Journal of Industrial Microbiology & Biotechnology (1999) 23, 497–502

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The effect of complex carbon and nitrogen, salt, Tween-80 and acetate on delta-endotoxin production by a *Bacillus thuringiensis* subsp *kurstaki*

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Delta-endotoxin production by a strain of *Bacillus thuringiensis* subsp *kurstaki* on complex media based on crude gruel and fish meal was investigated. High proteolytic activities were concomitantly produced with the bioinsecticide. In such complex media, the repressive regulation due to readily consumed carbon sources was partially overcome. In order to improve substrate assimilation, 0.5 g L^{-1} sodium chloride and 0.1% Tween-80 were supplemented to the production medium, increasing delta-endotoxin yields when using gruel concentrations below 59 g L^{-1}. At and beyond 75 g L^{-1} gruel, delta-endotoxin yields were not affected in the presence of 0.5 g L^{-1} NaCl and 0.1% Tween-80, but proteolytic activity yields were remarkably reduced. Thus, the use of sodium chloride and Tween-80 allowed reduction of the initial gruel concentration to 42 g L^{-1} for the production of 3350 mg L^{-1} delta-endotoxin, while it was only 3800 mg L^{-1} with 92 g L^{-1} gruel. Moreover, similar to 0.5 g L^{-1} NaCl and 0.1% Tween-80, the use of 10 g L^{-1} sodium acetate significantly improved delta-endotoxin production and also reduced the proteolytic activity to 250 U ml^{-1}.

Keywords: *Bacillus thuringiensis*; delta-endotoxin; gruel; fish meal; proteases; sodium chloride; sodium acetate

Introduction

Bacillus thuringiensis (Bt) is a Gram-positive, spore-forming, insecticidal crystal protein-producer. The crystals are composed of proteins named delta-endotoxins which exhibit larvicidal toxicity upon ingestion by susceptible insect larvae, dissolution and activation by larval gut-juice proteases [1]. Delta-endotoxins are synthesized concomitantly with sporulation. The bioinsecticide is composed of spores and crystals. Bt represents 90% of the bioinsecticide used for the control of pests in agriculture, forestry and veterinary medicine [8]. In previous work [17], we investigated the nutritional requirements of a newly isolated *B. thuringiensis* subsp *kurstaki*, which is toxic to Lepidoptera (S Tounsi, Centre de Biotechnologie de Sfax, Tunisia, unpublished results). These results showed several nutritional limitations, especially related to organic and inorganic nitrogen sources, amino acid needs and, particularly, carbon metabolic repression which regulated toxin production. Gruel hydrolysate, prepared by alpha-amylase and protease hydrolysis, is efficient for bioinsecticide production, but in order to improve the final concentration of delta-endotoxin, it is important to overcome the repressive regulation. One way could be to use complex substrates which need preliminary endogenous hydrolysis before assimilation. This alternative could be useful because it is well-known that bacteria related to the genus *Bacillus* produce many hydrolases such as proteases and amylases [12] which are involved in assimilation of proteins and starch-based compounds, respectively [11]. Moreover, several media based on complex substrates such as corn steep liquor [4], peptones [16] blackstrap molasses and Great Northern White Bean concentrate [8] are efficient for Bt bioinsecticides production, but, the role of hydrolytic activities, produced during the growth cycle, in the assimilation of complex carbon or nitrogen sources was not elucidated. However, we have characterized the metalloproteases produced by a selected *B. thuringiensis kurstaki* strain, BNS3 [19]. Gruel, a cheap by-product of semolina factories, is available in large quantities. Fish meal has been widely used as a nitrogen source for Bt production [14].

In order to overcome carbon catabolite repression, we opted for the combination of crude gruel and fish meal for the formulation of media supporting growth, sporulation and delta-endotoxin production. First, we determined the production of extracellular proteolytic and amylolytic activities by strain BNS3 on several media used for delta-endotoxin production. Since we observed overproduction of proteolytic activities by our strain when it was grown on crude or hydrolyzed gruel, we studied the implication of such activities in the assimilation of crude gruel and fish meal for bioinsecticide production. Moreover, we investigated culture conditions which reduce the proteolytic activities to levels just enabling assimilation of the complex substrates. This alternative is environmentally safer than the approach of Donovan *et al* [3] who improved crystal protein stability and yield by genetic deletion of specific proteases from genetically modified *B. thuringiensis*.

Materials and methods

Bacillus thuringiensis strain

The strain BNS3 was *Bacillus thuringiensis* subsp *kurstaki* (serotype H 3a, 3b, 3c) from our collection of Bt strains

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Received 5 November 1998; accepted 19 August 1999

isolated at our Institute according to Jaoua *et al* [5]. This strain was selected for large-scale production of bioinsecticides because of its high toxicity to Lepidopteran insect larvae, including *Prays olea*, *Ephestia kuehniella*, *Spodoptora exigua* and *Ostrinia nubilalis* (S Tounsi, Centre de Biotechnologie de Sfax, Tunisia, unpublished results). The nutritional requirements of this strain were studied [17]. The acrystalliferous strain BNS3 cry^- spo^+ was obtained by plasmid curing from the wild strain BNS3 (S Tounsi, unpublished results).

Gruel and fish meal sources

Gruel was obtained from a local semolina factory processing durum wheat to produce mainly 65% semolina, 10% flours, 14% gruel and other by-products. Gruel contained 60% starch, 5% other carbohydrates, 1.5% cellulose and 12% gluten. Fish meal was obtained from the Office National de la Pêche, Mahdia, Tunisia. It contained 43% proteins.

Bioinsecticide production

The strain was grown in liquid media under conditions optimized for delta-endotoxin production [17]. The carbon source was either total sugars from gruel hydrolysate prepared according to Zouari *et al* [17] or from crude gruel (Table 1). The media also contained ammonium sulfate, yeast extract and fish meal as indicated in the Results section. The following minerals were used (g L^{-1}): KH_2PO_4, 1; K_2HPO_4, 1; $MgSO_4$, 0.3; $MnSO_4$, 0.01 and $FeSO_4$, 0.01. The pH was adjusted to 7 before sterilization. In each shake flask, 1 g of $CaCO_3$ was added for pH stabilization. Carbon source and $CaCO_3$ were sterilized separately. Inoculated flasks, containing 50 ml of culture medium, were incubated 72 h at 30°C in a rotary shaker set at 200 rpm.

Supplemental sodium chloride, Tween-80 and sodium acetate

The strain was grown in the crude gruel media as indicated in the Results section. Sodium chloride, Tween-80 (polyoxythelene sorbitan monostearate for bacteriology, Fluka AG, CH-9470 Buchs, Switzerland) and sodium acetate were added as supplements. Concentrations and combinations of these chemicals are mentioned together with the results.

Table 1 Growth, delta-endotoxin production and proteolytic and amylolytic activities in culture media based on different preparations of gruel. Enzymatic hydrolysis of gruel was performed according to Zouari *et al* [17]

Gruel	Endotoxin (mg L^{-1})	Proteolytic activity (U ml^{-1})	Amylolytic activity (U ml^{-1})	CFU (10^9 ml^{-1})	Toxin/TS[a] (mg g^{-1})
Centrifuged hydrolysed	954 ± 48	850 ± 34	3.5 ± 0.1	5.3 ± 0.2	63 ± 3
Crude hydrolysed	750 ± 37	1715 ± 68	0.5 ± 0.1	6.5 ± 0.2	50 ± 2
Crude	1267 ± 63	1917 ± 77	4.5 ± 0.1	4.1 ± 0.2	84 ± 4

[a]TS, total sugars.

Biomass and delta-endotoxin determination

After 72 h, the cultures contained a mixture of spores, crystals and minor cell debris. The number of spores was estimated by colony forming units (CFU), at the end of the experiments. Samples were heated at 70°C for 10 min and appropriate dilutions were plated on solid LB medium [15]. Delta-endotoxin was determined as follows [17]: 1 ml of culture medium was centrifuged for 10 min at 10 000 × *g* and the pellet was washed twice with 1 M NaCl and twice with distilled water. The pellet was then suspended in 1000 μl of 50 mM NaOH (pH 12.5). After 3 h incubation at 30°C, total proteins in the supernatant were measured using Bio-Rad reagent (Bio-Rad Protein assay, cat. 500–0006, München, Germany). The acrystalliferous strain BNS3 cry^- spo^+, obtained by plasmid curing from the wild strain BNS3 was used as a negative control in order to take into account contaminating dissolved proteins from spore coat, cell debris and particulate or insoluble matter. The negative control value was determined for every experiment related to each cultivation condition. We considered the effect of growth conditions on insoluble protein content and thus the contribution of particulate matter to the protein levels measured. Moreover, the high purity of the crude solubilized crystal proteins from gruel- and fish meal-based media was proven by SDS-PAGE analysis as described by Zouari and Jaoua [18]. We showed by Coomassie blue gel coloration that the electrophoretic pattern contained only the two expected major bands, corresponding to the protoxin and the activated toxin forms of BNS3 delta-endotoxins [18]. Statistical treatment of the results showed maximal deviations of 5%. The presented values are the average results of three determinations for two separate experiments carried out for each cultivation condition. Delta-endotoxin production was calculated as: delta-endotoxin (mg L^{-1}) divided by the initial gruel concentration (g L^{-1}).

Proteolytic activity assay

Proteolytic activity was determined according to Kembhavi *et al* [6]: 1% of casein substrate was prepared in 0.1 M Tris-HCl buffer at pH 7.0. The reaction mixture consisting of 1 ml of casein and 1 ml of diluted enzyme preparation was incubated for 20 min at 60°C. This incubation temperature (60°C) corresponds to the optimal temperature for the proteolytic activity in the supernatant of BNS3 culture media [19]. The reaction was terminated with 3 ml of 5% TCA (trichloroacetic acid). The absorbance of the filtrate was measured at 280 nm. Parallel blanks were prepared with inactivated enzyme samples treated for 5 min in boiling water. The enzyme was diluted to give linear reaction product after 20 min incubation. The appropriate dilutions were those which ensured a final absorbance of almost 0.5. One proteolytic activity unit was defined as the amount of enzyme preparation required to liberate 1 μg of tyrosine per minute under the experimental conditions. The statistical treatment of the results showed maximal deviations of 4%. The values presented are the averages of three determinations of two separate experiments for each cultivation condition. The yield of proteolytic activity in the culture media was: proteolytic activity (U L^{-1}) divided by initial gruel or fish meal concentration (g L^{-1}).

Amylolytic activity assay

Amylolytic activity was measured according to the Miller method [7]: 0.5 ml of 1% starch in 0.1 M Tris-HCl buffer at pH 6.0 was mixed with 0.5 ml of appropriately diluted enzyme sample and incubated for 30 min at 40°C. The reaction was stopped with 3 ml 3,5-dinitrosalicylic acid and boiled for 10 min. Distilled water (20 ml) was added to the reaction medium and the OD at 545 nm was determined. Parallel blanks were run with inactivated enzyme samples treated during 5 min in boiling water. One amylolytic activity unit was defined as the amount of enzyme preparation required to liberate 1 μg of glucose per min under the experimental conditions. The values presented are the averages of three determinations for two separate experiments carried out for each cultivation condition.

Results and discussion

Evidence of delta-endotoxin production on different gruel preparations

To evaluate the capacity of strain BNS3 to grow and to produce delta-endotoxin on gruel-based media, gruel was used in different forms: enzymatically hydrolyzed gruel according to Zouari *et al* [17], the supernatant of centrifuged hydrolysate of gruel, and crude gruel. Total sugars from these preparations (15 g L^{-1}) were supplemented by 5 g L^{-1} yeast extract, 5.4 g L^{-1} ammonium sulfate and mineral salts according to the procedure described in Materials and Methods. The role of hydrolytic activities in the assimilation of the principal components of gruel, mainly gluten and starch, was investigated through the determination of proteolytic and amylolytic activities in the supernatant of each medium at the end of the culture. In spite of different spore counts, crude gruel gave the best delta-endotoxin production compared to the hydrolysates (Table 1). Although amylolytic activities were low in the centrifuged hydrolysate and in the crude gruel, they were negligible in the crude hydrolysate. This may be explained by different induction events of amylase gene expression due to different concentrations and compositions of inducers. Proteolytic activities were high: 1715 U ml^{-1} and 1917 U ml^{-1}, respectively, in crude hydrolysate and crude gruel, but only 850 U ml^{-1} in the centrifuged hydrolysate. These differences in proteolytic activity levels could be explained by strong protease induction by insoluble gluten components in addition to induction due to products of their proteolysis. Thus, the strain produced the bioinsecticide efficiently on complex carbon sources by producing high hydrolytic activities, mainly proteolytic activities. On 25 g crude gruel, the yield was 84 mg delta-endotoxin g^{-1} total sugars, while it was 64 mg g^{-1} total sugars from centrifuged gruel hydrolysate. We have previously shown for the same strain that the delta-endotoxin yield was only 49 mg delta-endotoxin g^{-1} glucose [17].

Production of delta-endotoxin on crude gruel and fish meal

Since strain BNS3 produced protease activities of almost 1800 U ml^{-1} and enough amylase activities to enable the assimilation of crude gruel components, we studied the possibility of substituting yeast extract and ammonium sulfate by a complex source of amino acids and nitrogen, fish meal (Table 2). With 25 g L^{-1} crude gruel, corresponding to 15 g L^{-1} starch, and 5 g L^{-1} yeast extract, the highest delta-endotoxin production was obtained with 5.4 g L^{-1} ammonium sulfate. These results are similar to those we obtained with gruel hydrolysate, showing the importance of the combination of yeast extract to a mineral nitrogen source [17]. When using fish meal at 5 g L^{-1} or 10 g L^{-1}, no more than 2.7 g L^{-1} ammonium sulfate were needed. With 20 g L^{-1} fish meal, ammonium sulfate reduced delta-endotoxin production. Regulation by nitrogen metabolic repression of delta-endotoxin production by BNS3 strain [17] and of spore formation in several Bt strains [9] has been reported.

Evaluation of the metabolic repression of bioinsecticide production by crude gruel and fish meal

BNS3 delta-endotoxin synthesis is regulated by carbon and nitrogen metabolic repression as demonstrated using readily assimilated substrates: glucose, yeast extract and ammonium sulfate [17]. In order to evaluate the importance of this repression when using complex substrates, increased crude gruel concentrations were tested with 20 g L^{-1} fish meal. The yield of delta-endotoxin production decreased significantly with increasing crude gruel concentrations up to 59 g L^{-1}. But beyond this concentration and up to 92 g L^{-1}, yields were stable (42 mg delta-endotoxin g^{-1} gruel) (Table 3). Previous results obtained with glucose

Table 2 Growth, delta-endotoxin production and proteolytic activity in culture media based on 25 g L^{-1} crude gruel, yeast extract, ammonium sulfate and fish meal

Organic and inorganic nitrogen source (g L^{-1})	Endotoxin (mg L^{-1})	Proteolytic activity (U ml^{-1})	CFU (10^9 ml^{-1})	Toxin/gruel (mg g^{-1})
Ammonium sulfate (2.7) + Yeast extract (5)	1180 ± 59	1736 ± 69	3.2 ± 0.2	47 ± 2
Ammonium sulfate (5.4) + Yeast extract (5)	1298 ± 65	1866 ± 75	2.1 ± 0.2	52 ± 2
Ammonium sulfate (8) + Yeast extract (5)	1157 ± 58	1545 ± 62	3.3 ± 0.2	46 ± 2
Ammonium sulfate (2.7) + Fish meal (5)	1220 ± 61	1496 ± 60	1.2 ± 0.2	49 ± 2
Ammonium sulfate (5.4) + Fish meal (5)	1178 ± 59	1756 ± 70	1.1 ± 0.2	47 ± 2
Ammonium sulfate (2.7) + Fish meal (10)	1351 ± 67	1650 ± 66	1.3 ± 0.2	54 ± 2
Ammonium sulfate (2.7) + Fish meal (20)	1381 ± 69	1685 ± 67	3.8 ± 0.2	55 ± 3
Fish meal (10)	1248 ± 63	1925 ± 77	2.7 ± 0.2	50 ± 2
Fish meal (20)	1584 ± 79	1585 ± 63	2.8 ± 0.2	63 ± 3

Table 3 Metabolic repression due to increased concentrations of carbon source from crude gruel on delta-endotoxin production. The experiments were carried out with 20 g L^{-1} fish meal

Gruel (g L^{-1})	Endotoxin (mg L^{-1})	Proteolytic activity (U ml^{-1})	CFU (10^9 ml^{-1})	Toxin/gruel (mg g^{-1})	Proteolytic activity/gruel (10^3 U g^{-1})
25	1584 ± 79	1585 ± 63	2.8 ± 0.2	63 ± 3	63 ± 3
42	2093 ± 105	1640 ± 66	4.7 ± 0.3	50 ± 2	39 ± 2
59	2488 ± 124	1775 ± 71	8.2 ± 0.3	42 ± 2	30 ± 1
75	3069 ± 153	2084 ± 83	15.8 ± 0.5	41 ± 2	28 ± 1
92	3841 ± 192	1842 ± 74	38.4 ± 0.6	42 ± 2	20 ± 1
108	4067 ± 203	1999 ± 80	43.1 ± 0.6	38 ± 2	18 ± 1

Table 4 Effect of increased fish meal concentrations on delta-endotoxin production and proteolytic activity in culture media based on 92 g L^{-1} gruel

Fish meal (g L^{-1})	Endotoxin (mg L^{-1})	Proteolytic activity (U ml^{-1})	CFU (10^9 ml^{-1})	Toxin/gruel (mg g^{-1})	Proteolytic activity/fish meal (10^3 U g^{-1})
0	2125 ± 106	875 ± 35	30.1 ± 0.6	23 ± 1	–
10	3128 ± 156	2393 ± 95	30.4 ± 0.6	34 ± 2	239 ± 9
15	3330 ± 167	2059 ± 82	35.4 ± 0.6	36 ± 2	137 ± 5
20	3901 ± 195	1862 ± 74	38.4 ± 0.6	42 ± 2	93 ± 4
30	4087 ± 204	1616 ± 64	35.5 ± 0.6	44 ± 2	54 ± 3
35	4030 ± 201	1401 ± 56	36.3 ± 0.6	44 ± 2	40 ± 2
40	4103 ± 205	1376 ± 55	36.1 ± 0.6	44 ± 2	34 ± 1

Table 5 The organic and inorganic nitrogen need for production of bioinsecticide on 92 g L^{-1} gruel

Nitrogen sources (g L^{-1})	Endotoxin (mg L^{-1})	Proteolytic activity (U ml^{-1})	CFU (10^9 ml^{-1})	Toxin/gruel (mg g^{-1})
Fish meal (20)	3982 ± 199	1888 ± 76	38.1 ± 0.6	43 ± 2
Fish meal (20) + YE[a] (2.5)	2420 ± 121	2000 ± 80	40.7 ± 0.6	26 ± 1
Fish meal (20) + AS[a] (2.7)	3781 ± 189	2500 ± 100	49.8 ± 0.7	41 ± 2

[a]YE, yeast extract; AS, ammonium sulfate.

Table 6 Effect of sodium chloride and Tween-80 on delta-endotoxin production and proteolytic activity. The experiments were performed with 92 g L^{-1} gruel and 20 g L^{-1} fish meal

NaCl (g L^{-1})	Tween-80 (%)	Endotoxin (mg L^{-1})	Proteolytic activity (U ml^{-1})	CFU (10^9 ml^{-1})	Toxin/gruel (mg g^{-1})
0.00	0.0	3929 ± 196	1848 ± 74	39.0 ± 0.6	43 ± 2
0.25	0.1	3797 ± 190	1353 ± 54	38.7 ± 0.8	41 ± 2
0.50	0.1	3889 ± 194	1120 ± 45	38.3 ± 0.6	42 ± 2
0.75	0.1	3807 ± 190	1134 ± 45	39.6 ± 0.6	41 ± 2
0.00	0.1	3964 ± 198	1831 ± 73	39.2 ± 0.6	43 ± 2

Table 7 Metabolic repression due to increased concentrations of carbon source from crude gruel, on delta-endotoxin production in the presence of 0.5 g L^{-1} NaCl and 0.1% Tween-80. The experiments were carried out with 20 g L^{-1} fish meal

Gruel (g L^{-1})	Endotoxin (mg L^{-1})	Proteolytic activity (U ml^{-1})	CFU (10^9 ml^{-1})	Toxin/gruel (mg g^{-1})	Proteolytic activity/gruel (10^3 U g^{-1})
25	2237 ± 112	1756 ± 70	2.8 ± 0.2	89 ± 4	70 ± 3
42	3338 ± 167	2436 ± 97	5.1 ± 0.3	79 ± 4	58 ± 3
59	3356 ± 168	2471 ± 99	8.2 ± 0.3	57 ± 3	42 ± 2
75	3100 ± 155	1350 ± 54	15.8 ± 0.5	41 ± 2	18 ± 1
92	3815 ± 190	1151 ± 46	38.4 ± 0.6	41 ± 2	13 ± 1
108	3996 ± 200	1095 ± 44	43.1 ± 0.6	37 ± 2	10 ± 1

Table 8 Effect of increased fish meal concentrations on delta-endotoxin production and proteolytic activity in the presence of 0.5 g L^{-1} NaCl and 0.1% Tween-80. The experiments were performed with 92 g L^{-1} crude gruel

Fish meal (g L^{-1})	Endotoxin (mg L^{-1})	Proteolytic activity (U ml^{-1})	CFU (10^9 ml^{-1})	Toxin/gruel (mg g^{-1})	Proteolytic activity/fish meal (10^3 U g^{-1})
0	2200 ± 110	909 ± 35	30.1 ± 0.6	24 ± 1	–
10	3709 ± 186	1803 ± 95	30.4 ± 0.6	40 ± 2	180 ± 9
15	3878 ± 204	1688 ± 82	35.4 ± 0.6	42 ± 2	113 ± 5
20	3995 ± 200	1119 ± 45	38.4 ± 0.6	43 ± 3	56 ± 2
30	3887 ± 194	1085 ± 44	35.5 ± 0.6	42 ± 2	36 ± 1

[17] showed strong reduction of BNS3 delta-endotoxin yields beyond 15 g L^{-1} glucose, indicating the importance of carbon catabolite repression in regulating delta-endotoxin synthesis by strain BNS3. Moreover, even with this concentration, the yield was only 49 mg delta-endotoxin g^{-1} glucose, significantly reduced compared to that obtained in the present work with 25 gruel (63 mg delta-endotoxin g^{-1} gruel). Thus the use of crude gruel and fish meal limited carbon catabolite repression and allowed production of 3841 mg L^{-1} delta-endotoxin when using 92 g L^{-1} gruel, compared to 1584 mg L^{-1} delta-endotoxin produced at 25 g L^{-1} gruel. Glucose, a preferred carbon source, represses a number of operons and genes in both Gram-negative and -positive bacteria by catabolite repression [10]. Components of the phosphoenolpyruvate-dependent sugar transport system are considered to play a central regulatory role in glucose-mediated repression of genes in bacteria. Regarding delta-endotoxin synthesis, glucose repressed *cry* IVA toxin protein induction in *B. thuringiensis* subsp *israeliensis* [2]. The *cry* IVA mRNA levels are suppressed in the presence of glucose [2]. In the present work, we partially overcame carbon catabolite repression of strain BNS3 delta-endotoxin synthesis by use of complex substrates. On the other hand, proteolytic activity levels were not strongly affected by varying gruel concentrations as shown by the continuous decrease of the proteolytic activity yields, showing the complex regulation of protease synthesis in complex media as well as the possible effect of media modifications on proteolytic activities.

A gruel concentration of 92 g L^{-1} was chosen to evaluate the importance of various fish meal concentrations. The highest yields of delta-endotoxin were obtained at and

beyond 20 g L^{-1} fish meal (Table 4). This concentration was used in further experiments. Moreover, the proteolytic activities were reduced with increased fish meal concentrations, demonstrated by a strong decrease in proteolytic activity, but when the production was conducted without fish meal, delta-endotoxin and proteolytic activity were poor. These results indicate that in crude gruel-based media, synthesis of delta-endotoxin depended on the composition and the concentration of the organic nitrogen sources. This conclusion may be supported by the fact that strain BNS3 is auxotrophic for several amino acids and that glutamic acid and cysteine improved bioinsecticide production [17]. The reduction in proteolytic activities could be explained by repressive regulation due to the nitrogen source [9].

Since the combination of organic and inorganic nitrogen sources improves bioinsecticide production [17], the effect of 2.7 g L^{-1} ammonium sulfate and 2.5 g L^{-1} yeast extract on delta-endotoxin and proteolytic activity production was tested at 92 g L^{-1} crude gruel and 20 g L^{-1} fish meal. The final CFU show that the composition of the nitrogen source affected strain BNS3's growth (Table 5). Yeast extract dramatically reduced delta-endotoxin synthesis but not proteolytic activity. Ammonium sulfate did not improve bioinsecticide synthesis but increased proteolytic activity. Nitrogen sources from gruel and fish meal were sufficient for *B. thuringiensis* bioinsecticide production. Nevertheless, this complex medium supported high protease activity which might have further effect on stability of the bioinsecticide. This effect could be predicted since Donovan *et al* [3] showed that crystal protein stability and yield may be improved by deletion of specific protease genes.

Involvement of sodium chloride and Tween-80 in delta-endotoxin and protease synthesis

To find conditions which reduce proteolytic activity in culture media, several chemicals were used. Sodium chloride was used in several complex media to enhance the availability of soluble proteins [8]. Tween-80 was used as a surfactant to increase bacterial cell membrane permeability and consequent nutrient uptake [8]. The results obtained with the addition of these chemicals to 92 g L^{-1} gruel and 20 g L^{-1} fish meal are summarized in Table 6. Spore counts and delta-endotoxin levels were not strongly affected by sodium chloride and Tween-80, but reduction in protease activity was noticed. Morries *et al* [8] showed that the increase of nutrient solubility increased the toxicity of *B. thuringiensis* subsp *aizawai* strain HD133 but they did not quantify delta-endotoxin and proteolytic activities. We attribute the increased toxicity to the stability of the bioinsecticide. For pilot plant production of *B. thuringiensis* subsp *galleria*, 0.02–0.05% NaCl was used in the medium [14].

Since sodium chloride and Tween-80 increase assimilation of culture nutrients, it was important to evaluate the gruel and fish meal concentrations to be used. Results shown in Table 7, compared to those of Table 3, clearly show that up to 59 g L^{-1} gruel, these chemicals significantly improved the yields of delta-endotoxin production. It is important to note that at and beyond 75 g L^{-1} gruel, the yields of delta-endotoxin were similar to those obtained in the absence of NaCl and Tween-80. This could be due to oxygen limitation and to pH effects due to increased gruel concentrations. Reduction of protease activities was not observed up to 59 g L^{-1} gruel which may be explained by an easier assimilation of nitrogen organics from gruel compared to several organics from fish meal. This conclusion was confirmed by the results shown in Table 8 showing that at 92 g L^{-1} gruel, sodium chloride and Tween-80 reduced the requirement for fish meal to 10–15 g L^{-1} but with no reduction in proteolytic activity.

It is clear from these results that the use of sodium chloride and Tween-80 could reduce the initial concentration of gruel from 92 g L^{-1} to 42 g L^{-1}. The choice should be based on the balance between delta-endotoxin and proteolytic activities.

Effect of sodium acetate on delta-endotoxin and proteolytic activity

Rowe and Margaritis [13] showed that *B. thuringensis* oxidizes carbohydrates aerobically to form organic acids, mainly acetate, causing a decrease of pH to 5.8. These acids are further oxidized to carbon dioxide causing an increase of the pH to 8. We investigated the effect of sodium acetate on bioinsecticide production in several media based on 42 g L^{-1} gruel and 20 g L^{-1} fish meal. We also studied the correlation between the effect of sodium acetate and that of sodium chloride and Tween-80 (Table 9). At 5 g L^{-1}

Table 9 Effect of sodium chloride, Tween-80 and sodium acetate on delta-endotoxin production and proteolytic activity in 42 g L^{-1} gruel and 20 g L^{-1} fish meal

Sodium acetate (g L^{-1})	NaCl (g L^{-1})	Tween-80 (%)	Toxin (mg L^{-1})	Proteolytic activity (U ml^{-1})	Toxin/gruel (mg g^{-1})	Proteolytic activity/fish meal (10^3 U g^{-1})
0	0.0	0.0	2080 ± 104	1607 ± 64	50 ± 2	80 ± 4
5	0.0	0.0	2213 ± 111	1342 ± 54	53 ± 2	67 ± 3
5	0.5	0.0	3351 ± 168	2154 ± 86	78 ± 4	108 ± 4
5	0.5	0.1	3235 ± 162	1200 ± 48	77 ± 3	60 ± 2
5	0.0	0.1	2360 ± 118	1370 ± 55	56 ± 3	69 ± 3
0	0.5	0.1	3355 ± 168	2400 ± 96	80 ± 4	120 ± 5
10	0.0	0.0	3008 ± 150	250 ± 10	72 ± 3	12.5 ± 0.5
10	0.5	0.0	2759 ± 138	350 ± 14	66 ± 3	17.5 ± 0.7
10	0.5	0.1	2846 ± 142	243 ± 10	68 ± 3	12.2 ± 0.5
10	0.0	0.1	2974 ± 149	134 ± 5	71 ± 3	6.7 ± 0.3

acetate, delta-endotoxin concentration was slightly improved, but proteolytic activities were reduced to 84%. Addition of 0.5 g L^{-1} NaCl gave similar delta-endotoxin production to that obtained without acetate, but with no significant reduction of proteolytic activities. Addition of Tween-80 to 5 g L^{-1} acetate and 0.5 g L^{-1} NaCl did not improve delta-endotoxin reduction, but reduced protease activities. At 10 g L^{-1}, sodium acetate reduced proteolytic activities and improved delta-endotoxin concentration up to 3000 mg L^{-1}. Addition of NaCl and/or Tween did not improve bioinsecticide production.

These results clearly show reduction in proteolytic activities in conjunction with an increase in delta-endotoxin production. This might be caused by improving cell nutrient assimilation, deviation of cell metabolism due to acetate, osmotic changes, modifications of the growth rate, pH or other interactions.

Conclusion

Media based on both crude gruel and fish meal were efficient for *B. thuringiensis* growth and delta-endotoxin synthesis, compared to glucose or gruel hydrolysates. New media were formulated for the production of more than 3300 mg L^{-1} delta-endotoxin, indicating that such complex media are able to overcome carbon catabolite repression. The high proteolytic activities produced concomitantly with the bioinsecticide could be reduced by sodium acetate or sodium chloride when using high concentrations of gruel. With low gruel concentrations, the reduction in proteolytic activity was not observed, even with high fish meal concentrations. Thus, the reduction of protease activities could be due to our particular culture conditions, without the need to proceed to genetic manipulation [3].

Acknowledgements

This work was supported by grants from the Tunisian Secrétariat d'Etat à la Recherche Scientifique et à la Technologie and from the International Center for Genetic Engineering and Biotechnology (ICGEB).

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