ISSN: 0976-3031

Available Online at http://www.recentscientific.com

CODEN: IJRSFP (USA)

International Journal of Recent Scientific Research
Vol. 10, Issue, 02(E), pp. 30999-31005, February, 2019

International Journal of Recent Scientific Research

DOI: 10.24327/IJRSR

Research Article

COST EFFECTIVE FERMENTATION TECHNIQUES FOR THE ENHANCED PRODUCTION OF BACILLUS THURINGIENSIS SUBSP. ISRAELENSIS

Gopinathan, C* and Elyas, K.K.,

Department of Biotechnology, University of Calicut, Tenjhipalam-673635, Malappuram District, Kerala, India

DOI: http://dx.doi.org/10.24327/ijrsr.2019.1002.3170

ARTICLE INFO

Article History:

Received 13th November, 2018
Received in revised form 11th December, 2018
Accepted 8th January, 2019
Published online 28th February, 2019

Key Words:

Bacillus thuringiensis subsp. Israelensis (Bti), Fed-batch fermentation, , Starchy substrates, Calcium chloride, Sporulation.

ABSTRACT

Bacillus thuringiensis israelensis is a biopesticide high in demand due to its eco-friendly nature and high efficacy. The use of Bti in developing countries is still limited mainly due to the bioprocess complexicity involved in scale-up and related high cost of production. In this research work, we have shown the fed-batch fermentation method using inexpensive alternatives for carbon and nitrogen /protein sources can effectively reduce the cost in large-scale production of Bti. We have also shown that with the addition of more than 3% starchy substrates there is no repression in growth compared to glucose and sucrose. The role of calcium chloride in inducing early sporulation as well as increasing toxicity is also well demonstrated. The role of various buffers and additives in increasing biomass production and early sporulation was also part of the study.

Copyright © Gopinathan, C and Elyas, K.K., 2019, this is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution and reproduction in any medium, provided the original work is properly cited.

INTRODUCTION

As vectors of various diseases likemalaria, filariasis, Chikungunya and dengue, mosquitoes are major source of threat to public health (Suh Kathryn N *et al.*) [1]. 3.7 billion people are at the risk of getting affected from malaria. 217 million new cases and 5, 38, 00 deaths were reported in 2018 alone as per WHO. Most of the death reports (approx 80%) are from just 15 countries especially in Africa and Asia (WHO)[2]. Hence, it is essential to control vectors that have decreased mosquito-borne diseases.

For more than four decades chemical pesticides has considerably increased both agricultural and horticultural crops by effective control of pests. Health maintenance in the fast deteriorating environmental conditions has considerably increased the costof living for people. Most insect pests have developed resistance to the chemical insecticides, including the disease causing vectors,. Due to rampant usage of chemical insecticides there is loss of natural predators and presence of insecticide residues in our food chain. In rare cases, it has even led to highly disastrous bioamplification(Weill M *et al.*)[3], (Vinod Bihari *et al.*) [4].

Recently, the use of biopesticides for controlling mosquitoes has considerably increased (Poopathi S *et al.*)[5]. Safety of Bioinsecticides products makes them attractive for application in cities and in rural areas. Inspite of its advantages, these bioinsecticides have not still come up as key players in global market mainly due to its high cost of production (Copping LG *et al.*) [6]. For the past 40 years, numerable strains of Bacillus thuringiensis have been in use. However, Bacillus thuringiensis var israelensis (Bti) is the most widely accepted strain. It produces four different crystal inclusions formed by Cry4Aa, Cry4Ba, Cry11Aa and Cyt1Aa and is reported to be highly toxic against Aedes aegypti, Culex and anopheles. Species of mosquitoes. These toxins show synergistic effect to prevent the insect resistance (Margalith Y *et al.*)[7], (Bravo A, *et al.*)[8], (Bravo A *et al.*)[9]. The intake of these toxins by mosquito larva leads to dissolving the Bti parasporal inclusion body in the alkaline gut juices. The protoxin is cleaved by midgut proteases to form active δ-endotoxin proteins. These active endotoxins damage the midgut wall by interfering with the function of midgut epithelial cell membranes (Boisvert M *et al.*)[10]

Though the high efficacy and specificity of Bti are useful in controlling mosquitoes, the cost to grow and produce Bti, through highly refined laboratory bacterial culture medium, is

Corresponding author:* **Gopinathan, C
Department of Biotechnology, University of Calicut, Tenjhipalam-673635, Malappuram District, Kerala, India

high. The cost of Bti production 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 (Ejiofor, *et al*. 1991)[11]. Therefore, the selection of growth medium or raw material is critical for commercial production of these biopesticides. In order to encourage the commercial production of biopesticides, utilization of less expensive raw material is advisable (Mummigatti & Raghunathan, 1990)[12]. Several raw materials (industrial and agricultural by-products) have been tested successfully in mosquito-control programme, as an alternative culture media, for the production of Bacillus sphaericus and Bti (Saalma *et al*., 1983)[13] ; (Obeta & Okafor, 1984, *et al*.)[14] ; (Poopathi *et al*., 2002)[15].

The feasibility of producing a given microbial product depends, to a large extent, on the cost at which the product can be produced. This, in turn, depends on the efficiency with which the organism produces this product, the fermentation parameters (temperature, pH, aeration, etc.), the means of recovery and formulation and finally, the cost of the medium used in the fermentation (Ejiofor and Okafor,1988)[16]. Although the introduction of cheap agro- industrial by-products in fermentation media of B. thuringiensis H-14 has not elicited enough interest many workers have produced very potent formulations using various raw materials as media components (Dulmage, 1981)[17]; (Hertlein *et al*., 1981)[18], (Obeta and Okafor, *et al*., 1984)[19]; (Okafor, *et al*., 1987)[20].

Agro industrial waste sugar cane bagasse was used as a suitable substrate for the production of Bti in combination with soya bean powder. Here water extract of bagasse was used, without delignification (Poopathi.S, *et al*. 2013)[21].Similarly a barley based medium was used for the production of Bti (P.S.Vimaladevi, *et al*. 2015)[22]Prabakaran and Balaraman (2006)[23] attempted to develop a medium based on raw materials including soybean flour (Glycine max), groundnut cake powder (Arachis hypogea), and wheat bran extract (Triticum aestivum) in a 100-L fermentor.

A novel cost effective medium for the production of Bacillus thuringiensis subspecies, israelensis for mosquito control was developed by Subbiah poopathi.et.al, 2012.[24]

In this study, our attempt was to make use of the Fed-batch fermentation method in order to increase the bacterial biomass as well as the δ-endotoxin production. Also as part of this study, we have identified a number of carbon sources, primarily based on its cost, and examined its technical feasibility of utilising it as a substrate for large-scale industrial fermentation. Substrates like, sucrose, Jaggery, cane sugar juice powder, and taro, tapioca peels, sweet potato and purple yam were individually examined for its potential to increase δ-endotoxin production under batch fermentation mode.

Bacillus thuringiensis subsp. *israelensis* (Bti) is a sporulating bacterium, which produces endospores during the stationary phase. The spore is a tough structure highly heat resistant in nature,and contains Calcium Di-picolinic Acid (DPA) as one of its integral component. So it is very important that the conditions, which induce very good early sporulation, should be investigated in detail. Early sporulation leads to less batch time, which in turn leads to more production and more profits. In this context, the experiments regarding the effect of calcium on sporulation and insecticidal activity of Bti culture was performed. This experiment assumes lot of importance, since most of the usual medias do not give early sporulation. In typical fed-batch condition, the total input of carbon per batch can be increased considerably by incremental feeding. This leads to very high biomass productivity and product formation, compared to batch fermentations. There will be a growth limiting stage in fedbatch culture, where even if the substrate concentration is increased there will not be any proportional increase in product/biomass formation. Usually two reasons can be attributed for this phenomenon: (1) metabolic product /byproduct repression (2) Oxygen limitation. Out of these two factors, the major limiting factor will be the oxygen limitation, since oxygen is a sparingly soluble gas in the fermentation broth. As part of this study, we have also investigated the effects of different buffering agents in improving overall productivity.

MATERIALS AND METHODS

Bacterial Culture and Maintenance

Bacterium used in this study was of *Bacillus thuringiensis* subspecies *israelensis,* serotype H-14 . Bacteria was grown in modified Glucose Yeast extract salt (mGYS) broth, containing glucose (0.3%), Ammonium sulphate (0.2%), Yeast extract 0.2%), Di potassium hydrogen phosphate (0.5%),Magnesium sulphate (0.02), Calcium chloride (0.008) at pH of 7.2 and temperature of 30°C. To prevent clumping of cells, all cultures were passed through the pre-culture step.

Pre-Culture Stage

The cultivation of bacteria began with a pre-culture stage. A loop, full of the refrigerated preserved culture, was transferred to 20mL of mGYS broth in 100mL conical flask and incubated stagnant for 12-15 hours. For further cultivation, 1mL of the pre-culture was used as the inoculum for 100mL of the medium.

Repression Studies in batch-Fermentation mode

In order to assess the maximum level of each of the carbon source, which can be used in batch-fermentation without catabolite repression, each of the experiments were done in batch fermentation using a variety of carbon sources like sucrose, taro powder, purple yam powder, tapioca peel powder and sweet potatopowder, cane sugar and jaggery. The concentrations of each of the carbon source ranged from 1-8%. In each of these media, peptone (0.5%) and yeast extract (0.25%) were added as additives.

Process

In order to assess the maximum level of initial carbon concentration, which *Bacillus thuringiensis* subspecies *israelensis* can tolerate in submerged culture, a number of media containing increased concentrations of particular carbon sources were prepared. The concentration of each carbonsource, giving rise to the catabolite repression and repression due to oxygen limitation effect was thus determined.

Preparation of the Different media and Inoculation

The quantities of the media additives are expressed in percentage. The starting pH for each of the medium was set to 7.3. All the media were autoclaved in 500mL conical flasks of at 15lbs [sq. inch]-1 for 15 minutes. After cooling, each

medium was inoculated with 1mLof the Bti pre-culture. The flasks were aerated by a rotary shaker at 180 rpm. The cells were allowed to grow for 36 hours before being harvested and further experiments conducted on.

Fed-batch Mode of Experiments

Various media compositions were prepared with different carbon sources like glucoseand sucrose, each starting with initial sugar concentration of 4% and additives, peptone (0.5%), Yeast extract (0.25%) and rock phosphate 0.1% to each of these media. To get rid of the catabolite repression and to get rid of problems due to increased viscocity to achieve increased cell density, and in turn higher toxin production, fed batch mode of operation was designed. The final concentration was achieved by additional increments of the particular carbon source at the rate of 1% at each 12 hours interval. Thus operation was done using variable volume fed batch mode.

Media Preparation and Inoculation Strategies

The quantities of the media additives are expressed in percentages. The starting pH for each of the medium was set to 7.3.

Procedure

For all the batch experiments all the media 150ml were autoclaved in 500mL flasks of equal diametrical dimensions at 15 lbs [sq. inch]**-1** for 15 minutes. After cooling, each medium was inoculated with 1mL of the pre-culture. The flasks were aerated (180 rpm, in rotary shaker) and the cultures were allowed to grow for a period of 48 hours. light microscopy was done to check the sporulation status of the culture., If the culture sporulated by that time, the cells were harvested by centrifugation, and if the culture was still growing as is evident due to the presence of vegetative cells only, then the next microscopy was done again after a gap of 12 hours.

For fed batch mode of experiments **a**ll the media 150ml were autoclaved in 500mL conical flasks of equal diametrical dimensions at 15 lbs [sq. inch]**-1** for 15 minutes. After cooling, each medium was inoculated with 1mL of the pre-culture. The flasks were aerated (180 rpm, in rotary shaker) and the cultures were allowed to grow for a period of 48 hours. Then, autoclaved 1% solution of the respective carbon source (glucose, sucrose) dissolved in 20 ml of distilled water, was added aseptically by using the laminar air -flow chamber to the respective flasks, and again allowed to grow for twelve hours before the next addition. After the final addition, the culture was allowed to grow for 24 h. light microscopy was done to check the sporulation status of the culture, at regular intervals. If the culture sporulated by that time, the cells were harvested by centrifugation, and if the culture was still growing as is evident due to the presence of vegetative cells only, then the next microscopy was done again after a gap of 12 hours. Bti is a sporulating bacterium, which produces endospores during which endotoxin production occurs. So, in order to produce delta-endotoxin in minimum time, the culture was tested for induction of early sporulation. Two experiments were done using 3%jaggery as the carbon source and 0.5% soya bean powder and peptone as nitrogen sources and 0.25%yeast extract as additives with 0.1% CaCO3as supplement. The potency of each formulation was determined by performing mosquitoe larvidal asssay and noting the killing time in 1/100 dilution.

Effect of Buffered Media (on Sporulation) in batch Fermentation

Usually, in fermentation with glucose or sucrose as the carbon source as part of the, various acids will be secreted leading to a decrease in the pH, which will have a negative effect on the biomass yield and delta-endotoxin production. In order to counter this pH drift, buffered media was used to neutralize the pH. A medium with 3% glucose, 0.5% peptone, 0.25% yeast extract and 0.1% calcium chloride was prepared in 100ml of phosphate buffer of pH 7.2. The biomass production and killing time as a measure of potency was observed.

Estimation of cell Density

The Bti cell density measurements were done using spectrophotometer 600 nm for the readings. The absorbance were noted at λmax =600nm.The zero correction in each case was achieved using the initial fermentation broth without inoculum. 1:10 dilution of the fermented broth was taken for each of the cases to note the absorbance value. The absorbance thus observed was multiplied by the dilution factor to arrive at the appropriate cell density values.

Harvesting of the cells from the Fermented broths and Determination of the wet and dry Weights of Bti cells

A round-bottom centrifuge tube of 50mL capacity was taken in each case, washed with detergent solution, rinsed with distilled water and autoclaved. The weight of the empty tube was taken using a digital weighing balance. 40mL of the Bti fermented broth was then transferred to the tube aseptically in the laminar airflow chamber. A centrifuge used was used to remove cells. centrifugion, was carried at 5000rpm (1900g) at 15°C for 30 minutes. After centrifugation, the supernatant was dispensed and the weight of the centrifuge tube was again taken using the digital weighing balance. The difference between the final and the initial weights gave the wet weight for 150mL of the fermented broth. The same was used for calculating the wet weight corresponding to 100mL of the fermented broth. The dry weight for 100mL of the fermented broth was calculated by multiplying the weight for the same volume by a factor of 0.4.

Bioassay

A mixed breed of larvae was collected from wastewater. First and second stages of larvae of the tiger mosquito, *Aedes aegypti*, was manually isolated from the mixed breed of larval population. Bioassay was done according to the WHO guidelines [27]. A few hours before setting up the bioassay, 3-4mm long larvae were removed using pipettes with at least 2 mm diameter and blunted orifice to avoid injury to the larvae. They were collected in large numbers in tap water to facilitate the quick transfer time difference between the first and the last test beakers. The wet cell mass obtained after centrifugation was suspended in 150 mL of tap water and mixed well. Ten larvae were taken in a 100mL beaker containing 99mL of tap water.1mL of the culture was dispensed to the beaker and the time was noted as t=t0. In addition to the test beaker, a similar setup was prepared, but without any culture being added to it (ten larvae suspended in 100mL tap water). This was used as the control. The mortality of the larvae was observed and the

killing time noted separately for each of the test culture being used. All the glassware was washed properly with distilled water and autoclaved to inactivate any residual Bti toxin from previous experiments and hence avoid the occurrence of any false positive results.

RESULTS

Table 1 Batch fermentations of Bti using glucose as the carbon source for catabolite repression studies

Percentage of Carbon (Glucose) + 0.25% YE + 0.5% Peptone	Absorbance at 600 nm	Wet weight (in grams) per 100 ml of broth	Dry weight (in grams) per 100 ml of broth	Killing time (in minutes) by Bioassay with 1:100 dilution of the harvested endotoxin
1	5.59	1.12	0.418	267
2	6.37	1.97	0.778	165
3	9.6	3.65	1.456	114
4	8.76	2.73	1.188	119

Table 2 Batch fermentation of Bti using sucrose as the carbon source for catabolite repression studies

Percentage of Carbon (Sucrose) + 0.25% YE + 0.5% Peptone	Absorbance at 600 nm	Wet weight (in grams) per 100 ml of broth	Dry weight (in grams) per 100 ml of broth	Killing time (in minutes) by Bioassay with 1:100 dilution of the harvested endotoxin
1	3.8	0.79	0.232	320
2	4.20	0.87	0.367	278
3	5.000	1.0940	0.437	239
4	7.3	2.00	0.8	211
5	7.564	2.54	1.112	176
6	6.63	1.62	0.648	232

Table 3 Batch fermentation of Bti using jaggery as the carbon source for catabolite repression studies

Percentage of Carbon (Jaggery) + 0.25% YE + 0.5% Peptone	Absorbance at 600 nm	Wet weight (in grams) per 100 ml of broth	Dry weight (in grams) per 100 ml of broth	Killing time (in minutes) by Bioassay with 1:100 dilution of the harvested endotoxin
1	5.6	1.23	0.495	178
2	7.8	2.38	0.9282	137
3	10.600	4.895	2.3580	73
4	10.250	3.564	1.4156	109

Table 4 Batch fermentation of Bti using cane sugar juice as the carbon source for catabolite repression studies

Percentage of Carbon (Cane Sugar Juice) + 0.25% YE + 0.5% Peptone	Absorbance at 600 nm	Wet weight (in grams) per 100 ml of broth	Dry weight (in grams) per 100 ml of broth	Killing time (in minutes) by Bioassay with 1:100 dilution of the harvested endotoxin
3	8.30	4.25	1.700	43
4	4.19	2.44	0.976	119

Table 5 Batch fermentation of Bti using tapioca peel powder as the carbon source for catabolite repression studies

Percentage of Carbon (tapioca powder) + 0.25% YE + 0.5% Peptone	Absorbance at 600 nm	Wet weight (in grams) per 100 ml of broth	Dry weight (in grams) per 100 ml of broth	Killing time (in minutes) by Bioassay with 1:100 dilution of the harvested endotoxin
1	3.9	0.8	0.32	311
2	4.7	2.12	0.847	127
3	6.780	5.680	2.272	83
4	6.180	6.240	2.496	63
5	7.710	6.690	2.784	48
6	8.230	12.15	4.860	39

Table 6 Batch fermentation of Bti using Taro powder as the carbon source for catabolite repression studies

Percentage of Carbon (Taro powder) + 0.25% YE + 0.5% Peptone	Absorbance at 600 nm	Wet weight (in grams) per 100 ml of broth	Dry weight (in grams) per 100 ml of broth	Killing time (in minutes) by Bioassay with 1:100 dilution of the harvested endotoxin
1	4.0	0.9	0.36	289
2	4.9	2.21	0.884	116
3	7.4	6.35	2.59	89
4	7.8	6.68	2.662	81
5	8.8	13.1	5.29	28
6	8.3	12.4	4.89	25

Table 7 Batch fermentation of Bti using Sweet potato as the carbon source for catabolite repression studies

Percentage of Carbon (Sweet potato) + 0.25% YE + 0.5% Peptone	Absorbance at 600 nm	Wet weight (in grams) per 100 ml of broth	Dry weight (in grams) per 100 ml of broth	Killing time (in minutes) by Bioassay with 1:100 dilution of the harvested endotoxin
1	3.7	0.83	0.3237	311
2	4.6	2.04	0.807	131
3	6.9	5.89	2.356	96
4	7.3	6.32	2.59	93
5	7.9	6.75	2.767	79
6	8.5	12.7	4.9	25
7	7.9	6.69	2.609	83

Table 8 Batch fermentation of Bti using Purple yam as the carbon source for catabolite repression studies

Percentage of Carbon (Purple yam) + 0.25% YE + 0.5% Peptone	Absorbance at 600 nm	Wet weight (in grams) per 100 ml of broth	Dry weight (in grams) per 100 ml of broth	Killing time (in minutes) by Bioassay with 1:100 dilution of the harvested endotoxin
1	3.5	0.78	0.304	321
2	4.3	1.94	0.777	93
3	6.3	5.52	2.205	98
4	6.8	5.73	2.205	90
5	7.5	6.11	2.566	79
6	8.1	11.7	4.69	33
7	7.7	6.83	2.69	92

Table 9 Fed-batch fermentation of Bti using glucose as the carbon source

Percentage of Carbon (Glucose) + 0.25% YE + 0.5% Peptone+ 0.1% rock phosphate	Absorbance at 600 nm	Wet weight (in grams) per 100 ml of broth	Dry weight (in grams) per 100 ml of broth	Killing time (in minutes) by Bioassay with 1:100 dilution of the harvested endotoxin
4	5.010	1.4530	0.68120	125
5	8.913	3.8460	1.53840	125
6	11.29	8.0600	3.22400	99
7	9.050	1.2032	0.48128	146
8	6.900	1.5458	0.61832	67

Table 10 Fed-Batch fermentation of Bti using sucrose as the carbon source

Percentage of Carbon (Sucrose) + 0.25% YE + 0.5% Peptone+0.1% rock phosphate	Absorbance at 600 nm	Wet weight (in grams) per 100 ml of broth	Dry weight (in grams) per 100 ml of broth	Killing time (in minutes) by Bioassay with 1:100 dilution of the harvested endotoxin
4	2.500	0.533	0.2134	148
5	4.450	0.706	0.2824	147
6	7.540	0.806	0.3224	134
7	8.341	2.026	0.8104	101
8	2.341	0.453	0.1812	200

Table 11 Effect of Calcium Carbonate on sporulation in batch fermentations of Bti

(a) With peptone as the nitrogen source and jaggery as the carbon sources:

Percentage of Carbon (Jaggery) + 0.5% Peptone 0.25% *YE* 0.1% *Calcium Carbonate*	Absorbance at 600 nm	Wet weight (in grams) per 100 ml of broth	Dry weight (in grams) per 100 ml of broth	Killing time (in minutes) by Bioassay with 1:100 dilution of the harvested endotoxin
3	14.72	5.230	2.092	29
3	10.600*	4.895*	1.958*	73*

* (denotes the corresponding values without addition of calcium carbonate)

(b) With soya bean powder as the nitrogen sources and jaggery as the carbon sources

Percentage of Carbon (Jaggery) + 0.5% Soyabean powder + 0.25% *YE* 0.1% *Calcium Carbonate*	Absorbance at 600 nm	Wet weight (in grams) per 100 ml of broth	Dry weight (in grams) per 100 ml of broth	Killing time (in minutes) by Bioassay with 1:100 dilution of the harvested endotoxin
3	16.900	6.62	2.648	17

Table 12 Effect of buffer on batch fermentations of Bti using glucose as carbon source

Percentage of Carbon (Glucose) + 0.5% Peptone + 0.1% *YE* 0.1% *Calcium Chloride*	Absorbance at 600 nm	Wet weight (in grams) per 100 ml of broth	Dry weight (in grams) per 100 ml of broth	Killing time (in minutes) by Bioassay with 1:100 dilution of the harvested endotoxin
3	9.99	3.78	1.512	97
3	9.60*	3.64*	1.456*	104*

* (denotes the corresponding values without addition of buffer)

DISCUSSION

In the present research work, we have focused on multi-parametric media optimization for improving the productivity of Bacillus thuringiensis subsp. israelensis serotype H14 as well as madea strategic design for fed-batch fermentations of Bti. We have investigated in detail on the possibility of use of crude sources of carbon and nitrogen in Bti production. We have also investigated the catabolite repression limits of different carbon sources in submerged batch fermentations. The research work also included investigation of the probable factors affecting the sporulation status of Bti cultures. Bti grows profusely in a defined media containing 1% glucose and 0.5% peptone and 0.1% Yeast extract, hence delaying sporulation. To ensure early sporulation, scientists had designed a media, which contained only 0.3% glucose (mGYS media). Spore and crystal synthesis is primarily is primarily based on the use of the amino acids derived from the breakdown of proteins, both within the cell and those available in the media. Spore protein tends to incorporate more from the media (Vinod Bihari, *et al.*)[,25] .

In this study, we have tested the tolerance levels of Bti to various carbon sources in submerged batch experimental designs. As evident from Table 1, Bti responded differently to each of the carbon source used. Available scientific literature has already reported that Bti shows catabolite repression above 3% glucose. But, here Bti has shown the same effect at 5% with sucrose (Table2).

In case of tapioca peel powder, purple yam , and sweet potato repression was not seen upto 6% and in the case of taro repression was observed after 5%.We could not increase the substrate any further due to the inherent viscosity problems associated with soluble or insoluble starch additives in the media. The resulting decrease in oxygen solubility meant that the bacterial growth would be hampered. It was difficult to circumscribe the viscosity problem with the use of impellers because the experiment was being carried out in the laboratory scale. With jaggery and sugar cane juice the repression was at 4%. But the active biomass produced with 3% sugar cane juice or jaggery was distinctively greater than that produced with the same concentration of glucose under identical fermentation conditions.

This was further proved by the marked increase in larvicidal activity as evidenced by the decreased killing time. Sucrose is a di-saccharide and is slowly hydrolysed. This may provide an insight to the high tolerance value of Bti for sucrose, Upto 5%. The yield was highest with 5% taro powder. Biomass yield was much higher with other starchy substrates like sweet potato, purple yam, and tapioca peel powder.

But harvesting and downstream processing, when using starchy substrates , is a cumbersome procedure. The challenges associated with the large-scale Bti-production are three folds: (a) Achieving high biomass (b) Achieving reduced killing time, i.e. increased larvicidal activity, and (c) Reduced cost of production. But, not much progress seems to have been achieved on the optimization of nutrient parameters for mass multiplication of Bti. Considering the aforementioned challenges and limitations of batch fermentations, we decided to go for strategic fed-batch designs. Fed-batch fermentation was carried out with increasing concentrations of glucose, sucrose with additional supplementation of rock phosphate, since otherwise phosphate may be rate limiting under high cell density fermentations. (Table 9 and 10). Given its serious viscosity limitations, no fed-batch was performed using tapioca peel powder, sweet potato, purple yam and taro as carbon source. As is evident from Table 5 to 9, we were successful in nullifying the catabolite repression effect.

Fed-batch with glucose showed the maximum growth at 6%, gradually falling thereafter. Even though, we got a decreased growth at 8% glucose (as is evident from the absorbance values at 6 and 8%), the killing time was less with 8% glucose (Table9).

This was shift from convention, as killing time directly correlates to the biomass being produced. We repeated the experiment and the results only reconfirmed our earlier finding. Both 6% and 8% fed-batch cultures were optimally sporulated at the time of bioassay. The reason for such a decreased killing time, without a proportional increase in biomass production may be the fact that the crystal protein gene and sporulation-specific gene expressions may be happening at two different times. Some literature has made an indirect hypothesis that there is a possibility of delinking of the sporulation and toxin production process (Saalma *et al.*)[26]. So, this is the first time that it is being experimentally proved that spore formation and toxin production is not correlated.In fed-batch fermentation of Bti using increasing concentrations of sucrose (Table10), repression was above 7%. But the killing time was drastically

more than with glucose, even with the biomass produced with 7% sucrose. Slow release of the monosaccharide moieties in sucrose fed-batch fermentations is the reason for such decreased sporulation and toxin production. Sucrose, as a carbon source, is highly favorable due to wide availability and low cost.

The pH of the medium has a great influence on the overall productivity. The pH ranges between 5.5-8.5 during fermentation, the optimum pH being 7.3.A shift from the optimum pH may lead to decreased growth and productivity. The control of pH is thus extremely essential to ensure optimal productive efficacy. A compound may be added to the media to serve specifically as a buffer or may be used as a nutrient source (Stanbury and Whittaker, 1995)[27]. Batch fermentation was designed with calcium chloride additive as buffering agent. Table 12 shows that addition of a buffering agent at a lab scale does not have far reaching consequences as far as productivity or larvicidal activity is concerned. But it may have consequences in industrial scale.

The experimental design should be such that the production time is shorter and the harvested product (sporulated culture) should be available within 72-96 hours. Flores. et. al[28] had shown that the sporulation depended on a specific DO value. ADO value less than the minimum required will result in decrease in sporulation. This may explain the finding that sparged aeration stimulates sporulation in batch fermentations of glucose.

There is a lot of existing confusion regarding the roles of glucose, minerals, and the other supplements in initiating the sporulation process. It has been reported that depletion of phosphate, magnesium and potassium ion initiates sporulation.
Some reports suggest depletion of the protein source as the stimulant for sporulation. Some papers report contradictory, the requirement of a residual glucose level for sporulation to commence.

Here, we have set-up an experiment to investigate the role of calcium carbonate in sporulation. Table 11a and 11b clearly details, result of these studies.

When jaggery was used in batch fermentations with calcium carbonate and peptone Table (11a), there was both an increase in biomass production and decrease in sporulation time, as is evident by the highly reduced killing time. When jaggery was used with soyabean, we got even better results (Table 11b). The reason is that soyabean powder may contain some natural biochemical additives, which enhance toxin protein production along with high biomass. Table 11 a and 11 b exhibits clearly the role of calcium carbonate in early and complete sporulation, which almost no other media composition can ensure. Though calcium chloride was used as a buffering agent in one of the media, it did not show any increment because calcium in calcium chloride is non-utilizable. Our work has convincingly proved that calcium carbonate (utilizable form) is must as a media additive to ensure complete and very quick sporulation.
The present work has showed the critical importance of batch-to-batch uniformity in submerged fermentations of Bti. This work has led to the optimization of the fermentation design, capable to achieve high endotoxin productivity. Considering the total inputs of the raw materials in the different media optimized, indications are high potentials for possible scale up to the industrial scale, using similar process parameters that has been designed and optimized by us.

CONCLUSION

The fed-batch fermentations of Bti, conducted by us, conclusively prove the following facts: (a) opting for fed-batch fermentation designs may circumvent catabolite repression problems, associated with batch fermentation of Bti with glucose and fructose. (b) Crude carbon sources (like jaggery, sugar cane, and tapioca peel powder, purple yam powder, sweet potato powder, taro powder) and nitrogen source (soyabean powder) can be effectively used as media components for Bti production without catbolite repression upto 6%. This will ensure lowcost of production with high productivity and increased larvicidal activity. (c) Sucrose is not a viable alternative to glucose as the carbon source. (f) Calcium carbonate (0.1%) ensures early and complete sporulation. Hopefully, this in turn will increase social acceptability of Bti , and will thus alleviate the socio-economic problems inflicted by disease-carrying vectors.

Acknowledgments

The authors gratefully acknowledge the financial support by Department of Biotechnology, Government of India.

References

1. Suh Kathryn N, Kevin C Kain, Jay S. Keystone. Mapping malaria. Canadian Medical Association Journal. 2004; 171(9):1023-1024.
2. World Health Organization, Global Malaria Programme. World malaria report 2012. Available at: http://www.who.int/malaria/publications/world-malaria-report-2015/en/. Accessed 18 August 2016.
3. Weill M, Lutfalla G, Mogensen K, Chandre F, Berthomieu A, Berticat C *et al*. Insecticide resistance in mosquito vectors. Nature. 2003; 423:136-137.
4. Vinod Bihari. Biopesticides from spore former bacillus cultures. Proc.from Short time course on industrial biotechnology equipment and Facility design at Birla institute of scientific research, Jaipur, 1998, 67-86.
5. Poopathi S, Archana B. A novel cost-effective medium for the production of Bacillus thuringiensis subsp. israelensis for mosquito control. Tropical biomedicine. 2012; 29(1):81-91.
6. Copping LG, Menn JJ. Biopesticides: a review of their action, applications and efficacy. Pest Management 3.
7. Margalith Y, Ben-Dov E. Biological control by Bacillus thuringiensis subsp. israeliensis. In: Rechcigl, J.E., Rechcigl, N.A. (Eds.), Insect Pest Management: Techniques for Environmental Protection. CRC Press, 2000, 243.
8. Bravo A, Gill SS, Soberón M. Bacillus thuringiensis mechanisms and use. In: Gilbert, L.I., Iatrou, K., Gill, S.S. (Eds.), Comprehensive Molecular Insect Science. Elsevier BV, ISBN 0-44-451516-X, 2005, 175e206.
9. Bravo A, Likitvivatanavong S, Gill SS, Soberón M. Bacillus thuringiensis: A story of a successful bioinsecticide. Insect biochemistry and molecular biology. 2011; 41(7):423-431. doi:10.1016/j.ibmb.2011.02.006.

10. Boisvert M, Boisvert J. Effects of Bacillus thuringiensis var. israelensis on target and nontarget organisms: a review of laboratory and field experiments. Ann Bioeth 2000; 10:517-61.
11. Ejiofer,A.O and N. Okafor Formulation of a flowable liquid concentrate of *Bacillus thuringiensis* serotype H-14 spores and crystals as mosquito larvicide; Journal of applied microbiology,september 1991.
12. Mummigatti & Raghunathan, 1990; Influence of media composition on the production of delta-endotoxin by Bacillus thuringiensis var. thuringiensis; Journal of invertebrate pathology,1990,Mar, 5592)147-51.
13. Saalma *et al*., 1983;Utilization of fodder yeast and agro-industrial by-products in production of spores and biologically active endotoxins from Bacillus thuringiensis; Zentralblatt für Mikrobiologie 138(7):553-63.
14. Obeta & Okafor, 1984;Medium for production of primary powders of Bacillus thuringiensis subsp; Applied and Environmental Microbiology 47(4):863-7 · May 1984.
15. Poopathi *et al*., 2002;world journal of microbiology and biotechnology;,Development of low-cost media for the culture of mosquito larvicides, *Bacillus sphaericus* and *Bacillus thuringiensis* serovar. *israelensis* Volume 18, Issue 3, pp 209–216 .
16. Ejiofor and Okafor,1988;Production of mosquito larvicidal Bacillus thuringiensis serotype H-14 on raw material media from Nigeria; Journal of Applied Microbiology 67(1):5 - 9 , March 2008.
17. Dulmage, 1981; Insecticidal activity of isolated of Bacillus thuringiensis and their potential for pest control; Microbial control of pests and plant pests, 34 (5), 118-127
18. *Hertlein et al. 1981 ;Field evaluation of* Bacillus sphaericus *strain 1593 as a mosquito biocide; Journal of invertebrate pathology, vol48, issue(2), 1981, pp 133-138.*
19. Obeta and Okafor, 1984; Medium for production of primary powders of Bacillus thuringiensis subsp; Applied and Environmental Microbiology 47(4):863-7 · May 1984.
20. Okafor, 1987; Use of unmalted sorghum as a brewing adjunct:world journal of microbiology and biotechnology., Volume 6, Issue 3, pp 318–322 .
21. Poopathi.S,2013;Potential of sugarcane bagasse (agro-industrial waste) for the production of Bacillus thuringiensis israelensis;tropical biomedicine, 2013, sept (01), 30(3),504-515.
22. P. S.Vimaladevi,Duraimurugan, P., Lakshminarayana, M. ,;Comparative efficacy of microbial, botanical and chemical insecticides against lepidopteran pests in castor;2015,The Ecoscan; 9(1&2);7-10.
23. Prabakaran, G. and Balaraman, K. (2006) Development of a Cost-Effective Medium for the Large Scale Production of Bacillus thuringiensis var israelensis. Biological Control, 36, 288-292.
24. Subbiah poopaathi.et.al, 2012;Optimization of medium composition for the production of mosquitocidal toxins from *Bacillus thuringiensis* subsp. *Israelensis*; IJEB,vol 50, (01),January, 2012, ,65-71.
25. Vinod Bihari. Biopesticides from spore former bacillus cultures. Proc.from Short time course on industrial biotechnology equipment and Facility design at Birla institute of scientific research, Jaipur, 1998, 67-86.

2. 26 Saalma *et al*., 1983;Utilization of fodder yeast and agro-industrial by-products in production of spores and biologically active endotoxins from Bacillus thuringiensis; Zentralblatt für Mikrobiologie 138(7):553-63.
3. 27. P.F. Stanbury,Allan Whittaker and stephan .J. Hall; Principles of fermentation technolgy,second edition,1995.media for industrial fermentations, 105.
4. 28. Flores, Elias Razo, Fermin Perez, Mayra de la Torre. Scale-up of Bacillus thuringiensis fermentation based on oxygen transfer. J of Fermentation and Bioengineering. 1997; 83(6):561-564.

How to cite this article:

Gopinathan, C and Elyas, K.K,. 2019, Cost Effective Fermentation Techniques for the Enhanced Production of bacillus Thuringiensis subsp. Israelensis. *Int J Recent Sci Res.* 10(02), pp. 30999-31005.
DOI: http://dx.doi.org/10.24327/ijrsr.2019.1002.3170

****