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Utilization of Dairy by Product in the Production of Bioinsecticide

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Abstract: Locally available sugar cane molasses was utilized in the production of bioinseticide by cultivating four strains of *Bacillus thuringiensis* (*B.t*) namely H-D 133, H-D 234, ENRC 60 and ENRC 63 under shaking culture. The highest yields obtained after 90 hour fermentation. Total viable counts, total spores and percent of spores were different between the four strains. The by product whey ultrafiltrate was utilized in spore crystal mixture recovery from the fermentation beer of the tested strains. Adjusted fermentation beer to pH 7.0 and pH 4.0 prior recovery showed the advantage in spore an crystal mixture recovered yield when the fermentation beer was adjusted to pH. 4.0 Comparison between lactose solution and whey ultrafiltrate contained the same amount of lactose for spores-crystals mixture recovery from fermentation beers showed increases in the yield where in whey ultrafiltrate was employed. Bioassay against the larvae of *Spodoptera littoralis, Spodoptera exigua Phthorimaea operculella* and *Earias insulana* were performed. The $LC_{50,}$ slopes, 95% confidence limits and potencies for both two spore-crystal mixtures obtained by lactose solution or whey ultrafiltrate of the four strains were determined. The bioassay showed that the larvae of potato tuber moth and spin boll worms were highly sensitive to the four produced pathogen than *S. littoralis* and *S. eixigua*. The spore-crystal mixture recovered in the presence of whey ultrafiltrate showed high toxic effect against the assayed larvae of *S. exigua*, *E. insulana* and *S. littoralis*, whereas, the recovered spore-crystal mixture in the presence of lactose solution was more toxic for *P. operculella*.

Key words: Bioinsecticides, *Bacillus thuringiensis,* whey ultrafiltrate, bioassay

Introduction

Agriculture has changed quite markedly over the last forty years as a consequence of the use of synthetic chemicals to control pests on different crops. The world consumption of pesticides jumped from less than 30 thousand metric tons to 3 million metric tons in 1985 (Prokopy, 1988).

Chemical control has made a significant contribution to the rapid and constant increase in agricultural production around the world but we must realize that it also contributed to a very rapid decrease of the plant health situation (Brader, 1991).

The over-dependence on chemical control, modern agriculture became extremely vulnerable with a host of undesirable side effects to human health and the environment. An estimated 25 million agricultural workers in developing countries are poisoned every year by pesticides (Jeyaratnam, 1990).

On the other hand, the effect of pesticides on the environment, although with well documented cases, is still lacking a global assessment. Among pesticides, insecticides stand out as the most important group when human health and the environment are considered. From the so-called "dirty dozen" (Georghiou and Lagunes, 1991).

The use of biological insecticides has been increasing largely due to their high specify without harmful side effects on man, animal and other environmental elements .So far *Bacillus thuringiensis* has proven to be the most applicable biological agent in compacting a wide number of insect pests (Huag, 1980). However, the expansion in the use of *B. thuringiensis* is still limited particularly in the developing countries due to economic reasons. The great interest for the production of *B.t* as a bioinsectside for biological control is due to the success of its application for numerous harmful pests e.g soft and hard tickes in males and females of *Arigas persicus* and *Hyalomma dromedarii* (Hassanain *et al.*, 1997), Colorado potato beetle (Colazza *et al.*, 1996; Dubois and Jossi, 1993; Schrod *et al.*, 1996) cotton boll worm (Carran *et al.*, 1995; Fadel and Sabour, 1998), maize stem borer, *Spodoptera frugiperda* (Alfonso *et al.*, 1994; All *et al.*,1996; Guerra, 1995; Khanna *et al.*,1995), tea pests (Unnamalai and Vaithilingam, 1995), olive pests (Delrio, 1995), Free living nematodes (Borgonie *et al.*,1996; Ismail and Fadel, 1997,1999), Cheepecto parasites (Levot *et al.*,1995), Cockroaches, *Blattele germanical*, (Zukowski, 1995), protection of apple in storage (Jakli and Lepoivre, 1995) and Leather Jackets (Smith *et al.*,1993).The objective of the present work other than the production of bioinsecticides is studying the possibility of utilizing whey ultrafiltrate the main dairy industry by products instead of lactose sugar in the spore-toxin mixture precipitation from *B. thuringiensis* fermentation beer to eliminate the *B.t* production costs.

Materials and Methods

Molasses treatment: Sugar-cane molasses was obtained from sugars and Integrated Company, EL – Hawamidia Giza – Egypt. To get rid of mud and insoluble substances crude molasses was diluted with water in ratio 1:1 (W/W). Five hundred percent of calcium super phosphate was added, then heated to 90 °C. The cooled diluted molasses was centrifuged at 3.000 rpm for 10 minutes. The supernatant was employed in bacterial growth medium as carbon and phosphorus sources.

Bacterial strains: *Bacillus thuringiensis (B.t)* var entomocidus H-D 133 and 234 were obtained from Pests and Plant Protection Department N.R.C, Egypt and *B.t* ENRC 60 and *B.t* ENRC 63 were locally isolated from Egyptian soil according to method of Travers *et al.* (1987) in Microbial Chemistry Lab. NRC. Egypt, the bacterial cultures are preserved on nutrient agar slants.

Larval insects: *Spodoptera littoralis*, *S.exigua*, *E.insulana* and *P.operculella*. were obtained from the farm of Pests and Plant Protection Department. National Research Center, Dokki, Cairo, Egypt.

Preparation of inoculum: The inoculum was prepared in 250 ml conical flasks contained 25 ml of a medium with (g/l) peptone, 6.0; yeast extract, 3.0; meat extract, 1.5; and glucose 1.0,. The flasks were autoclaved at 121°C for 15 min. The flasks were inoculated with a loop of 3 days bacterial culture on nutrient agar medium, then incubated on rotary shaker 200 rpm at 29°C for 48h. The activated culture was used as an inoculum at 10%(V/V).

Fermentation medium: The bacterial cultures were prepared in 500ml Erlenmeyer flasks each contained 60ml of fermentation medium had the following composition (g/L) 2.0% total sugars cane molasses, yeast extract 2.0 and 1.0 peptone. The medium

was adjusted to initial pH 7.0 using diluted sodium hydroxide before autoclaving. The flasks were autoclaved at 121°C for 15 minute. The cooled sterilized flasks were inoculated by the above activated culture at 10% (V/V). The inoculated flasks were incubated in shaking incubator adjusted to 200 rpm and 30 °C for 3 days .

Recovery of spore-crystal mixtures: Small quantities of *B.t* recovered by the lactose acetone coprecipitation procedure of Dulmage (1970). The flow - sheet for this procedure is given blow:

Whole beer pH 8.4 – 8.7
Adjust to pH 7.0 or pH 4.0 with HCl

Supernatant	Centrifuge
(discard)	Residue
	Suspend in 1/10 – 1/20 vol.

4.6% lactose or whey ultra filtrate
(vol. Based on original beer)
Add slowly while stirring
1. 5% vol. Acetone
Stir 30 minutes
Let stand 10 – 30 minutes

Filtrate	Filter with suction
(discard)	Residue
	Stir with small-volume
Filtrate	acetone
(discard)	Filter with suction
	Residue
Filtrate	Stir with small-volume
(discard)	acetone
	Filter with suction
	Residue
	Dry overnight

Flow sheet for laboratory-scale recovery process for spore-crystal mixtures of *Bacillus thuringiensis*.

1) Centrifugation: A description of a centrifuge useful for the recovery of small quantities of bacteria is centrifuged at 27, 300 G for 30 minutes.
2) First filtration: The creamy residue from the centrifuged beer is suspended in 1/10 volume of 4.2% lactose or whey ultrafiltrate (4.2 lactose) and coprecipitated with acetone. The suspension is filtered with suction using Whatman No. 2 filter paper and a 12-14 cm Buchner funnel. The filtration will go faster if the acetone lactose or acetone whey ultrafiltrate *B.t* suspension is allowed to stand 10-30 minutes after stirring.
3) Second and third filtrations: If the first filtration has proceeded properly, the *B.t* will easily come off the filter paper. The purpose of the second and third filtration is primarily to remove remaining water from the *B.t* preparation. Sufficient acetone should be used in each wash to accomplish this purpose. On rare accessions, a third wash may be necessary.
4) Drying: The *B.t* from the final acetone wash is placed in an evaporating dish, covered lightly with a piece of filter paper and allowed to dry on the laboratory bench at room temperature and a relative humidity of less than 40% overnight.

Total counts and total spores: Plate pouring method (Wilson, 1922) was used for determination of total counts and total spores.

Protein determination: The protein contents of spore-delatoxin mixture precipitate was determined by Micro kejldahel method according to AOAC. (1980)

Bioassay: The assay procedure proposed by Dulmage *et al.* (1971) was adopted. Serial dilutions of the test samples and of standard preparations of known potency are incorporated into artificial diet used. Proper preparation of the diet was considered to ensure an accurate assay. With emphasis on using completely dissolved agar. The initial suspensions and all dilutions of the test samples are made in a saline consisting of the following ingredients (g/l): NaCl, 8.5: $K_2 HPO_4$, 6.0 and $K H_2 PO_4$, 3.0: 1 ml of 1% tween 80 solution to aid welting the test sample. The endotoxin samples were diluted at ratios of 1:10 or 1:50 in the diet. The samples prepared are such that a 1:10 dilution of the endotoxin suspension in the diet will give a concentration of 500 μg endotoxin /ml diet. The diet-toxin added was throughly mixed in the diet which was then dispensed into small plastic cups before handling. The cups infested with the test larvae were incubated at 26 $\pm$ 2 °C for 7 days which they were examined and the mortality percentage was recorded.

Results and Discussion

Production of bioinsecticides: Table 1 shows the viable cell density, spore fermentation count, changes of pH value over course of fermentation time for four strains of *B. t* . Maximum vegetative cell counts production were produced in 36-54 hours with the onset of spore count. Maximum spore in fermentation beers were attained after 72h for all tested strains. Data show the behavior of *B.t* strains under study in the fermentation medium where an initial pH drop take place in the early phases of growth. This is due to the formation of some acids from the sugar in the molasses. Amount of acids formation, resulting in lowering pH medium were differed between tested strains.

The data shows a variation between strains in total count, as the highest yield was produced by *B.t* H-D133 and the lowest was produced by *B.t* ENRC 60. Similarly the highest spore count was formed by *B.t* H-D 133 and *B.t* ENRC respectively. Also there was a variation in the percent of spores to total count between the cultivated strain in the fermentation medium. The highest spore percent was achieved with the two local isolates *B. t* ENRC 60 and *B.t* ENRC 63, as it were 98 and 97.5% respectively. No significant difference between *B.t* H-D 133 and *B.t* H-D 234, since the spores percent were 91.6 and 91.0% respectively.

Various data has been published in literature about total count pH variation, spore percent and the optimum incubation time for obtaining the suitable yield from spore crystal mixture There criteria was shown it differences according to strains medium composition as well as cultural conditions (Brabosa, 1993; Dulmage, 1989 , Jones, 1993) The biomass yield data reported in literature are very different and even an author reports very wide intervals, there are values higher and lower.

Extremely high values indicate that *B. t* is using different carbon sources while lower values suggest the accumulation of some metabolic products. Rowe (1990) found that *B.t*

Table 1: Effect of incubation period on the total count and spores counts on four serotype of *B. thuringiensis (B.t)* namely H-D 133, H-D.234, ENRC 60 and ENRC 63

Incubation period (h)	*B.t* H-D 133			*B.t* H-D 234			*B.t* ENR C 60			*B.t* ENR C 63		
	Total count ($x10^9$)	Spore count ($x10^9$)	Final pH	Total count ($x10^9$)	Spore count ($x10^9$)	Final pH	Total count ($x10^9$)	Spore count ($x10^9$)	Final pH	Total count ($x10^9$)	Spore count ($x10^9$)	Final pH
0	0.002	-	7.0	0.002	-	7.0	0.017	-	7.0	0.21	-	7.0
18	9.0	1.8	6.6	9.1	1.1	6.4	7.8	0.9	6.8	7.8	0.70	6.5
36	25.5	4.1	7.6	24.6	4.8	7.3	26.3	5.6	7.5	23.9	6.1	7.6
54	28.1	19.6	8.1	27.3	22.1	7.9	24.2	15.8	8.6	27.6	22.4	8.4
72	19.4	14.2	8.8	16.9	14.3	8.2	20.6	19.2	8.8	21.1	18.7	8.9
90	10.7	9.8	9.1	8.9	8.1	8.6	15.1	14.8		12.1	11.8	9.2

Table 2: Effect of incubation period on the biomass and protein yields of four serotypes of *B.t* namely H-D 133, H-D.234, ENRC 60 and ENRC 63

Incubation	*B.t* H-D 133		*B.t* H-D 234		*B.t* ENRC 60		*B.t* ENRC 63	
period (h)	Yield g/L	% protein	Yield g/L	% protein	Yield g/L	% protein	Yield g/L	% protein
18	1.10	46.1	1.19	42.6	1.32	52.1	1.21	40.2
36	3.65	44.3	3.52	41.5	3.96	84.7	4.01	40.1
54	5.43	42.8	5.08	38.2	5.11	45.6	5.83	37.9
72	7.21	40.2	7.16	37.1	6.86	42.5	7.26	36.4
90	6.81	38.6	6.01	36.8	5.65	40.8	6.12	35.4

* Dry precipitate of spore-detatoxin mixture.

Table 3: Comparison of recovery spore and crystals from fermentation beer of four *B.t* strains using 4.2% (w/v) lactose solution or 4.2% (w/v) total sugars whey ultrafiltrate when beer adjusted to pH 4.0

	B.t strain			
Item	H-D 133	H-D 234	ENRC 60	ENRC 63
Volume (ml)	1000	1000	1000	1000
Spore count/ ml	$17.8x10^{9}$	$15.5x10^{9}$	$20.2x10^{9}$	$20.6x10^{9}$
Yield(g) recovery (spore crystal mixtures) with 4.2% lactose solution	7.21	7.16	6.86	8.21
Yield(g) recovery (spore crystal mixtures) with 4.2% total sugars whey permeate	7.26	7.66	6.88	8.65
Spore count/g recovery with lactose solution	$46.8x10^{22}$	$32.1x10^{22}$	$37.9x10^{22}$	$27.5x10^{22}$
Spore count/g recovery whey ultra filtrate	$46.9x10^{22}$	$32.6x10^{22}$	$37.9x10^{22}$	$27.8x10^{22}$
%soluble protein in recovery with lactose solution	28.2	25.7	30.5	24.4
%soluble protein in recovery with whey ultra filtrate	28.9	26.1	31.8	25.9
Fermentation period (h)	90	90	90	90
Harvest pH	9.1	8.9	8.9	8.6

Table 4: Comparison between spores and crystals mixture recovery for fermentation beer of four *B.t* strains using 4.2% (w/v) lactose solution and 4.2% (w/v) total sugar whey ultra filtrate when beers were adjusted to pH 4.0

	B.t strain			
Item	H-D 133	H-D 234	ENRC 60	ENRC 63
Volume (ml)	1000	1000	1000	1000
Spore count/ ml	$17.8x10^{9}$	$15.5x10^{9}$	$20.2x10^{9}$	$20.6.10^{9}$
Yield(g) recovery (spore crystal mixture) with 4.2% lactose solution	7.32	7.20	7.05	8.44
Yield(g) recovery (spore crystal mixture) with 4.2% total sugars whey permeate	7.76	8.27	8.64	8.56
Spore count/g recovery with lactose solution	$47.2x10^{22}$	$32.1x10^{22}$	$38.3x10^{22}$	$27.6x10^{22}$
Spore count/g recovery whey ultra filtrate	$47.6x10^{22}$	$32.4xo10^{22}$	$38.3x10^{22}$	$27.8x10^{22}$
%soluble protein in recovery with of lactose solution	28.9	26.8	31.2	24.9
%soluble protein in recovery with whey ultra filtrate	29.4	27.6	31.8	25.7
Fermentation period (h)	90	90	90	90
Harvest pH	9.1	8.9	8.9	8.6

Table 5: Potency of different spore-crystal mixtures recovered form fermentation beers of four *B.t.* strains in the presence of lactose or whey ultrafiltrate against *P. operculella*

Source of spore-crystal mixtures	Spore-crystal mixtures recovered in the presence of lactose solution. in				Spore-crystal mixtures recovered the presence of whey ultrafiltrate			
	LC_{50} (ug/ml)	Slope	95% Confidence limit	Variance	LC_{50} (ug/ml)	Slope	95% Confidence limits	Variance
B.t. H-D 133 Aizawai	38.81	1.58	18.5 - 57.7	0.0001	29.19	1.59	19.2- 47.1	0.0001
B.t H-D 234 Galleriae	26.03	1.26	11.9 - 43.3	0.004	27.51	1.16	11.9 –23.7	0.006
B.t ENRC 60	43.03	1.37	12.8 – 52.3	0.004	40.02	1.36	27.5 - 66.3	0.004
B.t ENRC 63	30.10	1.29	17.7 – 51.3	0.004	29.31	1.28	11.8 – 43.9	0.004

Table 6: Potency of different spore-crystal mixtures recovered form fermentation beer of four *B.t.* strains in the presence of lactose or whey ultrafiltrate against *E. insulana*

Source of spore-crystal mixtures	Spore-crystal mixtures recovered in the presence of lactose solution.				Spore-crystal mixtures recovered in the presence of whey ultrafiltrate			
	LC_{50} (ug/ml)	Slope	95% Confidence limits	Variance	LC_{50} (ug/ml)	Slope	95% Confidence limits	Variance
B.t H-D 133 Aizawai	47.57	1.21	27.5-67.4	0.008	46.82	1.16	26.5-62.4	0.006
B.t H-D 234 Galleriae	56.87	1.07	31.1-68.8	0.009	53.71	1.08	39.1-62.1	0.009
B.t ENRC 60	65.33	1.59	36.9-87.8	0.009	49.55	1.22	27.5-69.4	0.008
B.t ENRC 63	39.17	1.13	26.3-42.5	0.006	38.27	1.12	28.5-45.9	0.006

Table 7: Potency of different spore-crystal mixtures recovered form fermentation beer of four *B.t.* strains in the presence of lactase or whey ultrafiltrate against *S. littoralis*

Source of spore-crystal mixtures	Spore-crystal mixtures recovered in the presence of lactose solution.				Spore-crystal mixtures recovered in the presence of whey ultrafiltrate			
	LC_{50} (ug/ml)	Slope	95% Confidence limits	Variance	LC_{50} (ug/ml)	Slope	95% Confidence limit	Variance
B.t H-D 133 Aizawai	179.60	1.22	101.6-257.6	0.008	159.62	1.09	92.1- 236.2	0.009
B.t H-D 234 Galleriae	166.64	1.09	101.6- 263.4	0.009	138.66	1.46	84.1-143.1	0.007
B.t ENRC 60	177.14	1.09	113.1-271.2	0.009	181.24	1.36	140.1-283.2	0.009
B.t. ENRC 63	66.22	1.18	73.4-182.5	0.009	58.51	1.41	70.5-145.5	0.008

Table 8: Potency of different spore-crystal mixtures recovered form fermentation beers of four *B.t.* strains in the presence of lactose or whey ultrafiltrate against *S. exigua*

Source of spore-crystal mixtures	Spore-crystal mixtures recovered in the presence of lactose solution.				Spore-crystal mixtures recovered in the presence of whey ultrafiltrate			
	LC_{50} (ug/ml)	Slope	95% Confidence limits	Variance	LC_{50} (ug/ml)	Slope	95% Confidence limit	Variance
B.t. H-D 133 Aizawai	189.50	1.21	11.6-267.5	0.008	168.62	1.46	94.1-306.2	0.007
*B.t.*H-D 234 Galleriae	170.3	1.22	114.6 –258.3	0.008	126.11	1.43	65.7-175.5	0.008
B.t. ENRC 60	74.3	1.33	131.1- 293.1	0.008	68.32	1.36	159.3- 315.4	0.008
B.t. ENRC 63	1710.5	1.04	78.3 –285.1	0.008	115.34	1.46	78.3-159.3	0.008

accumulates piruvate, butirate, Lactate, acetate and ploy beta hydroxy butirate under different culture condition, even more, it is able to metabolize them as well as amino acids Dulmage (1993) showed that there was a considerable range in yields between the different stereotypes.

Recovery of spore-crystal mixture from fermentation beer: Table 1 shows the data obtained when lactose solution or whey ultrafiltrate utilized in spore-crystal mixture recovery from fermentation beer adjusted to pH 7.0 at the end of fermentation period and before applying the recovery method. Similarly when the fermentation beer acidified to pH 4.0 at the end of fermentation period and before carried out the spores-crystal mixture recovery (Table 2).
Data show that whey ultrafiltrate was more efficient in spores-crystal recovery than lactose solution for *B.t* H-D 234 and *B.t* ENRC 60 as about 7.0 and 5% increase in the recovery were achieved respectively. On the other had no advantage for whey ultra filtrate or lactose solution in recovery of spores-crystal mixture from the fermentation beer of *B.t* H-D 133 or *B.t* ENRC 63 (Table 1). There was advantages however, in spores-crystal mixture recovery yield when the fermentation beers were adjusted to pH 4.0 before conducted the recovery (Table 2). The advantages were higher with utilizing whey ultra filtrate and the highest increases were attired with *B.t* H-D 234 and *B.t* ENRC 60 as it were 8 and 11% respectively compared to the opposite for pH 7.0. The advantage obtained by applying whey ultrafiltrate instead of lactose solution may be due to presence of some components aids in a flocculent of the product (cell debris, spores, crystals and cells) which upgrade the critical sedimentation rate (Villafana, 1990). This effect was previously reported by Cords and Fisher (1966). For recovery purposes, broth was acidified till pH 4.0 and this process did not modify the toxicity of the product (Villafana, 1990) Rodrignez *et al.* (1993) showed that the recovery of crystal and spores was enhanced when some insoluble proteins remained in the broth if pH is adjusted to pH 4.0. **The mortality effect of the spore-crystal mixtures:** Tables 5, 6, 7, and 8 show the potencies of different spore-crystal mixtures recovered from the fermentation beer of four *B.t* strains utilizing either lactose solution or whey ultrafiltrate. The potency was varied among the tested strains as well as the potency was varied according to the agent used in the recovery of spore-crystal mixture. The larvae of potato tuber moth *(P. operculella)* and spin boll worm *(E. insulana)* were highly sensitive the pathogen in spore-crystal mixtures for all tested *B.t* strains than *S. littoralis* and *S. exigua*. Tables 5 and 6 show that spore-crystal mixtures recovered were more effective on *P. operculella* larvae than the larvae of *E. insulana.* The recovered spore-crystal mixtures from the fermentation beers of *B.t* H-D133 and *B.* ENRC 60 in the presence of whey ultrafiltrate were more toxic against *P. operculella* than the recovered in the presence of lactose solution as LC_{50} was 29.19 and 38.8 for *B.t* H-D133 and 40.02 and 43.03 for *B.t* ENRC 60 respectively (Table 5). On the other hand no significant potencies were shown against the bests when the spore-crystal mixtures of recovered from fermentation beer of both *B.t* H-D 234 and *B.t* ENRC 63 in the presence of either lactose solution or whey ultrafiltrate.
Significant difference in toxic effect for the spore-crystal mixtures recovered from fermentation beer of *B.t* ENRC 60 in the presence of whey ultrafiltrate and that obtained in the presence of lactose solution against larvae of *E. insulana* as LC_{50} were 49.52 and 65.31 ug / ml respectively (Table 6). Nearly equal effect for spore-crystal mixtures recovered using lactose solution or whey ultrafiltrate to recover spore-crystal mixtures from the fermentation beers of both *B.t* H-D133 and *B.t.* ENRC 63 also, spore-crystal mixtures recovered from fermentation beer of *B.t* ENRC 63 was effective on *S. littoralis* larvae than that recovered from fermentation beers of other tested strains (Table 7). Whereas, spore-crystal mixtures recovered from fermentation beer of *B.t* ENTÝRC 60 was more effective on *S. exigua* than that recovered from fermentation beers of other strains under study (Table 8).
Tables 6 and 7 show promising results were achivied when whey ultrafiltrate was replaced lactose solution for recovering spore-crystal mixtures from fermentation beers of the cultures of *B.t* H-D133, *B.t* H-D 234 and *B.t* ENRC 63 , when LC_{50} was taken a critiria. Whereas, the same was not for the recovery of spore-crystal mixture of *B.t* ENRC 60.

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