

## **INFLUENCE OF SODIUM GLUTAMATE, BUBBLING N<sub>2</sub>- GAS AND SUPERFICIAL AERATION ON TETANUS TOXIN PRODUCTION IN *Clostridium tetani* CULTURES**

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### **ABSTRACT**

The influence of sodium glutamate as a supplement to Latham Mueller medium, while using bubbling nitrogen flow as an anaerobic agent and superficial aeration as an inducer of cell lysis and as a mechanism for the haulage of gases in the fermentation processes was evaluated. Using the *Clostridium tetani* Massachusetts's strain, several five (5) liter batch fermentations were carried out for tetanus toxin production under the following conditions: Latham Mueller medium, with or without sodium glutamate, nitrogen flow and superficial aeration. The results demonstrated that the addition of sodium glutamate (2.5 g/l), combined with a bubbling nitrogen flow (0.33 l/min) and superficial aeration (0.33 l/min), produced a significant increase in cell concentrations, repressing the tetanus toxin formation; while the gas flow (nitrogen and superficial aeration) without sodium glutamate improved the toxin production by approximately 49%, providing conditions for the following outcomes: a maximum toxin level of 73 Lf/ml; a toxin formation rate of 1844.0 Lf/l.h; and, an over-all productivity of 833.5 Lf/l.h.

**Key words:** tetanus toxin, fermentation, N<sub>2</sub>, sodium glutamate.

### **RESUMEN**

Se estudió la influencia del glutamato de sodio como suplemento del medio Latham Mueller, combinado con la utilización de un flujo de burbujeo de nitrógeno gaseoso como agente causante de anaerobiosis así como la aireación superficial como agente inductor de la lisis celular y como mecanismo de arrastre de los gases en el proceso de fermentación. Utilizando la cepa de Massachusetts de *Clostridium tetani*, se desarrollaron fermentaciones de cinco (5) litros para la producción de la toxina tetánica, bajo las siguientes condiciones: Medio Latham Mueller, con o sin glutamato de sodio, flujo de nitrógeno y aireación superficial. Los resultados demostraron que la adición de glutamato de sodio (2.5 g/l), combinado con un flujo de burbujeo de nitrógeno (0.33 l/min) y aireación superficial (0.33 l/min), produjo un incremento significativo en la concentración celular; mientras que el flujo de gas (nitrógeno y aireación superficial) sin adición de glutamato de sodio incrementó la producción de toxina en un 49%; estas condiciones permitieron la obtención de un nivel máximo de toxina de 73 Lf/ml, una tasa de formación de toxina de 1844.0 Lf/l.h y una productividad global de 833.5 Lf/l.h.

**Palabras clave:** toxina tetánica, fermentación, N<sub>2</sub>, glutamato de sodio.

### **INTRODUCTION**

*Clostridium tetani* produces an exotoxin - tetanospasmin - which is the causative agent of the disease, tetanus. This illness is characterized by muscular rigidity, trismus,

arching of the back (opisthotonus), arm flexion, and intercostal muscle spasms which together produce the patient's death, due to an insufficient ability to breathe (Bizzini y Germanier 1984; Reyes y Flores 1986; Bleck y Brauner 1997). Tetanus is,

however, fully preventable through simple vaccination; nonetheless, tetanus still has a significant incidence, mainly in its neonatal tetanus form (TN). As of 1989, TN prevention, control and epidemic surveillance efforts have been strongly enforced in Colombia. Such activities have been carried out through a vaccination strategy consisting of at least two doses of tetanus toxoid (TT2) applied to women of child-bearing age (FEW) living in high-risk areas. Vaccination of FEW has been considered as a complement to the routine activities already carried out (vaccination of pregnant women, childbirth institutional care, and support for midwives to be qualified under the clean childbirth approach) (MNS, 1997).

According to the evaluation of the TN-elimination plan, as presented in 1996 (MNS 1997), Colombia has met the goal of eliminating the illness as a public health problem, as reflected in the 87% reduction of cases within the 1989-1996 period. Currently, the Colombian National Institute of Health, produces the anti-tetanus vaccine in three different presentations: Tetanus toxoid (TT); Diphtheritic and tetanus toxoid for adults (Td); and both toxoids combined with *Bordetella pertussis* (DPT); however the demand for the vaccine has intensified, requiring the use of new technological tools that make increased production and an improvement in the quality of the various vaccine presentations possible.

## MATERIALS AND METHODS

Fermentations were carried out with *Clostridium tetani* Massachusetts's strain, maintained at 4°C. The inoculates were prepared in thioglycolate liquid medium, previously gassed with nitrogen (95% purity) for 30 minutes, and incubated at 35°C for 24h. the Latham Mueller (LM) culture medium was used (NZ-Casein 33.3 g/l; CaCl<sub>2</sub>

0.8 g/l; K<sub>2</sub>HPO<sub>4</sub> 1.1 g/l; glucose 8.0 g/l; MgSO<sub>4</sub> · 7H<sub>2</sub>O 0.1 g/l; NaCl 2.5 g/l; thiamine hydrochlorate 0.00025 g/l; riboflavin 0.00025 g/l; pyridoxine 0.00025 g/l; calcium pantothenate 0.001 g/l; biotin 2.5 x 10<sup>-6</sup> g/l; cyanocobalamine 2.5 x 10<sup>-6</sup> g/l; nicotinic acid 0.00025 g/l; uracil 0.00125 g/l; L-cystine 0.125 g/l; sodium glutamate 2.5 g/l; FeCl<sub>3</sub> · 6H<sub>2</sub>O 0.032 g/l), with or without 2.5 g/l of sodium glutamate. To facilitate understanding, various variants were set up to summarize the fermentation conditions (I, II, III, IV). These were designated as follows: Variant I: LM medium without sodium glutamate, without nitrogen flow and without aeration flow. Variant II: LM medium without sodium glutamate, with a bubbling nitrogen flow of 0.33 l/min, for 30 minutes before the microorganism inoculation and from 30 minutes after the inoculation until the end of the exponential phase of growth, and superficial aeration of 0.33 L/min after the exponential phase until the end of fermentation. Variant III: LM medium supplemented with 2.5 g/l of sodium glutamate, without nitrogen flow and without aeration flow. And, Variant IV: LM medium supplemented with 2.5 g/l of sodium glutamate, with bubbling nitrogen flow of 0.33 l/min, for 30 minutes before microorganism inoculation and resuming 30 minutes after the inoculation, until the end of the exponential phase of growth, and superficial aeration of 0.33 l/min after the exponential phase until the end of the fermentation process.

A seven liter New Brunswick Bioflo IIC and III laboratory bioreactor was used with a working volume of 5 liters; the operating conditions were: 35°C; 100 r.p.m.; variable pH starting at 7.1 ± 0.2. This fluid was then inoculated 1% (v/v) with a growing culture of *Clostridium tetani*. Growth kinetics were followed via pH, dry weight, (g/l) and glucose concentration (g/l) (Antrona method). Tetanus toxin concentration was measured via a Flocculation-reaction titer

(Lf/ml) using the Ramón's Flocculation technique and a potency test. To calculate the Minimum Lethal Dose, the toxin potency was measured through the inoculation of 1ml of toxin into laboratory mice (strain NIH, weight ranging from 18 to 20g) (DML), (WHO 1978). To follow the Biomass concentration, a calibration curve of dry weight (g/l) vs. optical density ( $DO_{650nm}$ ) was prepared by using a Milton Roy spectrophotometer (model Genesys 5).

The specific growth rate of the *Clostridium tetani* was calculated for each of the fermentation processes, using the following definition:

$$\mu = \frac{1}{X} \frac{dx}{dt} \quad (1)$$

Where X is the biomass (g/l), and t is time in hours.

The results were analyzed statistically with the SAS 6.12 program, INSTITUTE INC., CARY, NC, USA, which permits the observation of significant differences among the assays at a 95% reliability level.

## RESULTS AND DISCUSSION

In fermentation Variant I, the adaptation phase was not observed (Figure 1a): the microbial growth began directly at an exponential rate, from  $t = 0h$  until  $t = 10h$ , and continued with a low acceleration growth phase, until  $t = 52h$ , when the microorganism reached a maximum biomass of 0.3524 g/l. This phenomenon was due to the exhaustion of some nutrient other than glucose, and which was more easily assimilated by the microorganism. Such an initially consumed substrate could be an amino acid, as Mead and Barker reported in 1971 and 1981, respectively. The pH showed a rapid decrease during the exponential growth phase; later, it diminished slowly down to

$6.9 \pm 0.2$ , when the microorganism reached its maximum biomass (Figure 1b). This decrease in the pH value is due to the metabolic activity of the *C. tetani*, which liberates considerable quantities of gases and acids, that usually decrease the pH (Nielsen 1967; Hepple 1968). The kinetics of glucose consumption had only one(??) phase (Figure 1b) during the microbial growth phase; at the beginning, the microorganism consumes the substrate slowly; later an accelerated decrease was observed in the glucose concentration; falling from 10.57 g/l to 7.51 g/l. The tetanus toxin was first detected at 48h of fermentation (Figure 1b); the toxin titer stabilized at the 91h mark of culture growth (Table 1) when a maximum toxin value of 40 Lf/ml was observed.

In Variant II, a maximum biomass of 0.7834 g/l was observed (Figure 2a). The increase in biomass is caused by the nitrogen flow, which guarantees complete anaerobiosis, which proves to be beneficial for bacterial growth (De Luca *et al.*, 1997); and by the superficial aeration, that acts as a gas-hauling mechanism, given that such gases are liberated into the medium during microbial metabolism (Quintero *et al.*, 1998). (Table 1).

pH and glucose consumption showed a behavior similar to that which was observed in Variant I. Tetanus toxin was first detected at 48 hours of culturing; a maximum toxin value of 73 Lf/ml was observed when this titer was stabilized at 82 hours of fermentation (Figure 2b); therefore, the increase in toxin concentration was also due to gas flow, since not only was the highest toxin value obtained, but also the best specific toxin formation rate (1844.0 Lf/l.h) and the best productivity reading (833.54 Lf/l.h). (Table 1).

In Variant III, a maximum biomass of 0.4188 g/l was observed at 32 hours (Figure 3a);

this is a shorter time, when compared to other fermentations. The time reduction was due to an excess of sodium glutamate, which accelerated the growth rate of the microorganism, thus, in turn, reducing the time necessary for autolysis (Mellanby, 1968; Bizzini, 1979) (Table 1).

The evolution of pH (Figure 3a) and glucose consumption (Figure 3b) followed a similar pattern to that observed for Variants I and II. Production of tetanus toxin started at around the 36h mark of fermentation, while the maximum toxin titer of 50 Lf/ml was observed at the 64h cultivation mark (Figure 3b) (Table 1).

On the other hand, Variant IV produced the highest biomass concentration (1.0535 g/l), as compared with the previous variants (Figure 4a); the best specific growth rate was also observed for this Variant (0.6291 h<sup>-1</sup>). These results reflect the addition of sodium glutamate, just as Quintero, *et al.* (1998) reported. They found that sodium glutamate in concentrations higher than 1.5 g/l, proportionally increases the specific growth rate, the biomass concentration and the rate of cell lysis. Consequently, Variant IV fermentation showed a high glucose consumption rate producing the smallest biomass–substrate yield (Table 1). However when comparing

**Table 1**  
Average results from the fermentations

PARAMETERS	FERMENTATIONS			
	VARIANT I	VARIANT II	VARIANT III	VARIANT IV
X <sub>max</sub> (g/l)	0.3524	0.7835	0.4188	1.0535
X <sub>max</sub> time (h)	56	48	32	52
μ <sub>x</sub> (h <sup>-1</sup> )	0.4411	0.4602	0.5218	0.6291
% glucose consumption	28.94	61.22	30.6	88.61
Y <sub>x/s</sub> (g biom/g gluc.)	0.2032	0.2810	0.3766	0.1663
First toxin detection in the culture (h)	48	48	36	36
Maximum toxin value (Lf/ml)	40	73	50	43
Maximum toxin value time (h)	91	82	64	82
Specific toxin formation rate (Lf/l.h)	871.7	1844.0	1489.6	966.3
Y <sup>P</sup> <sub>x</sub> (Lf/g biom.)	237123	177987	297619	85386
Productivity (Lf/l.h)	416.371	833.545	658	528.390

Variant IV with Variant III, was found that in the latter, the biomass–substrate yield was better (Table 1); a fact that shows that sodium glutamate and gases separately increase the microorganism growth rate, but when sodium glutamate is combined with gas flow, they contribute to the poor biomass-substrate yield that was observed. In the four variants studied, an exponential growth phase was observed (Table 1), followed by a low growth acceleration. Glucose consumption kinetics showed two consumption phases, accelerating (0–50h) and non accelerating (~50h up to the end of culture) (Figure 4b). High concentrations (2.5 g/l) of sodium glutamate possibly sustain the exponential growth

phase. During the non accelerating consumption phase, it is possible that the substrate(s) consumed might be one of the amino acids present in the medium, such as aspartic acid, serine, asparagine, threonine or histidine (Pickett 1943; Andreesen *et al.*, 1989; Porfirio 1996; Porfirio *et al.*, 1997).

The tetanus toxin was first detected at 36h of fermentation, 12h earlier than in Variants I and II, but the maximum toxin titer was 43 Lf/ml (Figure 4b); this poor toxin titer was also due to the interaction between sodium glutamate and gas flow, which produces some bacterial metabolic changes, inhibiting toxin production (Mellanby, 1968; Bizzini, 1979).

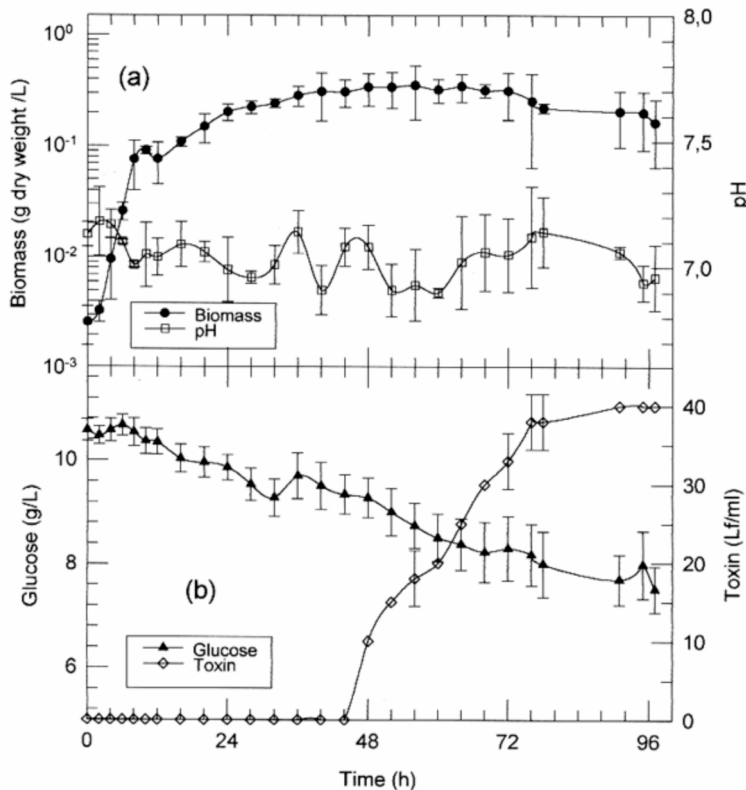


FIGURE 1. Kinetics of fermentation: Latham Mueller medium, without sodium glutamate - without bubbling gas flow (Variant I). Curve a: Biomass (g/l) and pH vs. time (h); Curve b: Glucose (g/l) and Toxin (Lf/ml) vs. time (h).

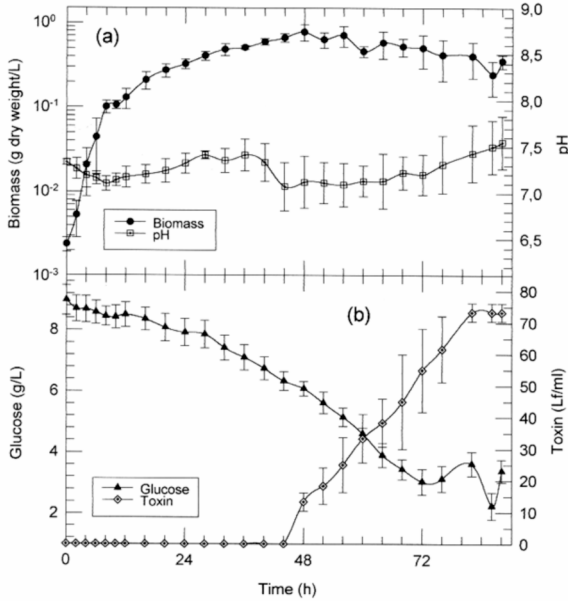


FIGURE 2. Kinetics of fermentation: Latham Mueller medium, without sodium glutamate - with flow of bubbling gases (Variant II). Curve a: Biomass (g/l) and pH vs. time (h); Curve b: Glucose (g/l) and Toxin (Lf/ml) vs. time (h).

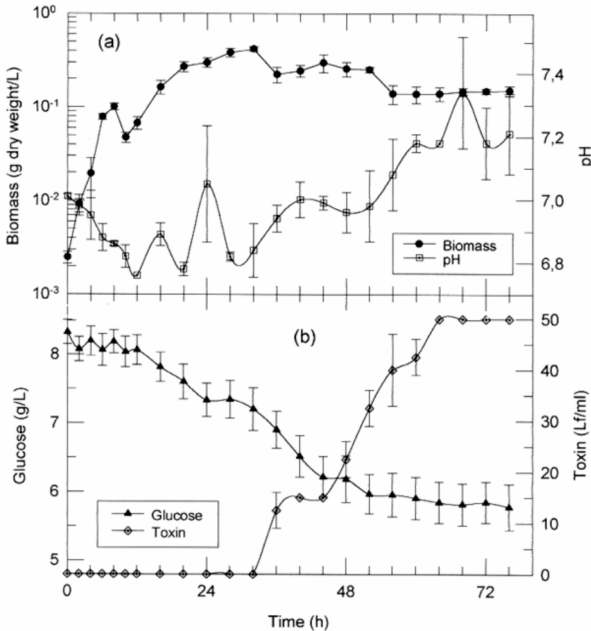


FIGURE 3. Kinetics of fermentation: Latham Mueller medium with sodium glutamate - without bubbling gas flow (Variant III). Curve a: Biomass (g/l) and pH vs. time (h); Curve b: Glucose (g/l) and Toxin (Lf/ml) vs. time (h).

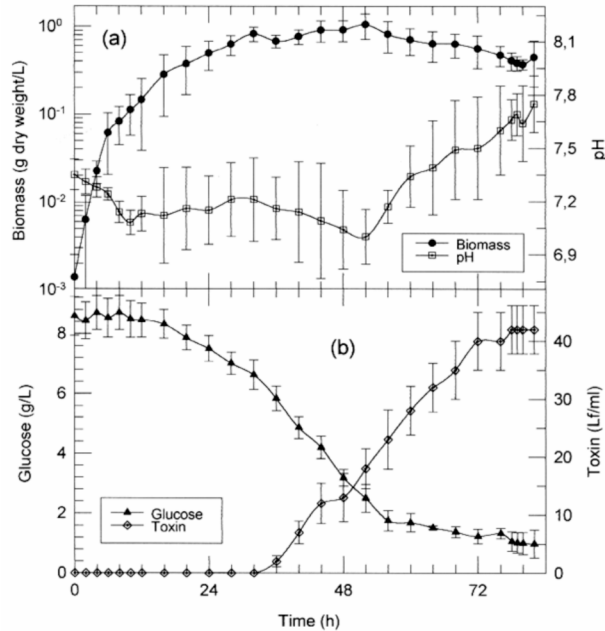


FIGURE 4. Kinetics of fermentation: Latham Mueller medium with sodium glutamate - with flow of bubbling gases (Variant IV). Curve a: Biomass (g/l) and pH vs. time (h); Curve b: Glucose (g/l) and Toxin (Lf/ml) vs. time (h).

## CONCLUSIONS

Based on the results, it is necessary to emphasize the importance that addition of sodium glutamate has, since it benefits bacterial growth; however, it does not necessarily have a positive influence on toxin production, while bubbling nitrogen flow and superficial aeration contribute separately to increases in toxin production, thus substantially improving productivity. It is also worth mentioning that the best conditions for tetanus toxin were production observed in Variant II. This fermentation process was carried out in LM medium without sodium glutamate, with a bubbling nitrogen flow of 0.33l/min, for 30 minutes before, and after the inoculation of the microorganism, until the end of the exponential phase of growth, (see corrector's note, pp 6 & 7) and with a superficial aeration of

0.33 L/min after the exponential phase until the end of fermentation.

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