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Novel techniques for cost-effective production of *Bacillus thuringiensis* Subsp. *israelensis*

C Gopinathan, Arindam Chaudhury and AT Vivek

Abstract
Bacillus thuringiensis is a desirable biopesticide because of its low cost and lack of toxicity. The use of Bt in developing countries is limited due to process complications and high cost of production. In the present study, we have shown the fed-batch fermentation method using inexpensive alternatives for carbon and protein sources can effectively reduce the cost in large-scale production of Bti. We have shown that with the alternative media supplements, neither the biomass production, nor larvicidal activity is hampered. In addition, we have also shown the positive effects of sparged aeration, and the indispensable role of yeast extract. We have provided the first experimental proof delineating the sporulation process and delta-endotoxin production. We have investigated the role of various buffering agents and additives in increasing biomass production and early sporulation.

Keywords: *Bacillus thuringiensis subsp. Israelensis* (Bti); biopesticide; larvicidal activity; biomass; alternative carbon and nitrogen sources; aeration; sporulation

1. Introduction
Mosquitoes are major source of threat to public health as vectors of various diseases like malaria, filariasis, Chikungunya and dengue [1]. According WHO, 3.2 billion people are at the risk of getting affected from malaria. 214 million new cases and 4, 38, 00 deaths were reported in 2015 alone. Most of the death reports (approx 80%) are from just 15 countries especially in Africa [2]. Hence, it is necessary to control vectors that have decreased impact on the reduction of mosquito-borne diseases.

Chemical pesticides are in abundant use, the world over, for more than three decades. This has considerably increased both agricultural and horticultural crops by effective control of pests. Maintaining good health in the fast deteriorating environmental conditions has considerably increased the per capita cost of living for human beings. Most insect pests, inclusive of the disease causing vectors, have developed resistance to the chemical insecticides. The chemical insecticides are also responsible for the loss of natural predators and presence of insecticide residues in our food chain. In some cases, it has even led to highly detrimental bio-amplification [3-4]. In recent years, the use of biological agents for controlling mosquitoes has rapidly increased [5]. Harmless nature of Bioinsecticides products makes them attractive for application in urban as well as rural areas. However, these bioinsecticides have not lived up the expectations of becoming key players in global market [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 adopted strain. It produces four different crystal inclusions formed by Cry4Aa, Cry4Ba, Cry11Aa and Cyt1Aa and is reported to be highly toxic against Aedes aegypti and Culex sp. species. These toxins show synergistic effect to overcome the insect resistance [7-9]. The ingestion by mosquito larva leads to dissolving the Bti parasporal 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 disrupting the function of midgut epithelial cell membranes [10].

In this study, we have attempted to make use of the fed-batch fermentation technique in order to increase the biomass as well as the δ-endotoxin production. As part of this study, we have selected a number of carbon sources, primarily based on its cost, and examined its feasibility of using it as a substrate for large-scale industrial fermentation. Substrates like glucose, sucrose, jaggery, cane sugar juice and starch were individually examined for its potential to increase δ-endotoxin production under batch and fed-batch conditions. Since in most of the industrial fermentations, there is an optimum ratio of carbon and nitrogen (C: N) to get

maximum output, we have also studied the effect of different ratios of carbon and nitrogen under fed-batch conditions in order to increase the overall productivity. *Bacillus thuringiensis* subsp. *israelensis* (Bti) is a sporulating bacterium, which produces spores during the stationary phase. The spore is a tough structure with heat resistance in nature, and contains Calcium Di-Picolini Acid (DPA) as one of its integral component. It is inevitable that the conditions, which induce very good early sporulation, should be investigated in detail. In this context, the analysis of the effect of calcium on sporulation and insecticidal activity of Bti culture was performed. This was done 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 substantially 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 fed-batch 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) Catabolite 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. In an experimental set-up, we have analyzed the effect on boosting the oxygen biomass productivity in an already growth-limiting fed-batch mode. As part of this study, we have also investigated the effects of different buffering agents in improving overall productivity.

2. Materials and methods

2.1 Bacterial culture and maintenance

Bacterium used in this study was serotype H-14 of *Bacillus thuringiensis* subspecies *israelensis*. The 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 ℃. To avoid clumping of cells, all cultures were passed through the pre-culture step.

2.2 Preculture stage

In all cases, 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 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.

2.3 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 glucose (1-4%), sucrose (3-6%), tapioca powder (3-8%), cane sugar (3-4%) and jaggery (3-4%). The concentrations of each of the carbon source ranged from 1-8%. In each of these media, peptone (0.5%) and yeast extract (0.1%) were added as additive.

2.3.1 Process

In order to assess the maximum level of initial glucose 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 carbon source, giving rise to the crab-tree effect was thus determined.

2.3.2 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 equal diametrical dimensions at 15lbs [sq. inch]$^{-1}$ for 15 minutes. After cooling, each medium was inoculated with 1mL of the pre-culture. The flasks were aerated through Blue Sky BS-310 Super Aerator GB 4706.1-92.The cells were allowed to grow for 36 hours before being harvested and further experimented on.

2.4 Fed-batch mode of experiments

Various media compositions were prepared with different carbon sources like sucrose, glucose, cane sugar and jaggery, each starting with initial sugar concentration of 3% and additives, peptone (0.5%), Yeast extract (0.1%) to each of these media. To get rid of the crab-tree effect and to get increased cell density, and in turn higher toxin production, fed-batch mode of operation was designed. In order to address the problem of catabolite repression, the operations were achieved started with an initial carbon concentration of 3%. The final concentration was achieved by additional increments of the carbon source at the rate of 1% at each 12 hours interval. Thus, the fed-batch mode was carried in the variable volume mode.

2.4.1 Media preparation and inoculation strategies

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

2.4.2 Procedure

All the media 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 and the cultures were allowed to grow for a period of twelve hours. Then, autoclaved 1% solution of the respective carbon source, dissolved in minimum volume of distilled water, was added aseptically in the laminar airflow chamber to the respective flasks, and gain allowed to grow for twelve hours before the next addition. After the final addition, the culture was allowed to grow for 24 h with intermittent light microscopy being done to check the sporulation status of the culture. If the culture sporulated by that time, the cells were harvested, and if the culture was still growing then the next microscopy was done again after a gap of 12 h.

2.5 Media alternatives for the parametric optimization of the fermentation media

In order to reduce the process cost, peptone was replaced by soyabean powder and glucose as carbon source and yeast extract as additive in fed-batch fermentation. Also, two different media were tested in batch fermentation with peptone (0.5%) as nitrogen source and for carbon source, cane sugar (3%) or jaggery (3%) was added without other additives.

2.6 Impact of Aeration

To assess the influence of oxygen, both batch and fed-batch fermentations were performed in media containing Glucose (3%), Peptone (0.5%) and Yeast extract (0.1%) with increased

oxygen supply. In fed-batch fermentation, initial concentration of 3% glucose was used.

2.7 Effect of buffered media and Calcium carbonate (on sporulation) in batch fermentation

Usually, in fermentation with glucose as the carbon source as part of the metabolism, various acids will be secreted leading to dip 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. 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 jaggery as the carbon source and soya bean powder and yeast extract as additives with 1% $CaCO_3$ as supplement.

2.8 Estimation of cell density

The cell density measurements were done using Spectronic $20D^+$ Milton Roy spectrophotometer using the 600-900 nm filter for the readings. The absorbance were noted at λ_{max} =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.

2.9 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 J8005907 top loading balance. 40mL of the fermented broth was then transferred to the tube aseptically in the laminar airflow chamber. The centrifuge used was Rota 4R-V/F_m Plasto Crafts. The rotor used was No.9 centrifuge, was carried at 5000rpm (1900g) at 15 ℃ for 30 minutes. After centrifugation, the supernatant was dispensed and the weight of the centrifuge tube was again taken using the J8005907 top loading 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.

2.10 Bioassay

A mixed breed of larvae was collected from wastewater. First and second stage 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 [11]. 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 100mL of tap water.1mL of the culture was dispensed to the beaker and the time was noted as $t=t_0$. 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 and autoclaved to inactivate any residual toxin from previous experiments and hence avoid the occurrence of any false positive results.

3. Results & Discussion

Table 1: Batch fermentations with different carbon sources of Bti for catabolite repression studies

Percentage of carbon source	Absorbance at 600nm	Wet weight per 100mL of broth (in grams)	Dry weight per 100mL of broth (in grams)	Yield factor (Y=X/S)
Glucose				
1	5.56	1.02	0.408	1.02
2	6.32	1.92	0.768	0.96
3	9.6	3.64	1.456	1.2133
4	8.71	2.72	1.088	0.9067
Sucrose				
3	5	1.094	0.4376	0.3646
4	7	1.7214	0.68856	0.4304
5	7.564	2.03	0.812	0.406
6	6.63	1.62	0.648	0.27
Tapioca powder				
3	6.78	5.68	2.272	1.8933
4	6.18	6.24	2.496	1.56
5	7.71	6.96	2.784	1.392
6	8.23	12.15	4.86	2.025
Jaggery				
3	10.6	4.895	2.358	1.6316
4	10.25	3.564	1.4156	0.891
Cane sugar juice				
3	8.3	4.25	1.7	1.4167
4	4.19	2.44	0.976	0.61

Table 2: Fed-batch fermentations with different carbon sources of Bti for catabolite repression studies

Percentage of carbon source	Absorbance at 600nm	Wet weight per 100mL of broth (in grams)	Dry weight per 100mL of broth (in grams)	Yield factor (Y=X/S)
Glucose				
4	5.01	1.453	0.6812	0.3632
5	8.913	3.846	1.5384	0.7692
6	11.29	8.06	3.224	1.3433
7	9.05	1.2032	0.48128	0.1719
8	6.9	1.5458	0.61832	0.1932
Sucrose				
4	2.5	0.533	0.2134	0.1332
5	4.45	0.706	0.2824	0.1412
6	7.54	0.806	0.3224	0.1343
7	8.341	2.026	0.8104	0.2894
8	2.341	0.453	0.1812	0.0566
Jaggery				
4	8.5	2.05	0.82	0.5125
5	9.03	2.52	1.008	0.504
6	9.35	3.38	1.352	0.5633
7	9.7	3.64	1.456	0.52
8	10.7	4.51	1.804	0.5638
9	15	4.61	1.844	0.5122
10	15.323	4.92	1.968	0.492
11	16	5.3	2.12	0.4818
12	9.2	2.91	1.164	0.2425
Cane sugar juice				
4	8.25	2.61	1.044	0.6525
5	9.025	2.83	1.132	0.566
6	10.9	3.35	1.34	0.5583
7	11.97	3.65	1.46	0.5214
8	14.02	5.27	2.108	0.6588
9	15.19	5.6	2.24	0.6222
10	15.98	6.31	2.524	0.631
11	16.67	6.99	2.796	0.6355
12	9.36	3.16	1.264	0.2633

Table 3: Protein substitution studies using soyabean (nitrogen source) and glucose (carbon source) in fed-batch fermentation

Percentage of Glucose	Absorbance at 600nm	Wet weight per 100mL of broth (in grams)	Dry weight per 100mL of broth (in grams)	Yield factor (Y=X/S)	Killing time (in mins)
7	14.672	8.63	3.452	1.2329	63
8	16	9.67	3.868	1.2087	56
9	18.968	11.24	4.496	1.2488	38
10	19.867	12.46	4.984	1.246	35
11	20.023	13.01	5.204	1.1827	31
12	17	10.23	4.092	0.8525	49

Table 4: Comparison of Batch fermentations of Bti using cane sugar juice and jaggery without yeast extract

Percentage of carbon source	Absorbance at 600nm	Wet weight per 100mL of broth (in grams)	Dry weight per 100mL of broth (in grams)	Yield factor (Y=X/S)	Killing time (in mins)
Cane sugar juice					
3	1.75	2.08	0.832	0.6933	137
Jaggery					
3	1.63	0.86	0.344	0.2867	249

Table 5: Aeration studies in batch and fed-batch fermentation using glucose (carbon source) with high aeration

Percentage of Glucose	Absorbance at 600nm	Wet weight per 100mL of broth (in grams)	Dry weight per 100mL of broth ((in grams)	Yield factor (Y=X/S)	Killing time (in mins)
Batch-fermentation					
3	12.46	5.73	2.292	1.91	41
Fed-Batch fermentation					
6	11.34	8.1	3.24	1.35	95

Table 6: Effect of buffer on batch fermentations of Bti using 0.1% Calcium chloride

Percentage of Glucose	Absorbance at 600nm	Wet weight per 100mL of broth (in grams)	Dry weight per 100mL of broth (in grams)	Yield factor (Y=X/S)	Killing time (in mins)
3	9.99	3.78	1.512	1.26	97
3	9.60*	3.64*	1.456*	1.2133*	104*

*Without addition of buffer

Table 7: Effect of Calcium carbonate on sporulation in batch fermentations of Bti using Jaggery as carbon source and peptone or soyabean as nitrogen source

Percentage of Jaggery	Absorbance at 600nm	Wet weight per 100mL of broth (in grams)	Dry weight per 100mL of broth (in grams)	Yield factor (Y=X/S)	Killing time (in mins)
When Peptone as nitrogen source					
3	14.72	5.23	2.092	1.7433	29
3	10.600*	4.895*	1.958*	1.6316*	73*
When Soyabean as nitrogen source					
3	16.9	6.62	2.648	2.2067	17

*Without addition of Calcium carbonate

Table 8: Comparative sporulation timings for different media used for submerged batch fermentations

Media composition and fermentation mode per batch	Time taken for sporulation to commence (hours)	Time taken for cell lysis to commence (Hours)
3% Glucose+0.5% Peptone+0.1% Yeast extract	37	53
3% Sucrose+0.5% Peptone+0.1% Yeast extract	60	Not Observed
3% Tapioca powder+0.5% Peptone+0.1% Yeast extract	49	61
3% Jaggery+0.5% Peptone+0.1% Yeast extract	31	49
3% Cane Sugar juice +0.5% Peptone+0.1% Yeast extract	27	35
3% Glucose+0.5% Peptone+0.1% Yeast extract + 0.1% Calcium chloride	23	29
3%Jaggery+1% Soyabean powder+0.1% Yeast extract + 0.1% Calcium carbonate	17	26

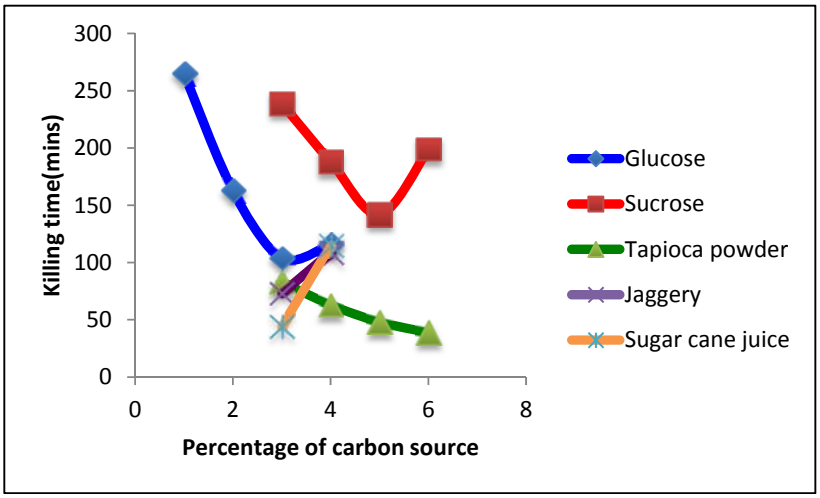
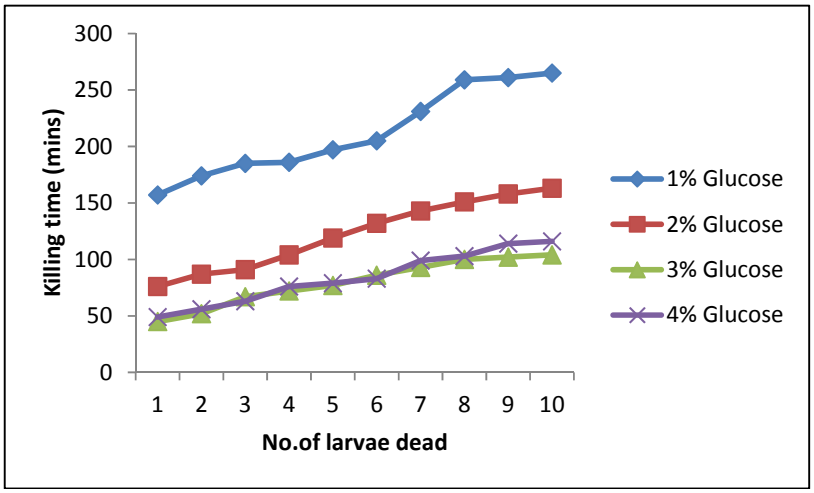
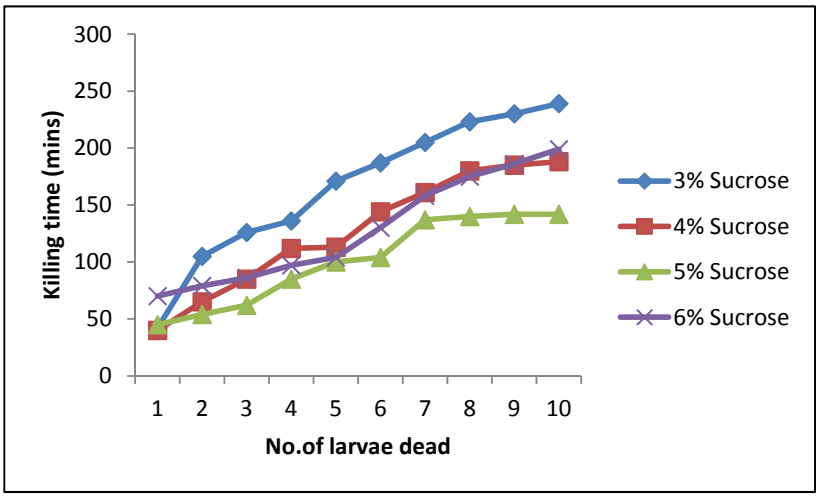


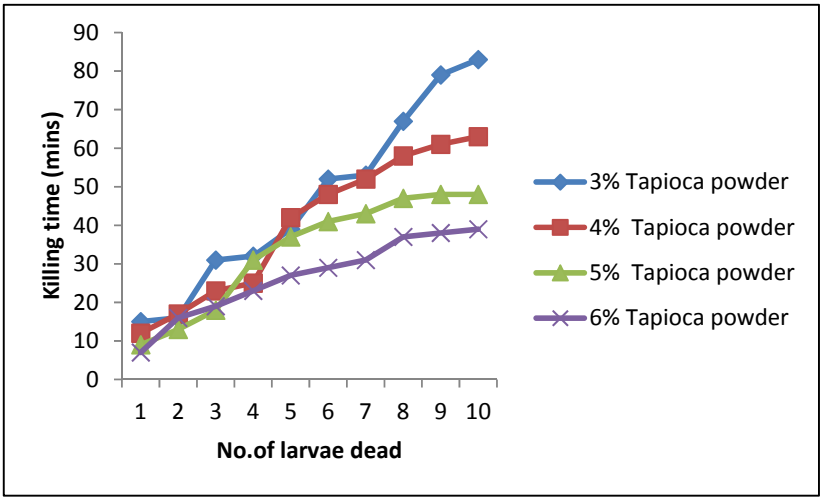
Fig 1: Larvicidal activity of *Bacillus thuringiensis* subsp. *israelensis* for different carbon sources in batch fermentations. The figure also shows the different concentrations for each carbon source, at which the 'crabtree effect' is observed. Larvicidal effect is maximum at different concentrations for each of the sources used.



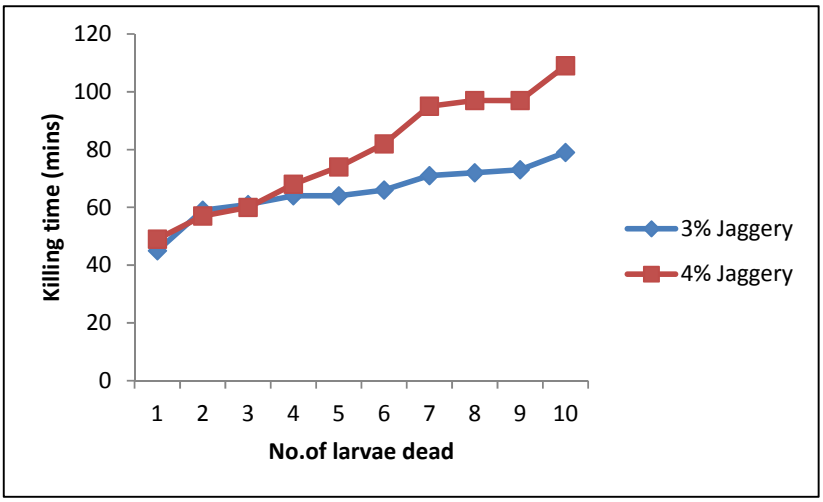
(a)


300
250
200
150
100
50
0
Killing time (mins)
1 2 3 4 5 6 7 8 9 10
No.of larvae dead
3% Sucrose
4% Sucrose
5% Sucrose
6% Sucrose

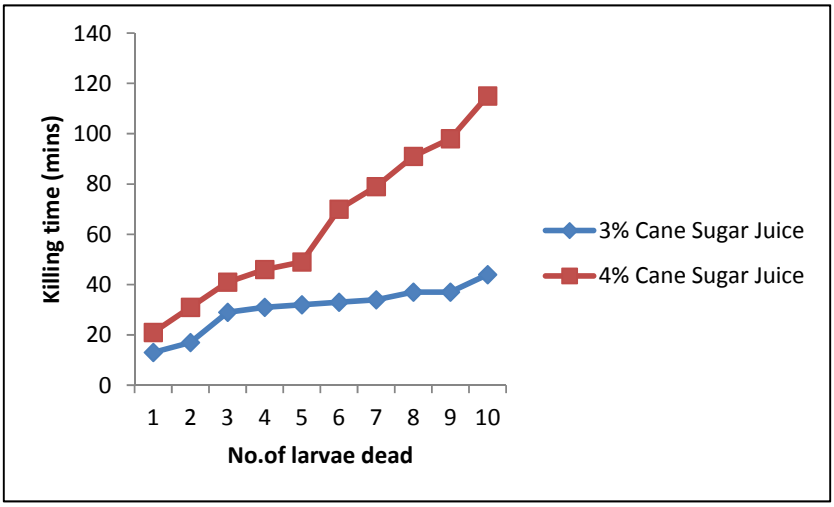
(b)


90
80
70
60
50
40
30
20
10
0
Killing time (mins)
1 2 3 4 5 6 7 8 9 10
No.of larvae dead
3% Tapioca powder
4% Tapioca powder
5% Tapioca powder
6% Tapioca powder

(c)


120
100
80
60
40
20
0
Killing time (mins)
1 2 3 4 5 6 7 8 9 10
No.of larvae dead
3% Jaggery
4% Jaggery

(d)



(e)

Fig 2: Bioassay results of batch fermentation studies using different carbon sources (a) Glucose (b) Sucrose (c) tapioca powder (d) Jaggery (e) Cane sugar juice

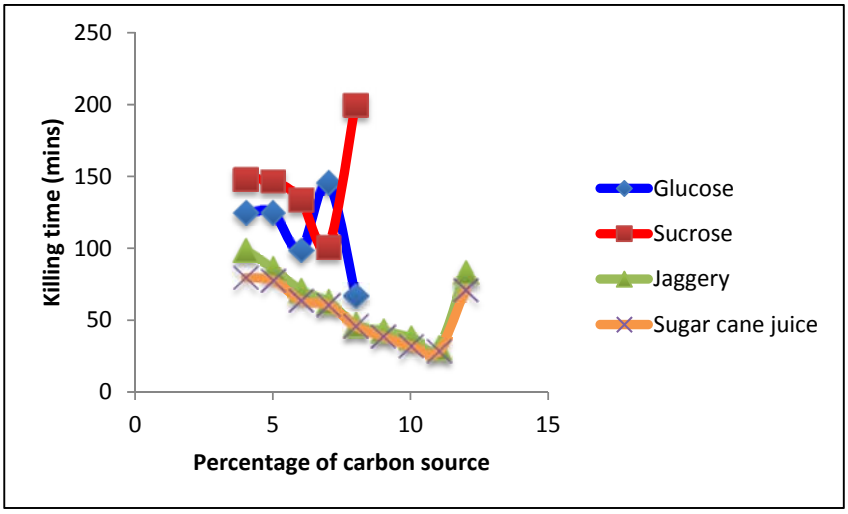
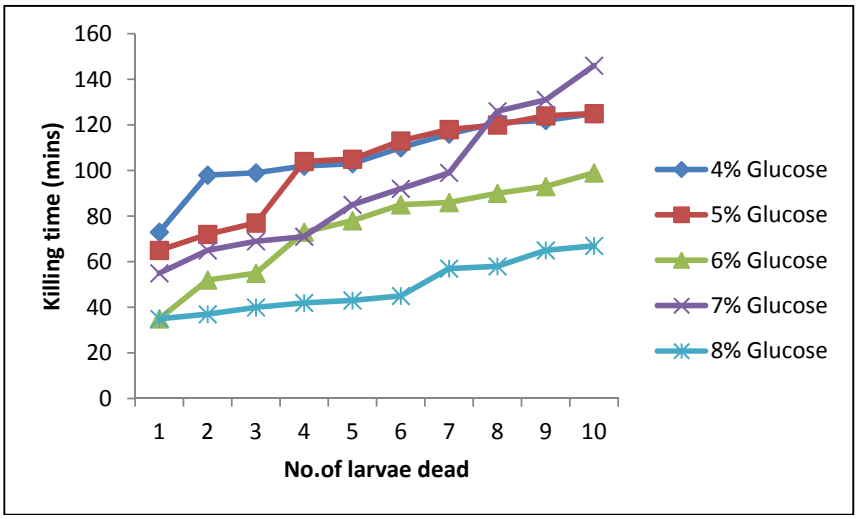
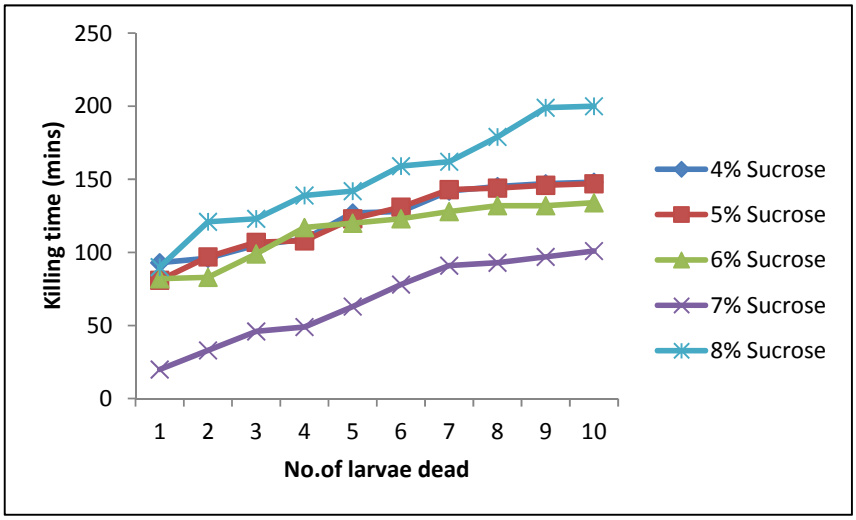


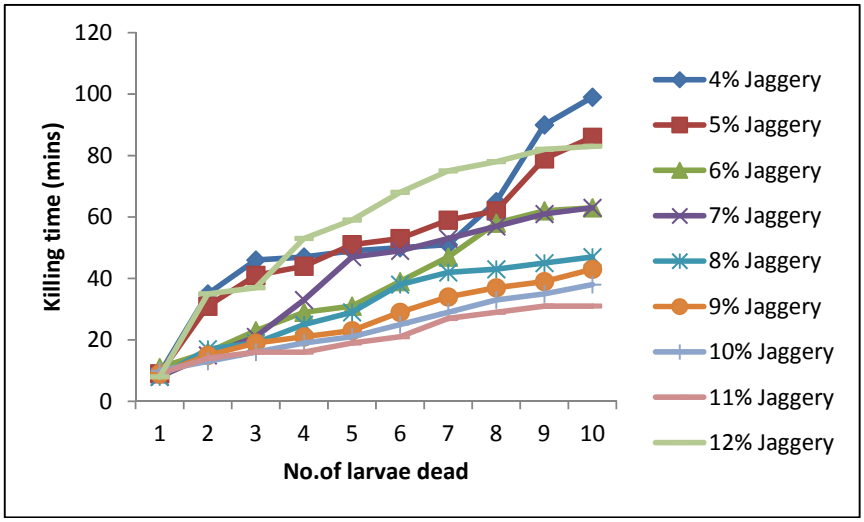
Fig 3: Larvicidal activity of *Bacillus thuringiensis* subsp. *Israelensis* for different carbon sources in fed-batch fermentation.



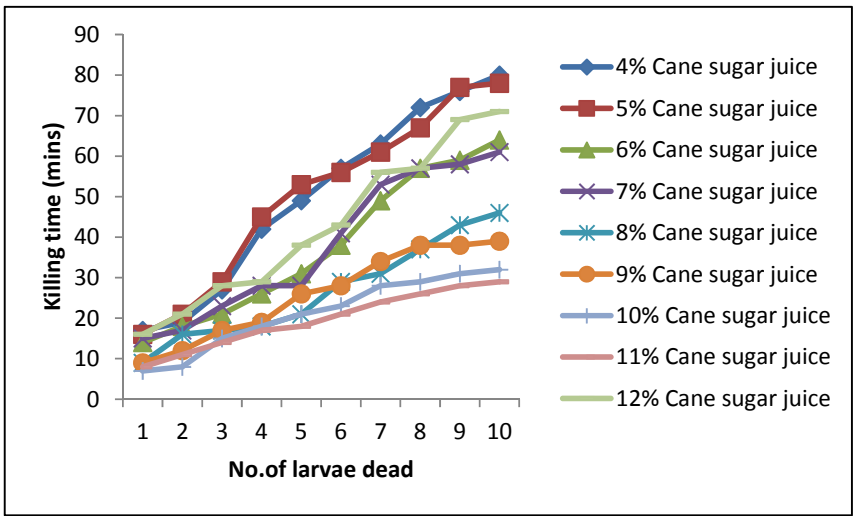
(a)



(b)



(c)



(d)

Fig 4: Bioassay results of fed-batch fermentation studies (a) Glucose (b) Sucrose (c) Jaggery (d) Cane sugar juice

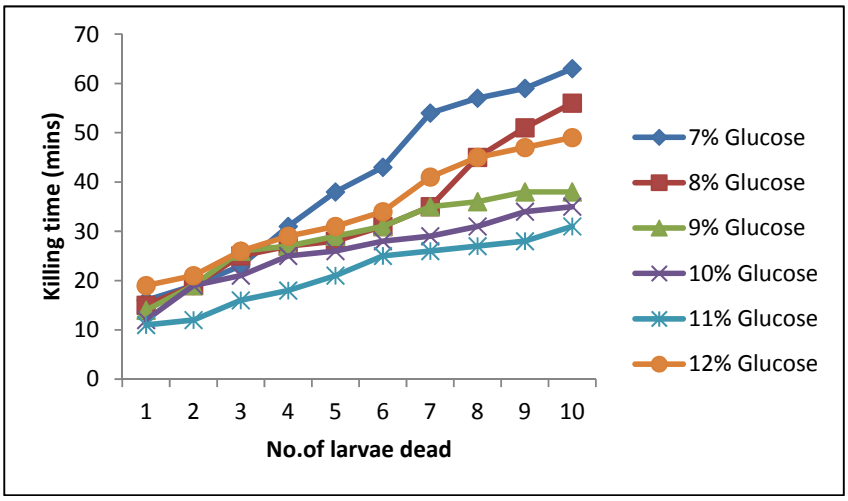


Fig 5 A: Bioassay results of fed-batch fermentations of Bti with different concentrations of glucose and 1% soyabean

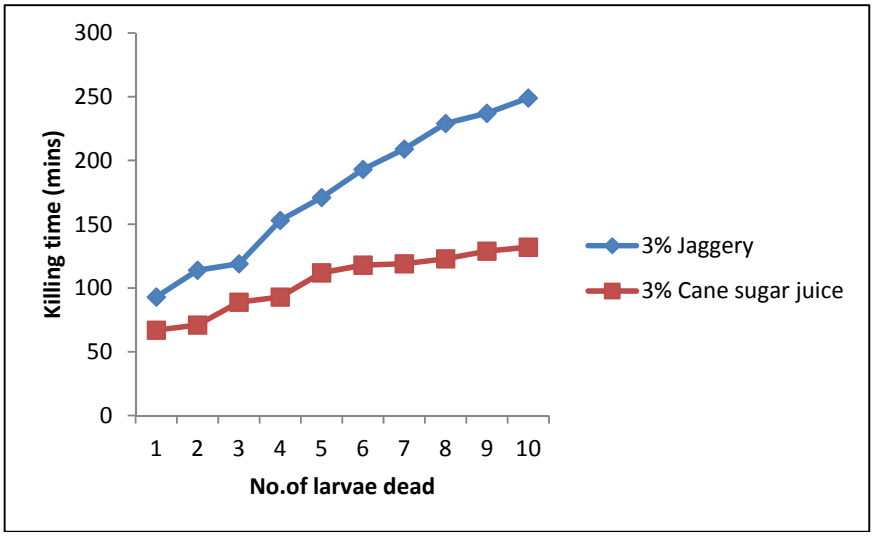


Fig 5 B: Bioassay results of batch fermentations of Bti with different carbon sources (3% Jaggery and 3% cane sugar juice) without yeast extract

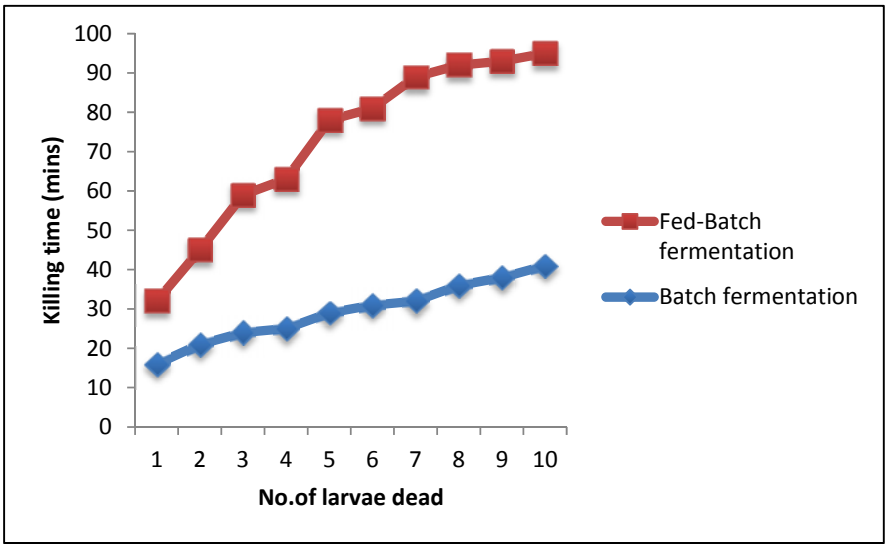


Fig 6: Bioassay results of aeration studies of Bti with different carbon sources (3% Jaggery and 3% cane sugar juice) without yeast extract

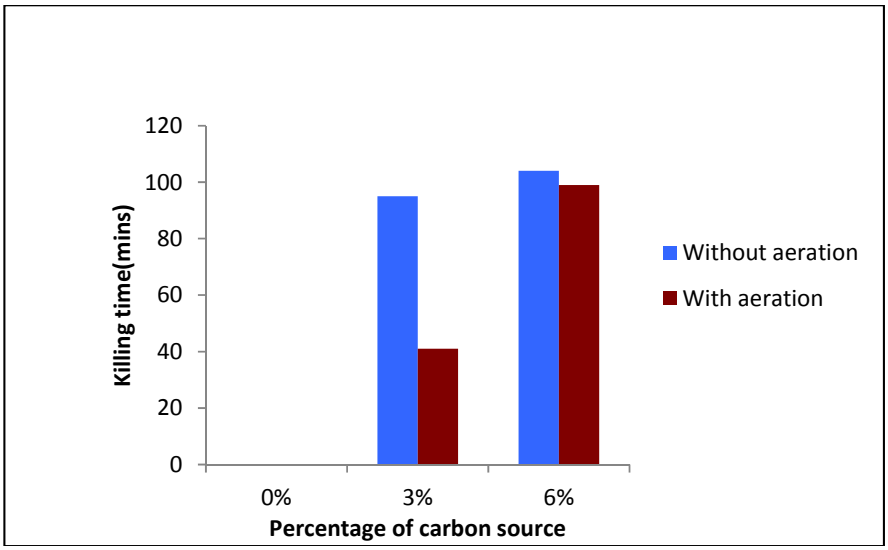


Fig 7: Comparison of larvicidal activity between biomass obtained through batch (left) and fed-batch (right) fermentations of *Bacillus thuringiensis* subsp. *Israelensis* using increased aeration (high Kla value), and that obtained through normal aeration.

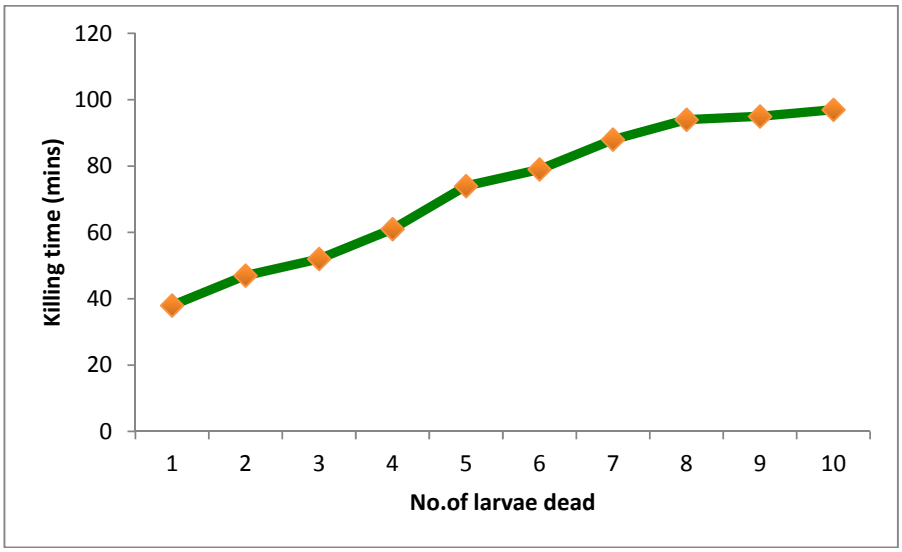


Fig 8: Bioassay result of media containing 0.1% Calcium chloride

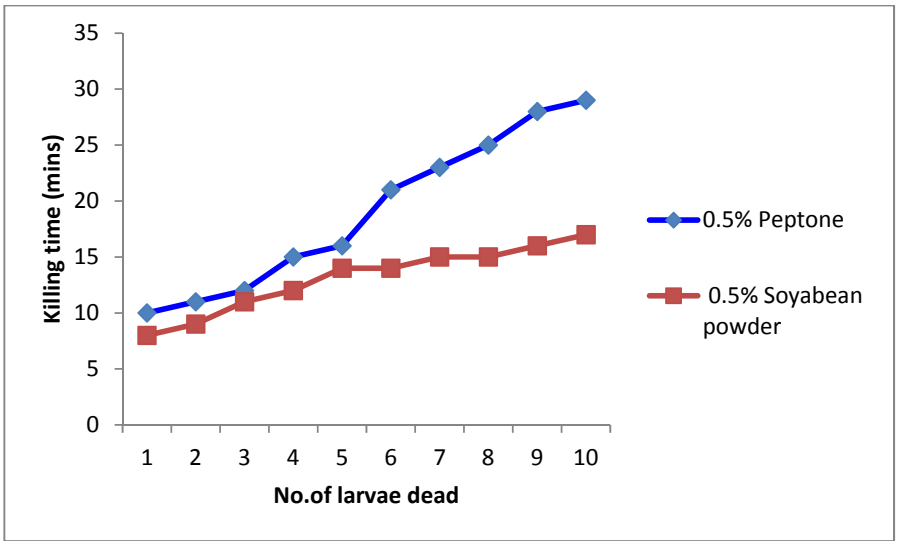


Fig 9: Bioassay results of batch fermentations of Bti for observing effect of 0.1% Calcium carbonate with two different nitrogen sources as additive on sporulation

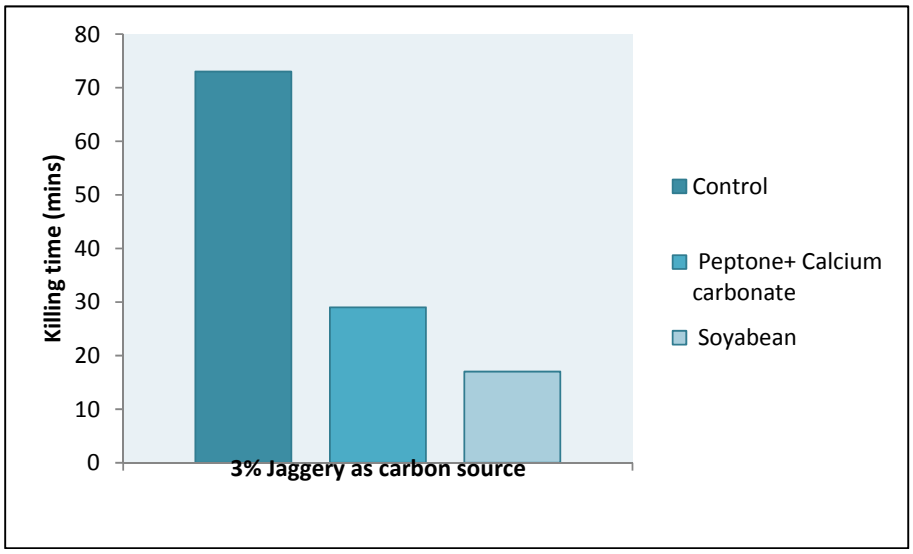


Fig 10: Comparison of larvicidal activity between biomass obtained in batch fermentations without and with calcium carbonate.

In the present study, we have focused on multi-parametric media optimization for improving the productivity of *Bacillus thuringiensis* subsp. *israelensis* serotype H14 as well as made a strategic design for fed-batch fermentations of Bti. We have investigated in details on the possibility of use of crude sources of carbon and nitrogen in Bti production. We have also investigated the catabolite repression limit of different carbon sources in submerged batch fermentations. The workflow also included investigation of the probable factors affecting the sporulation status of *Bti* cultures. Bt 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 [4].

In this study, we have tested the tolerance levels of Bti to various carbon sources in submerged batch experimental designs. As evident from Fig 1 and 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 (Table 1, Fig1 and Fig 2(a-e)). In case of tapioca powder, repression was not unto 6%. We could not increase the substrate concentration 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 disaccharide and is slowly hydrolysed. This may provide an insight to the high tolerance value of Bti for sucrose. The yield factor (Y=X/S) was highest with 6% tapioca powder. But harvesting and downstream processing, when using starch, is a cumbersome procedure. The high wet weight achieved with the tapioca powder is not valid.

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 [12]. 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 Jaggery and sugar cane juice (Table 2). Given its serious viscosity limitations, no fed-batch was performed using tapioca powder as carbon source. As is evident from Fig. 3 and Table 2, 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 Fig 4(a).

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 [13]. 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 (Table 2 & Fig 4(b)), repression was above 7%. But the killing time (Figure 3) 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.

Fed-batch fermentations with jaggery (Table 2 and Fig 4(c)) and sugar cane juice (Table 2 and Fig 4(d)) gave us many interesting results. Whereas even in fed-batch fermentations, 6% and 7% are the limiting concentrations for glucose and sucrose, jaggery was effectively utilized as the carbon source as high as 11%. The same result was obtained with sugar cane juice-based fed batch fermentation. As Figure 3 shows achievement of a positive result in co-optimizing increased biomass production with increased larvicidal activity. This may lead to reduction in production cost. The increased biomass production may be attributed to the probable presence of growth factors in natural sugar cane and the processed jaggery. But one shortcoming of using jaggery may be seasonal variations, and process fluctuations, due to different extraction procedures of jaggery. This in turn may make process repeatability a difficult aspect. Some growth factors and protein supplements present in natural cane sugar may be denatured or filtered out during the extraction of jaggery from cane sugar juice. This may provide the answer to the little difference observed between the behaviour of the two as carbon supplements.

The repression was found above 6% in fed-batch fermentations with glucose (as has already been discussed). There were two possible reasons for it: (a) Protein limitation-The optimal C: N ratio for maximum biomass production is generally around 10:1.The fed-batch media used in this study was containing 7% glucose and 0.5% peptone, i.e., a C: N ratio of 14:1. (b) Oxygen limitation-due to increased biomass, oxygen distribution may have decreased, which would have adversely affected production beyond 7% glucose level.

To determine the exact cause, we went for fed-batch fermentations of glucose with higher concentration of protein (nitrogen). Since peptone is very expensive media component, increasing the peptone concentration will result in steep increase in overall cost of production. Thus, to effectively manage the production cost, we opted for alternative cheaper sources of organic nitrogen. Soyabean powder was used, which contains approximately 46% of the available protein. It was used at a final concentration of 1%, so that the C:N ratio becomes < 10:1.As reported in Table 3 and in Figure 5 A, we got an increase in the biomass production with increasing concentrations of glucose up to a final concentration of 11%.Comparing Table 3 with Table 2, we see that the wet weight increased by 7.4268g/100mL of fermented broth for glucose concentration of 7%.The wet weight achieved at 11% was even higher than those achieved with jaggery and cane sugar at 11%.Glucose is costlier than either jaggery or cane sugar. But soyabean powder used to get high biomass with glucose is also comparatively far cheaper than peptone being use in fed-batches with cane sugar and glucose. Feasibility studies in large-scale fermenters will clearly tell about the more appropriate option.

To confirm that product limitation was really due to protein scarcity, we designed fed-batch fermentations of glucose with increased KL a/DO, but without increased nitrogen concentration. We used a specially designed commercial sparger in order to increase the oxygen availability. There was hardly any difference with sparged air (Table 5 and Fig 6). As is clear from Figure 7 (right), there is no substantial decrease in the killing time. This finding conclusively proved that protein limitation was the root cause for repression above 6% for fed-batch fermentations of Bti using glucose as the carbon source. Thus, repression at 11% may be explained by the accumulation of toxic metabolic wastes, which are detrimental to the microbe.

The importance of fed-batch fermentation in industry is that it gives improved biomass, as well as, increased product. In addition, fed-batch method also reduces down time, which gives huge savings in labor and utilities like sterilization and cleaning of the fermentor. But, when air was sparged to a batch fermentation of 3% glucose, there was marked increase in biomass production and simultaneous decrease in killing time. Figure 7 (left) clearly depicts the increased larvicidal activity with increased aeration. This was an interesting result because it substantiated certain literatures and opposed a majority of those.

Given the excellent biomass production with jaggery and cane sugar-based fed batch fermentations, we designed an experiment to find whether the previously mentioned carbon sources can also act equally well in the absence of yeast extract. We were conducting all our experiments with media containing 0.1% yeast extract. Still, we opted for this experiments because 0.1% yeast extract can be avoided, it will act as a tremendous means of curtailing down the production cost. As documented in Table 4 and Figure 5B, neither jaggery, nor sugar cane, performed well in the absence of yeast extract. Yeast extract constitute a rich source of amino acids, which are all very much required for the sporulation process. Our experiments even proved that yeast extract supplements the normal bacterial growth. The data from Crueger and Crueger, 1998 illustrates the various constituents of commercially available yeast extract. An analysis of the table will help to understand the indispensability of YE in *Bti* production [14]. But we were successful to trim down the usage of yeast extract from the generally used 0.3-0.5% to 0.1% (which was the normal concentration in all our media.

The pH of the medium has a great influence on the over all 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) [15]. Batch fermentation was designed with calcium chloride additive as buffering agent. Table 6 and Fig 8 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 [16]. Flores *et al* had shown that the sporulation depended on a specific DO value. A DO value less than the minimum required will result in decrease in sporulation [17]. This may explain the finding that sparged aeration stimulates 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 [18]. Here, we have set-up an experiment to investigate the role of calcium carbonate in sporulation. Table 7, Figure 8, Table 8 and Figure 9 clearly details, result of these

studies. When jaggery was used in batch fermentations with calcium carbonate and peptone (Fig 10), 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 7). The reason is that soyabean powder may contain some natural biochemical additives, which enhance toxin protein production along with high biomass. The result is also figured in figure 10 (right). Table 8 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. Moreover, the work stands out in that we have shifted from the general protocol of single-factor method of media optimization. We have successfully tried in multi-factorial media optimization for fed-batch fermentation of *Bti*. 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.

4. 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. (b) Crude carbon sources (like jaggery, sugar cane, and tapioca powder) and nitrogen source (soyabean powder) can be effectively used as media components for Bti production. This will ensure low cost of production with high productivity and increased larvicidal activity. (c) Sucrose is not a viable alternative to glucose as the carbon source. (d) Sugar cane and jaggery are highly profitable alternatives for glucose, even in fed-batch. (e) Sporulation process and crystal protein production is not linearly correlated. (f) Calcium carbonate (0.1%) ensures early and complete sporulation. (g) Sparged aeration induces more production in batch fermentations of Bti. (h) Yeast extract is an indispensable supplement, but can be used at a final concentration of 0.1% only with excellent results. Hence, this work will allow lowering the cost of production of active Bti product. Hopefully, this in turn will increase its social acceptability, and will thus alleviate the socio-economic problems inflicted by disease-carrying vectors.

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