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The effect of aeration conditions, characterized by the volumetric mass transfer coefficient K_La , on the fermentation kinetics of *Bacillus thuringiensis* kurstaki

Jihane Rahbani Mounsef^{a,b,c,*}, Dominique Salameh^c, Nicolas Louka^c, Cedric Brandam^{a,b}, Roger Lteif^c

^a Université de Toulouse, INPT, UPS, Laboratoire de Génie Chimique, 4 Allée Emile Monso, F-31030 Toulouse, France

^b CNRS, Laboratoire de Génie Chimique, F-31030 Toulouse, France

^c Université Saint Joseph de Beyrouth, Centre d'Analyse et de Recherche, Unité de Technologie et Valorisation Alimentaire B.P. 11-514 Riad El Solh, Beyrouth 11072050, Lebanon

A B S T R A C T

The aeration is a key factor for *Bacillus thuringiensis* growth, sporulation and δ -endotoxins production. The objective of our work was to study the effect of aeration on the fermentation kinetics of *Bacillus thuringiensis* kurstaki (Btk), cultivated in a cereal milling byproduct (CMB) mono-component medium, in order to improve the δ -endotoxins productivity. Aeration conditions were systematically characterized by the volumetric mass transfer coefficient K_La . In the 6% CMB culture medium, different values of the maximal specific oxygen uptake rate were obtained at different values of K_La . For K_La of 7.2 h^{-1} , the growth was inhibited and the sporulation was defective. There was a linear increase of the average specific growth rate and faster sporulation and liberation of spores and δ -endotoxins crystals when K_La was increased between 13.3 h^{-1} and 65.5 h^{-1} . Similar kinetic was observed in cultures performed at K_La equal to 65.5 h^{-1} and 106.2 h^{-1} . The highest toxins productivity of $96.1 \text{ mg L}^{-1} \text{ h}^{-1}$ was obtained in the 9% CMB culture medium for K_La of 102 h^{-1} . It was possible to track the evolution of the bacterial cells between vegetative growth, sporulation and liberation of mature spores by following the variation of the CO_2 percent in the effluent gas.

Keywords:

Aeration

Bacillus thuringiensis

Kinetics

K_La

1. Introduction

Bacillus thuringiensis (Bt) is a facultative anaerobe, gram positive, spore forming bacterium. The biotechnological importance of this bacterium resides in its ability to produce, during sporulation, insecticidal crystal proteins known as δ -endotoxins toxic to specific species of insects. Despite its safety to public health and its minor impact on the environment, the Bt insecticide still represent a small fraction of the global crop protection market (Sanchis and Bourguet, 2008). Commercialization of Bt products depends on the low cost production that can be achieved by improving the crystal protein yield (Ben Khedher et al., 2011). Little information, concerning the

kinetics of the fermentative process involving *B. thuringiensis*, is available. On commercial scale the objective is to complete the fermentation in minimal duration, without compromising on the amount of the δ -endotoxins produced, which will reduce the cost of energy consumption involved in running the bioreactors and also greater number of batches could be completed ultimately becoming cost effective. The oxygen supply is a decisive parameter in *B. thuringiensis* fermentation. Several papers have been published on the effect of aeration on the growth, sporulation and crystal production of different strains of Bt but reports are sometimes contradictory. In fed batch cultures under constant dissolved oxygen concentration (DO) of 20% for 24 h followed in batch mode for additionally 24 h at different Oxygen Transfer Rate (OTR) values (0, 20, 100 and 250 mmol/L/h), Sarrafzadeh and Navarro, (2006) studied the effect of oxygen on the sporulation phase of *B. thuringiensis* H 14. The highest sporulation rate (100%) was observed in the absence of oxygen while a partial inhibition of sporulation but improved toxicity was obtained in conditions of excessive aeration. As well, by keeping the DO concentration at 50% during the vegetative and

* Corresponding author at: CNRS, Laboratoire de Génie Chimique, F-31030 Toulouse, France.

E-mail addresses: gihane.rahbany@net.usj.edu.lb (J.R. Mounsef), dominique.salameh@usj.edu.lb (D. Salameh), nicolas.louka@usj.edu.lb (N. Louka), cedric.brandam@ensiacet.fr (C. Brandam), roger.lteif@usj.edu.lb (R. Lteif).

Table 1
Aeration conditions.

Agitation speed (rpm)	340	500	500	500	500
Aeration flow (v/v/m)	0.033	0.033	0.165	0.33	1
$K_L a$ (h^{-1})	7.2	13.3	40.7	65.5	106.2

transition phases then raising the DO to 100% of saturation throughout sporulation and cell lysis, [Boniolo Fabrizio et al., 2012](#) obtained a higher toxicity of *B. thuringiensis israelensis* (Bti) (IPS-82). While, [Ghribi et al. \(2007\)](#) observed a lower spore yield but higher δ -endotoxin synthesis by a *B. thuringiensis kurstaki* strain BNS3, when 60% and 70% oxygen saturation were ensured during the first 6 h, then decreased to 40% up to the end of fermentation. Also, in semi continuous processes combined with batch processes for sporulation, [da Silva et al., 2011](#) reported an increase of 53.6% in sporulation of Bti IPS 82 under aerated conditions, but the toxicity was about four times higher under non aerated conditions.

The aim of the present work is to analyze the effect of aeration on growth and sporulation kinetics of a *B. thuringiensis kurstaki* strain cultivated in a cereal milling byproduct (CMB) mono-component medium, in relation with toxin synthesis, in order to determine the optimal conditions for improving the toxin proteins productivity. A kinetic study is presented at different oxygen transfer rates and for different ratios of CMB in the culture medium.

2. Materials and methods

2.1. Microorganism and culture media

A bacterial strain *B. thuringiensis* variety *kurstaki*, registered as Lip^{MKA}, designated by "Lip", isolated from the Lebanese soil, was used. "Lip" showed a higher insecticidal activity against *Ephestia kuehniella* larvae compared to that of the reference strain HD1 (LC50 of Lip and HD1 were 33.27 and 128.61 μ g toxin/g semolina, respectively) ([El Khoury et al., 2014](#)). The culture medium was a mono-component medium containing only a cereal milling byproduct (CMB) in distilled water. This medium was shown efficient for the production of Bt in a previous work ([Rahbani-Mounsef et al., 2014](#)). In the results section we indicate the ratio of CMB (w/v) used in each experiment. The CMB was provided by arcenciel NGO; a sustainable agricultural lead developer in Lebanon.

2.2. Preparation of seed culture

The first seed culture was prepared by inoculation of a 500 mL Erlenmeyer flask containing 100 mL Luria Bertani broth by a loopful of bacteria from the slant. The flask was incubated on a rotary shaker at 30 °C and 340 rpm for 8 h. At this time 2% (v/v) of the first passage seed was used to inoculate a 500 mL flask containing 98 mL of tryptose -phosphate broth, which was incubated at the same conditions as the first passage seed for 12 h.

2.3. Culture conditions

A New Brunswick "Bioflo 110" 2 L bioreactor was used. The volume of the culture medium was 1.45 L and the bioreactor was inoculated by 50 mL of a second stage seed. The pH and the DO were not controlled but they were monitored continuously. The temperature was maintained at 30 °C. The volumetric mass transfer coefficient $K_L a$, (K_L is the mass transfer coefficient; a is the gas/liquid interface area per liquid volume) that describes the efficiency with which oxygen can be delivered to a bioreactor for a given set of operating conditions, ranged between 7.2 h^{-1} and 106.2 h^{-1} by varying aeration rate and agitation speed ([Table 1](#)). Foaming was controlled by adding a natural antifoaming agent. Concentrations

of O₂ and CO₂ in the exit gas were measured with a Servomex oxygen and carbon dioxide analyzer. Harvest time was typified by 90% free spores and δ -endotoxins crystals.

$K_L a$ was determined by a static gassing out technique ([Dunn and Einsele, 1975](#)). In this technique, the O₂ concentration of the solution is lowered by gassing the liquid out with the nitrogen gas. Aeration is then initiated at a constant air flow rate and the increase in DO concentration is monitored using dissolved O₂ electrode. The plot of $\ln(C_L^* - C)$ versus t will produce a straight line where the slope is equal to $K_L a$. (C is the concentration of dissolved O₂ in the fermentation broth, C^* is the saturated dissolved O₂ concentration).

2.4. Analytical methods

2.4.1. Cell, spore and crystal counts

Cell, spore and crystal counts were microscopically determined in a Petroff hausser counting chamber (cell depth: 0.02 mm, ruling pattern: Improved Neubauer, 1/400 mm²) using high power, oil immersion objective of an Olympus microscope. The cells were counted as vegetative cells including cells containing spores. Spore count represented the free spores. Serial dilutions with thorough sonication were used to avoid cells and spores crystals clumping. Result is expressed in number of cells, spores or crystals per mL.

2.4.2. Toxin protein estimation

A 1 mL sample of lysed cell suspension was centrifuged at 13000 rpm for 5 min. The supernatant was discarded and the pellet was washed two times with NaCl 0.14M–0.01% Triton X-100 solution and four times with cold water. The crystal protein in the pellet was dissolved with 0.05 N NaOH for 3 h with stirring. The suspension was centrifuged at 13000 rpm for 5 min and the pellet, containing spores and cell debris was discarded. The concentration of the crystal protein in the supernatant was determined by the Bradford method ([Bradford, 1976](#)) using the bovine serum albumin as a standard. The toxin protein yield was calculated as mg of toxin protein produced per g of wheat bran in the culture medium.

2.4.3. Determination of oxygen transfer rate (OTR), oxygen uptake rate (OUR) and specific oxygen uptake rate QO₂

The oxygen transfer rate was calculated as $OTR = K_L a (C_L^* - C)$ where C is the concentration of dissolved oxygen measured in the bioreactor, with a C_L^* value of 7.71 mg L⁻¹ (maximal dissolved oxygen concentration in water at 30 °C and atmospheric pressure). The oxygen uptake rate was calculated using the following equation:

$$OUR = OTR - \frac{dc}{dt}$$

The specific oxygen uptake rate was calculated by dividing the oxygen uptake rate by the biomass concentration:

$$QO_2 = \frac{OUR}{X}$$

2.4.4. Determination of specific growth rate, productivity and cell yield based on oxygen

The average values of the specific growth rate were calculated using the following equation:

$$\mu = \frac{(\ln X_{max} - \ln X_0)}{(t_f - t_0)}$$

t_0 and t_f : times corresponding to the beginning of the fermentation and the end of the vegetative growth, respectively. X_0 and X_{max} : the cell concentrations at t_0 and t_f , respectively.

Productivity was defined as the ratio of toxin protein produced to the fermentation time. The cell yields based on oxygen was

calculated as concentration of cells produced per mg of oxygen consumed up to the moment when maximum cell concentration was reached. Total oxygen consumption was calculated by an integrating method.

2.4.5. Statistical analysis of results

All the results related to determination of cells and bipyramidal crystals counts, spore counts and toxin protein concentration were the average of two replicates of two separate experiments for each cultural condition. Mean values were compared by a Student's *t*-test at the 0.05 level.

3. Results and discussions

3.1. Growth, sporulation and δ -endotoxin production in a 6% CMB culture medium at different values of $K_L a$

The variations of the DO throughout the fermentations showed that the following values of $K_L a$ of 7.2 h⁻¹, 13.3 h⁻¹, 40.7 h⁻¹ and 65.5 h⁻¹ all correspond to oxygen limited conditions with an oxygen limitation period decreasing from 28.5 h to 1 h as $K_L a$ increased from 13.3 h⁻¹ to 65.5 h⁻¹. For $K_L a$ equal to 106.2 h⁻¹, the maximal oxygen transfer rate meets the maximal oxygen demand of "Lip": the dissolved oxygen concentration decreased after inoculation to reach a value of 0% at the end of the vegetative growth and increased immediately afterwards (Fig. 1c). The growth was inhibited and the maximum cell concentration obtained was much lower in cultures performed at $K_L a$ of 7.2 h⁻¹ in comparison with those obtained for other values of $K_L a$ studied (Fig. 1a). This inhibition may be due to the small amount of oxygen available in the culture medium as it may be due to the low pH of 5.4 that was reached in these conditions. We followed this culture until 120 h, the dissolved oxygen concentration did not increase and the microscopic observation showed a defective sporulation. The inhibition of spores and crystals synthesis could also be attributed to the low pH (Yousten and Rogoff, 1969).

The method used for the determination of cell and spore concentration by microscopic count, using the Petroff Hausser counting chamber, allowed us to distinguish five phases in the growth of *Btk* as it is shown on the Fig. 2: I_a lag phase, I_b exponential growth, I_c deceleration of growth, II phase between end of vegetative growth and beginning of mature spores liberation, III phase between beginning of mature spores liberation and end of fermentation identified by the liberation of most of the spores. For $K_L a$ equal to 13.3 h⁻¹, growth continued after the dissolved oxygen concentration reached a value of 0% (Fig. 2). It is well known that the oxygen demand of *B. thuringiensis* decreases during sporulation (Amicarelli et al., 2010) but in these aeration conditions, the oxygen uptake rate (OUR) reached a maximum value of 3.2 mmol/L/h after 8 h of fermentation (Fig. 1d) and the microscopic observation showed that there was no notable fall of the OUR until maturation of most of the spores. This suggests that an oxygen transfer rate of 3.2 mmol/L/h is critical for the sporulation of "Lip" in the process conditions employed, and could explain the defective sporulation for $K_L a$ of 7.2 h⁻¹ where the maximal oxygen transfer rate is 1.7 mmol/L/h.

For $K_L a$ equal to 40.7 h⁻¹ DO reached a value of 0% in the deceleration phase of growth and reincreased before the beginning of the decline phase. The oxygen limitation period of 1 h was observed during the transition to sporulation in $K_L a$ conditions corresponding to 65.5 h⁻¹. For $K_L a$ of 106.2 h⁻¹, we can consider that no oxygen limitation was observed. Thus, it has not been possible to correlate the concentration of the dissolved oxygen in the culture medium with the growth phase of "Lip" for the different values of $K_L a$.

Couch, (2000) described *B. thuringiensis* as voracious users of oxygen and reported that care should be taken to prevent falling of the DO below 20%. Boniolo Fabrízio et al., 2012 observed a maximum increase of 16% of the biomass measured as g dry weight L⁻¹ between batch runs of *B. thuringiensis israelensis* in a synthetic medium (GYS), performed at a constant DO of 5% and those performed at a DO of 50%. But in the present work, the *Bt* production was not affected even with an oxygen limitation period up to 28.5 h. A spore count and a toxin protein concentration of 1.9 × 10⁹ spore/mL and 2.19 g L⁻¹, respectively, were obtained. The maximum cell concentrations reached in the CMB mono-component medium, for different values of $K_L a$ ranging from 13.3 h⁻¹ to 106.2 h⁻¹, were of the same order (Table 2) suggesting that the oxygen was not the growth limiting factor. Whereas, the time needed to reach these concentrations varied. It was 8.5 h for both $K_L a$ of 65.5 h⁻¹ and 106.2 h⁻¹, while for $K_L a$ equal to 40.7 h⁻¹ and 13.3 h⁻¹, maximum cell concentrations were reached after 10.5 and 12 h of cultivation, respectively. Similarly, when *Escherichia coli* which is also an anaerobe facultative bacteria, was cultivated in TB medium (TB medium is similar to buffered LB-glycerol but contains a smaller amount of glycerol and a higher amount of complex compounds (yeast extract and tryptone)), oxygen limitation resulted in a longer culture time but did not cause a loss in cell yield (Losen et al., 2004).

For all $K_L a$ values in the range of 13.3–106.2 h⁻¹, the percentage sporulation exceeded 98%. No significant difference was observed between the spores concentrations obtained in the aeration conditions corresponding to $K_L a$ ranging from 13.3 h⁻¹ to 106.2 h⁻¹. A spore concentration of 1.9 × 10⁹–1.99 × 10⁹/mL was achieved. These values are comparable or greater than those obtained in *Btk* cultures where the agitation speed and aeration rate were varied in order to keep the dissolved oxygen values in the broth above 70% of saturation (Bihari et al., 2002) or above 30% of saturation value at all time (Yezza et al., 2005). The crystal concentrations obtained for the different values of $K_L a$ were not significantly different but a maximum increasing toxin protein concentration of 13.7% was observed when $K_L a$ increased from 13.3 h⁻¹ to 65.5 h⁻¹ which suggests that the crystals formed for $K_L a$ equal to 65.5 h⁻¹ have a higher amount of protein. According to Avignone-Rossa et al. (1992) the production of *B. thuringiensis israelensis* δ -endotoxins was 50% lower under oxygen limitation than the production in non-limited aeration conditions. Boniolo Fabrízio et al., 2012 reported that the effect of aeration was much more important on the δ -endotoxin production than on sporulation.

3.2. Variations of the CO₂ percent in the effluent gazes

Variations of the CO₂ percent in the exit gas were monitored throughout the fermentations at different values of $K_L a$. It was notable that the growth curves and the CO₂ percent variation curves, for the different values of $K_L a$, were related (Fig. 3). Except for $K_L a$ equal to 7.2 h⁻¹, the CO₂ percent variation curves could be divided into three domains I, II, III. The first domain was characterized by the increase of the CO₂ percent to reach a maximal value. This domain corresponded to the vegetative growth of *Bt* and the maximal value of the CO₂ percent was reached by the end of this growth phase. In the second domain, there was an irregular variation of the CO₂ percent and a tendency to stagnation. The third domain, characterized by a continuous decrease of the CO₂ percent, started with the beginning of liberation of *Bt* mature spores in the culture medium. For $K_L a$ equal to 7.2 h⁻¹, where the sporulation was defective, the third domain of the curve described for the other values of $K_L a$ was not observed. The CO₂ percent in the effluent gas reached a maximum value at the end of the vegetative growth, then decreased and stabilized. These results show that it was possible to determine the time intervals of *B. thuringiensis* kurstaki growth

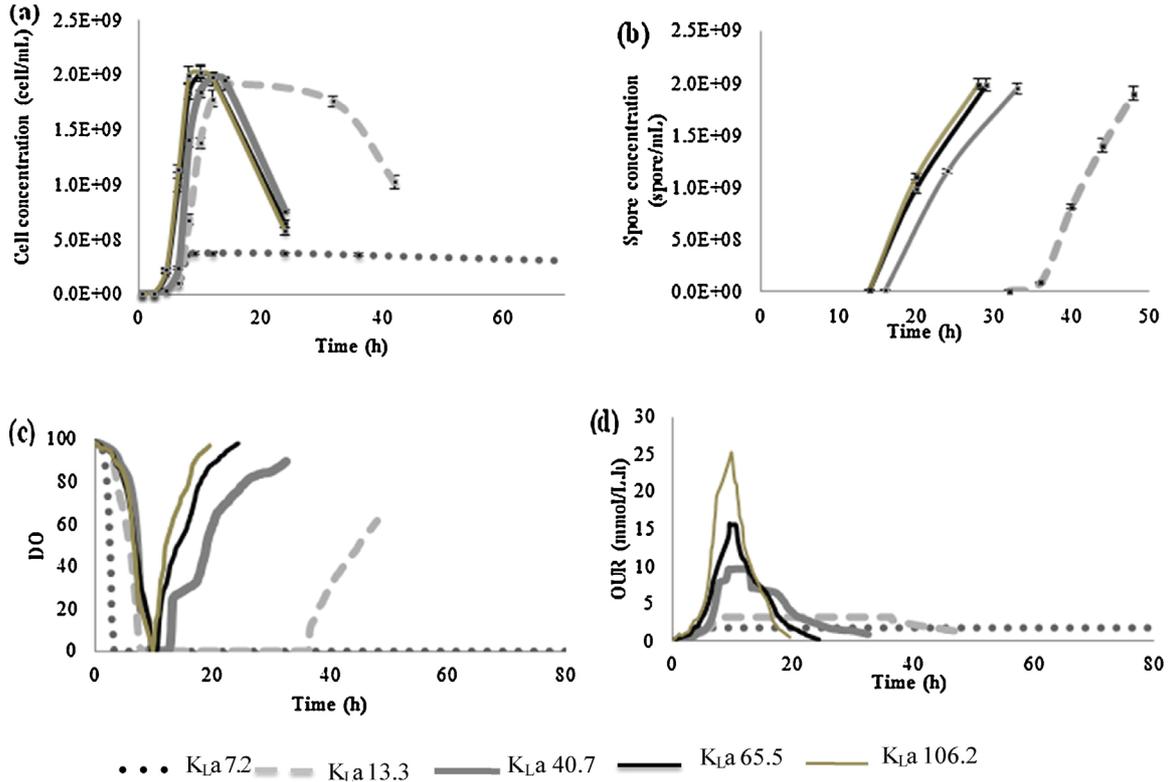


Fig. 1. Variations of *Bt* cell concentration (a), *Bt* spore concentration (b), DO (c) and OUR (d) at different values of K_{La} .

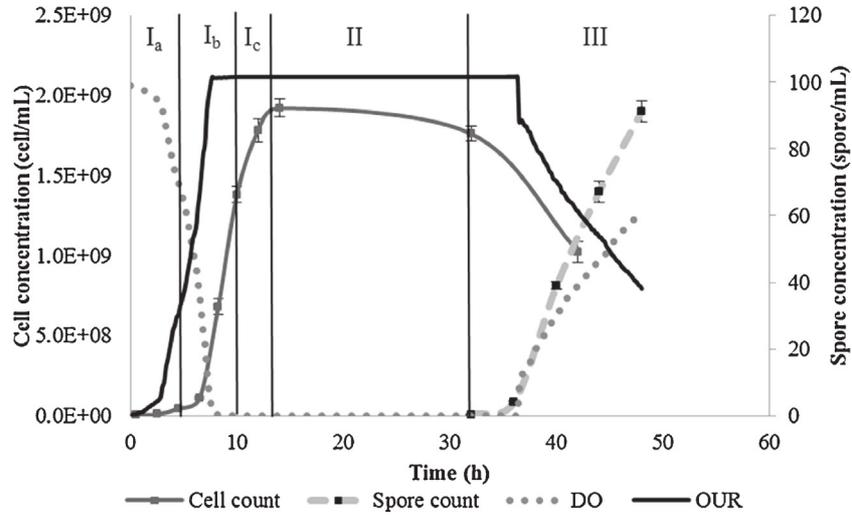


Fig. 2. Variations of cell concentration, spore concentration, DO and OUR for K_{La} of 13.3 h^{-1} .

Table 2
Cell, spore, crystal and toxin protein concentrations at different aeration conditions.

$K_{La}(\text{h}^{-1})$	Cells ($\times 10^9/\text{mL}$)	Spores ($\times 10^9/\text{mL}$)	Crystals ($\times 10^9/\text{mL}$)	Toxin protein (g L^{-1})
7.2	0.38 ^(a)	–	–	–
13.32	1.92 ^(b)	1.9 ^(a)	1.88 ^(a)	2.19 ^(a)
40.68	1.97 ^(b)	1.95 ^(a)	1.92 ^(a)	2.35 ^(b)
65.52	2 ^(b)	1.98 ^(a)	1.94 ^(a)	2.49 ^(c)
106.2	2.03 ^(b)	1.99 ^(a)	1.95 ^(a)	2.44 ^(c)

Values in the same column that are followed by the same letter are not significantly different ($p < 0.05$).

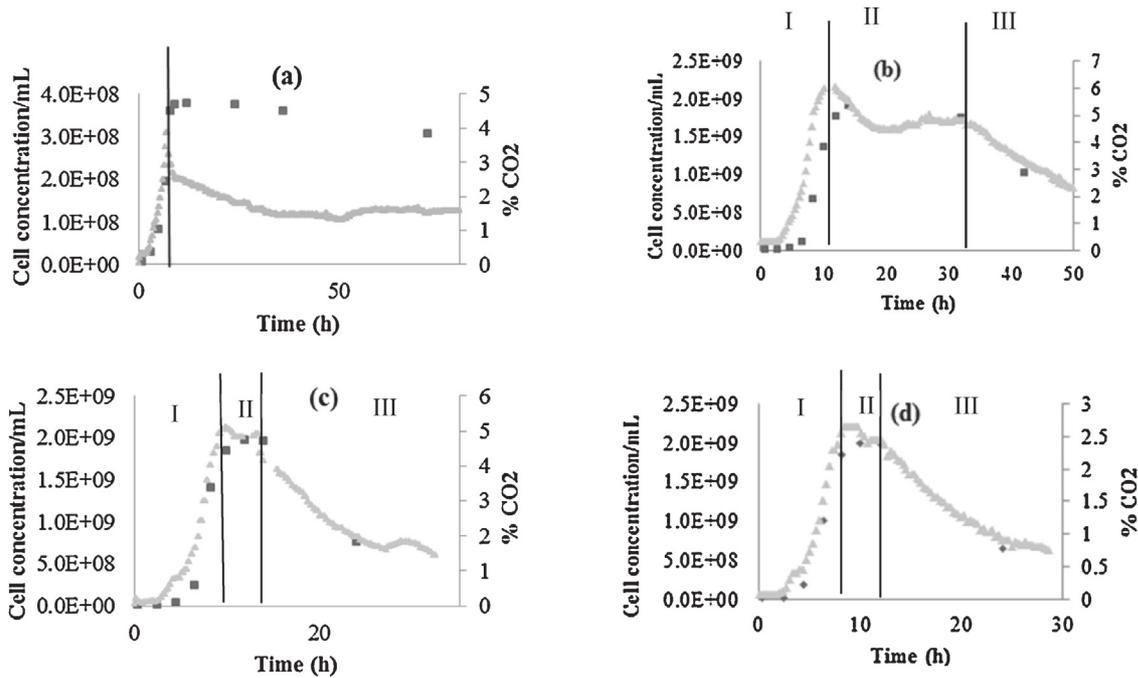


Fig. 3. Growth and CO₂ percent variation curves (a) K_{La} 7.2 h⁻¹; (b) K_{La} 13.3 h⁻¹; (c) K_{La} 40.7 h⁻¹; (d) K_{La} 65.5 h⁻¹. ■ Cell concentration, ▲ % CO₂.

phases by following the variations of the CO₂ percent in the effluent gas.

3.3. pH variations at different values of K_{La}

The initial pH was 6.2 and slight variations were observed in all studied conditions with a pH in the range of 5.6–6.8 throughout the fermentation, except for K_{La} equal to 7.2 h⁻¹ where the pH decreased to reach a low value of 5.4 and did not increase afterwards. Foda et al. (1985) suggested that elevated aeration enhances the acid metabolite consumption and avoids the excessive pH descent, and Kraemer-Schafhalter and Moser (1996) observed a promotion of acetic and lactate acid synthesis under oxygen limitation. This could explain the lower values of pH reached at K_{La} of 7.2 h⁻¹. The pH decreased at the beginning of the fermentation to reach a minimum value after 6 h, 5.34 h and 4.34 h for K_{La} equal to 13.3 h⁻¹, 40.7 h⁻¹ and 65.5 h⁻¹, respectively. This indicates a faster consumption of sugars and could be another indication of faster biomass build up as K_{La} increased.

3.4. Variations of the specific growth rate and the maximum specific oxygen uptake rate for the different values of K_{La}

The curves of variation of the maximum specific oxygen consumption rate QO_{2max} and the average specific growth rates μ for the different values of K_{La} had the same allure: a linear increase in the K_{La} range 13.3–65.5 h⁻¹ followed by a plateau (Fig. 4). The higher values of QO_{2max} obtained as K_{La} increased from 13.3 to 65.5 h⁻¹, show how depending on the aeration conditions the respiratory metabolism of *Bt* would be more or less favored. The increase of the specific growth rate could be then attributed to the increased production of energy with the increase in the maximum specific oxygen uptake rate. Yezza et al. (2005) also found higher specific growth rates when the oxygen transfer rate was increased as a result of the reduction of the working volume in the fermenter from 10 to 8 L.

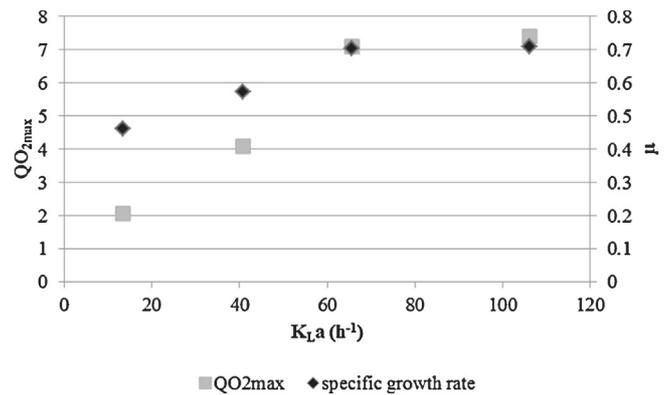


Fig. 4. Variations of specific growth rate and maximum specific oxygen uptake rate at different values of K_{La} .

3.5. Productivity

The time cycle for K_{La} equal to 65.5 h⁻¹ was 29 h while for K_{La} equal to 13.3 h⁻¹ lysis of many of the sporangia liberating mature spores and crystals did not start until 35 h of fermentation (Fig. 1b). While Sarrafzadeh and Navarro, (2006) observed a faster rate of *Bt* mature spore liberation in the absence of oxygen, the spores and the toxin proteins productivities in the present work were increased by 78.6% and 94.9%, respectively, between K_{La} equal to 13.3 h⁻¹ and K_{La} equal to 65.5 h⁻¹ (Fig. 5). The values of the spores productivity and those of the toxin proteins productivity obtained for K_{La} of 65.5 h⁻¹ and 106.2 h⁻¹, compared by Student's test at $p < 0.05$, were not significantly different.

3.6. Cell yields based on O₂

The cell yields based on O₂, calculated by evaluating the total consumed O₂ from the beginning of the fermentation until maximum cell concentration was reached, decreased from 2.63.10¹² to 1.38.10¹² cell/g consumed O₂ when K_{La} was increased from 13.3 h⁻¹ to 106.2 h⁻¹. These results are in agreement with reports

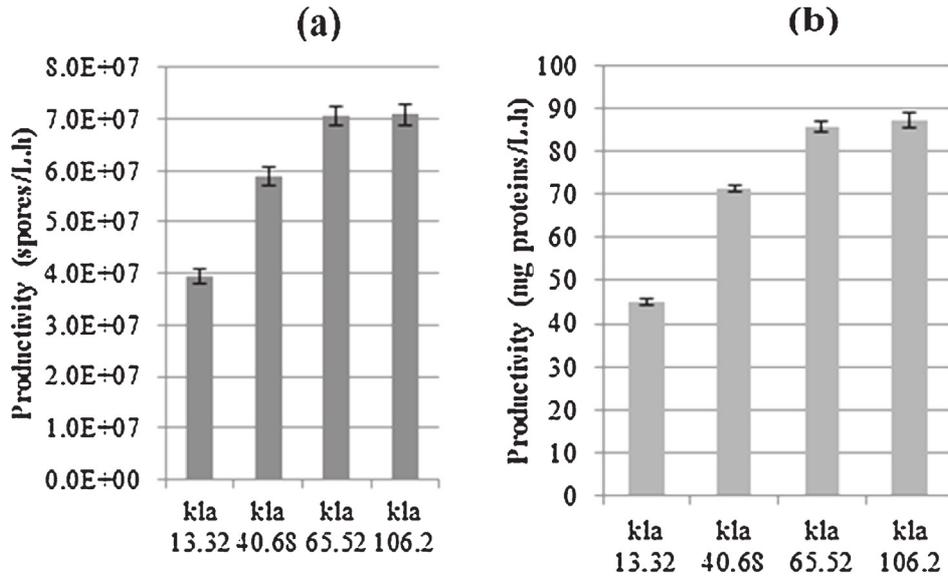


Fig. 5. (a) Spores productivity, (b) toxin proteins productivity obtained for different values of K_{La} .

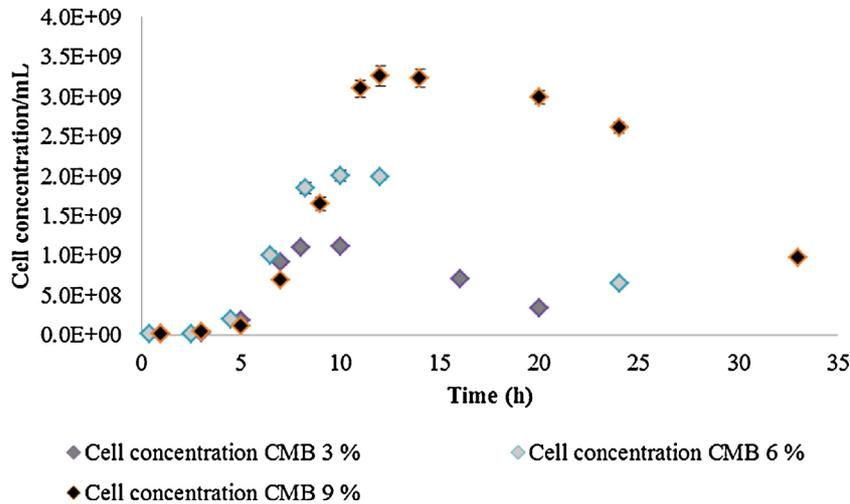


Fig. 6. Growth curves at different ratios of CBM.

given by other authors (Rizzi et al., 1989; Avignone-Rossa et al., 1992) who suggested a better efficiency of oxygen consumption under oxygen limitation.

3.7. Growth, sporulation and δ -endotoxin production for different CMB ratios

In the aeration conditions (500 rpm, 0.33 L/L/min) corresponding to a K_{La} of 65.5 h⁻¹ in the 6% CMB medium, we studied the growth kinetics of the local strain of Btk at different ratios of CMB (3, 6 and 9% (w/v)). K_{La} decreased in the CMB 9% thick culture medium to 52.9 h⁻¹, while the measured K_{La} in the 3% CMB medium was 72.4 h⁻¹. The growth curves are presented in Fig. 6.

The values registered for the dissolved oxygen observed in the three different batch fermentations runs showed that the DO did not fall lower than 43.5% when the CMB ratio in the culture medium was 3%. Oxygen limitation periods of 1 h and 12 h were observed when the CMB ratio increased to 6% and 9%, respectively. The exponential growth phase was prolonged, sporulation and lysis were delayed and the time cycle was increased to 36 h for the CMB ratio of 9%. The maximum cell concentrations as well as maximal spore

and crystal concentrations reached were proportional to the CMB ratio in the culture medium indicating that the high CMB ratio of 9% did not inhibit growth and sporulation. But, the toxin protein yield decreased from 41.5 to 31.44 mg protein/g CMB when the CMB ratio in the culture medium increased from 6 to 9%. The best toxin proteins productivity (85.7 mg L⁻¹ h⁻¹) was reached in the 6% medium. While the highest crystal productivity was attained in the 9% medium but with crystals less concentrated in proteins than those obtained at 6%.

These results show that the oxygen demand of the bacteria for the growth and sporulation are not identical to its oxygen demand for optimal toxin protein synthesis. Maldonado-Blanco et al. (2003) also found that high concentrations of cells and spores do not guarantee high concentrations of Bt toxins.

In an attempt to improve the toxin protein production in the 9% CMB culture medium, a batch was run at the maximal flow rate of the bioreactor (1.34 v/v/m) with an agitation speed maintained at 500 rpm. The K_{La} value corresponding to these aeration conditions was 102 h⁻¹ and an oxygen limitation period of 5 h was still observed. A toxin protein concentration of 3.17 g L⁻¹ was obtained and the toxin yield achieved was 35.2 mg protein/g CMB. The high-

est toxin proteins productivity of $96.1 \text{ mg L}^{-1} \text{ h}^{-1}$ was obtained in these conditions. However this air flow (and K_{La}) seems difficult to be reached on an industrial scale.

A linear correlation was found between the amount of oxygen consumed to reach the maximum cell concentrations and the maximum cell concentration obtained at different CMB ratios in the culture medium with a correlation coefficient of 0.99.

4. Conclusion

An oxygen limitation period throughout the fermentation, in the 6% cereal milling byproduct mono component medium, up to 28 h did not harm the production of *B. thuringiensis* kurstaki. For reasonable values of the air flow rate, the best toxin proteins productivity was reached in the 6% CMB culture medium for K_{La} of 65.5 h^{-1} . At the industrial scale a higher productivity increases the economical yield of the production. From a practical point of view, the determination of the optimal value of K_{La} , giving a higher productivity, is of great importance since K_{La} is a criterion that could be used for the scale-up of the production.

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