

**Guidelines
for
Production
of
Bacillus thuringiensis H-14
and
*Bacillus sphaericus***

edited by:

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*UNDP/World Health Organization/WHO Special Programme
for Research and Training in Tropical Diseases (TDR)*

**Guidelines for Production of
Bacillus thuringiensis H-14 and *Bacillus sphaericus***

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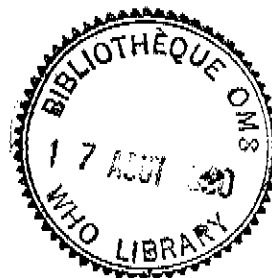
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1

Introduction

During the past few years, there have been major advances in the development and use of microorganisms as microbial insecticides. Although several types of microorganisms can affect insects, only three types are being produced commercially at the present time: viruses, fungi, and bacteria. Of these, only fungi and bacteria can be grown away from the insect in fermentations; viruses can be grown in large quantities only in host insects. The present discussion is restricted to organisms that can be produced in normal fermentation processes.

Throughout the fermentation process, it is important to maintain strict quality control. The fermentation processes are effective only in the hands of skilled workers. The production of microorganism must be supervised by someone with a high degree of training in microbiology. Monitoring of the fermentation will require bioassay by a skilled entomologist.

In the past, man has successfully conducted many fermentations with only limited knowledge of their processes. However, these traditional fermentations contain their own controls. For example, quality control in wine is maintained by colour, taste, and odour. If a fermenter is faulty, if a starter culture is spoiled, the winemaker can detect it. Furthermore, in these fermentations, the danger of contamination - ie. the growth of unwanted organisms - is reduced by some property of the fermentation. For example, some fermentations grow anaerobically (in the absence of air), while many potential contaminants require air to grow; other fermentations produce a high degree of acidity, while many contaminants will not grow in acid, etc. Conditions such as these make it difficult for contaminating microorganisms to compete in a fermentation, and so help to ensure the success of the traditional fermentations and to explain their long-term successes.

Modern fermentations are different. Developments in fermentation technology now make it possible to grow microorganisms under conditions that are not natural to them; however, these may be highly favourable to the growth of contaminants. Many benefits have been derived from these developments, which allow the production of agents that could not be produced under traditional technologies, thus lowering the costs of important fermentation products. However, the artificial nature of these improved fermentation processes renders them very sensitive to minor changes in fermentation conditions, in the nutrient composition or in the microorganism itself. This document examines the various aspects of fermentation as they apply to the production of microbial insecticides, and what is required to undertake this important and potentially very valuable manufacturing process.

In any manufacturing process it is essential to be able to measure (assay) the quality and quantity of the product. Assays requiring tests against living organisms are defined as "biological assays" or "bioassays". Bioassays are essential to the production of microbial insecticides, and, before discussing fermentation, the philosophy and limitations of these assays

must be understood. Therefore, discussion must begin by defining the procedures and requirements of bioassays. Second, it is necessary that the source material - the microorganism to be used in the fermentation - be authentic. Thus the culture maintenance must be well understood.

Many - if not most - of the efforts to produce and utilize microbial insecticides have foundered, not on the limitations of the microorganism, but on the failure of the producers of the microbial formulations to recognize the importance - indeed the absolute necessity - of careful, accurate, and reliable bioassay and culture maintenance techniques.

2

Bioassay of Microbial Insecticides

2.1 Philosophical and Interpretive Differences between Bioassays of Chemical and Microbial Insecticides

The procedure for monitoring the production of a chemical insecticide is relatively simple. The pure product has already been evaluated and its insecticidal activity is known. The production process can be followed chemically, and intermediate products can be measured and evaluated so that changes in yields during any step in the process can be quickly observed and corrective measures taken. Impurities in the final product are generally minor. One can weigh the insecticide produced and test it for purity. With this data one can evaluate the activity and the quality of the product and the guarantee the "percent active ingredient" statement on the product's label. The production process does not depend in any way on bioassays.

This is not the case when one monitors the production of microbial insecticides. There is no tangible active product to be weighed. The level of impurities in the final product is very high; in fact, the active ingredient may represent only a small percentage. Furthermore, the level of impurities can vary widely from fermentation to fermentation. Of course, there is an active ingredient: the spores of a bacterium or fungus; one of the toxins produced by various strains of *Bacillus thuringiensis* (*B.t.*); or a combination of spores and toxin as may be the case with *Bacillus sphaericus* (*B.s.*). But even if it were possible to purify and weigh this active ingredient, it might be meaningless to do so. For example, spores of a fungus can vary in virulence, depending on variations in the strain of fungus or in the fermentation.

This affects the conduct and design of the bioassay. The reliability demanded from bioassays of microbial insecticides must be much greater than that demanded when bioassay of chemical insecticides is done. Bioassays are not accessories to the production of micro-organisms - they are

central and vital to it; and monitoring the entire production process and the product itself is dependent upon these bioassays.

2.2 Design of Bioassays of Microbial Insecticides

In any bioassay one is evaluating the response of a group of living organisms that, no matter how inbred they may be, are not perfectly homogeneous. Thus the results can only be an estimate. The accuracy of this estimate depends upon many things: how precisely one can measure the response that one is observing; how many individual insects are in the population that are being tested; the quality of the insect population; and how many times the bioassay is repeated. In this section, an attempt has been made to describe precise details of a bioassay.

2.2.1 The LC50

Most bioassays of microbial insecticides have been designed to determine how much material is required to cause death in a population of target insects. Statistically, the most accurate measurement of a death-response of an insect population is that concentration of toxin which will kill 50% of the insect population.

Determining the LC50 requires considerable experimental effort. One must expose randomly selected members of the test insect population to a series of dilutions of the microbial insecticide in their diet for a predetermined period of time and then calculate the percentage kill of the population at each dilution. Then, either by use of log-probit graph paper, or preferably through computer analysis, the theoretical LC50 can be calculated.

The method used to expose the insect to the toxin and the length of the time of exposure will depend upon the insect being tested and upon its response to the microbial insecticide being assayed; for example, *B.t.* H-14 assays are complete in 24 hours; *B.s.* assays are complete in 48 hours and assays of *B.t.* H-3a, 3b require 7 days. It is important that the assay is standardized and that a minimum of five, and preferably seven, dilutions be tested. The maximum kill of any dilution must be <90% and the minimum, >10%. The dilutions should be selected so that the calculated LC50 will fall in the middle range of the dilution series. The number of insects used for each concentration will depend on the insect and upon the accuracy needed. For example, 25 to 50 larvae of *Trichoplusia ni* are used per dilution to standardize *B.t.* H-3a, 3b for use against lepidopterous insects, while 100 larvae of *Aedes aegypti* per dilution are used to standardize *B.t.* H-14, for use against mosquitos and blackflies. *Ae. aegypti* was chosen for a variety of reasons. A suitable species of the *Culex pipens* complex for which comparative data are available may also be used.

A bioassay to determine the LC50 of a microbial insecticide demands considerable numbers of insects, and this requires rather large insect colonies. Furthermore, if the assays are to be accurate, the insect colony must be vigorous and disease-free. This requires clean, protected rearing areas with good control of temperature, humidity, and lighting. The cost of maintaining such insect colonies must be considered in evaluating the economics of producing these insecticides.

2.2.2 The international unit or IU

The LC50 is a valuable tool, but no matter how carefully an insect colony is bred and reared, it will vary in its susceptibility to a microbial insecticide. The LC50s for a single formulation can vary from day to day. For example, Dulmage (1973) analyzed the response of *Heliothis virescens* to HD-1-S-1971, the primary US standard for *B.t.* H-3a, 3b. As shown in Table 1, the LC50 measured for the standard in 18 bioassays run over a 3-month period varied from 6.0 to 17.5 $\mu\text{g/ml}$ of the diet. In the past few years, over a very large number of bioassays, this LC50 has varied from as low as 5 μg to over 40 $\mu\text{g/ml}$ diet. Obviously, no accurate measurement of potency can be obtained from the LC50 alone.

The solution has been to compare the LC50 determined for a test sample with the LC50 of a standard formulation tested on the same day against the same population of insects. The presumption is made that under these conditions the ratios between the LC50s determined for these test samples and the LC50s of the standard will remain constant, regardless of any changes in the condition or response of the insects. Therefore, the preparation of a standard formulation is required for bioassaying a new microbial insecticide.

Table 1. Distributions of LC50s of HD-1-S-1971, a Standard Preparation of the Delta-Endotoxin Produced by *B. thuringiensis*, H-3a,3b as Measured in Bioassays vs. *H. virescens*

Range of LC ₅₀ s as $\mu\text{g/ml}$ diet	No. of times LC ₅₀ within range
5.0-7.5	2
7.6-10.0	3
10.1-12.5	8
12.6-15.0	3
15.1-17.5	2

It would be perfectly valid to use these ratios to express activity. For example, if the LC50 of a test sample is one-half that of the standard, one can say that the test sample is twice as active as the standard or that the ratio of its activity to that of the standard is 2.0, according to the following formula:

$$\text{Ratio of activity, sample to standard} = \frac{\text{LC}_{50} \text{ standard}}{\text{LC}_{50} \text{ sample}}$$

In practice, this is awkward. One must deal with decimal numbers, many of them less than 1.0, and one must define the standard each time. This can be avoided by assigning units of potency to the standard and then expressing the activity of the test sample in similar units. This has been done in the

case of *B.t.* and *B.s.* formulations, and, by agreement, no matter which standard is defined, one expresses these units as "International Units" or "IUs". The formula thus becomes:

$$\text{Potency of Sample, IU/mg} = \frac{\text{LC}_{50}^{\text{standard}}}{\text{LC}_{50}^{\text{sample}}} \times \text{Potency of standard}$$

Several standard formulations have been used in the assay of bacterial larvicides. The names of these standards and the potencies assigned to them are given in Table 2.

Table 2.

Bacteria	Name of standard	Serotype	Potency -IU/mg
<i>B.t.</i>	HD-1-S-1971	H-3a,3b	18,000
<i>B.t.</i>	HD-1-S-1980 ^a	H-3a,3b	16,000
<i>B.t.</i>	IPS-78	H-14	1,000
<i>B.t.</i>	IPS-82 ^b	H-14	15,000
<i>B.s.</i>	RB-80	H-5a,5b	1,000
<i>B.s.</i>	SPH-88 ^c	H-5a,5b	1,000

^aThis is the current international standard for lepidopteran-active *B. thuringiensis* larvicides.

^bThis is the current international standard for *B. thuringiensis* larvicides active against diptera (mosquito and black fly).

^cThis is the current international standard for *B. sphaericus* larvicides, active against mosquitos.

The convention of using the term "International Unit" can easily lead to confusion. There is not necessarily a correlation between the IUs of one standard and those of another. For example, as seen in Table 2, HD-1-S-1980 has been assigned 16,000 IU/mg whereas IPS-78 is 1,000 IU/mg. These two materials affect different insects; HD-1-S-1980 has very little activity against mosquitos whereas IPS-78 is very active against them. The assay insect and the standard used in the bioassay to interpret potencies expressed in international units must be known.

2.2.3 Evaluating the accuracy of a bioassay

Dulmage (1982) has listed some of the requirements used to determine if a bioassay should be considered valid. These are as follows:

- i. There must be <10% dead in the control larvae - those that were grown on the same diet, but which were not exposed to toxin.
- ii. Dilutions must be selected so that at least 5 concentrations of each sample and 7 concentrations of the standard are "valid" ie. with no more than 90% or less than 10% kill).

- iii. Slopes of the regression curves must be within the ranges expected by experience, and they must be parallel to each other within the error of the day's assays. Non-parallel curves are rejected.
- iv. If computer analysis is available, the 95% Confidence Limits around the LC50 should be determined. They should be such that the Maximum Limit/Minimum Limit <2.0 . If not, the assay is rejected.
- v. Similarly, the 95% Confidence Limits around the IUs should be determined and the Maximum Limit/Minimum Limit should be <2.0 . If not, the assay is rejected.
- vi. Assays should be replicated on 3 separate days, and the mean and standard deviation (SD) between the 3 assays should be determined. The Coefficient of Variation (CV) of each assay series should be determined ($CV = SD/\text{mean}$). The CV should be <0.2 . If the CV is >0.2 , one or two more assays should be run. If the CV becomes <0.2 , the assays are accepted. If it remains >0.2 , the assay is rejected. If this occurs with most of the samples being tested, it is evidence that something has gone wrong with the bioassay procedure. Otherwise, one must suspect that the sample being tested is not homogeneous.

The confidence that we can place in a bioassay depends on the reproducibility and the reliability of the IU determined for each sample. Thus the CV is the key to the trust that we can place in our assays. The criterion that the CV should be <0.2 was based on many years of experience with the *B.t.* bioassays against lepidoptera insects, which has demonstrated that if the assay procedures are being followed correctly and the test insects are of good quality, the CVs determined on the various sets of *B.t.* samples falls below <0.2 . Experience with bioassays of *B.t.* H-14 against mosquitos indicates that mosquito bioassays are more accurate than lepidopterous assays and that the requirement for the CVs in these assays might be reduced to 0.15. The bioassay of *B.t.* H-14 is described in detail in Section 2.3 and that of *B.s.* in Section 2.4.

Bioassays are used in many different situations with different kinds of products. It is generally considered that if the error in the assays can be held to 20%, the assays will permit good standardization of the product and will adequately support research to increase production. To achieve this goal with the *B.t.* bioassay, assuming a CV of 0.2, we will need to replicate the assay between 3 and 4 times.

2.3 Bioassay Protocol for *Bacillus thuringiensis* H-14 Preparations¹

2.3.1 Standard bacterial preparation

Use the material "IPS-82" as the standard, obtainable from the Pasteur Institute. It should be stored and handled according to the suppliers' recommendations. These should include the necessity of storage at 2-5°C,

¹Based on Annex 5 of the report of the Fifth meeting of the Scientific Working Group of Biological Control of Vectors, TDR/VEC-SWG(5)/81.3. UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, World Health Organization.

equilibration at room temperature before opening the plastic box to avoid condensation and, after the taking of a sample, resealing the container and returning it to 2-5°C in darkness.

2.3.2 Assay species

Use early 4th instar larvae of the Bora-Bora strain of *Aedes aegypti*. Eggs to start breeding colonies can be obtained from the Pasteur Institute, or one of several other centres.

To obtain larvae at an equal developmental stage, induce eggs to hatch by a stimulant such as the addition of 100 mg ascorbic acid/litre of water. This deoxygenates the water and all the eggs hatch very quickly. Purchase a large stock of suitable mosquito food to avoid variation due to the use of different batches of food in the course of time. Store this food in dry, cool (5°C) conditions to prevent infestation by storage pests or infection by moulds. Feed larvae with standardized optimal quantities of food, using a standardized breeding routine at a constant temperature selected from a range between 22° and 28°C. Do not vary this temperature for different experiments. Harvest the larvae on filter-paper, with a strainer or with a pipette, at a pre-selected age when all have just moulted into the fourth instar. If *Ae. aegypti* is unacceptable in a country for safety reasons, use *Culex quinquefasciatus* or *C. pipiens*.

2.3.3 Preparation of bacterial suspensions

The bacterial preparation to be assayed may be either a liquid suspension or a dry powder. If it is a liquid suspension, it is necessary to determine the dry weight per milliliter of the preparation. This is done by heating a small, known volume in a 100 - 110°C oven. The dry weight value is used later in calculation of the LC50. Whether the preparation is a liquid or a dry powder, it must be thoroughly homogenized prior to being added to the assay cups. Homogenize a weighed quantity of powder or a measured volume of liquid in a known volume of chlorine-free water, preferably deionized or distilled (or tap water can be aerated in shallow trays for 24 hours). A sonifier, glass homogenizer, and ball mill or blender with rotating knives are suitable homogenizing equipment. Place a loop full of this initial suspension on a microscope slide, apply a cover slip and check under phase contrast illumination that all clumps have been broken into individual spores and crystals. Agitate the initial suspension thoroughly (eg. by vortex mixer) and prepare at least five serial dilutions at, for example, x 0.8 intervals in chlorine-free water. Reagitate every time a fresh dilution is made. For the standard IPS-82, a top concentration of 0.04 mg/litre is likely to be suitable.

A sufficient range of concentrations should be used for the test samples in order to obtain a good mortality rate distribution. Shake each dilution (eg. 20 circular shakes by hand), then apply early 4th instar larvae at a suitable density such as one larva per 8 ml of bacterial suspension (eg. 25 larvae per 150 or 200 ml is suitable). The larvae should not be fed during the assay. Wax cups should not be used as they may trap the crystals. Keep at a constant temperature selected from the range of 23° to 27°C in

darkness or dim light: they must be kept away from sunlight. An exposure time limited to 24 hours is desirable. It should extend over an active feeding period, while avoiding the prepupal period and pupation.

2.3.4 Numbers of insects and replicate assays

Sufficient insects and replicate assays should be used to achieve a coefficient of variation not exceeding 20%. For instance, four cups of 25 larvae per concentration, seven concentrations per test bacterial product are suitable, and with the assay repeated on three different days. The objective is to have at least two mortality rates on either side of the LC50 within the range of 10 - 90% mortality. It is essential that, when the assays are repeated on different days, they be based on a freshly-weighed batch of powder.

2.3.5 Reading the assay

After an exposure period of exactly 24 hours, count the numbers of live larvae (the results are based on counts of live larvae because of the cannibalistic tendency of larvae at lower concentrations). If the control mortality exceeds 10% discard the assay. Calculate percentage mortalities and obtain LC50 values by plotting on log-probit graph paper, or by using a computer programme with a probit or logit transformation of mortality against log concentration.

2.4 Bioassay Protocol for *Bacillus sphaericus* Preparations

2.4.1 Standard bacterial preparation

The standard powder "SPH-88" can be obtained from the Pasteur Institute. For handling and storing see 2.3.1.

2.4.2 Assay species

Use early 4th instar larvae of the *Culex pipiens* complex (*C. quinquefasciatus*). In assays with *Anopheles* sp. (eg. *An. stephensi*) which are less susceptible than *Culex*, third instar larvae should be used. *Ae. aegypti* should not be used because this species is relatively unsusceptible to the toxin of *B.s.*

2.4.3 Preparation of the bioassay

The directions given in Section 2.3.3, 2.3.4, and 2.3.5 for assay of *B.t.* H-14 are generally applicable to assay of *B.s.* preparations. For bioassay of preparations of unknown activity, time can be saved by first making a range-finding assay with widely spaced (eg. 10 fold) concentrations. The SPH-88 standard powder is prepared for the assay as follows. A 50 mg L⁻¹ stock solution of the SPH-88 powder is prepared by thorough blending (sonication, homogenization) in chlorine-free water. To each plastic cup containing 150 ml of water, add up to 25 larvae. A total of 50-100 larvae should be used at each dilution. Add 120 μ l, 90 μ l, 60 μ l, 30 μ l, 24 μ l, and 15 μ l of the bacterial stock solution to the cups to obtain final concentrations of

0.04, 0.03, 0.02, 0.01, 0.008, and 0.005 mg L⁻¹ respectively of SPH-88. Fifty to 100 larvae in cups containing only water are held as controls. A small amount of food (eg. ground mouse biscuit or a few drops of 10% w/v autoclaved bakers yeast) is added to each cup. The assay should be incubated at 25° - 27°C.

2.4.4 Reading the assay

After an exposure of 48 hours, count the number of live larvae and proceed as directed in section 2.3.5.

2.5 Calculation of Potency in International Units (IU)

The relative potency of the test preparation with respect to the standard is given by the equation:

$$\text{Potency (I.U./mg)} = \frac{\text{LC}_{50} \text{ standard}}{\text{LC}_{50} \text{ test preparation}} \times \text{IU/mg standard}$$

2.6 Summary of Discussion on Bioassays

If the use of a microbial insecticide is to gain widespread acceptance, it must give reproducible control of insects. Microbial insecticides are attractive as ecologically safe, non-polluting entities, but they will not be used unless they are reliable. This means that we must be able to identify and standardize the insecticidal activity of each lot or formulation that is sold. The only way we have of doing this with *B.t.* or *B.s.* formulations is through the use of bioassays in which we measure the killing power of the product. This requires determining the LC50 of the product against test insects. LC50s alone can be misleading, since the susceptibility of insect populations to a particular microbial insecticide can vary from day to day. Therefore, it is preferable to compare the LC50 of each test sample with that of a standard formulation tested at the same time on the same population of insects. For convenience, a potency is assigned to the standard in International Units or IUs, and the activity of the test sample is then also expressed in IUs compared to the standard. Spore counts are fairly meaningless in determining the potency of a microbial insecticide.

Culture Maintenance and Preservation

3.1 Isolates of *B. sphaericus* and *B. thuringiensis*

There are over a thousand strains of *B.t.* in the Howard Dulmage collection (HD-) at the USDA Culture Collection in Peoria, Illinois, USA, active against agriculturally important insects. Similarly there are several hundred isolates of *B.s.* (Singer, 1982), 25% of which are larvicidal to mosquito larvae. Insecticidal bacilli cultures may be obtained from the following laboratories:

- a) Northern Regional Research Center, ARS, USDA,
1815 N. University, Peoria, Illinois 61604, USA.
- b) Department of Biological Sciences, Western Illinois University,
Macomb, Illinois 61455, USA.
- c) Unité des Bactéries Entomopathogènes, Institut Pasteur,
28 rue du Dr Roux, 75724 Paris Cedex 15, France

3.2 Problems of Culture Variability

The most important single need for the production of microbial insecticides is a supply of reproducible, reliable, authentic cultures of the microorganism. The principle bacteria used in the control of insects (*B.t.* and *B.s.*) are relatively easy to maintain. Nevertheless care and skill are needed, and the person in charge of the culture collection should be a trained microbiologist.

At one time, supervision by a skilled microbiologist did not seem to be an important aspect of maintaining isolates *B.t.* and *B.s.* The production of their toxins was considered to be a highly stable characteristic, so that it was believed that anyone with a minimum of training in sterile techniques could maintain them. However, the more that we learn about these bacilli, the more complex the picture appears, and the greater the demand for a thorough knowledge of microbiology.

This imposes very strict standards on the maintenance of the cultures to ensure that the various isolates are kept separate from each other without any cross-contamination. Otherwise, unwanted and unrecognized changes could occur that would affect future fermentations.

3.3 Differences in the Production of Toxin between Isolates

The previous section showed that the type of toxin produced and which insects it killed depended upon the isolate. It also showed that the medium used in the fermentation could affect yields of toxin by the same isolate. Even natural isolates of the same serovar can differ in how much toxin they can produce. Table 3 shows the differences in potencies and yield between different isolates of *B.t.* H-14, as well as between fermentations. The four cultures shown are from different sources. Three of the isolates are distinctly different in behaviour: HD-500 produces more delta-

endotoxin than does HD-519, but neither produces very high activity. On the other hand, yields of toxin with HD-563 and HD-567, which closely resemble each other, are about 15-times higher than those from the less potent isolates. Similar results have been obtained with isolates of *B.s.*

Any isolate, no matter how well protected, may weaken in storage in its ability to produce toxin. The isolates shown in Table 3 were therefore compared by growing them under the normal fermentation conditions and determining how well they produce toxin. It is important to monitor the quality of key cultures. The scientists who are responsible for culture maintenance must have access to laboratory-scale fermentation equipment and must routinely test any cultures being used to produce larvicidal formulations to be sure that they have not lost any ability to produce insecticidal activity. However, unless some problem arises, such tests are not needed more than 3 or 4 times/year.

3.4 Methods of Preserving Microbial Cultures

3.4.1 Equipment

Specialized and major equipment desirable for culture maintenance is listed below:

a. *Laminar Flow Hood*

To keep larvicidal bacilli uncontaminated, it is best to conduct all transfers of culture material in a germ-free environment. A laminar flow hood furnishes this. Essentially, a laminar flow hood takes in normal room air, passes it through air-conditioning filters to remove the larger particles of dust, etc. in the air, then passes it through high-efficiency filters to remove bacteria in the air, and finally passes it through a series of baffles that remove the turbulence in the air flow and pushes a wall of sterile air over the bench top within the hood. In a few minutes, the area within the hood is essentially germ-free, and transfers of cultures can be made safely. Several sizes of hoods are available and one that is about 1.8 m long and 0.6 m deep should prove adequate.

b. *Steam Sterilizer or Autoclave*

All pipettes and other glassware, all lyophilizing tubes, and all agar and liquid media used in culture maintenance must be sterilized. Sterilizations are best carried out in a steam autoclave at 121°C. It is more economical to buy an autoclave large enough to be used in shake-flask research (see section 5.2.1) as well as by the culture maintenance group. Autoclaves need a supply of high pressure steam. Models that will generate their own steam supply are available, but if a central steam supply is available (and, as will be discussed later, production and experimental fermenters require a central steam supply), it is preferable to buy an autoclave that can be connected to this supply. Both circular and rectangular shaped autoclaves are available. An autoclave with dimensions of 50 x 50 x 90 cm will be large enough in most cases but, if a larger one is desired, dimensions of 50 x 90 x 90 cm may be used.

Table 3. Potencies of Formulations of Various Isolates of *B. thuringiensis* H-14 (Dulmage, unpublished data)

Isolate Number	Formulation Number	Potency in IU/mg
HD-500	R-179	206
HD-500	R-966-A	680
HD-519	R-933-B	179
HD-519	R-966-B	152
HD-563	R-967-B	2,170
HD-563	FR-201-AB-AP	3,250
HD-567	R-967-C	1,970
HD-567	FR-201-CD-AP	2,910

temperatures higher than ambient. However, they regulate temperature well. Several types of these incubators are available, but one of the least expensive types - and one that will give good temperature control - is a gravity convection incubator. Temperatures within the incubator should be maintained at 28°-30°C. Culture studies often require large numbers of test tubes and Petri dishes. An incubator with interior dimensions of about 48 x 50 x 66 cm should be adequate.

d. Refrigerator

Agar slants and lyophilized stocks of *B.t.* and *B.s.* should be kept refrigerated at about 4°C. A refrigerator is therefore needed, but it does not have to be elaborate and any household-style refrigerator can be used.

e. Miscellaneous

If cultures are to be preserved as lyophilized stocks, vials or tubes in which to conduct the lyophilization are needed, along with a high-vacuum pump which can reduce pressures to 0.1 mm. An oxygen torch is needed to seal the vials. There are many satisfactory ways of preparing lyophilized stocks, one of which is described in section 3.4.4. Most of these procedures do not require expensive equipment.

3.4.2 Culturing and Maintaining Isolates on Agar Slants

The simplest way of maintaining cultures is to grow and store them on agar slants in screw-top test tubes. It is important to use an agar medium which will support good growth and sporulation of the isolates. Difco Nutrient Agar (NA), which is available as pre-mixed powder (Table 4), will suffice for most isolates of *B.t.* Tryptose-phosphate agar (TPA) is frequently used for growing *B.t.* but TPA was found to be less satisfactory than NA for this organism. *B.s.* isolates can be grown satisfactorily on NYSM agar (Myers & Yousten, 1980) or on MBS agar (Kalfon *et al.*, 1983). Supplementation of nutrient agar with manganese salt is particularly important to achieve good sporulation which is important for successful

c. Incubator

B.t. and *B.s.* isolates grow best between 28° and 32°C, and close temperature control is needed for good growth of the cultures in agar or liquid media. Thus a bench-top incubator is needed in which to grow these organisms on slants and agar plates. There are incubators that regulate temperature by heating or cooling as needed. Less expensive incubators regulate temperature only by heating and thus can maintain only

storage of stock cultures on slants. *B.t.* isolates will also grow well on these media. Formulae are given in Table 4.

Cultures of *B.t.* are usually considered to be quite stable and to maintain their identity well under continuous transfer on agar slants. However, there is one serious risk; the production of delta-endotoxin by *B.t.* appears to be controlled by plasmids (Gonzales, *et al.*, 1981) which can be lost on transfer. In spite of the supposed stability of *B.t.*, repeated transfers on agar slants may carry a significant risk of loss of activity. Lyophilization is safer, but more time-consuming. If a laboratory uses the same isolates on a regular basis, they may prepare a months supply of slants from one tube from their lyophilized stock. To preserve the supply of lyo-stock tubes, it is possible to subculture enough slants from the first series of slants for a second months use. This procedure is a practical compromise between the necessity of maintaining large numbers of lyophilized tubes of each culture and the risks of repeated transfers on slants.

3.4.3 Maintaining Isolates in Soil

B.t. grows and survives well in soil. Many alkaline to neutral soils have been used with success. Sterile soils offer a means of preserving *B.t.* Such soils have been used at Wuhan in the People's Republic of China. There they prepare a mixture of river silt loam and clay and distribute in 1 gm portions into test tubes. The tubes are sterilized 3 times by dry heat at 160°C. Tubes are incubated for about 24 hours between sterilizations. To preserve a culture, a suspension of the isolate is prepared by adding 10ml water to an agar slant of the bacillus in a 250 ml bottle. Then 0.2 ml of the suspension is added to each test tube of soil. The tubes are incubated for about 1 week and then dried under vacuum. The scientists of the People's Republic of China keep each soil stock for only 1 year, but this is only a precaution: they believe that the stocks could probably be kept safely for 5 to 10 years. However, note should be made that loss of plasmids has been observed in isolates of *B.t.* kept in soil.

Based on various reports the soil procedure seems better than continuous transfer on agar slants, but is not as good as lyophilization.

3.4.4 Maintaining Isolates as Lyophilized Stocks

For over 50 years, many micro-organisms have been preserved by lyophilization or freeze drying. Lyophilization appears to be the safest way of maintaining *B.t.* and *B.s.* In this procedure, spores of the isolate are dried and stored under vacuum. There are many different methods used, with cultures being dried as spores of the organisms on filter paper strips, or as frozen suspensions of the cultures in lactose or in reconstituted dry, fat-free, milk. Simple, mostly "home-made" equipment can be used in most of these processes, although more elaborate (and much more expensive) equipment can be bought. The basic device is simply a large stainless steel manifold which is connected to a dry ice trap and thence to a vacuum pump capable of reducing pressures to 0.1 mm. Connected to the main manifold by rubber vacuum tubing are a series of small manifolds, each of which, in turn, is connected by lengths of rubber vacuum tubing to the lyophilization tubes in which the cultures will be dried.

Table 4. Agar Media Used for Preparing Stock Cultures of *B. thuringiensis* and *B. sphaericus*^a

	Ingredient	Grams/Liter
1. Nutrient Agar (NA)	Peptone, bacteriological	3.0
	Beef extract	5.0
	Agar	20.0
2. Tryptose-Phosphate Agar (TPA)	Tryptose	20.0
	Dextrose (glucose)	2.0
	NaCl	5.0
	Na ₂ HPO ₄	2.5
	Agar	20.0
	3. Modified Nutrient Agar for <i>B. sphaericus</i> (NYSM) ^b	Nutrient Agar (Difco) or as formulated in #1
	Yeast extract	5.0
	MnCl ₂ · 4 H ₂ O	0.01
	CaCl ₂ · 2 H ₂ O	0.1
	MgCl ₂ · 6 H ₂ O	0.2
	Agar	As needed to achieve 2% w/v
4. Modified <i>B. sphaericus</i> (MBS) ^c	Tryptose	10.0
	Yeast Extract	2.0
	KH ₂ PO ₄	6.8
	MgSO ₄ · 7 H ₂ O	0.3
	MnSO ₄	0.02
	Fe ₂ (SO ₄) ₃	0.02
	ZnSO ₄ · 7 H ₂ O	0.02
	CaCl ₂	0.2
	Agar	20.0
		Adjust pH to 7.2

^a NA and TPA media can be purchased commercially in a premixed form. However, in this form they only contain 15 g/l of agar. In all cases the final agar concentration should be brought to 20 g/l. This is particularly important if it is desired to obtain isolated colonies of *B. sphaericus* on plates. The motility of this bacterium is restricted at the higher level (2%) of agar.

^b Yousten & Wallis, 1987

^c Kalfon et al., 1983

The lyophilization tubes used to dry and store the cultures are constructed from 100 mm lengths of 9 mm borosilicate glass tubing which have been sealed at one end by means of an oxygen torch, plugged at the other end with cotton, and sterilized at 121°C in a steam autoclave. A milk suspension of the culture to be lyophilized is prepared. The cotton plug to the lyophilization tube is removed, and sufficient suspension is added to the tube by means of a Pasteur pipette so that the depth of the suspension in the

tube is about 6 mm. The cotton plug is replaced, and the suspension is immediately frozen in a dry ice-alcohol mixture at about -50°C . The tubes are now ready for the lyophilization process. It is essential to keep the milk suspensions frozen during the drying procedure. Normally, materials being lyophilized under high vacuum do not melt, being kept frozen by the absorption of heat by the vaporization of the water being removed. However, the impediments to the flow of water vapour imposed by the narrow size of the lyophilization tubes and by the fibres in the cotton plugs are such that the milk suspensions will not remain frozen by themselves. To correct for this, the tubes are partially immersed in a dry ice bath. The bath is allowed to slowly warm from -40°C to $+2^{\circ}\text{C}$ over a 4-hour period. When the drying is complete, the tubes are cut free from the manifolds and simultaneously sealed using an oxygen torch. (The purpose of using rubber vacuum tubing for connections between the manifolds and between the manifolds and the lyophilization tubes is to furnish enough flexibility to allow the operator to position the tubes for cutting.) With proper care, the glass tubing left connected to the manifolds is sealed at the same time, and no loss of vacuum occurs during the cutting process.

The tubes are reconstituted for use by tapping them to loosen the dried pellet, wiping the outside with 70% alcohol to minimize the chance of contamination when the tubes are opened, scoring the tube to allow easy breakage, and then dropping the pellet of the tube into 1 to 2 ml of sterile nutrient broth. The newly reconstituted suspension is then transferred to the surfaces of agar slants and is ready for use after 48 hours incubation.

If the cultures just reconstituted are to be used for large-scale fermentations, it will be very important to test their biological activity before using them. Such fermentations are expensive, and the precaution is necessary. However, the tests need not be elaborate. First, one can test the serology of the culture to make sure that it is the right serotype. Second, one can use one slant from the lyophilized stock as an inoculum for a shake-flask fermentation (Section 5.2.1) and see if the yields of toxin are typical.

Lyophilization appears to be a very safe procedure. Some cultures (not *B.t.*) have been stored as lyophilized stocks for several decades. Some of the lyophilized stocks of *B.t.* have now been stored for nearly 15 years with no apparent change.

3.5 Summary of Discussion on Culture Maintenance

Both *B.t.* and *B.s.* appear to be very stable microorganisms, and it is possible to maintain this culture by serial transfer on agar slants, by storing in sterile soil, or by lyophilization. The safest procedure is probably lyophilization, followed by storage in sterile soil. While storage on agar slants appears to be safe, there is always some risk in serial transfers of any culture, and therefore this procedure is the least satisfactory.

Submerged or Deep-Tank Fermentations

4.1 Basic Principles

A fermentation is a means of feeding suitable nutrients to a micro-organism in order to obtain useful or valuable end-products. It needs water, an adequate supply of carbon and nitrogen. If it is an aerobic micro-organism, as are *B.t.* and *B.s.*, it needs a plentiful supply of air. It will usually be very particular about the temperature and pH of its environment. It must grow alone. The proper equipment and adequate support from various utilities are required.

In this section, some of the equipment and utilities will be discussed and methods of using them in shake-flask and deep-tank fermentations will be described. Also, some of the recovery procedures will be reviewed.

4.2 Equipment and Utilities

4.2.1 Water

a. Hardness

The presence of excess concentrations of minerals in the water supply is perhaps the most serious and the most common problem faced in the use of water. A fermentation plant consumes great volumes of water. High pressure steam is needed to operate autoclaves, to sterilize large fermenters, and, in a less demanding but equally important service, to lubricate bearings and to protect valves and other similar openings to the fermenters against contaminating microorganisms. Large quantities of chilled water are needed, as will be discussed in the next section. If the mineral content is high, there can be a damaging and rapid build-up of scale, harmful not only to the steam generators but also to the many delicate valves involved in a fermentation process. One of the prime considerations in deciding where to locate a fermentation plant is the hardness of the water.

Mineral levels in hard water can be reduced - at a cost. If the water supply is very hard, such reduction is imperative. In ARS Laboratories in Brownsville, Texas, for example, the minerals in water supply range from 900-1800 ug/ml (ppm). It would be impossible to protect the equipment against such hardness if the water were not pre-treated. Brownsville Laboratories used a reverse-flow osmosis system which is probably the most satisfactory method of treating this end of hardness. The water must be acidified, flocculated, and passed through a sand filter before passing through the osmotic membranes, but the process reduces the solids in the water to about 90 ug/ml, a level that is satisfactory for the operation of their equipment.

b. Temperature

Another serious problem with many water sources is that they are too warm. Fermentations are exothermic. Large amounts of water are needed

to keep fermentations cool. *B.t.* and *B.s.* are usually grown at 28-30°C. To maintain close control over such temperatures in a fermenter requires that the temperature of the cooling water be about 15°C. Some areas of the world have ready access to water of this temperature, but in most cases - especially in sub-tropical and tropical climates - the only way to obtain such cold water is through the use of water chillers or cooling towers.

This problem should not be underestimated. Solving it can greatly reduce the cost of a fermentation. A chiller can adequately cool the water, but it is expensive to operate. However, once the cooled water has passed through the fermenter cooling coils, it has absorbed heat. This warming is particularly great during the cooling cycle after sterilization, and this poses a question: is it less costly to re chill the warmed water or to use fresh influent reverse flow water and chill it? The cost of the water becomes important in answering this question.

Similar combinations may need to be developed even where the water supply is cold. Depending on the cost of water, it may prove more economical to recirculate some water and chill it. In deciding where to locate a fermentation plant, one must calculate the amount of influent water required and the cost of cooling.

c. Pollution of influent water

Water supplies can be polluted, either with microorganisms or with chemicals. Polluted water can cause considerable problems in a fermentation plant, and in some cases may greatly interfere with the growth of the desired microorganism. Water that is badly contaminated with microorganisms is difficult to sterilize. Chemically polluted water may inhibit the growth of the microorganisms used in the fermentation. Before choosing a site for a fermentation plant, samples of the water supply should be tested to be sure that it will support the desired fermentation.

d. Pollution of effluent water

The first step in recovery of a *B.t.* or *B.s.* product is to centrifuge the fermentation beer and save the sediment or cream -- which is a rather small percentage of the beer volume. The rest is discharged to the sewers of the fermentation plant. The waste may increase the biological oxygen demand of the sewage, perhaps beyond the capacity of the treatment system. Also it may inhibit the growth of desirable organisms in the sewage that ordinarily decompose the wastes. At the very least, if any significant volume of supernatant fermentation beer reaches the sewage treatment plant, it upsets the ecology of the sewage treatments. The wastes or their degradation products may generate undesirable odours.

The seriousness of the problem depends on local conditions, and it must be considered in designing and locating a fermentation plant. One solution would be the construction of a sewage treatment plant to treat the beer before discharge into the local system.

4.2.2 Utilities

a. General specifications

The designer of a fermentation plant must take into consideration utilities such as steam, water, air, and electricity. The specific demands will depend on the size of the plant, local conditions and equipment. Typical data are given in Table 5. These data should help in estimating the utilities needed for a given fermentation plant.

Table 5. Specification for Support Equipment for Different-Sized fermenters¹

Size of Fermenter	50 litre	500 litre	5000 litre
Specification			
<i>Steam</i>			
kg/hour	23	90	640
kg/m ² (Guage pressure)	4.0-5.5	5.5-7.0	7.0-8.8
<i>Water</i>			
litres/min	15	45	150
kg/m ² (Guage pressure)	4.0-5.5	4.0-5.5	7.0-8.8
<i>Air</i>			
m ³ /min. (STP)	0.079	0.59	3.8
kg/m ² (Guage pressure)	3.9-5.5	5.5-7.0	7.0-8.8
<i>Electricity</i>			
Voltage (single phase)	115, 208 or 240	240	---
(3 phase)	----	--	240 or 460
kilowatts	4	11	40

¹ To give some idea of the needs of different-sized fermenters, the New Brunswick Scientific Co., of Edison, N.J., who manufacture fermenters, provided their specifications for 3 different-sized fermenters.

b. Air supplies

Fermenters demand large volumes of air, as shown in Table 5. It is not difficult to find air compressors that can supply these demands. However, the air from many types of compressors is contaminated by oil. This oil will eventually destroy the air filtration system of the fermenter, and, if it reaches the beer, it will damage the fermentation. It must be removed. fluctuations in air pressure within the fermenter system can permit contaminating microorganisms to enter. Therefore, it is important to install a pressure tank because the volume of air passing through the fermentation should be kept constant.

c. Electric power

Fermentations are frequently spoiled due to the failure of electric power. This not only allows a sharp drop in air supply and pressure, it also stops the impeller or mixer used to stir the fermentation beer. If the local electrical supply is not highly reliable, it would be wise to install a back-up generator at the plant with an automatic start-up in case of power loss.

Fluctuations in voltage can lead to contaminated fermentations due to fluctuations in air supply and agitation, or possibly to burned-out motors or damaged electronic components. The solutions to fluctuating voltage are expensive: for example, portions of the equipment may need to be protected with constant voltage transformers. The frequency and seriousness of such fluctuations should be considered in evaluating the local power supply.

4.2.3 Major fermentation and recovery equipment

Many kinds of equipment are used in the fermentation and recovery of microorganisms or microbial products. Four types (shakers, fermenters, centrifuges, and spray driers) are commonly used in the fermentations of *B.t.* and *B.s.* and some companies are attempting to replace the centrifuges by filter presses to separate cells and crystals from the beers. Before constructing any fermentation facility, the selection of equipment will have to be studied in depth (Solomons, 1969; Wang, *et. al.*, 1979). This discussion is restricted to the specific models that were used in the Brownsville Laboratories. They are typical of the equipment needed in any fermentation facility. Before building any fermentation plant, it would be best to examine models from several companies. We do not intend to recommend any one brand over another.

a. Rotary shakers

In any aerobic fermentation, it is important to get an intimate mixture of microorganism, nutrient, and air. The beer must be continuously agitated during the fermentation. This is important, even when the cultures are grown in test tubes or flasks. Flask fermentations are used for two purposes: (1) to build up inoculum for starting fermenters; and (2) to allow study of fermentation variables. The flasks are clamped to the platform of a shaking machine and aerated by shaking. In shake flask fermentations, aeration is the principal limiting factor to good growth of the microorganism. One must remember that the solubility of oxygen in the medium under the ordinary conditions used in a fermentation is about 10 mg/litre. In contrast, a carbon source such as glucose may be present in concentrations as high as 40 g/litre.

There are several choices to be considered in selecting a shaker. First, should one use a reciprocal or a rotary shaker? The platform of a reciprocal shaker moves back and forth in a straight line, causing considerable splashing of the liquid in the flask, which superficially seems an ideal way to aerate. The platform of a rotary shaker moves in a circular manner, creating a vortex in the liquid. Reciprocal shakers are valuable for many types of mixing, but they are not as good as rotary shakers for fermentation studies. There are several reasons for this. First, if a cotton plug is used in the

mouth of the flasks to protect against contamination, this is effective only as long as the plug remains dry. It is easy for the plug to get wet by the splashing action of a reciprocal shaker - particularly by the initial large splash when the shaker is turned on. Second, there is considerable evaporation of the water in the fermentation medium in flasks on a reciprocal shaker. This effectively changes concentrations of nutrient ingredients. Third, the mixing action of the rotary shaker seems to be more uniform than that of a reciprocal shaker, particularly as the viscosity of the beer changes during a fermentation. Finally, by the proper choice of flask size and of the stroke and rpm of the shaker, it is possible to achieve higher aeration in a rotary shaker than in a reciprocal shaker (Anon., 1975).

Rotary shakers usually have exchangeable platforms, so that a wide variety of flask sizes can be used. The choice of flask depends mostly on the preference of the user. A useful combination might be 500 and 2000 ml Erlenmeyer flasks. Some researchers prefer to use flasks with indented walls as baffles. This markedly increases aeration. However, such flasks also increase foaming, which may be a problem, particularly in *B.t.* fermentations.

Platform sizes also vary with different shakers. In a large operation, a multiple-tiered shaker might be selected. At Brownsville Laboratories they preferred to use several single-tiered shakers, each with the choice of two platforms 45x75 cm in size: one that could hold 24 500-ml Erlenmeyer flasks and the other, 12 2000-ml flasks. In considering the size of the shaker one should remember that fermentations generate much heat, and stagnant air is not an effective coolant; therefore, the flasks in the middle of the platform may be much hotter than those on the outside unless fans are used to circulate the air.

Rotary shakers are available either contained within an incubator box or as open platforms. The incubator shaker most commonly used can control temperatures only by heat. It offers the advantage that, by keeping all shakers in a single room that is cooled to a temperature about 6°C below the minimum temperature that will be used for any shaker, a series of temperatures can be studied merely by varying controls on the individual shakers. It is especially important, when selecting an incubator shaker, that it has proper ventilation and an adequate-sized fan.

The open-air platform shaker is of course restricted to ambient temperature. Since temperature control is important, this type of shaker must be kept in an incubator room. If the effects of a series of temperatures are to be compared, several incubator rooms will be needed - an expensive undertaking. However, the open-type shaker costs much less than the incubator type and, if incubator rooms are available with sufficiently accurate temperature controls, then the open-type shaker is preferable.

Specifications for a rotary shaker giving satisfactory performance are shown in Table 6. For example, the stroke should trace a rotary motion describing a circle fairly close to 25 mm. This is a practical stroke for good aeration. Also, sufficient speed is needed. In the Brownsville Laboratories most shake flask fermentations were grown at 340 rpm and, if a single speed shaker is selected, one may recommend one with about that speed. Some shakers have variable speed controls; if such a shaker is used, the range of speeds should be at least between 150 and 400 rpm, to ensure good aeration.

Table 6. Specifications for Model G25 Gyrotary Shaker^a

Temperature Range	5°C above ambient to 60°C
Stroke	Rotary motion describing a 25 mm circle
Speed Range	40 to 400 rpm
Dimensions	115(width)x72(depth)x77(height) cm
Platform Size	45 x 75 cm
Drive Motor	0.127 metric Hp
Power	115 v 50/60 Hz, 1-phase

^a Courtesy New Brunswick Scientific Co., Edison, N.J.

b. Fermenters

A fermenter is basically a deep tank to hold a liquid medium through which air is pumped. Several accessories are needed to achieve this protection from contamination. Each fermentation will have specific requirements for temperature and aeration. The fermenter must be able to control these and keep them at the settings ordered by the operator. Several monitors and controls are needed to observe and regulate the progress of the fermentation. Some of the accessories and controls needed to fulfill the requirements for a fermentation tank are described below.

i. Shape and construction of the tank

Laboratory-scale fermenter tanks are often constructed of glass. They have the advantage of letting the operator observe what is going on inside the fermenters. There is some danger of breakage of a glass jar, which could be serious if it happened when the medium was still hot from autoclaving, and stainless steel tanks are safe. The 14-litre fermentor tanks used at Brownsville were constructed with a glass window so that one can see at least some of what was happening inside the jar.

Small fermenters of the size used at Brownsville were constructed from 3-5 mm stainless steel. Large tanks have an inner lining of type 316 stainless steel, with parts not in contact with the culture medium being made from type 304 stainless steel. (A steel very resistant to galvanic corrosion is frequently used in fermentation conditions.) All tanks that are designed to be sterilized *in situ* are rated for a design pressure of 2.2 - 2.5 kg/m² (gauge pressure) and are equipped with suitable safety pressure release valves.

The height:diameter ratio (H:D) of a fermentation tank should be about 2.0. In very small fermenters, this H:D is sometimes about 1.0; however, this H:D ratio would not be practical in larger fermenters. Agitation and the use of impellers is discussed below. Most fermentation tanks, from the smallest to the largest, include baffles to improve turbulence and aeration. According to Solomons (1969), the most effective baffling comes from the

use of 4 equally-spaced baffles running vertically from top-to-bottom of the tank, with the width of each baffle being about 10% of the diameter of the tank. The design and the use of baffles seems to be more or less standard throughout the industry.

ii. Agitation

Air enters a fermentation tank through a sparger placed centrally in the bottom of the tank. The most common in the past has been a ring sparger in which air exited to the tank medium through equally-spaced holes around a ring-shaped tube of stainless steel. Single-hole spargers placed directly under the impeller shaft are used today.

It is essential to get homogeneity throughout a fermentation medium. This can be obtained through agitation by a stirrer or impeller aided by the baffles. The design of the impeller has changed over the years, ranging from propeller-like impellers with adjustable blades, to vaned disks, to the presently widely-adopted turbine impeller. The diameter of the impeller needed for optimum agitation varies according to the fermentation and, in a very viscous medium such as may occur during the growth of fungi, the diameter of the impeller should be about half the diameter of the tank. However, in a relatively non-viscous medium, typical of the media in which *B.t.* is grown, the diameter of the impeller should be about 0.33 that of the tank. It is almost impossible to get adequate agitation with a single impeller; thus two, and sometimes three, impellers should be used. These should be placed at least one, and perhaps two, impeller diameters apart of the shaft. The number and positioning of impellers may be particularly important in a fermentation with a high oxygen demand such as occurs in *B.t.* fermentations.

The shaft of the impeller can enter the tank through either the top or the bottom. Top entry shafts have been the most common, although there are some advantages to a bottom entry shaft. First, it reduces the number of attachments needed on the top of the fermenter and allows the construction of bigger portholes for entering and maintaining the fermenter. Second, the shaft can be much shorter. The Brownsville Laboratory had a 200-litre pilot plant fermenter with a bottom drive and has had very few maintenance problems with the shaft assembly.

A good seal in the stirrer shaft assembly is important. This has proved to be one of the major problems in constructing and maintaining fermentors which seems to have been solved with the development of a mechanical seal, lubricated and cooled by sterile water from condensing steam. Springs are used to hold the seal tightly together and to compensate in this manner for wear. The pilot plant fermenter used at Brownsville had a duplex seal with continuous steam bleed. This seal had served them without replacement over several years.

Magnetic drives have been developed as alternates to the mechanical drive. The use of a rotating magnet outside the fermenter to turn the impeller inside the fermenter eliminates the need to design a shaft assembly that can go through the tank wall. If the volume of medium to be stirred is high (>150 to 200 litres), or if the medium is viscous, magnetic drives may not work well.

iii. Power input-impeller drives

Solomons (1969) has listed six essential features to the design of a good power and transmission unit:

- The set power provided should be constant.
- Variations in stirrer speed should be easily made.
- The connexion of the drive assembly to the fermenter shaft should be easily connected and disconnected to allow the removal from the fermenter for washing and assembly, etc.
- The whole unit should be rigid and the stirrer bearing assembly should permit high stirring speeds without shaft whip.
- The speed of the stirrer should be measured.
- Preferably the power uptake by the impeller should be measured.

As the size of the fermenter increases, the efficiency of the power input increases. Table 7 shows the variation of power (in HP) vs. size in smaller-scale fermenters. Note that the specifications call for 1/2 HP for a 40-litre fermenter, 5 HP for a 400-litre fermenter, but only 30 HP for a 4000-litre fermenter. The efficiency continues to improve in still larger fermenters. Solomons (1969) cited a large fermenter of 170,000 litres which was powered by a 450 HP motor. This represents about a 3-fold improvement in efficiency over the power needed for the 4000-litre fermenter. This, of course, has an important bearing on costs and should be considered when deciding what size fermenters should be used. Larger fermenters are proportionately less costly to operate.

Table 7. General Specifications - Industrial Pilot Plant fermentors^a

Model	IF-40	IF-400	IF-4000
Total Volume (Litres)	40	400	4000
Working Volume (Litres)	30	300	3000
Vessel Diameter (cm)	30	66	140
Vessel Height (cm)	61	130	295
Impeller Speed (rpm)	50-500	40-400	25-250
Horsepower	1/2	5	30
Aeration Rate (litres/min)	5-45	48-480	200-2000

^a Courtesy of New Brunswick Scientific Co., Edison, N.J.

iv. Impeller speeds and aeration rates

One of the important aspects of impeller speed is the tip speed of the impeller blade. Therefore, impeller speeds will depend upon the size of the fermenter and the diameter of the impeller being used. Thus the larger the tank, the slower the impeller speeds. For example, the impellers in a 14-litre tank are run at 700 rpm, and in a 250-litre, at 400 rpm. It is estimated

that an equivalent agitation in a 4000-litre tank would be 200 rpm.

The aeration rate used in most fermentations is about 1.0 volume of air/volume of medium/minute (v/v/m). Oxygen available to the microbe depends on the efficiency of the distribution of the air stream throughout the medium. There is high demand for air during the early stages of *B.t.* fermentations. Inefficient aeration could lead to an inadequate supply of oxygen to the bacillus and interfere with the progress of the fermentation.

Aeration is complex, and the solution of how to provide the proper balance of air flow, impeller size, and impeller speed, can only come from experimentation. Scale-up can pose problems. Considerable attention is being given to the mathematics of mixing as applied to fermentations (Wang, *et al.* 1979).

v. Treatment of influent and effluent air

The air incoming to a fermenter must be sterile. Air is almost universally sterilized by filtration. This filtration must reduce air-borne particles by more than 99.999%. The problems of air filtration have been effectively solved.

Air entering small fermenters is usually sterilized by filtering it through a tube of glass wool or a combination of glass wool and activated carbon. In larger fermenters, the air is usually filtered through bacteriological filters made either from glass fibre paper or from ceramic. The pore size of these filters should be no more than 0.5 microns. The filters are sterilized with high pressure steam at the same time that the fermenter is sterilized. A filter used at Brownsville Laboratories² did not fail in over two years. Solomons (1969) used a ceramic filter in his laboratory which served for over 10 years without failure. The effectiveness can be shortened by the presence of water or oil droplets in the air stream which must be removed before the air enters the fermenter filtration system.

It is a common procedure to filter also the effluent air from the fermentations. The filters used for this are not too different from those used for the influent air. The air coming from the fermenters is saturated with water.

Seals and gaskets are not always perfect and leaks could allow non-sterile air to enter the fermenter. The air exhausts, whether filtered or not, should contain valves to allow for a positive pressure within the fermenter.

vi. Sterilization

(1) Shake flasks and laboratory-scale fermenters

The nutrient medium used in any fermentation must of course be sterile. Media in shake flasks are readily sterilized in an autoclave. Fermenters ranging in size from approximately 1 to 30 litres can be divided into two different groups according to the manner in which they are sterilized. Laboratory-scale fermenters are designed to be sterilized within a steam autoclave; pilot-scale fermenters are sterilized *in situ*. Flasks, and the

² Made by Domnick Hunter Inc., Durham, England

smallest fermenters, can be sterilized in an autoclave such as described in Section 3.4.1, b.

Frequent sampling is needed to follow the course of a fermentation, and, in the case of the *B.t.* and *B.s.* fermentation, the sample size must be large enough for bioassays. If a sample is large relative to the volume of beer, sampling can alter this volume enough that the act of sampling can significantly affect the course of a fermentation. This constraint led the Brownsville Laboratores to choose a 14-litre fermenter for laboratory work. It was large enough to furnish them with the sample that they wanted without too great a loss in volume of the fermentation beer. To sterilize these fermenters, Brownsville used an autoclave approximately 600 x 750 x 760 cm in size with a wagon or cart which could be rolled into and out of the autoclave and to the fermentation site.

There is no agitation of the medium within the fermenter during autoclaving. Solid ingredients tend to settle to the bottom of the tank and heat penetration of the sediment is slow. Sterilization times must be checked by experimentation. Media containing relatively low levels of solids require about 1.5 hours to be sterilized, while media with high concentrations of solids can require 2 or more hours to sterilize. Solomons (1969) stated that unagitated fermenters containing high levels of starch or protein gels were "virtually impossible to sterilize, the presence of these insoluble materials inhibiting adequate heat penetration of the sediment they formed, even after 3 hours exposure time."

(2) Pilot and production-scale fermenters

In the past, batch sterilization has been the traditional method of sterilizing media. When the fermenter is relatively small (below 4000 litres), batch sterilization is the most convenient. If a plant has large fermenters, continuous sterilization offers many advantages.

In batch sterilization the medium is prepared inside the fermenter, after which high pressure steam is allowed to flow through the heating and cooling coils of the fermenter (see Section 4.2.3, b, viii). The medium is heated to 121°C under pressure. To avoid settling of solid components, the media is agitated during the entire sterilization process. The speed of agitation is kept to the minimum needed to avoid settling of the solids. During the cooling process, air is continuously pumped into the fermenter to maintain a positive pressure.

To summarize, in batch sterilization the fermenter becomes the autoclave. There are drawbacks to this type of sterilization - the primary one being that it takes a long time to heat the medium to sterilization temperature: as much as 90 minutes for a 60,000-litre fermenter (Solomons, 1969). Wang, *et al.* (1979) plotted the heating, holding, and cooling temperature-time profile for a typical batch sterilization. The entire profile covered 4 hours, with 50% of the time being consumed in heating the medium to 121°C; 17% in holding it at that temperature for sterilization; and 33% in cooling it back to the desired incubation temperature. This profile is similar to the cycle for the Brownsville 250-litre fermenter, where a 90-minute sterilization times is divided, 40% for heating; 30% for holding; and 30% for cooling.

Continuous sterilization is based on two key principles:

- (1) The higher the temperature to which a medium is raised, the shorter the time needed to sterilize it.
- (2) The smaller the amount of medium being sterilized, the quicker it can be heated to sterilization temperature.

In continuous sterilization, the medium is heated to a much higher temperature than in batch sterilization, but the medium is exposed to this high temperature for only a short time. There are several advantages to this. In continuous sterilization, the medium is sterilized while being piped to the fermenter. The fermenter and its accessories are sterilized separately. The medium can be heated in several ways: it can be passed through tubular or plate-and-frame heat exchangers, or it can be heated by direct steam injection into the medium. After being held at the desired temperature for a specified period of time, the medium can be quickly cooled in a heat exchanger or by flash cooling by releasing into a vacuum tank. All this is being done while the medium is in transit from a mixing tank to a sterilized fermenter. The design is not simple, and the choice of pipe diameter, flow rates, type of heating and cooling, etc., must be analyzed and plotted by a skilled engineer. Most media contain solid particulate matter, and it must be remembered that while the surrounding medium may be heated to the selected sterilization temperature in seconds, it may take minutes for the particles to reach the same temperature. Also, there is danger that the pipes will become plugged if the flow rate is too slow to keep the particulate matter in suspension or if there is a build-up of carbonaceous matter on the heated walls of the pipes from the burning particles allowed to adhere to them.

There are several advantages to continuous sterilization. There is as a rule less destruction of medium ingredients in the short heating times associated with continuous sterilization.

It is not known how much improvement there would be in yields in *B.t.* or *B.s.* fermentations if continuous sterilization were substituted for batch sterilization. Continuous sterilization has other advantages to offer. The demand for steam from the generating plant would become more uniform, and the plant would not have to be built with excess capacity to allow for surges in demand. The entire media sterilization process is shortened. Sterilization would be more reproducible and there should be less variability in media autoclaved at different times. When designing a fermentation plant, serious consideration should be given to the use of continuous sterilization.

It must be remembered that no matter which process is used to sterilize the medium, the fermenter and its accessories must also be sterilized. In the batch process, these are sterilized together with the medium; in the continuous process, they are sterilized separately. The fermenter must be designed so that high pressure steam is directed to all connexions or pockets in the interior wall. There are inoculating and sampling ports, lines supplying nutrients that are being autoclaved separately, lines carrying antifoam or acid and base, and there are pH and dissolved oxygen probes. All these accessories and their connections to the fermenter must be sterilized with high-pressure steam. As the fermenter cools, filtered

air must be continually fed into it so that there will be no bleeding of non-sterile air through gaskets and seals into the fermenter (see Section 4.2.3,c,v). Furthermore, the fermenter must be designed so that any valves, such as sampling ports, which will be opened or closed during the fermentation are connected with high-pressure steam so that they can be resterilized after each use.

(3) Separate sterilization of glucose

Glucose is readily caramelized by such minerals as iron and phosphate. The amount of caramelization increases with the concentration of glucose and with sterilization time. Caramelization can be minimized by sterilizing the glucose separately in distilled or deionized water. It is recommended that this be done whenever glucose is used in fermenter media.

vii. Temperature control

Temperature is an important facet of the fermentation process. Micro-organisms are often very strict in their demand for a proper temperature. The growth of an organism or its production of a desired metabolic product can be markedly delayed or lowered by temperatures that deviate from the micro-organism's demands. The optimum temperature for the growth of a particular microorganism will have to be determined by experimentation.

Temperature should be controlled within a range of $\pm 0.5^{\circ}\text{C}$. Modern equipment allows strict control in fermenters where the temperature is controlled by coils within the fermenter. Temperatures are monitored by a thermistor and controlled by alternating chilled water and hot water or steam through the coils. In small fermenters (up to 100 litre capacity), the water is heated by electrical heating elements. The major requirement in temperature regulation in fermenters is usually cooling, and the water should be cold enough to do this quickly.

Deviations from a specified temperature can be serious. It is therefore important that a continuous record be kept of fermentation temperatures so that any unexpected deviation can be observed and monitored. Thermistors and modern electronic equipment can detect deviations of as little as $\pm 0.25^{\circ}\text{C}$, and, coupled with strip recorders, can do an excellent job of monitoring and controlling fermentation temperatures.

viii. Foam control

Many fermentations produce a considerable amount of foam. If foaming is left unchecked, the consequences can be disastrous. Excess foam lowers the oxygen transfer rate (OTR) with a direct effect on growth and yields. Foam will flow over into the air exhaust filters, clogging them and forming easy pathways for contaminants to enter the fermenter. Beer will be carried over as foam into the sewer lines, resulting in large losses in the volume of the beer. The same conditions that improve aeration - the presence of baffles, the use of multiple impellers - all stimulate foaming. Foaming can be controlled by mechanical devices and chemical antifoam agents which are added as needed during the fermentation process.

(1) Mechanical foam control

There are several types of mechanical foam breakers available. They are usually attached to the impeller shaft. Most of the foam breakers combine a disk that has a beating action with a cone-shaped device that will carry the foam back down the impeller and back into the medium. Ultra-sonic and centrifugal foam controllers have also been proposed.

Mechanical foam breakers may be useful if the foaming problem is not too severe, if the organism being grown is sensitive to chemical antifoams, or if a chemical antifoam would interfere in the recovery of the desired fermentation product. However, unless there is a specific need for a mechanical foam breaker, it is recommended that chemical antifoams be used.

(2) Chemical antifoams

In the past, there was little choice of chemical antifoam. The most common were lard oil, peanut oil and sesame oil. In the last few years, there has been a rapid expansion in antifoam technology, and there are now a variety of chemical antifoams available. The manufacturers of antifoams do not specify the ingredients used in their formulations, except to describe them as "silicone" or "organic" antifoams. Both can control foaming in *B.t.* fermentations and, presumably, in *B.s.*

Antifoam agents change the characteristic of fermentation media, affecting surface tension and oxygen solubility. It is a general practice to use as little antifoam as possible. A low level of antifoam is generally incorporated into the fermentation medium when it is prepared, and sterilized. Thereafter, the need for additional antifoam is determined by means of a probe held at a selected distance above the surface of the medium being agitated in the fermenter. The probe used is usually a conductance type and operates very simply. When foam rises above the medium and touches the probe, it completes an electrical circuit. This causes a short burst of sterile antifoam to be added to the medium. If, after a designated period of time, the circuit continues to exist, a second burst of antifoam is added - and so forth, until the foam drops below the tip of the probe, and the circuit is broken.

In laboratory-scale fermenters, the antifoam is sterilized in an autoclave along with the fermenter. Rubber tubing leads from the antifoam flask to the fermenter. The tubing is passed through a peristaltic pump which is activated upon demand. In production- and pilot plant-scale fermenters, designed to be autoclaved in place, the antifoam is sterilized separately in a stainless steel tank at the same time that the fermenter is sterilized. Stainless steel tubing connects the two tanks and is sterilized along with the antifoam tank. The antifoam tank is pressurized, and antifoam is added to the fermentation tank upon demand by means of solenoid valves.

The antifoams available today are generally water-soluble or emulsifiable. They can be safely sterilized at 121°C. They are active at low concentrations. They are not toxic and do not interfere in the fermentation or recovery processes. They are inexpensive. These characteristics make chemical antifoams satisfactory tools for controlling foaming in fermentations.

ix. pH monitoring and control

With the development of steam sterilizable electrodes, it became practical to monitor and to control the pH within a fermenter. Each fermentation has a distinctive pH curve. One of the most sensitive indicators in a fermentation is a change in the pH curve.

Sterilizable pH probes are sheathed in stainless steel housing to protect them from breakage. Only the tip of the electrode is in contact with the medium. There are two categories of pH probes for use in fermentations: pressurized and unpressurized. Pressurized probes are designed for use in fermenters that are sterilized *in situ*. During the sterilization, the pressure inside the vessel rises to about 1.0-1.5 kg/m² above atmosphere, while the external parts of the pH probe remain at atmospheric pressure. This pressure differential could lead to a blowout or to leakage through the O-ring assemblies used to seal the junctions to the probe housing and to the tank. To protect against this, this type of probe is designed to be pressurized by a hand-pump to 1.5-2.0 kg/m².

Probes used on fermenters that are designed to be sterilized within a steam autoclave do not face a pressure differential. These probes also need to be protected from leakage. O-ring assemblies are used to protect against leakage into the probe housing, and threaded fittings are used to prevent leakage through the junction on the fermenter wall or top.

In some fermentations, it may be very valuable to be able to control pH by the automatic addition of acid and/or base. In such fermentations, pH controllers are desirable. There are two types of controllers, a mono-functional probe that can add either acid or base, but not both; and dual-functional that can add acid or base as needed. The mono-functional type is less expensive and is perfectly satisfactory for the regulation of many fermentations.

In either case, the acid or base must be sterilized, using the same procedures as those used for sterilizing antifoam agents. However, some precautions must be taken in the case of sterilizing acids. The stainless steel reservoir used for sterilizing acid must be glass-lined if the acid is at all corrosive to the metal. Otherwise, the acid can damage the tank and weaken the walls, creating a very dangerous situation.

Control of the pH may not be essential in the *B.t.* fermentation. At Brownsville the pH in *B.t.* fermentations was controlled by manipulating the initial concentrations of glucose and nitrogen source. By maintaining a proper balance between sugar and nitrogen source, it is possible to maintain what seems to be a typical pH progression in a wide variety of media. These balances could be changed and, in particular, the initial concentrations of sugar increased to control the acid formed. It is preferable to use pH controllers on fermenters, even though experience so far indicates that satisfactory production of *B.t.* can be achieved without pH monitoring devices. The problems of pH control are different for the two types of fermentation, as discussed in Chapter 5.

x. Dissolved oxygen monitoring and control

A dissolved oxygen (DO) monitoring system based on a probe developed by Johnson *et al.* (1967) is available on the market. It consists of

a lead anode and a platinum cathode. A Teflon membrane 1 to 2 mm thick separates these from the medium to avoid poisoning of the electrodes by the medium ingredients. The device produces a millivolt signal which is directly proportional to the rate of oxygen diffusion through the membrane. The diffusion rate, in turn, is proportional to the oxygen partial pressure in the medium.

DO probes are quite rugged; however, they are very sensitive to pressure changes which can damage the thin membrane. They are useful in studying the dynamics of the metabolic processes involved in the fermentation. They were helpful to Brownsville during the early stages of their development of the *B.t.* fermentation, for they showed that the input of air into the fermenter and the agitation rate being used were inadequate.

4.3 Recovery Equipment

4.3.1 Shake flask and other small-scale recoveries

A general description of the method for small-scale recovery of *B.t.* from fermentation beers is given in section 5.4.1. Except for routine laboratory supplies, only two pieces of equipment are needed in this procedure: a refrigerated centrifuge and a pump that will furnish a rapid vacuum for vacuum filtration. Several satisfactory models of both the centrifuge and the vacuum pump are available from different manufacturers. The description of the equipment used in Brownsville is given to illustrate the capability needed in these apparatus.

a. Refrigerated centrifuge

Most of the samples from fermentation studies with *B.t.* range from 500-3000 ml in volume. To recover samples of this size, Brownsville used a Sorvall Model RC-5 Superspeed Centrifuge with a GSA rotor that will hold six 350-ml stainless steel bottles with caps. They use a rotor speed of 13,000 rpm which generates 27,300 G. to achieve an excellent recovery of spores and crystals after 30 minutes of centrifugation.

b. Vacuum pump

The recovery process for *B.t.* from small volumes of beer requires filtration using a vacuum flask, a Buchner funnel, Whatman No. 2 filter paper and suction. In order for the filtration to proceed rapidly and smoothly, there must be no delay in obtaining a vacuum in the filter flask, once the filter paper is wet. The pump does not need to generate a high vacuum, but it does need to have a high capacity. An industrial-type oilless vacuum pump is commonly used, and it is useful to connect a single central pump to the various laboratories in the area. To avoid troublesome fluctuations in vacuum, one should connect a holding tank to the system. Since acetone is a component of the materials being filtered, it is important to vent the pump to the open air to eliminate any possible fire hazard.

4.3.2 Pilot and production-scale fermenter recoveries

It is impractical to centrifuge large volumes of beer in batches. A continuous-flow centrifuge is needed. In addition, the precipitation process

used in small-scale recoveries, described in Section 4.3.1 and 5.4.1 would be impractical because of the cost of the large volume of acetone that would be needed. An alternate procedure is to spray-dry the cream from the centrifugation. Some alternative systems will be described to illustrate the capabilities needed in the equipment used for these processes.

a. Centrifuge

There are several sizes and types of continuous flow centrifuge available for the large-scale recovery of *B.t.* How well they function depends upon several factors:

- the diameter of their centrifugation chambers;
- the length of the path that the beer takes to pass through the chamber;
- the speed of the centrifugation (RPM);
- the pressure (number of Gs) to which the beer is subjected (this is primarily due to a combination of 1 and 3);
- how long the beer is subjected to the action of the centrifuge (this is due to a combination of the rate of flow of the beer into the centrifuge (process rate) and the length of the centrifuge chamber);
- the solid content of the beer;
- the sediment capacity of the centrifuge.

The spinning tube (or tubular cylinder) type of centrifuge will satisfactorily recover *B.t.* spores and toxin, provided that the quantity to be recovered is within the capabilities of the centrifuge. Table 8 lists the specifications of 4 tubular cylinder centrifuges of differing sizes³. The four vary in speed, centrifugal effect (g), size of cylinder, process rate, and sediment capacity. It is tempting to select a model according to the process rate but, if the solid content of the medium is high, the sediment capacity may be a more important criterion.

Table 8. Specifications for Different Tubular Cylinder Centrifuges^a

Specification/Model	Z41	Z61	Z80	Z101
Speed (rpm)	20,000	17,000	16,000	14,000
Centrifugal Effect (g)	16,950	17,670	18,200	15,760
Process Rate (litres/hr)	50-250	100-500	150-1500	200-2000
Size of Cylinder:				
Length (mm)	437	730	730	700
Internal Diameter (mm)	76	105	125	143
Sediment Capacity (litres)	2	6	7.9	10

^aCourtesy New Brunswick Scientific Co. Inc., Edison, N.J.

³Marketed by the New Brunswick Scientific Co. Inc., Edison, N.J.

The principle of a spinning tube centrifuge is very simple. The beer is fed into the bottom of the centrifuge and enters a rapidly spinning tube. The spinning motion helps to carry the liquid up and over the top of the spinning tube, while the solid particles, trying to travel in a straight line, are forced to the side of the tube. Since there is a finite time for the particles to reach the wall, depending upon the size and density of the particles, how thoroughly the beer is clarified and how many particles are lost through the top, depends upon the feed rate or process rate of the beer. It is important for the diameter of the tube to be wide enough, relative to the sediment deposited on the wall, to ensure that the build-up of sediment does not significantly narrow the tube and reduce the efficiency of the centrifugation.⁴

There are other types of centrifuges which will work for *B.t.* One of the best ways to buy a suitable centrifuge is to work with the manufacturer to test the beer in his equipment. In such a test, it is important to test a beer with a high solids content to give the centrifuge as severe a test as possible.

b. Spray dryer

If the goal of the fermentation is to produce a water-dispersible powder or an encapsulated formulation, then the next major step after centrifugation of the beer is to dry the product through the use of a spray dryer. A spray dryer is a simple and effective piece of apparatus.

The principle of a spray dryer is simple. A slurry of the material to be spray-dried is fed into the drying chamber as a fine spray. These small drops fall through a layer of heated air. The water in the droplets are finely divided and thus impart a high surface area to the total volume of the material being dried. Evaporation of the water is very rapid - 5 to 20 seconds is common - and the dried particles fall out of the heated air before they can become hot. The dried powder is picked up by a blower and carried to cyclone collector. The speed of drying is regulated by the droplet size, the inlet temperature of the heated air, and the outlet temperature to which the powder is cooled. When a nozzle system is used, the droplets tend to be larger and to fall more rapidly through the chamber. Alternatively, a centrifugal atomization system can be used. This system forms the spray by accelerating the liquid off the edge of a wheel spinning at 8,000 - 20,000 rpm. The resulting spray is extremely fine. Brownsville laboratories has used both systems, and found that the centrifugal atomization feed markedly improved the recovery and formulation of *B.t.*

Several companies manufacture spray dryers. Brownsville laboratories used the "Spray-Aire" model.⁶ Feed to the spray dryer is through an AT-4

⁴The New Brunswick Scientific Co., the Sharples-Stockes Division of the Pennwalt Corporation, Warminster, Pennsylvania, and the Alfa Laval Group, Stockholm, Sweden, also market spinning tube centrifuges.

⁵For example, Sharples markets a Multi-disc Centrifuge under the Trade Name "Nozjector", the Alfa Laval Group, Stockholm, markets a solid bowl disc centrifuge.

⁶Manufactured by Stork-Bowen Engineering Inc., Somerville, New Jersey, a member company of Bmf-Stork, headquarters in the Netherlands.

centrifugal atomizer. This model is designed for laboratory or pilot-plant use. It handles the centrifuged cream from a 250-litre fermenter without difficulty. Table 9 describes the apparatus and how it is used.

Selecting the correct spray dryer for a given process takes experience and careful analysis. For the most effective use, the size of the dryer and the type of feeding mechanism must be selected according to the type and volume of product. Before choosing a dryer for recovering water-dispersible powders or encapsulated formulation of *B.l.* or *B.s.*, it is

recommended to read the relevant literature⁷ (Published by Bowen). Several companies, including Stork-Bowen, maintain testing laboratories where they will test the use of their dryers in a proposed process and advise on the type and model to use. It is very important to take advantage of such services when available.

Table 9. Typical Pilot-Plant Operation of a Spray Dryer

Model	Spray-Aire
Centrifugal Atomizer	AT-4
Feed Rate	131 ml/min.
Inlet Temperature	150°C
Outlet Temperature	75°C
Recovery Rate ^a	1,600 g/hr

^aBased on recovery from a slurry containing 20% solids produced on a soy flour-corn steep fermentation medium (Dulmage. Unpublished data.).

⁷This includes reprints of papers by Belcher, *et al.* (1963a, 1963b) and by Dittman and Cook (1977) and a pamphlet "Spray Drying Technology" printed by Stork-Bowen.

5

Fermentation Procedures

5.1 Selection of Media Ingredients

It is not possible to recommend specific nutrients for every laboratory to use. The choice of nutrients depends on three factors: availability, cost, and how well the microorganism can utilize them. The factors must be balanced against each other. The media ingredients used at Brownsville are discussed here. They are readily available locally and are relatively inexpensive. For example, one of the more useful nitrogen sources is cotton-seed flour. However, it would do no good to recommend cotton-seed flour where this is not locally available. Commonly available potential nutrients are shown in Table 10. Not all of these have yet been tried in the *B.l.* or *B.s.* fermentations.

Table 10. Examples of Potential Inexpensive Media Available in Developing Countries

Liquids

Coconut milk* (waste product)
 Crude sugar* (eg. jaggery)
 Whey* (waste product)
 Molasses*
 Corn steep liquid*

Materials of plant origin

Legumes and other seeds, chick peas*, peanuts, lima beans*, cowpeas*, soya beans*, bambara beans*, kidney beans*, cotton seed meal*, peanut cake
 Cereals, corn, guinea corn, millets etc., wet mash from breweries
 Tubers, cassava, yams, sweet potatoes etc.
 Yeast powder*, wet yeast ex brewery (waste)

Materials of animal origin

Fishmeal*
 Blood*

* *Tried and found useful: the others either have not been tried or have given poor results but may be useful in combination with other ingredients.*

Local waste materials could be valuable and could markedly lower the cost of production of these microbial insecticides. Any change in fermentation conditions or nutrients must be evaluated in light of the following analyses:

(1) *Media costs vs. yields.* As a rule, media costs represent only a small portion of the costs. The energy input involved in running the fermentors, water chillers, steam generators, and centrifuges represent major costs. The equipment used is expensive, and overhead is high. There is cost of screening or grinding the final product or of processing it into a flowable formulation. Finally, there is the cost of packaging and distributing the material. Since so much of the cost of a microbial insecticide is fixed by these other factors, increases in potency or yield must be relatively high before they make a major impact on the unit cost of the product.

(2) *Interference in the recovery process.* Whether the final product is a water-dispersible powder or a flowable formulation, it will be applied in the field as a spray, either from the ground or from the air. In either case,

the product must be finely enough divided to ensure that it will pass freely through the spray apparatus. This will require that the material be finely divided enough to pass through a 200 mesh screen. This factor plays a major role in dictating the nutrients used in the fermentation medium: a significant portion of the solids present in a harvested beer represent residual, unused, media ingredients. If these are coarse, grinding will be necessary. Grinding not only adds an extra and costly step to the recovery process, it results in a significant loss of active ingredient because of the heat and dust generated in the grinding equipment. This loss can be avoided by using nutrients that are either fine by nature (for example, corn-steep liquor) or have already been ground (cotton-seed flour instead of cotton-seed meal). This requirement for using finely divided nutrients is unique to the production of microbial insecticides and must be taken into consideration in any programme for developing suitable fermentation media.

(3) *Effect on the final formulation.* There can be direct or indirect effects of fermentation ingredients on the final formulation. For example, water-dispersible powder formulations of *B.t.* are stable only when the moisture content of the powder is less than 4%. Therefore a water-dispersible powder should not be strongly hygroscopic. All formulations can absorb moisture under certain conditions and therefore should be kept in sealed containers.

5.1.1 Carbon Sources

Glucose, starch, and molasses have all been used as carbon sources in *B.t.* fermentations. The level of carbohydrate must be carefully chosen for two reasons: first, all the strains of *B.t.* that were examined so far produce acid from glucose. If the concentration of sugar is too high, the pH of the medium will drop below pH 5.6-5.8, and the acidity will inhibit or stop growth. It is difficult to define "too high". This depends on the concentration of the nitrogenous nutrients in the medium. *B.t.* tends to produce alkaline materials from these nitrogenous nutrients that can neutralize the acidic compounds produced from the sugar. Too low a level of sugar can terminate growth too soon. One should try to have a relative balance of sugar and nitrogenous materials so that the initial pH of the fermentation will drop to about 5.8-6.0, after which it will rise slowly and steadily to a level of pH 8.0. Proper concentrations are not difficult to determine by experimentation (see also Section 5.3.2).

B.s. does not use most of the common carbohydrates such as glucose, molasses, or starch as carbon sources (Russell et al. 1989). The carbon for growth and energy production must be provided by amino acids present in proteinaceous substrates such as soybean flour, cottonseed flour, or fish meal. It has been shown that partially hydrolyzed protein substrates are utilized more efficiently than crude protein substrates. Glycerol has also been shown to be a useful addition to media containing peptones (partially hydrolyzed proteins) as the primary source of carbon (Klein, et al., 1989). Dharmsthiti et al. (1985) reported growing *B.s.* on a medium containing 7%

hydrolyzed liquor by-product from a monosodium glutamate factory. Each batch of liquor varied somewhat in composition and this variability might require some readjustment of each batch of a medium using it as a component.

5.1.2 Nitrogen sources

Several nitrogen sources are used in *B.t.* fermentations. Three are proteins: soybean flour (soy flour), cottonseed flour (Proflo^R), and fish meal. The soy flour and the cottonseed flour are both very good nutrients. Fish meal is also effective, but its use is restricted, partly because the meals contain significant levels of residual fish scales, which make it difficult to obtain a uniform product, and partly because of the residual odour carried over into the final formulation. Corn-steep is very useful as an additive to these media, and it may be satisfactory as the sole nitrogen source. (Corn-steep liquor is rich in many types of nutrients and its value in a fermentation is not restricted to serving as a nitrogen source.) Corn-steep is very acid, being derived from the acid treatment of corn in the commercial production of starch, and it is important to neutralize any medium containing corn-steep. Such media should be adjusted to pH 6.8-7.0 before autoclaving.

While these materials are all proteins or polypeptides in fundamental composition, they differ in nitrogen levels, in amino acid content, and in other nitrogenous or non-nitrogenous content. The composition of soybean flour, cottonseed flour, and a typical corn-steep, are given in Table 11. It should be noted that, while corn-steep is an inexpensive and a very useful nutrient, it is quite a variable mixture.

Nitrogen for the growth of *B.s.* is provided by the amino acids which are also used as the carbon source. It does not appear to be necessary to add additional nitrogen in the form of ammonium salts.

5.1.3 Minerals

Minerals are essential in the nutrition of organisms. Five metallic ions are considered to be particularly important in the growth and sporulation of bacilli: Mg⁺⁺, Mn⁺⁺, Fe⁺⁺, Zn⁺⁺, and Ca⁺⁺. These are all normally present in the carbon and nitrogen sources used in fermentations and there may be no need to include these ions in the fermentation media.

In many media 0.3 g/l MgSO₄·7H₂O; 0.02 g/l MnSO₄·H₂O; FeSO₄·7H₂O; ZnSO₄·7H₂O; and 1.0 g/l CaCO₃ are added. Adding them to a medium will not damage a fermentation, even if there are already sufficient levels of these minerals present. They are also high enough to correct any deficiencies.

5.1.4 Formulae of selected media

Table 12 lists the formulae of several selected media used in *B.t.* fermentation. Some of these media are particularly significant in work with *B.t.* Medium B-4c is a simple, basic medium that was routinely used in the

Table 11. Composition of three nitrogenous fermentation ingredients

A. Nutrient composition.

Per cent of total weight

Ingredient:	Soybean flour ^a	Cottonseed flour ^b	Corn-steep liquor ^c
Nutrient			
Protein	52.0	61.0	23.0
Carbohydrate	30.0	23.2	5.8
Fat	1.0	4.1	0
Fiber	3.0	03.2	0
Ash	6.0	6.7	7.8

B. Amino acid composition.

Per cent of protein content

Ingredient:	Soybean flour ^a	Cottonseed flour ^b	Corn-steep liquor ^c
Amino acid			
Alanine	2.62	4.13	6.5
Arginine	3.78	7.15	5.8
Aspartic acid	-	8.01	6.5
Cystine	0.41	1.45	1.8
Glutamic acid	-	15.99	14.2
Glycine	-	2.75	4.9
Histidine	1.35	1.98	3.1
Isoleucine	2.46	2.42	3.1
Leucine	4.08	6.46	8.0
Lysine	-	-	4.0
Methionine	0.60	1.40	2.7
Phenylalanine	2.60	4.17	4.9
Proline	-	2.68	5.8
Serine	-	3.77	4.4
Threonine	-	2.46	4.9
Tryptophan	2.17	0.86	0.9
Tyrosine	0.34	2.53	3.1
Valine	-	3.25	5.3

^a Analysis of Toasted Nutrisoy Flour^R. Data from the producer, Archer Daniels Midland Co., Decatur, Illinois.

^b Analysis of Proflo^R. Data from the producer, Traders Protein Division, Trader Oil Mill Co., Fort Worth, Texas.

^c Data on a typical lot of corn-steep liquor (condensed fermented corn extractive) from Corn Refiners Association Inc., Washington D.C.

Table 12. Examples of Media Used in Fermentation of *B. thuringiensis* and *B. sphaericus*

Medium No.:	Level - grams/litre					
	B-4c	B-8b	B-12	B-13	Megna ^a	Singer ^b
Ingredient						
Cottonseed flour	10.0	30.0	-	-	16.7	-
Soybean flour	-	-	20.0	40.0	-	10.0 ^c
Fish meal	-	-	-	-	-	10.0
Corn Steep (wet weight)	-	30.0	20.0	20.0	33.4	-
Peptone	2.0	2.0	-	-	-	-
Dextrose	15.0	45.0	30.0	30.0	-	-
Molasses	-	-	-	-	18.6	-
Yeast extract	2.0	2.0	-	-	-	-
Vitamin mix ^d	-	-	-	-	-	1.0 ^d
MgSO ₄ ·7H ₂ O	0.3	0.3	0.3	-	-	-
FeSO ₄ ·7H ₂ O	0.02	0.02	0.02	-	-	-
MnSO ₄ ·H ₂ O	0.02	0.02	0.02	-	-	-
ZnSO ₄ ·7H ₂ O	0.02	0.02	0.02	-	-	-
CaCO ₃	1.0	1.0	1.0	-	-	-
CaCl ₂	-	-	-	-	-	0.1

^a Megna, (1959)

^b Singer, (1981)

^c Singer used soybean meal instead of soybean flour.

^d Singer's table, from which the data on Singer's medium was taken, stated that 1.0% of a vitamin mix was included. However, his table does not specify the mix used.

Brownsville screening programme searching for more active or different isolates of *B.t.* Medium B-12 is a useful for fermentation studies in fermentors. Medium 8-b has yielded improved activity with several isolates, and Medium B-13 has supported the production of the highest insecticidal activity against *Heliothis virescens*. Yields from Medium B-13 are not always high. The yields refer to results from a combination of media studies and the use of a particular strain of *B.t.* The "Megna medium" (Megna, 1963) was cited as an example in one of the important early patents on *B.t.*

Less information is available about production of *B.s.* because this bacterium has not been produced on a large scale. However, both NYSM and MBS broths (see Table 4) have been used to produce this bacterium in small fermentors for field trials. Another useful medium contained (g/l): Humko-Sheffield HySoy (15.0), yeast extract (2.0), MgSO₄ (0.3), FeSO₄ (0.02), ZnSO₄ (0.02), MnSO₄ (0.02) set to pH 7.5 (Davidson & Titus, 1987). A medium used at the pilot level (150 l) contained (g/l): Humko-Sheffield

peptonized milk (10), Amber Laboratories autolysed yeast extract (2), $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ (0.3), $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$ (0.02), $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (0.02), $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$ (0.02) (Davidson *et al.*, 1984).

There is no ideal way to get reproducible inocula, so a compromise is made by inoculating a "seed flask", usually an Erlenmeyer flask containing tryptose-phosphate broth (*B.t.*) or NYSM (*B.s.*) (see Table 4); placing this flask on a rotary shaker for a designated period of time; transferring a small volume of this first-passage seed to a second seed flask; incubating this for another designated period of time on the shaker; and finally using this second-passage seed to inoculate the production in experimental shake-flasks or fermenters. If the fermenter is very large, a third-passage seed, produced in a fermenter and in a less expensive medium, may be used.

5.2.1 Shake-flask fermentations

A loopful of culture is taken from a nutrient agar slant (Table 4) to inoculate a 500-ml Erlenmeyer flask containing 100 ml of tryptose-phosphate broth. The flask is placed on rotary shaker and incubated at $30^\circ \pm 1^\circ \text{C}$ at 340 rpm for 12 hours. At this time, 2 ml (2% by volume) of the first passage seed is used to inoculate a similar flask, which is incubated under the same conditions as the first-passage seed for 12 hours. Finally, 2% (by volume) of this second-passage seed is used to inoculate shake flasks in the fermentation series. This procedure works very well for all *B.t.* fermentations, as well as with *B.s.*

5.2.2 Fermentations in pilot-plant fermenters

To inoculate pilot-plant scale fermenters, a loopful of culture is taken from a nutrient agar slant and used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of tryptose-phosphate broth. The flask is placed on a rotary shaker and incubated at $30^\circ \pm 1^\circ \text{C}$. However, in the case of seeds used for fermenters the primary seed flask is incubated for only 6-8 hours. At this time, about 13 ml of the first-passage seed is used to inoculate 670 ml of tryptose-phosphate broth contained in a 2000-ml Erlenmeyer flask (2% by volume inoculum size). This second-passage seed is incubated under the same conditions as the primary seed for 6 hours and then used to inoculate the fermenters. Fermenters are always inoculated with approximately 0.5% by volume of this second passage seed. Fourteen litre fermenters contain 10 litres of medium. To inoculate these, one should use a sterile 50-ml syringe to remove 50-ml aliquots from the seed flask and inject the seed into the fermenter through a rubber diaphragm that seals the port. A 200-litre fermenter contains 125-150 litres of medium, and an entire seed flask is used to inoculate this fermenter, approximating the 0.5% inoculum size.

5.3 Fermentation conditions

5.3.1 Shake-flasks

Shake-flasks are useful tools for screening large numbers of isolates of

B.t. They are not as useful in studies of fermentation media or other aspects of fermentation. The reason for this seems primarily due to the limitations of air supply in shake flasks. The larger the shake flask and the smaller the relative level of nutrient medium in the flask, the greater the similarity between shake flask data and fermenter data.

It might be useful to restrict usage to 500-ml and 2000-ml flasks. The 500-ml flask is useful for screening isolates. It is recommended that 125 ml medium be used per flask. The high oxygen demand of *B.s.* will be best satisfied by placing no more than 10% of a flask's volume as liquid eg. 50 ml in a 500 ml flask. The volume will yield about 30-35 g of product from one shaker platform board of 24 flasks - a weight that is needed for screening programmes.

2000-ml flasks are rarely used except the production of seed but, with this sized flask one should attempt to get as high aeration as possible by not using more than 50-100 ml medium per flask.

The limitation of aeration in shake-flasks limits the choice of media. Best growth in shake-flasks occurs when the nutrient content of the medium is kept low. Thus Media B-4c and B-12 function well in shake-flasks, but Media B-8b and B-13 do not (Table 12).

Table 13. Conditions for Shake-Flask Fermentations of B. thuringiensis and B. sphaericus

Condition	<i>B. thuringiensis</i>	<i>B. sphaericus</i>
Size of flask	500 to 2000 ml	500 to 2000 ml
Volume medium/flask	50 to 125 ml	50 to 125 ml
Incubation temperature	20 - 30°C	28 - 30°C
Agitation	340 rpm	340 rpm
Starting pH	6.8 - 7.2	6.8 - 7.2
Harvest pH	7.8 - 8.5	8.5 - 9.2
Age at Harvest	24 - 48 hours	48 - 72 hours
Usual spore count/ml beer	10 - 50 X 10 ⁹	1 - 10 x 10 ⁹

5.3.2 Fermenters

The 14 litre fermenter is a useful tool for beginning fermentation research. The only limitation is the very large jump in size experienced when one attempts to extrapolate data from the 14-litre fermenters to production-sized equipment, which may be 40,000 times larger or more. The 200-litre fermenter solves much of this difficulty. It is large enough to predict and solve many scale-up problems.

Table 14 and comments following the table discuss typical conditions encountered when 14-litre and 200-litre fermenters were used.

The most common pattern of pH seen in *B.t.* fermentations reflects the

production of acid from glucose by this organism. After sterilization, the pH of the fermentor is pH 6.8 - 7.2. Immediately after inoculation, the pH falls steadily as the glucose is utilized, reaching a pH of about 5.8 - 6.0 after 10 - 12 hours. At this point, the pH starts to rise at the same rate that it fell, reaching pH 7.5 by 25 hours. The rise in pH slows gradually, reaching a pH of 8.0 at about 30 hours. The pH may continue rising, reaching pH 8.8 after 50 - 60 hours. With some cultures, the initial drop in pH may only reach pH 6.5 - 6.6. In such fermentations, there may be little or no rise in pH as the fermentation continues.

Table 14. Conditions for Fermentations of *B. thuringiensis* with 14-litre and 200-litre Fermenters.

Conditions	14 litre	200 litre
Volume of medium ^a	10 litres	125 - 150 litres
Aeration	1 v/v/m	1 v/v/m
Agitation	700 rpm	400 rpm
No. of Impellers ^b	2	3
Incubation temperature	28 - 30°C	28 - 30°C
Starting pH ^d	6.8 - 7.0	6.8 - 7.0
Final pH	8.0 - 8.5	8.0 - 8.5
End of log-phase of growth ^d	16 - 18 hours	16 - 18 hours
End of sporulation phase	20 - 24 hours	20 - 24 hours
Completion of lysis	35 - 40 hours	35 - 40 hours
Age at harvest	40 - 72 hours	40 - 72 hours

^a Volume of medium: 10 litres of medium are placed in 14-litre fermenters and 125-150 litres in 200 litre fermenters. These volumes are, of course, below the capacity of the tanks.

^b Impellers: While agitation in rpm differs from 14-litre fermenter to 200-litre fermenter, the different speeds mainly reflect the different diameters of the impeller blades. However, there are differences in behaviour during scale-up, and it has been found that, while 2 impellers are satisfactory for the smaller 14-litre fermenter, 3 impellers are needed for adequate dispersion of air and nutrients in the 200-litre fermenter. Before adding the third impeller, there were considerable difficulties in maintaining an adequate air supply to the fermenters.

^c pH: The pH patterns of fermentations mainly depend upon the medium and the organism. For example, the pH patterns observed in *B.t.* and *B.s.* fermentations differ markedly. It is very important to monitor pH continuously during a fermentation.

The most common pattern of pH seen in *B.t.* fermentations reflects the production of acid from glucose by this organism. After sterilization, the pH of the fermentor is pH 6.8 - 7.2. Immediately after inoculation, the pH falls steadily as the glucose is utilized, reaching a pH of about 5.8 - 6.0 after 10 - 12 hours. At this point, the pH starts to rise at the same rate that it fell, reaching pH 7.5 by 25 hours. The rise in pH slows gradually,

reaching a pH of 8.0 at about 30 hours. The pH may continue rising, reaching pH 8.8 after 50 - 60 hours. With some cultures, the initial drop in pH may only reach pH 6.5 - 6.6. In such fermentations, there may be little or no rise in pH as the fermentation continues.

In *B.t.* fermentations the pH can be controlled to some extent by maintaining a proper balance of sugar to nitrogenous compounds - the sugar causing a drop in pH, and the nitrogenous materials partially balancing the drop by producing basic compounds. If too high a sugar level is used without an adjustment in the nitrogen source, the pH of the fermentation can drop below pH 5.6 - 5.8. At this pH, the organism will stop growing. Too much sugar may also inhibit the sporulation of the bacillus, even though the pH never drops to a low level.

In contrast to *B.t.*, the pH in *B.s.* fermentations moves continuously upward throughout the growth and sporulation of the bacteria. Since the bacteria do not use sugars as a source of carbon, acids are not formed. Rather, ammonia accumulates in the broth, probably due to deamination of amino acids. The final pH may range from 8.0 to 9.0 depending upon the protein content of the medium. It is possible to control the pH by the addition of acid, and this may enhance toxin production by some strains but not by others.

5.3.3 Duration of various growth phases

The "log-phase" of any bacterial fermentation is that period during which the organism is vigorously growing and rapidly dividing. This first phase lasts 16 - 18 hours. Sporulation is complete by 20 - 24 hours after inoculation, although the cells have not yet lysed. Lysis is complete by 35 - 40 hours.

5.4 Recovery procedures

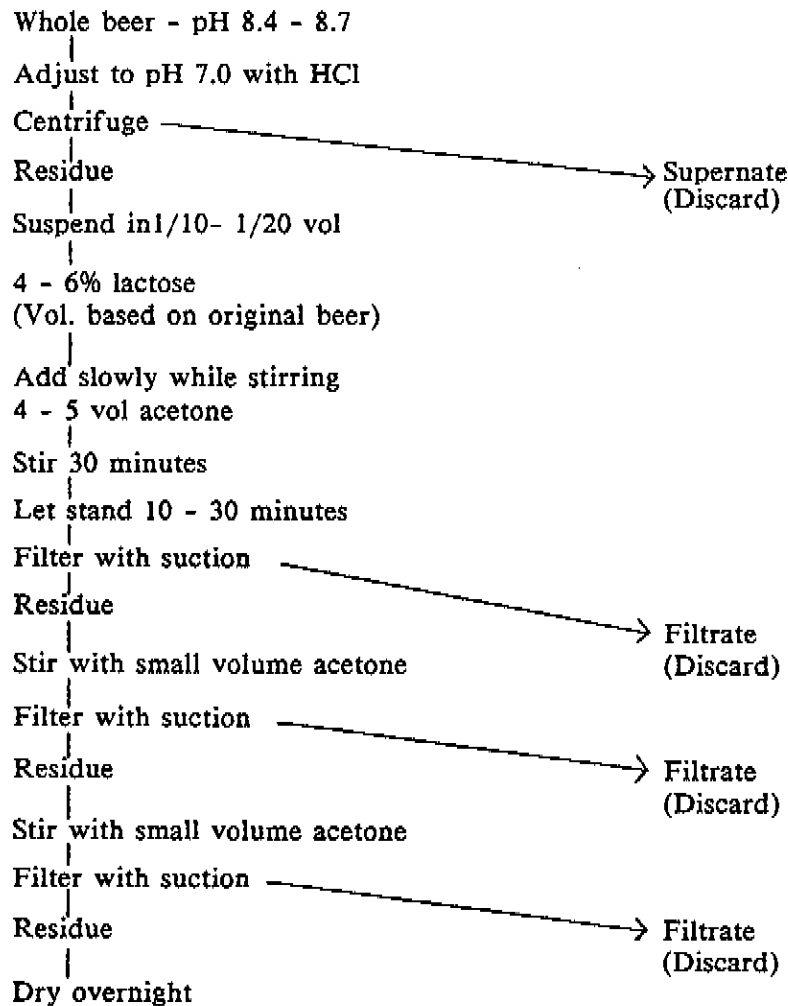
5.4.1 Recovery of small quantities of *B.t.* and *B.s.*

Small quantities of *B.t.* or *B.s.* can easily be recovered by the lactose-acetone coprecipitation procedure of Dulmage, et al. (1970). The flow-sheet for this procedure is given in Table 15. Specific comments have been omitted from the Table for brevity, and some further explanation follows.

1) *Centrifugation:* A description of a centrifuge useful for the recovery of small quantities of bacteria is given in Section 4.3.1.a. As specified, the beer is centrifuged at 27,300 G for 30 minutes.

2) *First filtration:* The creamy residue from the centrifuged beer is suspended in 1/10 volume of 4 - 6% lactose and coprecipitated with acetone. The suspension is filtered with suction using Whatman No. 2 filter paper and a 12 - 14 cm. Buchner funnel. The filtration will go faster if the acetone-lactose-*B.t.* suspension is allowed to stand 10 - 30 minutes after stirring. With the suction on, a portion of the supernatant is poured into the Buchner to wet the paper. Before the liquid can pass through the paper, the rest of the suspension is quickly slurried and poured into the Buchner. Under these conditions, filtration proceeds rapidly and smoothly.

Table 15. Recovery process for spore-crystal complex of
B. thuringiensis and *B.sphaericus*.
Flow sheet for pilot plant and laboratory scale.



3) *Second and third filtrations:* If the first filtration has proceeded properly, the *B.t.* will easily come off the filter paper. The purpose of the second and third filtrations is primarily to remove remaining water from the *B.t.* preparation. Sufficient acetone should be used in each wash to accomplish this purpose. On rare occasions, a third wash may be necessary.

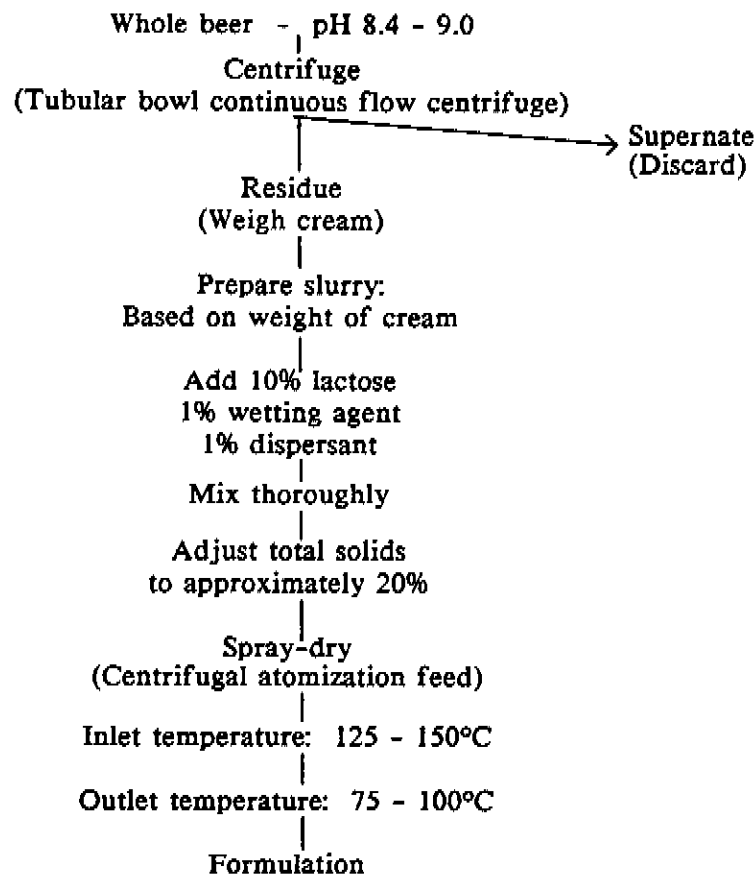
4) *Drying:* The *B.t.* from the final acetone wash is placed in an evaporating dish, covered lightly with a piece of filter paper and allowed to dry on the laboratory bench at room temperature and a relative humidity of less than 40% overnight. It may be necessary to use a warm incubator to

achieve this low humidity. The next morning, the powder is transferred to a mortar and lightly ground with a pestle to break up loose aggregates. No real grinding should be needed.

5.4.2 Pilot plant scale recovery of *B. thuringiensis* and *B. sphaericus*

Large quantities of the bacteria are readily recovered by spray drying. A flow-sheet for this procedure is given in Table 16. A continuous-flow type centrifuge is used to separate the *B.t.* from the beer. Specifications for such a centrifuge are given in Section 4.3.2.a. It is very important that the feed-rate to the centrifuge is adjusted. Too slow a feed will result in clogging the spinning tube prematurely by not utilizing the full length of the tube for the collection of the *B.t.* or *B.s.* Too fast a feed means that much of the product will pass through the centrifuge and be lost.

Table 16. Spray drying recovery process for spore-crystal complex of *B. thuringiensis* and *B. sphaericus*.



It is also very important to select the proper feed rate into the spray drier. Specifications for the spray drier are given in Section 4.3.2.b. If the drying temperature is too cool or if the spray falls through the heated air too fast, drying will be incomplete, and material will be seen sticking to the sides of the spray drier. With proper handling, a spray drier will give a high-quality, finely-divided product.

5.4.3 Flowables

One can avoid precipitation or spray drying by formulating the final product as a flowable formulation. In this case, the cream from the centrifugation step is reconstituted in oils, preservatives, and dispersing agents as required. A flowable formulation is easy to mix into spray tanks, it is somewhat easier to handle than a dry material, it can be applied in a much more concentrated form than a water-dispersible powder. Flowables, however, have one major disadvantage: they are not as stable as powders. They are much more susceptible to heat and other conditions of storage than are water-dispersible powders.

Flowables play an important role in the utilization of bacterial larvicides and their production should be considered seriously by anyone planning to produce these materials.

6

Semi-Solid Fermentations

The oldest method of aerobic fermentation has been to grow the microbe on a wet matrix (ie. a semi-solid fermentation). As early as the 1890s in the United States, Takamine Laboratories successfully marketed fungal amylase and bacterial protease produced via semi-solid fermentation. Attempts have been made to produce the *B.t.* delta-endotoxin in semi-solid conditions, and one company (Nutrilite Products), produced and marketed on a small scale a wettable powder of a lepidoptera-active *B.t.* to control the cabbage looper (*Trichoplusia ni*) on cabbage and lettuce.

The semi-solid procedure combines the advantages of growing a microorganism on the surface of a solid medium such as nutrient agar with the improved efficiency in aeration and nutrient supply obtained in submerged fermentation. Under the semi-solid procedure, a coarsely divided matrix is moistened with a nutrient medium that has been inoculated with the microorganism to be grown. As a result of this mixing and moistening, the surface of each particle contains a small droplet of the medium on which the microorganism can feed.

6.1 Principles of Semi-Solid Production

6.2.1 Media

Semi-solid fermentations are conducted on a loosely aggregated matrix of rather coarse particles. These particles not only support the physical positioning of the droplet-particle mixture, they may also supply some nutrient material themselves. Several inexpensive agricultural products have been used as matrices, most commonly bran, but also corn, rice hulls, or a combination of these. A fluffy mineral, such as Perlite, has been used, sometimes alone, but more often in combination with one of the above, especially with bran.

Experience with the growth of *B.t.* in semi-solid fermentation, as reviewed by Dulmage & Rhodes (1971) has been based on a patent by Mechalas (1963), which used a mixture of bran and Perlite as a matrix on which to support the fermentation. Their data are used below to illustrate a typical fermentation.

The nutrients used for semi-solid fermentations are essentially the same as those used for submerged fermentation, but the quantities have to be adjusted for the differences in procedure. The moisture content of the final bran-nutrient-bacillus mixture must be kept within fairly close limits. Too little water, and the microorganism will not grow well; too much, and the medium will clump and soften, and the passage of air will be severely reduced.

The Mechalas procedure first describes the production of a seed with which the bran will be inoculated. This is prepared in submerged fermentation. The semi-solid medium was composed of a mixture of 545 g wheat bran, 380 g expanded Perlite, 62 g soybean meal, 36 g dextrose, 3.6 g lime, 0.9 g NaCl, 0.29 g CaCl₂ and 160 ml water. This mixture was sterilized in flowing steam for 60 min, and then inoculated with 400 ml of seed from the fermentor. The final mixture had a pH of 6.9 and a moisture content of 60% by weight. Such a medium would be satisfactory to use in most of the various semi-solid fermentation procedures.

6.1.2 Aeration

a. Shallow trays or small containers

Adequate aeration is important in semi-solid fermentations. Several methods are used. The oldest and the simplest is to spread the bran-nutrient-microorganism mix on shallow trays in a humidified chamber. This is fermentation at its most rudimentary level, since no mechanical devices are used in the fermentation, adequate contact with air being achieved by the shallowness of the layers in the trays.

This procedure does not work well when bacteria are being grown. One of the major problems is keeping the bran layers moist. Also, this procedure is not practical where labour costs are high and the use of trays renders the fermentation very susceptible to contamination.

b. Aeration in static bins

Space and manpower can be saved if the bran mixture is heaped in piles in deep containers. The limitation here is aeration, and many companies have attempted to devise simple, inexpensive devices to achieve good aeration. While there are many variations of this procedure, the Mechalas patent typifies the methodology and the goals in the use of static bins (Mechalas, 1963).

Under the Mechalas procedure, the bran-nutrient-microorganism mix is placed in bins with perforated bases through which air at 30-35°C and 95-100% R.H. is passed. A low air flow is used in the first three hours of the fermentation (0.4 - 0.6 vol air/vol inoculated medium/min.), but thereafter the flow rate is increased to 1.0 - 1.2 vol. The bacteria multiply rapidly and generate considerable amounts of heat. This is accentuated by the tendency of air to form channels in the bran piles, resulting in inefficient control of temperature in parts of the piles. This can be countered to some extent by digging into the medium and stirring thoroughly at least once during the fermentation. As described by Mechalas, the bins were incubated for about 36 hours, during which time the pH rose to 7.5 and the moisture content dropped to about 53%.

This is a relatively inexpensive way to grow *B.t.*, and it does not involve elaborate equipment. However, there are problems involved. The air used in the fermentation, while clean, is not sterile, with the result that large numbers of potentially contaminating organisms reach the bottom of the piles. The bottom layer of the bran in the bin acts as an air filter, and contamination might normally be restricted to this layer. However, the necessity for stirring the bran during the fermentation can easily spread these contaminants throughout the bran. Second, the channelling referred to above results in very uneven air supplies throughout the bran. This leads to uneven production of *B.t.* and its toxins within the bran pile. Finally, even under the best of conditions, air is not a highly efficient heat exchanger, and the temperatures will vary throughout the bin, even though the pile is stirred. All these factors tend to decrease yields. The problems of semi-solid fermentations in static bins make submerged fermentation procedures preferable for the production of *B.t.* A summary of some of the drawbacks is given in Section 6.3.

c. Production in revolving bins

Most of the problems of semi-solid fermentation can be overcome through the use of revolving bins with perforated walls through which sterile humidified air is passed. Under this procedure the bran is being continually tumbled, so aeration becomes highly efficient. Unfortunately, this tumbling action also makes the bran mixture as vulnerable to contamination as in a fermentor. Consequently, the requirements of this procedure are as stringent as those for a submerged fermentation, and the chief value of the semi-solid fermentation - low cost - is lost. It would not be practical to use this procedure to produce *B.t.*

6.1.3 Harvest

Harvesting semi-solid fermentations is nothing more than changing the air supply from humidified air to dry air. The bran mixture is dried *in situ* in the same container in which it was fermented. However, as discussed in Section 5.1, the final formulation must be finely divided to use in application equipment. The use of finely-divided nutrients in the semi-solid procedure is precluded because the fine particles would decrease aeration and increase channeling. Thus the dried bran is unsuitable for use and must be ground. As discussed in that Section, grinding results in a significant loss of active ingredient, a factor that must be taken into account in considering semi-solid fermentation.

6.2 Problems associated with Semi-Solid Fermentation

Perhaps the biggest problem with semi-solid fermentations is that the very simplicity of their operation tends to lead many scientists to think that the rules and strictures about the quality control devised for materials produced in submerged fermentation do not apply to products produced by semi-solid fermentation. This is not true. If successful semi-solid fermentations are to be conducted, a qualified microbiologist must supervise the process. This may be even more important in the case of semi-solid fermentations because contaminants may more easily go unnoticed.

Proper bioassays of the product for insecticidal activity are necessary. Table 17 shows the amount of insecticidal activity produced per 10^6 spores in six semi-solid fermentations. The yields varied over a 7-fold range.

Table 17. Insecticidal Activity of a Single Strain of B. thuringiensis in Six Semi-Solid Fermentation Runs^{a,b}

Assays on harvested bran			
Run no.	Spore count x $10^9/g$	IU/mg dry wt	IU/ 10^6 spores ratio
1	6	240	40
2	17	1400	82
3	7	940	130
4	3	840	280
5	11	840	76
6	5	650	130

^aMedia for all runs were identical. Assay insect: *Estigmene acrea*

^bFrom Dulmage & Rhodes (1971).

Formulation of *Bacillus sphaericus*

The efficacy of unformulated primary powders and whole fermentation beers of several isolates of *B.s.* under a variety of field conditions is well documented (Silapanuntakul *et al.*, 1983; Mulla *et al.*, 1984a, 1984b, 1985; Hougard *et al.*, 1985; Lacey *et al.*, 1988a). However, formulation of the primary powders and fermentation beers will make them more suitable for use and more effective under a variety of environmental conditions. The formulation requirements for *B.s.* are essentially the same as for *B.t.* H-14. Formulation objectives are outlined in Table 19, and commercial formulations of *B.t.* H-14 are presented in Table 20.

7.1 Factors influencing formulation of efficacy

Several factors influence the effectiveness of a particular larvicide formulation. These begin with the choice of a patent bacterial isolate and its growth under fermentation conditions that result in a high yield of toxin. The method of cell recovery and of drying (if this is done) will also affect the toxin content and the particle size in the end product. The particle size in suspended formulations can exert considerable influence on the efficacy of the larvicide. Molloy *et al.* (1984) reported that the settling rate of a formulation correlated directly with its mean particle size. Mullens & Hinkle (1988) observed that the flowable concentrate Teknar TM (Zoecon-Sandoz), the *B.t.* H-14 formulation with the smallest mean particle size, settled more slowly than two wettable powders and consequently provided more prolonged control.

7.2 Formulations of *Bacillus sphaericus*

Although *B.s.* is not being produced commercially at present, experimental formulations have been made in granular, flowable concentrate and sustained release forms similar to those available for *B.t.* H-14 have been made.

7.2.1 Granules

Vegetative canopy may impede conventional spray applications of wettable powder and flowable concentrate formulations from reaching target larvae. Granular formulations (in which bacteria are coated upon or incorporated within a carrier) allow penetration of canopy and provide even distribution within the larval habitat (Lacey & Inman, 1985). Modifying the carrier to increase flotation will extend contact time with surface feeding species such as the anophelines whereas sinking pellets may be more effective against bottom feeding species. An experimental granular

formulation of *B.s.* similar to the Bactimos(R) formulation of *B.t.* H-14 in terms of physical characteristics and inert constituents, facilitated penetration of vegetation and provided excellent control of *Culex* and *Psorophora* larvae. However, this formulations was much less effective against *Anopheles* spp. (Lacey *et al.* 1988b). Berry *et al.* (1987) report effective use of other experimental granules for effective control of *C. pipiens* and *Ae. trivittatus*.

7.2.2 Flowable concentrates

Flowable concentrate formulations have provided the greatest number of options in terms of application and ease of handling for *B.t.* H-14 (Lacey & Undeen, 1986). A flowable concentrate of *B.s.* 2362 (Biochem Products) applied using a Beecomist ULV spray head provided nearly complete control of *Ps. columbiae* at fairly low dosage rates and achieved moderate control of *An. quadrimaculatus* breeding in rice fields (Lacey *et al.*, 1986).

7.2.3 Sustained release formulations

Incorporating primary powder of *B.s.* within a matrix that enables both sustained-release of inoculum as well as flotation, may extend residual control in habitats where conditions are not favorable for recycling and/or where the toxin settles from the larval feeding zone. Briquette and sustained-release pellets evaluated by Lacey *et al.* (1984, 1988b) provided extended control under these conditions and in full sunlight. Pradeepkumar *et al.* (1988) report sustained control of *Mansonia* mosquitoes in natural habitats with a briquet formulation of *B.s.*

7.3 Storage

B.s. toxin remains extremely stable under optimal storage conditions of neutral pH and 4°C (Lacey 1985) and fairly stable at room temperature (Mian & Mulla, 1983; Hertlein *et al.*, 1980). Toxin that is exposed to high pH (10.8) will become denatured immediately (Lacey, 1985). Prolonged storage under even slightly basic conditions at room temperature results in an accelerated decline in larvicidal activity. However, a rapid decline in spore viability is observed during storage under acidic conditions (Lacey 1985). Although spores are not required for larvicidal activity (Burke *et al.*, 1983) recycling cannot occur in the absence of viable spores. Thus formulation additives that prevent secondary fermentation during storage should not necessarily affect spore viability.

7.4 Environmental considerations

In addition to the impact of environmental parameters on the effectiveness of the larvicide, consideration must be given to the impact of the formulation components on the environment. These components should be innocuous to non-target organisms, both plant and animal.

Table 18. Formulation objectives for microbial control agents of mosquito larvae^a

I. Ease of Handling

1. Storage - space considerations
2. Mixing - flowability, optimally little or no mixing or dilution required
3. Application - equipment compatibility (avoid clogging of nozzles, etc.); permit variety of application options

II. Ensure Stability

1. Long shelf life - compatibility of adjuvants with toxin; prevent secondary fermentation and contamination
2. Field - UV protection where needed; protection from microbial denaturing of toxin; minimize settling of toxin

III. Maximize Contact with Target Larvae

1. Penetrate foliage - granular and pelleted formulations
2. Optimize dispersal - maximal distribution with minimal drift
3. Maintain toxin in larval feeding zone - sustained release floating formulation
4. Attract or arrest target larvae - inclusion of feeding stimulants/arrestants

IV. Increase Residual Activity

1. Maximize prolonged contact with larvae (see III)
2. Optimize field stability (see II-2)

V. Considerations

1. Cost
2. Adjuvant compatibility with toxin and environment
3. Ensure normal larval feeding

^a modified from Lacey (1984).

7.5 Assessing formulation potency

Toxicity ratings should be provided for each batch of formulated material. A standardized bioassay protocol suitable for use in standardizing *B.s.* and using 48h old larvae of *C. quinquefasciatus* as the assay insect is shown in Paragraph 2.2. The Institut Pasteur has prepared a proposed international standard of *B.s.* (Rb-80, isolate 1593.) for use in this assay.

Table 19. Commercially produced formulations of B. thuringiensis H-14 and their uses (Modified from Lacey, 1984).

Wettable Powders

Conventional aerial and ground application to unobstructed breeding sites

Liquids

Oil and aqueous base flowable concentrates, conventional aerial and ground application, point source and low volume aerial application

Granules

Botanical and clay carriers, aerial application, especially to breeding sites with dense foliage (eg. rice fields and salt marsh)

Briquettes, pellets

Provide sustained larvicidal activity in containers and small impoundments

8

Safety and Quality Control

8.1 Chemical contamination

Care is required to ensure that the final products do not contain unwanted chemical contaminants. Occasionally however, an applicator will use a dirty tank or a barrel, thus contaminating the *Bacillus* formulation *in situ*. There is no good method of preventing or detecting this.

8.2 Microbial contamination

Any breakdown in the fermentation process can lead to contamination. The formulation that is applied to a field contains, in addition to the *B.l.*

toxin, viable spores of the bacilli which, in theory, could spread and multiply in the environment. These bacilli do persist over a few months, but they have never been known to spread away from the treatment area or to have harmed anything in the environment. However, while research over twenty years has demonstrated that these spores of *B.t.* are harmless, there maybe other contaminating microbes. Microbial contaminants in a formulation are much harder to detect than are chemical contaminants. Therefore, since living cells are being applied in any *Bacillus* treated plots and fields, it is prudent to examine all commercial batches to be sure that they are free from harmful contaminants. To ensure this, four types of test are used.

8.2.1 In vitro tests

a) Microscopic examination

The fermentation beers, the development of the vegetative stages, maturing cells with spores, and the final product should be carefully examined and monitored using a microscope (1000 magnification). If a microscope with phase contrast is available, good differentiation of spores and crystals can be seen. Otherwise, several stains are available that will differentiate spores and crystals. (Spores of *B.t.* are oval; spores of *B.s.* are round or spherical).

2) Plate tests

Comparison tests should be run to confirm the absence of microorganisms that could be infective to man. For safety purposes, it is absolutely essential that local production methods and products be as free of contaminating microorganisms as normal food products. The main bacterial pathogens to be tested by standard plating techniques are the following:

- Staphylococcus* spp.
- Streptococcus* spp. (eg. *S. faecalis*)
- Eschericia coli*
- Pseudomonas* spp.
- Shigella* spp.
- Salmonella*
- Clostridium* spp.
- Bacillus* spp. (other than *B.t.* or *B.s.*)

8.2.2 In vivo tests

a) Mouse test

To be sure that a formulation does not contain bacteria other than *B.t.* or *B.s.*, six 20g mice are subjected to a single subcutaneous injection of 10^6 spores of the test bacilli, after which they are observed for 7 - 21 days. If a mouse dies, lesions develop, or any signs of sickness are seen, the batch should be rejected until further studies have been done to see if the death or disease reflected a bad formulation, or was due to an experimental error.

b) Bioassays

A basic and important tool in safety tests is a review of the potency as measured in bioassays. Procedures for the assay of *B.t.* H-14 are given in Sections 2.2 and 2.3. If the potency of a formulation deviates much from the expected (either higher or lower), it should be a warning that the abnormal potency is due to contamination.

It is extremely important that a high standard of quality be maintained in all these studies, and that no bad lots be released. This is particularly important in the development of a novel form of insect control such as the use of microbial insecticidal agents.

8.3 Safety and quality

To ensure the development of adequate quality control procedures for local *B.t.*, *B.t.* H-14, and *B.s.* production, technical cooperation with WHO Collaborating Centres should be encouraged. These centres could provide expertise, reference materials, and general guidance. In exchange, local industries could offer new concepts of production, develop new products, and explore new fields.

It should be emphasized that local scientists should initially be trained to ensure that they acquire adequate background in microbiology, entomology, and fermentation technology.

It is recommended that WHO play a pre-eminent role in promoting an efficient exchange of information and techniques among interested parties in developed and developing countries.

9

Packaging and Distribution

As a dry powder, *B.t.* is quite stable. If it is protected from water and not subject to extremes of heat, it can be stored indefinitely. Thus packaging water-dispersible powders, granular formulations and dusts, is not difficult; the only requirement is that they should be packed in water-proof drums or bags.

Flowable formulations of *B.t.* are more difficult to prepare and stabilize. They tend to be quite sensitive to heat and must be protected in storage. They have a tendency to settle and form a sludge at the bottom of the container. If the user does not adequately agitate the container, the active material may be left at the bottom of the drum. Shelf-life of the flowables is much shorter than that of dry materials. Thus, care must be taken both in the distribution and in storage of flowable materials. Flowables, being liquids, are packaged in plastic bottles or drums.

To Ferment or to Buy Microbial Insecticides

The whole tenor of the discussions in this document has been to recommend the use of submerged fermentations to produce *B.t.* or *B.s.* The initial outlay for equipment is high, and there is a strong need for well-trained supervisors. However, the procedures are straightforward and require no difficult or complicated methodology or equipment. If the government or the organization making the decision already operates a fermentation facility - and if this facility has vacant fermenters that could be used for the production of these larvicides - then it might be a wise decision to initiate production. It might also be observed that the fermentation is a safe procedure. It requires dedication and skill from those workers who will be carrying out the physical operation of the fermentation.

On the other hand, if a plant is to be constructed only for the production of larvicide, careful consideration must be given to the projected demand for the product. Because of high initial capital outlay for fermentors, steam generators, water chillers, etc., there must be a high utilization of the fermentation equipment to spread this overhead cost. Otherwise, the product will be too expensive.

The effectiveness and the use of *B.t.* H-14 has increased so much during the last several years that one may assume the potential usefulness of this organism has only just begun to be exploited. With the development of the agricultural strains, *B.t.* moved from being a laboratory curiosity to a commercial product - albeit one that was expensive and not too effective. The development of the HD-1 strain showed that at least on some crops, *B.t.* could compete in effectiveness and cost with chemicals. The high potencies achieved after such a short period in the development of fermentations of *B.t.* H-14, coupled with their unique activity against mosquitos and blackflies, adds another major facet to the *B.t.* picture. Recently, several new strains of *B.t.* have been found that appear, in the laboratory at least, to be much more active. The future of *B.t.* fermentation seems bright.

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