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Effect of Carbon: Nitrogen Ratio on the *Bacillus thuringiensis* Spore Production

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Abstract: The production of Cry protein and spore by *Bacillus thuringiensis* is related to the formulation of the culture medium and operating conditions of the bioreactor, such as agitation and aeration. Cry proteins are responsible for the bioinsecticide activity of *B. thuringiensis*. In this work the effects of carbon to nitrogen (C:N) ratio and carbon concentration on spore production of *B. thuringiensis* HD23 are reported. The spore production was determined at C:N ratios from 4 to 10, and at carbon source concentrations of 10 g/L, 20 g/L and 30 g/L for each C:N ratio. The nitrogen source was soybean meal (hydrolyzed and non-hydrolyzed); while carbon source was glucose. Experiments were carried out in shake flasks. The results shown that with the non-hydrolyzed soybean meal, the highest spore concentration (3.7×10^{12} espores/L) and the highest spore productivity (1.54×10^{11} spores/L h) were achieved at a C:N ratio of 10 and at a glucose concentration of 20 g/L; whereas with the hydrolyzed soybean meal, the spore concentration (4.3×10^{12} spores/L) and spore productivity (1.79×10^{11} spores/L h) were higher than with the non-hydrolyzed soybean meal, and these were also achieved at a C:N ratio of 10 and at a glucose concentration of 20 g/L. At these conditions the count of crystals produced was 3.0×10^{12} crystals/L. Also, the maximum specific growth rate was 0.6 h^{-1} at the better conditions of C:N ratio and glucose concentration. The inoculums growing in log phase and cultured on the complex medium allow reducing considerably the lag phase. Finally, when *B. thuringiensis* HD23 is grown using the best formulation of the culture medium obtained in

this work and a bioreactor with a high oxygen transfer, may be possible that the productivity can be even higher.

Keywords: Bioinsecticide, C:N ratio, Spore productivity, Glucose concentration, Shake flask.

INTRODUCTION

Bacillus thuringiensis is a spore-forming bacterium that during sporulation process produces one or more crystalline bodies of proteinaceous nature known as δ-endotoxins or Cry proteins. These proteins are responsible for the bioinsecticide activity of *B. thuringiensis.* Active δ-endotoxins have been found versus lepidoptera insects, coleoptera, diptera and hymenoptera. *B. thuringiensis* requires a carbon source, amino acids and some salts to grow and produce spores and Cry proteins.

The effect of the C:N ratio on growth and metabolite formation has been studied in different species of microorganisms and plants, for example: *B. thuringiensis* HD73 had the highest spore production at C:N ratio of 4, whereas the highest Cry protein production[1] was at C:N ratio of 7. In other hand, the fungus *Cordyceps militaris* had a maximum cordycepin production and productivity of 345.4 ± 8.5 mg/L and 19.2 ± 0.5 mg/L per day in medium with optimized[2] C:N ratio of 17. Also, it was demonstrated that the use of sugarcane blackstrap molasses and yeast extract, at a C:N ratio of 10 in the culture of *Saccharomyces cerevisiae*, provided the highest glucose-6-phosphate dehydrogenase activity (P_{max} = 5180 U/L), with a three-fold increase in comparison to the original culture medium[3]. There are a lot of cases where the influence of the C:N ratio on the microorganism metabolism has been demonstrated; this knowledge it could be used to control the growth of a microorganism and the metabolite production.

The objective of this work was to evaluate the spore concentration produced by *Bacillus thuringiensis* HD29 when it was cultivated on different carbon to nitrogen ratios and at different glucose concentration.

METHODS

Microorganism: *B. thuringiensis* HD23 was provided by the CIIDIR-Sinaloa (México), and was propagated on nutrient agar and preserved it on sterile filter paper at 4ºC in eppendorf tubes. A disc filter paper was used to inoculate nutrient agar slant tubes, which were incubated at 30ºC during 2 days.

Culture media: The inoculum was prepared using the following medium: soybean meal 14 g, glucose 10 g, $CaCO_3$ 0.1 g, $MgSO_4$ $7H_20$ 0.00011 g, $MnSO_4$ H_2O 0.04 g, $FeSO_4$ $7H_2O$ 0.028 g, $CaCl_2$ $4H_2O$ 0.03 g, tap water 1 L. In the fermentation experiments were used the same mineral salts in the medium, in which the amounts of glucose and soybean meal were varied as required, to obtain different carbon to nitrogen ratios. Hydrolyzed soybean meal was prepared by adding sulfuric acid to a pH 1, and heating at 121ºC for 30 min.

Fermentation experiments: The experiments carried out at different C: N ratios were as follows: the inoculums were obtained from a subculture growing at log phase, which was obtained from a first culture growing at log phase too. The inoculums concentration was approximately 4 x 10^{10} cell/L and the shake flasks were inoculated with 10% v/v from this inoculums. It is important to emphasize that in these cultures

the complex medium was used as is indicated above. *B. thuringiensis* was cultured in shake flasks at 200 rpm, pH 7 and 30°C for 24 h. Each experiment was done in triplicate.

Analytical methods: Spore and cell counts were microscopically determined with a Neubauer chamber. The biomass concentration was calculated from the cell count considering 2.3 x 10^{-12} g as the weight of one cell[4].

RESULTS AND DISCUSSION

The spore and Cry protein concentrations obtained in *B. thuringiensis* cultures are dependent of the C:N ratio, culture conditions such as agitation and aeration and culture media composition[5]. In this work, for glucose concentration of 20 g/L, when the C:N ratio increases from 4 to 7, the spore concentration decreases 0.45 times; but when the C:N ratio continues increasing from 7 to 10, the spore concentration increases 20 times, reaching 3.7 x 10^{12} spores/L (Fig. 1). This result is some different to that reported in the literature [3], where the C:N optimum ratio for a total solids concentration of 60 g/L (near concentration to that used in this work) was 7. However, for a total solids concentration of 150 g/L, the C:N optimum ratio was 4 to produce spores[3]. Effectively, this difference can be attributed to strains of different type and different combination of total solids in the culture medium.

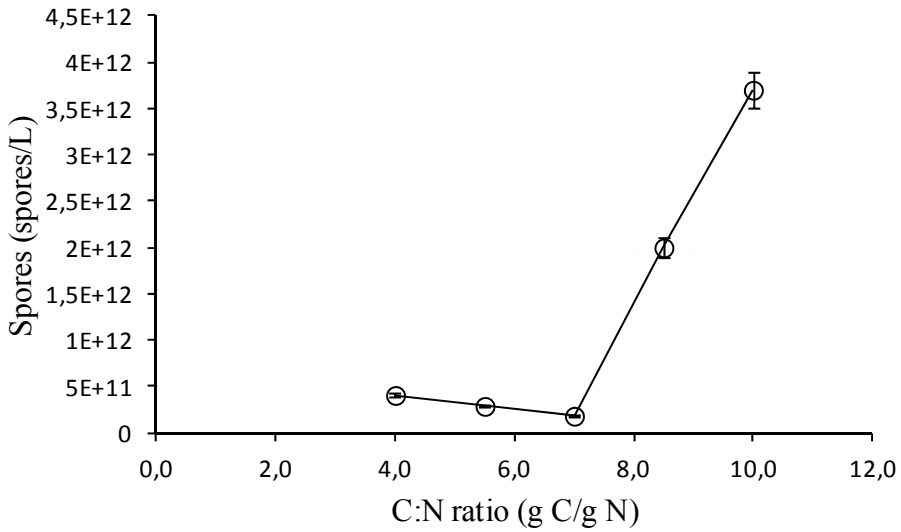

Fig.1: Spores produced by *Bacillus thuringiensis* HD29 as a function of carbon to nitrogen ratio at glucose concentration of 20 g/L Bars errors are shown.

The change in the metabolic response of *B. thuringiensis*, when increases the C:N ratio, is similar to that presented for the production of arachidonic acid by the fungus *Mortierella alpine*, where the cellular yield increased markedly at C:N ratios below 7; carbon utilization was switched from cellular growth to lipid biosynthesis in the C:N ratio range of 7‑15. However, for C:N ratios higher than 15, the mycelial concentration decreased due to nitrogen limitation but the lipid yield still increased[6].

Once the effect of the C:N ratio on the production of *B. thuringiensis* spores was determined, it was also of interest determining the effect on the production of said spores, when increasing concentrations of the carbon and nitrogen sources, maintaining constant the C:N ratio. The Fig. 2 shows the spore concentration obtained for glucose concentrations of 10, 20 and 30 g/L for each C:N ratio of 4, 7 and 10.

The spore concentration produced by *B. thuringiensis* remained approximately constant with increasing glucose concentration from 10 to 30 g/L for C:N ratios of 4 and 7; while the spore concentration increased with increasing glucose concentration from 10 to 20 g/L and decreased with increasing glucose concentration from 20 to 30 g/L at a C:N ratio of 10. The highest spore concentration (3.7×10^{12} spores/L) was obtained at a C:N ratio of 10 and a glucose concentration of 20 g /L.

These results suggest that at C:N ratios of 4 and 7, the increase in the concentrations of carbon and nitrogen sources does not influence on the metabolism of *B. thuringiensis* that directs the spore formation. However, at C:N ratio of 10, the increase concentrations of carbon and nitrogen sources, change the metabolism that directs the spore formation. This effect is greater at glucose concentration of 20 g/L and a soybean meal concentration of 21 g/L. These results agree with those obtained in experiments where the media were supplemented with 6‑12 g/L of carbon source, the highest spore yield attained was at a C:N ratio of 160, when the carbon concentration was 8 g/l for the fungus[7] *Lecanicillium lecanii* CA-1-G.

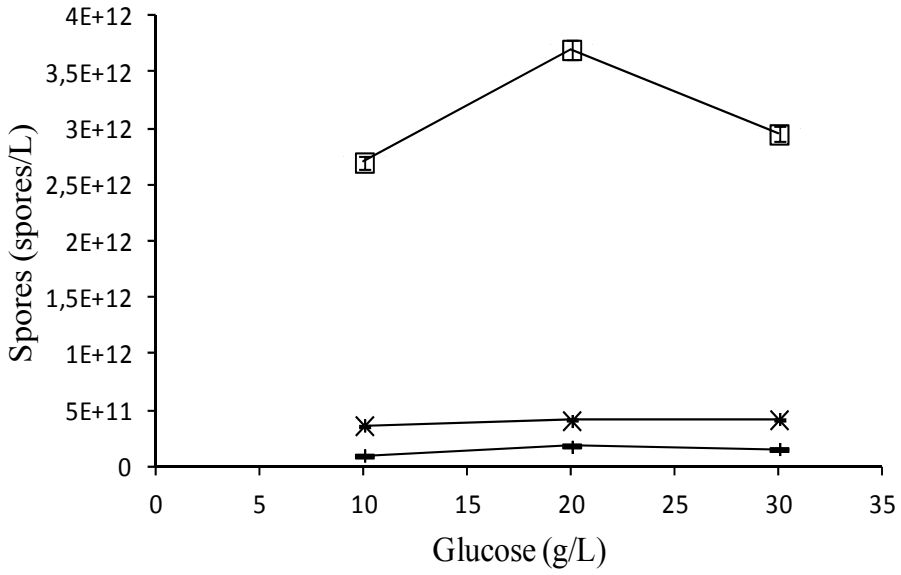

Fig.2 Spores produced by *Bacillus thuringiensis* HD29 as a function of glucose concentration and C:N ratio of 4.0 (X), 7 (-) and 10 (□). Bars errors are shown.

The highest spore concentration was obtained at a glucose concentration of 20 g/L and a soybean meal concentration of 21 g/L, at a C:N ratio of 10. The kinetics of this fermentation is shown in Fig. 3. Practically,

there was no lag phase because the inoculum used was in log phase and was obtained from a subculture in which glucose and soybean meal were used, as mentioned in the Methods section.

The maximum specific growth rate was 0.6 h^{-1}, this value is between the values reported for *B. thuringiensis*, which is from 0.4 to 1.1 h^{-1}. This wide range of values is explained because the microorganism uses different substrates and metabolic intermediates, and is exposed to different fermentation conditions (mixing and oxygen transfer, pH) that control the metabolic state of the organism [4].

The spore liberation starts just when the growth of the microorganism reaches the maximum cell concentration, and when decrease the cell concentration increases the spore formation. The decrease of pH during growth of the organism is associated with the production of organic acids, as is reported in the literature[8].

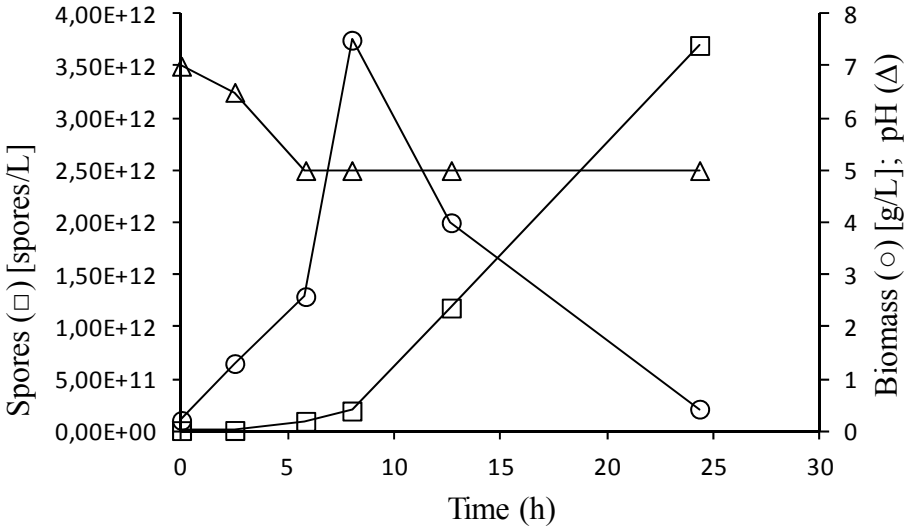

Fig.3: Growth and sporulation of *Bacillus thuringiensis* HD29.

As mentioned, among others factors, the spore production is associated with the concentration and chemical composition of the carbon and nitrogen sources. Therefore, it was of interest investigate the effect of use hydrolyzed soybean meal on spore production. Table 1 shows the productivity (spores/L h) obtained using hydrolyzed and non-hydrolyzed soybean meal, at C:N ratios from 4.7 to 10, and a glucose concentration of 10, 20 and 30 g/L. In most of the combinations presented in Table 1, the productivities obtained with hydrolyzed soybean meal were higher that when soybean meal was not hydrolyzed. The highest productivity was obtained with hydrolyzed soybean meal at a C: N ratio of 10 and a glucose concentration of 20 g/L.

When soybean meal is hydrolyzed to free amino acids, these may be in more amount and more readily available to the microorganism, that those present in the non-hydrolyzed soybean meal. In fact, although it has been reported that *B. thuringiensis* produces proteases[9], it may be possible that with hydrolyzed soybean meal there may be a greater amount of free amino acids available for growth and sporulation of *B. thuringiensis*. It is remarkable that with hydrolyzed and non-hydrolyzed soybean meal, the highest productivities were achieved at a C: N ratio of 10 and a glucose concentration of 20 g/L.

Table 1: Spore productivities obtained from soybean meal (hydrolyzed and non-hydrolyzed) at different C:N ratios and glucose concentrations.

C:N ratio (g C/g N)	Glucose (g/L)	A: Hydrolyzed Productivity (spores/L h)	B: Unhydrolized Productivity (spores/L h)	A/B (-)
4	10	1,56E+10	1,52E+10	1,03
7	10	8,33E+09	3,92E+09	2,13
10	10	1,06E+11	1,13E+11	0,94
4	20	1,44E+10	1,70E+10	0,85
7	20	7,92E+09	7,67E+09	1,03
10*	20*	1,79E+11*	1,54E+11*	1,16
4	30	1,75E+10	1,73E+10	1,01
7	30	7,96E+09	6,33E+09	1,26
10	30	1,50E+11	1,23E+11	1,22

***C.N ratio and glucose concentration corresponding to the highest spore productivities.**

Another important factor that contributes in the growth and the sporulation is the dissolved oxygen concentration. *B. thuringiensis* is an aerobic bacterium that requires high supply of oxygen, so when *B. thuringiensis* is cultivated in shaking flask, the growth and spore production can be limited by oxygen; as was pointed out in literature, where a lineal relation between spore production and volumetric coefficient of oxygen transfer was found[1].

B. thuringiensis was cultivated in shake flasks at 200 rpm. At this shaking frequency the volumetric oxygen transfer coefficient (k_La) was of 12 h^{-1} (data not shown). In fact, *B. thuringiensis* is an aerobic bacterium with high oxygen supply requirements, so for the commercial cultivation of *B. thuringiensis,* bioreactors with k_La of approximately 500 h^{-1} are employed. Therefore, in this work the microorganism growth was limited by oxygen. This fact suggests that when *B. thuringiensis* is cultivated in the better medium attained in this work, but using a bioreactor with high transfer of oxygen, the spore concentration could be even higher.

Finally, it is noteworthy that the biopesticide activity of *B. thuringiensis* is in the cry protein that is produced in the form of a crystal and although there is evidence that a high efficiency of sporulation does not define whether the culture reaches an optimal level of protein production[1], it is also known that both processes are dependent[10]. In this paper, at the end of the fermentation shown in Fig. 3, a count of 3.0 x 10^{12} crystals/L was reached. This number is 83% of the spore count.

CONCLUSIONS

The spore production of *Bacillus thuringiensis* HD29 in shacking flask was improved when the carbon to nitrogen ratio was 10, reaching similar concentrations attained in industrial processes: 3.7 x 10^{12} spores/L for non-hydrolyzed soybean meal and 4.3 x 10^{12} spores/L for hydrolyzed soybean meal, which were produced at a C:N ratio of 10 and a glucose concentration of 20 g/L.

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