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Nutritional Requirements of a Strain of *Bacillus thuringiensis* subsp. *kurstaki* and Use of Gruel Hydrolysate for the Formulation of a New Medium for δ-Endotoxin Production

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ABSTRACT

Nutritional requirements of a strain of *Bacillus thuringiensis* (Bt) subsp. *kurstaki* were elucidated for δ-endotoxin production. The effect of some principal nutrients was deeply investigated, showing several nutritional and metabolite limitations in Bt growth and δ-endotoxin synthesis. This led us to formulate a new medium based on the hydrolysate of gruel, a cheap and abundant byproduct of semolina factories, supporting growth and δ-endotoxin synthesis. After hydrolysis of gruel by α-amylase, followed by proteolysis using alcalase, the resultant soluble material substituted glucose very well for Bt δ-endotoxin production. Indeed, 15 g/L total sugars coming from that hydrolysate, supplemented by 5.4 g/L ammonium sulfate as nitrogen source and either 5 g/L yeast extract or 3 g/L peptone from casein or 3 g/L casaminoacids or 0.25 g/L cysteine or aspartic acid, were the principal components of this new medium in which almost 1 g/L of δ-endotoxin in 4.5 g/L total dry biomass was produced.

Index Entries: *Bacillus thuringiensis*; δ-endotoxin; gruel hydrolysate; metabolite repression; optimization of bioinsecticide production.

INTRODUCTION

Insecticidal activity of *Bacillus thuringiensis* (Bt) is based on spores and crystals. The crystals are synthesized concomitantly with sporulation, and are composed of proteins named δ-endotoxin. Upon ingestion by susceptible

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insect larvae, the crystals are dissolved in the high alkaline larval gut-juices, and involve the disruption of the epithelial cells, which leads to larvae death *(1)*. Crystals are most responsible for the toxicity and spores are not directly involved, but a qualitative effect on mortality can be assessed *(2)*. Indeed, large quantities of spores with high insecticidal activity are required for practical applications *(3)*. This means that when producing Bt as bioinsecticide, a high spore count is not sufficient to ensure toxicity, and it is necessary to reach high δ-endotoxin titers. One of the most underreported aspects of Bt is that of production and formulation, although there are certain works in connection with Bt growth on several synthetic or complex media *(2–6)*. In general, Bt medium consisted of carbon (C) and energy sources, such as glucose *(2–7)* and corn steep liquor *(8)*, and nitrogen (N) sources, such as peptones, yeast extract, and ammonium sulfate and minerals. Sikdar et al. *(9)* optimized phosphate, carbonate, and magnesium concentrations, and showed also that iron, manganese, and copper are required at low levels for toxin production. Arcas et al. *(6)* reported that yeast extract is essential as an amino acid source, but not as a vitamin source. Goldberg et al. *(8)* indicated that media supporting high vegetative cell growth may not be adequate for sporulation. They also reported that vigorous vegetative growth was not always followed by good sporulation. In addition, it is likely that the synthesis of the toxin compounds is affected when no exponential growth is allowed. Moreover, several reports mentioned the use of subproducts as potential media for low-cost production of Bt insecticides. Even so, optimal conditions are not always reported. The ability of Bt to grow and sporulate easily on starch, and on several carbohydrates as C source, led us to study the formulation of a new medium based on gruel, a cheap byproduct of semolina factories. Indeed, durum wheat is industrially milled to produce semolina (65%), flours (10%), gruel (14%), and other byproducts (11%). Semolina factories, widely distributed throughout the world, are producing large quantities of gruel with no significant added value. Such gruel contains 50–70% starch, 5% other carbohydrates, 1.5% cellulose, and 8–12% proteins.

A new, simple, and cheap medium containing hydrolysate of gruel as C source is proposed to support production of Bt bioinsecticides. Data on growth of Bt and on biomass and δ-endotoxin production are given.

MATERIALS AND METHODS

Microorganism (BNS3) and Inocula

The microorganism employed was a strain of Bt subsp. *kurstaki* (serotype H 3a, 3b, 3c) from our collection of Bt strains from Tunisia isolated at our institute, according to Jaoua et al. *(10)*. This strain was selected for its toxicity to the Lepidoptera *Prays oleae, Ephestia kuehniella, Spodoptora*

exiga, and *Ostrinia nubilalis* (S. Jaoua, unpublished results). Inocula were prepared by transferring cells from nutritive agar slants into 5 mL of culture medium, incubated 6 h at 30°C, and transferred into 1 L Erlenmeyer flasks containing 50 mL of medium. After 8 h of incubation at 30°C, the culture was employed to inoculate culture media in shake flasks.

Media and Culture Conditions

The culture media were based on glucose or hydrolysate of gruel as C sources, either yeast extract or peptone from casein or casamino acids as source of amino acids, and ammonium sulfate or urea as N source. In the Results section, we indicate the corresponding source and its concentration for each experiment. The following minerals were used (g/L): 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 7.0 before sterilization. In shake flasks, 1 g of $CaCO_3$ was added for pH stabilization. C source, glycerol, and $CaCO_3$ were sterilized separately. Flasks were incubated 72 h at 30°C in a rotary shaker set at 200 rpm.

Analytical Methods

As commonly carried-out for Bt *(3,6,11)*, cell mass was spectrophometrically determined by absorbance measurement (OD) at 600 nm after 72 h incubation. We microscopically observed that after 72 h, the cultures were composed only of a mixture of spores, crystals, and cell debris. Then, the relationship between OD and biomass was determined. The OD of 1.0 corresponded to 0.21 g/L of dry biomass (spores, crystals, and cell debris). The number of spores was estimated by the determination of colony forming units (CFU), at the end of the experiments. Culture samples were heated at 70°C for 10 min and appropriate dilutions were plated on solid Luria Broth (LB) medium *(12)*. Glucose was monitered by using glucose oxydase (Biomaghreb, Tunisia, GOD, PAL). Total and reducing sugars were determined by using DNS reagent *(13)*. Total Kjeldahl N (TKN) and mineral N were measured by using the conventional method of Kjeldahl. δ-endotoxin was determined as follows (Aronson, A.I., personal communication): 1 mL of culture medium was centrifuged for 10 min at 10,000g, and the pellet was washed twice with 1*M* NaCl and twice with distilled water. The pellet was then suspended in 200 µL of 50 m*M* NaOH, pH 12.5. After 3 h of incubation at 30°C, total proteins in the supernatant were measured by using BioRad reagent (BioRad Protein assay, cat. no. 500-0006, Munich, Germany). The values presented are averages of the results of three determinations and two separate experiments. The statistical treatment of the results showed deviations of 5%. The strain BNS3 cry^-, spo^+, obtained by plasmid curing from the wild strain BNS3 (Tounsi, S., not published), was used as negative control, in order to take into account, in δ-endotoxin determinations, the possibly contaminat-

ing dissolved proteins from spore coat and cell debris. This evidenced the statement that 90–95% of the soluble proteins were from totally dissolved crystals produced by the strain BNS3.

Hydrolysis of Gruel

Gruel was obtained from a local semolina factory. It contained 65% starch and 12% proteins (from TKN determination). One kg of gruel was suspended in water (total vol 3.5 L) and heated at 110°C for 25 min. Then, 240,000 IU of α-amylase (Thermamyl, NOVO) were added and incubated at 90°C for 60 min with gentle agitation. When performed, hydrolysis with amyloglucosidase (AMG) was carried-out by incubating the total volume with 40 IU of AMG (AMG, NOVO) during 4 h at 60°C pH 4.7. Proteolysis was performed by using 4 mL of alcalase (2.4 L, NOVO) per kg of gruel at 50°C, pH 8.0, with continuous correction of pH using 4 *N* NaOH. The proteolysis degree was estimated with the following formula: PD = 54.149 (B/PM) (%), B is the volume (mL) of 4 *N* NaOH used to correct the pH; PM (g) is the quantity of proteic matter used in the experiment. We obtained PD of almost 19%. Hydrolysate of gruel was the supernatant layer obtained after centrifugation during 20 min at 8,000 *g*.

RESULTS

Optimization of Culture Parameters for δ-Endotoxin Production

The optimization of the strain BNS3 culture parameters supporting growth, sporulation, and δ-endotoxin synthesis was realized by using a synthetic medium composed of (g/L): glucose, 20; yeast extract, 3; ammonium sulfate, 7; glycerol, 5, and minerals. The indicated concentrations were chosen according to previous works *(14–16)*. Series of experiments were carried out in order to study, in each one, the effect of one individual parameter. Table 1 gives the experimental range in which each parameter was studied and the optimal values of cultural parameters corresponding to the highest δ-endotoxin production. In order to lighten Table 1, results concerning the evolution of δ-endotoxin and biomass production are not shown, but they are discussed. They show that the optimal vegetative cell inoculum was 5%. The temperature chosen was 30°C. Indeed, we found that at higher temperatures, biomass production increased, but δ-endotoxin dramatically decreased. Taking into account glucose, glycerol, and ammonium sulfate, respectively, as C and N sources. C:N equal to 7 was adequate. Yeast extract used as amino acids source was absolutely necessary for Bt growth. Beyond 5 g/L, growth continued to increase, but δ-endotoxin decreased. Addition of glycerol up to 5 g/L improved 2.5-fold toxin synthesis. Thus, with the indicated conditions of Bt culture, 735 mg of δ-endotoxin in 4.5 g total dry biomass were produced per liter of culture broth.

Table 1
Optimal Conditions for Biomass and δ-Endotoxin Production on Glucose-Based Medium

Culture parameters	Experiments range	Optimal
Inoculum size (%)	0.5–7.0	5
Temperature (°C)	26–37	30
C/N ratio	5.5–9.0	7
Yeast extract (g/L)	0–11	5
Glycerol (g/L)	0–8	5
Glucose (g/L)	10–200	15

Table 2
Effect of Initial Concentration of Glucose on Growth and δ-Endotoxin Synthesis

Glucose concentration (g/L)	10	15	30	60	90	120	150	180	200
Residual glucose (g/L)	0	0	0	0	0	27	36	55	57
Biomass (g/L)	3.3	4.6	7.5	10.2	10.3	10.1	10.3	7.4	7.6
δ-endotoxin (mg/L)	586	735	700	688	686	685	690	632	641
Yield (mg Toxin/g Gc[a])	58.6	49.0	23.3	11.5	7.6	7.1	6.1	5.1	4.5
Yield (g Biomass/g Gc[a])	0.33	0.31	0.25	0.17	0.11	0.10	0.09	0.06	0.05

[a]Gc, Glucose consumed.

In order to improve the production of δ-endotoxin in the culture broth, the initial concentration of glucose was increased. The results, in Table 2, were obtained at the conditions mentioned in Table 1. They show that when the glucose concentration was increased to 60 g/L, increase in biomass and δ-endotoxin concentrations was not proportional. Beyond 60 g/L of glucose, biomass and δ-endotoxin were not affected, and glucose was entirely consumed. Residual glucose was observed with and beyond 120 g/L of initial glucose concentration. These results clearly show that the initial concentration of C source significantly regulates the bioinsecticide production, and that 15 g/L of C source seems to be quite adequate. The reduction, clearly observed, in the yield of biomass and δ-endotoxin proves a phenomenon of metabolite repression named glucose effect, caused by increased glucose concentrations *(17)*. This phenomenon is characterized by a repressive regulation of many enzymes, caused by an increase of the intracellular concentration of ATP *(18)*. This metabolite

Table 3
Bt Growth and δ-Endotoxin Production on Several C Sources

C source (15g/L)	Endotoxin (mg/L)	CFU ($\times 10^8$/mL)
Glucose	715	3.6
Sucrose	393	4.4
Molasses	425	4.8
Cellulose	370	1.9
Starch	588	2.1
Gruel hydrolysate	965	3.2

repression affecting biomass and δ-endotoxin production is caused by an increase of the number of metabolic pathways, which could explain the consumption of glucose beyond 60 g/L *(17)*.

Evaluation of Insecticidal Production on Several Carbohydrates

The influence of the media composition on growth and δ-endotoxin production by the strain BNS3 was realized on several carbohydrates. The results are shown in Table 3. In such experiments, the sporulation counts were estimated at the end of the culture by the determination of the CFU, rather than biomass, because of residual insoluble particles of cellulose and starch plus the dark color of molasses, inferring interferences in the OD determination. Taking into account both CFU and δ-endotoxin production data, the gruel hydrolysate could be considered as the best C source for the production of BNS3 insecticide. The gruel hydrolysate used in this experiment was the one obtained by α-amylase and alcalase proteolysis of gruel.

Choice of Gruel Hydrolysate

With the idea of finding a simpler and cheaper medium than that based on glucose, gruel was hydrolyzed by α-amylase, and the resultant hydrolysate was subject to AMG and/or protease (alcalase) hydrolysis. Different hydrolysates were obtained after the successive steps of hydrolysis indicated in Materials and Methods and used for δ-endotoxin production. Ammonium sulfate was added at 5.4 g/L (corresponding to a C:N equal to 7, by taking into account only glycerol and total sugars as C source) to evaluate the necessity of a complementary N source. The experiments were carried out with 15 g/L TS, 5 g/L yeast extract, 5 g/L glycerol, and minerals. Table 4 shows the results of the use of these different hydrolysates, compared to glucose, in order to produce biomass and δ-endotoxin. These results show that addition of ammonium sulfate improved biomass and particularly δ-endotoxin production. On the other hand, it is clear that gruel hydrolysis by AMG does not significantly improve the insecticidal production. Thus, hydrolysis of gruel with α-amylase followed by alcalase (PD equal to 19%), generated good medium (named H2) for biomass and particularly δ-endotoxin produc-

Table 4
Use of Different Hydrolysates of Gruel as C Source

Hydrolysates	With 5.4 g/L ammonium sulfate		Without ammonium sulfate	
	Biomass (g/L)	Toxin (mg/L)	Biomass (g/L)	Toxin (mg/L)
Glucose	4.5	735	–	–
H1 (gruel not treated)	3.6	819	3.1	478
H2 (gruel treated with alcalase)	4.6	929	3.8	580
H3 (gruel treated with AMG)	4.2	876	3.2	455
H4 (gruel treated with AMG+alcalase)	3.7	851	3.2	581

Note: Each hydrolysate was obtained after α-amylase hydrolysis prior to the mentioned hydrolysis.

Table 5
Effect of the Initial Concentration of TS on Growth and δ-Endotoxin Synthesis

T.S.[a] concentration (g/L)	10	15	20	25	30	40	60	80	100
Residual TS[a] (g/L)	1.0	1.8	2.0	2.4	2.7	3.7	5.8	6.9	13.8
Biomass (g/L)	4.1	4.6	4.9	5.6	6.1	7.3	11.6	14.3	15.9
δ-endotoxin (mg/L)	784	965	1053	1141	1255	1444	1112	960	677
Yield (mg toxin/g TSc[b])	87.1	73.1	58.5	50.5	46.0	39.8	20.5	13.1	7.8
Yield (g biomass/g TSc[b])	0.46	0.35	0.27	0.25	0.22	0.20	0.21	0.20	0.18

[a]TS, Total sugar.
[b]TS, Total sugar consumed.

tion. Further experiments were carried out with gruel hydrolysate H2, containing (g/L): total sugars, 198; TKN, 4.45; proteins, 19.3; and glucose, 10.

Effect of Initial Concentration of Gruel Hydrolysate

In order to assert the C metabolite repression that regulates growth and δ-endotoxin production in Bt, which we have already observed with glucose

Table 6
Effect of Yeast Extract and Glycerol on BNS3 Bioinsecticide Production

Concentration (g/L)	0	2	4	5	7
Yeast extract	755/3.4	854/3.9	921/4.2	997/4.6	923/5.0
Glycerol	736/3.4	860/4.0	1017/4.2	1084/4.4	925/4.1

Note: The results were obtained with variable concentrations of yeast extract (using 5 g/L glycerol) and glycerol (using 5 g/L yeast extract) in the H2 medium containing 15 g/L total sugars and C:N equal to 7, and are presented as δ-endotoxin (mg/L)/biomass (g/L).

Table 7
Effect of the Substitution of Yeast Extract by Variable Concentrations of Peptone from Casein, Casaminoacids, or Casein, on BNS3 Bioinsecticide Production

Concentration (g/L)	1	2	3	4
Peptone from casein	801/4.2	864/4.4	1056/4.4	993/4.1
Casaminoacids	811/4.4	908/4.5	960/4.7	993/4.9
Casein	818/3.6	820/3.7	790/3.5	768/3.6

Note: The results were obtained in the H2 medium containing 15 g/L total sugars, 5 g/L glycerol, and C:N equal to 7, and are presented as δ-endotoxin (mg/L)/biomass (g/L).

(Table 2), the effect of the initial concentration of total sugars from gruel hydrolysate was elucidated (Table 5). Compared to glucose (Table 2), by increasing the initial concentration of the C source, higher biomass concentrations were obtained, but the yield of δ-endotoxin and biomass production dramatically decreased. The concentration of 15 g/L of total sugars from gruel hydrolysate H2 seems to be adequate to reach good concentration of BNS3 insecticide (965 mg/L) with reasonable yield (73 mg δ-endotoxin/gTS).

Formulation of New Media Based on Gruel Hydrolysate for Bioinsecticide Production

The final formulation of the medium, considering the influence of its components on the yield of biomass and δ-endotoxin production, was possible through the optimization of their adequate concentrations. Thus, the effect of variable concentrations of each component on both final biomass and δ-endotoxin concentrations was elucidated using the optimal conditions of Table 1, except for glucose, which was substituted for with 15 g/L TS from gruel hydrolysate H2. Table 6 clearly shows that 5 g/L of yeast extract gave the best δ-endotoxin synthesis with good growth. Moreover, up to 5 g/L, glycerol improved biomass and δ-endotoxin production. Table 7 illustrates the possible substitution of yeast extract. Indeed, it shows that 3 g/L of either peptone from casein or casaminoacids substituted very well for the 5 g/L of yeast extract (shown in Table 6). By contrast, 1 or 2 g/L of

Table 8
Effect of Variable C:N Ratios on BNS3 Bioinsecticide Production

C:N ratio	3	5	6	7	9
Ammonium sulfate (g/L)	12.5	7.5	6.2	5.4	4.2
	838/4.2	981/4.2	982/4.2	965/4.4	894/4.3

Note: The results were obtained in the H2 medium containing 15 g/L total sugars, 5 g/L yeast extract, 5 g/L glycerol, and variable concentrations of ammonium sulfate, and are presented as δ-endotoxin (mg/L)/biomass (g/L).

Table 9
Effect of the Substitution for Ammonium Sulfate by Urea on BNS3 Bioinsecticide Production

Urea (2.6 g/L) + yeast extract (5g/L)	535/2.5
Urea (2.6 g/L) + peptone from casein (5g/L)	524/2.5

Note: The results were obtained in H2 medium containing 15 g/L total sugars, 5 g/L yeast extract, 5 g/L glycerol, and 2.6 g/L urea, corresponding to C:N equal to 7, and are presented as δ-endotoxin (mg/L)/biomass (g/L).

casein poorly substituted for yeast extract, but, at higher concentrations, casein negatively affected the δ-endotoxin synthesis. Results of Table 8, studying the effect of C:N by using variable concentrations of ammonium sulfate, show that a C:N between 5 and 7 was adequate. Urea used as N source did not substitute well for ammonium sulfate (Table 9), when used either with yeast extract or peptone from casein. It negatively affected growth and δ-endotoxin production. Table 10 shows that not more than 0.3 g/L of magnesium sulfate were needed to substantially increase the production of the bioinsecticide. In order to substitute for the amino acid source by a cheaper one, we determined the auxotrophy of the strain BNS3. We found that this strain was auxotrophic to alanine, serine, histidine, valine, leucine, and aspartic acid. To evaluate the need of several amino acids in the culture medium, yeast extract was substituted for with 0.25 g/L of single amino acid (Table 11). The most relevant substitution for the amino acid sources was that obtained with the 0.25 g/L of cysteine or aspartic acid, which gave similar results in term of δ-endotoxin production. With several other amino acids, biomass production was slightly affected when δ-endotoxin synthesis was dramatically reduced.

DISCUSSION

From the elucidation of the nutritional requirements of Bt strain BNS3, in order to produce the toxic agent (δ-endotoxin) of the bioinsecticide, we noticed that this strain grows easily at a relatively broad range of

Table 10
Effect of Magnesium Sulfate on BNS3 Bioinsecticide Production

$MgSO_4$ (g/L)	0.00	0.15	0.30	0.60	1.20	1.80
	999/3.9	1115/4.3	1052/4.4	1060/4.4	1066/4.4	963/4.3

Note: The results were obtained in the H2 medium containing 15 g/L total sugars, 5 g/L yeast extract, 5 g/L glycerol, and 5.4 g/L ammonium sulfate corresponding to C:N equal to 7, and are presented as δ-endotoxin (mg/L)/biomass (g/L).

Table 11
Effect of Substitution for Yeast Extract by 0.25 g/L of Single Amino Acid

Cysteine (0.25 g/L)	930/3.2
Aspartic acid (0.25 g/L)	992/4.0
Alanine (0.25 g/L)	391/2.3
Histidine (0.25 g/L)	591/3.8
Serine (0.25 g/L)	546/3.9
Valine (0.25 g/L)	484/3.8
Leucine (0.25 g/L)	517/3.4
Isoleucine (0.25 g/L)	517/3.4

Note: The results were obtained in the H2 medium containing 15 g/L total sugars, 5 g/L yeast extract, 5 g/L glycerol, and 5.4 g/L ammonium sulfate, corresponding to C:N equal to 7, and are presented as δ-endotoxin (mg/L)/biomass (g/L).

cultural conditions. But, simultaneously, it seems that its optimal growth conditions were not necessary those for optimal δ-endotoxin synthesis. Since the latter is highly correlated with the final concentration of biomass, there is a compromise between the high level of growth and that of the δ-endotoxin synthesis during sporulation. The optimal bioinsecticide production conditions defined for the strain BNS3 take into consideration the balance between biomass and δ-endotoxin production. Indeed, we were careful in the determination of C source usage, and we attempted to correlate sugar metabolism with δ-endotoxin production.

We found that the addition of 0.15 g/L of magnesium sulfate to BNS3 culture medium improved the δ-endotoxin production. Although Sikdar et al. *(9)* optimized mineral concentrations in Bt culture media, it is generally accepted that minerals such as manganese are required for efficient sporulation. As δ-endotoxin gene promoters are generally sporulation specific *(1)*, it is not surprising that crystal production increases when such minerals are added to the medium. The addition of 5 g/L glycerol to glucose- or gruel hydrolysate-based media improved the toxin synthesis 2.5-fold and 0.5-fold respectively. Such result agrees with the findings of Fridlender et al. *(16)*, who showed that addition of glycerol in some culture media of the bacterium *Bacillus sphaericus* improved toxin production. They also found

that consumption of glycerol correlated well with toxin yield. On the other hand, we found that cell growth and δ-endotoxin production by our strain of Bt were regulated by C metabolite repression. This was clearly marked when the corresponding yields significantly decreased with increasing glucose or total sugars concentrations. In the literature, a wide range of C source concentrations were used, but the quantitative correlation between biomass and δ-endotoxin synthesis was not elucidated. High N concentrations did not improve cell growth and δ-endotoxin synthesis. The ratio C:N was important to take into consideration. When between 5 and 7, C:N did not significantly affect the bioinsecticide production. With low C:N, the toxin synthesis was negatively affected, but the biomass production was not improved. This agrees with the findings of Pearson and Ward *(3)* which show that spore formation and protease production by various strains of Bt appear to be regulated by N metabolite repression.

In gruel hydrolysate, the use of ammonium sulfate as sole N source without yeast extract did not greatly affect cell growth and δ-endotoxin synthesis. This could be because of the proteolysis of gruel components by alcalase, which supplies some amino acids and peptides, but their amount present is not high enough for attaining high δ-endotoxin concentration. These results demonstrate that in gruel hydrolysate-based medium, a combination of an amino acids source and ammonium sulfate seemed to be the most convenient for the strain BNS3. Moreover, the nature of the N and amino acids sources significantly affected the bioinsecticide production. Indeed, urea did not substitute the ammonium sulfate; casein used as amino acids source gave poor growth of the Bt strain BNS3. This agrees with the result of Pearson and Ward *(3)*. Thus, it is clear that this Bt strain requires some amino acids or peptides that are essential for its growth. Cysteine and aspartic acid are necessary for δ-endotoxin synthesis. This is in agreement with Arcas et al. *(6)*, who proved that cystine is an essential component of Bt medium. The optimization of the nutritional parameters in Bt media for the bioinsecticide production revealed the importance of the C:N, the combination of the most appropriate N and amino acid source, and particularly the metabolite repression of the C and N sources, which regulate both growth and δ-endotoxin synthesis. This limitation must be considered for the large-scale production of the bioinsecticide, and the final process must overcome the metabolic repression.

Gruel, a cheap byproduct of semolina factories, represented a good alternative to provide simple and cheap medium for this purpose. Gruel hydrolysis by α-amylase and alcalase was enough to supply adequate gruel hydrolysate for δ-endotoxin production. Here, we treated gruel with amylase and alcalase to obtain gruel hydrolysate containing sugars and peptides from starch and gluten, respectively, as principal components of gruel. This was necessary to carry out, to avoid residual particles of gruel

in the medium, inferring aisance in the determination of growth and δ-endotoxin parameters during the optimization step. We showed, earlier, the ability of several Bt strains to grow and adequately produce their δ-endotoxins on crude gruel. Indeed, many species of bacilli produce amylases, proteases, and several hydrolytic enzymes, which make the bacterium able to use starch and gluten from gruel as substrate/nutrient.

We consider these findings as basic knowledge needed to define Bt media based on side products of agro-industries available at industrial quantities and cheap prices, like gruel. We project to carry into effect a fermentation process to produce Bt insecticide in media based crude gruel, taking into account the optimized nutritional requirements and the metabolic mechanisms of Bt.

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