



Technological Development of Biomass and the Challenge of Complete and Efficient Sporulation of *Bacillus Coagulans* BVB5

Magali Uono^{a*}, Sibylle Hacker^a, Marco Antonio Stephano^a, Vitória C. Viana^b, Catarina V. Manfrinato^a, Cristina S. B. Bogsan^{a*}

^aDepartment of Biochemical-Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, University of São Paulo

^bBiomedical Sciences, University of São Judas Tadeu
cris.bogsan@usp.br

The present study aims to develop an efficient industrial fermentation process that promotes the increase of sporulated biomass in a single batch of *Bacillus coagulans* BVB5. Preliminary studies of various culture media were performed in Erlenmeyer flasks for 96 hours in a shaker at 44 °C and 240 rpm, and the degree of sporulation was analyzed. The culture media tested were glucose, yeast extract, peptone based medium (GYP), GYP with salts (GYPs), glucose, yeast extract, tryptone based medium (GYT), soy protein, yeast extract, sodium chloride based medium (FNY), 50% FNY (FNY50), glucose, soy protein, potassium salts based medium (DSK) and 50% DSK (DSK50). The results had shown that the FNY50 medium obtained a higher degree of sporulation of 100% followed by FNY with 99.99%, whereas media containing relatively large amounts of glucose such as GYP, GYPs and GYT did not exhibit satisfactory sporulation. The reduction of the cost and consequent increase of the efficiency in the preparation of a probiotic microorganism of BVB5 in the DVS format are the main benefits of reducing the time of the fermentation/sporulation process. For this, more studies are needed,

1. Introduction

The concept of functional foods involves the benefit of health gain if consumed periodically by both, humans and animals (Ashwell, 2012). Among so-called functional foods are the ones incorporated with probiotics that are "living microorganisms, which when administered in adequate amounts confer benefits to the health of the host" (Hill et al., 2014).

Since the European Union banned the use of antimicrobials in animal feed in 2006, alternatives are being sought to replace them, and the probiotic microorganism comes into focus as one of these alternatives. Some *Bacillus* species could be characterized as probiotic with the technological differential due to the ability to form spores, whose characteristic is inherent to the genus.

The thermal resistance of the spores confers technological advantages over non-spore-forming probiotic bacteria, as resisting to a thermal process used in the food industry, for example, spray dryer and pelletizing processes. The prolonged shelf life also is acquired due to spores. *Bacillus coagulans* reunites all those characteristics and furthermore, depending on the culture medium, can produce a great quantity of lactic acid as a metabolite, thus being considered as a lactic acid bacterium (Juturu & Wu, 2018). However, compared with other *Bacillus* species, *Bacillus coagulans* shows difficulties in promoting sporulation (Amaha et al., 1956). Chen and colleagues (2016) present a long list of reagents aiming a high-density growth of *Bacillus coagulans* and Amaha and colleagues (1956), minerals that could be responsible for sporulation, such as manganese and calcium, followed by conditions of depletion of nutrients such as phosphor and carbon source, and also, addition of soil extracts (Amaha & Ordal, 1957) to change the sporulation pattern.

Some sporulation processes take a long time to be considered technologically efficient, there are also processes that take days to obtain only a low degree of sporulation (Laflamme et al., 2004). This study carried out experiments in several culture media to attempt an efficient BVB5 fermentation/sporulation technological

Paper Received: 2 June 2018; Revised: 13 November 2018; Accepted: 8 February 2019

process, aiming at its complete sporulation in one single batch fermentation in a shorter time, to promote a low cost of the process in general. The present study aims to develop an efficient industrial fermentation process that promotes the increase of sporulated biomass of BVB5.

2. Material and Methods

2.1 *Bacillus* strain

The strain of BVB5 was provided by the VitaBridge Company (São Paulo, Brazil) for the development of a food additive based on sporulated probiotic bacterium. The food additive will be added in the feed of broilers. The BVB5 strain was inoculated onto nutrient agar plates and stored in a refrigerator at 5 °C as workbench.

2.2 Culture media and inoculum medium

The culture media tested were GYP, GYPs, GYT, FNY, DSK, FNY50 and DSK50 which were prepared according to the table below (Table 1). The GYPs medium was also used as inoculum medium after verifying this medium does not produce spores. The inoculum of the experiment is the result of two activations in the GYPs medium, and the first activation was performed by inoculating 5 colonies of the workbench plate in 10 mL of GYPs medium. The medium of the second activation was inoculated with 1% of the broth resulting from the first activation and both were brought to the shaker at 44 °C for 24 hours. The broth resulting from the second activation was used as inoculum for the culture media of the sporulation kinetics of BVB5. For the sporulation experiment, the inoculated amount in each medium was 1% of GYPs broth of BVB5, relative to the total volume of each medium (FNY, FNY50, DSK, DSK50) to be inoculated, contained in the Erlenmeyer flask (Table 1) (Adapted from Zhang et al., 2014).

2.3 Analytical methods

Enumeration of the fermented broth of BVB5.

Volumes of 100 µL of each sample were serially diluted in 900 µL of 0.1% peptone water and 5 µL of each diluted solution were inoculated in triplicate into Petri dishes containing nutrient agar. The plates were incubated in BOD at 44 °C for 24 hours and their number of colonies counted, and the results were calculated in CFU/mL. The results were plotted in diffusion and logarithmic graphs. The resulting curves were analyzed in conjunction with the results malachite green/safranin (MG/SF) slides

Smears of fermented broth of BVB5 stained with MG/SF.

Glass slides of smears of a volume of 5 µL of the collected samples were fixed by flame, stained with MG/SF according to the Wirtz-Conklin method (Hamouda et al., 2002), and the samples were observed and photographed in Light microscope (Olympicus X-40) to verify the presence or absence of spores and make an empirical estimate of the number of spores in relation to the vegetative cells. The observed results were used to compare with the results of the enumeration plot.

2.4 Experimental design

a. Preliminary test of sporulation

Preliminary studies of the different culture media (Table 1) were performed in Erlenmeyer flasks during 96 hours in shaker at 44 °C and 240 rpm, and the degree of sporulation of each broth was analyzed by microscopic observations of smears of fermented broth stained with MG/SF. Culture broths showing spores were chosen to proceed with studies of sporulation kinetics.

b. Activation of BVB5

Activation of BVB5 was performed inoculating 5 colonies of BVB5 from the workbench (cultivated in nutrient agar plate for 24h in BOD at 44°C) in 10 mL of GYPs medium and incubated in shaker at 44°C and 240 rpm for 24h. Enumeration was performed, and glass slide of smear stained with MG/SF was prepared.

c. Sporulation kinetics of BVB5

The sporulation kinetics of BVB5, inoculated with 300 µL of activation broth in 30 mL of each chosen medium, were performed in a shaker at 44°C and 240 rpm. Samples were collected at 0 hour after inoculation and every 3 hours until 24 hours, and every 24 hours until 96 hours, ie, 0 h, 3 h, 6 h, 9 h, 12 h, 15 h, 18 h, 21 h, 24 h, 48 h, 72 h and 96 h respectively. For each sample, the glass slides of smear stained with MG/SF were prepared, and enumeration was also performed.

Table 1 – Composition of the different culture media for both, preliminary tests and kinetics of sporulation of BVB5

Reagents	GYP %	GYPs %	GYT %	DSK %	DSK50 %	FNY %	FNY50 %
Glucose	2.0	2.0	4.0	0.39	0.195	–	–
Tryptone	–	–	1.3	–	–	–	–
Soy Protein	–	–	–	0.24	0.12	2.0	1.0
Yeast extract	0.5	1.0	1.3	0.08	0.04	1.0	0.5
Peptone	0.5	1.0	–	–	–	–	–
CH ₃ COONa	–	1.0	0.067	–	–	0.0335	0.0168
K ₂ HPO ₄	–	–	–	0.1	0.05	–	–
KH ₂ PO ₄	–	–	–	0.1	0.05	–	–
NaCl	–	0.001	0.0015	–	–	1.2	0.6
MnSO ₄	–	0.001	0.0015	0.00086	0.00043	0.18	0.09
CaCO ₃	0.01	–	2.0	0.1	0.05	–	–
MgSO ₄ .7H ₂ O	–	0.02	0.0013	0.00086	0.00043	0.012	0.006
FeSO ₄ .7H ₂ O	–	0.001	0.024	0.0001	0.00005	0.012	0.006
ZnSO ₄	–	–	–	0.00086	0.00043	0.012	0.006
CaCl ₂	–	–	–	–	–	0.06	0.03
pH	6.0	6.0	6.0	6.0	6.0	6.0	6.0

3. Results and discussion

- Smears stained with MG/SF of preliminary studies of sporulation

According to the figure 1, spores can be seen on the slides of DSK (fig. 1D) and FNY (fig. 1E) broths smear stained with MG/SF, whereas media containing relatively large amounts of glucose such as GYP (fig. 1A), GYPs (fig. 1B) and GYT (fig. 1C) did not exhibit satisfactory sporulation. The morphology of the vegetative cells of the GYP broth is the most diverse among them, presenting cells that are filamentous (Amaha et al., 1956) and longer than others in which have shorter cells. This is possibly due to the absence of salts in the composition of GYP, which are present in other media and all of them showed shorter vegetative cells (Chen et al., 2016). Chen and colleagues (2016) observed that the chemical alterations in the medium could increase the production of the metabolites, as lactic acid and other bio-chemicals, but didn't look for the sporulation with biomass increased in a single batch. Thus, DSK and FNY were the culture media chosen to carry out sporulation kinetics.

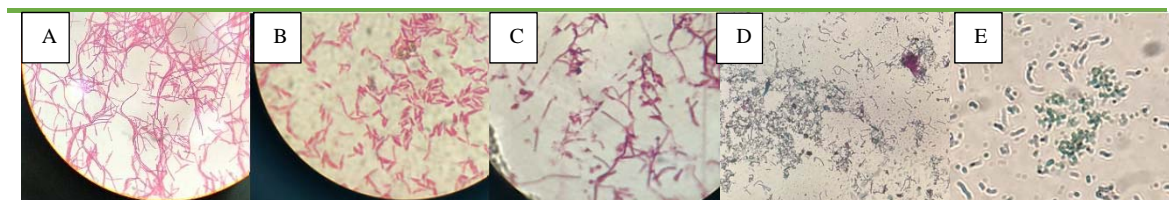


Figure 1: Smears slides stained by MG/SF. A-Light microscope image of *B. coagulans* BVB5 in GYP media (40x); B- Light microscope image of *B. coagulans* BVB5 in GYPs media (40x); C- Light microscope image of *B. coagulans* BVB5 in GYT media (40x); D- Light microscope image of *B. coagulans* BVB5 in DSK media (40x); E- Light microscope image of *B. coagulans* BVB5 in FNY media (40x).

Sporulation kinetics

The smear of GYPs with MG/SF (Figure 1) shows mostly red colored short vegetative cells which culture was chosen as the inoculum seed for the sporulation kinetics. The number of bacteria inoculated in each medium of sporulation kinetics was calculated from the result of enumeration of activation broth showed in the table 2. Therefore, the inoculation of 300 μ L of activation broth (GYPs) represents the amount of 1.54×10^8 bacteria inoculated in 30 mL of each sporulation test media.

Table 2: Result of enumeration of activation broth in GYPs

GYPs(CFU/mL)
5.13E+08

Table 3–BVB5 enumeration of sporulation kinetics

	FNY(CFU/mL)	FNY50(CFU/mL)	DSK(CFU/mL)	DSK50(CFU/mL)
0 h	4.37E+06	4.78E+06	6.02E+06	5.70E+06
3 h	1.48E+08	8.83E+07	1.33E+08	1.19E+08
6 h	4.57E+08	4.67E+05	2.99E+08	7.28E+07
9 h	9.29E+06	4.92E+05	2.64E+08	1.38E+08
12 h	6.50E+06	1.25E+06	3.48E+08	2.18E+08
15 h	8.01E+04	0.00E+00	3.18E+06	2.45E+06
18 h	6.41E+03	4.88E+05	1.31E+07	8.91E+06
21 h	5.21E+03	1.36E+06	1.91E+07	1.64E+07
24 h	1.06E+04	1.20E+06	3.70E+07	1.87E+07
48 h	2.33E+04	2.87E+04	4.30E+07	2.57E+07
72 h	2.47E+05	4.89E+06	1.78E+08	1.07E+07
96 h	1.12E+05	1.00E+04	1.56E+06	4.32E+06

The sporulation of *B.coagulans* is not as simple as other *Bacillus* species (Amaha et al., 1957). The degree of sporulation was calculated by dividing the result of the subtraction of the maximum and minimum values of CFU/mL by the maximum value of CFU/mL of each medium (Table 3). The results had shown that the DSK and DSK 50% media obtained higher degree of sporulation, 96.40% and 98.88% respectively, FNY50 presented highest degree of sporulation of 100.00% followed by FNY with 99.99%, whereas media containing relatively large amounts of glucose such as GYP, GYPs and GYT did not exhibit satisfactory sporulation (Figure 2 and table 3).

The graph of well-ordered curves showing a normal frequency, allows the points of maximum sporulation to be verified for the all culture media, located before 21 hours of fermentation: 21 h for FNY and 15 h for FNY50, DSK and DSK50 (Figure 2). These results are justified by the respective images shown in figure 3, in which FNY50 and FNY possess mostly green spores in their totality, while DSK and DSK50 have green spores and red vegetative cells. The result of FNY50/15h in the experiment is zero CFU/mL, so the adoption the value of 1.00E+00 CFU/mL was made to enable logarithmic calculation of that point in the graph (Figure 2).

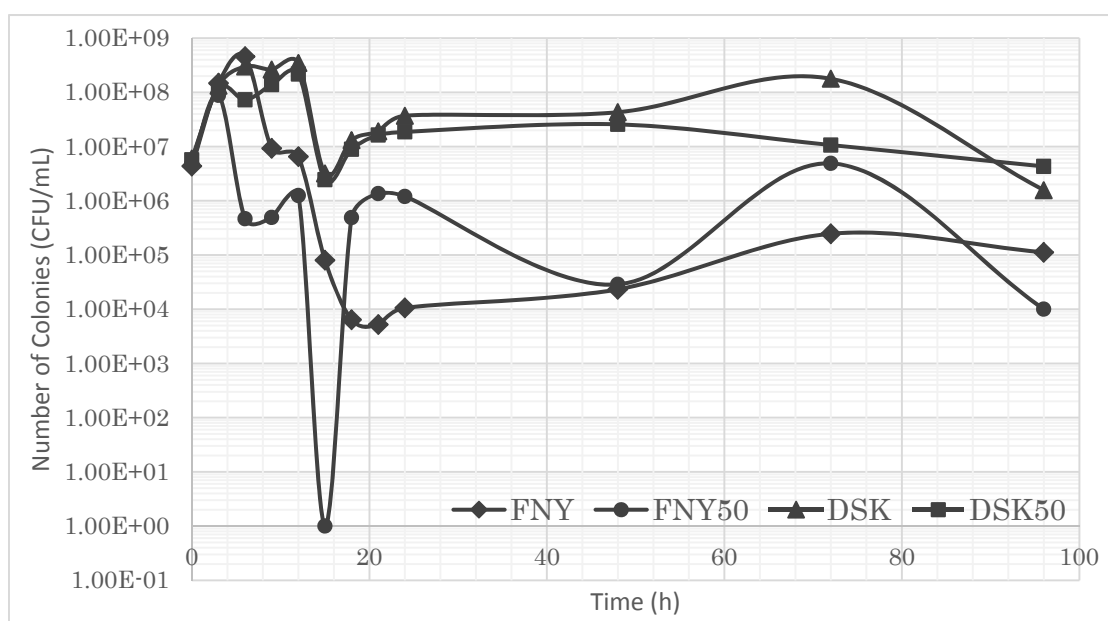


Figure 2 – Graph of number of colonies CFU/mL x time (hours).

The present study tested culture media of half the original nutrient concentration, FNY50 and DSK50, to verify whether the nutrient depletion condition could be a key to sporulation (Amaha et al., 1956; Lima & Souza, 2014), and the graph in figure 2 shows that FNY50, compared to FNY, seems to follow this condition. However, it did not happen to be the same with DSK50 compared to DSK, where both presented almost the same minimum value of CFU/mL. Thus, nutritional depletion is not a general condition valid for all sporulation culture media for BVB5. The presence or absence of certain minerals may be a sporulation condition, since the morphology of BVB5 cells showed filamentous and absence of spores in the GYP medium, and the other media

with presence of minerals had shorter cells with spores in FNY, FNY50, DSK, DSK50 and some in GYPs (Figure 1). Among all culture media, sporulation occurred in FNY and DSK and their correlated media (Figure 1). Comparing its nutrients with other culture media (Table 1), the major difference lies in the presence of soy protein that is not hydrolyzed and therefore not soluble in the culture media.

Abhyankar et al. (2016), shows that the industrial form to generate *Bacillus industrialis* spores is in two steps, the first one in liquid medium to grow the biomass followed by preparation in solid agar plates. The FNY and FNY50 media are technologically interesting due to the short time of maximum sporulation (<24 h) and the respective degrees of sporulation in a single batch in a liquid medium.

Analyzing only the degree of sporulation, FNY50, at first seems to be more interesting, but when maximum numbers of colonies are considered it is concluded that FNY, which reached the value of 4.57×10^8 CFU/mL is actually more interesting by the total number of vegetative cells that this medium achieve in a total of 21 hours, of which 99.99%, through the calculations in figure 3, are transformed into spores. This value is higher than the number of spores of 8.87×10^7 CFU/mL of FNY50. As FNY50 showed the highest degree of sporulation, it is interesting to follow studies to increase the biomass of vegetative cells that could be totally transformed into spores.

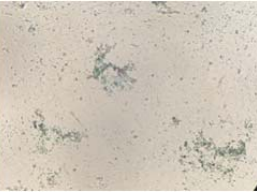
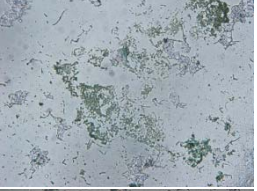
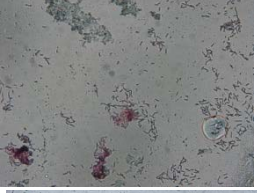

Medium	Minimum (CFU/mL)	Maximum (CFU/mL)	Sporulation time	Degree of sporulation	Images of the smears stained with MG/SF
FNY	5.21E+03	4.57E+08	21 h	99.99%	
FNY50	0.0E+00	8.83E+07	15 h	100.00%	
DSK	3.18E+06	3.48E+08	15 h	96.40%	
DSK50	2.45E+06	2.18E+08	15 h	98.88%	

Figure 3: Maximum and minimum point of CFU/mL of each media and their respective images

4. Conclusion

The culture media of FNY and FNY50 showed values of time/degree of sporulation that are technologically more interesting comparing to others: DSK, DSK50, GYP, GYPs and GYT. For aiming the reduction of costs of the fermentation sporulation process of BVB5 in the Direct Vat Set (DVS) format, more studies are needed, e.g., increasing of biomass with reproducibility of sporulation degree of FNY/FNY50. Thus, the next step of our group will be to increase sporulation efficiency in FNY/FNY50 by inserting some new parameters such as addition of carbohydrates, some salts, control of both aeration and pH, which will be performed in bioreactors to increase efficiency of the fermentation/sporulation process of *Bacillus coagulans* BVB5.

Acknowledgements

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001, and VitaBridge Scientific Development Co.

References

- Abhyankar W. R., Kamphorst K., Swarge B. N., van Veen H., van der Wel N. N., Brul S., de Koster C.G., de Koning L. J. 2016. The Influence of Sporulation Conditions on the Spore Coat Protein Composition of *Bacillus subtilis* Spores. *Front. Microbiol.* V.7, p.1636.
- Amaha, M., & Ordal, Z. J. 1957. Effect of divalent cations in the sporulation medium on the thermal death rate of *Bacillus coagulans* var. *thermoacidurans*. *Journal of bacteriology*, v.74, n.5, p.596.
- Amaha, M., Ordal, Z. J., & Touba, A. 1956. Sporulation requirements of *Bacillus coagulans* var. *thermoacidurans* in complex media. *Journal of bacteriology*, v.72, n.1, p.34.
- Ashwell, M. 2012. Concepts of functional foods. VVB Laufersweiler.
- Chen, Y., Dong, F., & Wang, Y. 2016. Systematic development and optimization of chemically defined medium supporting high cell density growth of *Bacillus coagulans*. *Applied microbiology and biotechnology*, 100(18), 8121-8134.
- Hamouda, T., Shih, A. Y., & Baker, J. R. 2002. A rapid staining technique for the detection of the initiation of germination of bacterial spores. *Letters in applied microbiology*, v.34, n.2, p.86-90.
- Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., ... & Calder, P. C. 2014. "Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic". *Nature Reviews Gastroenterology and Hepatology*, v.11, n.8, p.506.
- Juturu, V., & Wu, J. C. 2018. Production of high concentration of l-lactic acid from oil palm empty fruit bunch by thermophilic *Bacillus coagulans* J12. *Biotechnology and applied biochemistry*, v.65, n.2, p.145-149.
- Laflamme, C., Lavigne, S., Ho, J., & Duchaine, C. 2004. Assessment of bacterial endospore viability with fluorescent dyes. *Journal of Applied Microbiology*, v.96, n.4, p.684-692.
- Lima, A. M., Souza, R. R. 2014. Use of Sugar Cane Vinasse as Substrate for Biosurfactant Production Using *Bacillus subtilis* PC. *Chemical Engineering Transactions*, v. 37, p. 673.
- Zhang, Y., Chen, X., Luo, J., Qi, B., & Wan, Y. (2014). An efficient process for lactic acid production from wheat straw by a newly isolated *Bacillus coagulans* strain IPE22. *Bioresource technology*, 158, 396-399.