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Improved Bacillus thuringiensisbased biopesticide production using cheap carbon and nitrogen sources by solid state fermentation technique.

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Summary*: Bacillus thuringiensis is the most widely used biopesticide in the world. Bacillus thuringiensis serovar.israelensis (Bti) is a proven biopesticide to control mosquitoes. It is critical for the biopesticide industry to achieve a high yield in the fermentation process in order to reduce its cost and compete with chemical pesticide market.We have studied the performance of submerged fermentation and solid state fermentations for its potential for improved biomass production and its ability to induce early sporulation.Solid-state fermentation (SSF) was found to be superior in biomass and delta endotoxin production.There is considerable reduction in batch time, when bacteria is grown using solid state fermentation ,due to early sporulation. The data generated will help to scale-up the Bti production process using SSF technique.*

Keywords*: Bacillus thuringiensis serovar. israelensis, Batch–time, Bengal gram, Biomass productivity, Soybean meal, Solid-state fermentations.*

I. Introduction

Mosquito borne diseases form a major component of communicable diseases(Malaria, Filariasis, Dengue fever, Chikungunya and Japanese encephalitis) in most of the developing countries of the world. Although chemical insecticides has been effectively used for the past several decades, its use has been restricted recently due to various factors including resistant development in the vectors, environmental pollution and harmful effects on target species. Bacillus thuringiensis (Bt) is the most widely used biopesticide .It is very critical for the Bt pesticide industry to be able to achieve a high yield in the fermentation process in order to reduce its cost and compete with chemical pesticides in the market. Bacillus thuringiensis based pesticides have special success in managed pest control programmes to reduce the usage of chemical pesticides (Yang and Shaw 1995).It has been applied to a variety of crops, including vegetables,cotton,corn,maize,potato,soybean, etc and also against disease causing vectors like mosquito. Bt is a gram-positive bacterium characterized by its ability to produce crystalline inclusions called endotoxin proteins during sporulation. Bt produces a group of endotoxins ,crystal shaped proteins, that can target specific group of insects (Luthy et al 1982; Lereclus et al 1989; Dulmega and Aizawa 1982).

Bacillus thuringiensis serovar israelensis (Bti) is the most effective microbial control agent active against mosquitoes that is available to date (de Bajrac 1978; Tyrell et al.1979; de Bajrac & Thiery 1984; Federici et al.1990; Mahmood 1998; Su&mulla 1999). It synthesizes intracellular crystal inclusions containing multiple protein components with molecular weights of 134,125,67 and 27 kDa (Sekar 1986; Hofte & Whiteley 1989;Wirth et al.1998). These proteins have also been cloned individually and are shown to be toxic to mosquito larvae (Sekar & Carlton 1985; Delecluse et al.1991, 1993). However a combination of these proteins seems to exhibit much higher toxicity than any of the individual components. After the insect digests Bt toxins and spores, the proteins dissolve in the alkaline and reducing environment in the midgut of insect larvae. This causes extensive damage to the larvae. The spores may germinate and propagate in the midgut. The insects usually stop feeding right after toxin affects their midgut and eventually dies.

Solid-state fermentations are those in which microbial growth and product formation occur on the surface of solid substratum. Solid state fermentation include a number of well known microbial processes, such as soil growth, surface culture, composting, wood rotting, mushroom cultivation and the production of western foods like Bread, mold ripened cheese and sausages, commonly known in the west as solid state fermentations, and in the east as koji fermentations. Koji process was used in Japan for the manufacture of fungal enzymes. Koji is probably the largest enzyme product on a worldwide basis (Barbesgaard 1977). Advantages of solid-state methods include simplicity, yield and homogeneity of spore preparations (Vezina.C and K. singh 1975). Solid-state methods have been used for the production of cellulases, amylase and Pectinase (Toyama.N.1976), Protease (Knapp.J.S and J.D.Howell 1980) and Lipases (Yamada.K. 1977).

Solid-state fermentations are popular with fungi. There are only few SSF processes based on bacteria. There is hardly any report regarding solid-state fermentations using Bti. Solid-state fermentations of Bacillus thuringiensis tolworthi grown on moist rice have been reported (Deise Maria et al 2001).

II. Materials and methods

BacteriaCultures of Bti strains H14 were obtained from ETH, Zurich, Switzerland.Bacteria culture and maintenanceBacteria used in this study were Bti serotype H-14.The growth temperature and the shaker speed were 30°C and 180 rev/min respectively.

The parent strain was maintained as sporulated cultures on sterile modified glucose yeast extract salt (mGYS) agar slants containing 0.3% glucose, 0.2% ammonium sulphate, 0.5% dipotassium hydrogen phosphate, 0.2% yeast extract, 0.02% magnesium sulphate, 0.008% calcium chloride and 0.005% manganese sulphate (w/v), all dissolved in 100 ml distilled water and pH is adjusted to 7.0 before the addition of agar.
In all the cases the cultivation of bacteria began with a preculture stage. A loopful of the refrigerated preserved culture was transferred to 20 ml of mGYS broth in 100 ml Erlenmeyer's flask and incubated stagnant for 12-15 hours. For further cultivation 1.ml of the preculture was used as an inoculum for 100.ml of the medium.

Bacterial culture medium
Preparation of liquid medium: The liquid medium for submerged fermentation experimentsis prepared by dissolving 3 gm of Glucose, 0.5 gm of Peptone, 0.1 gm yeast extract and 0.1 gm calcium chloride (w/v) in 100 ml of distilled water. The pH was adjusted to 7.0 before autoclaving. After transferring to 250 ml Erlenmeyer's flask, it was autoclaved at 121°C for 15 minutes. The cooled medium was inoculated with 0.1 ml of Bti preculture and incubated for 48 hours by aerating the culture at 180 (rev/min) on a rotary shaker. The sporulation status of the culture was done by microscopy.

Preparation of solid media: Solid media for the growth of Bti (SSF) were prepared by dissolving 3.gm. of glucose, 0.5.gm.of peptone, 0.1.gm.of yeast extract and 0.1.gm. of calcium chloride (W/V) in 100.ml. of distilled water. The pH was adjusted to 7.0 before adding 2.0.gm (W/V) of agar to the medium. After transferring the media to 250 ml. Erlenmeyer flask, it was autoclaved at 121degree celsius for 15 minutes. Before solidification, 20 ml. of the molten agar medium is poured into sterile petriplates, placed inside laminar flow chamber. After solidification, 0.1ml of Bti preculture was added to the plate and it was spread uniformly over the solid medium using a sterile glass spreader. The plates were then incubated at 30 degree celsius for 48 hours for the growth of Bti. The sporulation status of the culture was done by microscopy.

Similarly different solid media compositions were prepared by varying the carbon and nitrogen sources. Instead of glucose and peptone, Wheat flour, Rice flour and Tapioca powder were used as alternate crude carbon sources and Bengal gram powder, Soybean meal and green gram powder were used as the alternative to peptone as the nitrogen source.

Biomass estimation: The total biomass produced in liquid culture under aerated conditions for48 hours was determined by centrifugation (8000Xg at 30 degree celsius for 5 minutes). The biomass production under Solid-state fermentation condition (SSF) was determined by harvesting the biomass from all the five petri plates, which contains 100.ml. of solid agar medium, using a sterile spatula. The biomass collected from both solid and liquid cultures is dried overnight at 80 degree celsius, in order to accurately determine the dry weight of the harvested biomass, using a digital weighing balance.

Determination of sporulation status: The sporulation status of the liquid and solid mediacultures were done by microscopy. Schaffer-Fulton method of staining was used to visualize the spores, and slides were observed with 100x oil-immersion objective. The liquid and solid media were incubated till the culture attains complete sporulation.

III. Results and discussion

Table.1 gives the comparative biomass production of liquid and solid media preparations of Bti, after 48 hours of growth. It is very much evident that the biomass production of Bti. in solid media is more , 0.61.gm.(dry weight.) compared to 0.50.gm in liquid culture. Although the total input of carbon (glucose) is 3%(W/V) in both cases, there is better biomass production in the case of solid-state fermentation. The reason for this phenomenon is not clear. It may be due to the lack of catabolite repression in solid media, due to the absence of high glucose concentration in proximity to the bacterial cell.

From the given data, it is conclusively proved that SSF method is superior in terms of biomass output for the economical production of Bti.

Table 1: Biomass production of Bacillus thuringeinsis serovar. isralensis in submerged and solid state fermentations.

Media Composition	Biomass production in 48 hours (dry. wt. in gms)
Liquid media Glucose – 3 gm Peptone – 0.5 gm Yeast extract – 0.1 gm Calcium Chloride – 0.1 gm Distilled water – 100 ml pH – 7.0	0.50 gm
Solid media Glucose – 3 gm Peptone – 0.5 gm Yeast extract – 0.1 gm Calcium Chloride – 0.1 gm Distilled water – 100 ml pH – 7.0 Agar – 2 gm	0.61 gm

In most of the industrial fermentations raw material costs account for 60-70% of the production cost, and the selling price of the product will largely be determined by the cost of the carbon source (Whitaker 1973;Moo young 1977). In order to reduce product cost, instead of glucose, SSF experiments were conducted with different crude carbon and nitrogen sources.

Table.2 gives the biomass production of Bti. grown in SSF with different crude carbon and nitrogen sources. Out of five different media combinations, wheat flour 3%(W/V) with soy bean meal gave maximum biomass (1.gm.Dry .wt), while wheat flour 3% (W/V) with 0.5% Bengal gram powder combination and Rice flour 3%(W/V) with 0.5%(W/V) soybean combination gave a total dry wt of 0.75 .gm.in 48 hours. Tapioca powder3% (W/V) with 0.5% (W/V) Bengal gram powder based combination-produced 0.70.gm., while wheat flour 3% (w/v) with 0.5% green gram combination yielded 0.66.gm.dry wt. of biomass. Soybeans contain approximately 50% of its dry wt. protein. The high concentration of protein in the medium may be beneficial for the luxurious growth of Bti, which is evident from the biomass production of Bti. using wheat flour and soybean media combinations.

Solid Media Composition	**Biomass production in 48 hours (dry. wt. in gms.)**
Wheat flour – 3 gm Soybean meal – 0.5 gm Yeast extract – 0.1 gm Calcium Chloride – 0.1 gm Distilled water – 100 ml pH – 7.0 Agar – 2 gm	1.00 gm

Table 2a: Biomass production of Bacillus thuringeinsis serovar. isralensis in Wheat flour andSoybean meal based solid media.

Table 2b: Biomass production of Bacillus thuringeinsis serovar. isralensis in wheat flour and Bengal gram based solid media.

Solid Media Composition	**Biomass production in 48 hours (dry. wt. in gms.)**
Wheat flour – 3 gm Bengal gram powder – 0.5 gm Yeast extract – 0.1 gm Calcium Chloride – 0.1 gm Distilled water – 100 ml pH – 7.0 Agar – 2 gm	0.75 gm

Table 2c: Biomass production of Bacillus thuringeinsis serovar. israelensis in Tapioca and Bengal gram powder based solid media.

Solid Media Composition	**Biomass production in 48 hours (dry. wt. in gms.)**
Tapioca powder – 3 gm Bengal gram powder – 0.5 gm Yeast extract – 0.1 gm Calcium Chloride – 0.1 gm Distilled water – 100 ml pH – 7.0 Agar – 2 gm	0.70 gm

Table 2d: Biomass production of Bacillus thuringeinsis serovar. israelensis in wheat flour and Green gram based solid media.

Solid Media Composition	**Biomass production in 48 hours (dry. wt. in gms.)**
Wheat flour – 3 gm Green gram powder – 0.5 gm Yeast extract – 0.1 gm Calcium Chloride – 0.1 gm Distilled water – 100 ml pH – 7.0 Agar – 2 gm	0.66 gm

Table 3: Sporulation status of Bacillus thuringeinsis serovar. israelensis in solid state and submerged fermentations.

Media Composition	Culture time (in hour)	Sporulation status of the culture
Liquid Media Glucose – 3 gm Peptone – 0.5 gm Yeast extract – 0.1 gm Calcium Chloride – 0.1 gm Distilled water – 100 ml pH – 7.0	56 hr	Completely sporulated
Solid Media Glucose – 3 gm Peptone – 0.5 gm Yeast extract – 0.1 gm Calcium Chloride – 0.1 gm Distilled water – 100 ml pH – 7.0 Agar – 2 gm	40 hr	Completely sporulated

Table.3 gives the comparative sporulation data of Bti grown in liquid and solid media. Bti being a spore former sporulates, when culture reaches stationary phase of growth. Delta endotoxins are synthesized only during the sporulation stage. The commercial value of Bti products are very much dependant on the quantity of delta endotoxins in the final preparations.

It is very much critical for the Bti fermentation industry tohave early sporulation of theculture, so that more batches can be run in a year, leading to increase in profits.

From Fig.3, it is evident that when Bti is grown in solid media, the sporulation time (the time taken for the culture to attain complete sporulation) is 40 hours compared to 56 hours ,when Bti is grown in liquid culture. This data is of very much importance, since the batch time can be considerably decreased in the industry, if Bti is grown using SSF method, without compromising in terms of biomass production.

Concluding remarks: In the present investigation, the superiority of solid-statefermentation (SSF) over submerged fermentation (SmF) has been established. Improved biomass production and early sporulation of the Bti culture in SSF has made this technique eligible for industrial scale-up of the process. Novel bioreactor design has to be thought of before venturing for large-scale production that will minimize contamination and need for labour.

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