0.20	g
NiCl	0.25	mg
$ZnSO_4 \cdot 7H_2O$	1.20	g
$CoSO_4 \cdot 7H_2O$	0.23	mg
KI	0.01	g
Add water to	1 liter	

Table 9-3.

Vitamin Stock Solution (100X)

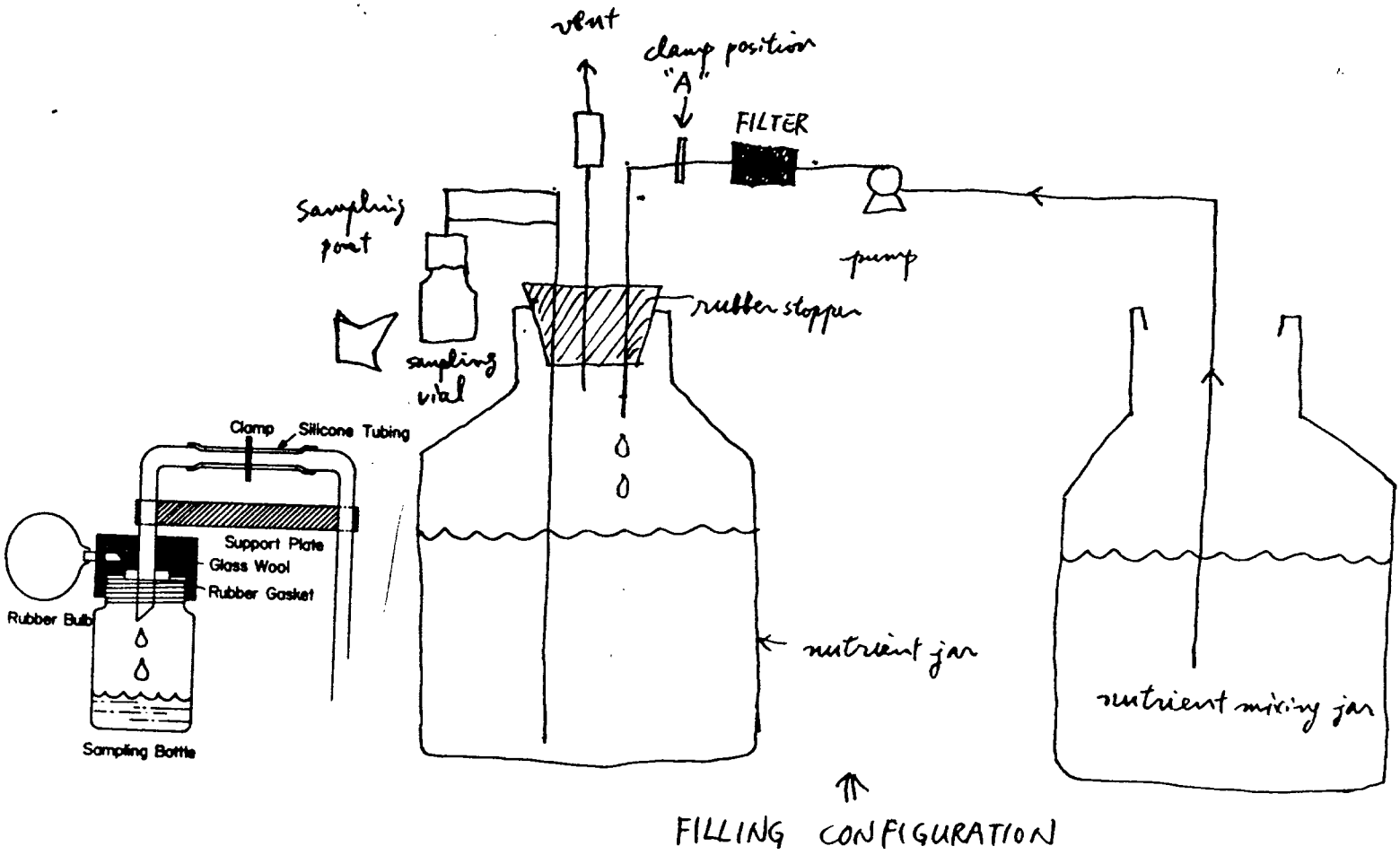
Compound	Weight-Volume	
myo-Inositol	12.5	g
Pyridoxine-HCl	0.625	g
Ca-n-Pantothenate	0.625	g
Thiamine-HCl	0.5	g
Nicotinic Acid	0.5	g
D-Biotin	0.0125	g
Add water to	1 liter	

Table 9-4.

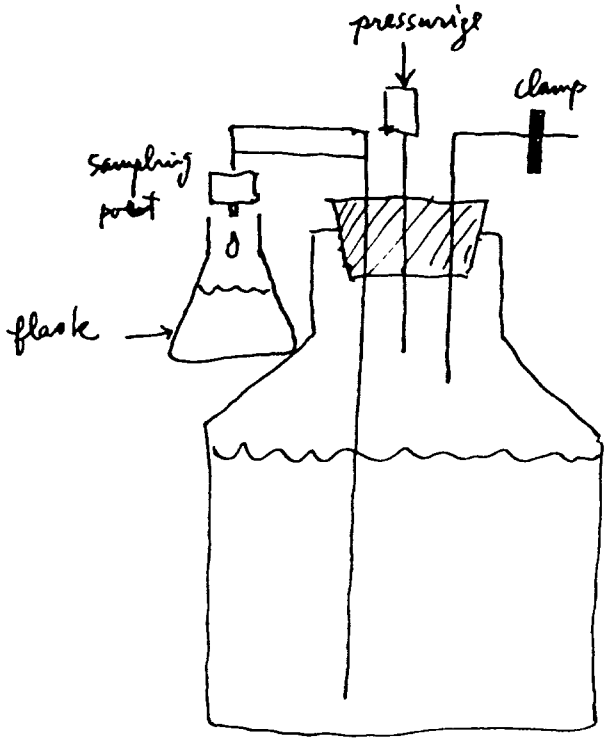
Normal Strength Working Nutrient Solution

Compound	Weight-Volume	
Phthalic acid, monopotassium salt	0.20	g
$MgCl_2 \cdot 6H_2O$	0.52	g
EDTA	0.1	g
$(NH_4)_2SO_4$	12.00	g
Mineral Stock Solution	10.	ml
$FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$	0.035	g
Vitamin Stock Solution	10.	ml
Carbon Source (e.g. Glucose)	0-50	g
KOH (for pH=5.0)	1.62	g
Add water to	1 liter	

Adjust pH to 5.00 with 1N KOH & 1N HCl solutions



FILLING CONFIGURATION



DISPENSING CONFIGURATION

Figure 1. Large scale filter sterilization of the medium

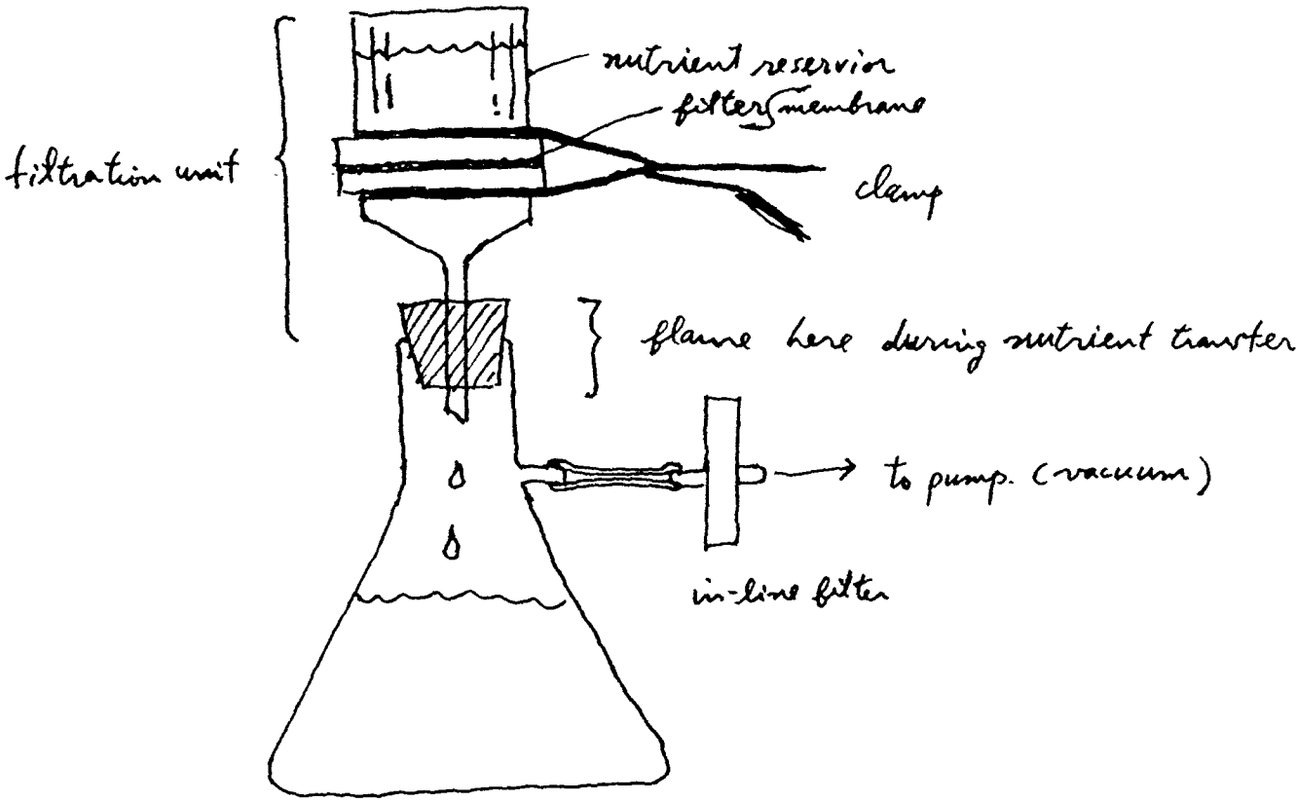


Figure 2. Small scale filter sterilization of the medium.

EXPERIMENT NO. 9 – SUPPLEMENT B

CELL DIFFERENTIATION BY GRAM'S STAIN

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ENCH 485, Spring 1988

Method

The Gram staining method, named after the Danish bacteriologist who originally devised it in 1844, Hans Christian Gram, is one of the most important staining techniques in microbiology. It is almost always the first test performed for the identification of bacteria. The *primary stain* of the Gram's method is crystal violet. Crystal violet is sometimes substituted with methylene blue, which is equally effective. The microorganisms that retain the crystal violet-iodine complex appear purple brown under microscopic examination. These microorganisms that are stained by the Gram's method are commonly classified as *gram positive* or *gram non-negative*. Others that are not stained by crystal violet are referred to as *gram negative*. (Note that the word *gram* is often not capitalized, indicating that the immense popularity of the method has made it a common name.)

Besides Gram's stain, there is a wide range of staining methods available. The procedures for these other methods follow quite closely those of Gram's stain. By using appropriate dyes, different parts of the cell structure such as capsules, flagella, granules, or spores can be stained. Staining techniques are widely used to visualize those components that are otherwise too difficult to see under an ordinary light microscope either because of the lack of color contrast between the object

under examination and the background or because of the limited resolving power of the light microscope. In addition, staining techniques are useful in detecting the presence or absence of certain cell components, thus allowing a relatively simple scheme of differentiation or identification of microorganisms. In this respect, Gram's stain ranks among the most important diagnostic tools in biological science.

In Gram's method, which is based on the ability of a cell in retaining the crystal violet dye during solvent treatment, it is the difference in the microbial cell wall that is amplified. The cell walls for gram-negative microorganisms have a higher lipid content than gram-positive cells. Originally, both kinds of cells are penetrated by the crystal violet. Iodine is subsequently added as a *mordant* to form the crystal violet-iodine complex so that the dye cannot be removed too easily. This step is commonly referred to as *fixing* the dye. However, the subsequent treatment with the decolorizer, which is a mixed solvent of ethanol and acetone, dissolves the lipid layer from the gram-negative cells. The removal of the lipid layer enhances the leaching of the primary stain from the cells into the surrounding solvent. In contrast, the solvent dehydrates the thicker gram-positive cell wall, closing the pores as the cell wall shrinks during dehydration. As a result, the the diffusion of the stain-iodine complex out from the cell is obstructed, and the cells remain stained. The actual mechanism of decolorization is currently not well understood and remains controversial. At any rate, if the decolorizer treatment is properly timed, there exists a period during which the crystal violet-iodine complex is effectively removed from the gram-negative cells but still retained in the gram-positive ones. Thus, the length of the decolorizer treatment is critical in clearly differentiating the gram-positive cells from the gram-negative cells. A prolonged exposure to the decolorizing agent will remove all the stain from both types of cells. The student is cautioned that some gram-positive cells lose the stain easily and therefore may appear gram negative.

Finally, although not essential, a *counterstain* of safranin is applied to the smear to dye the decolorized gram-negative cells with a pink color. Thus, the size and shape of both types of cells can be more easily observed under a microscope. At the same time, they can be differentiated by the imparted color. If desired, the slides can be permanently mounted and preserved for record keeping.

A mixture of *S. cerevisiae* and *E. coli* will be examined in this experiment, although Gram's stain is usually applied to differentiate bacteria, which are usually too small to be seen clearly under a light microscope. *S. cerevisiae* cells will be stained by the crystal violet-iodine complex and should appear purple-brown in color. In contrast, the much smaller *E. coli*, cells should appear pink, the color of safranin. Repeat the entire process until a satisfactory slide is prepared and properly focused in a microscope for the approval of the instructor. Note the improvement in cell visualization as compared to a plain unstained slide.

List of Reagents and Instruments

A. Equipment

Bunsen burner

Microscope

Slide

Cloth pin

Water bottle

B. Reagents

Crystal violet

Methylene blue

Ethanol, 95%

Acetone

Ammonium oxalate

Iodine

Potassium iodide

Sodium bicarbonate

Safranin O

Procedures

1. *Prepare Reagents:*

   a. *Gram Crystal Violet Solution:* Dissolve 20 g of crystal violet, 95 % dye content, in 100 ml of ethanol to make a crystal violet stock solution. Similarly, dissolve 1 g of ammonium oxalate in 100 ml of water to make an oxalate stock solution. Working solution is obtained by mixing 1 ml of the crystal violet stock solution with 10 ml of water and 40 ml of the oxalate stock solution. Store the working solution in a drop bottle.

   b. *Methylene Blue Solution:* Dissolve 1 g of methylene blue, 90 % dye content, in 100 ml of ethanol; this is Solution A. Mix 0.03 g of KOH in 300 ml of water; this is Solution B. Mixing Solutions A and B yields the working solution.

   c. *Gram Iodine Solution:* Dissolve 1 g of iodine, 2 g of potassium iodide, and 3 g of sodium bicarbonate in 300 ml of water.

   d. *Gram Decolorizer Solution:* Mix equal volumes of 95 % ethanol and acetone.

   e. *Gram Safranin Solution:* Dissolve 2.5 g of safranin O in 100 ml of 95 % ethanol to make a stock solution. Working solution is obtained by diluting one part of the stock solution with five parts of water.

2. *Prepare a Slide Smear:*

a. Transfer a drop of the suspended culture to be examined on a slide with an inoculation loop. If the culture is to be taken from a Petri dish or a slant culture tube, first add a drop or a few loopful of water on the slide and aseptically transfer a *minute* amount of a colony from the Petri dish. Note that only a very small amount of culture is needed; a visual detection of the culture on an inoculation loop already indicates that too much is taken.

b. Spread the culture with an inoculation loop to an even thin film over a circle of 1.5 cm in diameter, approximately the size of a dime. Thus, a typical slide can simultaneously accommodate 3 to 4 small smears if more than one culture is to be examined.

c. Hold the slide with a cloth pin. Air-dry the culture and fix it or over a gentle flame, while moving the slide in a circular fashion to avoid localized overheating. The applied heat helps the cell adhesion on the glass slide to make possible the subsequent rinsing of the smear with water without a significant loss of the culture. Heat can also be applied to facilitate drying the the smear. However, ring patterns can form if heating is not uniform, e.g. taking the slide in and out of the flame.

3. *Gram Staining:*

a. Add about 5 drops of crystal violet stain over the fixed culture. Let stand for 60 seconds. Note that a clothes pin is used to hold the slide during the staining procedure to avoid staining one's hand.

b. Pour off the stain and *gently* rinse the excess stain with a stream of water from a faucet or a plastic water bottle. Note that the objective of this step is to wash off the stain, not the fixed culture.

c. Add about 5 drops of the iodine solution on the smear, enough to cover the fixed culture. Let stand for 30 seconds.

d. Pour off the iodine solution and rinse the slide with running water. Shake off the excess water from the surface.

e. Add a few drops of decolorizer so the solution trickles down the slide. Rinse it off with water after 5 seconds. The exact time to stop is when the solvent is no longer colored as it flows over the slide. Further delay will cause excess decolorization in the gram-positive cells, and the purpose of staining will be defeated.

f. Counterstain with 5 drops of the safranin solution for 20 seconds.

g. Wash off the red safranin solution with water. Blot with bibulous paper to remove the excess water. Alternatively, the slide may shaken to remove most of the water and air-dried.

4. Liberally wash off any spilled stain immediately with water to avoid leaving permanent marks in the sink, lab bench, or glassware.

5. Examine the finished slide under a microscope.

Reference Readings

1. Bartholomew, J. W. and Finkelstein, H., Relationship of cell wall staining to Gram differentiation, *J. Bacteriol.*, **75**, 77, 1958.

2. Syndney M. Finegold and William J. Martin, *Diagnostic Microbiology*, Chapt. 3, Mosby Co., St. Louis, 1982.

3. Salton, M. R. J., *The Bacterial Cell Wall*, Elsevier Press, Amsterdam, 1964.

4. Gregersen, T., Rapid method for distinction of gram-negative from gram-positive bacteria, *Eur. J. Appl. Microbiol. Biotechnol.*, **5**, 123, 1978.

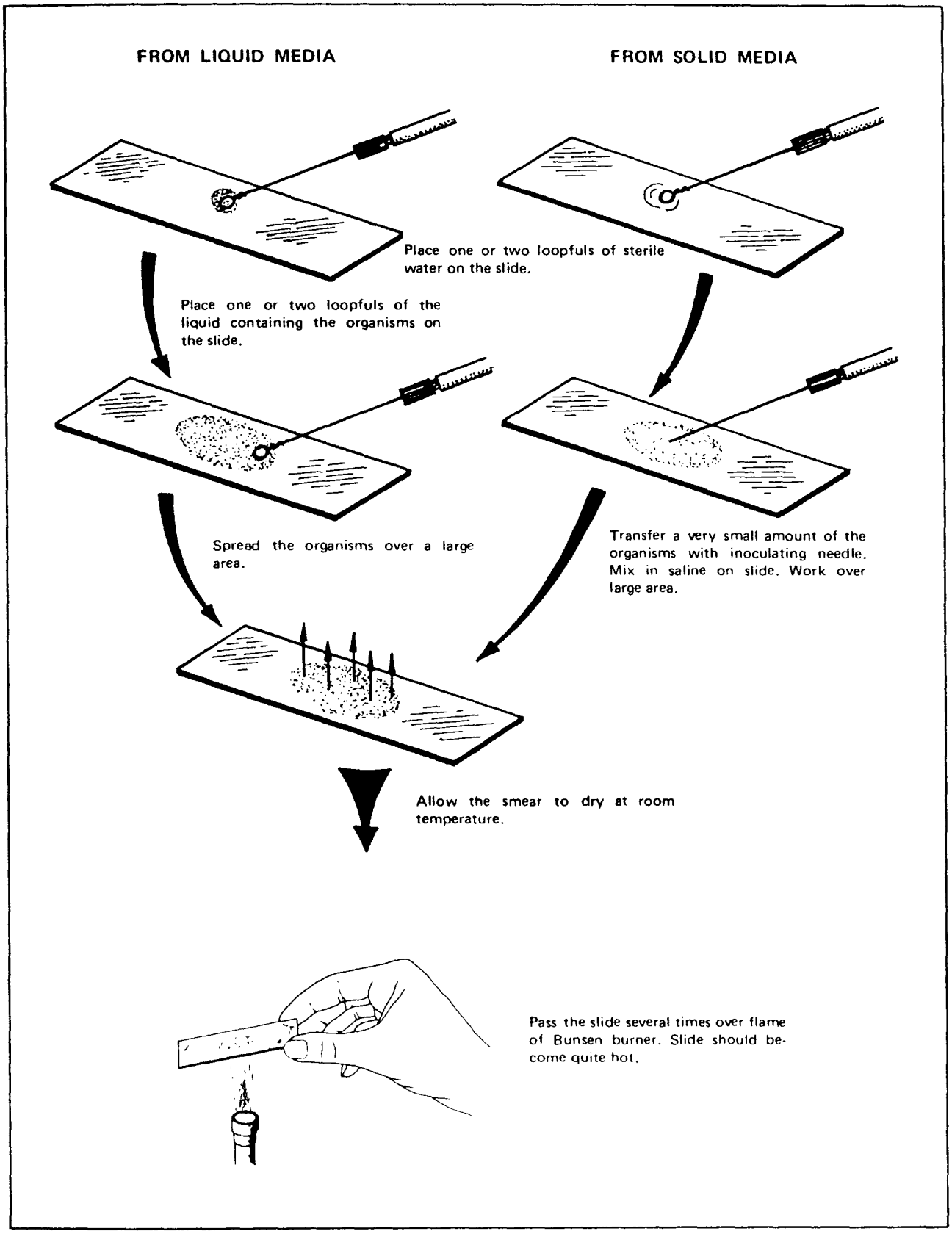
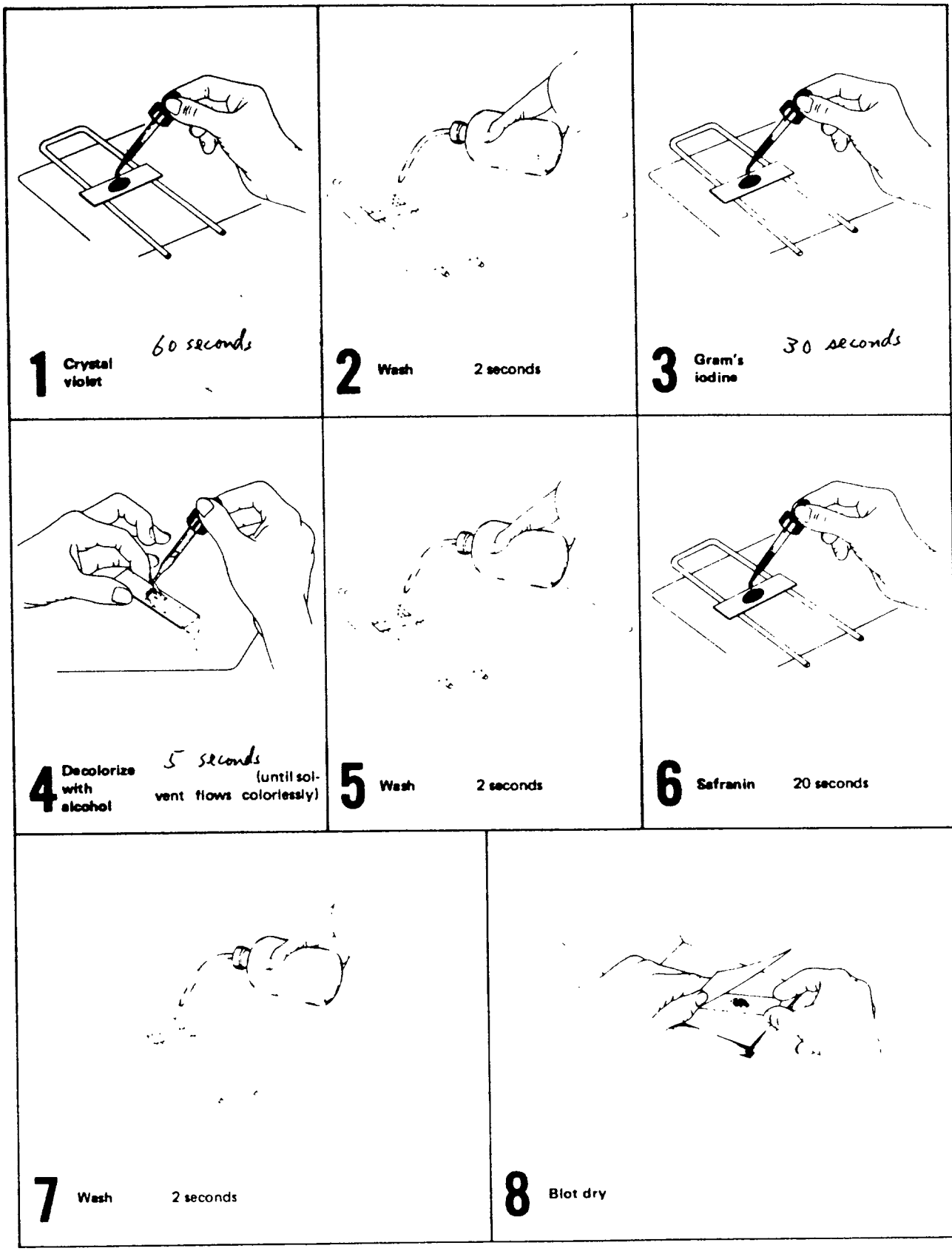


Figure 11-1 Steps in preparing bacterial smear



Gram-staining routine

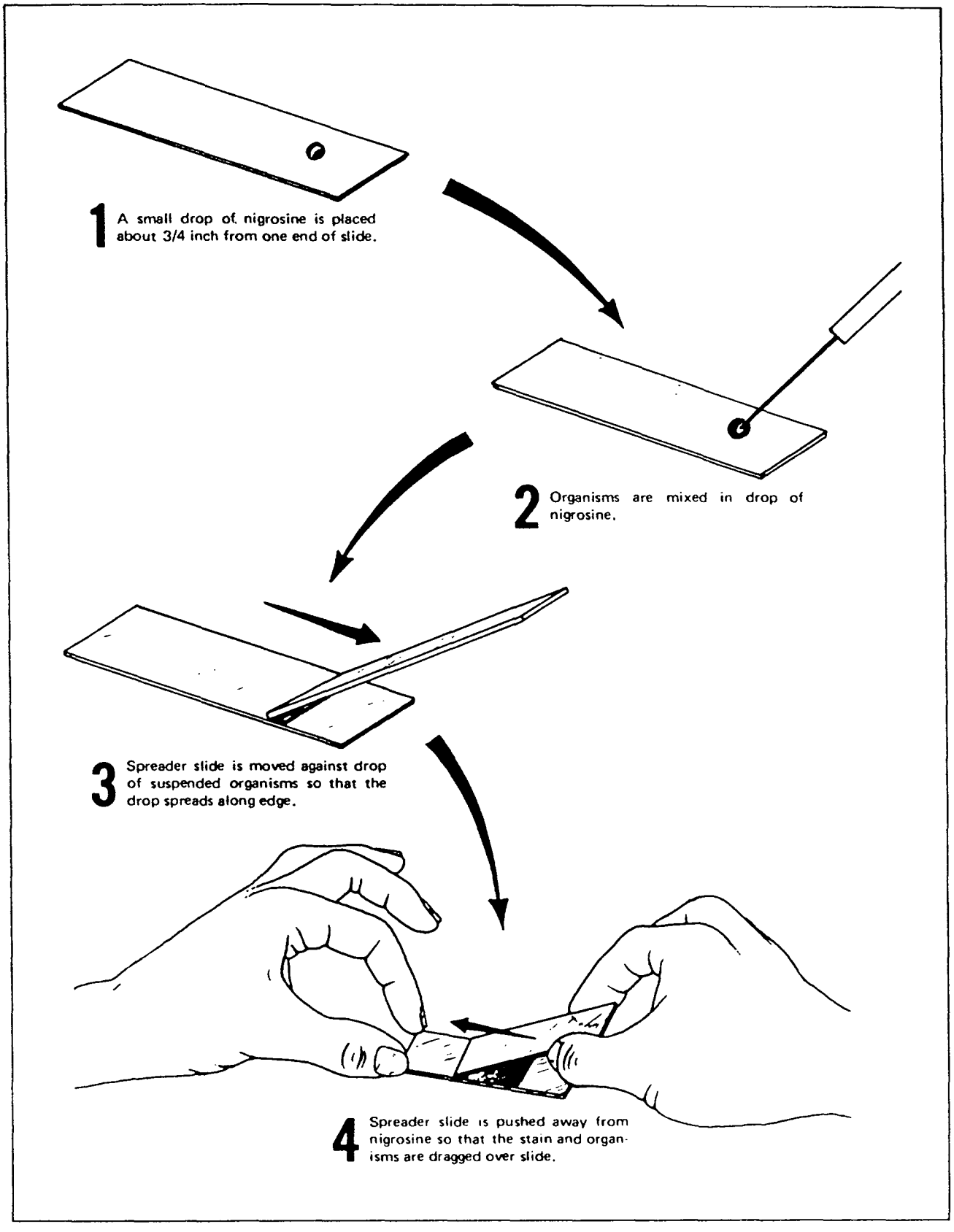


Figure 10–1 Negative staining process

EXPERIMENT NO. 9 – SUPPLEMENT C

MEASUREMENTS OF CELL BIOMASS CONCENTRATION

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ENCH 485, Spring 1988

Introduction

The cell density can be quantified in two basic ways: as grams of dry or wet weight per liter of sample, or as number of viable/dead cells per ml. The cells in a sample can be separated from the broth and weighed while they are wet, or the cells may be thoroughly dried before weighing. The dry weight measurement usually gives a much more consistent result than the wet weight. Alternatively, the number of cells can be counted either by successively diluting the original sample and plating on a Petri dish, with the help of a microscope and a counting chamber, or with an automated cell counter such as a Coulter counter or a cytoflowmeter. The plating method detects only the viable cells; whereas, the automated cell counters can only detect the total number of cells.

All of the above methods either require the availability of expensive equipment or the substantial investment of time. In reality, the most often used method simply monitors the optical density of the sample. The absorbance of the sample measured in a spectrophotometer is correlated to either the dry weight or the number of cells per volume.

Biomass concentration is one of the most critically needed measurements in fermentation studies. It is also one of the most difficult and unreliable ones. For example, all the above dry/wet weight methods and all the automated counting equipment fail completely if the broth contains other insoluble particulate matter, which is often the case in a practical fermentor. Similarly, the optical density measurement only has limited usefulness if the fermentation broth is not clear. In addition, these methods cannot distinguish the viable cells from the dead ones. On the other hand, the standard plate count can detect viable cells among other particulate matters. However, the method requires elaborate preparations, and it takes 24-48 hours for the cells to be incubated and counted; the cost of Petri dishes and media can also be prohibitive. Consequently, the direct plate count is useless in feedback control of a fermentation process; it is mainly used industrially to countercheck other measurements, especially the optical density.

In this experiment, the cell density of a given sample will be measured with the following five methods: wet weight, dry weight, optical density, direct cell counting with a chamber, and successive dilutions followed by plating.

List of Reagents and Instruments

A. Equipment

Flasks

Graduated cylinder

Filtration unit with vacuum pump

Filter membrane or weighing pan

Centrifuge

Oven, 100°C

Balance

Spectrophotometer

Cell counting chamber

Microscope

Petri dish, 4 per standard plate count

Sterile pipets, 4 per standard plate count

Sterile bottles, 3 per standard plate count

B. Reagents

Flask of culture

Nutrient (YPG) agar

Sterile water

Procedures

1. *Dry/Wet Weight Measurement:*

   a. Dry in an oven an empty aluminum weighing pan or a sheet of cellulose acetate filter membrane, 47mm in diameter, 0.45μm in pore size. Weigh them and store them in a desiccator lined with Drierite (anhydrous $CaSO_4$).

   b. Stir the flask to suspend the culture evenly. Pour out 100 ml of the culture into a graduated cylinder.

   c. Separate the cells from the broth either by centrifugation at 10,000 g for 5 minutes or by filtration. In the case of centrifugation, carefully discard the clear broth and scrape the cell paste from the centrifuge tube into a weighing pan. Rinse the centrifuge tube with a few ml of water. Pour the rinse water into the weighing pan, as well. In the case of filtration, the culture is poured into the holding reservoir fitted on the filter membrane. A vacuum is applied to pull the liquid through the membrane. Rinse the reservoir with a few ml of water and scrape any paste adhering to the glassware. The wet weight of the culture is measured immediately after

all the water has been pulled through.

d. Dry the cell paste in an oven set at 100 °C. The cells will be charred and the filter membrane will be burned if the temperature of the oven is set too high. Measure the weight of the pan/filter plus the cell paste periodically until there is no further decrease in the dry weight. It will take 6-24 hours to dry the sample completely, depending on the oven temperature and the thickness of the paste. Calculate the difference in the weight, and express the dry weight in g/l.

2. *Optical Density:* Dilute the sample to appropriate concentrations as needed, and measure the absorbance of the sample with a spectrophotometer at 550 nm. Other wavelengths may also be used, but one must be consistent. Generate a calibration curve to relate the absorbance with cell dry weight. The usual rules of operating a spectrophotometer apply here, as well. For example, the accuracy of the method is the highest when the absorbance is between 0.1 and 0.5. For a given culture sample, a good spectrophotometer should yield a linear relationship between the number of cells and the absorbance. However, the optical density is also a function of cell morphology such as size and shape, because the amount of transmitted or scattered light depends strongly on these factors. Consequently, an independent calibration curve is required for each condition in accurate research work, as the cell size and shape depend on the specific growth rate and the nutrient composition. As a rule of thumb, an optical density of 1 unit corresponds to approximately 1 g/l of dry cell. This is also commonly referred to as the turbidity measurement.

3. *Cell Counting Chamber:* This is a standard method for counting the number of microorganisms in milk. It is also widely used in blood counts and vaccine counts; thus, a counting chamber is also commonly called a *hemocytometer.*

But the technique is not very popular among biochemical engineers. Two thin rails of a well defined height of 0.02mm are attached to the surface of a glass slide that is marked with evenly spaced lines at 0.05mm intervals. Thus, each square represents a volume of 5.0×10^{-8}ml.

a. Add 0.1 ml of methylene blue and 1 ml of the suspended culture to a test tube. Mix well. Cells are stained for 3 minutes to enhance visualization. Add water to dilute the sample as needed. This introduces a dilution factor which must later be incorporated into the calculation of the original cell density.

b. Clean all the grease from the counting chamber with ethanol so that cells can be clearly counted later.

c. Place a piece of reinforced cover glass on top of the rail. There should be a small space, 0.02 mm to be exact, between the platform and the cover glass.

d. Fill a capillary pipet with the stained sample. Gently touching the tip of the pipet against the edge of the cover glass will attract the sample to fill the space under the cover glass solely by capillary actions.

e. The number of cells enclosed in each square is counted visually under an ordinary light transmission microscope. One may need to raise the oil immersion lens slightly to shift focus at the space under the cover glass. Repeat for at least 20 squares. Take the average number of cells per square.

f. Calculate the number of cells per ml by multiplying the number of cells per square by the dilution factor introduced in Step 3a by $1/(5.0\times10^{-8})$, or 2.0×10^{7}.

4. *Standard Plate Count:* The major part of the procedure deals with a series of

successive dilutions of the original culture in sterile bottles with sterile water. The diluted culture is poured into Petri dishes along with the nutrient agar. The number of colonies is counted after incubation.

a. Shake the flask containing the culture, and pipet 1 ml of the culture aseptically into a capped sterile bottle marked "A," which contains 99 ml of sterile water. Vigorously shake the bottle A to mix the culture and break any flocculating clumps of microorganisms. The dilution factor in bottle A is 1:100. A dilution factor of 1:101 is often more easy to work with physically than 1:100; in this case, the subsequent dilution factor is also similarly modified without any loss in the accuracy.

b. With the second sterile pipet, transfer 1 ml from the bottle A into a similar bottle marked "B," which also contains 99 ml of sterile water. Shake and mix. The dilution factor in bottle B is 1:10,000.

c. With the third sterile pipet, transfer 1 ml from the bottle B to a Petri dish marked 1:10,000. Thus, the number of colonies from this dish is multiplied by 10^4 to give the number of cells in 1 ml of the original sample.

d. With the same pipet as Step 4c, transfer 0.1 ml from the bottle B to a Petri dish marked 1:100,000. The number of colonies from this dish is multiplied by 10^5 to give the number of cells in 1 ml of the original sample.

e. With the same pipet as Step 4c, transfer 1 ml from the bottle B into a third bottle marked "C," which contains 99 ml of sterile water. Shake and mix. The dilution factor in bottle C is 1:1,000,000.

f. With the fourth sterile pipet, transfer 1 ml from the bottle C to a Petri dish marked 1:1,000,000. Thus, the number of colonies from this dish is multiplied by 10^6 to give the number of cells in 1 ml of the original sample.

g. With the same pipet as Step 4f, transfer 0.1 ml from the bottle B to a Petri dish marked 1:10,000,000. The number of colonies from this dish is multiplied by 10^7 to give the number of cells in 1 ml of the original sample.

h. Heat the capped culture tube containing 50 ml of agar to boil for 10 minutes. The heating both melts and sterilizes the agar.

i. After the agar is cooled to 45°C, pour 12 ml to each of the four Petri dishes. The culture will be killed if the agar is too hot; it will solidify if it is cooled for too long. Swirl the plates very gently to mix the culture with the agar.

j. Allow the agar to solidify.

k. Incubate the plates in the inverted position at 37°C for 48 hours.

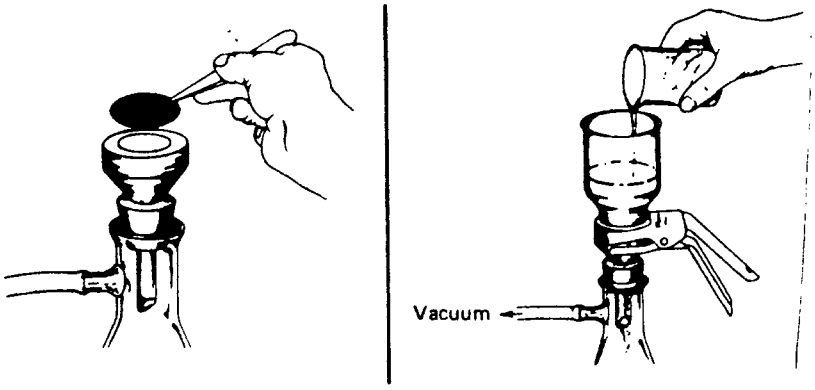
l. Select those plates that have 30-300 colonies. Count every colony, large and small. To keep track of the counted colonies, dot the colonies with a permanent marker pen as one counts them. Different colored pens may be used for a mixed culture.

Statistically, the most reliable results are given by plates with between 30 and 300 colonies. Only about two significant figures can be obtained from this method. The accuracy can be improved if multiple plates can be prepared. This, however, is rarely done due to the cost.

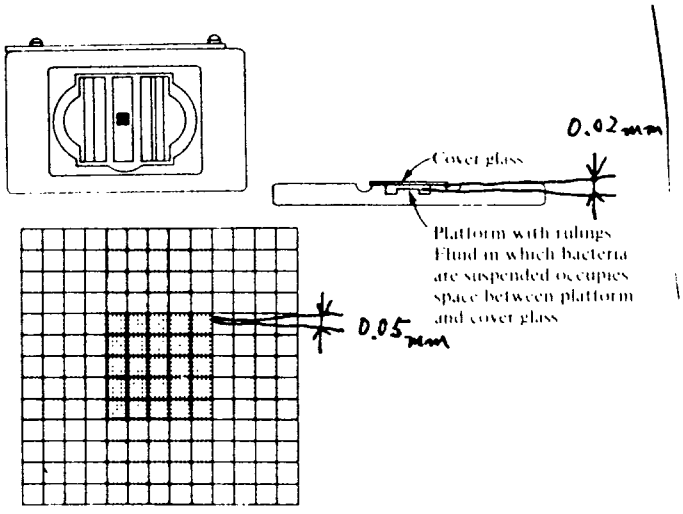
m. Some of the plating may be omitted if the number of cells per ml can be estimated to the order of magnitude. Finally, a cell counting chamber can be used in conjunction with cell plating to distinguish the number of viable and nonviable cells.

Questions

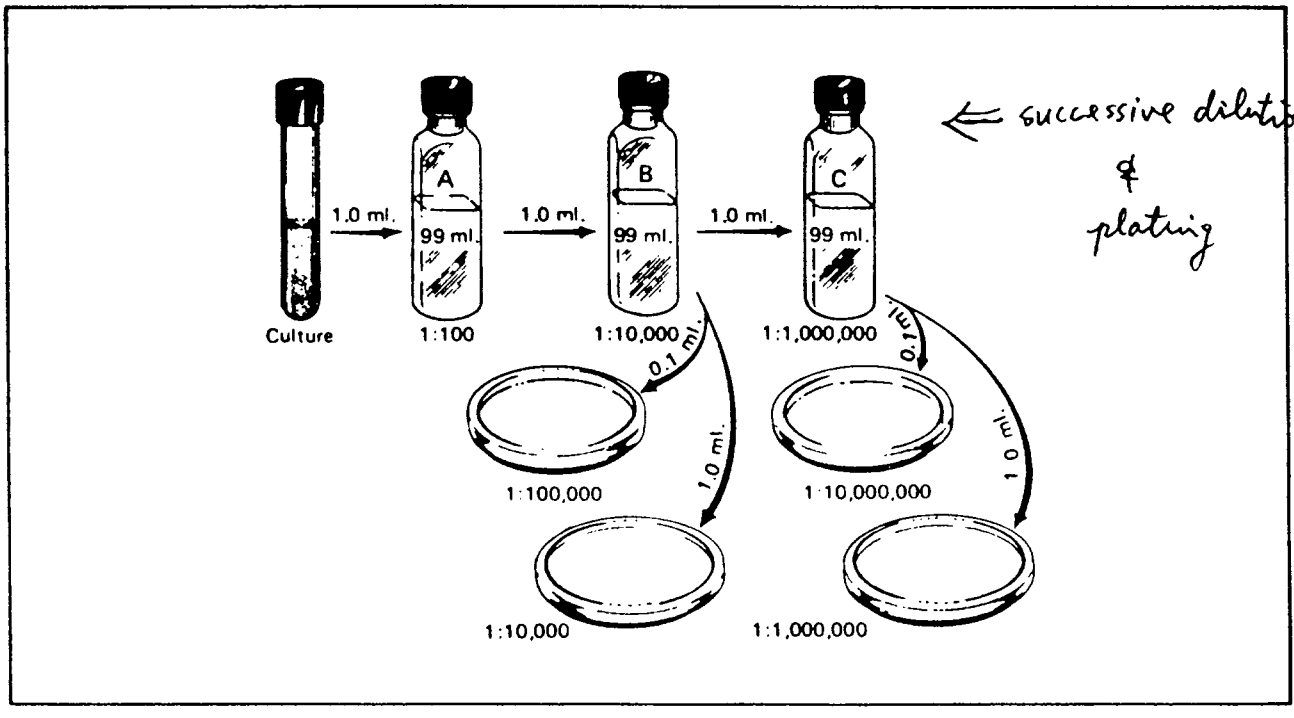
1. Report the cell density in appropriate units for the given sample.



⇐ dry/wet weight



⇐ cell counting chamber



⇐ successive dilutions & plating

EXPERIMENT NO. 9 – SUPPLEMENT D

SUCROSE ASSAY
BY THE DINITROSALICYLIC COLORIMETRIC METHOD

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ENCH 485, Spring 1988

Method

Unlike other carbohydrates, sucrose is the only non-reducing common disaccharide. Consequently, most tests for sugar detection utilizing such reagents as Benedict's solution, Fehling's solution, and DNS (3,5-dinitrosalicylic acid) solution result in negative readings for sucrose. (The student should convince himself of this fact by performing the test with a pure sucrose solution.) However, these methods can still be applied if sucrose is first hydrolyzed in an acid solution to yield glucose and fructose. This method is a straightforward modification of the original DNS method for glucose analysis.

List of Reagents and Instruments

A. Equipment

Test tubes

Pipets

Spectrophotometer

B. Reagents

Dinitrosalicylic Acid Reagent Solution, 1 %

Dinitrosalicylic acid: 10 g

Phenol: 2 g (optional, see Note 1)

Sodium sulfite: 0.5 g

Sodium hydroxide: 10 g

Add water to: 1 liter

Potassium sodium tartrate solution, 40%

HCl, concentrate (37.3%, 11.9 N) solution

KOH, 5N solution

Procedures

1. Add 1 drop, or 20 μl, of concentrate HCl solution to 1 ml of the sucrose solution. Allow the hydrolysis to proceed at 90°C for 5 minutes.
2. Add 3 drops, or 0.05 ml, of the 5 N KOH solution to neutralize the acid, because the DNS method must be applied in an alkaline condition to develop the red brown color which represents the presence of reducing sugars.
3. Add the DNS reagent and follow the DNS method henceforth.
4. Generate a calibration curve to correlate the absorbance to the sucrose concentration.

Discussion

The DNS method can be applied twice to measure the individual concentrations of a mixture of glucose and sucrose. First, a small part of the original sample is consumed in measuring the glucose concentration by following the original DNS procedure. Another part of the sample is hydrolyzed and subsequently subjected to the same DNS procedure. The difference in the absorbance between the acid treated sample and the untreated sample is due to the presence of sucrose. The

sucrose concentration can then be calculated from a calibration curve based on that difference in the absorbance.

EXPERIMENT NO. 10

CELL FRACTIONATION BASED ON DENSITY GRADIENT

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ENCH 485, Spring 1988

Objective

To simulate cell fractionation based on density gradient.

Introduction

Centrifugation is a common separation technique that accomplishes separation based on the the density and size differences in a mixture of components. In the absence of Brownian motion and thermal mixing, a centrifuge is often not absolutely necessary in separating most of the particles that a novice student is accustomed to. For example, yeast cells or most chemical precipitates, given enough time, will eventually settle at the bottom of a container at 1 g (gravity unit). However, the process can be sped up considerably with a centrifuge.

There are two stages of separation with centrifuges. The first stage takes advantage of the difference in the terminal velocities of different particles as determined by Stoke's law:

$$v_t = \frac{2}{9}\frac{R^2}{\mu}(\rho_s - \rho)a$$

where v_t is the terminal velocity of the particle, R the radius of the particle, a the centrifugal acceleration of the centrifuge, μ the viscosity of the medium, ρ_s the

density of the particle, and ρ the density of the medium. From the above equation, it is apparent that the terminal velocity is a function of the particle radius and density. Thus, on the average, bigger and heavier particles will travel through the medium faster and settle at the bottom of a centrifuge tube in a shorter time. Because a typical mixture of cell homogenate contains organelles of varying sizes and densities, as well as shapes, they can be separated according to the sedimentation speed. The decrease in the sedimentation time in a centrifuge over gravitational settling is mainly due to the considerable increase in the variable a in Stoke's equation.

In the study of cell organelles, different fractions of the subcellular particles are routinely separated with a centrifuge. It was by this type of isolation technique that mitochondria were discovered to be responsible for the entire tricarboxylic acid cycle and ribosomes accountable for protein biosynthesis.

In a typical separation scheme, an isotonic 0.25-0.35M sucrose solution is mixed with cells. The mixture is placed in a bead homogenizer, an ultrasonic cell disrupter, or a simple kitchen blender and the cell membranes are broken to spill out the cell contents. The resulting mixture of subcellular organelles can be placed in a centrifuge and spun at 1000g for 10 minutes. Intact cells and heavy nuclei are collected at the bottom of the centrifuge tube as a pellet. After the supernatant is further centrifuged at 10,000g for 20 minutes, subcellular particles of intermediate terminal velocities such as mitochondria, lysosomes, and microbodies may be collected. The smaller and lighter particles (ribosomes, endoplasmic reticulum fragments, cell membranes, and microsomes) can be further separated from the supernatant of the preceeding stage by centrifugation at 100,000g for 60 minutes. The final supernatant may be considered to be the soluble portion of the cell cytoplasm. Notice the increasingly longer time and especially the exponentially increasing centrifugation speed required to effect separation. The last speed is achievable in an ultracen-

trifuge. This mode of separation is commonly called *differential centrifugation.*

Each fraction obtained through differential centrifugation contains quite a few different types of organelles which have similar sedimentation velocities, i.e. similar values of $R^2(\rho_s - \rho)$. Because this factor is a combination of both the size and the density, the fraction can be further separated based on density alone irrespective of the sizes. This second stage can be accomplished by a process known as *density gradient centrifugation.*

The density gradient may be established naturally by simply placing sucrose crystals at the bottom of a test tube. The sugar dissolves in the solution and diffuse toward the top. However, the time required to establish a sugar gradient in this manner is unacceptably long, and the process is not well regulated. A more practical method of establishing a density gradient is by placing layer after layer of sucrose solutions of different concentrations, thus, densities, in a test tube, with the heaviest layer at the bottom and the lightest layer at the top. The cell fraction to be separated is placed on top of the layer. A particle will sink if the density of the particle is higher than that of the immediate surrounding solution. It will continue to sink until a position is reached where the density of the surrounding solution is exactly the same as the density of the particle. A centrifuge can be highly helpful to accelerate this process of reaching the quasi-equilibrium point; however, unlike the differential centrifugation technique used during the first stage of cell separation, the length of centrifugation for this second stage does not matter too much, as long as the system is permitted to come to quasi-equilibrium.

In the following steps, organelles are substituted by colored plastic beads to demonstrate separation according to the particle density in a density gradient.

List of Reagents and Instruments

Size and density of some typical organelles*

Organelle	diameter (μm)	Density (g/cm^3
Nuclei	5-10	1.4
Mitochondria	1-2	1.1
Ribosomes	0.02	1.6
Lysosomes	1-2	1.1

**Carolina Tips*, Nov. 1, 1973.

A. Equipment

Graduated cylinder

Test tube

B. Reagents

Sucrose

Plastic beads of various densities

Procedures

1. Prepare 50 ml of 15% and 50 ml of 40% sugar solutions. Ink or food coloring may be added to each solution to enhance the visual identification of the different layers of sugar solutions.
2. Measure the density of each sugar solution with a hydrometer.
3. Pipet 20 ml of the 15% sugar solution and position the pipet tip at the bottom of the test tube containing 20 ml of water. Carefully let the 15% sugar solution flow out beneath the water layer.
4. Following the above step, pipet 20 ml of the 40% sugar solution and discharge it beneath the 15% solution. There should be three layers: 0%, 15%, and 40%, counting from the top. Thus, one has effectively created a density gradient.

5. Drop a few plastic beads and record the positions where the beads settle.
6. Separate subcellular organelles instead of plastic beads if an ultracentrifuge is available.

Discussion

Note that inexpensive testers of antifreeze and battery acid that have multiple plastic beads of various densities contained in an oversized eye dropper are also based on similar density concepts. For example, when the sulfuric acid content in a battery is low, the density of the electrolyte solution is too low to cause all the beads to float. All the beads should float when the sulfuric acid concentration is just right. Actually, the charging state of the battery is only indicated by the hydrometer indirectly.

Questions

1. Based on the level at which the beads float, estimate the density (or the windows of densities) of each type of bead.
2. If 10 cm of 2% sugar solution is placed at the bottom of 10 cm of water in a cylindrical test tube, the equilibrium sugar concentration should be 1%. Find the diffusion coefficient of sugar from literature sources, and calculate the time needed for the surface of the solution to reach 0.95% if the system is left undisturbed.
3. When establishing a density gradient in a test tube, is it better to build from the heaviest layer up or the lightest layer down? Why?
4. Comment on ways to improve the experiment.

Reference Readings

1. Martin, D. L. and Sampugna, J., *Molecules in Living Systems: A Biochemistry Module*, Harper and Row, 1973.

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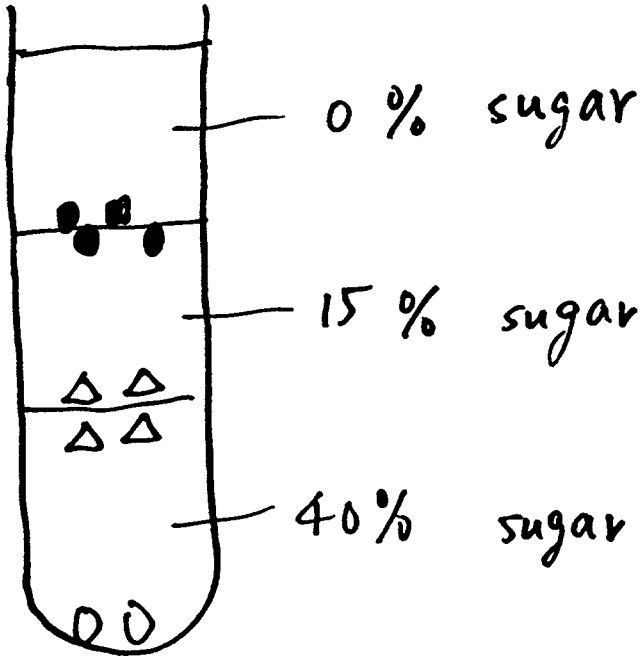

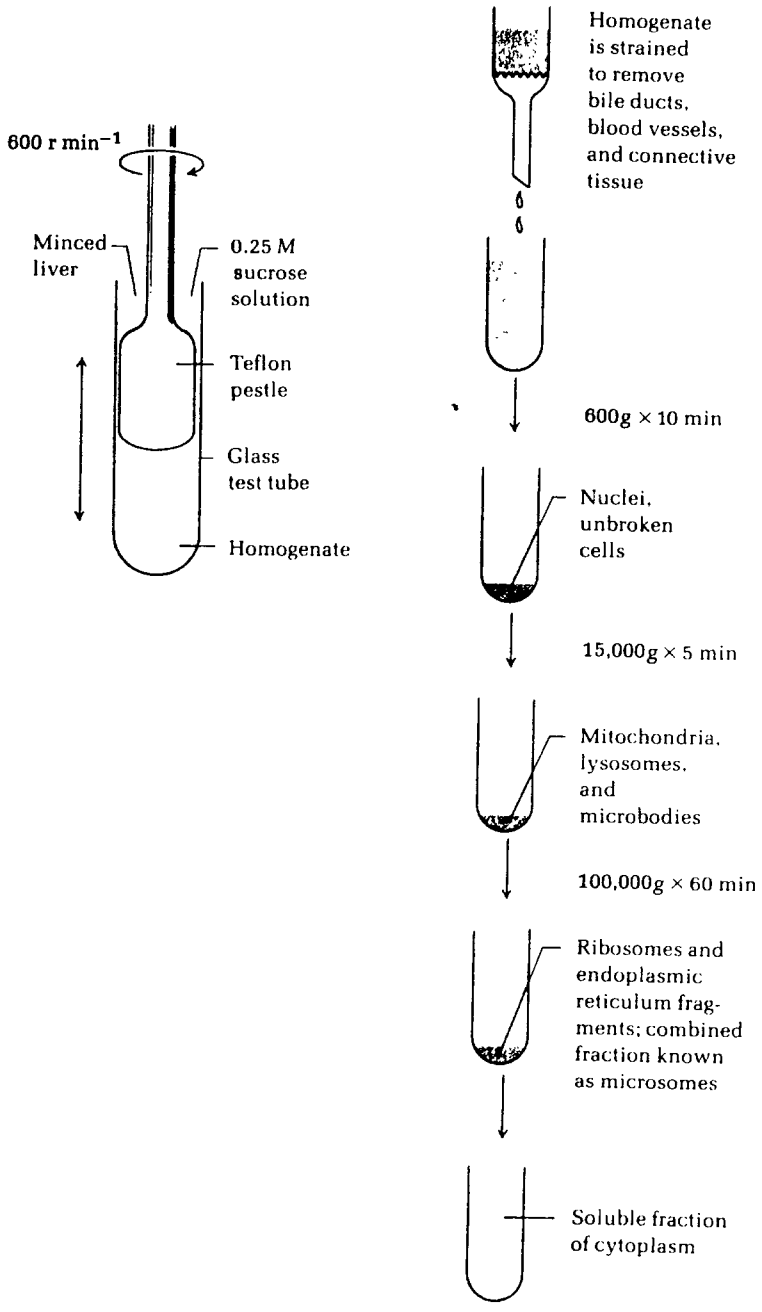

Figure 14-11
Isolation of intracellular structures from rat liver by differential centrifugation. The cell membranes are ruptured by the shearing forces developed by the rotating homogenizer pestle. Following removal of connective tissue and fragments of blood vessels and bile ducts by a stainless steel sieve, the cell extract is centrifuged at a series of increasing rotor speeds.

EXPERIMENT NO. 11

CELL IMMOBILIZATION WITH CALCIUM ALGINATE

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ENCH 485, Spring 1988

Objective

To investigate the conversion of glucose to ethanol by entrapped yeast cells in a continuous reactor.

Introduction

This experiment introduces the students to an immobilized cell fermentor. Yeast cells will be entrapped in calcium alginate gels by using the similar techniques as in enzyme immobilization. Other cell entrapment media that have been previously attempted include polyacrylamide, gelatin, chitosan, and κ-carrageenan gels.

Due to the constraint in the available equipment to carry out the immobilization procedure aseptically, the experiment will be conducted without autoclaving. The immobilized cell reactor will be employed to convert glucose into ethanol anaerobically. The reasons for choosing this system of microorganism and product are many folds. First, the anaerobic condition will eliminate the need for aeration, which causes many technical problems. Secondly, the lack of oxygen will prevent the uncontrolled growth of aerobic contaminants in an unsterilized fermentor. The

presence of high levels of ethanol should also discourage most microorganisms from taking over the fermentor. To reduce further the chance of contamination by bacteria, the pH of the fermentor will be kept low; a value of 4.0 should drastically slow down the growth of most bacteria but only slightly affect the yeast's ethanol producing capacity.

The production of ethanol in an immobilized bioreactor is a relatively well studied process. As high as 95% of the theoretical yield of alcohol based on glucose (8.5 % ethanol from 14defined as the volume of nutrient feed per hour per gel volume, of 0.4-0.5 hr^{-1} is commonly used to maximize the ethanol productivity. An ethanol productivity of 20 g/l-hr can be achieved.

Both the steady state response and the transient approach to the steady state will be studied in this experiment .

List of Reagents and Instruments

A. Equipment

Beakers

Graduated cylinder

Balance

Pipets

Magnetic stirrer

Syringe & needle

Spectrophotometer with flow through cell

pH probe and controller

Microcomputer with data acquisition capabilities

B. Reagents

Growth medium, see the recipe in Experiment No. 9.

Alginic acid, sodium salt

$CaCl_2$

Yeast culture

NH_4OH

Reagents for glucose analysis

Reagents for ethanol analysis (or a GC)

Procedures

1. *Immobilized Cell Preparation:*

   a. Dissolve 9 g of sodium alginate in 300 ml of growth medium, following the same procedure adopted in enzyme immobilization to avoid clump formation. Stir until all sodium alginate is completely dissolved. The final solution contains 3% alginate by weight. See Note 1.

   b. Thoroughly suspend about 250 g of wet cells in the alginate solution prepared in the previous step. Let air bubbles escape. See Note 2.

   c. Drip the yeast-alginate mixture from a height of 20 cm into 1000 ml of crosslinking solution. (The crosslinking solution is prepared by adding an additional 0.05M of $CaCl_2$ to the growth media. The calcium crosslinking solution is agitated on a magnetic stirrer. Gel formation can be achieved at room temperature as soon as the sodium alginate drops come in direct contact with the calcium solution. Relatively small alginate beads are preferred to minimize the mass transfer resistance. A diameter of 0.5-2 mm can be readily achieved with a syringe and a needle. The beads should fully harden in 1-2 hours. Note that the concentration of the $CaCl_2$ is about one fourth of the strength used for enzyme immobilization.

   d. Wash the beads with a fresh calcium crosslinking solution.

2. *Immobilized Cell Reactor Construction:*

   a. Construct an immobilized cell reactor with a 500ml Erlenmeyer flask fitted as shown in Figure 1. Place the hardened beads in the flask and seal it with a rubber stopper with appropriate hose connections.

   b. Make all necessary connections. Start the experiment by filling the flask with the growth media (100g/l glucose) to the working volume of 350ml.

3. *Immobilized Cell Reactor:* Then following sequence of events will be monitored both on-line and off-line. The responsibilities of on-line data acquisition and off-line sample collection and analysis will be shared by the entire class; the exact assignment will be determined in class. A microcomputer will be programmed to take data on the glucose concentration and the rate of NH_4OH addition needed to maintain the pH at 4.0. The off-line samples will be analyzed for the optical density (for free cell concentration), glucose concentration, and ethanol concentration. Furthermore, the liquid and gas flow rate will be measured with a graduated cylinder as indicated in Figure 2.

   a. The reactor will be operated in a batch manner until no more glucose is utilized. This can be detected with the leveling off in the glucose concentration.

   b. Substrate feeding will then commence at the rate of 0.4 hr^{-1}. Record the substrate flow rate. The approach to the first steady-state during the start-up will be followed.

   c. Various parameters (nitrogen consumption rate, carbon dioxide evolution rate, glucose concentration, ethanol concentration, and free cell level) at the high steady state are recorded.

   d. Decrease the substrate feeding rate to 0.2 hr^{-1} Measure the substrate flow

rate and follow the transient approach to the new low steady state.

e. Repeat part 2c) for the new steady state.

4. If time permits, continue shifting the flow rate and obtain more information on steady states. Continue operating the bioreactor until noticeable deterioration in the performance is detected due to gel swelling, cell death, or severe contamination.

Notes

1. To avoid the premature gel formation, the phosphate concentration in the medium must be adjusted to less than 100μM.

2. Because cell growth can break the bead and is generally considered undesirable beyond what is needed to compensate for the endogenous decay, the cells used for immobilization ideally should have just entered the stationary phase. An equivalent amount of dried cell culture may also be used in lieu of wet cell paste. The actual cell loading may be varied according to the substrate concentration in the feed and the desired product levels. The ratio of wet weight to dry weight is approximately 4 for most cells.

Discussion

Basically, immobilization of live cells is very similar to the enzyme counterpart. In the past, various cells have been immobilized: bacteria, yeasts, fungi, plant tissues, mammalian tissues, and insect tissues. However, true successes are limited to only a few cases. One of the problems is the mass transfer resistance imposed by the fact that the substrate has to diffuse to the reaction site and inhibitory or toxic products must be removed to the environment. Oxygen transfer is often the rate limiting step in a suspended cell culture, and it is more so in an immobilized cell culture. Oxygenation in an immobilized cell culture is one of the major technical

problems that remain to be solved. In light of the oxygenation problems, immobilization techniques have been mainly confined to anaerobic processes in which either obligate (strict) anaerobes are employed or only the anaerobic components of the facultative metabolic mechanisms are selectively utilized.

The lower microorganisms (bacteria, yeasts, and fungi) can be rather easily immobilized with a number of methods: entrapment, ion exchange adsorption, porous ceramics, and even covalent bonding. In terms of dollar values, chemicals of plant origin account for the lion's share of the market. Some examples of plant extracts are drugs, flavors, and perfumes. Despite the recent surge in research activities in animal cell culture throughout the country, few applications actually exist beyond the production of monoclonal antibodies. Immobilized insect tissues have been used in pesticide research and has a potentially quite large commercial market in agriculture.

Most of the principles involved in enzyme immobilization are directly applicable to cell immobilization. Covalent bonding, affinity bonding, physical adsorption, and entrapment in synthetic and natural polymer matrices. The most popular and practical immobilization technique deals with cell recycle with an ultrafiltration membrane or a hollow fiber cartridge. Although this process is not ordinarily viewed as cell immobilization at all, it is functionally equivalent, the cell recycle devices effectively retaining the catalysts in a bioreactor and accomplishing the same objective as cell immobilization.

An immobilized cell bioreactor is well suited for those cells whose growth phases and product formation phases are uncoupled. Cell biomass and primary metabolites are growth associated products, but secondary metabolites such as antibiotics and various enzymes are produced during the stationary phase. The uncoupling of the phases means that productive cells cannot compete with the nonproductive

cells in a continuously operated suspension fermentor because the productive cells spend the nutritional and energy resources producing chemicals in quantities far above the amount necessary for their survival, instead of reproducing themselves to propagate further. On the contrary, cell growth in an immobilized cell reactor must be severely limited if gel swelling or breakage is to be avoided. However, once the cells are immobilized, the cell viability must be concomitantly sustained over a long period of time. Thus, immobilization is advantageous for sustaining slowly growing cells, especially plant tissues. In summary, one wishes to keep the immobilized cells alive without multiplying.

Questions

1. Considering the reaction stoichiometry, calculate the theoretical yield of ethanol from glucose. What is the steady-state productivity of the bioreactor? Would you conclude that the productivity of an immobilized cell reactor is higher than a continuously operated suspension type?
2. Plot the response of the reactor (glucose concentration, productivity, etc.) as a function of time after a shift-up in the substrate flow rate. Was there any delay/lag in the response? How long did it take to reach the steady state? Repeat for a shift-down in the flow rate.
3. If immobilization procedure can be carried out aseptically, how long do you expect an immobilized cell reactor to operate between down times? What are some of the reasons for terminating a continuous fermentor.
4. Comment on ways to improve the experiment.

Reference Readings

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3. Nagashima, M., Azuma, M., and Noguchi, S., Technology developments in biomass alcohol production in Japan: continuous alcohol production with immobilized microbial cells, *Ann. N.Y. Acad. Sci.*, **413**, 457, 1983.

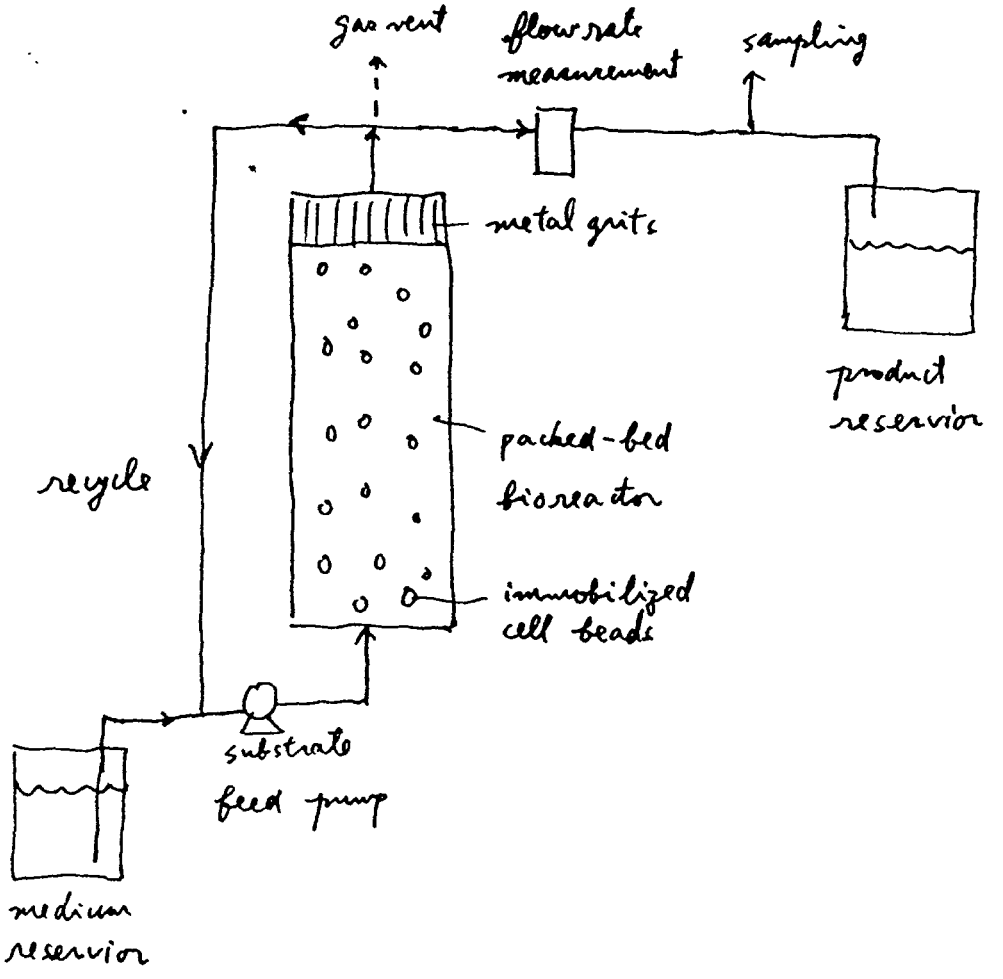

Figure 1a. Packed-bed immobilized cell reactor

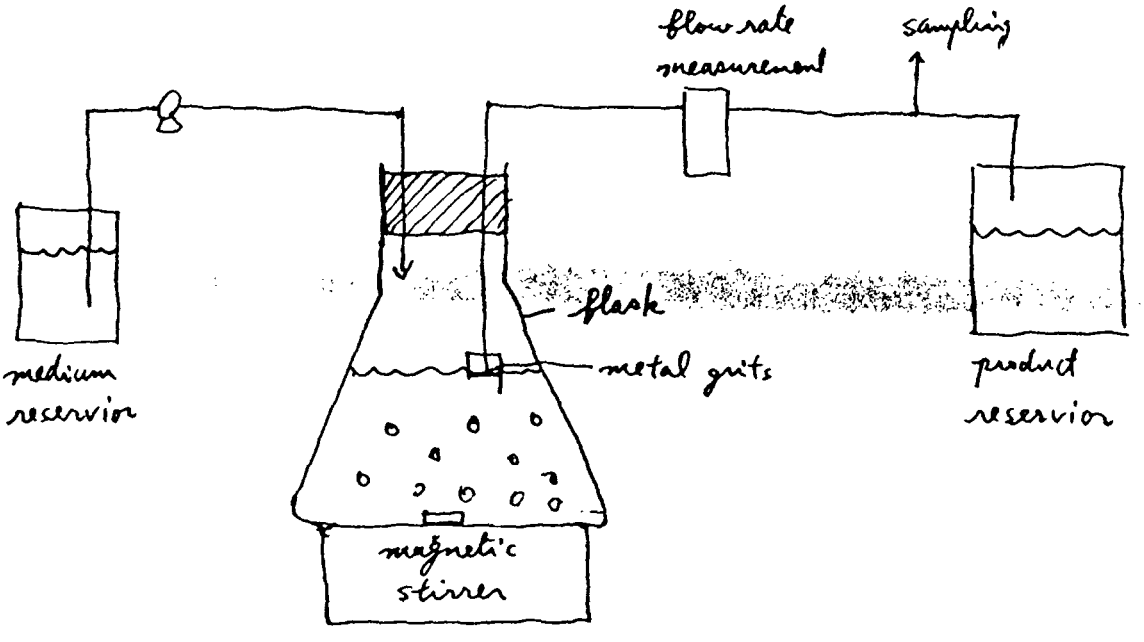

Figure 1b Continuously stirred immobilized cell reactor

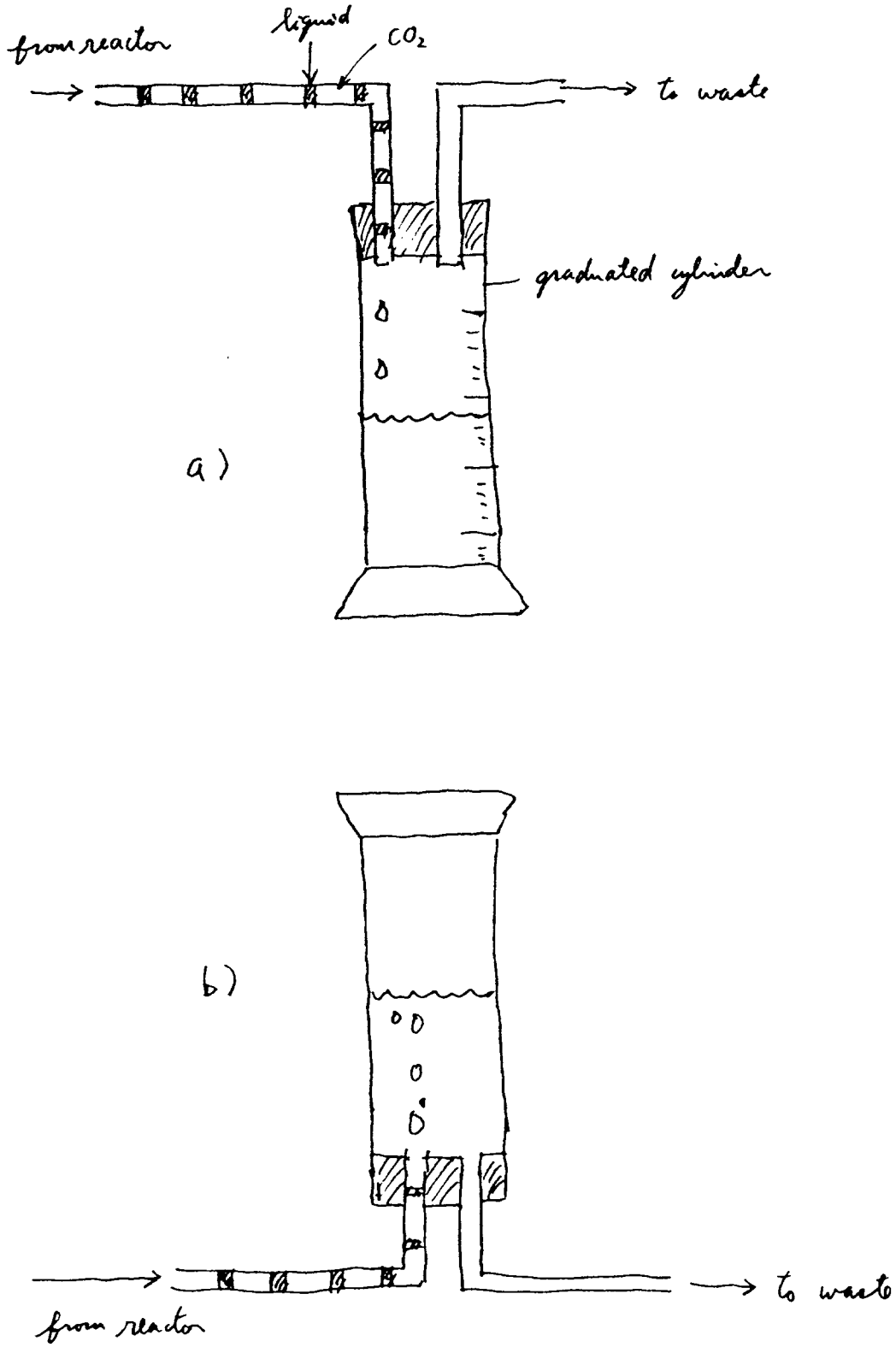

Figure 2 a) Use a graduated cylinder in the up-right position to measure the liquid flow rate. b) The graduated cylinder is then inverted to measure the gas flow rate.

EXPERIMENT NO. 12

WINE FERMENTATION

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ENCH 485, Spring 1988

Objective

To study the effect of sugar content on wine fermentation.

Introduction

Wine making is still very much an art rather than science. Since many books are devoted to the subject of wine making and wine tasting, the topic will not be elaborated upon here. Although grapes are by far the most often used fruit, various other fruits such as peaches and prunes may also be used to make wines.

The procedures of making grape wine at home are quite straight forward. Grape juice is simply inoculated with a package of yeast starter culture purchased from a supermarket. Primary fermentation lasts for approximately one week; during that time most of the sugar originally present in the juice is converted to ethanol and yeast cells, with the evolution of carbon dioxide. The excess yeast cells are then removed from the juice along with other sediment, and a slower secondary fermentation is allowed to proceed to develop the final flavor. Sugar may be added to the original must to achieve the desired alcohol content or to modify the flavor. The type of wine can be classified according to the color of the wine. Another classification is based on the starting sugar content, as listed in Table 1.

Table 1. Classification of wines according to the sugar content.

Type	Specific Gravity	Sugar Content (wt %)
Dry Wine	1.085 - 1.100	21 - 25
Medium Sweet Wine	1.120 - 1.140	29 - 33
Sweet Wine	1.140 - 1.160	33 - 37

Use of a Hydrometer

A hydrometer is a floatation device used to measure the density of a liquid. Because the liquid exerts a buoyancy force equal to the weight of the volume displaced by the hydrometer, the meter will float higher in a denser fluid than in a lighter fluid. In general, a denser solution has more dissolved solids. A conventional hydrometer costs approximately $10-20 each and can easily measure the specific gravity of a liquid to an accuracy of 0.001. Many scales are available on a hydrometer. A typical one employed in the alcoholic beverage industry allows readings in both the Balling/Brix scale and the Potential Alcohol (PA) scale, as well as the usual specific gravity scale. The Balling, or Brix, scale is calibrated based on the *weight* percent of sugar in solution; whereas, the PA scale is an indication of the *potential* (*volume*) percent of alcohol that may be produced through fermentation based on the complete conversion of the sugar originally present in the solution. Of course, this represents the maximum value that is rarely reached. Usually, residual sugar is present and is sometimes desirable for taste. Note that for historical reasons, the alcohol content is commonly measured in units of volume percent or proof, while the sugar content is expressed in weight percent. Both units are widely used in wine industries.

The proper way of using a hydrometer is to spin it gently in the grape juice, must, or wine for which the specific gravity is to be measured. Twisting of the hydrometer removes most of the air bubbles from its surface which can invalidate

the measurement. Do not drop the hydrometer into a container from a height, for the hydrometer is likely to be broken. A graduated cylinder is usually used for this purpose. The sugar content of the sample in weight percent is read from the meter which in turn indicates the potentially achievable alcohol content in the final fermented product. Two readings are required to estimate the alcohol content in a fermented wine. The first reading is taken at the beginning of fermentation. The second reading will tell one the amount of the remaining sugar at the time of the reading. Thus, the current alcohol content is simply the present PA value minus the starting PA value.

Because the density of a liquid is a function of the temperature, Table 2 gives the correction that one should add to the specific gravity readings to obtain the corresponding values at 60 °F, the temperature at which the meter is calibrated.

Table 2. Temperature correction to the specific gravity reading.

Temperature (°F)	Correction (sp.g.)
50	-0.0005
60	0.000
70	+0.001
77	+0.002
84	+0.003
95	+0.005
105	+0.007

List of Reagents and Instruments

A. Equipment

Graduated cylinder

Test tubes

Erlenmeyer flasks or bottles

Rubber stoppers

Tygon tubing

Hydrometer

Balance

B. Reagents

Grape juice

Sucrose

Active dry wine yeast, strains of *Saccharomyces ellipsoideus*

Montrochet, Redstar brand

Universal Foods Corporation

Milwaukee, Wisconsin, 53201

Procedures

1. *Prepare Starter Yeast Culture*

   a. Mix 1 g of dry wine yeast culture in 100 ml of grape juice. See Note 1.

   b. Let the yeast grow in a loosely capped container at room temperature for 24 hours.

2. *Primary Fermentation*

   a. Add enough sugar to grape juice to prepare the following 4 substrates, about 1 liter each:

   b. Measure the specific gravity and PA value for each of the starting substrates with a hydrometer. This is the initial PA value which will be used later to estimate the alcohol content.

   c. Inoculate each bottle with 20 ml of the starter yeast culture prepared in

Run	Conc. of Extra Added Sugar
A	0.0g/l
B	100.0g/l
C	200.0g/l
D	300.0g/l

the previous step. See Note 2.

d. Plug the juice bottle with a rubber stopper. A piece of Tygon tubing is extended from the stopper to provide a vent for the evolved carbon dioxide. The other end of the tubing is dipped in water in a small test tube taped to the bottle. The water prevents the entry of oxygen, which alters the metabolism of the yeast and spoils the wine. At the same time, carbon dioxide can escape from the bottle.

e. Ferment at room temperature for one week.

3. *Secondary Fermentation*

a. At the end of one week, decant the juice from the bottle to clean individual temporary containers.

b. Measure the PA values for each of the substrate with a hydrometer. Estimate the alcohol content by subtracting the present PA value from the initial PA value.

c. Discard the sediment and wash each bottle with water.

d. Pour the juice back into the cleaned bottle. Put back the cleaned assembly of rubber stopper and Tygon tubing.

e. Ferment slowly for another 4-6 weeks.

f. Measure the PA values as before when it is ready for consumption.

4. Taste the wine and celebrate the end of the course in biochemical engineering laboratory!

5. Try out other fruit juices.

Notes

1. Fresh grapes may be crushed to obtain the juice. Most wineries add sulfur dioxide either as a gas or as a solid salt to prevent the growth of other yeasts and bacteria which cause spoilage. Sulfur dioxide is also produced by *Saccharomyces* during fermentation. The simplest source of juice is the supermarket shelf. Use the "All Natural" variety with no added preservatives or sugar.

2. Dry wine yeast may be directly added to grape juice from a package at the level of 1 g per 4 liter. For best results, first suspend 1 g of dry wine yeast in 10 ml of warm water at about 35°C. Then add the suspended culture to grape juice.

Questions

1. Compare the estimated values of alcohol content with those measured independently either by a wet chemistry method or a gas chromatograph. Does the hydrometer indeed give a good estimation for the ethanol content in wine?

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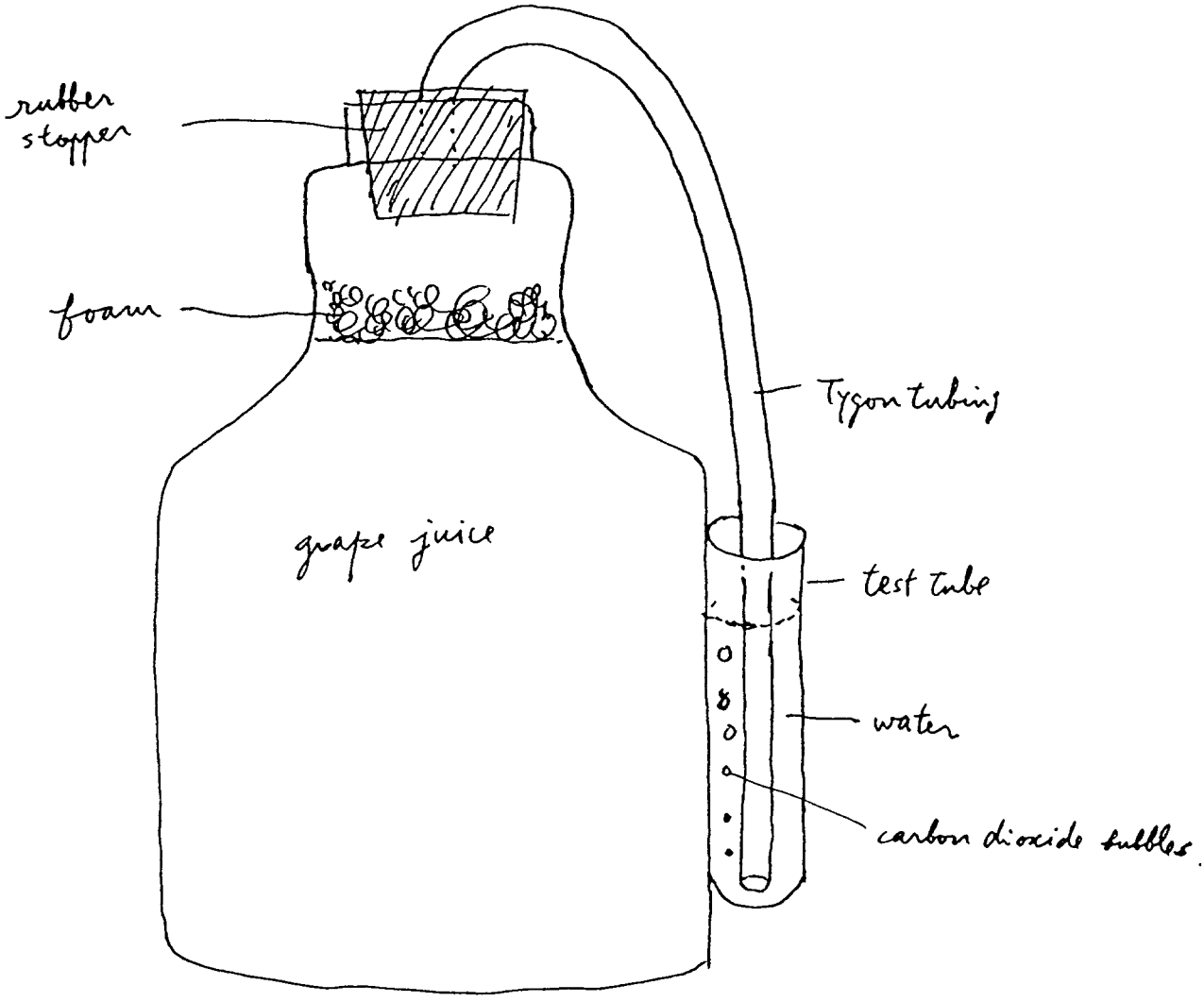
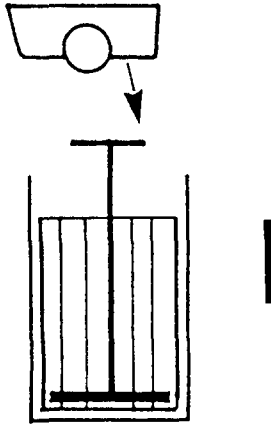


Home-made wine fermentor.

BASIC WINE MAKING

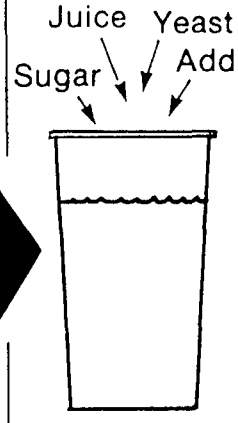
SCALE: 1 or 5 gallon batches

1 FRUIT PREPARATION



2 FERMENTATION

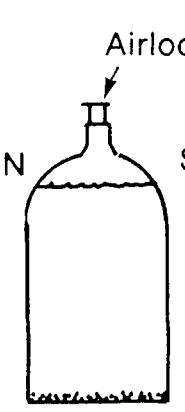
PRIMARY
Open container



SIPHON

When foam drops

SECONDARY
Closed container

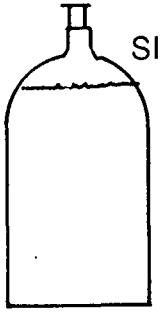


3 CLARIFICATION & BULK AGING

SIPHON

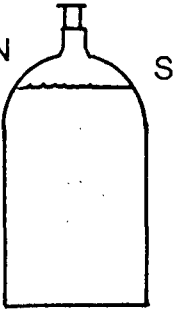
when clear

1st Racking



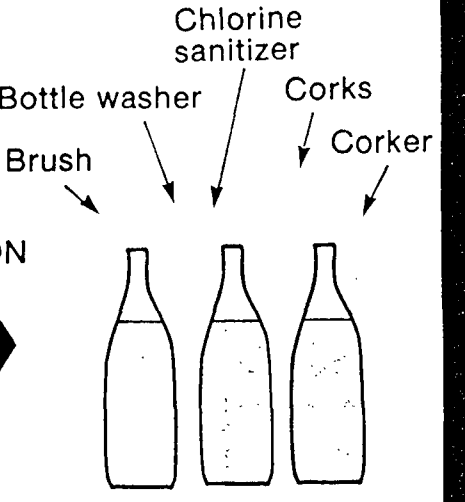
SIPHON

2nd Racking



SIPHON

4 BOTTLING



5 BOTTLE AGING

Whites - 90 days

Reds - 180 days

55°

	1	2	3	4
TIME:		4-7 days	30 days	30+ days	30+ days
SPECIFIC GRAVITY:		1.090-0.040	1.040-0.995	1.000-0.990	1.000-0.990
TEMP:		70°	70°	60°	60°

12-X