JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2016. Vol. **10**(2), p. 871-878

# Scale-up from a Shake Flask to a Bioreactor, Based on Oxygen Transfer for the Production of Spore-crystal Complexes from *Bacillus thurungiensis*

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https://doi.org/10.22207/JPAM.10.2.04

(Received: 20 February 2016; accepted: 17 April 2016)

**To reduce the cost of *Bacillus thuringiensis* production, we used an industrial by-product made of molasses added to soybean flour. In addition, we scaled-up the production from a shake flask to a bioreactor. The results obtained with these cultures carried out in a 7-L bioreactor with 4-L of working volume were 5.68 ± 0.68 gDW/L, 8.9 x $10^9$ spo/mL and 1.1 mg/mL of total protein. With these results, we produced 1.7-fold more biomass than a shake flask and a 15-fold higher spore count than a shake flask; statistically, we produced the same concentration as that obtained using a shake flask. Therefore, we have achieved a successful scale-up process.**

**Keywords:** $K_L$a, spore-crystal, kinetic, *Bacillus thuringiensis*, scale-up, bioreactor.

The use of an effective biotechnological process to develop bioinsecticides to combat the pest population in agricultural sectors has increased in recent years due to the development of resistance in insects against chemical insecticides. In México and Latin American, it is expensive to produce bioinsecticides[1-3]; therefore, the use of inexpensive, locally available ingredients for the production of bioinsecticides could help to reduce the cost of the product[4].

Studies on the production of bioinsecticides using various *Bacillus thuringiensis* subspecies have been reported[5, 6]. This aerobic spore-forming bacterium produces a variety of plasmid-encoded crystal proteins, known as d-endotoxins, and virulence factors, which exhibit high toxicity against agricultural pests[7]. The spore-crystal complex of *B. thuringiensis* strain GP139 has been successfully used as a microbial control agent for *Bemisia tabaci*[8].

Several authors have proposed to use soya flour and a molasses sugar cane-like raw material to develop a cheap culture media (9, 10). In previous work developed in our laboratory (11), various industrial by-products were investigated in shake flasks for their ability to support spore-crystal complex production by strain GP139 of *B. thuringiensis*, toxic to *B. tabaci*. Our results showed that the best media culture was soya flour and molasses medium, which achieved 3.28 gDW/L, 5.9 x $10^8$ spo/mL and 1.36 mg/mL of total protein, while maintaining toxicity against *B. tabaci*.

Shake flasks have been widely used, allowing experiments to be carried out with minimal costs and material. However, there is little known regarding the influence of the operating conditions on mass transfer and hydrodynamics in shake flasks[12,13]. Nevertheless, scaling-up from a shake flask to a bioreactor is used to produce large quantities of the final product[14] and some attempts

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have been reported to try to scale up from a flask to a stirred tank[15, 16, 17].

In aerobic cultures, agitation and aeration are involved in mass transfer during the process flow. An inadequate supply of oxygen is one of the major problems in the industrial and lab-scale production of microbial metabolites[18]. To evaluate if a bioreactor is able to supply oxygen at a non-limiting rate, it is essential to have a good estimate of the oxygen transfer capacity of the vessel; this can be measured in terms of the oxygen mass transfer coefficient ($K_L a$). The $K_L a$ often serves to compare the efficiency of bioreactors and their mixing devices and is an important scaling-up criterion in bioprocesses[17, 19, 20].

The aim of this study was to establish the culture conditions in a laboratory bioreactor for obtaining similar concentrations of spore-crystal complex to that produced in a shake flask; a practical strategy of scaling up is presented based on experimental $K_L a$ data. The success of the scaling-up strategy was demonstrated by the final biomass and spore-crystal complex concentration of strain GP139 of *B. thuringiensis*.

## MATERIALS AND METHODS.

### Microorganisms

The *B. thuringiensis* strain GP139 was kindly supplied by the Guadalupe Peña group. It was isolated from a *B. tabaci* corpse[8] and maintained in the spore phase on filter paper in sterilized Eppendorf tubes.

### Inocula and medium preparation

The inocula were prepared as follows: three strips of filter paper with spores were placed in 5 mL of Nutrient Broth medium and incubated overnight at 30° C. The 5 mL were transferred to a 500-mL Erlenmeyer flask containing 200 mL of medium containing 50 g/L molasses supplemented with 50 g/L soybean flour like medium and incubated for 8 h at 30° C in a rotary shaker (Labtech LSI-100SR, Korea) at 150 rpm. We inoculated the bioreactor with a concentration of 5% v/v.

**Fermentation procedure.** The experiments were performed in a 7-L bioreactor (Applikon Biotechnology®, USA) with a working volume of 4-L. The fermentation runs were carried out in batch mode. Details of bioreactor dimension are summarized in Table 1. The fermentation runs were carried out in batch mode.

The aeration rate was 1 vvm and the dissolved oxygen (DO) concentration was measured using a polarographic electrode (Applikon Biotechnology®, USA). The temperature was controlled by a thermocouple (Applikon Biotechnology®, USA) that maintained the temperature at 30° C throughout all of the runs. The medium pH was controlled to 7 ± 0.2 using a sterile pH electrode (Applikon Biotechnology®, USA) and by adding NaOH 1 M and $HC_L$ (30%) solutions by an on-off control (BC electronic 7615, Italy). We used three different agitation speeds 250, 650 and 1000 rpm using an ADI 1032 controller speed (Applikon Biotechnology®, USA) to generate three different conditions for the oxygen transfer rate (OTR) and oxygen uptake rate (OUR). Three replicates were performed for each kinetic variation.

### Biomass, spore count, specific growth rate and doubling time determination

Samples 6 mL were taken during the first 24 h at regular 2-h intervals. To determinate biomass dry weight, 6 mL from each time interval was centrifuged at 10000 rpm for 5 min; the supernatant was discarded and the pellet containing only cells was recovered, washed three times and dried in an incubator at 80° C overnight. Spore counts were determined in a Neubauer chamber at 24 h and 48 h. Specific growth rates were calculated from a linear regression of natural logarithm of biomass concentration versus time; the slope corresponded to the specific growth rate value (μ). Doubling times were calculated with the following formula:

$$td = \frac{ln(2)}{\mu} \quad ...(1)$$

### Experimental determination of $K_L a$, oxygen transfer and uptake rates for the shake flask and bioreactor.

In the shake flask, we used the dynamic method proposed by (12) and in the bioreactor, we used the dynamic method proposed by (21). The rate of oxygen accumulation in a well-mixed liquid phase on batch culture can be described by:

$$\frac{dCO_2}{dt} = (OTR) - (OUR) = k_L a\,(C_L^* - C_L) - QO_2X \quad ...(2)$$

### $K_L a$ determination

The $K_L a$ was evaluated by considering the final steady state of dissolved oxygen

concentration after re-oxygenation Figure 1a, assuming $K_L a$ is constant with the time the equation 2 could be integrate in the time using the integration rules. The equation for $K_L a$ is given as:

$$\ln \frac{(C_L^* - C_{L1})}{(C_L^* - C_{L2})} = K_L a \cdot t \quad ...(3)$$

The $K_L a$ value was calculated from slope by plotting ln $(C_L^*-C_{L1})/(C_L^*-C_{L2})$ against time according to equation 3.

**OUR determination**

The air supply was interrupted at a certain time during fermentation for the determination of OUR (Figure 1b). The dissolved oxygen values decreased linearly with the cultivation time due to cellular respiration and slope by plotting DO versus time represented OUR or $QO_2X$.

**OTR determination**

When the aeration is turned on again (Figure 1b), the dissolved oxygen concentration increases until it reaches the steady oxygen concentration, and by using the estimated OUR value, $K_L a$ can be determined from the measured profile of dissolved oxygen concentration, using Equation (2) again. Under these conditions, for a given biomass concentration, X, and once $QO_2$ value is known, equation (2) can be integrated, taking into account the time at which the aeration of the culture is restored, and the following equation can be applied:

$$QO_2X \cdot \Delta t + \Delta C_L = K_L a \cdot \int_{t1}^{t2} (C_L^* - C_L)\, dt \quad ...(4)$$

The equation (4) can be used to determine the $K_L a$ several times during the production process, solving this equation by a numeric method for each data set of experimental values of DO vs. time, the equation (4) can be now expressed as:

$$\frac{\Delta DO}{\Delta t} = A - K_L a \cdot DO \quad ...(5)$$

The $K_L a$ value was calculated from slope by plotting $\Delta OD/\Delta t$ against OD and A constant is given by:

$$A = K_L a \cdot C_L^* - QO_2X \quad ...(6)$$

The values of OTR, OUR and $K_L a$ were investigated at three different agitation speed 250, 650 and 1000 rpm in the bioreactor.

**Statistical analysis**

All biomass, spore count and $K_L a$ measurements in the shake flask and the bioreactor were made at least in triplicate and were compared by analysis of variance (ANOVA); the means of significant effect were determined using Tukey's test at $P > 0.05$ level.

## RESULTS AND DISCUSSION

The $K_L a$ was the criterion used for scaling up the culture process from the shake flask to the 7-L bioreactor. We estimated the $K_L a$ occurring in the shake flask using the dynamic method proposed by[12] and the $K_L a$ occurring in the 7-L bioreactor using the dynamic method proposed by[21]. We observed that with an agitation rate of 250 rpm, we obtained a $K_L a$ value of 37.1 $h^{-1}$ in a conventional 250-mL Erlenmeyer flask containing 100 mL of culture medium. To obtain a similar value of $K_L a$ (37.1 $h^{-1}$), the 7-L bioreactor needs to be agitated at 650 rpm (maintaining an aeration of 1 vvm and 4-L of working volume) by using an interpolation of $k_L a$ values measured by the dynamic method (Figure 2).

There are no known reports about scaling up from a shake flask to a bioreactor using $K_L a$ values in *B. thuringiensis* cultures. However, various authors observed that the determination of the oxygen mass transfer coefficient in a bioreactor establishes the efficiency of aeration

**Table 1.** Dimensions of shake flask and bioreactor.

| Bioreactor | Dimensions | Shake Flask | Dimensions |
|---|---|---|---|
| Total volumen (L) | 7 | Total volume (L) | 0.25 |
| Working volume (L) | 4 | Working volume (L) | 0.1 |
| Impeller (six-blade Rushton turbine) | 1 | Ratio of media volumen | 0.4 |
| Impeller diameter (m) | 0.06 | | |
| Tank diameter (m) | 0.15 | | |
| Liquid height (m) | 0.27 | | |

**Table 2.** Main fermentation results obtained in *Bacillus thuringiensis* strain GP129 carried out under different initial $K_La$ and OTR values

| Agitation Speed (rpm) | $K_La$ ($h^{-1}$) | OTR ($mgO_2/m^3$ s) | OUR ($mgO_2/m^3$ s) | Biomass (gDW/L) | Spore count (#/mL) | Protein (mg/mL) | $t_x$ [a] (h) | $t_f$ [b] (h) | $\mu$ [c] ($h^{-1}$) | $t_d$ [d] (h) |
|---|---|---|---|---|---|---|---|---|---|---|
| 250 (shake flask) | 37.1±0.06 b | 63.50 | - | 3.28±0.24 c | $5.9x10^8$±0.9 b | 1.36±0.1 a | 24b | 72 a | 0.107±0.009 b | 6.6±0.61 a |
| 250 | 11.16±0.05 c | 19.50 | 12.94±0.56 a | 4.93±0.11 b | $7.1x10^8$±2.5 b | 0.61±0.1 c | 32 a | 48b | 0.095±0.003 b | 7.3±0.22 a |
| 650 | 38.5±0.03 b | 65.90 | 12.55±0.34 a | 5.68±0.02 a | $8.9x10^9$±4.9 a | 1.16±0.1 a | 12 c | 24 c | 0.167±0.001 a | 4.1±0.04 a |
| 1000 | 75.6±0.01 a | 129.30 | 13.38±0.94 a | 4.95±0.38 b | $7.4x10^9$±4.2 a | 0.90±0.1 b | 18d | 24 c | 0.158±0.003 a | 4.4±0.08 a |
| | 0.5* | - | 8.75* | 7.59* | 11.87* | 5.55* | 0* | 0* | 3.09* | 4.54* |

[a] time to achieve the maximum cell concentration, [b] fermentation time, [c] specific growth rate, [d] doubling time, * variant coefficient. Different letters indicate significant differences by Tukey's test ($P$ Â 0.05).

and helps quantify the effects of operating variables (agitation speed) on the provision of oxygen[22, 23, 24]. Therefore, in this work, we use the oxygen transfer scaling-up criterion by interpolating experimental data as a new way to produce large amounts of spore-crystal complexes by strain GP139 of *B. thuringiensis*. Under this scaling-up process, high cell and spore-crystal complex concentrations were achieved.

The effect of scaling up based on the $K_La$ value in strain GP139 is shown (Figure 3). As observed, significant differences were obtained between the cultures carried out in a shake flask (250 rpm and 37.8 $h^{-1}$) and a bioreactor (650 rpm and 38.52 $h^{-1}$) (3.28 ± 0.24 gDW/L and 5.68 ± 0.02 gDW/L, respectively). There are no reports about scaling up from a shake flask to a bioreactor with *B. thuringiensis*. However, (25) reported the growth of *B. thuringiensis* in a bioreactor with an agitation speed of 650 rpm and an OTR of 214.6 $mgO_2/m^3$ s. They produced 3.20 gDW/L, and we reported 5.68 ± 0.02 gDW/L, 77.5% more with a similar agitation speed (650 rpm) but a different OTR (65.88 $mgO_2/m^3$ s). The solubility of oxygen could be affected by solutes; therefore, the commercial medium and the soybean flour and sugar-cane molasses medium would have different dissolved oxygen concentrations. In addition, other authors have proposed that the nutrients can affect growth and metabolite production[9, 10, 26].

The successful scaling-up strategy was also confirmed by measuring the spore-crystal complex concentration, time to achieve the maximum cell concentration, and fermentation time; specific growth rate and doubling time are shown in Table 2.

Upon scaling up this culture up to a 7-L bioreactor, a maximum biomass of 5.68 ± 0.02 gDW/L was observed; significant differences were found between the shake flask and the bioreactor in spore-crystal complex concentration and kinetic values. We produced 8.88 x $10^9$ spo/mL in the bioreactor, 15 fold more than the shake flask (5.9 x $10^8$ spo/mL). In addition, we produced 1.16 mg/mL of protein, which is statistically the same amount as in shake flask. Moreover, the time corresponding to the maximal biomass production was reduced two-fold; kinetic time dropped by 2 days. We obtained a specific growth rate of 0.167 $h^{-1}$ and a doubling time of 4.1. For this work, we reported 10-

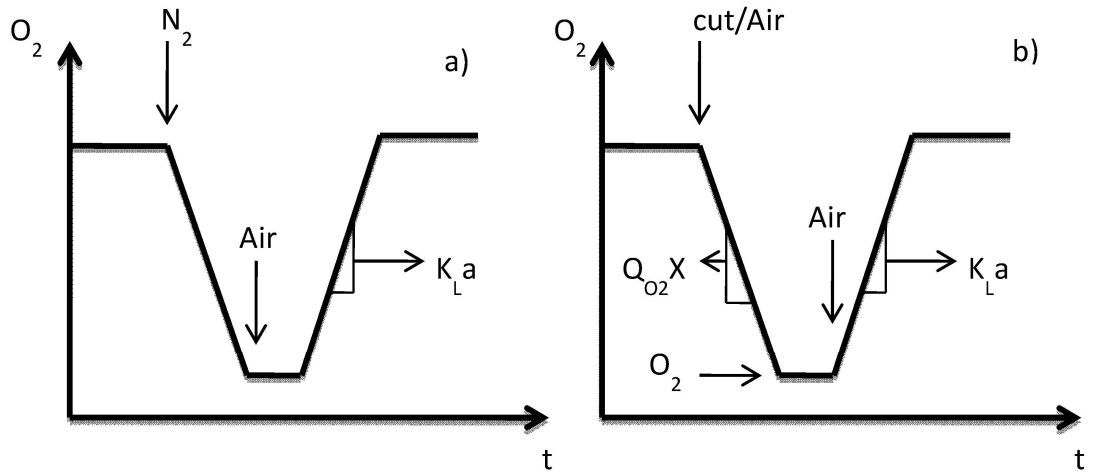


**Fig. 1.** a) Schematic description of the dynamic method without cell. b) Schematic description of dynamic method in a bioprocess is black

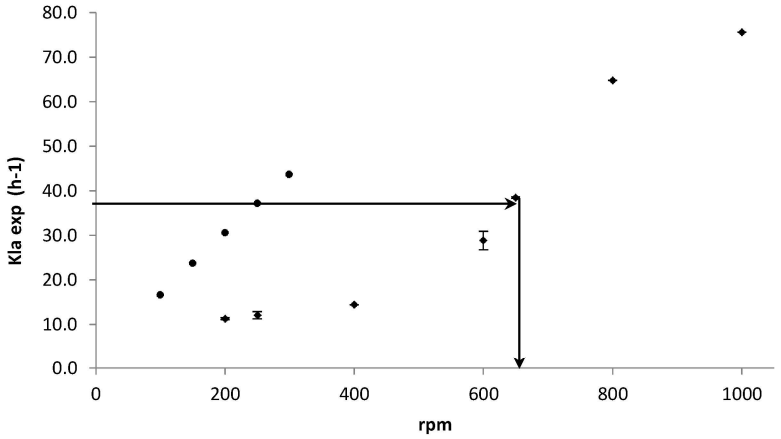


**Fig. 2.** Oxygen mass transfer coefficient ($K_La$) as a function of agitation speed. (Ï%) $K_La$ values in a shake flask were determined using a dynamic method proposed by (12). (f&) $K_La$ values in a 7-L bioreactor were determined using a dynamic method proposed by (21)

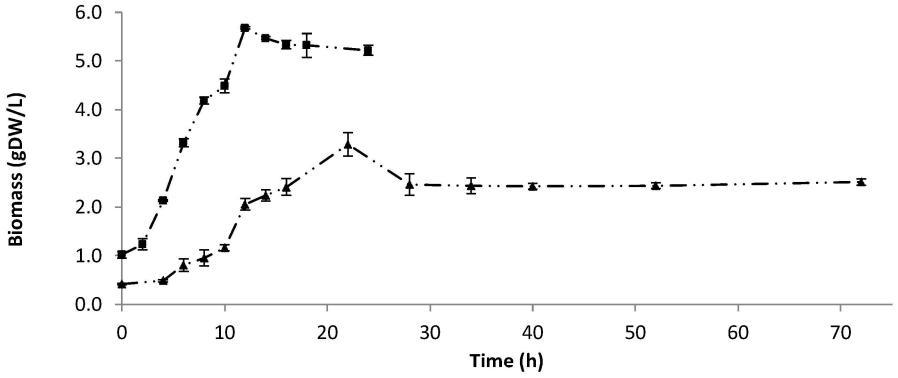


**Fig. 3.** Effect of scaling up from a shake flask to a bioreactor based on $K_La$ values in *Bacillus thuringiensis* strain GP129. ²%shake flask (250 rpm and 37.8 $h^{-1}$), % bioreactor (650 rpm and 38.5 $h^{-1}$)

fold more spore count and 3-fold more protein than[25]. However, we used different culture media and different $K_L a$ values but similar work volume and agitation speed.

In addition, to analyze whether oxygen limitation affects spore-crystal production during fermentation[5, 9, 13, 14, 15, 16, 17], two experiments were performed with in both $O_2$-limited (250 rpm, 11.16 $h^{-1}$ and 63.5 $mgO_2/m^3 s$) and non-limited (1000 rpm, 75.6 $h^{-1}$ and 129.3 $mgO_2/m^3 s$) conditions. In Figure 4, we show the effect of agitation speed on dissolved oxygen. We observed that at an agitation speed of 250 rpm, the dissolved oxygen value decreased by approximately 15%. We noted that at an agitation speed of 650 rpm, there is a decrease in the oxygen concentration during the first 4 hours and then maintained at 25%; later, there is an increase in the value of saturation. Finally, at 1000 rpm, dissolved oxygen concentration descends and ascends rapidly. These profiles are similar to those reported by several authors[2, 27, 28, 29].

Regarding biomass in $O_2$-limted cultures, we obtained 4.93 gDW/L, which is significantly different than that obtained in the shake flask (3.28 gDW/L). Furthermore, we produced the same biomass amount under $O_2$-limited and non-limited

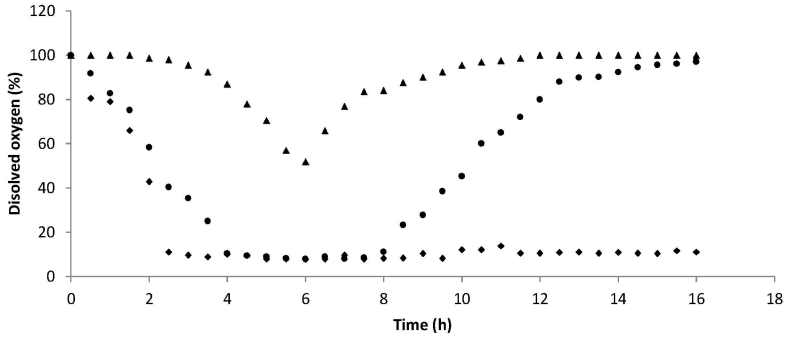


**Fig. 4.** Effect of agitation speed on dissolved oxygen concentration. ¨ 250 rpm, · 650 rpm and ²%1000 rpm

(4.95 gDW/L) conditions but obtained the maximum cell concentration at different times. There is an effect of oxygen transfer rate on the production of the spore-crystal complex. Once the $K_L a$ value was reached in both the shake flask and the bioreactor, we did not find a significant difference in spore count among the shake flask ($5.9 \times 10^8$ spo/mL) and $O_2$-limited conditions ($7.13 \times 10^8$ spo/mL). However, we produced 100-fold more spores in a non-limited ($7.36 \times 10^9$ spo/mL) culture than in an $O_2$-limited culture. Furthermore, in the $O_2$-limited culture, we produced $0.61 \pm 0.05$ mg/mL of total protein, which is significantly different from that obtained using the shake flask; in the non-limited culture, we produced $0.90 \pm 0.002$ mg/mL of total protein, which is also significantly different from that obtained using the shake flask. This behavior can be described as dependent on the oxygen transfer and culture media[2, 6, 10, 19, 28, 30, 26].

Therefore, in this work, we use the oxygen transfer scaling-up criterion by interpolating experimental data as a new way to produce large amounts of spore-crystal complexes by strain GP139 of *B. thuringiensis*. Under this scaling-up process, high cell and spore-crystal complex concentrations were achieved.

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