BIOLOGICAL CONTROL

Use of By-Products Rich in Carbon and Nitrogen as a Nutrient Source to Produce *Bacillus thuringiensis* (Berliner)-Based Biopesticide

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Produção de Biopesticida à Base de *Bacillus thuringiensis* (Berliner) Usando Meios Alternativos Ricos em Carbono e Nitrogênio como Fontes de Nutrientes

RESUMO - A quantidade de carbono e nitrogênio usados para produzir biopesticidas à base de *Bacillus thuringiensis* (Berliner) pode influenciar a qualidade final do produto. O objetivo deste trabalho foi testar meios com diferentes níveis de carbono e nitrogênio: meio 1 - glicose de milho a 1.5% + farinha de soja a 0,5%, meio 2 - glicose de milho a 3,0% + farinha de soja a 1,0%, meio 3 - glicose de milho a 1,0% + farinha de soja a 3,0% e meio 4 - Luria Bertani (LB) + sais ($FeSO_4$, $ZnSO_4$, $MnSO_4$ e $MgSO_4$). O inóculo semente foi produzido usando 150 ml de meio LB mais sais incubados por 18h a 30ºC, sob agitação de 200 rpm. A cepa utilizada foi 344 (*B. thuringiensis* var *tolworthi* – pertencente ao Banco de Microorganismos da Embrapa). O pH foi medido a intervalos regulares. Após 96h de cultivo, o pH de todos os meios testados tenderam ao básico (entre 6,91 e 8,15), o maior número de esporos foi de 4,39 x 10^9 sporos/ml no meio 3, onde o teor de proteína usado foi o mais alto. A produção de massa celular foi maior no meio 3, com total acumulado de 39,3 g/l. A mortalidade de larvas de *Spodoptera frugiperda* (J.E. Smith) de dois dias de idade nos meios 3 e 4 foi de 100%. A CL_{50} para o meio 3 foi de 8,4 x 10^6 esporos/ml. Os meios alternativos usados promoveram crescimento satisfatório de *Bt*, sendo o meio 3 o mais promissor para ser usado na produção de biopesticida à base de *Bt*.

PALAVRAS-CHAVE: *Spodoptera frugiperda*, biocontrole, manejo de pragas, inseticida microbiano

ABSTRACT - The amount and sources of carbon and nitrogen used to produce *Bacillus thuringiensis* (Berliner)-based biopesticide may influence the quality of the final product. The objective of this research was to test different levels of carbon and nitrogen: medium 1 - 1.5% maize glucose + 0.5% soy flour, medium 2 - 3.0% maize glucose + 1.0% soy flour, medium 3 - 1.0% maize glucose + 3.0% soy flour and medium 4 - Luria Bertani (LB) + salts ($FeSO_4$, $ZnSO_4$, $MnSO_4$, $MgSO_4$). The seed culture was produced in LB medium plus salt, under agitation (200 rpm) for 18h at 30ºC. The strain 344 of *Bt* was used (*B. thuringiensis* var *tolworthi* – belonging to the Embrapa´s Bt Bank). The pH was measured at regular intervals. and After culturing for 96h, the pH of the four tested media was basified (6.91 and 8.15), the number of spores yielded 4.39 x 10^9 spores/ml in medium 3, where the amount of protein is high. The dry biomass weight accumulated in media 3 was 39.3 g/l. Mortality of 2-day-old larvae *Spodoptera frugiperda* (J.E. Smith) was 100% when using *Bt* produced in media 3 and 4. CL_{50} for medium 3 was 8.4 x 10^6 spores/ml. All tested media were satisfactory to *Bt* growth, and medium 3 wass the most promising to be used on a large scale *Bt*-based biopesticide production.

KEY WORDS: *Spodoptera frugiperda*, biocontrol, insect pest management, microbial insecticide

Global use of chemical insecticides for insect pest control has caused environmental pollution. The use of chemicals is effective to control insect pests, however they present some disadvantages: they can kill non-target organisms and cause human intoxication. Growing awareness towards environmental problems caused by the use of chemical insecticides has resulted in an urgent need to develop new environmentally acceptable products (Tirado Montiel *et al*. 2001).

The use of microorganisms such as bacteria, virus, protozoa and fungi as biological control agents may minimize the problems caused by the excessive use

of chemicals. Among these microorganisms, *Bacillus thuringiensis* (Berliner) (*Bt*) is the most widely used biopesticide (Yang & Wang 1998, Glare & Callaghan 2000). *B. thuringiensis* is a Gram-positive soil bacterium characterized by its ability to produce crystalline inclusions called "endotoxin proteins" during sporulation. The crystalline inclusions along with the spores have a high potential to control a great number of insect pests belonging to the order Lepidoptera, Diptera and Coleoptera (Vidyarthi *et al*. 2002).

B. thuringiensis grows in culture media containing sources of nitrogen, carbon and mineral salts. Various agricultural and industrial by-products, such as maize glucose, soybean flour, peanuts, cane molasses and liquid swine manure, are carbon and nitrogen rich and may be used as raw materials in biopesticide production. Tirado-Montiel *et al*. (2001) first tested the use of wastewater sludge for biopesticide production, although the entomotoxicity level reported was low. Many authors report the use of by-products and raw materials to develop *Bt* based biopesticides (Salama *et al*. 1983, Obeta & Okafor 1984, Morris *et al*. 1997). Moreover, the low cost of by-products as nutrient source in fermentation media for *Bt* biopesticide production has received little attention. However, a higher level of entomotoxicity is desired to reduce the production cost of biopesticides.

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith), is one of the most important corn insect pests in Brazil and its damage may reduce yield production up to 34% (Cruz 1995). The use of chemical insecticides to control S. *frugiperda* has increased over the years, reaching 10 to 14 sprays in some areas. Regarding the use of biopesticicdes, there are differences in Bt specificity, as some pest species are more prone to Bt infection. These differences were found among species, i.e. *Spodoptera* spp. are difficult to control with Bt-based biopesticide (strain HD1), while *Heliothis virescens* (Boddie) and *Plutella xylostella* (L.) are not (Baum *et al*. 1999). Beegle & Yamamoto (1992) also confirm the results that *Bt* is not much efficient against S. *frugiperda*.

The use of *B. thuringiensis*-based biopesticides is limited due to the high production costs (Dulmage 1981). -However it may become feasible and cheap, if affordable ways for mass production of theis entomopathogen were developed. The objective of this research was to evaluate the use of low cost raw materials rich in carbon and nitrogen as a nutrient source to produce *Bt*-based biopesticides.

Material and Methods

Bacterial strain. *B. thuringiensis* sv *tolworthi,* strain 344, belonging to the Embrapa Maize and Sorghum Microbial Bank was isolated from a soil sample. The sample was mixed with salt solution (0.8% NaCl) and the flask was incubated on a rotary shaker for18h. One milliliter of this suspension was heated in a water bath at 80°C for 30 min and cooled in ice for 15 min, and plated on Luria Bertani (LB) enriched with salts (all expressed in $g.l^{-1}$ - 0.002 g of $FeSO_4$, 0.02 g of $ZnSO_4$, 0.02 g of $MnSO_4$, 0.3 g of $MgSO_4$) and 2 g of glucose (pH adjusted to 7.2) to sporulate. After 24-72h of incubation at 30 ± 1°C, colonies were observed on a phase contrast microscope.

Media preparation. Strain 344 was subcultured on Luria Bertani (LB) enriched with salts (all expressed in $g.l^{-1}$, 0.002 g of $FeSO_4$, 0.02 g of $ZnSO_4$, 0.02 g of $MnSO_4$, 0.3 g of $MgSO_4$) and 2% glucose, with pH adjusted to 7.2. After subculturing, strain 344 was streaked on sporulating medium containing the same salts plus 12 g of Bacto-Agar (Difco), 8.0 g of nutrient broth, and was incubated for 24h at 30 ± 1°C and preserved at 4°C for future use.

Treatments were composed of four media. Medium 1 was composed of 1.5% maize glucose and 0.5% soy flour, medium 2 was composed of 3.0% maize glucose and 1.0% soy flour, medium 3 of 1.0% maize glucose and 3.0% soy flour and medium 4 of lab commercial Luria Bertani (LB) plus salts ($FeSO_4$, $ZnSO_4$, $MnSO_4$ and $MgSO_4$) and glucose. All four media were sterilized at 121°C for 30 min.

Seed culture preparation. A loopful of strain 344 from Bacto-Agar plates was used to inoculate 150 ml of sterilized LB medium enriched with salts, followed by incubation on a rotary shaker at 250 revolutions per minute (rpm) at 30°C for 12-16h. The actively growing cells were used as a second inoculum for the production of a second batch.

Growth of *B. thuringiensis tolworthi* in four different media. Flasks containing 150 ml of sterilized media 1, 2, 3 and 4 were inoculated with strain 344 grown in the seed culture. A 5% (v/v) inoculum from this flask was used to inoculate 150 ml of sterilized media. The shaking flasks were incubated at a stirrer speed of 200 rpm for 96h at 30°C. Culture samples were withdrawn from the flasks at different intervals to determine viable cell (VC), viable spore (VS) and entomotoxicity.

Cell mass. Samples of 100 ml were centrifuged at 10.000 rpm for 20 min. The supernatant was discarded and the pellet was lyophilized. Dry weight was calculated and expressed in gram per liter. The same sample was used for the spore count and toxicity test.

Spore count. Samples were withdrawn at different intervals and analyzed for colony forming units (CFU) of viable cells (VC) by the serial plate technique. The culture samples were heat treated at 80°C for 15 min, serially diluted and plated on LB enriched with salts and glucose agar plates. Plates were incubated at 30 ± 1°C for 24h to form fully developed colonies. The colonies counted in the plates were between 10 and 300.

pH. The pH of all media was adjusted to 7.2 ± 0.1 with NaOH after sterilization, and was measured at regular intervals during 96h of the fermentation process, using a pH meter.

Toxicity test. The spore crystal complex produced in different media was assayed against two- day-old laboratory-reared *S. frugiperda* larvae. First-instar larvae were raised on an artificial diet for two days prior to the bioassay. In order to determine the entomotoxicity the culture was harvested 24, 48, 72 and 96h after the fermentation process started, and used for larval treatment and larvae mortality was compared with the progress of the toxin production. The corn leaf surface method was used to test for entomotoxicity, by preparing the strain 344 produced on all tested media in sterile distilled water, and applying 18 µl of each suspension on the corn leaf surface provided to each larvae. Corn leaves were washed once with 0.5% sodium hypochlorite and two times with distilled water. Each bioassay included four doses wiht two replicates each, along with the appropriate control. Larval mortality was scored up to eight days after larval infection. Probit regression analysis was carried out to calculate LC_{50}.

Results and Discussion

Chemical analysis (Silva 1999) of the raw material showed that the amount of nitrogen in maize glucose is below the detectable level, however the amount of carbon is 619,900 mgl^{-1}. The amount of nitrogen in soybean flour is 58,800 $mgkg^{-1}$ and the amount of carbon is 675,900 $mgkg^{-1}$. Carbon is present in LB medium, 23,803.79 mgl^{-1}, as well as nitrogen, 18,883.24 mgl^{-1}. Table 1 shows the results of carbon, nitrogen and micronutrients found in the LB medium and the raw material components of the other three media tested. According to Yang and Wang (1998), glucose is used in most large-scale Bt fermentation; however, high levels of glucose (> 40 g/liter) can cause growth inhibition. The amount of K, Mg, Ca, Fe, Mn and Cu found in the four tested media were enough to promote Bt growth (Sikdar *et al*. 1991). Avignone Rossa *et. al*. (1990) showed that the higher the yeast extract concentration, the higher were the biomass dry weight values.

Table 1. Quantitation of macro and micronutrients in maize glucose, soybean flour and LB medium.

Raw material	N	C	P	K	Ca	Mg	S	Zn	Fe	Cu	Mn	Na
	……..……mg/l……..…….											
LB + Salts	18,883.24	23,803.796	219.98	770.00	2.92	32.08	99.18	7.23	6.30	0.23	9.33	163.33
Maize glucose	0.00	619,900.0	236.31	408.33	0.00	36.46	0.00	5.83	29.17	0.00	11.67	1108.33
	……..……%……..……							……..….mg/kg……..……				
Soybean flour	5.88	67.59	0.48	1.75	0.17	0.20	0.16	0.00	0.01	0.00	0.00	0.00

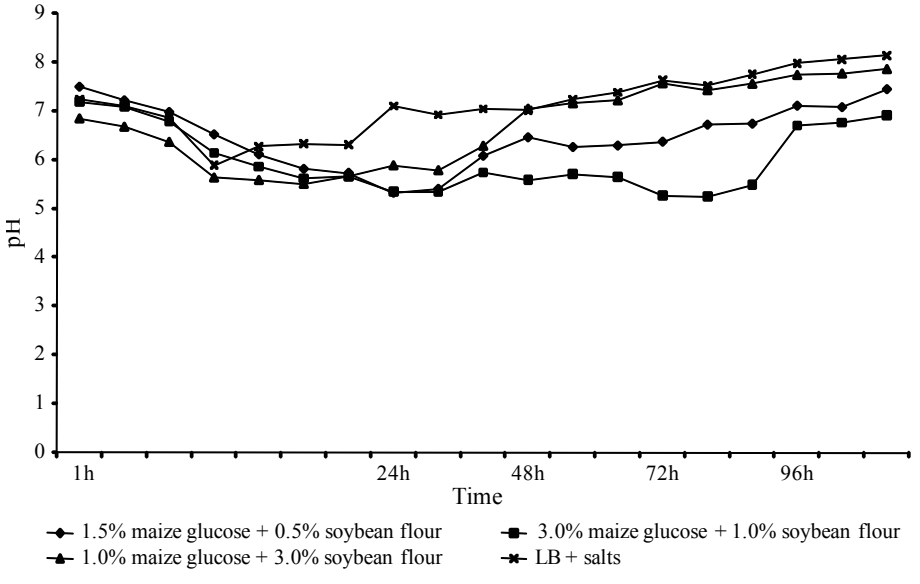

Fig. 1. The pH variation during the fermentation of *B. thuringiensis* var. *tolworthi* in four media.

Our results followed the same tendency where medium 3 had the highest yeast extract concentration (3.0%).

The pH had little variation during the fermentation process for the four tested media. Since there was no pH control during the experiments, a pH decrease was observed at the beginning of the fermentation for all tested media (Fig. 1). All media decreased the pH to around 6 in the first 10h to 20h of fermentation, and after this period, media 3 and 4 maintained pH above 7 (Fig. 1). Medium 2 maintained a low pH, around 5.4, after the first 24h, reaching a minimum value of 5.25 after 72h of fermentation. Medium 2 contained the highest amount of glucose (30g/l). pH increase in media 3 and 4 occurred only 24h after fermentation, remaining between 7.0 and 8.07, while in media 1 and 2 pH was between 7.0 and 7.5 (Fig. 1). The pH attained at media 3 and 4 was possibly due to the utilization of the carbohydrates before the sporulation phase was reached (Tirado Montiel *et al*. 2001). These patterns in pH variation were similar to those found by Tirado Montiel *et al*. (2001) and Vidyarthi *et al*. (2002). They reported the final pH in the sludge samples stabilized between 8 and 8.5.

Ejiofor and Okafor (1989) and Abdel-Hameed *et al*. (1991) concluded that low pH can inhibit growth, sporulation and crystal formation of *Bt*. Abdel-Hameed *et al*. (1991) also stated that if the pH of a culture medium is not between the range 6.5-7.5, sporulation and δ endotoxin formation could be adversely affected. All tested media yielded an increasing amount of spores when harvested in the first 20h (Fig. 2), although pH was decreasing.

Medium 3, containing the highest amount of protein, yielded a maximum spore count of 4.39 x 10^9 spores/ml within 96h of fermentation, but had already produced 3.07 x 10^9 spores/ml within 48h of fermentation. The commercial LB media yielded the lowest amount of spores, 1.14 x 10^9 spores/ml, after 72h of fermentation (Fig. 2). *B. thuringiensis* subsp. *tolworthi* produced an appreciable amount of spores in all tested media after 48h of fermentation, producing 10^9 c.f.u/ml of viable cells (Table 2), however the number of heat resistant cells decreased substantially (Table 3). According to Tirado Montiel *et al*. (2001), the availability of carbon can influence the yield of viable cells, spores and toxins in the *Bt* production process. This fact is confirmed by our results on medium 3 (1.0% maize glucose and 3.0% soy flour), probably because the carbon source from maize glucose is more readily available than the carbon in the soy flour, the major nitrogen source. Also, Yang & Wang (1998) stated that the critical glucose levels vary among different strains and it should be identified for each specific strain.

The biomass produced by different media varied (Fig. 3). Medium 3 had the highest biomass production with high toxicity compared to the conventional and commercial lab medium (Table 2), similarly to what was observed by Prabakaran & Balaraman (2006). All media showed a decrease in cell mass production around 72h of fermentation (Fig. 4), however medium 3 showed the highest cell mass production during the whole process of fermentation (Fig. 4).

Mortality of two- day-old *S. frugiperda* larvae fed with *Bt* withdrawn at regular intervals was above 93% in medium

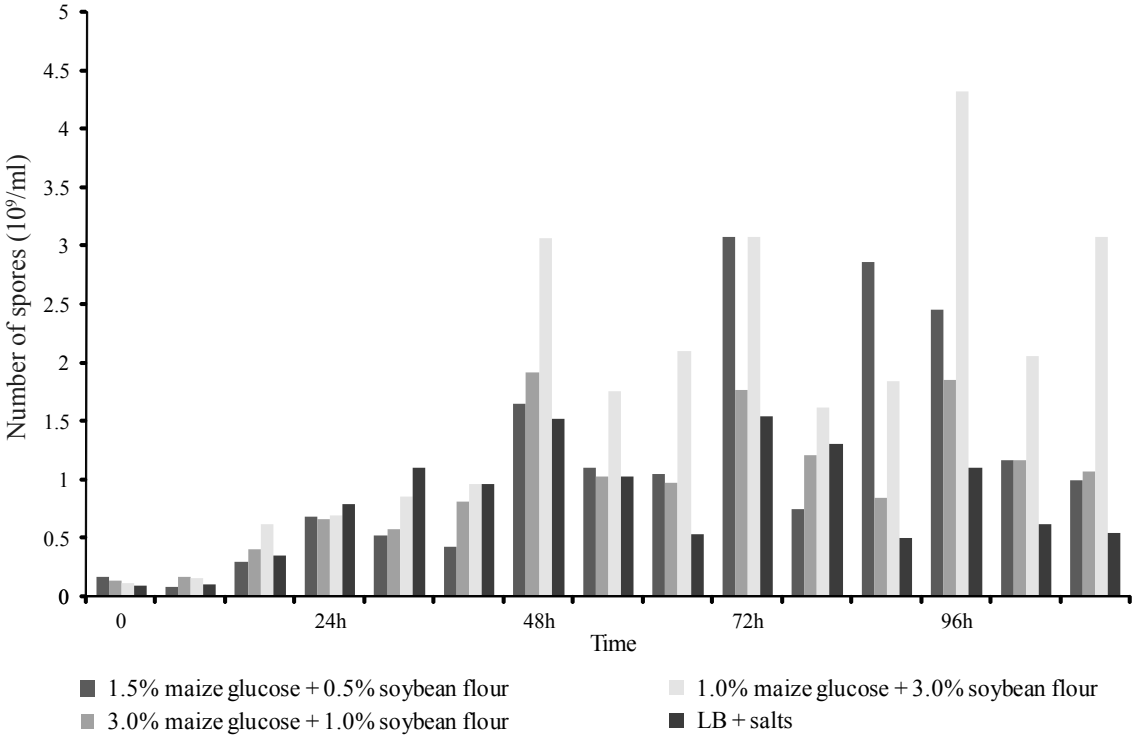

Fig. 2. Number of spores of *B. thuringiensis* var. *tolworthi* in four media.

3 (high carbon content), after 48h of fermentation, showed with CL_{50} of 8.4 x 10^6 spores/ml (Table 2). Mortality was low in media 1 and 2 after 96h of fermentation, and the highest mortality was achieved after 72 h and 48 h for media 1 and 2, respectively. The age of the *Bt* culture is very important in a large-scale production system, where the reduction of the fermentation period is desirable.

Viable cell and viable spore counts of *Bt* grown in agricultural by-products media were similar to *Bt* grown in commercial medium; however, entomotoxicity (Table 3) observed in medium 3 was higher, even after 48h of fermentation. The use of maize glucose and soybean in the production of *Bt* has many advantages. They are available throughout the year and allows for faster sporulation and high biomass dry weight. Besides, the cost of production of a medium containing carbon and nitrogen sources using agricultural by-products is feasible and cheap.

Table 2. Mortality of *S. frugiperda* two-day-old larvae caused by *B. thuringiensis* var. *tolworthi* in four media harvested at different intervals (1, 24, 48, 72 and 96h).

	1h		24h		48h		72h		96h	
	Spore concentration	Mortality (%)	Spore concentration	Mortality (%)	Spore concentration	Mortality (%)	Spore concentration	Mortality (%)	Spore concentration	Mortality (%)
1.5% maize glucose + 0.5% soybean flour	1.73 x 10^5	0	6.84 x 10^5	0	1.61 x 10^6	2.08	3.10 x 10^6	2.12	2.47 x 10^6	8.51
	1.75 x 10^6	2.08	6.84 x 10^6	8.33	1.61 x 10^7	25.00	3.11 x 10^7	10.41	2.46 x 10^7	4.17
	1.72 x 10^7	27.08	6.83 x 10^7	12.51	1.64 x 10^8	33.33	3.08 x 10^8	37.50	2.47 x 10^8	38.29
	1.74 x 10^8	20.83	6.87 x 10^8	85.10	1.65 x 10^9	82.97	3.09 x 10^9	93.61	2.44 x 10^9	69.56
3.0% maize glucose + 1.0% soybean flour	1.37 x 10^5	4.16	6.72 x 10^5	6.25	1.92 x 10^6	12.50	1.77 x 10^6	4.17	1.86 x 10^6	4.17
	1.33 x 10^6	2.12	6.74 x 10^6	2.08	1.93 x 10^7	39.58	1.78 x 10^7	10.41	1.87 x 10^7	16.66
	1.33 x 10^7	8.33	6.75 x 10^7	33.33	1.96 x 10^8	53.19	1.78 x 10^8	31.25	1.89 x 10^8	47.91
	1.31 x 10^8	4.16	6.79 x 10^8	79.16	1.91 x 10^9	83.33	1.71 x 10^9	52.08	1.89 x 10^9	76.59
1.0% maize glucose+ 3.0% soybean flour	1.16 x 10^5	2.08	7.02 x 10^5	2.08	3.07 x 10^6	12.51	3.08 x 10^6	8.88	4.32 x 10^6	18,75
	1.13 x 10^6	17.02	7.10 x 10^6	2.08	3.11 x 10^7	31.91	3.09 x 10^7	33.33	4.37 x 10^7	16.66
	1.11 x 10^7	10.41	7.09 x 10^7	4.16	3.02 x 10^8	58.33	3.12 x 10^8	95.65	4.33 x 10^8	82.97
	1.19 x 10^8	16.66	7.05 x 10^8	18.75	3.07 x 10^9	93.75	3.02 x 10^9	100	4.39 x 10^9	100
LB + salts	9.25 x 10^4	0	7.90 x 10^5	0	1.52 x 10^6	8.33	1.55 x 10^6	2.08	1.10 x 10^6	6.52
	9.24 x 10^5	0	7.91 x 10^6	8.33	1.53 x 10^7	10.63	1.58 x 10^7	4.17	1.11 x 10^7	16.66
	9.28 x 10^6	0	7.98 x 10^7	4.25	1.53 x 10^8	4.16	1.52 x 10^8	8.51	1.15 x 10^8	51.06
	9.21 x 10^7	21.73	7.93 x 10^8	16.68	1.59 x 10^9	25.00	1.54 x 10^9	38.29	1.14 x 10^9	100

Table 3. Number of heat resistant cells, expressed as colony forming units (c.f.u./ml) harvested at different intervals during the fermentation of *B.thuringiensis* var. *tolworthi* in four culture media.

Media	24h	48h	72h	96h
Medium 1- 1.5% maize glucose + 0.5% soybean flour	4.7 x 10^8	2.2 x 10^8	3.6 x 10^7	8.7 x 10^6
Medium 2- 3.0% maize glucose + 1.0% soybean flour	3.0 x 10^8	3.8 x 10^7	2.3 x 10^7	1.27 x 10^8
Medium 3- 1.0% maize glucose+ 3.0% soybean flour	3.1 x 10^8	3.1 x 10^7	8.3 x 10^7	3.9 x 10^7
Medium 4- LB + salts	3.0 x 10^8	3.1 x 10^7	3.1 x 10^7	2.2 x 10^7

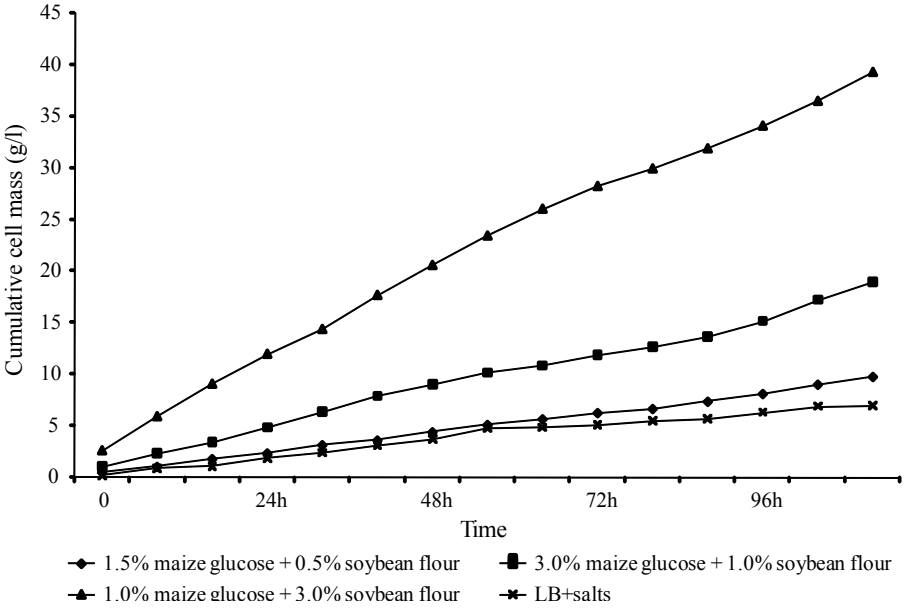

Fig. 3. Cumulative freeze dried powder weight (dry biomass-gl^{-1}) of *B. thuringiensis* var. *tolworthi* in four media.

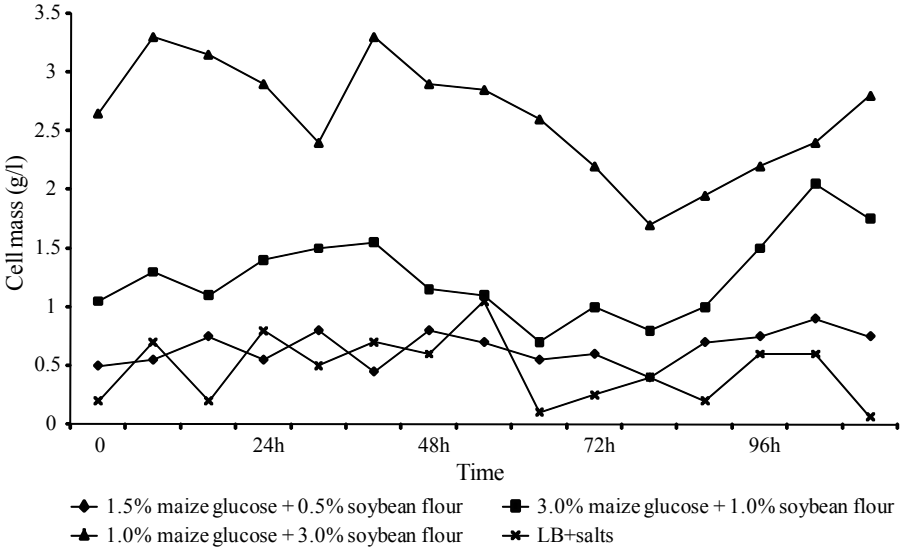

Fig. 4. Fluctuation of freeze dried powder weight (dry biomass-gl^{-1}) of *B. thuringiensis* var. *tolworthi* in four media, harvested at different intervals.

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