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Reduction of sporulation time by solid state fermentation of Bacillus thuringiensis subspecies israelensis using tapioca peel as the carbon source.

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Abstract: *Mosquito-borne diseases not only cause a loss of lives but also impose heavy health and economic burdens. Extensive use of chemical insecticides for the control of malaria and other mosquito-borne diseases has led to the development of resistance in mosquitoes to these insecticides and hazards to the environment. Biolarvicides of the strain Bacillus thuringiensis israelensis, serotype H-14 is highly effective against mosquito larvae. But the large scale use of Bti is impeded by its high cost of production. Solid State Fermentation (SSF) is more viable than Submerged Fermentation for Bti production on a large scale. It brings early sporulation of the bacteria, which is desirable in biopesticide industry based on these bacteria. Tapioca peel is an abundant waste which can serve as an excellent substrate for any fermentation process after pretreatment. This freely available waste can lower the media cost of Bti production when scaled up.*

I. Introduction

Mosquito-bites not only cause nuisance and discomfort but also transmit some very dreadful diseases such as Malaria, Dengue fever, Japanese encephalitis, etc. The massive uses of insecticides in the past few decades for the control of mosquitoes and other pests have resulted in environmental problems and health hazards [1]

Bacillus thuringiensis israelensis , a rod-shaped, gram positive, spore-forming bacterium has already been proven as a potent control for mosquito larvae since its discovery by Goldberg and Margalit in 1977. Bti is highly toxic to dipterans, at the same time safe to non-target organisms and environment [2]. The larvicidal activity of Bti resides in at least four major crystal protoxins, of 134, 128, 72 and 27 kDa, encoded by cry4Aa, cry4Ba, cry11Aa and cyt1Aa respectively [3]. The risk of development of resistance in mosquitoes to Bti based products is very low, due to the synergistic interactions within its multi-toxin complex [4].

Bti products contain spores and parasporal crystals of Bti H-14 serotype which must be ingested by the larval stage of the mosquito to cause mortality. Following ingestion, the parasporal crystals are solubilised in the alkaline larval midgut, followed by a proteolytic activation of the protoxin into active toxin. The toxin binds to a receptor on the midgut epithelium resulting in pore formation in the cell, which leads to the death of the larva [2]. Even though Bti products are efficient controls for mosquito and black fly larvae, their use in developing countries is limited by their cost. Thus, there is a need to reduce the overall production cost of Bti in order to make it competitive in the market. It depends on many factors; however, the raw material cost is one of the most important criteria, which may comprise >70% of the overall production cost [5].

Conventionally Bti has been produced by Submerged fermentation. But Solid State Fermentation (SSF) can help reduce the production cost. SSF is distinguished from Submerged fermentation (SmF) by the fact that microbial growth occur at or near the surface of solid materials with low moisture content in contrast to that taking place in continuous aqueous phase in case of Submerged fermentation [6]. SSF offers numerous advantages over SmF. It is cost effective due to the use of simple growth and production media comprising agro-industrial residues, uses little amount of water, which consequently releases negligible or considerably less quantity of effluent, thus reducing pollution concerns. SSF processes are simple, use low volume equipment (lower cost), and are yet effective by providing high product titres (concentrated products). There is a very less chance of contamination in SSF due to low moisture content[7]. Extraction of products from SSF is easy and cheap compared to submerged fermentation which requires costly methods like ultracentrifugation, microfiltration, vaccum filtration, etc [8]. Hence, SSF comes out with a solution to the additional consumption of power in the maintenance of the culture and during the harvest and post- harvest processes.

Local agro-industrial wastes when used as raw materials not only help in cleaning the environment but also generate wealth. Tapioca is one such crop which has a huge industry in India. Cassava peels (contain large amounts of cyanogenic glucosides) and Pomace are the main byproducts of this industry. The peels constitute about 20-30% of the weight of the tuber. Consequently, a large amount of tapioca peel waste is generated, which causes environmental problems when left as such in the surroundings of processing plants, burnt or carelessly disposed of in surface waters [9,10,11]. Sun-drying is considered to be the most effective method for reducing the toxicity of tapioca peel so that it can be used as a Carbon source for Bti.

II. Materials And Methods

Materials used : Bti stock culture, Glucose, Tapioca starch, Freshly collected tapioca peels, Peptone, Yeast extract, $CaCl_2$, Water, Agar, Grinder, 250 mL Conical flasks, Petriplates, Micropipette and tips, Glass spreader, Autoclave, Weighing balance, Microscope, Slides, Butter paper, Spatula, pH paper.

Methods : 1) Comparative biomass production and sporulation time of Bti in Submerged and Solid State Fermentation using Glucose as the Carbon source.

A comparison was made between SSF and Submerged fermentation in order to assess which one of the two is more economical for the production of Bti based biopesticides. Hence, Bti was grown in the two media (solid and broth) with equivalent parameters. Both the media contained same composition (in grams): Glucose-1, Peptone- 0.1, Yeast extract- 0.1, $CaCl_2$- 0.1, Agar (only for SSF) - 1.5, Water- 100mL. The ingredients were dissolved in two separate 250 ml conical flasks. The pH was adjusted to 7.2 and agar was added in one of the conical flasks. The media were sterilized at 121^0 C and 15 psi pressure. After cooling at around 50^0 C, the media containing agar was poured in 5 pre-sterilized petriplates (approximately 20 ml in each) and allowed to solidify. The broth was inoculated with 0.1 ml preculture of Bti and aerated on a rotary shaker at 150 rpm for 48 hours at 30^0 C. The solid plates were inoculated with 0.02 ml each of Bti preculture and incubated at 30^0 C. Samples were taken at regular intervals for microscopic observation of spores. After all the cells were sporulated, the culture broth was centrifuged at 8000 rpm for 30 minutes. The supernatant was discarded and the pellet of bacterial cells was collected and weighed. The lawn of bacteria grown on the petriplates were collected by scraping out the biomass with the help of a presterilized spatula on a butter paper and the biomass was weighed.

2) Biomass production and sporulation time of Bti with Tapioca starch as the Carbon source in SSF.

SSF was chosen as the mode of fermentation in the subsequent experiments. In the media, Glucose was replaced with Tapioca starch as the carbon source. The compostion of the media (in grams) was as follows: Tapioca starch- 1, Peptone- 0.1, Yeast extract- 0.1, $CaCl_2$- 0.1, Agar-1.5. All the ingredients were suspended in a 250 ml conical flask and pH was adjusted to 7.2. after that agar was added and mixed. The media was sterilized at 121^0 C, 15 psi pressure. After cooling at around 50^0 C, the media was poured in 5 pre-sterilized petriplates (approximately 20 ml in each) and allowed to solidify. The solid plates were inoculated with 0.02 ml each of Bti preculture and incubated at 30^0 C. Samples were taken at regular intervals for microscopic observation of spores. After all the cells were sporulated, biomass was scraped out with a sterilized spatula on butter paper and weighed.

The solid media containing Glucose as the carbon source was taken as control.

3) Biomass production and sporulation time of Bti using Tapioca peel as the carbon source in SSF.

Next, the bacteria was grown using a similar media in which Tapioca starch was replaced with Tapioca peel. Fresh tapioca peel was collected from local bakeries and washed thoroughly to remove soil and debris. Excess water was drained off. The material was then shredded to small pieces using a grinder. The resulting material was then sun dried to remove all the cyanogenic glycosides as well as moisture. The dried tapioca peel was again ground to a fine powder, which was then used in the media as the carbon source. All other media components as well as growth parameters were kept constant. After all the cells were sporulated, biomass was weighed.

Solid media containing glucose as the carbon source served as the control.

III. Results And Discussion

1) Comparative biomass production and sporulation time of Bti in Submerged and Solid State Fermentation using Glucose as the Carbon source.

In SSF, Bti gives almost four times more biomass compared to submerged fermentation (biomass was taken at 15^{th} hour) due to more surface area. In terms of yield factor, SSF has given a value of 1.1 compared to 0.25 with submerged fermentation which will be highly profitable when scaled up.

In submerged fermentation, the bacteria takes more time to sporulate (48 hours) because the bacteria is free to move and has an easy access to nutrients. While in SSF mode, Bacillus thuringiensis israelensis sporulates early (15 hours) due to water stress and localized availability of nutrients. This property can be exploited for production of Bti based biopesticides because early sporulation reduces batch time. Thus more number of batches can be taken in a year with the same cumulative downtime and substrate input, which ultimately will tremendously improve the overall output and the profits of a biopesticide industry.

Table 1(a) and 1(b) show that SSF is more viable as it gives more biomass and early sporulation of the bacteria.

2) Biomass production and sporulation time of Bti with Tapioca starch as the Carbon source in SSF.

Table 2(a) shows the biomass production of Bti using tapioca starch as the carbon source in SSF mode. It shows that the biomass productivity is almost equal to glucose. Comparing the cost of pure glucose and abundantly available tapioca starch, there is a huge price variation with respect to the main carbon source which ultimately decides the profitability of any fermentation process.

Table 2(b) shows the sporulation time of Bti in tapioca starch based medium compared to glucose based medium. From the data, it is evident that with tapioca starch based medium, almost equal number of batches can be run in a year, although the inputs in terms of substrate cost is negligible compared to the cost of pure glucose.

3) Biomass production and sporulation time of Bti using Tapioca peel as the carbon source in SSF.

Table 3(a) and 3(b) show the biomass production of Bti using tapioca peel as the carbon source in SSF mode. Since tapioca starch has got various industrial applications and tapioca as such is used as a food item, its usage as carbon source for Bti production may not be cost effective. Diverting large amounts of tapioca or tapioca starch for the production of such microbial metabolites may initiate new issues and questions concernig food security, especially when millions are starving in this world due to lack of food availability. In this context, usage of non edible, at the same time industrial agro wastes like tapioca peel can be thought of as fermentable substrate for the large scale production of this highly sought after biopesticide.

Table 3(a) shows that tapioca peel based medium gives almost equal biomass output compared to glucose based medium, proving that tapioca peel can be used as an efficient fermentable substrate for the large scale production of Bti based biopesticides. Table 3(b) shows that Bti when grown in the tapioca peel based medium in SSF mode sporulates in just 13 hours compared to 16 hours with glucose based medium. This is because Tapioca peel is rich in lignocellulose. The bacteria finds it difficult to breakdown this polymeric structure and hence goes into sporulative phase once the metabolizable sugars are depleted in the vicinity.

IV. Conclusion

This study brings out the fact that Solid State Fermentation is highly economical for the production of Bti based biopesticides due to the many advantages associated with it. One of the most important being 'early sporulation' of the bacteria, which is desirable since Bti is a sporulating bacteria and the crystalline inclusions reside within the spore. SSF cuts down the expenses and power consumption associated with Submerged fermentation. It can be scaled up by solving the problems of heat and mass transfer and aeration extent. Instead of thick beds, thin layer or coating of media can be done and the bacteria grown on it.

Tapioca peel is a waste but rich in nutrients and hence if diverted to fermentation process, will lower the media cost which is often a big burden for any bioproduction. Prior to use, the peel should be properly sun-dried to remove the toxic cyanogenic glucosides.Tapioca peel with its polymeric structure induces early sporulation, so it is an appropriate carbon source for production of Bti based larvicides.

Comparative Biomass production and sporulation time of Bti in Submerged fermentation and SSF using glucose as the carbon source.

Table 1(a)

Mode of fermentation	**Biomass (in grams)**	**Yield factor [Y=X/S]**
Submerged	0.25	0.25
SSF	1.1	1.1

Table 1(b)

Mode of fermentation	**Sporulatation time (in hours)**
Submerged	48
SSF	16

Biomass production and sporulation time of Bti using Tapioca starch as the carbon source in SSF.

Table 2(a)

Carbon source	**Biomass (in grams)**	**Yield factor [Y=X/S]**
Control (Glucose)	1.1	1.1
Tapioca starch	1.0	1.0

Table 2(b)

Carbon source	**Sporulation time (in hours)**
Glucose (control)	16
Tapioca starch	15

B**iomass production and sporulation time of Bti using Tapioca peel as the carbon source in SSF.**

Table 3(a)

Carbon source	**Biomass (in grams)**	**Yield factor [Y=X/S]**
Control (Glucose)	1.1	1.1
Tapioca peel	1.2	1.2

Table 3(b)

Carbon source	**Sporulation time (in hours)**
Glucose (control)	16
Tapioca peel	13

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