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A N I N T E R N A T I O N A L J O U R N A L

Lab-Scale Production of *Bacillus atrophaeus*' Spores by Solid State Fermentation in Different Types of Bioreactors

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ABSTRACT

Studies were conducted to evaluate Bacillus atrophaeus spores' production by solid-state fermentation (SSF) using sugarcane bagasse as support and soybean molasses as substrate at lab-scale in column bioreactors (forced aeration), plastic bags and Erlenmeyer flasks (aeration by diffusion). Different moisture contents (84%, 86% and 88%; 89%, 91% and 93%) and aeration rates (30mL/min, 45mL/min, 60mL/min and 90mL/min) were studied. The best condition for spore production ($3.3x10^{10}$ CFU.g^{-1}dry matter) in column bioreactor was 80% of initial humidity and no aeration. In Erlenmeyer flasks and plastic bags the best sporulation production reached 1.7 up to $4.7x10^{10}$ CFU.g^{-1}dry matter with 88-93% of initial moisture. The aeration rate had no significant effect on the spore yield. The initial moisture had a significant effect depending on the bioreactor type. Sporulation kinetic's assay was carried out and it showed the possibility to reduce the time of spore formation in two days.

Key words: *Bacillus atrophaeus*, spores, solid state fermentation, column bioreactor, biofilm

INTRODUCTION

Bacterial spores are differentiated cell types, highly specialized, and designed for the survival on adverse conditions. Its dormancy and resistance are responsible for some serious problems in food-, medicare-, paper-, and space-industry. *Bacillus atrophaeus* is a Gram-positive, facultative anaerobic rod that makes endospores. It has been used as a biological indicator of sterilization assurance (Christensen and Kristensen, 1979; AAMI, 1994; Fritze and Pukall, 2001; USP 29, 2005), evaluation of agents for cleaning and disinfection (Blakistone et al., 1999; Penna et al., 2001), as well as an indicator organism in drinking water treatment (Gale et al., 2002). Due to its similar particle size and dispersal characteristics to those of *Bacillus antrhacis*, has been used as a biological tracer for anthrax (HSTAT, 2002).

It has been well established that bacterial spore properties are affected by the conditions during sporulation. In most studies, spores are routinely produced from fortified agar. Sella et al. (2008) had demonstrated the use of solid state fermentation (SSF) with agro-industrial residues as a cost-effective method for *Bacillus atrophaeus'* heat resistant spores' production.

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SSF process can be defined as microbial growth on solid particles without the presence of free water. The water present in SSF systems exists in a complexed form within the solid matrix, as a thin layer either absorbed to the surface of the particles or less tightly bound within the capillary regions of the solid (Raimbault,1988**).** Environmental factors such as concentrations of nutrients, moisture, water activity and oxygen levels significantly can affect microbial growth and product formation (Mudgett, 1986). Usually aeration also has very important effects on hydration properties and heat regulation in SSFs. The effect of defined SSF conditions and media to link specific bioreactor use with *Bacillus atrophaeus* sporogenesis has not been studied systematically.

The aim of the present work was to evaluate the influence of aeration and moisture on *Bacillus atrophaeus*' spores' SSF production using different types of bioreactors to define the best parameters for growth kinetic study.

MATERIALS AND METHODS

Microorganism

A strain of *Bacillus atrophaeus* ATCC 9372, Bach-1403349, obtained from standard strains supplied by *Instituto Nacional de Controle de Qualidade em Saúde* (INCQS/MS, Brazil), was used. Stock cultures and stored at −80°C in 20% glycerol. Working cultures were maintained on standard tryptone soy agar (TSA) and were sub cultured periodically at 36 ºC for 24-48 h and were stored a 4ºC.

Inoculum

For inoculum preparation, 100 µL of spore suspension—batch: 01/06-CPPI—was inoculated in three tubes, each one with 30 mL of tested medium. These media were incubated for 18 h at 36 °C. The media used were standard medium: tryptone soy broth – TSB (USP 29, 2005) and complex medium: soybean molasses (~ 75ºBrix), 2.0% w/v and tryptone, 4.0% w/v.

Sporulation

For SSF sporulation sugarcane bagasse was used as support. It was supplied by COCAMAR (Cianorte-Brazil), milled, washed once with tap water and twice with distillated water. The washed bagasse was dried on trays for 24 h at 90ºC in an air oven. The dry bagasse was sieved to obtain a particle size between 0.85 mm to 1.18 mm. The substrate consisted of: soybean molasses 2.0%, supplemented with sporulation inductor salts (K_2HPO_4. H_2O, 0.005%; $MnSO_4$. H_2O, 0.004%; $CaCl_2.6H_2O$, 0.004%; $MgSO_4.7H_2O$, 0.005%). Soybean molasses (~ 75ºBrix) was supplied by IMCOPA (Araucária-Brazil) with the composition: total sugar, 50.8%; protein, 5.1%; lipids, 0.3%; pH, 6.1. The initial substrate pH was adjusted to 8.0 before sterilization. The moisture content was adjusted with distilled water before autoclaving. The flasks with SSF medium were autoclaved at 121ºC for 15 min. The inoculum size was 3% (v/v substrate) or 10^7 CFU.g^{-1}dry matter. Calculated inoculum volumes were mixed thoroughly with the autoclaved medium in a sterile laminar flow chamber. SSF was carried out at 36ºC. Different quantities of dry substrate were used for the different bioreactors: 5 g for Erlenmeyer flasks; 10 g for column bioreactors and 12g for plastic bags. The fermented bagasse was mixed with 0.02M calcium acetate solution with Tween 80 (0.01%) pH adjusted to 9.7 and sterile glass beds for 1 h, before filtration. All spores' suspensions were filtered through gauze tissue and, subsequently, washed three times by centrifugation at 2,500 rpm (1.048g) for 20 min at 4ºC with cold, sterile 0.02M calcium acetate solution adjusted to pH 9.7 (Hoxey et al., 1985; Penna et al., 1996). Spore suspensions kept in flasks were subjected to a heat shock (80 ºC, 10 min) and were stored at 4 ºC. Viable spore counts were done by serial decimal dilutions in distilled sterile water and 50 µL of each dilution was inoculated on a tryptone soy agar plate surface, in duplicate. Plates were incubated overnight at 36 ºC. For the media and the results analyses were considered the U.S. Pharmacopeia requirements that allowed a 50% up to 300% as permissible variation of viable spores' counts enumeration.

SSF in column bioreactor

It was carried out in glass columns with a 4cm diameter and 20cm length (volume of 250 mL) filled with the inoculated SSF medium. The temperature of the water bath was maintained at 36 °C and the columns were connected in the bottom with a water saturated air flow. Fermentation was carried out for nine days. In the first assay a mixed 2 and 3 levels factorial experimental design with 2 factors and 1 central point was used in order to study the effects of

aeration rate -30 up to 90 mL/min- and the, moisture content- 86 to 90% (Table 1). In the second assay a 2^2 factorial experimental design - with 2 factors, 2 levels and 2 central points - was used in order to study the aeration rate - 0 up to 90 mL/min - and the initial humidity of the substrate - 84 up to 88% - effects (Table 2). As control the same conditions of fermentation was carried out in 250mL Erlenmeyer flasks without aeration.

Table 1 – Mixed 2^2 and 2^3 experimental design for testing *Bacillus atrophaeus* spores production by SSF in columns with differents initial moisture and aeration rates.

Run	Replicate	Moisture (%)	Aeration rate (mL/min)
1	Central point 1	88	60
2	1	90	90
3	1	90	30
4	1	86	30
5	1	86	90
6	1	88	90
7	1	88	30
8	Central point 2	88	60

Table 2– 2^2 experimental design for testing *Bacillus atrophaeus* spores production by SSF in columns with differents initial moisture and aeration rates

Run	Replicate	Moisture (%)	Aeration rate (mL/min)
1	1	88	90
2	1	88	45
3	1	88	0
4	Central point 1	86	45
5	Central point 2	86	45
6	1	84	90
7	1	84	45
8	1	84	0

Effects of bioreactor, substrate concentration, inoculum medium and incubation time

This study aimed at determining the bioreactor influence, the best substrate concentration (2% or 3%), inoculum medium (standard or complex) and incubation time (7 or 9 days) (Table 3). A 2^2 full factorial experimental design with 2 factors and 2 levels was used. The initial humidity was 91% and a polyethylene bags (39 x 50 cm), where the culture medium was prepared and autoclaved, was tested as bioreactor. The bags were sealed after inoculation.

Kinetics

Kinetics of spore production was followed for 9 days with the conditions: 91% initial moisture content: soybean molasses, 2.0% (w/v) and tryptone, 4.0 % (w/v) as inoculum medium, Erlenmeyer flasks and plastic bags as bioreactor.

Table 3- Effects of bioreactor, substrate concentration, inoculum medium and incubation time assay's for testing *Bacillus atrophaeus* spores production.

Run	Bioreactor	Inoculum medium	Soybean molasses (%)	Incubation time (days)
1	Erlenmeyer flask	Standard	2	7
2			3	7
3			2	9
4			3	9
5		Complex	2	7
6			3	7
7			2	9
8			3	9
9	Plastic bag	Standard	2	7
10			3	7
11			2	9
12			3	9
13		Complex	2	7
14			3	7
15			2	9
16			3	9

Analytical Methods

Reducing sugars were measured by Somogyi and Nelson (Nelson, 1944). The parameters pH and moisture were determined as described by Soccol et al., (1995). The water activity (a_w) measurements were determined at 21-22ºC, in triplicate, using an AquaLab CX-2 water activity meter (Decagon Devices, Pullman).

Statistical Analysis

Analyses were done using the software Statistica 6.0 (StatSoft Inc.). All experiments were realized in duplicate under aseptic conditions. The results were compared by ANOVA. Significant differences were accepted at the level of $p<0.005$.

RESULTS

For optimum SSF sporulation in column, differents relations between aeration rate -30 up to 90 mL/min- and moisture content were studied. In the first column fermentation assay free water was observed during incubation time when the moisture content exceeded 88%, due to saturation capacity of the solid matrix. This weakened the SSF process that is defined by Raimbault (1988) as microbial growth on solid particles without the presence of free water. Results revealed that at the end of the incubation time the water activities at all substrate moisture contents wet-basis were essentially identical (0.970). For the fermented mass from the columns the pH of the culture had an increase from 0.6 up to 2.0 in response to metabolic activities, yet the amount of moisture content (wet-basis) differed from 1% up to 5% and the maximum spore yield observed was 6.1×10^7 $CFU.g^{-1}$ dry matter in the no aerated column and 88% as initial moisture. For the fermented mass from the Erlenmeyer flasks the pH of the culture had an increase from 1.2 up to 2.7, the amount of moisture content (wet-basis) differed from 3% up to 27% , the maximum spore yield observed was 1.7×10^9 $CFU.g^{-1}$ dry matter in 90% as initial moisture and no free water formation were observed (Table 4).

Table 4 - *Bacillus atrophaeus* spores production by SSF in columns and Erlenmeyer flasks with differents initial moisture and aeration rates.

Assay	Moisture$_i$ (%)	Aeration rate (mL/min)	pH$_i$	Moisture$_f$ (%)	pH$_f$	Spores (CFU.g^{-1})
C1	88	60	6.4	87	8.0	2.6×10^7
C2	90	90	6.1	87	7.3	2.1×10^6
C3	90	30	6.1	87	6.6	1.8×10^6
C4	86	30	6.0	81	6.9	3.7×10^6
C5	86	90	6.0	83	7.8	3.9×10^6
C6	88	90	6.4	87	7.3	3.1×10^7
C7	88	30	6.4	84	6.6	2.0×10^7
C8	88	0	6.4	85	7.9	6.1×10^7
E1	86	-	6.4	61	7.9	1.1×10^8
E2	88	-	5.9	78	8.8	6.9×10^8
E3	90	-	6.1	87	7.3	1.7×10^9

C=column.
E=Erlenmeyer flask.
pH_i = pH after substrate sterilization (the pH was adjusted only before sterilization to avoid contamination).
pH_f = final pH.

The main effects plot of columns biomass (Fig 1) presents 88% for moisture content optimum value and no detectable differences in total spore formation between the 30 and 90mL/min aeration rate.

Under the studied conditions for forced aeration from 30 up to 90 mL/min, and aeration by diffusion in Erlenmeyer flask, the SSF sporulation results in the box plot analysis (Fig 2) showed that aeration by diffusion gave better spore yield in approximately 2 log (100 times) higher than the production in column, that may have been caused by partial loss of bacterial growth due to the formation of free water.

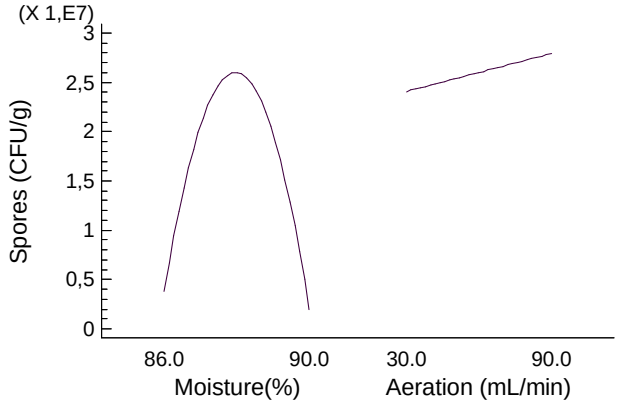

Figure 1 - Main effects of moisture and aeration rate on columns spores' production (forced aeration).

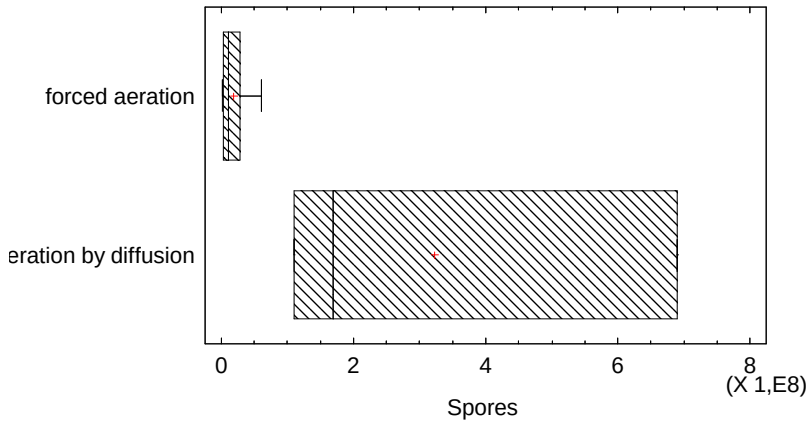

Figure 2 - Comparative *Bacillus atrophaeus* spores production in column bioreactors with forced aeration (30 up to 90 mL/min) and Erlenmeyer flasks (aeration by diffusion) with differents initial moisture (86 up to 90%).

In the second column fermentation experiment, over the range of initial moisture contents of 84–88% (w/w wet basis) and aeration rate of 0-90 mL/min, the highest spore content 3.7 x 10^{10} CFU. g^{-1} dry matter occurred at 88% initial moisture and no aeration (Table 5). The moisture content fell from 2% up to 6% and pH increased from 1.3 up to 1.9 during the fermentation time.

Table 5 - *Bacillus atrophaeus* spores production by SSF in columns and Erlenmeyer flasks with differents initial moisture and aeration rates

Assay	Moisture$_i$ (%)	Aeration rate (mL/min)	pH$_i$	Moisture$_f$ (%)	pH$_f$	Spores (CFU.g^{-1})
C1	88	90	5.7	86	7.0	1.7 x 10^{10}
C2	88	45	5.7	84	7.6	1.7 x 10^{10}
C3	88	0	5.7	82	7.0	3.7 x 10^{10}
C4	86	45	5.7	82	7.2	1.7 x 10^{10}
C5	86	45	5.7	80	7.0	6.7 x 10^{9}
C6	84	90	5.7	82	7.6	6.8 x 10^{8}
C7	84	0	5.7	77	7.0	3.3 x 10^{7}
C8	84	45	5.7	80	7.6	6.5 x 10^{7}
E1	84	-	5.7	75	7.5	3.9 x 10^{7}
E2	86	-	5.7	75	7.2	1.2 x 10^{7}
E3	88	-	5.7	73	7.0	1.9 x 10^{10}
E4	90	-	5.9	73	7.0	2.0 x 10^{10}

C=column.
E=Erlenmeyer flask.
pH$_i$ = pH after substrate sterilization (the pH was adjusted only before sterilization to avoid contamination).
pH$_f$ = final pH.

The analysis of variance (ANOVA), of these results showed statistically significant positive effect for substrate moisture content (p = 0.0073), no significant effect for aeration rate (p = 0.1483) and R^2 94.6.

In Erlenmeyer flasks (control) the pH of the culture had an increase from 1.1 up to 1.9, the amount of moisture content (wet basis) differed from 9% up to 15%, and the maximum spore yield

observed was 2.0 x 10^{10} CFU.g^{-1} dry matter at 90% initial moisture.

Under the studied conditions for forced aeration in columns, 0 up to 90 mL/min, aeration by diffusion in columns and in Erlenmeyer flasks, the SSF sporulation resulted in the box plot analysis (Fig 3) showed no differences in spore yield.

The effects of bioreactor's type and its relation with nutrient concentration level, inoculum medium and duration of cultivation on total end spore formation are presented in Fig. 4.

The results were stratified by the variables and runs a t-test were applied to compare the means. There were not a statistically significant difference between the means of the Erlenmeyer and plastic bags (*p*-value = 0.0921), standard and complex inoculum medium (*p*-value = 0.1491), substrate molasses concentration (*p*-value = 0.0826) and incubation time (*p*-value = 0.781165).

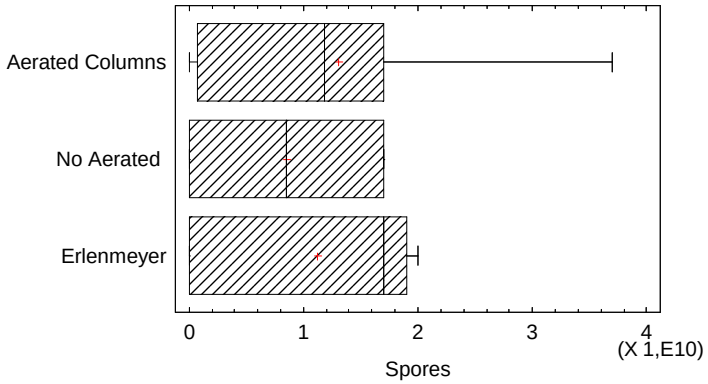

Figure 3 - Comparative *Bacillus atrophaeus* spores production in column bioreactors (forced aeration - 45 up to 90 mL/min), column bioreactors without forced aeration and Erlenmeyer flasks (aeration by diffusion).

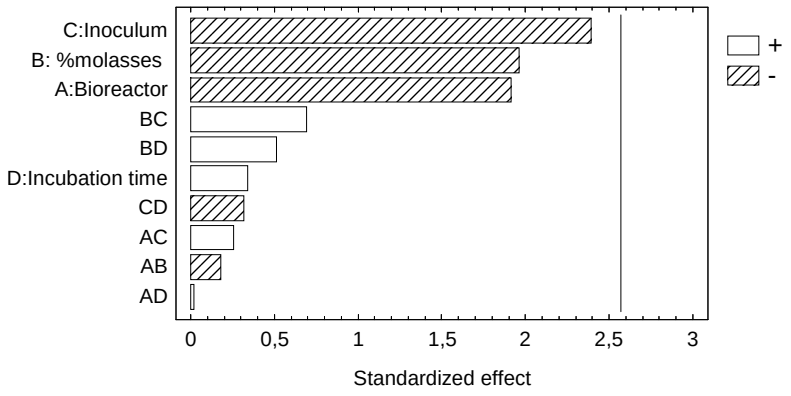

Figure 4 - Pareto Chart of Effects for *Bacillus atrophaeus* spore production without forced aeration. Variables: inoculum medium (industrialized and complex), % molasses (2 and 3%), bioreactor (plastic bag and Erlenmeyer flask) and incubation time (7 and 9 days).

The mean of all results showed that the pH decreases after sterilization process from 8.0± 0.1 up to 5.8 ±0, 1, and reached at 8.0±0.3 before 7 days of fermentation and decrease again to 7.8 ± 0.4 at the ninth day, the amount of moisture content (wet-basis) no differed, and the sugar consumption were 1.1 ± 0.4 g in 7 days and 1.3± 0.5g in 9 days incubation time.

Knowing that the growth rate of the microorganism depends on the local environmental conditions (Redmond et al., 2004; Vries et al., 2005), the evaluation of spore's yields from Erlenmeyer flasks and plastic bags fermentation was done by comparative kinetic studies. During fermentation kinetic in Erlenmeyer flasks the pH of the culture had an increased 1.6 in seven days incubation and decrease 0.5 from the 7th to the 9th incubation day, the water activity of the substrate (0.97) did not change, the amount of moisture content (wet-basis) has remained at 87%, the difference between the calculated moisture (91%) and the obtained (87%) is due to loss of water during sterilization process. The reducing sugars consummation was 0.83g. Fermentation kinetic in plastic bags showed that the pH of the culture had an increase 1.4 in seven days incubation and decrease 0.2 from the 7th to the 9th incubation day, the water activity of the substrate (0.7) did not change too, the amount of moisture content (wet basis) has remained at 89%, and the reducing sugars consummation was 0.89g, showing similarity in both processes.

The kinetic results demonstrated an increase in total biomass after 24 h of incubation of 3 up to 4 logs (thousand to ten thousand times), which corresponds to the exponential growth phase of vegetative cells. The sporulation began approximately 24 h after incubation and was stabilized after 168 h (7 days). The observed increase in the production of spores between the seventh and ninth days of incubation (276 h) was less than 1 log, confirming the data obtained previously and allowing the reduction of incubation time in 2 days, without compromising process productivity (Fig 5 and Fig 6).

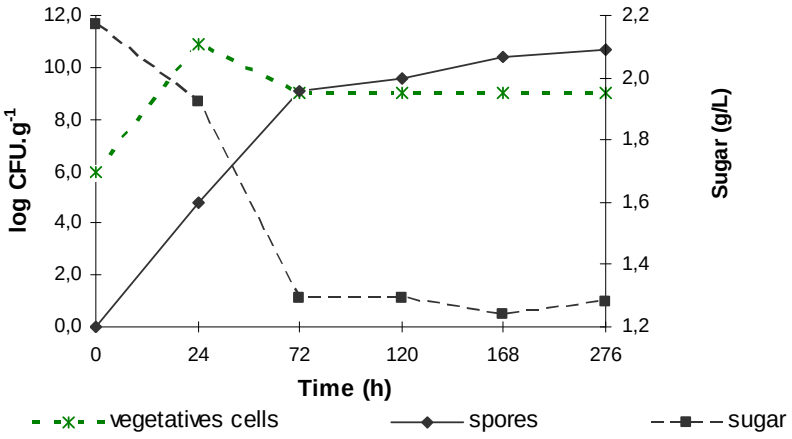

Figure 5 - *Bacillus atrophaeus* kinetics' spores production by SSF in plastic bags

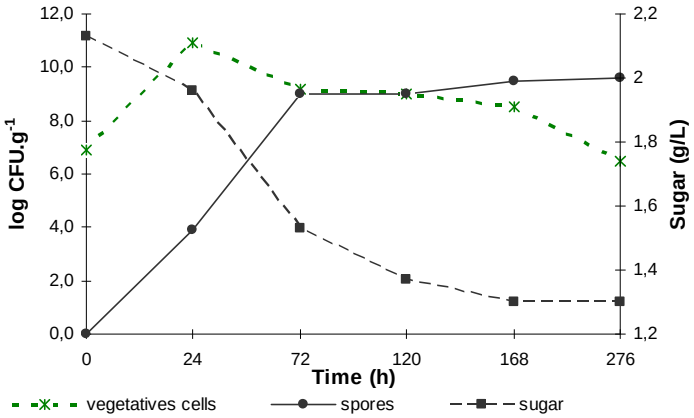

Figure 6 - *Bacillus atrophaeus* kinetics' spores production by SSF in Erlenmeyer flasks.

The spores obtained from both cultures, when germinated in tryptone soy agar (TSA) have mucoid colonies, large, and no delimited. The morphology was different from that obtained from standard strain spores cultivate in sporulation agar which are smaller and well defined (Fig. 7). Branda *et al* (2001) described this formation of colonies in wild strains of *Bacillus subtilis*, and joined its morphology with the production of surfactin and exopolysaccharides. These characteristics are also related to the use of a metabolic pathway that offers additional growth in structured communities, called biofilms, and the formation of spores through air structures called fruiting bodies. The 72 h in liquid medium tryptone soy broth (TSB) confirmed the ability of these cells to germinate and reproduce it in the form of biofilm, through the formation of a thick film on the surface and the formation of spores within 24 h of cultivation. The microscopic observation of this growth, stained by Gram method, showed the formation of chains and isolated cells, while the colonies from spores grown on agar for sporulation have only isolated cells. All this observations suggested the biofilm formation.

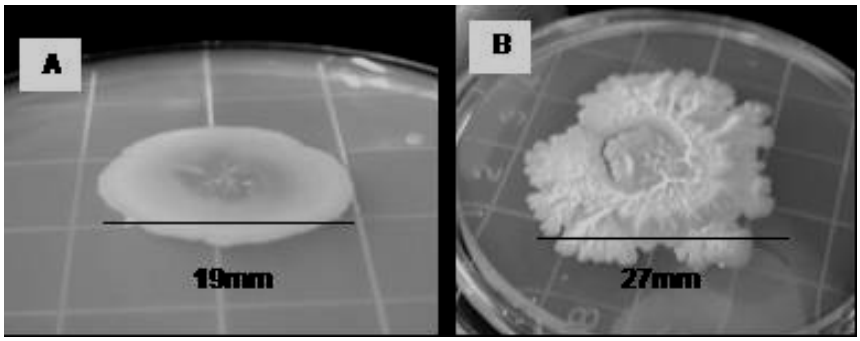

Figure 7 - *Bacillus atrophaeus* growth (TSA, 36ºC, 5 days) from spores produced (A) by sporulation in agar (B) by SSF.

DISCUSSION

The ability of the *Bacillus atrophaeus* for growing on a SSF probably is due to a function of its requirements of water activity, their capacity of adherence and their ability to assimilate the complex substrates used. Substrate moisture content is a significant process condition, especially for bacteria, because these organisms are considered the most suitable for growth in higher moisture contents. In SSF the aeration has essentially the functions: oxygen supply for aerobic metabolism, removal of CO_2, heat, water vapor and volatile components produced during the metabolism; regulate the substrate temperature and the moisture level. The exchange of O_2 and CO_2 between the solid and the gas phase depends on those factors that increase the contact surface between the phases like: additional aeration generated by forced step of sterile air, agitation and moisture level of the substrate (Gervais and Molin, 2003). In a column bioreactor, the substrate humidity changes during fermentation owing to the saturated air passing through the medium, consequently, it was very important to define optimal conditions for these parameters in order to attain high growth and sporulation (Vandenberghe et al., 2004; Prado et al., 2005). Bacterial performance often occurs at high moisture levels. However, studies in SSF involving strains of *Bacillus* for the production of enzymes, as have been observed as in aerobic SSF fungal studies, that increasing water content beyond the optimal level resulted in slowed growth and limited product formation, suggesting that the diffusion of gaseous oxygen into the larger liquid phase (within the interparticle spaces) was not adequate to support effective microbial respiration (Babu and Satyanarayana, 1996; Mamo and Gessesse, 1999). The observed results suggested that the efficiency of nutrients and oxygen transfer processes was sufficient to allow a good diffusion of solutes and gas, and promote the same cellular growth in the differents conditions: aerated columns, no aerated columns and flasks.

The water activity of the substrate did not change with moisture content and the fermentation time in all experiments. Knowing that water activity measures the thermodynamic potential of water in the system, and is a function of the type and amount of solutes present in the water and the water adsorption properties of the substrate and that moisture content is a direct measurement of the amount of water in the system (Chinn et al., 2007) it could be considered that the moisture content treatments investigated in this study had identical thermodynamic states because the water activities were identical. It was observed by Grajek and Gervais (1987) with sugar-beet pulp and by Chinn et al. (2007) with paper pulp sludge as support.

It was largely cited that the beginning of sporulation can be triggered by multiple environmental signals, like nutrient starvation and high cell densities, which cause a specific subpopulation of cells to switch on an elaborate genetic program resulting in spore formation (Sonenshein, 2000; Piggot and Losick 2002; Errington, 2003; Veening, 2007). In this kinetic study it was observed that the total sugar consumption was 0.83 up to 0.95 g and it was noticed that the sporulation started when there were still large supply of carbohydrates, indicating that in this case, the reduction or the lack of the carbon source was not the inducing sporulation factor. An increase in pH until the seventh day of incubation (between 1.4 and 1.6), was observed, followed by a slight decrease between the seventh and ninth day (0.2 to 0.5). Warriner and Waites (1999) also observed an increase in pH during sporulation of *Bacillus subtilis* on agar, attributing it to the absorption of acids formed for the production of new proteins. In plastic bag fermentation it was found almost exclusively the presence of spores at the end of the process, which can be justified by a higher rate of sporulation or by the absence or reduction of the partial germination of produced spores, but this factor gave no significant difference in total number of spores produced, in relation to the process implemented in a flask bioreactor.

The best conditions for spore production ($3.3\ 10^{10}$ CFU.g dry matter^{-1}) in column bioreactor was 80% of initial humidity and no aeration. In Erlenmeyer flasks and plastic bags the best sporulation production reached 1.7 up to $4.7\ 10^{10}$ CFU.g dry matter $^{-1}$ with 93-91% of initial moisture, respectively. No similar report about *B.atrophaeus* kinetic's spore production in differents bioreactors and conditions of aeration were found in the literature to compare the obtained results.

The spores obtained from SSF showed growth characteristics as biofilm formation. Nagel et al. (2002) cited by Plomp et al. (2005) stated that:

"Although very little is known about the mechanistic basis of differences observed is well known that gene expression in SSF may be different from that in other kind of fermentation". A new category of SSF was proposed by Gutierrez-Correa and Villena (2003): fermentation by the accession to the surface or fermented by biofilm. This category has been proposed in the application of the concept of biofilm (community of microorganisms attached to surfaces) in the solid state fermentation. The membership and subsequent differentiation of gene expression, creating different phenotypes observed in the microorganisms were not adhered cited as characteristics of this type of fermentation and additional studies should be done to elucidate this induction pathway.

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RESUMO

Estudos foram conduzidos para avaliar a produção de esporos de *Bacillus atrophaeus*, em escala laboratorial, por fermentação em estado sólido (FES) em biorreatores de coluna (aeração forçada), sacos plásticos e frascos tipo Erlenmeyer (aeração por difusão), usando bagaço de cana como suporte e melaço de soja como substrato. Diferentes teores de umidade (84%, 86% e 88%, 89%, 91% e 93%) e taxas de aeração (30mL/min, 45mL/min, 60mL/min e 90mL/min) foram estudados. A melhor condição para a produção de esporos no biorreator de coluna (3.3×10^{10} CFU.g^{-1} matéria seca) foi 80% de umidade inicial, sem aeração. Em frascos Erlenmeyer e sacos de plástico a melhor esporulação foi na faixa de 1.7 a 4.7×10^{10} CFU.g^{-1} matéria seca, com 88-93% de umidade inicial. A taxa de aeração não teve efeito significativo sobre o rendimento da esporulação. A umidade inicial apresentou efeito significativo relacionado ao tipo do biorreator. O estudo da cinética da esporulação demonstrou a possibilidade de reduzir o tempo de incubação para esporulação em dois dias.

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