# 44. Mass Production of *Bacillus thuringiensis* and *Nomuraea rileyi*

With the ever-increasing awareness of the harmful effects of chemicals on man and his environment, the immediate need for sustainable, eco-friendly pest management has been felt very strongly for providing an impetus to research and development of microbial pesticides. The approach to pest management has seen a significant change over the years from chemical control to integrated pest management, with emphasis currently on bio-intensive integrated pest management. The shift in this paradigm is the outcome of the search for eco-friendly pest-management strategies driven by the impact of the ill-effects of injudicious use of chemical pesticides on human health and environment. Among the different microbial agents developed and tested, bacteria, viruses and fungi are considered promising for the management of insect-pests. Mass production of the promising entomopathogens holds the key for promoting their large-scale field use for insect-pest management. Mass production of the insecticidal bacterium *Bacillus thuringiensis* and the entomopathogenic fungus *Nomuraea rileyi* are discussed hereunder.

## *BACILLUS THURINGIENSIS*

*Bacillus thuringiensis* (*Bt*) is the most successful microbial pesticide ever registered. It is a gram-positive bacterium forming elliptical spores, contained in unswollen sporangia, and a parasporal body (crystal) which appears in varied shapes in different isolates, viz. bipyramidal, rhomboidal, rounded, etc. It is a complex species divisible into subspecies and H-serotypes by serological and biochemical tests. *Bt* isolates/strains produce several insecticidal toxins, two of which are used in agriculture. The relative activity of each isolate against different insect species "spectrum activity" arrives partly from the combined effects of the potencies of the varying concentrations of the different insecticidal toxins that it produces. The δ-endotoxin of different isolates of *Bt* can kill different insect species or differ in the degree of their activity towards them. The maximum toxin production can be achieved only by careful attention to the interaction of fermentation conditions, media and the isolates involved, for example, no one medium is best suited to all isolates.

### Culturing on media

*Bacillus thuringinsis* (*Bt*) is easily multiplied on Luria-Bertani broth (LB), nutrient agar and T3 agar media. Pure culture of *Bt* is maintained on the agar slants while it is multiplied in the broth (medium without agar) of these media in flasks for testing against insects. It is essential to store the pure culture of *Bt* in 10% glycerol at -80°C and revive the cultures on Luria-Bertani agar (LA) slants when required. It is desirable to restrict sub-culturing of *Bt* on media to two sub-cultures at 15-day intervals to avoid occurrence of mutations in the cultures. After inoculation, the flasks are plugged with sterile cotton and placed on a shaker at 30°C. Time taken for complete growth, sporulation and lysis is 48–72 h depending on the strain/isolate. The *Bt* spores and

crystals are then recovered from the broth through centrifugation, the resultant pellet is dried and milled to get a powder, which is used for bioassays.

### LB agar (Luria-Bertani Medium) (*p*H 7.0–7.2) composition

Tryptone 10.0 g, yeast extract 5.0 g, sodium chloride 5.0 g, agar 15.0 g, water 1,000 ml

**T3 agar (*p*H 6.9) composition**

Tryptone 3.0 g, tryptose 2.0 g, yeast extract 1.0 g, magnesium chloride 0.005 g, sodium dihydrogenphosphate 6.9 g, di-sodium phosphate 8.9 g, agar 20.0 g, water 1,000 ml

**Nutrient agar (*p*H 7.2) composition**

Peptone 10.0 g, sodium chloride 5.0 g, yeast extract 5.0 g, agar 15.0 g, water 1,000 ml

### Growth phases

After inoculation there is an initial lag phase followed by the exponential phase during which intensive growth of the culture takes place. This phase persists up to the 16th–18th hour. At the end of this exponential phase, sporulation is initiated and the cells enter into the stationary phase. Spores start appearing within the cells, together with parasporal inclusions of the crystalline toxin. Sporulation is complete by 48–50 hours. This is followed by the lytic/ death phase wherein the cells are subject to lysis, liberating spores and crystalline inclusions into the fermentation liquid. Nearly 90–98% of all spores and inclusions are liberated after 60–72 hours. The changes of *p*H during the fermentation depend on the composition of the medium used. After sterilization of the fermentation medium the *p*H should ideally be 6.8–7.2. After inoculation, during the exponential phase, acids being formed from the saccharides, *p*H will drop to 5.8–6.0 (after 10–12 hours), will rise again to about 7.5 (after 25 hours), further rise to 8.0 (after 30–35 hours) and ultimately to 8.8 (50–60 hours). The pH during the fermentation period is maintained around 7.5 by the addition of 1N sodium hydroxide/ 1N hydrochloric acid.

### Medium Development

Whichever type of fermentation is chosen, nutrients must be provided, so that the micro-organism can grow. Chosen nutrients markedly affect how fast the organism grows, how much is produced and often, how infective the final product is. Nutrients to be provided include a carbon source, e.g. glucose or molasses; nitrogen source, e.g. soybean (*Glycine max*) meal or yeast extract, and some trace elements. The selection of nutrients for cultivation depends on availability, price and suitability for organism. The media used, however, represent only a small share of the costs of equipment, servicing, and utilities required for operation.

**Source of carbon**

When formulating the nutrient medium, carbon is provided by mono, di, and polysaccharides such as glucose, starch, molasses, etc. If their concentration is too

high, the *p*H will drop below 5.6–5.8, and acidity may prevent growth. It depends on balancing the level of saccharides and the sources of nitrogen, in as much as *Bt* is producing alkaline components from the nitrogen-bearing material and these can neutralize the acidic products. As long as the medium is properly chosen, the initial *p*H will drop from neutral to 5.8–6.0 and then will rise slowly toward 8.0- 8.3.

**Sources of nitrogen**

In the fermentation of *Bt* there are variegated sources of nitrogen; these can include albuminous material containing vitamins and various factors such as yeast autolyzate, yeast extract, dried yeasts, peptone, soya meal, maize (*Zea mays*) meal, maize extract, residues from the production of alcoholic beverages, fish meal, etc.

## Trace elements

Some ions which supplement nutrition are indispensable for the growth and sporulation of *Bt*. Mostly, they are supplied in the form of $MgSO_4.7H_2O$, $MnSO_4.4H_2O$, $FeSO_4.7H_2O$, $ZnSO_4.7H_2O$, $CaCO_3$ (0.001–0.005%).

## Fermentation techniques for Mass Production

After the growth and sporulation processes have been thoroughly investigated and tested at laboratory level, mass production of *Bt* can be undertaken on an industrial scale with various raw materials. The standard method of production of microorganisms is the process of fermentation. There are many types of fermentation; the two most common are submerged and solid. For optimal growth of *Bt,* important factors are *p*H, temperature and oxygen. It is possible to control these parameters in fermenters. Hence *Bt* is traditionally produced in liquid media in large-capacity industrial fermenters, employing the method of submerged or deep-tank fermentation, which is as the name implies growth of microorganism in a fully liquid system.

Submerged or deep-tank fermentation is, as the name implies, a growth of micro-organisms in a fully liquid system. There are a number of advantages for using complete liquid systems which include the ability to hold temperature and *p*H constant, the ability to pump large quantities of air into the system and disperse it by means of stirring impellers, and the ability to generate reasonably homogeneous conditions to maximize the growth of micro-organism. In the beginning of small-scale processing, the fermenters used are the seed fermenters of 20–40 litres capacity which contain 10–25 litres of nutrient medium. Culture from the shaker or from laboratory fermenter (1–3 litres), to the amount of 1–3% of volume of the medium, serves for inoculation.

The seed tanks can be made of glass or stainless steel; the fermentation tanks are made of stainless steel. An aeration ring is used to aerate the culture, together with an agitator or an air outlet under a propeller. The air volume used for aeration should correspond to between 1/2 and full volume of the cultivation medium.

Unless foam-suppressing agents are used, the fermentation liquid will be subjected to intensive foaming at the beginning of fermentation and also at the sporulation time (after about 24 h). Silicone de-foaming agents can be used to mitigate foam formation. Solid-state fermentations (SSF) are relatively easy to develop on a small scale. Scaling them up to the sizes necessary for commercial production presents numerous problems, aeration becomes a major difficulty as the volume of the solid mass increases more

rapidly than the available surface area. Substrates like wheat (*Triticum* sp.) bran and cotton (*Gossypium* sp.) seed meal have been employed successfully for multiplication *Bt* through SSF.

Low-cost mass production of *Bacillus thuringiensis* var. *kurstaki* isolate on the principle of solid-state fermentation can be successfully undertaken on a solid medium containing wheat bran (150 g), molasses (3.6 g), yeast extract (0.72 g), potassium salts (0.36 g of di-potassium hydrogen orthophosphate and potassium di-hydrogen phosphate) and 250 ml distilled water. The medium (*p*H 7.2–7.5) is autoclaved in polythene covers and dispensed aseptically into sterile plastic tubs after cooling. Seed culture of the isolate (multiplied in nutrient broth) is added to the medium, mixed thoroughly and closed air tight with a polythene sheet. The tubs are incubated for a period of 72 h at 30°C with intermittent aerations in a laminar airflow. After complete lysis, sterile distilled water is added to the fermented medium and filtered through a muslin cloth. The filtrate is centrifuged to remove the water-soluble β-exotoxin, which remains in the supernatant that is discarded. The pellet is mixed with the filtration residue, shade-dried, powdered and passed through a sieve to get the formulation.

## NOMURAEA RILEYI

*Nomuraea rileyi* is an entomopathogenic fungus of cosmopolitan occurrence, primarily infecting Lepidoptera and particularly the economically important, polyphagous noctuid pests. Preliminary identification of *N. rileyi* is possible by looking for malachite-green colouration on the insect surface. When viewed under the microscope, conidiophores are seen bearing dense whorls of phialides, i.e. conidiogenous cells that are short-necked. Conidia of *N. rileyi* are broadly ellipsoidal to cylindrical with a size of 3.5–4.5 × 2.0–3.1 µm. *Nomuraca rileyi* is a fastidious fungus and can be best multiplied on Saboraud's dextrose agar or maltose agar fortified with yeast extract. It is recommended that cultures be stored under 10% glycerol in liquid nitrogen (–196°C) for long-term preservation without genetic change. Slants overlaid with sterile mineral oil can be held at 25°C for 6–12 months without loss of viability. However, regular passage through host insect is essential for maintenance of virulence.

Solid-state fermentation is very common method for mass production of fungal bio-agents under laboratory conditions. Various agricultural wastes and by-products could be used for mass production, e.g. grain bran, wheat straw, wheat bran, wheat bran-saw dust, wheat bran-peat, sorghum (*Sorghum* sp.) grain etc. The *N. rileyi* can be cultivated on different media – sorghum, barley (*Hordeum vulgare*), wheat bran, etc. Aerial conidia produced are indistinguishable in morphology and infectivity from those produced on the surface of insect cadavers.

### Production of *N. releyi* on Sorghum

Crush the sorghum grains in a mixer grinder to get broken pieces. Weigh 25 g broken sorghum in a 250 ml conical flask and add 22.5 ml of 1% yeast extract solution. Soak overnight at 25°C. Plug the flasks with non-absorbent cotton and autoclave. Immediately after cooling, break the clump of sorghum aseptically using a sterile blunt forceps. Add dry spore of *N. rileyi* to the sorghum using a sterile micro-spatula. Shake well to disperse the spore evenly in the medium and incubate the flasks at 25°C in the dark. Mycelial growth is initiated on days 4th or day 5th and continues for 3–4 days.

Sporulation is initiated at 7–8 days after inoculation and continues for 3–4 days. The flasks are then transferred to refrigerator and stored for 4–5 days (sporulation continues in the refrigerator). The substrate along with the fungus is the shade-dried and sieved through a muslin cloth to obtain pure conidia of *N. rileyi*. Scale-up of production can be undertaken in polypropylene bags plugged with non-absorbent cotton.

### Production of *N.releyi* on barley

Crushed barley (5 g) is taken in a 250 ml conical flask and 45 ml distilled water containing 0.125 g yeast extract. The flask is plugged with non-absorbent cotton and autoclaved. After cooling, the flasks are inoculated aseptically with dry spore of *N. rileyi*, mixed well using a sterile spatula. The flask is plugged and incubated at 25°C in the dark till mycelial growth and sporulation is complete. For scale-up production, the medium can be poured into glass trays, transferred to a polythene cover plugged with non-absorbent cotton at the open end and sterilized. After cooling the medium can be inoculated with *N. rileyi* conidia aseptically, mixed well with a sterile spatula. The open end is again plugged with the cotton and the bag is incubated at 25°C in the dark till mycelial growth and sporulation is complete.

### Production of *N. releyi* on polished rice grains

Put 300 g boiled rice (for 10 min) into a bag with a synthetic sponge in its opening or a mixture of raw rice and water in 1:2 ratio, sterilize and cool. Cut the other end, inoculate dry spore aseptically and seal. Incubate at 25°C. Shade dry the substrate and sieve to get the conidial powder.

### Production of *N. releyi* On insects

The *N. rileyi* can also be multiplied without much difficulty on 7–8-day-old *S. litura* larvae on castor (*Ricinus communis*) leaves treated with conidia of *N. rileyi*. Larval death results in 6–7 days after exposure. Cadavers should be placed in a moist chamber to further progress of the mycelial growth and sporulation. Mycelial growth on mummified cadavers occurs within 24 h followed by sporulation 1-3 days later. Each larva yields about 24 or 2–4 $\times$ $10^{10}$ conidia. Sporulating cadavers can be dispersed in the field to increase the inoculum before the peak incidence of the pest. The feasibility of mass multiplication on insects needs to be explored.